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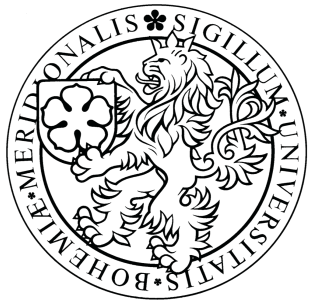

Glycobiology of Ticks and Tick-Borne Pathogens
Glycans, Glycoproteins, and Glycan-Binding Proteins

PhD. Thesis

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Annotation:

The proposed thesis brings new information on several aspects of tick glycobiology – tick *N*-glycans, tick lectins, and glycosylation of the tick-borne pathogen, Lyme disease spirochetes *Borrelia burgdorferi* s.l.

Declaration [in Czech]:

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Mgr. Ján Štěrba

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List of papers and author's contribution

The thesis is based on following papers (listed chronologically):

I. Štěrba, J., Vancová, M., Rudenko, N., Golovchenko, M., Tremblay, T.-L., Kelly, J.F., MacKenzie, R.C., Logan, S.M., Grubhoffer, L., 2008. Flagellin and outer surface proteins from *Borrelia burgdorferi* are not glycosylated. Journal of Bacteriology 190, 2619-2623 (IF = 3.726).
Ján Štěrba prepared the borrelia fractions, was responsible for electrophoretic and blotting analyses, writing of the manuscript.

II. Man, P., Kovář, V., Štěrba, J., Strohalm, M., Kavan, D., Kopáček, P., Grubhoffer, L., Havlíček, V., 2008. Deciphering Dorin M glycosylation by mass spectrometry. European Journal of Mass Spectrometry 14, 345-354 (IF = 1.103).
Ján Štěrba participated in data evaluation and writing of the manuscript.

III. Štěrba, J., Dupejová, J., Fišer, M., Golovchenko, M., Rudenko, N., Grubhoffer, L., 2009. Identification and characterisation of lectins in several tick species. FEBS Journal 276 (Suppl. 1), 88 (IF = 3.129). (published conference abstract)
Ján Štěrba participated in mRNA isolation, PCR analyses, phylogenetic analyses and was responsible for preparation of the abstract..

IV. Grubhoffer, L., Hajdušek, O., Vancová, M., Štěrba, J., Rudenko, N., Glycobiochemistry of ticks, vectors of infectious diseases: carbohydrate-binding proteins and glycans. FEBS Journal 276 (Suppl. 1), 141 (IF = 3.129). (published conference abstract)
Ján Štěrba participated in analysis of the presence of glycans in tick salivary glands and was responsible for preparation of the abstract..

V. Dupejova, J., Sterba, J., Vancova, M., Grubhoffer, L., 2011.

Hemelipoglycoprotein from the ornate sheep tick, *Dermacentor marginatus*: structural and functional characterization. Parasites & Vectors 4, 4 (IF = 2.130). *Ján Štěrba participated in mRNA isolation, PCR analyses, electrophoretic and blotting analyses, and was responsible for mass spectrometric analyses of HLGP glycans and was responsible for preparation of the abstract.*

VI. Sterba, J., Dupejova, J., Fiser, M., Vancova, M., Grubhoffer, L., 2011. Fibrinogen-related proteins in ixodid ticks. Parasites & Vectors 4, 127 (IF = 2.130). *Ján Štěrba participated in sample preparation, electrophoretic and blotting analyses, mass spectrometric analyses, and was responsible for preparation of the abstract.*

Content

1 Preface

It does not matter, if you are tanning on the grass behind your village, in the park in your home town or just walking in the wood in your preferred vacation locality, everywhere, you should be aware of ticks. Ticks, feeding for several hours or even several days; sometimes only one of them, sometimes large numbers waiting for their prey on the same place. Except of the inconvenience of the feeling, when such a parasite is sucking your blood without being noticed, there is another issue associated with ticks – they are vectors of pathogens, some of them very dangerous not only for humans but also for domestic animals such as dogs or cattle.

The proposed dissertation combines two "hot-topics" (ticks with their pharmacological as well as vectorial potential and emerging infectious diseases) with a third one – glycosylation. Today, we are far from thinking that glycosylation is present only in animals. Glycosylation of proteins is a widely present phenomenon found in all possible organisms, much more complicated then ever thought, affecting all types of molecule-molecule, molecule-cell, cell-cell, and cell-tissue interactions. Today, we are at the dawn of the glycome era – instrumentation allows us to study glycans in one organism, or on one protein, or even at one particular glycosylation site of the protein.

2 Introduction

The occurrence of tick-transmitted diseases itself would be an important reason to study ticks; anyway, if nothing else, there is also other factor – decreasing of the livestock weight and production and thus economic consequences [1].

The currently observed climate change causes a shift in the spatial and temporal distribution of ticks [2-4] and subsequently an increase of incidence of the tick-borne infections [5-7]. For some time (in the second half of the last century), the latitude of 500-700 m.s.l. was considered the upper limit for tick presence. However, the onset of climatic changes moved the limit for occurrence of different species (both plant and animal) from equator to north but also from lower to higher altitudes. Ticks (namely the most common tick in Czech Republic, the castor bean tick *Ixodes ricinus*) are currently observed above the former "limit" and reach as much as 1270 m.s.l. [2]. Similarly, tick occurrence above the limit was observed in Switzerland in altitudes above 750 m.s.l. [8].

Together with ticks, tick-borne pathogens occurrence shifted as well; tick-borne encephalitis virus was detected in ticks as far as 720 m.s.l. and *Borrelia* spirochetes in altitudes of 1100 m.s.l. [9, 10]. Similarly to ticks in lower altitudes, also here, multiple *Borrelia* infections were observed.

Tick-borne diseases (Lyme borreliosis caused by spirochetes of the genus *Borrelia* and tick-borne encephalitis and others) belong to the so-called emerging diseases gaining increasing attention in last years [5-7]. It is not simple to decide, which pathogen to include in this group. According to the simplest definition, emerging pathogens (diseases) are those which "emerge" in some aspect – either in a new locality (e.g. due to traveling of humans or migration of the vectors), or in new characteristics (based on changes in the genome) and subsequently, these new aspects bring changes in the infectivity, pathogenicity, morbidity, and mortality [11].

2.1 Ticks and tick-borne pathogens

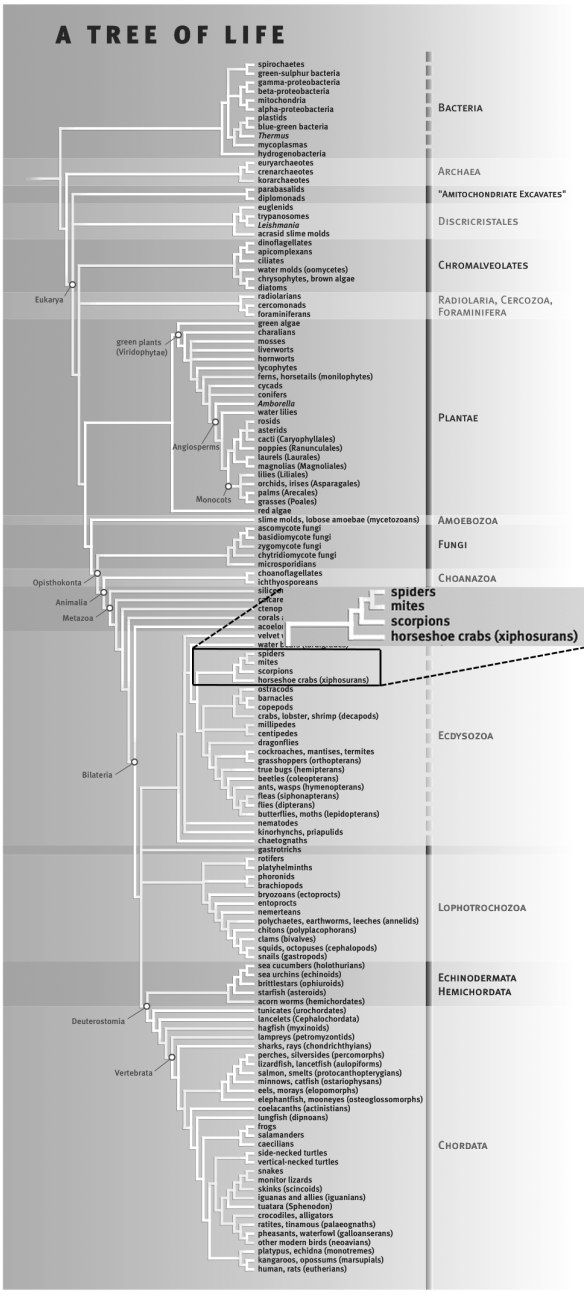

Figure 1. Tree of life, ticks (Ecdysoa, mites) are highlighted. Reproduced from [12], with permission.

Ticks (Ixodida) are arthropods belonging to the class Arachnida, subclass Acari, and superorder Parasitiformes. Three families belong to this order: Argasidae (also called soft ticks), Ixodidae (hard ticks), and Nuttalliellidae (Fig. 1).

Just a single species is known in the order Nuttalliellidae – *Nuttalliella namaqua*. Only few individuals of the species have been collected which does not allow its further investigation. Some specimens are currently deposited in museums, but due to their infection by fungi they are useless for phylogenetic analysis and thus, the phylogeny of ticks is still not completely resolved [13].

The family Argasidae comprise 186 species in several genera (*Argas*, *Antricola*, *Carios*, *Ornithodoros*, and *Otobius*). Lastly, Ixodidae is the biggest family which includes 720 species in genera *Dermacentor*, *Hyalomma*, *Ixodes*, *Rhipicephalus* (now including also the former genus *Boophilus*), and other. Ticks of the genera

Ixodes are sometimes called prostriate ticks and the other genera of the Ixodidae family metastriate ticks; this separation corresponds to some morphological differences [13].

Purely Australian presence of some tick lineages suggests the origin of ticks in Cretaceous (roughly 100 MYA) possibly together with evolution of the first birds and mammals. However, the tick phylogenetics is not resolved clearly and the result could be still absolutely different (some suggest the origin of ticks sometimes in Devonian around 350 MYA). Similarly, origin of blood-feeding in ticks is not clear. The widely accepted opinion suggests entomophagous ancestor and feeding on bodily fluids of dead organisms. In time, ticks evolved mechanisms which allowed their feeding on endothermic animals [14].

Ticks need to penetrate the host's skin to be able to feed, and they use their mouthparts to damage the blood vessels which results in bleeding. The leaking blood collects in the place of injury and the tick feeds on it (rev. in [14]). The bigger the damage, the faster the feeding. Thus, it is not surprising, that fast-feeding soft ticks cause bigger injuries, which can bleed even after detachment of the tick [15].

Ticks transmit several dangerous human and animal pathogens – they are the so called vectors of these diseases. From this point of view, the most common tick in Czech Republic is the castor bean tick, *I. ricinus*. Together with *I. persulcatus* it is one of the most common ticks in Eurasia. Other ticks of the genera are common in America – *I. scapularis* (eastern and middle part of the U.S.) and *I. pacificus* (western parts of the U.S.). Moreover, the genus *Ixodes* is spread over all continents. Other medically important tick genera are for example *Hyalomma* (dry areas of the Eurasia and Africa), *Amblyomma* (Americas), *Dermacentor* (Eurasia, Africa, Americas, New Guinea), *Rhipicephalus* (Africa and Eurasia), or *Ornithodoros* (all continents except Australia) [16].

Blood-feeding parasites represent an ideal way of transmission for pathogens infecting the host organisms. Evolution of arthropod-transmission was studied

for example in *Yersinia pestis*, where several thousand years were needed for the pathogen to acquire the necessary genetic apparatus for utilization of flea as the vector. At the same time, the pathogen increased its virulence for the hosts due to low transmission rate of *Y. pestis* by fleas or perhaps to counteract it [17].

Ticks also transmit pathogens, similarly to other blood-feeding parasites. The pathogens' life cycle is of course highly dependent on the tick's life cycle and they shuttle between the arthropods and the vertebrate hosts. Thus, the basic life-cycle for tick-borne pathogen has three stages: the primary host – the vector – the secondary (receiving) host.

The tick- (and arthropod-) borne pathogens exhibit both vector- and host-specificity. A nice example are the Lyme disease spirochetes, *B. burgdorferi* sensu lato (s.l.) – *B. afzelii*, *B. japonica*, *B. lusitaniae*, and some serotypes of *B. garinii* are associated with rodents as reservoirs, *B. valaisiana* and other serotypes of *B. garinii* are found in birds, while *B. burgdorferi* sensu stricto (s.s.) is found in both birds and rodents [18]. Regarding ticks, *Borrelia* are associated primarily with several species of the genus *Ixodes* (predominantly *I. ricinus*, *I. persulcatus*, *I. scapularis*, *I. pacificus*). Inoculation of *B. burgdorferi* s.s. into a non-vector tick *D. variabilis* resulted in clearance of spirochetes from the infected ticks showing the tick-specificity of pathogens [19, 20]. The different genospecies are also associated with different clinical outcome of the disease in humans [21].

Few or more pathogens are transmitted by certain tick species. One of the most potent pathogen-transmitters in Czech Republic and Europe is the castor bean tick, *I. ricinus*. Pathogens which are associated with this tick include bacteria such as *B. burgdorferi* s.l. or relapsing fever spirochetes (*B. recurrentis*), rickettsiae (*Rickettsia helvetica*, *R. slovaca*), *Anaplasma phagocytophilum*, ehrlichiae (*Ehrlichia phagocytophila*), and *Francisella tularensis*. Also, protozoa (*Babesia divergens*, *B. bovis*, *B. microti*) and viruses (tick-borne encephalitis virus, Eyach virus, Tribec virus, Uukuniemi virus) are transmitted by *I. ricinus*. Of the other ticks found abundantly in the Czech republic and Central Europe, *Dermacentor* ticks are the

vectors of babesiosis (*B. canis*), tularemia (*F. tularensis*), Q-fever, rickettsia (*R. slovaca*, *R. helvetica*), anaplasmosis (*A. marginale*) [21, 22].

Interaction between ticks and the transmitted pathogens has been studied both competent and non-competent ticks. Vector competence of ticks for some pathogens seems to be dependent on the innate immunity – for example defensins of the non-competent tick *D. variabilis* kill *B. burgdorferi*, while the spirochetes survive in *I. ricinus*, a competent vector [19, 20]. Protein expression patterns were compared between the infected and non-infected ticks for example in *I. ricinus* infected by *B. burgdorferi* [23]. Thioredoxin peroxidase, glutathione-S-transferase as well as defensin and other immunity related proteins were found to be up-regulated after *B. burgdorferi* infection. Other example is infection of *Rhipicephalus* ticks by several pathogens [24]. Here, several down-regulated genes were found – actin (as the effect of the remodeling of the actin cytoskeleton), enolase (changes in lipid metabolism), guanine nucleotide-binding protein and an unknown larval protein. Among others, guanine nucleotide-binding protein was found down-regulated also in *B. bovis* infected *R. microplus* tick midguts. The other up-regulated proteins were related to metabolism and translation and surprisingly, putative salivary secreted proteins were also identified in the infected tick midguts [25].

Infection related gene expression was studies also in ovaries of *R. microplus* after *Babesia* infection. Some of the findings correspond to the other studies; however, identification of hemoglobin (*Bos taurus*) or flagellin (*B. burgdorferi*) as the tick down-regulated protein raise doubts on the results of the study as the effect of co-infections was not considered [26].

2.2 Selected aspects of tick feeding

Blood-feeding is essential for survival of ticks, their molting into the next stage or egg-laying. During feeding on hosts, ticks undergoe substantial changes

in the structure and size of their tissues. Ingestion and digestion of blood during the feeding is enabled by changes in protein expression in all its tissues; changes in composition of the hemolymph influence the innate tick immunity, that has to react to potential ingestion of pathogens in the blood meal. Newly expressed biologically active molecules of salivary glands (SG) affect the success of tick feeding and pathogen transmission (saliva activated transmission) to the host as well [27-29]. Interestingly, ticks not only change the protein expression pattern in dependence on the feeding stage and life stage, but also depending on the host, as shown for susceptible versus non-susceptible hosts [29].

Generally, feeding induces expression of metabolism related genes, genes involved in detoxification and defense, protein degradation, or post-translational modification. Proteins involved in translation, cytoskeletal proteins, proteins involved in transport and storage are up-regulated. In *I. ricinus* salivary glands, a whole group of secreted and putative secreted proteins were up-regulated, including protease inhibitors, histamin-binding proteins, defensins, lectins, or metalloproteases [27]. In *D. variabilis* midgut, blood-meal digestion-associated enzymes were up-regulated, as well as peptidases, peptidase inhibitors, defense proteins, stress-associated proteins, and iron transport/storage proteins [28].

Blood-feeding itself differs between the argasid and ixodid ticks. Argasids compared to ixodids are frequent feeders. Ixodid ticks usually feed once in each of the three life stages (larvae, nymph, adult); the female feeds only once and after that it lays thousands of eggs; on the other hand, argasid nymphs feed several times (two to eight nymphal instars), the argasid female feeds several times, and after each feeding it lays hundreds of eggs. Of course, there is some variation such as in adult male feeding. For example the *Ixodes* males usually do not feed while males of the *Dermacentor* species (both are ixodids) feed quite often even though they ingest lower amounts of blood compared to females. Also, length of the feeding differs between the two tick groups – argasids usually feed for several minutes to several hours while ixodids for several hours to several days. Other

differences in feeding can be found between the two groups having an impact on the physiology of the ticks in whole.

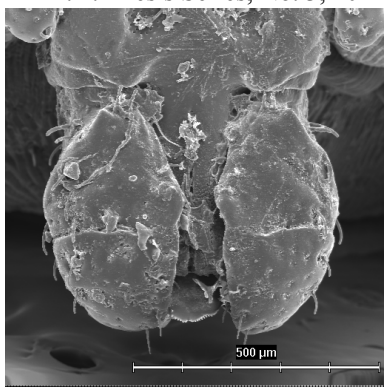

Figure 2. Capitulum of the partially fed female D. marginatus. Courtesy of Jarmila Dupejová.

On the anterior part of the tick, the tick "head", capitulum, is located (Fig. 2). It consists of the hypostome (which penetrates the skin and helps the tick to anchor in it), the chelicerae (which cut the skin), and palps (these do not enter the skin) [30, 31]. Chemo- and mechano-sensilla (sensory organs) are located on mouthparts, in Haller's organ, and on legs and help the tick to locate the "right" host and place for feeding [30]. Once in place, the chelicerae cut the epidermis and the hypostome is inserted into the wound where it keeps the tick in place. The tick SG produce cement which is both an adhesive and an anchor for the tick mouthparts in the host. The shapes and the extent of the cement cone differs among the species as does the length of mouthparts (usually, shorter mouthparts are combined with more cement and vice versa) [31]. After attachment and fixation, the tick SG start to secrete large amounts of pharmacologically active molecules and release them into the wound (several thousands secreted and putative secreted salivary proteins identified to date) [32].

2.2.1 Tick salivary glands

Salivary glands (Fig. 3, 4, and 5) are located anterolaterally in all stages of ticks. These paired organs serve important functions during the feeding (homoeostasis regulation, secretion of host defense-modulating molecules) and correspondingly undergo significant remodeling in the process. Each gland is composed of several acini attached to ducts (Fig. 3); both main salivary ducts join to form a salivarium.

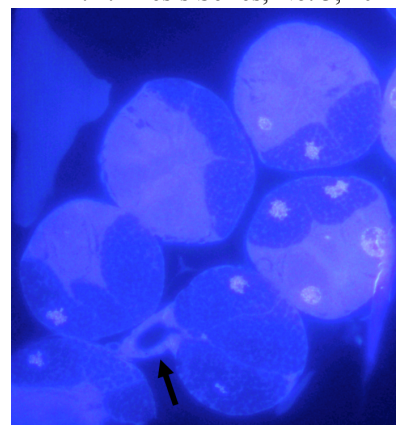

Figure 3. FM image of salivary gland acini, DAPI staining of cell nuclei. Attachment of acini to the salivary duct is highlighted (arrow). Courtesy of J. Dupejová.

Considering the presence of granula (which are believed to contain pharmacologically active molecules) inside the acini cells, the acini are distinguished as granular and agranular in both the argasid and ixodid ticks. While only one type of agranular acini (type I) is present in ixodid and argasid ticks, the number of granular acini is different. In ixodid ticks, type II and III granular acini are present in females and type IV in males; in argasid ticks, one type of granular acini is found [33, 34]. Cell types occurring in granular acini differ between tick species but depend also on the phase of the feeding. Several basic cell types were described [35, 36].

However, classification of both acini and cell types based on the presence and extent of granules and their morphology, electron density or histological staining is not a sufficient criterion as was showed by immunolocalization of SG proteins [37].

The agranular acini secrete a hygroscopic fluid and thus they are believed to be involved in water uptake [38]. In ixodid ticks, they possibly serve for storage of secreted molecules in the first phases of the feeding; later they produce pharmacologically active molecules [39].

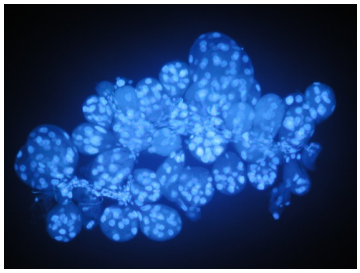

Figure 4. I. ricinus nymph salivary glands. FM image of DAPI-stained cell nuclei. Courtesy of Marie Vancová.

Cement and its proteins are also secreted by the granular cells [40]. During feeding, acini undergo changes in structure and size, some acini degenerate while new are formed [41]. Notable changes were observed for example in acini type III, where epithelium serving for fluid transport is formed and the overall shape and structure of the acinus is

changed [42, 43]. Uptake of fluid from hemolymph increases the size of type III acini and the subsequent contraction of acinal cells helps the secretion of fluid into the salivary duct [34, 43].

Ixodid males SG (Fig. 5) contain only one type of granular acini (type IV) in some ticks (such as *I. holocyclus*); in other species the exact number of granular acini is not known. Type IV acini contain only one type of granular cells [44]. Extensive salivation was observed in males during the spermatophore transfer. Thus, type IV acini have probably important functions during the mating process [45].

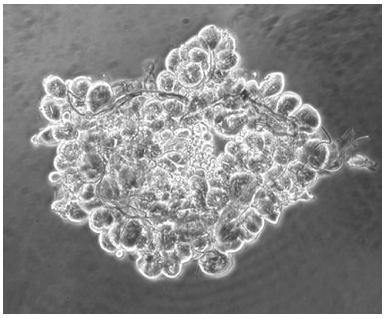

Figure 5. I. ricinus male (20x mag.) salivary glands. Light microscope images. Courtesy of M. Vancová.

Besides the physiological functions of SGs during the tick feeding, salivary glands are important also for the transfer of pathogens as they are the route of transmission and they produce the substances mediating the host's immune response [32]. Furthermore, toxic substances are secreted, causing toxicoses or allergic reaction in some individuals [46].

2.2.2 Biologically active tick salivary proteins

All hematophagous (blood-feeding) parasites have to overcome an important obstacle – the host's defense mechanisms. Penetration of the skin is only the first step in the whole process of the feeding. Damage caused to blood vessels triggers blood coagulation, aggregation of platelets and vasoconstriction. The body starts to form scars in the site of the damaged skin and the immune system (both humoral and cellular) attacks the intruder. The immune reaction is more furious in the case of previous contact of the host with the tick. Skin damage and the body healing responses trigger also itch. Thus, tick (similarly to other blood-feeding arthropods) developed a whole range of pharmacologically active

molecules counteracting all the defense mechanisms of the possible host organisms (a number of species ranging from reptiles, through birds to mammals).

Hemostasis

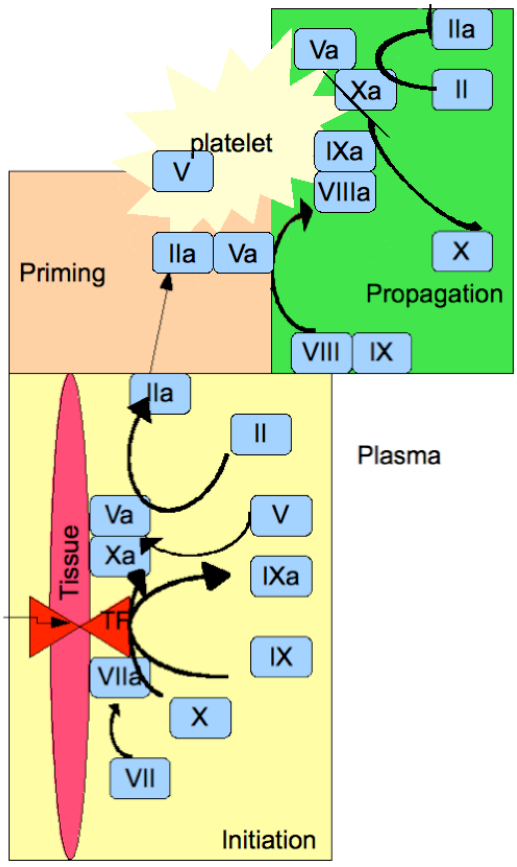

Figure 6. Blood-coagulation cascade. The initiation phase takes place in the site of injury, where cells with tissue factor on their surface are exposed to plasma. The priming and propagation phases take place on the surface of platelets. "a" next to the number of the factor means "activated". Activation of fVIII is simplified; in reality, inactive fVIII is bound to von Willebrand factor (vWf). During activation, vWf is released and fVIIIa is produced.

The vertebrate organisms reaction to any injury is immediate. First, vasoconstriction ensures, that blood flow (and blood-loss) is minimal. Injury exposes cells with surface molecules which are usually not in contact with the blood and these start two processes – blood coagulation and platelet activation/ aggregation. Furthermore, activated platelets release signals which activate other platelets, thus spreading the "information" about the injury. The clot formed by the aggregated platelets is further stabilized by fibrin fibers [47].

Regarding the blood-coagulation (Fig. 6), injury of tissues exposes cells bearing tissue factor (TF) on their surface to plasma. A quick reaction takes place, activated factor VII (fVIIa) binds to it. The complex TF-fVIIa activates factors IX and X (fIXa, fXa). fXa bound to tissue cells activates factor V (fVa) which binds to fXa and this complex starts the activation of the most important molecule – prothrombin (fII) is cleaved to make the active thrombin (fIIa) [48].

All these reactions of the initiation phase take place near the place of the injury and only limited amounts of thrombin are produced. These are not enough to make the blood clot; however the small amounts of thrombin enable the priming phase on the surface of activated platelets, where fIIa activates fV and the fIIa/fVa complex activates the factors VIII and IX in high amounts. The newly formed fVIIIa/fIXa complex then activates fX (again in much higher amounts then during the initiation phase) and the newly formed fXa/fVa complex then starts production of thrombin in huge amounts. Thrombin then activates fibrinogen and the fibrin mesh is formed (through action of the fXIIIa) [48]. Besides, glycosylation of some coagulation factors have been shown to be important for their activity and for the process [49-51].

To overcome the host defense, ticks evolved molecules affecting virtually all the steps of the cascade and the clot formation. First, vasoconstriction is inhibited by a prostacyclin-degradation product which acts as vasodilator (even though not so potent as prostacyclin itself) [52].

2.2.2.1 Tick salivary inhibitors of fX activation and of fXa action

The initial phase of blood coagulation is the target for quite a number of tick salivary proteins. Naturally, tissue factor pathway inhibitor (TFPI) is found in blood and ticks evolved its homolog. It was isolated from *I. scapularis* salivary glands and named Ixolaris. Both native and recombinant forms bind fX and fXa and inhibit the TF/fVIIa complex with IC50 of 30-420 pM. The binding of fXa occurs at its heparin-binding exosite instead of its active site (as does the TFPI). The activity of the recombinant Ixolaris was examined also *in vivo* in the rat model where it resulted in decreased thrombogenesis but did not result in bleeding. Thus, Ixolaris is a potentially safe antithrombotic agent for use in human medicine [53-55]. In the amino acid sequence of Ixolaris, three putative *N*-glycosylation sites were identified [53]. Another TFPI-homolog, Penthalaris, was

identified in the SGs of *I. scapularis*. The 35 kDa protein contains five Kunitz domains and 12 cysteine bridges. The recombinant protein maintained its activity and inhibited the activation of fXa by TF/fVIIa complex with IC50 of 100 pM. It inhibited also fIX activation in high concentrations [56].

Even if the TF/fVIIa complex activates fX, ticks can still stop the coagulation cascade. In *I. scapularis* saliva, fXa inhibitor family was found. Till now, two proteins were identified – Salp14, an immunodominant protein from fed *I. scapularis* nymphs saliva and the Salp9Pac protein [57].

The fXa inhibitors were found also in the soft ticks. Tick anticoagulant peptide (TAP), which is a serine protease inhibitor, was purified from the tick *O. moubata*. Its amino acid sequence shows similarity to Kunitz type inhibitors, and inhibits fXa action in high specificity while not inhibiting the other coagulation factors [58]. TAP was also prepared in recombinant form and experiments were performed *in vivo*. The results showed lower inhibition compared to heparin but it showed its potential for therapy [59]. However, even though the recombinant form was prepared by a private company (MERCK), the human trials were not completed [60]. Other fXa inhibitors were also identified, for example in *O. savignyi* salivary glands, where a protein similar to TAP was identified [61].

2.2.2.2 Tick salivary thrombin inhibitors

As we have seen, ticks possess molecules which can stop the coagulation in two steps of the initiation phase. This is clever, as the amount of activated coagulation factors produced in the beginning of the cascade is low and thus also the efficient amount of inhibitors could remain low. However, if anything would go wrong, ticks have a solution – thrombin inhibitors.

The first efficient thrombin inhibitor from tick saliva/salivary glands was isolated from *O. moubata*. It was named Ornithodorin and it is a highly potent and highly selective thrombin inhibitor. The protein was co-crystallized with thrombin which allowed the solution of its crystal structure. Analysis showed

that Ornithodorin consists of two domains with structures similar to Kunitz-type BPTI. Both N- and C-termini of the protein bind thrombin [62]. A similar protein was identified in *O. savignyi* and it was named Savignin [63].

Three other inhibitors were found in *B.* (*R.*) *microplus*. BmAP (*Boophilus microplus* anticoagulant protein) is a 60 kDa protein with IC50 of 100 nM to 1.1 μM and effectively inhibits thrombin but not fXa [64]. The second, Microphilin, has only 3 kDa. The isolated protein inhibited thrombin in IC50 of 5.5 μM. The protein and its function is temperature resistant and possibly binds to exosite I of thrombin [65]. Lastly, Boophilin was identified in *B. microplus*. The recombinant protein allowed its extensive study. Boophilin contains two Kunitz-type domains similarly to Ornithodorin and is not only an effective inhibitor of thrombin activity but, surprisingly, also of other serine proteases such as trypsin [66].

Further inhibitors were identified in tick of the genus *Amblyomma*. In *A. americanum* SG, thrombin inhibitor was identified and named – Americanin. The protein is a specific and competitive inhibitor of thrombin, with slow and reversible binding [67]. The naming nomenclature continued in *A. hebraeum*, as the thrombin inhibitor from this tick was named Amblin. However, this protein was not found in SG or saliva but in the hemolymph. It shows high sequence similarity to both Boophilin and Ixolaris [68]. *A. variegatum* salivary gland extract (SGE) also contained a thrombin inhibitor – Variegin. It is a very small protein as it is composed of only 32 amino acids. Even in a protein so small, separate domains responsible for thrombin binding (central region) and the kinetics of binding (N-terminus) were identified [69].

2.2.2.3 Tick salivary inhibitors of platelet aggregation and clot formation

Tick salivary proteins are targeting not only the blood coagulation but the platelet aggregation as well. It is also an important process, which takes place immediately after the blood-vessel injury. One of the molecules which activates

the platelets and starts the aggregation is adenosine diphosphate (ADP). Thus, one way how to stop the aggregation is to destroy ADP.

Apyrase activity (the enzymatic degradation of ADP) was observed in *I. dammini* saliva [70] and *O. savignyi* SGE inhibited platelet aggregation induced by several factors such as ADP, collagen, and thrombin [71]. Furthermore, the *O. savignyi* SGE was able to disaggregate the already aggregated platelets which suggests the presence of other anti-platelet-aggregation factors [71]. Also, the platelets which were disaggregated by the *O. savignyi* apyrase were able to aggregate again after addition of excess of thrombin. Two apyrase isoforms were identified in the cDNA from *O. savignyi* SGs and both seem to belong to the 5'-nucleotidase family [72].

Non-apyrase platelet aggregation-inhibitors were identified in *O. moubata* – the 6 kDa peptide Disagregin was able to inhibit platelet aggregation induced not only by ADP but also other factors (thrombin, collagen, epinephrin, platelet-activating factor). Its binding to platelets did not differ between the non-stimulated and ADP-stimulated platelets. It was purified and showed binding to both subunits of the platelet fibrinogen receptor (glycoproteins IIb and IIIa). Next, inhibition of platelet binding to fibrinogen by Disagregin was showed. In contrast to other fibrinogen antagonists the protein did not contain the Arg-Gly-Asp (RGD) motif [73]. Similar protein was identified in *O. savignyi* and the name Savignygrin was chosen. Similarly to Disagregin, it inhibits platelet aggregation induced by different molecules. The inhibition was efficient even in case of successful platelet activation. The protein contains the RGD motif [74].

Another platelet aggregation inhibitor was found in *D. variabilis* and called Variabilin. Its binding specificity was studied and the fibrinogen receptor glycoprotein IIb-IIIa was identified as its target as in the case of Disagregin and Savignygrin. Similarly to Savignygrin, it contains the RGD motif [75].

Tick saliva contain collagen-induced platelet aggregation inhibitor as well. *O. moubata* saliva contain Moubatin, which is a 17 kDa inhibitor of this process.

Moubatin does not inhibit the aggregation induced by other factors (including thrombin). However, it does not inhibit the binding of platelets to collagen [76]. An enzyme responsible for blood clot disintegration was identified in *I. scapularis*, where a salivary metalloprotease showed proteolytic activity towards fibrin, fibrinogen, gelatin, and fibronectin but not towards collagen and laminin [77].

2.2.2.4 Other biologically active molecules in tick saliva

Vertebrate hosts demonstrate yet other defense mechanisms. For example, pain or edema in the place of injury. One of the mediators for these reactions is bradykinin [78]; *I. scapularis* saliva contains the enzyme kininase, which destroys bradykinin, thus shutting down this type of the host's defense [79]. Ixodid tick saliva contain also other secreted proteins affecting the host's immune system such as inhibitors of neutrophil activation, inhibitors of the alternative complement pathway (such as Salp20), immunomodulators affecting NK cells function, inhibitors of T lymphocytes proliferation, or IL-2 binding proteins [80-84]. As an example, potential *N*-glycosylation sites were identified also in these proteins as was shown for Salp20 [81]. Inflammation mediated by tumor necrosis factor α (TNFα) is inhibited by both saliva and SGE from *I. ricinus* [85]. Antibody response is also counteracted by immunoglobulin-binding proteins for example in *R. appendiculatus* ticks [86, 87].

A whole family of anti-complement proteins was identified in *I. scapularis* SG cDNA library – Isac (*I. scapularis* anticomplement) and its family. Subsequently, its homologs were found also in *I. ricinus* and *I. pacificus*. Isac proteins interact with C3 convertase of the classical and alternative pathway [82, 88].

Studies of tick salivary transciptomes revealed much information; some new protein families were identified with no known function so far [27, 88] but we can still await the discovery of new potentially useful molecules.

Not all the biologically/pharmacologically active molecules from tick saliva and salivary glands were mentioned here and papers with information on new

molecules continue to be published. Several reviews tried to complete the information on tick (and arthropod) salivary pharmacy, see for example [32, 60] .

2.3 Tick circulatory system

The tick hemolymph serves some similar functions to vertebrate blood – it surrounds the internal organs, functions as water reservoir, and helps to maintain the osmotic pressure, transports the nutrients to all body parts, transports hormones, inorganic molecules, and of course, the metabolic products as well. It also contains molecules that fight the invading pathogens and heal injuries. The defense reactions of hemolymph components are helped by hemocytes. Nowadays, the researchers concentrate on identification of molecules with potential practical application. Therefore, growing amount of information is coming on antimicrobial peptides, tick immune system, or transport and storage proteins.

Even scientists working with ticks do not realize, that ticks have heart; or aorta; or arterial vessels. The tick heart was described as a sac-like structure, composed of a layer of muscle tissue. The suspensory muscles of the heart were found to be connected with nerve endings and thus nerve-innervation of heart contraction was suggested. Hemolymph flow and pressure (regulated partly by the heart) allows for example movement of chelicerae and hypostome [89].

2.3.1 Hemocytes

Considerable part of the tick (and arthropod) immune system is connected with hemocytes. Hemocyte (cell) content of hemolymph is subject to changes in response to life stage, feeding, and especially pathogen invasion. In uninfected ticks, the hemocyte count is around 10^3 cells/μl hemolymph [19, 90]. After inoculation of bacteria into ticks, the number of hemocytes increases and they migrate towards the pathogens [91]. The response is remarkable; infection of *D.*

variabilis with *Bacillus subtilis* resulted in more than 6-fold increase in hemocyte count in 2 days [92]. Infection with *B. burgdorferi* resulted in yet quicker response – more than 3-fold increase in hemocyte count in only one hour. Slow decrease in hemocyte counts followed [19]. Also, *Escherichia coli* were rapidly cleared of the ticks (within 6 hours) and the *E. coli*-"immunized" hemolymph exhibited borreliocidal activity [20]. Differences were observed in reaction to infection by *B. burgdorferi* of the competent vector *I. scapularis* and the non-competent tick *D. variabilis*. While borrelia freely circulated in *I. scapularis* and some were associated with hemocytes, in *D. variabilis* only remnants of borrelia were found and *Dermacentor* hemolymph itself was able to kill the bacteria [20].

The mode of action of hemocytes includes production of antimicrobial peptides/proteins, phagocytosis, encapsulation [93], and nodulation [90]. A number of antimicrobial peptides was identified in ticks; however, not all of them were localized and identified in hemocytes. Tick hemocytes produce mainly two families of antimicrobial peptides, defensins and cystatins, usually several different molecules in each tick species. Cystatins were identified for example in hemocytes of the ticks *O. moubata* or *H. longicornis* [94, 95]. One and more defensins were identified in hemocytes of the ticks *B. microplus*, *D. variabilis*, or *I. scapularis* [96-98]. In *D. variabilis*, storage of defensins in hemocytes' granules was suggested as was their secretion after bacterial infection [97].

The primary phagocytic cells are plasmatocytes [99]. Opsonization by blood-meal components enhances the phagocytosis [100]. The ability of tick hemocytes to neutralize the invading pathogen together with the pathogen-recognizing molecules and production of antimicrobial peptides influence the vector competency of tick species for different pathogens [101].

Phagocytosis of borrelia by ixodid hemocytes has two mechanisms – except the conventional also the so-called coiling phagocytosis was observed [102, 103]. On the other hand, there is no cellular reaction towards the intracellular parasite

R. ricketsii. This bacteria are engulfed, but not destroyed by plasmatocytes, and these then serve as a route of dissemination for the bacteria [104].

Lysosomal compartments were found in plasmatocytes and in lower extent also in granulocytes of both type I and II (corresponding to phagocytosis primarily by plasmatocytes) [100]. Up-regulation of lysozyme expression was also shown in hemocytes after bacteria infection in *D. variabilis* and *H. longicornis* [105, 106] but not after blood-meal itself in *O. moubata* [95]. Other ways of their destruction is the production of reactive oxygen species as in *B. microplus* hemocytes [107].

Recognition of bacteria triggers other cellular defense mechanisms of arthropods, as well. Plasmatocytes cluster around the pathogens and the bacteria-hemocyte aggregates adhere to different organs in hemocel; the process is called nodulation [90]. Nodulation serves as a very quick way for clearing of bacteria from hemolymph in arthropods (up to 90%) [108].

2.3.2 Hemolymph

Hemolymph is composed of plasma and circulating cells – hemocytes. Tick hemolymph represents approximately 10-25% of the whole tick body (fed or unfed) but in some ticks it can reach as much as 37.5% of the tick body which corresponds to 3-15 μl volume [109]. The variation of hemolymph proportion in the body is limited also during the blood-feeding. It contains relatively high concentrations of proteins, lipids, carbohydrates, amino acids, and other organic and inorganic molecules [110]. Its composition is readily altered during the feeding regarding all the components but also in response to pathogen invasion [23, 92]. Similarly, the osmotic pressure of tick hemolymph decreases during the feeding [109].

Sugars are present in hemolymph not only as the source of energy (such as glucose or mannose) but also as cryoprotectants against injuries caused by both low and high temperatures as was shown for glycerol and sorbitol [111]. Protective

function can be attributed also to antioxidants in the hemolymph which counteract the oxidative stress molecules originating from the metabolism and the presence of high amounts of heme in the tick [112]. Heme-related damage is further limited by the presence of heme-binding proteins; thus, free heme is not detected in tick hemolymph [113].

As mentioned earlier, hemolymph clotting was also observed as response to injuries or pathogen infection. One of the mechanisms can include granulocyte degranulation [93]. Several potential clotting factors have been already described in ticks [114].

Tick hemolymph proteins

Plasma/hemolymph proteins represent a considerable part of tick hemolymph. The most abundant are the proteins related to vitellogenesis (vitellins and vitellogenins) and heme-binding or heme-storage proteins (HeLps), also called carrier proteins (CPs) [115-117]. Important are also tick antimicrobial proteins including defensins (Chapter 2.3.1), lectins (Chapter 2.4.3), or proteins of host origin [118]. However, knowledge on the tick hemolymph proteins is still very limited [118]. Some of the proteins are described below.

Vitellogenins (Vgs) are the precursors of vitellins (Vns), the main yolk proteins. At least some of the ticks rely on the blood as the solely source of heme (which is indispensable for the eukaryotic metabolism) [119] and heme-storage proteins are important for the tick survival and yet more for the egg-production. The major egg proteins, Vns, thus have exactly this function – they bind and store heme for utilization in the tick embryo [120]. Still, this question is not solved as evidence for some heme-producing pathway was found in *D. andersonii* [113]. Vgs are very large proteins (several hundreds kDa), present in tick genomes in two copies, composed of several subunits; the exact number of subunits differs between ticks [121]. Vgs are produced by the fat body, the gut, and ovaries and are released to hemolymph, where they bind heme and are ingested by oocytes as

Vns [121]. Vgs contain conserved domains – the N-terminal lipoprotein domain and the C-terminal vWf domain as well as two conserved motifs – the GLCG motif and one or more RXXR convertase cleavage sites [122].

Independently of feeding and egg-production, other heme-binding proteins were identified in tick hemolymph and named carrier proteins (CPs), hemelipoproteins, or hemelipoglycoproteins. Through heme-binding, they are involved in protection against heme-induced oxidative damage. They show sequence similarities to Vgs and contain the lipoprotein domain, the vWf domain, as well as the RXXR and GLCG motif. The best studied are these proteins in *D. variabilis* [116, 123] and *R. microplus* [117].

Similarly to Vgs, CPs are very large proteins with molecular weight of more than 200 kDa. First biochemically characterized CP was the R. *microplus* Hemelipoprotein (HeLp, or RmCp – *Rhipicephalus microplus* carrier protein). The 354 kDa is composed of two subunits with molecular weights of 92 and 103 kDa. The molecule contained carbohydrates (3%, predominantly mannose) and lipids (33%) – thus the term hemelipoglycoprotein was introduced. In addition, the protein was present in hemolymph of unfed males and females [117].

Another CP was characterized in *D. variabilis* tick (DvCP – *Dermacentor variabilis* carrier protein). Even though it has high sequence similarity to HeLp, its size is smaller – only 210 kDa. Again, two subunits form the native protein and have molecular weights of 92 and 98 kDa. Its molecule also contains carbohydrates and lipids and its expression was confirmed in fat body, SGs, ovaria, muscle tissues, and hemolymph. Higher expression was observed in partially fed females [116, 123, 124]. Hemelipoglycoprotein was found and characterized also in *O. parkeri*. Its size was even bigger – 668 kDa and it contained three subunits with molecular weights of 48, 93, and 114 kDa. In *O. parkeri*, the protein was not found in fat body and salivary glands and its heme-binding capacity seems to be limited [116, 124].

Major proteins with N-terminal sequence similar to carrier proteins from *R. appendiculatus* and *D. variabilis* were found also in saliva of *A. americanum* and *A. maculatum* [125] and indirect results suggest their presence also in the cement cone of the ticks *R. appendiculatus* and *D. variabilis* [126, 127]. In *D. variabilis*, the expression of CPs was shown in different tissues such as hemolymph, hemocytes, and salivary glands (SGs). Furthermore, the production of the proteins was shown also in extracted SGs when they were incubated in organ cultures [128].

A number of host proteins has been identified in tick hemolymph – such as immunoglobulins [129], hemoglobin, histones, tubulin, and actin [130]. In the case of immunoglobulins, they persist in the tick hemolymph for several months after the blood meal [130] and through molting into the next stage [131] with specific mechanisms for their uptake [132]. Species specific uptake of some of the host proteins was reported in *A. americanum* and *I. scapularis* [130]. Furthermore, the host-derived immunoglobulins remained active in the tick [132].

Only some of the other tick hemolymph proteins were identified. An example is the tick α_2-macroglobulin (TAM) from *O. moubata* [133] or *I. ricinus* [134] which are glycoproteins similar to α_2-macroglobulin from the horseshoe crab *Limulus polyphemus* and seems to be involved in antibacterial defense [135].

2.4 Glycosylation in ticks and tick-borne pathogens

2.4.1 About protein glycosylation in general

Posttranslational modification (PTM) of proteins confers a huge extending of the possibilities provided by the information encoding and transfer according to central dogma (DNA-RNA-protein). PTMs allow further broadening of structural and structure-derived functional aspects of the proteins.

In the case of proteins, we recognize several of different PTMs, and the usage and the extent of the modification depends on the cell cycle, cell type,

tissue, and organism. Glycosylation is only one of them. Five types of protein glycosylation are recognized, with the *N*- and *O*-type as the two main types.

Glycosylation and its importance are known for about half a century and for a long time only eukaryotic glycosylation was believed to be possible. However, recent studies conducted on prokaryotes revealed not only the ability of these organisms to glycosylate proteins, but also new types of monosaccharide units used in the process and new types of sugar attachment to the protein backbone. This brings questions on glycosylation evolution – is the glycosylation as old as the life itself? Did bacteria and our ancestors incorporate a few sugars first which remained unchanged from that time in their successors (Eukaryotes) and later on, bacteria evolved utilization of new sugars over the ages? Some answers can be obtained from the sequences of the enzymes involved in *N*-glycosylation, which is the best studied for now, especially the enzyme, responsible for the transfer of the *N*-glycan precursor to the protein – STT3 protein and its homologs. STT3 is highly conserved throughout all organisms – bacteria, archaea, lower and higher eukaryotes (Chapter 2.4.1.1). This suggests one common ancestor which was able to perform such process. Later on, higher eukaryotes evolved two isoforms of the protein; one of the isoforms is similar to the proteins from the amoeba *Dictyostelium*, the other to the fungal and nematode enzymes [136].

Glycosylation of proteins confers to a number of functions in the cell and in the organisms; for some reviews see [137-139]. The eukaryotic glycans and glycoproteins and their role are quite well known and the amount of information is ever growing. Bacterial glycans are studied as well, mostly of the pathogenic bacteria. The roles of glycans are described below and summarized in Table 1.

Glycosylation of proteins takes place co-translationally (*N*-glycans) and post-translationally (*O*-glycans and *N*-glycans processing). Its effects show immediately in the endoplasmic reticulum (ER). The presence of the glycan greatly affects the folding of proteins as was shown in many cases. One of the most studied glycoproteins is Ribonuclease B (RNase B, which is a glycosylated form of RNase

A). Extent of its glycosylation greatly affected the formation of dimers by its N- or C-termini [140]. The correct protein folding was dependent on the extent and type of glycans also in the case of the HIV surface protein gp120 [141]. Human proteins are also affected by the presence/absence of glycosylation as shown in the case of human phospholipid transfer protein. Mutation of each of its six *N*-glycosylation sites resulted in lowered production and secretion of the protein. The protein mutated in all six positions was not detected at all [142]. *In silico* modeling confirmed the importance of glycosylation for protein folding but showed also another conclusion – for some proteins, the glycosylation site is more important than the extent of glycosylation itself. Studies on the mechanism of protein folding showed that glycosylation rather destabilizes the unfolded state than stabilizes the folded state – the newly formed glycoprotein has thus higher chance to find the right conformation and stay in it [143]. In this context, it is important to note, that glycosylation precedes the protein folding [144].

Table 1. Some examples of glycans functions in different organisms. Only the basic N- and O-type glycans are mentioned.

function	glycosylation type	organism
protein folding	*N*	eukaryotes, bacteria, viruses
protein stability, degradation	*N*	eukaryotes
protein translocation	*N, O*	eukaryotes, bacteria, viruses
cell adhesion	*N*	eukaryotes, bacteria
protein activity	*N, O*	eukaryotes, bacteria, viruses
cancer proliferation	*N, O*	eukaryotes
immune reactions	*N, O*	eukaryotes
pathogen binding	*N, O*	eukaryotes, bacteria, viruses

Thus, glycosylation is directly involved in the correct protein folding. But glycans have yet other functions; glycans participate also in the folding quality control performed by the lectins calnexin and calreticulin system in the endoplasmic reticulum (ER). These lectins recognize the *N*-glycosylated proteins in the ER, or more specifically, the five terminal saccharides of the donor glycan (Chapter 3.4.1.1) [145]. Through interaction with both the glycan and the protein part of the glycoprotein they control the folding – the unfolded proteins are then either transported to cytosol and degraded or they are partially deglycosylated, re-glycosylated, and again checked for the correct folding [146]. Calnexin/ calreticulin system is associated also with thiol oxidoreductase and thus the glycosylation is involved in disulfide bond formation in glycoproteins [147].

Glycosylation also affects the activity of proteins as was shown for some insect antimicrobial peptides. *O*-glycosylation either increased or decreased their activity and the presence of glycosylation affected the stability of these peptides in insect hemolymph (increased) versus mammalian serum (decreased) [148]. Furthermore, human interleukin IL-1β, nonglycosylated under native conditions, expressed in yeast was produced as both nonglycosylated and glycosylated. The glycosylated protein showed lower activity compared to the native interleukin [149]. Incomplete glycan processing and the subsequent incorrect folding may also result in lower activity of enzymes – in melanocytes, unnatural glycosylation of tyrosinase near the active site did not affect the transport of the protein to melanosomes but did result in lower ability of the enzyme to bind copper and to loss of its activity [150]. Not only activity of proteins, but also their chemical stability and resistance against temperature and chemical denaturation is conferred by glycans attached to the proteins. This was shown in the case of ribonuclease A (RNase A) which is not glycosylated when compared to RNase B, which is *N*-glycosylated. RNase B undergoes slower degradation in the body compared to RNase A [151]. Similar results were shown for N-terminal glycosylation of peptides, which resulted in longer survival of the glycosylated

peptides in human serum [152]. A specific glycan type, containing fucose α1,6-linked to the proximal saccharide of the *N*-glycan increases the survival of the proteins in the serum. The mechanism is mediated through the reduced flexibility of the glycan [153].

Protein trafficking in the cell is usually dependent on the so-called signal peptides. Thus, we now know for example the signals for translocation of proteins to Golgi or to mitochondria. Similarly, glycosylation can be used as the signal – for example in higher eukaryotes, phosphorylated mannose in the *N*-linked high-mannose glycans serves as the signal for transport of hydrolases into lysosomes [154]. Phosphorylation of *N*-glycans in human procathepsin L (lysosomal protease) serves also the role of signal for translocation to lysosomes [155]. Glycosylation of human organic anion transporter hOAT4 is essential for its plasma membrane insertion. The presence of complex glycans instead of high-mannose type glycans greatly increases its interaction with the substrate molecules [156].

Once the glycoproteins are correctly folded and in place, they can exert their functions. Except the cell adhesion and migration mediated by a whole group of the so called matricellular proteins [157], glycan-protein interactions were shown for example in the recognition of sperms by egg receptors [158] or cancer invasion and metastasis [159]. Glycans mediate the interaction between the cell surface recognition molecules and among others allow the formation of molecular interaction between neural cells. Therefore, these interactions can lie behind the flawless development of the nervous system with all its complexities [160].

As mentioned, lower organisms, including bacteria and archaea are also capable of glycosylation, and they have also their own recognition mechanisms for saccharides. It is therefore not surprising, that bacterial (and viral) pathogens use them for their own benefit. The interaction between the pathogen and the host's immune system is reciprocal as seen in the so-called lectinophagocytosis. Even though we hear everywhere about the ability of bacteria to rapidly change

their genomes and thus be always a step before the infected organisms, here is an example of the ability of the host (for example us, humans) to fight bacteria back. Bacteria and higher organisms differ in the type of glycans (more about it in Chapters 2.4.1.1 and 2.4.1.2), thus it is just obvious that phagocytes have specific lectins (sugar-specific proteins which are not immunoglobulins) on their surface which recognize bacterial glycans. Furthermore, bacteria have their own lectins, which recognize the host's glycans and which usually help them in host colonization. In the case of interaction with phagocytes, this can be really harmful for these bacteria – once the bacteria binds to the phagocyte, it is recognized as the evil and immediately destroyed. Both the binding mediated by the phagocyte's or bacterial lectins lead to the same result – destruction of the bacteria by lectinophagocytosis [161].

However, not all the host cells are phagocytes; therefore bacterial lectins play an important role in recognition of the suitable cell, which the bacteria can infect/utilize. One of the examples are the enteric bacteria binding to the gut, most profound being *Helicobacter pylori* recognition of Le^b antigens (at neutral pH) or sialylated glycans (at acidic pH occurring in the gut) [162, 163]. For some bacteria able to recognize sialic acids (a group of acidic saccharides), lectins specific for different conformations or different sialic acids were identified. The existence of these lectins partly explains the bacterial tropism towards different hosts (as different hosts produce different sialic acids) and the resistance/susceptibility of hosts to bacterial infection [164].

Protein-carbohydrate interactions involving bacteria serve also good (from the point of view of us, humans) – they allow the symbiosis of nitrogen-fixing bacteria with plants. A specific plant lectin as well as the corresponding bacterial polysaccharide are both required for the successful infection, or in this case symbiosis with the host [165, 166].

Application of the scientific knowledge is very important, and therefore glycobiologists apply the new results from glycobiology, mainly in two areas –

production of recombinant glycoproteins and fighting infections. As an example, the already mentioned *H. pylori* adhesion and infection can be inhibited by intake of specific carbohydrates in either free form or as glycoproteins [167].

Production of recombinant proteins is facing problems such as protein degradation, misfolding, inclusion body formation etc. [168, 169]. Activity of such proteins and their immunogenicity (as the bacteria- or yeast-produced proteins are modified by non-human glycans) is also the problem [170]. Some recombinant proteins such as the recombinant rabies virus glycoprotein are secreted only if they are glycosylated [171]. Also other recombinant proteins production in yeast was enhanced by the introduction of *N*-glycosylation sites. This effect was enhanced if the glycosylation site was introduced on the N-terminal side of hydrophobic regions (which otherwise cause protein aggregation) [172]. Activity of hormones or proteins for human disease treatment is affected by the presence of glycosylation. As an example, the glycosylated form of the recombinant human granulocyte colony-stimulating factor had markedly higher activity *in vivo* when compared to its non-glycosylated form [173]. However, glycosylation of antibodies does not seem to have significant effect on the pharmakokinetics [174]. Recombinant hormones, survive in circulation for prolonged time if glycosylated, too [175].

Some features of the two most important and most abundant types of glycosylation are listed below.

2.4.1.1 N-glycosylation

Asn-linked or *N*-glycosylation confers attachment of different oligosaccharides to the protein. All *N*-glycans share common (trimannosyl) core, composed of the proximal GlcNAc, attached to the protein, one more GlcNAc and three mannoses (see below). This core is further modified and three basic *N*-glycan types are thus recognized:

– high-mannose (or oligomannose) type, which contains only mannose residues attached to the trimannosyl core (Fig. 7a, b),

– complex type which does not contain any other mannose residue except the trimannosyl core; two, three or more saccharide branches (antennae) are attached to these mannoses (biantennary to pentaantennary glycans) (Fig. 7d, e, f, g),

– hybrid type which contains one or more mannose residues attached to the core as well as one or more antennae (Fig. 7c).

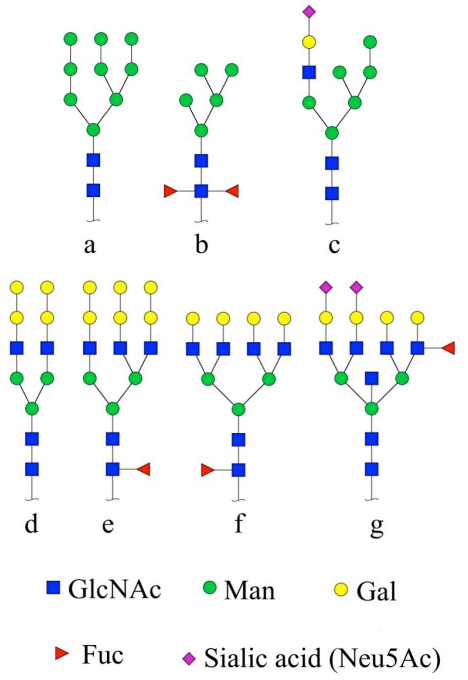

Figure 7. Examples of N-glycans. a, b - high-mannose type, c - hybrid type, d, e, f, g - complex type. e, f - mono- and b - difucosylated glycans. Saccharide symbols and colours according the Consortium for Functional Glycomics (CFG) nomenclature ([176], http://www.functionalglycomics.org/static/consortium/Nomenclature.shtml. This nomenclature will be used further on in the text.

Some other specific structures are also recognized – core-fucosylated (fucose is attached to the proximal GlcNAc by either the α1,3- or α1,6- bond; Figure 8b, e, f) or poly-*N*-acetyllactosamine glycans (a branch of repeating Galβ1,4-GlcNAcβ1,3 units are attached to the core mannose) [138].

N-glycosylation signal sequences

The enzymatic machinery responsible for the attachment of *N*-glycans to the proteins recognizes a specific amino acid triplet, called sequon. The consensus sequence is Asn-Xxx-Ser/Thr, where Xxx is any amino acid except for proline; asparagine is glycosylated in this sequon [177, 178]. This consensus sequence is used by a wide variety of organisms from bacteria and archaea to Eukaryotes [179]. In *Campylobacter jejuni*, the three-amino acid sequon could not fully

describe the glycosylation site and it was broadened to five-amino acid sequence Asp/Gly-Yyy-Asn-Xxx-Ser/Thr [180]. In the bacterium *Halobacterium halobium*, another difference in the consensus sequence arose; one of its proteins contains the N-terminal sequence Ala-Asn-Ala-Ser with the Asn-2 glycosylated; the possibility of the presence of two different oligosaccharyltransferases in this archaebacterium was suggested [181]. Furthermore, six human proteins are *N*-glycosylated in an atypical sequon Asn-Xxx-Cys, where, again, Xxx cannot be proline [182-188].

The eukaryotic glycosylation signal sequence seems to be more complicated as well. Regarding the middle, Xxx amino acid, the presence of proline totally inhibits the modification. In the case of tryptophan in this position, glycosylation was also not observed or only in very low ratio (5%) [189]. Other amino acids do not contribute to the successful glycosylation either; aspartic or glutamic acids (20%) or leucine (40%) in the middle position still do not assure modification of the sequon by an *N*-glycan. On the other hand, serine in the middle of the sequon ensured glycosylation of almost all sequons. The presence of the other amino acids leads to approximately glycosylation degree of more than 70% [189]. In archaea, valine was shown to be the middle amino acid ensuring the highest glycosylation ratio [190].

This seems to be complicated enough, but it is still not all. Proline located on the C-terminus of the sequon after threonine shows also total inhibition of glycosylation. In this case as well as in the case of proline in the middle of the sequon, hindering of the loop-formation by these prolines is believed to be the reason [177]. Except proline, methionine was found to be amino acid preceding the sequon which hinders the glycosylation process and methionine together with tryptophan and arginine are the least present amino acids preceding the *N*-glycosylation sequon Asn-Ser-Thr in the non-redundant protein databases [191]. Overall, eukaryotic sequons are preceded by aromatic amino acids in the -1 and -2 positions and Asp and Glu are disfavored. Prokaryotes prefer the presence of

hydrophobic residues, Asn or Phe in position -1 and Asp and Gly in -2 position [192]. In proteins with known structures, hydrophobic amino acids are found nearby the actually glycosylated sequons on both sides (-6 to +4). Aromatic residues are found usually before the sequon, in the +1 position usually small hydrophobic and in the position +3 larger hydrophilic amino acids. Also, aromatic and hydroxyl amino acids are found nearby the glycosylated sites in the glycoproteins structure [193].

Studies on the mechanisms lying behind the *N*-glycan transfer revealed that formation of a loop structure by the glycosylation sequence Asn-Xxx-Ser/Thr is essential for the reaction performed by oligosaccharyltransferases. The reason is the formation of a hydrogen-bond interaction between the asparagine and the hydroxyl amino acid (Ser, Thr), which is required for the catalysis [144, 194]. Even though the theoretical sequon contains both Ser and Thr as the third amino acid, also here, the reality is a little different. Majority (70%) of actually glycosylated sites in proteins with known structures contains threonine in the third position. On the other hand, the unoccupied glycosylation sites contain half-to-half serine and threonine as the third amino acid [193].

In agreement with these findings, evolution of *N*-glycosylation sites in the HIV surface glycoprotein gp120 was observed. While potential *N*-glycosylation sites in the transmembrane regions evolved towards sequences containing Ser as the third amino acid, the outer domain of the protein (which is heavily glycosylated and mediates the interaction with the host cells) over the years contained more sequences with threonine and thus the virus has evolved in the way, which ensures the higher glycosylation ratio for the outer domain [195].

Regarding the localization of glycosylation sites in proteins, most of the actually glycosylated sequons lies in the middle parts of proteins and the glycans in the terminal parts are more likely to be present in the N-terminus of the protein, compared to the C-terminus [195]. The negative effect of sequon localization near either of the termini was further confirmed [149] and increasing

glycosylation rate correlation with increasing distance of the glycosylation site from the C-terminus was also shown [191].

Initial steps in N-glycosylation enzymatic pathways

Enzymatic pathways involved in *N*-glycosylation of eukaryotic proteins will be described more in depth.

All the processes linked to transfer of the oligosaccharide to the Asn in the *N*-glycosylation sequon are related to ER (in eukaryotes). In contrast to *O*-glycosylation, *N*-glycans are not built up on the protein; rather, the whole oligosaccharide is attached to the protein. This oligosaccharide is formed first on the cytoplasmic side of the ER and throughout the whole process it is attached to a lipid carrier named dolichol (or to be chemically correct, dolicholpyrophosphate, Dol-PP) [137-139]. Two GlcNAcs are attached to Dol-PP and next five mannoses are added – thus, the *N*-glycan core is formed (Fig. 8). The heptasaccharide attached to Dol-PP is then flipped to the inner side of the ER membrane. Here, the synthesis of the Dol-PP attached precursor is completed and the final structure is a tetradecasaccharide containing two GlcNAcs, nine mannoses, and three glucoses (Fig. 9). In Prokaryotes, dolichol-type (polyisoprenyl phosphate) lipid from the cell membrane was suggested to be the anchor, which takes place in the cytoplasm [196].

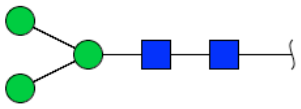

Figure 8. The N-glycan core - $GlcNAc_2Man_3$.

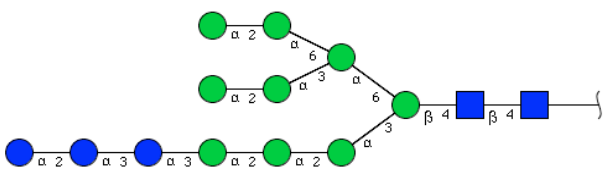

Figure 9. Tetradecasaccharide attached to the protein by the OST. Linkage for each saccharide is depicted. The three terminal branches are named A -, B-, and C-branch, starting at bottom.

This precursor is the oligosaccharide, which is attached to the protein. The reaction is catalyzed by the oligosaccharyltransferase (OST) which is a complex of

several subunits in higher eukaryotes but has only one component in lower eukaryotes and bacteria – the STT3 protein in lower eukaryotes and yeast, PglB in the bacterium *Campylobacter*, and AglB in the archaebacterium *Pyrococcus* [136, 196, 197]. In eukaryotes, two isoforms of the STT3 protein are present (STT3A, STT3B), both of them participating in the complex formation and enzymatic reaction. The purified mammalian OST complex (composed of the STT3 and ribophorin I) is able to recognize even hexapeptides or tripeptides. The catalyzed reaction is dependent on the presence of Mn^{2+} cations. The recognition of the substrate and the subsequent reaction is also dependent on the terminal saccharides of the donor oligosaccharide structure [198]. Tissue-specific expression of the isoforms was shown with different activities; thus, the existence of the STT3 isoforms can be related to regulation of protein glycosylation [199].

STT3 in all organisms (including eukaryotes, bacteria, and archaea) is highly conserved especially in its catalytic domain (the middle part of the enzyme) suggesting development of protein glycosylation early in the evolution of life on Earth. However, while the enzymes are similar, differences arise when comparing the glycan which is attached to proteins by STT3. The glycan HexNAc-(Pent)-HexA-(Pent)-Hex-Hex-HexNAc is used in Archae *Pyrococcus furiosus* [190], as well as other glycans. Archaeal systems utilize several proximal sugars (the ones, which are attached to the protein) – except the GlcNAc (similarly to Eukaryotes) they use also GalNAc and hexoses such as Glc [200-202].

Bacterial STT3 such as the one in *Campylobacter* use the glycan GalNAc-α1,4-GalNAc-α1,4-(Glc-β1,3)-GalNAc-α1,4-GalNAc-α1,4-GalNAc-α1,3-Bac with the bacillosamine (Bac) as the proximal sugar moiety [203].

On the other hand, eukaryotic glycans contain sole proximal sugar – GlcNAc, which was probably common for the shared ancestor. Still, we find differences in the STT3-attached sugar coming with the further evolution. Generally, Eukaryota use GlcNAc2Man9Glc3 as the glycan precursor, which is true for representatives of Animalia, Plantae, Amoeboza, Fungi [204], as well as

Chromalveolata such as the pathogenic protist *Phytophthora infestans* (based on the KEGG database) [205]. Differences arise when representatives of the kingdom Excavata come in the light. *Tetrahymena* use the GlcNAc2Man5Glc3 as the precursor, *Trypanosoma* and *Leishmania* glycans without the terminal glucose residues (GlcNAc2Man9 and GlcNAc2Man6, respectively), while *Giardia* utilize a residual GlcNAc2 glycan [204, 206-209]. For Rhizaria, such information is not available. Secondary loss of genes encoding the glycosyltransferases participating in the glycan precursor synthesis was suggested in the Excavata (but also in Fungi) during evolution [206].

For a long time, STT3 was believed to contain one conserved/catalytic motif – the widely used WWDYG motif. Later, another motif was found – the DXXK motif, which is localized in proximity to the WWDYG in the native protein and was shown to be essential for catalytic activity of the yeast OST [210]. However, studies of the active sites of a number of OSTs from different organisms revealed a much more complicated picture. The DXXK motif was further expanded and based on its presence in different Archaea three motifs were distinguished: the DK motif (D-X-X-K-X-X-X-M/I), the DM motif (D-X-X-M-X-X-X-K/I), and the MI motif (M-X-X-K/I-X-X-X-W or M-X-X-I-X-X-X-I/V/W). Furthermore, third motif, complementing the other two in the catalytic site was revealed (D/E/X-X-D). Considering the combination of the three motifs, three different catalytic sites are distinguished and named A-, B-, and E-type. The first, A-type is present only in Archaea (W-W-D-Y-G + DM motif + X-X-D), the B-type in Archaea and bacteria (W-W-D-Y/W/N/F-G + MI motif + X-X-D), and the last E-type in Archaea and eukaryotes (W-W-D-F/W-G + DK motif + E/D-X-D). More interestingly, in organisms containing more than one isoform of the STT3 protein, these isoforms belong to the same catalytic domain type [211].

Trimming and elongation reactions

Thus, as the first step of *N*-glycosylation, the tetradecasaccharide is attached to the protein (in Eukaryotes). Without any other reactions, this could not result in the wide variety of structures which we observe. Further reactions are required – trimming reactions (cleaving off the monosaccharides) and elongation/ branching reactions. The first trimming reactions are part of the quality control process in the ER employed by calnexin/calreticulin system. The terminal α1,2-glucose (of the so-called A-branch; Fig. 9) is cleaved off almost immediately after the transfer of the glycan precursor to the protein by ER glucosidase I. Further, ER glucosidase II cleaves off the second glucose and the glycoprotein is checked for the right folding. If the folding is right, the last glucose is also cleaved off. If not, the protein is re-glucosylated, allowed to refold, and checked again. The ER-residing α1,2-mannosidase cleaves the terminal mannose in the middle B-branch and the protein is transferred to the Golgi apparatus (GA) [153]. In GA, the three remaining α1,2-mannoses in the A- and C-branches are cleaved off. Here starts the actual production of diverse structures which we observe. The GA-based glycosyltransferases are responsible for these processes; interestingly, some of them modify both *N*- and *O*-glycans [153].

Another mechanism of deglucosylation of the precursor glycan was shown by Zuber and colleagues [212] – instead of removal of the last glucose residue by the ER glucosidase II, the glycoprotein could be processed by an endomannosidase in the GA. This could mean the possibility of another quality control check in GA.

The initial step in formation of complex (Fig. 7d, e, f, g) and hybrid glycans (Fig. 7c) is the attachment of *N*-acetylglucosamine (GlcNAc) by the enzyme α1,3-mannosyl-glycoprotein 2-β-*N*-acetylglucosaminyltransferase (MGAT1). Some glycosyltransferases work only if an exact saccharide is attached or cleaved off the glycan but also, they can be inhibited by addition of some structures. One of the examples is the core-α1,6-fucosyltransferase, which adds fucose in the α1,6-linkage only to the proximal core GlcNAc and only after cleaving off all the α1,2-

mannoses and after addition of GlcNAc by MGAT1. In this step, also other glycosyltransferases start to work [153].

Another GlcNAc can be attached to the α1,6-linked terminal mannose by another glycosyltransferase, MGAT2 – the basic form of a biantennary complex glycan is thus formed (Fig. 7d). Another GlcNAc can be attached to the same mannose forming triantennary glycan (Fig. 7e) by the enzyme MGAT5 (two isoforms A and B) or to the core mannose (MGAT3) producing bisecting glycan; however, the later reaction inhibits MGAT 5 (which means that either bisecting or triantennary glycan branching is formed). On the other hand, MGAT3 action does not inhibit other *N*-acetylglucosaminyltransferases such as MGAT4 which attaches another GlcNAc to the α1,3-linked mannose. Triantennary glycans and tetraantennary glycans thus formed (Fig. 7f) [153].

In humans, further saccharides can be attached to the terminal GlcNAcs. Namely, galactoses are added by the action of β1,3- and β1,4-galactosyltrasnferases (B3GT and B4GT, respectively). These enzymes act nonspecifically, recognizing terminal GlcNAcs irrespective of the source protein and glycan type. Yet further saccharides can be added and these are responsible for the actual functional aspect of the glycans [153].

In humans, fucose residues can be added as the terminal saccharide by the function of the corresponding fucosyltransferases in the α1,2-linkage (FUT1, FUT2), α1,3-linkage (FUT3 to FUT7, FUT9), or α1,4-linkage (FUT3 and FUT5). Yet before the extension of the complex glycan, FUT8 is responsible for addition of α(1,6)-linked fucose to the proximal GlcNAc in the core. In vertebrates, α1,3-linked core-fucose can be also added, but this is exceptional and α1,3-core-fucosylated glycans are mostly present in lower eukaryotes (*Caenorhabditis*, *Schistosoma*; FUT1), arthropods, or plants (KEGG database) [213].

Another way how the *N*-glycans termini are decorated is the addition of sialic acids. In humans, only one type is present, the *N*-acetylneuraminic acid (Neu5Ac), although other vertebrates, including apes, produce another type, the

N-glycolylneuraminic acid [214]. Altogether almost 50 different sialic acids are known to be present in glycans from different organisms including the derivatives of 2-keto-3-deoxynononic acid (Kdn) [215]. Sialyltransferases usually transfer sialic acids to terminal galactoses in several possible linkages – α2,3- (SIAT6, SIAT10), α2,6- (SIAT1), or α2,8-linkage (SIAT8). Further sialyltransferases exist, which transfer differentially modified sialic acids in a number of linkages [153, 215].

Still other saccharides are used in glycan modification such as glucuronic acid (glucuronyltransferases), but also modifications of the saccharides such as sulfation (sulfotransferases) [153].

The resulting glycans can differ for each glycosylation site of the same protein as well as for the same glycosylation site of proteins produced in different cells, cells of different tissues, and of different organisms. The actual set of glycans attached to proteins depends on the expression pattern of the different glycosyltransferases in the cell and to a lower extent also on the protein substrate [153, 216, 217]. This so-called microheterogeneity of glycans attached to the same glycosylation site has been observed for example in horseradish peroxidase [218]. Only some exceptions are known – among them the soybean agglutinin containing solely Man9GlcNAc2 glycans [219].

Typical monosaccharides comprising *N*-glycans in animals are hexoses (Man, Gal, Glc), *N*-acetylhexosamines (GlcNAc, GalNAc), deoxyhexoses (Fuc), and sialic acids (NeuAc, NeuGc). In plants, another sugar, Xyl is frequently used and plants use also different sugar bonding (Fig. 10). Insects (and arthropods) use the same carbohydrates as animals; the bonding is sometimes different. This is true also for nematodes [204].

The situation is different in microorganisms. Bacteria use yet other glycans, containing Bac, pseudaminic acid and its derivatives, Kdo (2-keto-3-deoxy-D-mannooctonic acid) which is derivative of sialic acid Kdn (2-keto-3-deoxy-D-nononic acid), or hexoses (Fig. 10). In Archaea, other "strange" sugars can be found, such as 2,3-diacetamido-2,3-dideoxy-β-glucuronic acid or 2-acetamido-2-

deoxy-β-mannuronic acid. Archaeal *N*-glycans can contain even threonine [200] or can be attached to the protein through glucose as the proximal saccharide [202]. Galacturonic acid, galactofuranose, glucuronic acid, and iduronic acids were also reported [220].

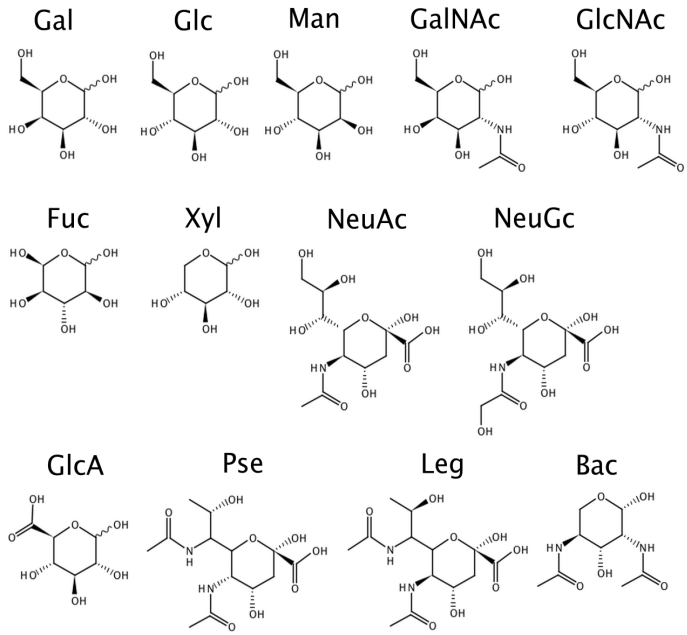

Figure 10. Some of the typical monosaccharides from the N- and O-glycans of Archaea, Bacteria, and Eukaryotes. Gal-galactose, Glc - glucose, Man - mannose, GalNAc - N-acetylgalactosamine, GlcNAc - N-acetylglucosamine, Fuc - fucose, Xyl - xylose, NeuAc - N-acetylneuraminic acid, NeuGc - N-glycolylneurmianic acid, GlcA - glucuronic acid, Pse - pseudaminic acid, Leg - legionaminic acid, Bac- bacillosamine.

2.4.1.2 O-glycosylation

In contrast to *N*-glycosylation, *O*-glycosylation is much more diverse in all its aspects – the diversity of structures, the enzymes, diversity of glycosylation sites (and lack of a common *O*-glycosylation site), and of glycosylation processes.

First, let start with the glycosylation sites. The only common thing for the *O*-glycans is that they are attached to either serine or threonine and the two amino acids are used in approximately the same ratio (there are some exceptions in particular proteins) [204].

O-glycosylation in Eukaryotes takes place on Ser/Thr of proline-rich domains [221]. Examination of the *O*-glycosylated proteins revealed the increased presence of Pro, Ser, and Thr, and to lower extent also Ala around the glycosylated sites. Ser and Thr are more present around the multiply glycosylated sites which is simply explained due to the fact, that these amino acid are the glycosylated ones. Pro seems to be the only amino acid, which has higher frequency around the *O*-

glycosylated sites, especially in the positions -1 and +3. *O*-glycosylation seems to be present especially in regions with the reverse turn secondary structures. Also, there is no difference in the location of the glycosylation site in the protein, thus not discriminating the termini. In search for *O*-glycosylation sites, the authors suggest scanning of the protein sequence in 30-residue windows and looking for 40% occupancy of these windows by Ser and Thr [204]. These results were confirmed in a more recent study, where glycine and valine were also found in higher occurrence around the *O*-glycosylation sites. Furthermore, proline is more frequently present in positions -2 and +2 if serine is glycosylated [222].

In bacteria, a consensus sequence was found for at least some bacteria and some proteins. D-S/T-A/I/L/V/M/T *O*-glycosylation sequon was observed in *Bacteroides fragilis* [223]. Similar sequence was observed as *O*-glycosylated in *Flavobacterium meningosepticum* and it was either D-S or D-T-T [224] while in *Acetogenium kivui* the sequence was V-T [225]. Regarding the specific *O*-glycosylation reactions, the *O*-xylosylation requires glycine following the modified serine and acidic residues on either side of the glycosylation site [217].

The actual *O*-glycans are also variable. Even in Eukaryotes (where *N*-glycans are attached only through one saccharide), a number of possible attachments for *O*-glycans was found. The reason is the lack of a common core-structure for *O*-glycans. In one of the possible groups of attached oligosaccharides using the GalNAc as the proximal sugar, eight basic core-structures were identified to date (Fig. 11) but the *O*-glycan diversity is further amplified by the possibility of attachment through other saccharides and by attachment of monosaccharides [138, 226].

The proximal saccharides of *O*-glycans are attached to proteins in the GA and in the case of *O*-mannosylation in ER; the corresponding OSTs show substrate-specificity (for peptides and proteins). Expression of these glycosyltransferases is tissue-specific. Additionally, glycan-modifying enzymes are expressed and further modifying the glycans by sulfation or acetylation.

Surprisingly, production of recombinant human erythropoietin with artificial *N*-glycosylation motif introduced near the *O*-glycosylation sites resulted in *O*-glycosylation of the *N*-glycosylation sequon. It is possible that *O*-glycosylation structural motif is favored at least in some proteins [144]. However, it is unexpected given that *O*-glycosylation usually does not take place in the ER (which is the place of *N*-glycan attachment) [138]. Thus, either the *O*-glycosylation motif spatial structure did not allow the STT3 to recognize the introduced *N*-glycosylation motif, or the *N*-glycan was attached and later removed. Another possibility is the attachment of at least the proximal saccharide of the *O*-glycan in the ER, as was shown for oligomannose glycans attachment in yeast [227]. Some of the *O*-glycan types are described below.

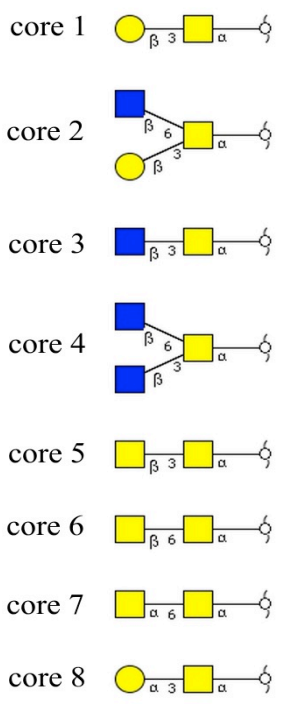

Figure 11. Core structures of the mucin-type O-glycans.

O-GalNAc

Mucins are an example of eukaryotic proteins extensively modified by *O*-glycosylation with GalNAc as the proximal (protein-linked) saccharide; thus,

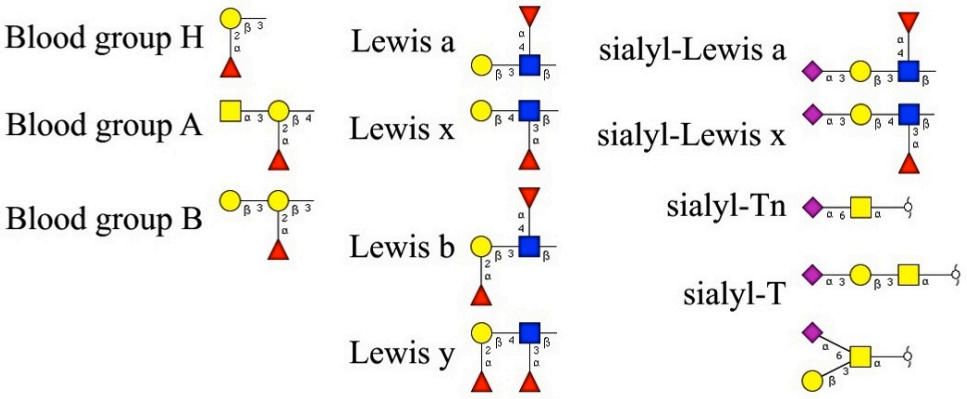

Figure 12. Terminal structures (antigens) of the O-GalNAc glycans.

these glycans are named also mucin-type. Eight core structures were identified differing in the saccharides which modify the proximal GalNAc (Fig. 11). Single GalNAc can be found attached to proteins, too. The biological activity of mucin-type glycans is dependent on the terminal part of the glycans. The possible termini are depicted on Figure 12 and can be further modified by methylation, sulfation, or acetylation [226].

O-GlcNAc

Attachment of GlcNAc seems to be correlated with activation/deactivation of proteins besides the phosphorylation of Ser and Thr. Proteins with a single GlcNAc attached to the protein are usually found in nucleus and cytoplasm. Cyclic addition and removal of phosphates or GlcNAc at the same amino acid was also suggested [226]. In bacteria, *O*-GlcNAcs were also identified as possible *O*-glycans in *Listeria monocytogenes* [228].

O-Man

α-linked mannose attached to proteins was first identified in yeast. Later, *O*-mannosylated proteins were found in other organisms with a variety of saccharides decorating the proximal mannose including GalNAc, GlcNAc, Fuc, sialic acids, Man, and others. However, *O*-mannosylation is present only at proteins produced in specific tissues such as neural tissue [226].

O-Fuc

Signal sequence for *O*-fucosylation was identified (and is the only clearly identified signal sequence for *O*-glycosylation with exception of the signal for *O*-glucosylation) and is related to conserved cysteine residues and the corresponding disulfide bridge formation such as in epidermal growth factor. In this protein, the sequence includes the second and the third conserved Cys and is constituted by C-X_{4-5}-S/T-C [226]. The corresponding fucosyltransferase in *Drosophila* functions also as chaperone and thus controls the protein fold in conjunction with *O*-

fucosylation as showed for Notch proteins [229]. GlcNAc (with Gal or sialic acid attached to it) or Glc were found as the extending saccharides for *O*-fucose [226].

Galactose, xylose or glucose attachment to Ser and Thr were also reported in Eukaryotes.

In bacteria, other saccharides can be used for attachment of *O*-glycans to the proteins. Presence of 2,4-diacetamido-2,4,6-trideoxyhexose linked *O*-glycans was reported in *Neisseria* [230] while in *Campylobacter*, *O*-linked pseudaminic acid, which also belongs to sialic acids, was detected. The pseudaminic acid was in some cases further modified with acetamidino and hydroxyproprionyl groups instead of the acetamido groups [231]. Other modifications of pseudaminic acid include *O*-acetylation, *N*-acetylglutamination, and di-*O*-methylglycerolylation [232]. Pseudaminic acid was found also in *H. pylori* [233]. In *Pseudomonas*, *O*-glycans are attached through rhamnose; the glycans contained hexoses, deoxyhexoses, pentahexoses, hexuronic acids, deoxyhexosamine, and unidentified residues [234].

2.4.2 Glycosylation in Arthropods

Model organisms are usually the most studied organisms and thus, not surprisingly, the best studied arthropod organism is the fly *Drosophila* – and this is true also in glycobiology. Recently, its *N*- and *O*-glycans, glycoproteins, and differences in its glycomes throughout its development were described. In other arthropods, several studies on glycans and glycoproteins were published. Nevertheless, the enzymatic machinery leading to glycosylation in arthropods is not well known, nor are the glycan structures in these organisms. Most common description of glycosylation of arthropod proteins is only as "putative glycosylation sites" (almost exclusive description of glycosylation in ticks).

Regarding the glycans, *N*- and *O*-glycans were described in *Drosophila*. *N*-glycans in these flies are less extensive (truncated) compared to mammalian ones,

and no tetra- or pentaantennary glycans were identified [235]. The most common *N*-linked structures are the high-mannose type glycans and the core-fucosylation is very abundant. The so-called paucimannosic *N*-glycans ($GlcNAc_2Man_{2-4}$ with possible core-fucosylation) are characteristic for arthropods but also for parasites, Nematodes, Molluscs (generally invertebrates), and plants [236-240].

In *Drosophila*, fucose bound to the proximal GlcNAc was found in both the α1,3- and α1,6-linkages. Mono- as well as di-fucosylated glycans were present. Hybrid and complex *N*-linked structures were also found in *Drosophila* tissues, even though they were a minor component of the glycome (comprising at most 12% of all glycans) [235]. Sialylated glycans were also identified in the *Drosophila* embryos, but not during the later stages of the fly development. The sialylated glycans lacked core-fucosylation, while difucosylated and α1,3-fucosylated glycans were only of high-mannose type. The abundance of complex glycans increased during the development in *Drosophila* [235]. The presence of non-fucosylated and α1,3-linked core-fucosylated glycoproteins was shown also in other arthropods such as the sand fly *Lutzomyia longipalpis* salivary gland lysates [241].

α1,3-linked core-fucosylation which is a common feature of insect (arthropod) as well as plant or parasitic N-glycans was revealed to be immunogenic [242, 243], leading to production of IgE antibodies and allergic reaction [244].

O-glycans in *Drosophila* are also more simple and truncated when compared to mammalian *O*-glycans. They comprise of core 1 and core 2 type glycans, *O*-Fuc glycans (GlcNAcβ1-3(GlcAβ1-4)Fuc), together with single HexNAcs and hexoses, variants of HexNAc-HexNAc glycans (modified by GlcA predominantly), and the Xyl-Glc structure. Some of the *O*-glycan structures are specifically distributed in the embryonic tissues [245, 246]. *Drosophila* embryonic hemocyte-like cell line produces Tn (GalNAc) and T-antigen (Galβ1,3GalNAc) mainly [247] and similar glycans were found also on *Vespula germanica* common wasp nest glycoproteins [248]. *V. germanica* wasps produced some of these glycans modified by terminal 2-

aminoethyl phosphates [248]. GalNAc as the proximal saccharide of *O*-glycans was identified in *V. germanica*) and in European hornet (*Vespa cabro*) with Gal, GalNAc, GlcNAc, and Fuc residues extending the glycans [249]. *O*-glycans are differentially expressed in different life stages as was shown in *Drosophila* [250].

2.4.2.1 Glycosylation in ticks

The majority of information on tick glycosylation was provided by indirect methods such as lectin staining of thin sections or lectinoblots of protein extracts. Only recently, mass spectrometry has been used for tick glycans studies.

The presence of glycosylated structures in tick salivary glands was first confirmed by Schiff staining in *R. appendiculatus*, *B. microplus*, and *D. variabilis* [42, 44, 251]. Lectin studies showed the presence of both *N*- and *O*-glycosylated proteins in tick samples and a considerable part of the identified glycoproteins have been shown to be antigenic determinants for the immune response of the host [252, 253]. Tissue-dependent glycosylation has been also shown [254, 255].

In whole-body homogenates of *I. ricinus* larvae, Man and Glc containing *N*-glycans were identified (ConA and PSA lectins staining), complex *N*-glycans containing terminal GlcNAc and Gal (WGA, PNA lectins) as well as *O*-glycans containing Gal and GalNAc (SBA, PNA lectins) [254]. Similar lectin reactivity was found also in the case of *I. ricinus* nymphs; also the lectin UEA recognizing Fuc in *N*-glycans gave positive reaction. Furthermore, several organs of the partially-fed *I. ricinus* females were analyzed for the presence of glycoproteins. Female integument contained mainly *N*-glycans of all types (ConA, PSA, PNA, WGA staining) and reactions with SBA (*O*-glycans) and UEA lectins (fucosylated *N*-glycans) were very weak. Female SGs gave similar staining pattern, but the SBA lectin recognized several glycoproteins more strongly. The midgut contained glycoproteins which were recognized by ConA, PSA, and WGA (*N*-glycans) and a small (approximately 12 kDa) glycoprotein gave strong reaction with PNA lectin (specific for Gal). SBA and UEA lectins stained very weakly the 12 kDa protein

(fucosylated *N*-glycans, *O*-glycans). Ovarian glycoproteins showed strong reaction with ConA, PSA, PNA, and WGA lectins, similar staining pattern was shown also for proteins from hemolymph and Malpighian tubules. In the fat body, only ConA, PSA, and WGA-reactive glycoproteins were found (suggesting the absence of *O*-glycans). A number of these glycoproteins were recognized also by rabbit sera from animals infested with *I. ricinus* larvae, nymphs, and adults [254]. Different acini types showed differential lectin staining in SG. While both acini type II and III were stained by ConA, PNA, and WGA lectins (Man-, Gal-, and Glc-specific), other Gal-reactive lectins stained only the type III acini (such as HPA and VVA lectins) [255].

In another study, heavy glycoprotein staining was observed in the granules of some cell-types of tick SGs, mainly *a*, *b*, and *c* cells of type II acini and *e* and *f* cells of type III acini. In unfed females SGs, mannose-specific lectins (ConA and GNA) stained the *a*, *b*, and *c* cells, granules of the *b*, *c*, and *e* cells were stained with GalNAc-specific (HPA), GlcNAc-, and NeuAc-specific lectins (WGA and MAA II) [256]. Partially-fed females SGs showed strong signal for Man identified in *b*, *c1*, *c2*, and *f* cells, but also GalNAc in *b*, *c2*, and *e* cells, and GlcNAc in all cell types. Procuticular and cuticular structures of ducts showed also positive signals in lectin affinity-staining (Man-, GalNAc-, and NeuAc-specific lectins) [256].

Moderate (in *b*, *c2*, and *e* cells) to strong signal (in *c1* cells) for NeuAc was present also in partially-fed females SGs, as assessed using lectins MAA II and SNA. Specificity of staining was confirmed using neuraminidase treatment (*V. cholerae*). Thiobarbituric acid colorimetric reaction was used for quantification of neuraminic acid in SG homogenates from fed females and showed approximately 75 nmol sialic acid per milligram of the sample [256].

Recently, Pedra and colleagues [257] isolated the *N*-glycans from some salivary proteins of the engorged *I. scapularis* nymphs and showed the presence of core-fucosylated structures by MS. High-mannose glycans with three to ten hexoses, core-fucosylated high-mannose glycans with up to four hexoses and

core-fucosylated complex glycans $GlcNAc_3Man_3Fuc$ and $GlcNAc_4Man_3Fuc$ were identified. Furthermore, they performed immunoblotting of the protein extracts from engorged nymphal salivary glands as well as midguts with anti-HRP antibodies which are specific for α1,3-linked core-fucose and core-xylose. Positive reaction of antibodies suggested the presence of α1,3-fucose. After RNA inhibition of α1,3-fucosyltransferase genes, α1,3-core-fucosylated glycans were not detected in nymphal salivary glands any more; however, the presence of α1,6-core-fucose in ticks after RNAi or overall the ratio of α1,3- versus α1,6-core-fucosylated glycans was not studied in this study [257].

Putative glycosylated components of tick saliva

The proteomes of SGs from several ticks and their life stages were studied to date. Some examples are the analysis of cDNA and proteins from homogenates and SDS-PAGE gels of partially-fed *I. scapularis* females [258], of cDNA libraries from nymphs and adult female *Ixodes scapularis* ticks [88], or different feeding stages of the *I. ricinus* females [27]. Hundreds of protein sequences were predicted, including several novel families unique for ticks. Several proteins sequences possess putative glycosylation sites. Some of the examples of these proteins are anticomplement proteins of the *I. scapularis* Isac (called Irac in *I. ricinus*) family, with serine- and threonine-rich C-termini. The Isac/Irac proteins act similarly to factor H and thus inhibit the complement activation [82, 88]. Tick mucins are also present in tick saliva and contain possible *O*-glycosylation sites [88]. Potential *N*-glycosylation sites were revealed for example in the deduced amino acid sequence of Ixolaris or 36-kDa from *D. andersoni* [53, 259].

Glycosylation in the host-parasite and parasite-pathogen relationships

Similarly to the importance of protein-carbohydrate interactions for infection of mammals by bacterial and viral pathogens, these interactions were

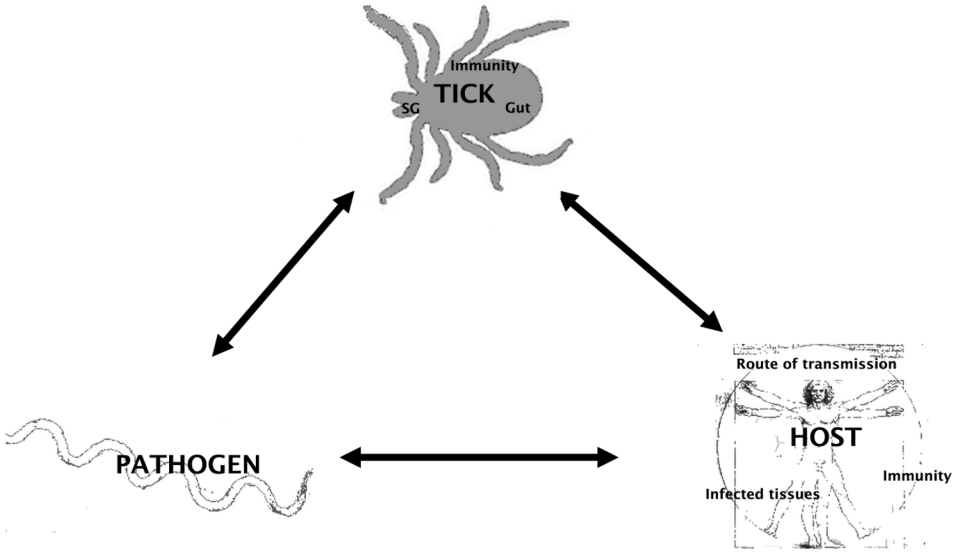

Figure 13. Tick-host-pathogen interactions.

shown to be involved also in parasite-pathogen and parasite-host relationships (Fig. 13).

For mosquitoes, glycan-specific adhesion of *Plasmodium gallinaceum* to the midgut cells of *Aedes aegypti* mosquitoes has been shown. Moreover, this interaction was shown to be tissue-specific, suggesting differential expression of *Plasmodium*-specific glycans in the midgut. The carbohydrates recognized by the malaria agent can possibly contain terminal sialic acid [260]. Binding of *P. falciparum* and *P. vivax* to GlcNAc of the *Anopheles tessellatus* mosquito midgut glycans has been suggested as well [261] and carbohydrates were used for inhibition of *P. vivax* oocyst development in *A. stephensii* [262].

In ticks, the susceptibility of acini type III of the tick *R. appendiculatus* to *Theileria parva* infection correlated with the differences in the surface glycan distribution of the different acini types [255]. Moreover, addition of tick hemagglutinins-inhibiting sugars (raffinose and mellibiose; containing reducing Gal) increased the infection rate of *R. appendiculatus* acini by *T. parva* in contrast to non-inhibiting sugars (Man and turanose) [263].

Furthermore, the requirement of α1,3-core-fucosylated glycans for successful *A. marginale* infection of the tick *I. scapularis* was shown – RNA inhibition of the responsible fucosyltransferases resulted in total absence of *Anaplasma* in the infected ticks. However, absence of α1,3-core-fucosylated glycans did not affect *Borrelia* infection [257].

Glycans as the future targets for pan-arthropod vaccines

Most of the currently studied anti-tick vaccine candidates are glycoproteins [264]. Furthermore, the glycosylation of the recombinant proteins used in vaccines affect the antigenicity and the glycosylated proteins have higher efficiencies compared to non-glycosylated proteins. Both the protein and the glycan parts are believed to contribute to antigenicity [265]. Tick glycans are believed to be the basis for future anti-tick vaccines which further raises the importance of tick glycan antigenicity studies [265, 266].

The *O*-glycans as well as some structural features of the *N*-glycans described in arthropods are similar to the glycans studied as pan-carcinoma antigens [267]. The possibility to develop pan-arthropod vaccine preventing the blood-feeding and transmission of arthropod-borne pathogens or even a combined pan-arthropod/pan-carcinoma vaccine further emphasizes the need for a more thorough and more precise characterization of glycan structures in ticks.

2.4.3 Glycan-binding proteins

The ability of glycans to influence the structure and interactions of proteins is allowed by the unique characteristics of glycans – high number of hydroxyl groups in their structures. Furthermore, in certain glycan structures, hydrophobic regions are formed by clustering of C-H groups. Carboxy-, sulpho-, and phospho-groups attached to carbohydrates can be further used in recognition of certain molecules.

As mentioned earlier, different organisms produce diverse glycans attached to proteins. Thus, it is obvious, that immune systems of higher organisms will produce antibodies directed against these molecules [240, 242, 244, 266].

However, other proteins with specific carbohydrate binding were discovered which do not originate in the immune system. These proteins were called lectins and were first discovered due to their ability to agglutinate cells (binding to the cell surface carbohydrates, as discovered later). First, they were discovered in plant seeds and in invertebrates, later on also in higher organisms. Nowadays, the low-abundant nuclear lectins are also known [268]. The binding potential of different carbohydrate structures is thus employed for various functions through lectins, serving as receptors, signal molecules, attachment molecules, etc.

Specific binding of lectins (more or less specific towards one carbohydrate molecule) was naturally used in research for detection and purification of carbohydrates and glycosylated molecules, but also in medicine – for example some cancer cells produce specific carbohydrates, which can be detected by lectins [269].

In invertebrates, lectins serve as pattern-recognition molecules (recognizing the so-called PAMPs – pathogen-associated molecular patterns) and thus recognize the foreign invaders and participate in immune reactions [270, 271]. Most lectins that have been isolated from arthropods are from the hemolymph; however, they are present also in other organs.

Lectins are a very variable group of proteins and a common classification does not exist; current most widely used classification is based on structural homologies rather than saccharide-binding specificity (with exceptions). Some of the lectin groups are:

•C-type lectins (calcium-binding), represented for example by the mammalian adhesion molecules selectins;

•E-type lectins originating from eels with specificity towards fucose;

•F-type lectins or ficolins containing fibrinogen/collagen domain;

•G-type lectins originating from snowdrop (*Galanthus nivalis*) or garlic, generally with specificity towards mannose;

•I-type lectins (members of the immunoglobulin superfamily), which include Siglecs – lectins with specificity towards sialic acid (another sialic acid-specific lectin is the blood coagulation factor H, but it structurally does not belong to the I-type);

•S-type lectins or galectins, specific for galactose and lactose;

•T-type lectins or tachylectins, produced by the horseshoe crab *Tachypleus tridentatus* [272].

2.4.3.1 Glycan-binding proteins in ticks

Lectins of ticks are known for relatively short time – the first works are dated back to 1991 [273, 274]. Hemagglutination activity (HA) of red blood cells by hemolymph from the ticks *O. tartakovskyi, O. papilipes, A. polonicus*, and *I. ricinus* was described as well as its inhibition. The inhibiting glycoproteins and saccharides included fetuin and bovine submaxillary mucin (but not the corresponding asialo forms), NeuAc, and GlcNAc. Sera reactive with the hemagglutination components of the hemolymph recognized 30 and 35 kDa proteins in the *O. tartakovskyi* hemolymph, 37 and 40 kDa proteins in the *O. papilipes* hemolymph, and 45 and 50 kDa proteins in the *I. ricinus* hemolymph. The antibodies recognized hemagglutinins also in *I. ricinus* hemocytes [273, 274]. The *I. ricinus* hemolymph lectin (85 kDa) was later localized in hemocytes (surface and granules) but also in other organs such as the midgut [275]. Another hemagglutinin/lectin was found in the *I. ricinus* midgut with several potential subunits (37, 60, 65, and 73 kDa). HA of the midgut was not detected in unfed ticks and the HA specificity was stated towards GalNAc, GlcNAc, rhamnose, and fetuin [254].

None of these lectins/hemagglutinins were purified; the first purified tick lectin was isolated from the *O. moubata* hemolymph and named Dorin M [276]. The hemolymph showed HA which was inhibited by NeuAc, GalNAc, GlcNAc, ManNAc, fetuin (but not asialofetuin), bovine submaxillary mucin, and ovomucoid. The protein in native state has 640 kDa with 37 kDa subunits (32 kDa after chemical deglycosylation) and shows specificity towards sialic acid [276]. The analysis of Dorin M amino acid sequence revealed its similarity to Tachylectins from the horseshoe crab *T. tridentatus* [277, 278] which are fibrinogen-related domain containing proteins (FRePs) belonging to T-type lectins. RT-PCR analysis of *O. moubata* tissues revealed the expression of Dorin M not only in hemocytes, but also in the salivary glands or the midgut [277].

The bioinformatics approach coupled with molecular techniques resulted in the identification of a similar fibrinogen-related molecule, OMFREP (*O. moubata* fibrinogen-related protein), in *O. moubata* hemocytes, which was 65% identical to Dorin M. Furthermore, two similar molecules were identified in *I. ricinus* and named Ixoderin A and Ixoderin B. Both showed significant homology to Dorin M. Ixoderin A shares a similar expression profile while Ixoderin B was constitutively expressed only in the SGs of *I. ricinus* with marginal expression in hemocytes. All these tick lectins were showed to be similar to vertebrate immune system proteins ficolins on the protein level [279]. Ficolins consist of a collagen-like domain, a C-terminal fibrinogen-related domain, and structurally resemble a mannose-binding lectin that is known to activate complement (lectin pathway) [280]. Yet other lectin was characterized in the *O. moubata* – OmGalec, which is a galectin as it binds lactosamin and similar carbohydrates via Gal and GlcNAc. The 37 kDa protein is expressed in all stages of tick life cycle and is abundant in hemocytes, the midgut, SGs, and reproductive organs [281].

Hemagglutination activity of *R. appendiculatus* and *R. pulchellus* hemolymph and midgut extracts was also observed. Interestingly, the HA increased in ticks infected by *T. parva* [255, 282].

2.4.4 Glycobiology of arthropod-borne pathogens

2.4.4.1 Protein-carbohydrate interactions of tick-borne viruses

Among the viruses transmitted by ticks, tick-borne encephalitis (TBE) virus (Flavivirus, Flaviviridae) causes the most serious infections of humans and of domestic animals. Its capsid is surrounded by the host cell-derived membrane with two viral structural proteins – the envelope (E) protein and the membrane (M) protein. M protein is the cleavage product of the prM (precursor of the M) protein formed during the maturation process. The E protein is important for the infection of the host cell as it participates in the recognition of host receptor(s). Also, it is important for the release of the virion after its endocytosis [283].

The viral RNA encodes seven non-structural proteins besides the three structural proteins. Three TBE proteins are glycosylated – the E protein, the pr part of the prM protein, and the non-structural NS1 protein. The E protein amino acid sequence contains two potential *N*-glycosylation sites, but only one of these seems to be glycosylated [284]. Chicken fibroblasts produced *in vitro* virions with protein E bearing complex biantennary *N*-glycans with core-Fuc and terminal Gal [285]. Mutation in the *N*-glycosylation sites of the E protein leads to decrease of its production and secretion but not the virus infectivity [286, 287]. Potential heparan sulfate (which is a glycosaminoglycan) binding was shown for virions produced in BHK-21 cell lines and other glycosaminoglycans such as heparin or chondroitins were able to inhibit virus replication in neural cells which suggests the involvement of protein-saccharide interactions in the host infection [288, 289].

The prM bears one glycosylation site in the pr part, which is cleaved off the protein during maturation. Mutation of this *N*-glycosylation sequon resulted in lower secretion of E protein in the virus-like particles [286]. The NS1 protein

contains two to three potential *N*-glycosylation sites in its sequence, and the function of its glycosylation is still not known [283].

The role of glycosylation for viral replication has been studied slightly more in other flaviviruses. Glycosylation of mosquito-borne West Nile virus (WNV) E protein leads to higher virulence for mice compared to non-glycosylated virions [290]. Furthermore, mutation of *N*-glycosylation sites of WNV prM and E proteins diminished the production of subviral particles [291]. However, while in one study the absence of E protein glycosylation resulted in higher infectivity rate of mosquito cells [291], in another the mutation of E protein glycosylation site resulted in decreased replication in mosquito tissues and lowered transmission of the mutated virus by mosquitoes [292]. Also, revertant viruses appeared in the infected mosquitoes suggesting strong selection towards the glycosylation of the E protein [292]. Activation of mammalian complement system by the mannose-binding lectin was shown to be important in fighting the flavivirus infection through recognition of mannosylated glycans at the surface of WNV virions produced in insect cells but also of Dengue virus (DENV) produced in both mammalian and insect cells. Furthermore, mutant viruses with the E protein

Karolína Šustrová: Cultivation of tick-borne encephalitis virus in the presence of inhibitor of glycosylation: its effect on nucleotide sequence of genes encoding viral proteins. BSc. thesis, 2008, Faculty of Science, University of South Bohemia (USB), České Budějovice. Supervisor: L. Grubhoffer, co-supervisor: J. Štěrba.

Passage of the TBEV virus in the presence of tunicamycin (the resulting strain called Hypr3TM), which is an inhibitor of *N*-glycosylation, resulted in three amino acid substitutions: $Ala_{317} \rightarrow Thr_{317}$ and $Val_{485} \rightarrow Gly_{485}$ in the E protein and $Gly_{580} \rightarrow Asp_{580}$ in the NS3 protein. The amino acids Gly_{485}nd Asp_{580} are present in the parental virus with low passage number in mice while the presence of Ala_{317} is unique for the Hypr3TM virus.

However, replication of the virus in the presence of tunicamycin in another work (Renata Strouhalová, MSc. thesis, 2011, Faculty of Science, USB. Supervisor: L. Grubhoffer, co-supervisor: V. Hönig) did not affect the nucleotide and amino acid sequence of the E protein. On the other hand, three subsequent passages in PS cells in the presence of tunicamycin resulted in increased titer of the virus (10^4-10^7 PFU/ml).

modified by two *N*-glycans (instead of one in the wild-type virus) were cleared from the infected organisms more rapidly [293]. E protein of DENV contains two glycosylation sites. Their mutation resulted in increased infectivity for mosquito cells but decreased the virion release from infected mosquito and mammalian cells [294]. Furthermore, mutation of one of the glycosylation sites stopped the replication of the virus in mammalian cells and mutation of both sites resulted in introduction of a novel glycosylation site when replicated in mosquito cells [295].

Differential virulence of TBEV for mammals was observed with virus passaged in tick or mammalian cell lines [296, 297]. In this regard, involvement of the different glycans and their role on the infectivity was suggested [298].

2.4.4.2 Protein-carbohydrate interactions of tick-borne bacteria

Protein glycosylation has been proved also in number of pathogenic bacteria. In fact, it is hard to find any microbial pathogen, whose successful invasion of host is not dependent on protein-carbohydrate interactions.

Glycoproteins in pathogenic bacteria were first identified in the glycosylated surface layer in *Clostridia* [299]. Furthermore, flagella proteins have been found to be glycosylated in *Campylobacter* and this glycosylation has been found to be essential for the bacteria pathogenicity [300].

Spirochetes *Borrelia burgdorferi* are gram-negative bacteria, causing a worldwide tick-borne emerging disease – Lyme borreliosis (LB). The life cycle of the bacterium involves the arthropod vector and the vertebrate host. The vectors for the transmission are ticks of the *Ixodes* family [301]. *Borrelia burgdorferi* s.l. is thus an example of a microorganism that has adapted during evolution to survive in different environments. The cytoplasm of *B. burgdorferi* cells is surrounded by an inner membrane, periplasmic space with periplasmic flagella, and finally the outer membrane. A weakly attached amorphous slime layer can be presumed at the cell surface [302]. This layer, together with the outer membrane, is covered with glycoconjugates [303].

In *B. burgdorferi*, two proteins, outer surface proteins A and B (OspA, OspB) were identified as glycoproteins [304, 305]. The two outer surface proteins were stained following periodate oxidation and they were sensitive to PNGase F (which cleaves off the *N*-glycans from the peptide backbone) [304]. Also, radioactively labeled GlcNAc was incorporated into these proteins, further confirming their glycosylation [305]. The presence of other minor glycosylated proteins have been described, too [304]. Surprisingly, *N*-glycosylation of recombinant OspA protein produced in mammalian cells showed alteration of the protein epitopes and its presentation to the immune system [306].

Furthermore, Ge and colleagues [307] showed one of the flagellin proteins, FlaA, to be glycosylated. In purified flagella, the protein was stained by Schiff staining and reacted with SNA and GNA lectins. Furthermore, PNGase F reduced the mass of the protein [307].

The other flagellar protein FlaB has been suggested to be post-translationally modified, even though the type and size of the modification was not specified further [308]. The protein (theoretical Mw of approximately 35kDa) showed an unusual migration in SDS-PAGE with 41 kDa [309], which can be attributed to PTMs.

Lectins recognized the SDS-PAGE separated and subsequently electroblotted borrelia proteins. WGA and ConA heavily stained outer membranes of *B. burgdorferi* cells and DBA, SBA, HPA, GNA, and UEA I lectins showed also positive staining. Additional SNA staining was observed electron microscopy of borrelia cryosections. Flagella were stained with WGA lectin. UEA I and DBA lectins stained the periplasmic space. Lectin staining was reduced after PNGase F and Endo H treatment. In the same work, anti-BSA and anti-rabbit serum (an additive in the cultivation medium) staining was observed on the surface of borrelia [303]. ConA, WGA and UEA I lectin labeling of bacterial surface and flagella was reported by Stoitsova and colleagues [310]. Sialic acid was detected at the surface of *B. burgdorferi* by lectin blotting also by other

authors [311]. A big variety of glycans was shown to be present at the surface of borrelia – glycans containing Man, Gal, Fuc, and NeuAc. As borrelia does not seem to be able to synthesize sialic acid [312], these results suggest either the presence of host-derived glycoproteins on the surface of borrelia, or the ability of borrelia to "scavenge" the glycans from the host proteins [313].

Some of the borrelia surface proteins participate in carbohydrate binding. Six strains of *B. burgdorferi* s.l. bind glycosaminoglycans heparin sulfate and dermatan sulfate [314]. Heparin molecules with a chain length of minimum 16 residues effectively inhibited binding of borrelia to Vero cells in correlation with the ability to inhibit hemagglutination [315]. Attachment of spirochetes to cultured endothelial and brain cells was inhibited by exogenous proteoglycans, by treatment of cultured cells with inhibitors of proteoglycan synthesis or sulfation, and by digestion with heparinase or heparitinase but not chondroitinase [316].

Of the known carbohydrate-binding proteins, the first is BgP (now named 5'-methyladenosine/S-adenosylhomocysteine nucleosidase, Pfs-2). It binds glycosamines, namely heparin and dermatan sulphate allowing the attachment of borrelia to host cells [317]. BgP was also revealed in the culture medium of *B. burgdorferi* [318]. *B. burgdorferi* s.s. bind gangliosides containing terminal NeuAc; however, this binding is not inhibited by sialic acid. Borrelia also bound the short-sugar-chain glycosphingolipids. Low-passage strains exhibited much higher binding compared to high-passage ones [319]. Inhibition of low-passage strains binding to human cells was observed for heparin, heparan sulfate, and dermatan sulfate *in vitro*, while attachment of high-passage (avirulent) strains was not affected by these glycosaminoglycans. Also, Chinese hamster cells deficient in heparan sulfate demonstrated similar binding by borrelia as the wild-type cells in inhibition experiments. Additionally, the charge of the GAGs, namely sulfation, was shown to be an important factor for borrelia binding. A potential GAG-binding receptor was isolated with Mw 39 kDa; however, it was not identified [320]. Two borrelial host-associated proteins DbpA and DbpB (decorin-binding

protein) mediate the binding to collagen-associated proteoglycan decorin [321]. Decorin occurs in the dermis and other vertebrate tissues. The binding to decorin is one of the borrelial mechanisms of penetration to the host and hiding against the immune system in the dermis [322]. DbpA/B bind purified heparin and dermatan sulfate [317]. Host-adapted spirochetes express DbpA together with OspC in higher amounts especially in ear, ankle, knees, and heart tissues [323].

Furthermore, a putative lectin molecule called B31LEC was identified in the genome of *B. burgdorferi* s.s. (strain B31) due to its similarity to lectin domains. The recombinant protein showed HA activity which was inhibited by fetuin, D-galactosamine, and D-mannosamine [324].

Glycosylation was identified also in other tick-transmitted bacteria. Similarly to *B. burgdorferi,* OspA and OspB proteins were found to be glycosylated in the

Martina Jonáková: Identification of glycoproteins in samples of the CB53 isolate of the spirochete *Borrelia burgdorferi* s.s.. BSc. thesis, Faculty of Science, USB, 2009. Supervisor: J. Štěrba, co-supervisor: L. Grubhoffer.

Several glycoproteins were stained in the outer membrane (OM) and periplasmic flagella (PF) fractions of *B. burgdorferi* s.s. spirochetes, strains B31 and CB53. Of these, two proteins were stained using MAA II lectin – the 40 kDa protein in PF of both strains and OM of CB53 and 20 kDa protein, which was present only in CB53 samples. SNA lectin recognized several proteins in the range of 60-100 kDa, approximately 200 kDa protein in PF, and OM of both strains, and the 20 kDA protein in PF and OM of the CB53 strain. Several other proteins were detected using GNA and DSA lectins; PNA lectin did not stain any proteins.

OM proteins (strain CB53) were further separated by isoelectrofocusing and glycoprotein detection was performed on these samples. Majority of the glycoproteins was present in the pH range of 5.4-7.0.

Lectin affinity chromatography aimed to isolation of sialylated glycoproteins using SNA lectin resulted in purification of the 20 kDa protein. Differential migration of OspC was observed – the 40 kDa band identified by MS as OspC was present in the pH range 4.6-5.4 after electrofocusing, while MS identified OspC in the 20 kDa band in the pH range of 5.4-7.0. These two protein bands were stained with MAA and SNA lectins. Also, the affinity-purified protein corresponding to MAA and SNA-stained protein were identified as OspC by mass spectrometry.

relapsing fever spirochetes *B. hermsii* and *B. turicatae* [304, 305]. In another relapsing fever spirochetes, *B. recurrentis*, HA activity was observed as well. Its HA was inhibited by glucosamine, galactosamine, and ManNAc [325].

Ehrlichia tandem repeat proteins showed altered migration with higher molecular weight than predicted; this difference was attributed to phosphorylation and glycosylation of these proteins, but it was not confirmed [326].

Adhesion of *A. marginale* to host/tick cells is mediated by Major surface proteins (MSP) 1a and 1b. These proteins are glycosylated (glycans size approximately 15 kDa for MSP1a) and contain Glc, Gal, and Man and in the case of MSP1a also xylose. The sugars seem to be *O*-linked to the *N*-termini of the proteins. Glycosylated proteins showed enhanced binding of tick cells compared to non-glycosylated proteins [327]. Cultivation of *A. phagocytophilum* in host cells deficient in production of sialyl-Le^x antigen (which they use as a receptor for their binding to host cells) resulted in differential glycan expression on the MSP2 protein – the protein produced by *Anaplasma* cultivated in normal host cells contained Glc, Xyl, and Man while glycans on the MSP2 from *Anaplasma* cultivated in sialyl-Le^x-deficient cells contained additional carbohydrates such as arabinose, Fuc, Gal, GlcNAc, and rhamnose. Thus, also for these *Anaplasma* proteins, *O*-glycans seem to be present. The appearance of GlcNAc in protein from deficient cell grown bacteria suggests the possibility of *N*-glycan attachment (the protein contains four putative *N*-glycosylation sites) [328].

Several glycosylated proteins were stained in 2D-separated membrane fraction proteins of *F. tularensis* ssp. *holarctica* by the Pro-Q Emerald 300 stain. Furthermore, 30 protein bands were stained by SNA, MAA, DSA, and PNA lectins; they were predicted to be localized to outer and cytoplasmic membranes but also to the cytoplasm and the periplasm. Some of the identified proteins as well as other proteins were identified after lectin-affinity chromatography using several lectins. Of the identified proteins, 18 contained the bacterial *N*-

glycosylation sites (and more the mammalian sequon); the other proteins probably contain *O*-linked glycans. However, the exact glycan structures modifying the proteins were not revealed [329]. In *Francisella*, except for common glycosylation, enzymes participating in sialic acid biosynthesis have been identified [312].

Currently, a carbohydrate-dependent infection of the tick was shown for *Anaplasma* bacteria. Previously, sialylated Le^x glycan moieties were shown to be important part of receptor molecules for *A. phagocytophilum* infection of mammalian neutrophils, namely NeuAc and α1,3-Fuc [330]. Similarly, α1,3-Fuc is required for *Anaplasma* infection of the tick. However, instead of Le^x epitope, the glycans recognized by *Anaplasma* on tick glycoproteins contain fucose bound to the core of *N*-glycans [257].

2.5 Methodological approaches to arthropod glycobiology

Several methods for direct studies of glycans/glycopeptides/glycoproteins are available – an example is mass spectrometry (MS); here, we can study the mass of actual molecules (abbreviated as MS), or to fragment them and thus study their structure in more detail (named as tandem MS, MS/MS, MS^n). Several indirect methods are also available; mainly lectins in combination with other procedures are used. These methods were separately or in combination used for analysis of glycans (glycomics), glycosylation sites (glycopeptidomics), and whole glycoproteins (glycoproteomics) of a number of organisms.

Some of these techniques which are related also to this thesis are described below; further methods such as SPR (surface plasmon resonance), lectinarrays, glycodendrimers and others are not mentioned.

2.5.1 Enzymatic and chemical ways to release glycans

One important thing is needed for analysis of any glycome – isolation of the glycans. For *N*-glycans, the task is quite simple. Commercially available enzymes can cleave off all the *N*-glycans. The most widely used is the *N*-glycosidase F or PNGase F from *Flavobacterium meningosepticum*. This enzyme cleaves the bond between the innermost GlcNAc of *N*-glycans and asparagine (which is changed to aspartic acid); however, the enzyme is not a general one – it is not able to cleave off the *N*-glycans with α1,3-bound fucose linked to the core (to the innermost GlcNAc). If you want to cleave off all the glycans (or just the α1,3-core-fucosylated after the PNGase F treatment), one can use *N*-glycosidase A (PNGase A) from almond (*Prunus amgydalus*) [331, 332].

Both these *N*-glycosidases are non-specific towards the different *N*-glycan types. More specificity is provided by the group of endoglycosidases (short name Endo). However, these have one disadvantage – they cleave between the two innermost GlcNAcs, which means, that it is not possible to determine the possible core-fucosylation of the glycans isolated using these enzymes.

Endo D is an endoglycosidase from *Streptococcus pneumoniae* (named after the former genus name of the bacterium, *Diplococcus*) with a narrow specificity towards high-mannose glycans which have one of the core mannose residues as terminal [333]. Three other enzymes were isolated from *F. meningosepticum*. Endo F1 cleaves high-mannose but not hybrid and complex structures and α1,6-core-fucosylation markedly decreases its activity [334]. Endo F2 cleaves biantennary glycans but does not prefer high-mannose glycans. Endo F3 cleaves bi- and triantennary glycans; its activity is lower than that of Endo F1 and F2 [335]. Endo H was isolated from *Streptomyces plicatus* and cleaves high-mannose structures and some hybrid glycans. α1,6-core-fucosylation does not inhibit its activity contrary to Endo F1 [334] and the enzyme works on glycans with bisecting GlcNAc [333].

Also other endoglycosidases were identified – Endo B from *Sporotrichum dimorphosporum* specific for some hybrid and high-mannose glycans [336], Endo

C2 from *Clostridium perfringens* with similar specificity as Endo H [337], Endo S from vegetative cells of *Dictyostelium discoideum* with the same specificity as in Endo C2 [338], and other [333].

In the case of *O*-glycans, there are no general enzymes, capable of releasing all of them. There are several enzymes with narrow specificity such as *O*-glycanase (or *O*-glycosidase) from *S. pneumoniae*, cleaving off only unsubstituted core 1 (and possibly core 8) mucin-type glycans (Fig. 12) [339]. To remove also substituted type 1 mucin glycans, previous treatment with neuraminidase, galactosidases, etc is needed. However, it means the glycan structures will be destroyed this way.

A great advance towards miniaturization and high-throughput was immobilization of glycosidases forming reactors with possibility of creation of a stream-lined system immobilized enzymes coupled to LC separation-MS detection [332]. There are also other efforts to improve the research of glycomes such as combination of different methods into one [340].

Except the enzymatic release of glycans, some chemical approaches are also available. One of them, reductive β-elimination, works due to the lability of the linkage between GalNAc and Ser/Thr in *O*-glycans under mild alkalic conditions. Sodium borohydride is used for reduction of the released glycans. Another chemical way to release the glycans (all of them) is the deglycosylation using trifluoromethanesulfonic acid in absence of water [341, 342].

Both the enzymatic and chemical methods for glycan releasing can be used for solubilized glycoproteins but also for release of glycans from gels separated by one dimensional SDS-PAGE or two dimensional (e.g. IEF/SDS-PAGE). Even glycans from the membrane-bound glycoproteins can be released (after SDS-PAGE and subsequent electroblotting) [343, 344].

However, all the above mentioned methods are capable of releasing only glycans with the already known attachment. In the case of exotic glycan linkages, digestion of glycoproteins with pronase has been shown to be beneficial. Pronase

digests result in total proteolytic cleavage of the peptide backbone and thus the glycan (and other PTM) structures remain attached solely to the linking amino acid. In the case of more extended structures, one or more amino acids are not cleaved from the linking amino acid; however, this is also of some use – it is possible to identify the glycosylated sequence. Pronase digestion have been used for preparation of bacterial or other glycans for MS as well as NMR analysis [200, 345, 346]. Immobilization of pronase onto Sepharose beads shows the possible use of this approach also in automation and for high-throughput studies; similarly, magnetic beads are possible to use. What is more important, the beads with immobilized pronase have been active for several weeks to months, which shows a great possibility in reducing the costs for such analyses [347].

2.5.2 Lectins – affinity staining and purification of glycans

As mentioned earlier (Chapter 2.4.3), two types of carbohydrate- and glycan-specific proteins exist: carbohydrate-specific antibodies and non-immune system originating lectins. Majority of lectins currently used are of plant origin. The commercially available lectins are usually recombinant proteins which allows their production in large enough quantities and purity.

First, lectin specificity was assessed by inhibition reactions, where mono- or di-saccharides were used as inhibitors. Even though this approach was helpful and brought basic knowledge on the affinity of the lectins towards glycosylated structures, the specificity of lectins towards more complex glycans and glycoproteins can differ, as can the binding affinity. While the binding affinity towards monosaccharides is in the range of millimoles, the affinity towards glycans and glycoproteins can reach micro- or even nanomoles [348]. Regarding the specificity, there are also differences between the recognition of monosaccharides and glycans by lectins. Fucose-specific lectins are an example – while all where inhibited by L-fucose to higher or lower extent in inhibition

reactions, their specificity towards fucose with differing linkages (α1,2-, α1,3-, α1,4-, α1,6-) varied markedly. Furthermore, some of them recognized only glycans with core-bound fucose in *N*-glycans (attached to the proximal GlcNAc), other recognized only terminal fucose, while still other are able to bind fucose with different linkages and localization in the molecule [349][1]. [349-351]

Specificity of lectins can be used for many applications in glycosciences. These include techniques for glycans and glycoproteins purification such as lectin affinity chromatography, isolation of cells producing specific glycans such as hemagglutination or cell sorting, or detection of the presence of glycans in cells or tissues such as lectin histochemistry and ELISA (these can be used also for diagnostics). In some cases when broad specificity is a problem, even antibodies targeting glycans can be used [348]. However, production of anti-glycan antibodies is possible only in organisms which do not produce similar glycans.

For lectin affinity purification, lectins recognizing broad range of structures are useful. Maybe the most widely used is the ConA lectin, which binds Man in high-mannose and hybrid *N*-glycans and to lower extent also complex *N*-glycans. As this lectin does not bind *O*-glycans, it can be used for purification of glycoproteins bearing *N*-glycan structures or specifically for purification of *N*-glycosylated glycopeptides. ConA in combination with GNA (also specific for Man), WGA (specific for GlcNAc and NeuAc predominantly with α2,3-linkage), and SNA lectins (specific for α2,6-linked NeuAc) can be used for purification of glycoproteins bearing almost all *N*-glycans.

Several ways for lectin affinity purification of glycoproteins can be used. The lectins can be directly coupled to a matrix such as agarose beads, activated silica, or magnetic beads and can be used for purification and fractionation of glycoproteins as well as glycopeptides [348, 352]. Furthermore, lectins conjugated

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[1] Some of the currently used Fuc-specific lectins include: AAA lectin which is specific for α(1-2)Fuc in *O*-glycans; AAL lectin specific for α(1-3)Fuc in Le^x antigen>core α(1-6)Fuc>core α(1-3)Fuc in *N*-glycans; LCA lectin specific for core α(1-6)Fuc>core α(1-3)Fuc in *N*-glycans; LTA lectin specific for α(1-2) and α(1-3)Fuc in *O*-glycans>Le^x>core α(1-6)Fuc in *N*-glycans [348-350].

to biotin can be used in combination with avidin/streptavidin coupled to a matrix. Still another option is the use of anti-lectin antibodies which can directly coupled to a matrix or protein A/G modified matrix can be used. If sufficient starting material is available serial lectin affinity chromatography can be used and thus even a complex glycan mixture can be separated [348].

Thus lectin affinity chromatography can be used for purification of glycosylated structures. The glycans can be released from the purified structures and analyzed for example by MS. Purification of glycopeptides from a protease digest of a protein further allows the identification of the glycosylation sites [348].

Furthermore, lectins can be used for lectin affinity blotting which is equivalent to the widely used western blotting using antibodies. Here, lectins are used instead of the primary antibodies for staining of the electroblotted proteins (usually after a previous separation by SDS-PAGE). Next, anti-lectin antibodies conjugated with enzyme producing colorimetric reaction are used to detect the binding. Another possibility is the use of lectins conjugated with biotin in the first step and avidin conjugated to an enzyme in the second step.

All the above mentioned techniques can be complemented by two approaches to further increase the specificity or to prove the specificity of binding/purification. The first is the use of endo- and exoglycosidases. Digestion of purified glycopeptides using the Endo enzymes greatly simplifies the identification of *N*-glycosylation sites by MS (only one saccharide unit remains attached to Asn instead of a great variety of structures). Exoglycosidase digestion can prevent or vice versa, allow the binding of lectins to glycoproteins. The second approach is the use of inhibiting saccharides (haptens) or glycoproteins – thus the specificity or reaction can be verified [348].

Lastly, lectins with more than one saccharide-binding site are used for agglutination of cells or hemagglutination (in the case of red blood cells). Cells with specific glycans on their surface can be agglutinated this way and purified

from a mixture of cells, but the technique is used also for isolation of lectins from samples [276, 348].

2.5.3 Purification and separation strategies for glycans, glycopeptides, and glycoproteins

Several methods can be used for specific enrichment of glycans and glycosylated molecules from complex samples along with lectin-affinity procedures. For removal of salts from the glycan samples, washing of the dried samples can be used (either during the process of the sample preparation let say in microtube or in the case of subsequent MALDI analysis directly on the MALDI plate). However, the high solubility of glycans in water can result in loss of the sample. Another simple way for removal of contaminants is dialysis [353].

Other basic techniques can be used for purification of carbohydrates (or glycopeptides) and their enrichment. Speaking of chromatographic techniques, gel filtration can be used. However, it separates molecules depending on their size and thus does not discriminate between carbohydrates and glycosylated molecules and the a-glycosylated structures. Similarly, reversed-phase liquid chromatography is not specific enough for separation of carbohydrates from peptides and proteins. Another method, called HILIC standing for hydrophilic interaction chromatography, is also used [353]. In this case, a hydrophilic matrix is used for retention of hydrophilic molecules (carbohydrates) in the hydrophobic solution. Silica derivatized with different groups is used for this purpose; the modifications include amine, amide, or sulfobetaine groups [354].

Yet another method with low specificity used for purification of sialylated (acidic) glycopeptides utilizes titanium dioxide (or ZrO_2) chromatography. Alkaline phosphatase digestion has to be performed prior the enrichment step, as TiO_2 binds also the phosphorylated peptides [355]. The resulting sample can be used for identification of glycosylated sites and glycan structures [356].

Glycans released from the proteins should be further purified before any other analysis. Solid-phase extraction (SPE) is widely used, especially with graphitized carbon (active charcoal) as the separation medium. Graphitized carbon was successfully used for purification of oligosaccharides from different samples including biological samples or reaction mixtures [353]. The interaction is based on hydrophobicity but the planarity of the carbohydrate molecules plays an important role in interaction with the carbon layers as well [357]. For low volume samples, disposable pipette tips or spin columns pre-packed with graphitized carbon (but also other media) were used [353, 358].

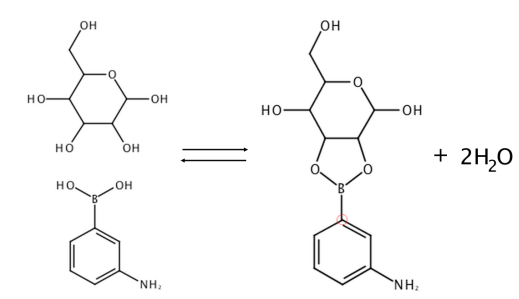

Figure 14. Reaction of saccharides with boronic acid.

Several modifications of the solid phase have been used in the enrichment of glycans and glycosylated molecules. 100-times improvement of detection limits can be reached using immobilized boronic acid (in the range of femto- and attomoles). In this approach, cis-diol groups of Man, Glc, or Gal are involved in formation of heterocyclic diesters with the boronic acid under alkaline conditions (Fig. 14). The immobilized carbohydrates/glycopeptides are then released by acidification of the solution. Recently, functionalization of magnetic beads with boronic acid brought the possibility to perform the reaction under native conditions or incorporate it into an automatic workflow [359].

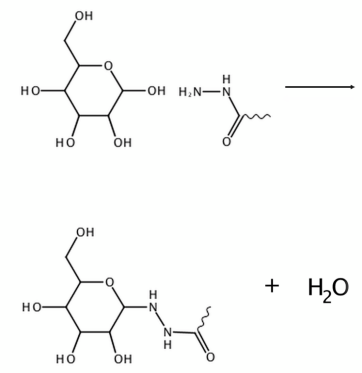

Figure 15. Immobilization of saccharides with hydrazide.

Saccharides and glycosylated molecules can be immobilized on solid phase via hydrazide groups. Again, the cis-diol groups of saccharides are involved. After oxidation of these groups into aldehydes these interact with the hydrazide groups to form hydrazone bonds (Fig. 15). As the reaction is irreversible, the

reaction is used for identification of glycosylation sites. The glycosylated peptides are released from the glycans using *N*-glycosidases. The released peptides contain Asp instead of the originally glycosylated Asn which results in the difference in the mass of the peptide of approximately 1 Da [360].

Similar reactions are used in the method called glycoblotting (it is not related to the electroblotting). Here, aminooxyl groups are used instead of boronic acid or hydrazine groups (Fig. 16). The aminooxyl groups are attached through a linker to either a phosphatidyl-related lipidic carrier molecules containing di-acetylene group (Fig. 17) or an acrylamide-derivative. In the first case, monomers form liposomes in water solutions and are polymerized by UV. The second approach produces water-soluble polyacrylamide polymers. These polymers coated with aminooxyl groups reacted with carbohydrates. The polymerized lipidic particles are separated from solution by centrifugation, while polyacrylamide polymers can be used for

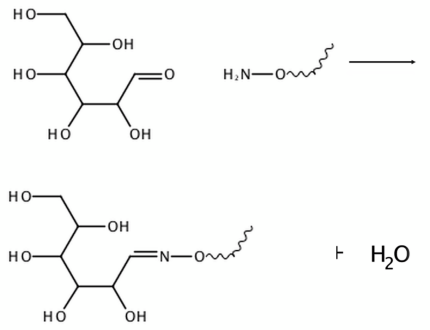

Figure 16. Reaction of carbohydrates with aminooxyl groups.

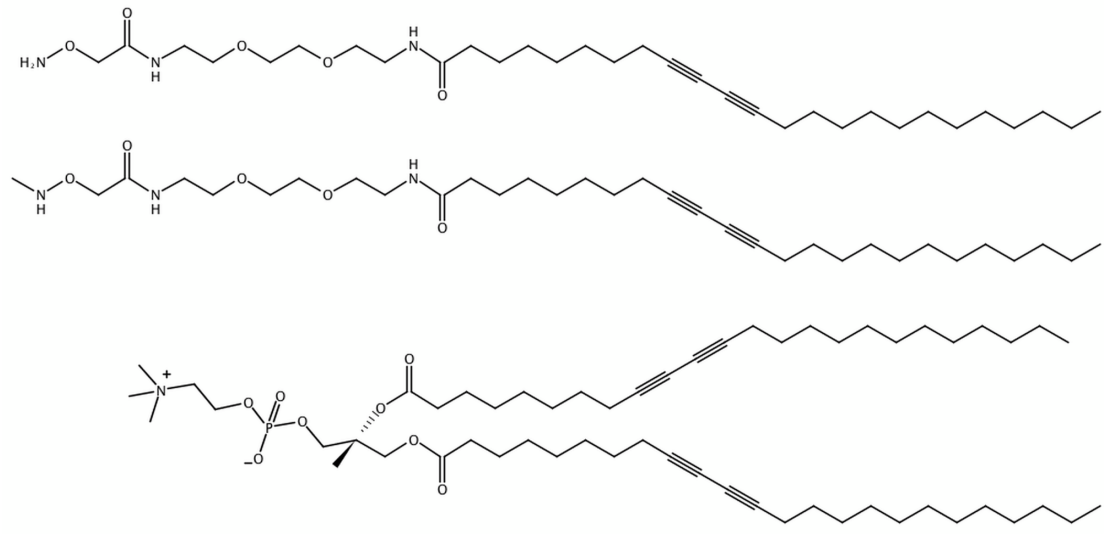

Figure 17. Lipidic carriers with diacetylene groups and terminal aminooxyl groups used for glycoblotting.

example for coating of the SPR chips. The carbohydrates can be released from the polymers by transoximization with chemicals containing aminooxy groups such as benzyloxyamine hydrochlorid [361].

The same interactions and the same matrices as in glycan enrichment can be used for their separation, in combination with the subsequent detection (fluorescence, mass spectrometry). Silica or aminopropylsilica resins have been used for glycan separation, supplemented with graphitized carbon columns and HILIC matrices; as the particle size is an important factor in separation, high-performance liquid chromatography is the commonly used technique [353, 362].

Furthermore, capillary electrophoresis (CE) can be used for separation of glycans. Here, the question of their detection arises (UV detection cannot be used). Labeling of glycans with fluorescent molecules proved to be useful and CE-LIF (laser-induced fluorescence) was shown to be a powerful technique for separation of glycans and their detection [353]. Some of the fluorescent labels used include APTS (8-aminopyrene-1,3,6-trisulphonate) or 2-AA (2-aminobenzoic acid) [363]. Combination of CE-LIF, fluorescent labeling of glycans, and exoglycosidase digestion can be used for sequencing of glycan structures. Furthermore, CE can be coupled to a mass spectrometer electrospray ionization [353].

Still other separation method is called FACE, standing for fluorophore-assisted carbohydrate electrophoresis. In this case, fluorophores such as 2-aminoacridone are attached to the glycans and the conjugates are separated in polyacrylamide gels [364].

2.5.4 Mass spectrometry of glycans and glycopeptides

Mass spectrometry (MS) allows analysis of complex samples in low quantities and high-throughput while providing sufficient structural information

of the glycans as well as glycopeptides. Thus, it is a method of choice in a number of studies. Several methods are available in this regard.

Rapid analysis of complex and low concentration glycan samples is allowed by MALDI (matrix-assisted laser desorption-ionization) usually in combination with TOF/TOF detector (time-of-flight). The method has also another advantage – MALDI produces only singly charged ions, which greatly simplifies the interpretation of the resulting spectra. ESI (electrospray ionization) or CID (collision-induced dissociation) producing also cross-ring fragmentation allow further structural analysis of glycans. However, if complete information on saccharide units linkage is needed, or if unknown linkages are present in the glycans, gas GC-MS (gas chromatography-mass spectrometry) is used [365].

MALDI-TOF permits the analysis of both neutral and acidic (NeuAc-containing) glycans. However, this has to be done separately, with the instrument operating first in positive mode and next, in negative mode. Analysis of sialylated glycans is complicated also by the relative instability of these glycans resulting in loss of the acidic component, especially in acidic solutions. Both these obstacles can be overcome by permethylation. In this reaction hydrogens in the hydroxyl groups are replaced by methyl groups. Permethylated glycans are more stable and the sialylated glycans can be analyzed in positive mode. Permethylation allows also the linkage analysis of the saccharide units; this is done usually using ESI or CID. For small amounts of sample, solid-phase permethylation is the method of choice, as it prevents the degradation of glycans [353].

Tandem (MS/MS) mass spectrometers brought the possibility to fragment selected glycan structures (with a selected m/z ratio) and analyze the resulting ions. As the fragmentation of glycans can occur at several sites of the molecule, generally accepted nomenclature was created by Domon and Costello [366]. Two basic types of fragmentation were described – the cleavage of the glycosidic bond (Fig. 18) and the cross-ring fragmentation.

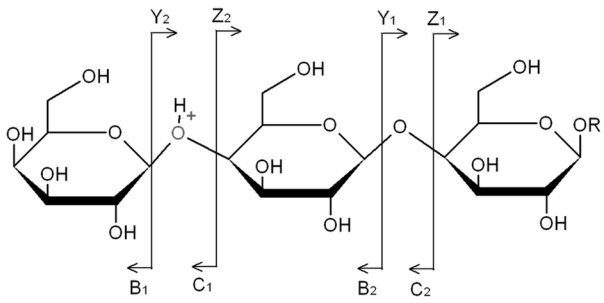

Figure 18. Glycan fragmentation in MS/MS instruments. B/Z and C/Z fragmentation is depicted.

As glycan ions usually contain only one charge in MALDI experiments, only one of the resulting fragments can bear a charge. For each cleavage, two types of ions are produced – ions resulting from the fragment on the reducing end or ions resulting from non-reducing end fragments.

If oxygen is retained on the reducing end side during the cleavage of the glycosidic bond, B and Y fragments are formed (Fig. 18) and depending on the retention of the positive charge either B ion (Fig. 19, upper row) or Y ion is formed (Fig. 19 lower row). Similarly, C and Z fragments are formed, when the oxygen remains at the non-reducing end fragment (Fig. 18) [366].

In both cases, the fragments/ions are numbered in subscript. For the B and C ions, the numbering starts at the glycosidic bond counted from the non-reducing end (B_1, B_2, ... B_n and C_1, C_2, ... C_n ions) and for the Y and Z ions/ fragments it is vice versa (Y_1, Y_2, ... Y_n and Z_1, Z_2, ... Z_n; Fig. 20). The bond between the reducing saccharide and the aglycone part of the molecule in the case of glycopeptides is numbered as zero. For branched structures, the units are numbered the same way but a Greek letter is added: α represents the largest branch, β the second largest, etc. For example, the fragments numbering can look something like $B_{1\alpha}$, $Z_{5\beta}$, $C_{1\delta}$ [366]. When cross-ring fragmentation occurs, again two ions can be formed – the A and the X ion. A represents the part of the molecule at the non-reducing end and the X ion the

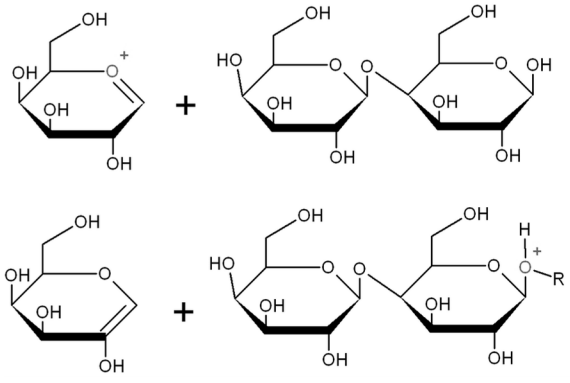

Figure 19. B and Y ions formed during the cleavage of the glycosidic bond in MS/MS experiments.

reducing end fragment, with the same numbering rule as above (Fig. 20). However, the fragmentation of the ring can occur through each bond in the saccharide ring. Therefore, additional numbering is introduced here, showing the bonds, which are fragmented. The numbering of bonds is shown in Figure 20 (blue numbers). Each fragment includes the two numbers of cleaved ring bonds in superscript, the letter showing the type of fragment and the number of the ring in the structure in subscript (such as $^{2,5}A_1$; Fig. 20) [366].

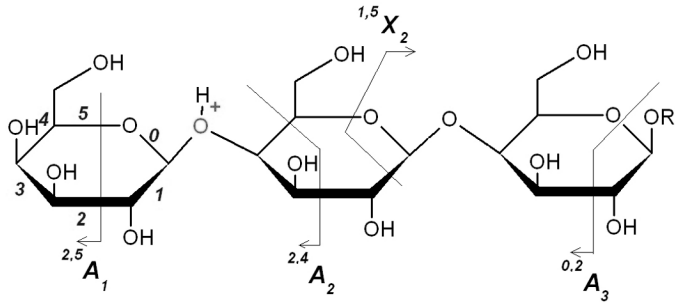

Figure 20. Cross-ring fragmentation designation in MS/MS experiments. Ring bond numbering is depicted. Examples of A and X ions designation are presented.

Liquid chromatography separation of glycans prior the mass spectrometric analysis has also its advantages – a complex mixture of glycans can be analyzed in more depth which allows the identification of higher number of glycan structures. Usually the combination LC-ESI-MS is used; however, LC-MALDI applications are also viable. In this case, small volumes of the LC-separated sample are dropped onto a MALDI plate and prepared for analysis.

The performance of LC separation and the subsequent MS detection of glycans is continually improving through use of smaller particle size, longer column lengths (and thus higher pressures), and the use of new separation matrices [353]. The choice of matrix in MALDI-MS experiments belongs to factors improving (or impairing) the analysis results. For glycan analysis, 2,5-dihydroxybenzoic acid (DHB) is used the most widely. Under normal pressure, a mixture of glycans, glycopeptides, and DHB in methanol/acetonitrile-water solution results in formation of long needles of matrix, containing the glycans in the central region and glycopeptides on the periphery. More homogeneous crystal

can be obtained through recrystallization of the needles in methanol or simply by crystallization under vacuum. Addition of other modified benzoic acids to DHB is also used and is referred to as super-DHB, but other compounds have been also used for glycan analysis with success [353].

Concerns on ionization efficiency of different glycans have been raised as well as the suitability of different ionization techniques for quantification of glycan structures. As shown by Harvey and colleagues [367], the intensity of glycan signals in MALDI corresponds to real abundances of the glycoforms. Furthermore, comparison of glycan quantitation measurements performed on an ESI-QTOF instrument in negative mode and two MALDI-TOF instruments in positive mode gave similar results [367]. However, authors do not exclude the possibility of different ionization efficiency for different glycans. Another study showed an even ionization of carbohydrates with mass above 1000 Da irrespective of their structure and gradual decrease of the intensities of smaller carbohydrates in MALDI-TOF, possibly due to saturation of the detector by the matrix ions [368].

For the analysis of the glycans structure and the linkage of the saccharide units, exoglycosidase digestion can be used – for example with specific galactosidases, mannosidases, neuraminidases, etc. Exoglycosidase digestion can be performed on whole glycoproteins, glycopeptides or the released glycans in microtubes, but on-plate (MALDI plate) digestion is also possible [369].

Derivatization and labeling of glycans have been also utilized for MS experiments besides the permethylation (or the similar method of peracetylation). Some of these methods are intended to introduce a charge into the glycan molecule or to enhance the fragmentation. Derivatization techniques introducing stable isotopes into the molecules have been also used for quantitation of glycans; for example through the introduction of deuterium atoms into the molecule or using specific compounds allowing specific incorporation of isotopes into glycan molecules. Methylation of hydroxyl groups

using methyliodide with zero, one, two, or three deuterium atoms further broadened the research possibilities of multiplexed comparative glycomics [353].

2.5.5 Electron microscopy – lectin and antibody staining

Electron microscopy (EM) allows visualization and localization of glycan structures directly in the sample – in the studied cell or tissue. For some applications in glycobiology, modifications to the regularly used procedures have to be applied. For example bacterial surface glycoproteins are the best preserved during sample preparation by adding a strong oxidant ruthenium red [303, 370].

Another way for bacterial glycoproteins preparation is the Tokuyasu method, where weak fixation (a combination of formaldehyde and glutaraldehyde is used). The fixed sample is then saturated with a cryoprotectant, frozen in liquid nitrogen, and finally cryosectioned. The sections are then lectin affinity stained or immunostained. This method results in higher labeling efficiency thanks to preservation the samples in conditions closer to their native state and through higher penetrability by lectins and antibodies [303, 371].

Fixation of the sample could be further improved by high pressure freezing, where the fixation occurs in several milliseconds. The sample is next chemically fixed, saturated by resin and embedded. However, the method has also its negative – lower permeability of the sections for lectins and antibodies [372]. These techniques, which allow preservation of glycan structures and efficient labeling, can be combined with digestion by glycosidases to process the glycans or their release from the proteins and subsequent lectin affinity staining [303].

The presence of glycosylation machinery enzymes can be also observed by EM using the in situ hybridization method [373]. Again, cryosectioning greatly improves the results through higher permeability of the sections.

2.5.6 Bioorthogonal chemistry

Bioorthogonal reporters are non-native molecules, which do not perturb the organism's internal environment and which can be modified by highly selective reactions with exogenous probes. Usually, they are modified natural molecules bearing only a small reactive group. The organism's enzymes recognize these molecules as “self” and incorporate them into their molecules. However, the resulting biomolecules with the incorporated bioorthogonal reporters are often inactive – and thus, their concentration has to be kept low enough to allow the survival of the organism and high enough to allow the detection of the marked molecules – proteins, lipids, carbohydrates, nucleic acids etc [374].

Depending on the nature of the exogenous probe, the system can be used for different applications. Reaction of bioorthogonal reporters with molecules bearing fluorescent dyes (or quantum dots) can be used for *in vitro* and *in vivo* imaging but also for blotting. Reaction with molecules modified by gold particles or quantum dots can be used for imaging using electron microscopy and again, blotting. In combination with the biotin-streptavidin system, bioorthogonally labelled molecules can be purified using affinity chromatography.

Azidosugars are the best suited for use in glycobiology, which after incorporation into native glycans and glycoproteins are reacted with phosphines or alkynes. The other possibility is to use alkynosugars which can be reacted with azides; however, in this case, only terminal alkynes can be visualized [374].

N-azido carbohydrate derivatives (NAz) currently used are: ManNAz, GlcNAz, GalNAz, and FucNAz. Of these, ManNAz is a derivative, which is further metabolized and the resulting product (SiaNAz, *N*-azidoacetyl sialic acid) is incorporated into glycans [375]. Sialic acid derivatives can be used as well [376]. Such derivatives were used not only for tissues sections imaging but also for *in-vivo* imaging [377]. GalNAz and GlcNAz are usually incorporated into *O*-glycans only [378, 379]. Exogenous metabolic pathways have to be introduced into the targeted organism to incorporate the GlcNAz into *N*-glycans [380]. *N*-azido

carbohydrates were used for labeling and imaging of glycoproteins in a broad range of organisms from nematodes [381] to mice [377].

2.5.7 Inhibition of glycosylation

Rather complementary approach to the above mentioned techniques is the use of glycosylation inhibitors. These can be for example analogues of the substrates used by enzymes participating in glycosylation. Some of the saccharide analogues used in research are the glucose analogues. The choice for this saccharide is obvious – incorporation of these analogues into the *N*-glycan precursor stops the production of *N*-glycans in the cell. However, the results of the use of glucose- or any other saccharide analogue should be carefully reviewed, as these analogues can affect also other processes in the cells.

Tunicamycin is a specific inhibitor of *N*-glycosylation which acts in the very first steps of the process – it inhibits the binding of GlcNAc to dolichol and thus the *N*-glycan precursor cannot be formed. Furthermore, it inhibits also some of the enzymes involved in bacterial glycosylation. Tunicamycin is a very specific inhibitor and a very potent one; its working concentration is in the range of μg/ml with some variation depending on the cell type. It can be used for *N*-glycosylation inhibition in both cell cultures as well as living organisms [382].

Other glycosylation inhibitors include for example nojirimycin and its derivatives (deoxynojirimycin, deoxymannojirimycin) or swainsonine which are analogues of mannose [382-384]. They inhibit α-mannosidases and stop the processing of *N*-glycans. Thus, high-mannose *N*-glycans are produced predominantly which hinders the functionality of glycoproteins in Eukaryotes. Several inhibitors of the *O*-glycosylation process have been also described to date [382].

3 Aims

This thesis aims to bring new knowledge on the glycobiology of ticks and tick-borne pathogens. Therefore, glycans of both ticks and tick-borne pathogens have been studied, as well as glycan-binding proteins (lectins) of ticks.

The individual aims were:

•To analyze the *N*-glycans of tick saliva, namely the saliva *I. ricinus* fed females using glycan-specific lectins and antibodies (lectinoblotting and immunoblotting) and by mass spectrometry (MALDI-TOF/TOF) of enzymatically released glycans.

•To analyze the *N*-glycome of tick salivary glands using lectinoblotting, immunoblotting, and mass spectrometry.

•To identify novel fibrinogen-related proteins and lectins in hemolymph of the ticks *D. marginatus*, *R. appendiculatus*, *R. pulchellus*, and *R. sanguineus*. Furthermore, investigate the *N*-glycosylation of tick lectins.

•To analyze the glycosylation of Lyme borreliosis spirochetes regarding the previously reported glycoproteins of *B. burgdorferi* s.s. Moreover, analyze the glycosylation of other *Borrelia* genospecies.

•To further analyze the *O. moubata* tick lectin Dorin M – its glycosylation and its expression in the OME tick cell lines.

4 Material and Methods

4.1 Material and methods described in published papers

2D electrophoresis (BN-PAGE/SDS-PAGE)

Separation of proteins by Blue native polyacrylamide gel electrophoresis (BN-PAGE) followed by SDS-PAGE is described in 5.5.1.1.

Electroelution

Electroelution of electrophoretically separated proteins is described in 5.5.1.1.

Electron microscopy

Labeling of cryosections using gold-antibody conjugates is described in 5.4.1.1.

Enzymatic deglycosylation

Enzymatic deglycosylation of glycoproteins using Endo H, PNGase A, and PNGase F is described in 5.4.1.1, 5.5.1.1, and 5.5.1.2.

Fluorescence microscopy

Immunofluorescence labeling of tick tissues thin sections is described in 5.5.1.2.

Glycan and peptide purification using spin columns

Purification of peptides using C18 spin columns and of glycans using spin columns with active charcoal is described in 5.5.1.1 and 5.5.1.2.

Hemagglutination activity (HA) analysis and HA inhibition

Hemagglutination activity analysis of tick lectins and the inhibition of this activity are described in 5.5.1.1.

Immune serum preparation

Preparation of mouse immune serum directed against tick proteins is described in 5.5.1.1 and 5.5.1.2.

Immunoblotting and lectinoblotting

Blotting of SDS-PAGE separated proteins using antibodies (immunoblotting) and lectins (lectinoblotting) is described in 5.4.1.1, 5.5.1.1 and 5.5.1.2.

Immunoprecipitation

Purification of proteins using immunoprecipitation is described in 5.5.1.1.

Mass spectrometry analysis of glycans and proteins

Mass spectrometric analysis of proteins (using ESI-QTOF) is described in 5.2.1.1 and 5.4.1.1, and MS analysis of glycans (using MALDI-TOF and MALDI-TOF/TOF) is described in 5.5.1.1 and 5.5.1.2.

Purification of B. burgdorferi outer membrane, periplasmic flagella, and cell cylinders factions

Preparation of outer membrane, and periplasmic flagella with the remaining cell cylinders from the *B. burgdorferi* spirochetes is described in 5.4.1.1.

Schiff staining of glycoproteins

Glycan-specific Schiff staining of glycoproteins is described and used in 5.4.1.1 and 5.5.1.1.

SDS-PAGE and protein staining

Sodium dodecylsulfate (SDS) PAGE separation of proteins and the subsequent Coomassie Brilliant Blue staining of proteins are described in 5.4.1.1, 5.5.1.1, and 5.5.1.2.

Solid-phase permethylation of glycans

Solid-phase permethylation of the purified glycans is described in 5.5.1.1 and 5.5.1.2.

Surface plasmon resonance

Analysis of lectin binding towards carbohydrates using surface plasmon resonance is described in 5.5.1.1.

Ticks

Rearing of ticks used in the experiments is described in 5.5.1.1 and 5.5.1.2.

Tick hemolymph, plasma, SGE, and tissue preparation

Preparation of tick hemolymph, plasma, salivary gland extracts, and other tissue homogenates is described in 5.5.1.1 and 5.5.1.2.

List of the materials and methods, which were used in preparation of the unpublished results and were not included in the papers or manuscripts are listed below.

4.2 Phylogenetic analysis

Oligosaccharyltransferase subunit STT3

Three regions are recognized in the STT3 subunit of the oligosaccharyltransferase. The medium M-region containing the conserved motif WWDYG, 20 amino acids on its N-terminal side and 70 amino acids extending to the C-terminus [210]; thus, the M-region contains 95 amino acids. The N- and C-regions contain the remaining N-terminal and C-terminal parts.

Tick STT3 genes were searched using the blastp and tblastn searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi). In both cases, tick (Ixodida) as well as Acari non-redundant databases were used. For blast searches, the sequence 'WWDYG' and the sequences of *D. melanogaster* STT3 homolog A (GenBank AAF50861) and STT3 homolog B (GenBank AAD27851) were used.

The M-region of the tick STT3 was aligned with the STT3 M-regions from archaebacterium, bacteria (three genes), and higher organisms – chromalveolates (four genes), plants (five genes), excavates (two genes), an amoeba, fungi (four genes), and animals (twelve gene) which included also insects (Table 2). Alignment was performed with the L-INS-i algorithm in the program MAFFT, which is freely available (http://mafft.cbrc.jp/alignment/server/; version 6) [385]. The suitable model (LG+I+G) was estimated using ProtTest (http://darwin.uvigo.es/software/prottest_server.html). Phylogenetic tree was computed using PhyML 3.0 with the selected model (http://www.atgc-montpellier.fr/phyml/) [386] and rendered using Archaeopteryx (http://www.phylosoft.org/archaeopteryx/) [387]. The archaebacterium *Metallosphaera* was used as the common ancestor.

Table 2. List of oligosaccharyltransferases. GenBank accession numbers are included.

organism	ref. No.	domain/ kingdom	organism	ref. No.	domain kingdom
Metallosphaera	ABP95959	Archaea	*Aedes* A	XP_001662467	Animalia
Campylobacter	AAD51383	Bacterium	*Aedes* B	XP_001648748	Animalia
Helicobacter	EES89644	Bacterium	*Anopheles* A	EAA06273	Animalia
Ixodes	EEC17161	Bacterium	*Anopheles* B	EAA08102	Animalia
Babesia	EDO05983	Chromalveolata	*Culex* A	XP_001865872	Animalia
Cryptosporidium	EEA06294	Chromalveolata	*Culex* B	XP_001850247	Animalia
Plasmodium	AAN35757	Chromalveolata	*Drosophila* A	AAF50861	Animalia
Toxoplasma	EEE26920	Chromalveolata	*Drosophila* B	AAD27851	Animalia
Arabidopsis	NP_174675	Plantae	*Gallus* A	CAG31486	Animalia
Oryza	EAY96032	Plantae	*Gallus* B	CAG31623	Animalia
Ostreococcus	CAL54049	Plantae	*Harpegnathor* A	EFN88856	Animalia
Ricinus	EEF47908	Plantae	*Harpegnathor* B	EFN85392	Animalia
Zea	ACN29281	Plantae	*Homo* A	AAH20965	Animalia
Leishmania A	XP_843223	Excavata	*Homo* B	AAL71884	Animalia
Leishmania B	XP_843220	Excavata	*Mus* A	AAH85313	Animalia
Trypanosoma	AAX70184	Excavata	*Mus* B	AAH52433	Animalia
Dictyostelium	EAL64892	Amoeba	*Rattus* A	AAI68223	Animalia
Aspergillus	CAK42956	Fungi	*Rattus* B	AAI66740	Animalia
Candida	EER34400	Fungi	*Salmo* A	ACI33169	Animalia
Pichia	CAY68350	Fungi	*Schistosoma* A	CAX74505	Animalia
Saccharomyces	EEU06314	Fungi	*Schistoma* B	XP_002577965	Animalia
Acyrthosiphon	XP_001943161	Animalia			

Similarly, oligosaccharyltransferase presence was analyzed in *B. burgdorferi* and other spirochetes (non-redundant databases of *Borrelia*, and Spirochetes). The *Campylobacter* pglB (AAD51383) and the conserved sequence WWDYG were used in the searches.

N-glycans modifying enzymes

Catalytic domains of each enzyme were estimated based on the literature; the databases were searched for *N*-acetylglucosaminyltransferases (MGAT1 to MGAT5), galactosyltransferases (B3GT and B4GT), fucosyltransferases (FUT 1 to FUT10, especially FUT6 and FUT8 related to *N*-glycan core-fucosylation), sialyltransferases (SIAT1 to SIAT 10), and other glycosyltransferases.

The already annotated glycosyltransferases sequences in ticks were used in the searches. In addition, the databases were searched for the glycosyltransferases using catalytic domains sequences, which were determined using published literature. MGAT1 and MGAT2 catalytic domains were estimated as the C-terminal 418 amino acids of the proteins [388], catalytic domain of the human MGAT3 was used for search for this enzyme, in the case of MGAT4 the bovine enzyme catalytic domain was used [389] and in the case of MGAT5 the enzyme from the rat [390]. Galactosyltransferases were searched using the *Drosophila* Brainiac protein [391]. Sialyltransferase family catalytic domain was used for identification of sialyltransferases in ticks [392] and the conserved motifs of the fucosyltransferase family for the search for FUT enzymes [213].

α2,3-sialyltransferase gene expression

α2,3-sialyltransferase (EEC07371) specific primers were designed for both RT-PCR and real-time RT-PCR detection of the sialyltransferase mRNA (Table 3). 150 bp product is amplified using these primers corresponding to the region of the α2,3-sialyltransferase mRNA within the positions 166 to 315. Primers were purchased from Generi Biotech (Czech Republic).

IRE/CTVM19 cells [393] were resuspended in the medium, pellted by centrifugation, washed, and mRNA was purified with Oligotex mRNA Mini Kit (Qiagen, Germany) according the instructions of the manufacturer. cDNA was prepared with oligo-dT18 primer using RevertAid First Strand cDNA Synthesis Kit (Fermentas, Thermo Fisher Scientific, Canada). Sialyltransferase gene

fragment PCR amplification was optimized for several commercial thermostable DNA Polymerases – PPP premix containing Taq Purple Polymerase (Top-Bio, Czech Republic), GreenTaq Polymerase (Promega, WI), Taq Polymerase (Invitrogen, CA), and Accu Polymerase (Ampliqon, Denmark). Annealing temperature optimization was performed in the range of 49°C to 56°C.

Table 3. Primers used for detection of sialyltransferase expression.

primer	sequence
IxSiaTransf F	GCT GTG GAC GGC TCG GAC TG
IxSiaTransf R	CAG GGC AGT CGT GGC GTA CC

4.3 Glycosylation in *Borrelia burgdorferi*

Growth of B. burgdorferi in the presence of tunicamycin

Tunicamycin (Sigma-Aldrich, MO, USA) was used as a general inhibitor of *N*-glycosylation, as it is able to inhibit the attachment of *N*-acetylglucosamine to the dolichol and thus stops the formation of the *N*-glycan precursor [382]. Tunicamycin was prepared as a sterile solution with concentration of 50 μg/ml PBS; this stock solution was used for preparation of the Barbour-Stoenner-Kelly (BSK-H) *Borrelia* growth medium (supplemented with rabbit serum) (Sigma-Aldrich) reaching the final concentration of 0.5 μg tunicamycin/ml of medium. *B. burgdorferi* s.s. (strain B31) were grown in tunicamycin supplemented BSK-H similarly to usual growth conditions [394].

Growth of B. burgdorferi in the presence of Triton X-100

The detergent removing the outer membranes, Triton X-100 (Sigma-Aldrich), was used to assess the ability of borrelia to attach host- and environment-derived glycoproteins to their outer membranes.

In first of the strategies, borrelia grown under normal conditions were used [394]. These were harvested by centrifugation, and one group was incubated in sterile PBS containing 0.1% Triton X-100 for 30 minutes at 37°C. The spirochetes without outer membranes were again pelleted, resuspended in sterile BSK-H medium (supplemented with rabbit serum), and allowed to grow for two weeks at 35°C. Control experiments were performed using bacteria grown under same conditions without the Triton X-100 treatment.

Another strategy used borrelia grown under normal conditions; these were harvested and allowed to grow in sterile BSK-H supplemented with rabbit serum and 0.1% Triton X-100 for two weeks at 35°C.

After two weeks, borrelia from both experiments were harvested by centrifugation and OM and PF fractions were prepared as published [394].

2D (IEF-SDS-PAGE) electrophoresis

Periplasmic flagella (PF) fraction prepared from *B. burgdorferi* s.s. (strain B31) was used for 2D gel electrophoresis and subsequent immunoblotting. For the first dimension separation, ReadyStrip IPG Strips with the pI range of 3 to 10 (Bio-Rad, PA, USA) were used according to instructions of the manufacturer (see below for short description), and after the isoelectrofocusing step, the proteins were separated in SDS-PAGE electrophoresis.

150 µl of sample (75 µg of proteins) in rehydration buffer (8 M urea, 2% CHAPS, 50 mM dithiothreitol, 0.2% BioLyte 3/10, 0.001% Bromophenol Blue; Bio-Rad) were prepared per each strip. 125 µl were pipetted into each lane in the rehydration tray (Bio-Rad) and the strip was gently placed on the solution. The strips were overlaid by mineral oil and rehydration was allowed overnight at room temperature. The rehydrated strips were used for IEF separation in a PROTEAN IEF focusing tray (Bio-Rad) using PROTEAN IEF System with 7 cm focusing tray (Bio-Rad). Focusing was performed with a total of 10000 Vh with 4000 V end voltage at 20°C (rapid ramp program).

After IEF, the strips were subjected to separation in second dimension by SDS-PAGE. Each strips was equilibrated in 2.5 ml of solution containing 6M urea, 0.375 Tris (pH 8.8), 2%SDS, 20% glycerol, 2% dithiothreitol for 10 minutes followed by incubation in 2.5 ml of solution containing 6M urea, 0.375 Tris (pH 8.8), 2%SDS, 20% glycerol, 2.5% iodoacetoamide for 10 minutes at room temperature. The strips were placed over a 12.5% polyacrylamide gel overlaid with 1% agarose (melted in 1x SDS running buffer). Protein marker (New England Biolabs, MA, USA) was run in the second dimension gel together with each strip.

After separation in second dimension, the gels were either stained in Page Blue Protein Stain (Fermentas, Thermo Fisher Scientific, Canada) or electroblotted to PVDF membrane. The blotted proteins were detected by SYPRO Ruby Gel Stain (Sigma-Aldrich) and the membranes were subsequently used for immunoblotting using mouse polyclonal serum directed against B31 and CB53 *B. burgdorferi* s.s. whole cell lysates.

Lectin affinity purification of glycoproteins

Lectin affinity purification was performed using all three lectins available in the Qproteome Sialic Glycoprotein Kit (Qiagen) – SNA, WGA, and MAA II. Short protocol based on manufacturer's instructions is provided below.

50 µl of outer membrane fraction of *B. burgdorferi* s.s. (strain B31) containing approximately 150-300 µg of total proteins were complemented with 500 µl of Binding buffer were used for each purification. Flow-through, wash, and eluate fractions were collected. Elution buffers provided by the manufacturer were used. All fractions were acetone precipitated, samples were centrifuged at 12 000 x g for 10 minutes (4°C), the pellet was dried and resuspended in ddH_2O.

4.4 FRePs in ticks

Dorin M in OME cell lines

The OME/CTVM21 (OME21) line was maintained in L-15 (Leibovitz) medium with 20% fetal bovine serum, 2 mM L-glutamine, 10% tryptose phosphate broth, and 1% antibiotic/antimycotic mixture (penicillin, streptomycin, amphotericin B; Sigma-Aldrich) at 28°C [395]. Cells were passaged at two-weekly intervals. Cells from one culture tube were harvested and centrifuged. The medium was collected and proteins were acetone-precipitated. The cell pellet was resuspended in lysis buffer containing SDS and sonicated. The cell lysate and the proteins from the medium were SDS-PAGE separated under reducing conditions. After separation, the proteins were electroblotted onto PVDF membranes and immunoblotted using anti-Dorin M rabbit serum [276] or rabbit serum directed against *I. ricinus* lectin [275]. The color reaction was developed using HRP-conjugated anti-rabbit antibodies (Vector Laboratories, CA, USA). Complementation of potential non-specific reaction with medium proteins was performed by pre-incubation of the immune sera with L15 medium.

Identification of FReP genes in ticks

Whole fed *D. marginatus* female was homogenized in DEPC-treated water with addition of RNALater (QIAGEN) in TissueLyser II (QIAGEN) at 30 Hz for 2 minutes in a pre-chilled adaptor. The homogenate was clarified by centrifugation and mRNA was isolated using Oligotex mRNA Mini Kit (QIAGEN).

Oligo-dT18 primer was used for preparation of cDNA using RevertAid First Strand cDNA Synthesis Kit (Fermentas). cDNA from *H. impeltatus* and *H. punctata* was obtained from N. Rudenko and M. Golovchenko (Institute of Parasitology, BC ASCR & Faculty of Science, USB, České Budějovice).

Degenerate primers (Dorin D F, Dorin D R1, Dorin D R2) specific for the fibrinogen domain of FRePs were used for amplification of the corresponding gene fragments from tick cDNAs at different temperatures (Table 4) [277]. New primers (Dm 1, 2, 3 F and R) were designed using the newly acquired sequences and were used for search of FRePs in ticks (Table 4).

The DNA fragments were separated using 1% agarose gel electrophoresis and the PCR products of acceptable quality were cut out the gel and the DNA was purified using Silica Bead DNA Gel Extraction kit (Fermentas) and sequenced.

Table 4. Primers used for the detection of FRePs in ticks.

primer	sequence
Dorin D F	GAG GCN TTY GCA AAY GTN GAR TGG
Dorin D R1	CAC RTT ATT TCC TAR CCA RTA YTC
Dorin D R2	CTT CAT CTC AAC RTT NGG RTA
Dm 1 F	GAA TAC TGG ATC GGC AAC CA
Dm 1 R	TGG TTG CCG TAC CAG TAT TC
Dm 2 F	CTA CTT TTA CAA GAA GTG GGC TG
Dm 2 R	GAC TCA TTG CTT TGT CTG AGG T
Dm 3 F	GAA CCC AAT CTA CGA CTT TTA C
Dm 3 R	GTC AGT TGA ATC CGA AGT GAC AT

5 Results

5.1 Tick glycosyltransferases

5.1.1 Unpublished results

5.1.1.1 Bioinformatic search for tick glycosyltransferases

Production of glycans is dependent on the presence of the appropriate enzymatic machinery in the cell and thus on the presence of corresponding genes in the organism's DNA. Therefore, the published tick genome (*I. scapularis*, GenBank ABJB000000000) and the known DNA sequences (NCBI) were searched for sequences similar to the known enzymes, participating in the *N*-glycosylation of proteins. The presence of several different enzymes (see below) in the tick genome was assessed. All these enzymes are associated with *N*-glycosylation.

First, the presence of the gene encoding the oligosaccharyltransferase (OST) has been investigated, as the *N*-glycosylation process is absolutely dependent on the ability of the cell to transfer the glycan precursor from dolichol or dolichol-like molecules to the future glycoprotein; in the case of eukaryotic organisms, the STT3 subunit of the OST has been analyzed.

Next, the ability of ticks to produce hybrid and complex *N*-glycans was tested (the ability to express high-mannose glycans is basically allowed by the presence of the corresponding OST). The databases were searched for already identified *N*-acetylglucosaminyl transferase genes (MGAT1, MGAT2, MGAT3, and MGAT5) which are necessary for the attachment of GlcNAcs to the *N*-glycan core.

The possibility of further modifications of glycans was assessed by searching for galactosyltransferases (attaching terminal galactoses to GlcNAcs) B3GT and B4GT, fucosyltransferases (attaching fucose in both α1,3- and α1,6-linkages) such as FUT1, FUT6, or FUT8, and finally genes encoding sialyltransferases (attaching

Figure 21. The Ixodes ricinus STT3 gene, GenBank EEC17161. The catalytic domain is highlighted in the box. Three conserved motifs are further marked by red lines: the WWDYG motif, the D/E/X-X-D motif, and the DK motif.

terminal sialic acid in α2,3-, α2,6-, and α2,8-linkages) SIAT1, SIAT6, SIAT8, and SIAT10.

In each case, additional search using the tick enzymes and conserved catalytic domains of the enzymes was performed in non-redundant Ixodida and Acari databases.

Tick oligosaccharyltransferase (OST)

The 95 amino acid catalytic domain (medium, M-region) of the yeast (*S. cerevisiase*, NP_011493) STT3 was used for the search of the tick (Ixodida) and Acari (this contains both tick and mites) sequences. The catalytic domain contains the highly conserved motif WWDYG [210].

The blastp (protein database search using amino acid sequence) and tblastn (translated nucleotide database search using amino acid sequence) searches identified only one protein – the putative oligosaccharyltransferase from *I. scapularis* (GenBank, EEC17161). STT3 genes in other tick species were not found.

Furthermore, searches were performed with WWDYG motif alone. However, these searches did not reveal any other STT3 gene.

The *I. scapularis* STT3 (Fig. 21) contains the combination of three conserved motifs which can be found in Eukaryotes – the WWDYG motif (525-529), the D/E/X-X-D motif (574-576), and the DK motif (592-599) [211].

Next, the *I. scapularis* STT3 M-region was aligned with the STT3 M-regions from other organisms, including

animals, insects, fungi, plants, an amoeba, excavates, chromalveolates, bacteria, and archaebacterium. Isoforms A and B were used for animal and insect genes. Furthermore, two *Leishmania* STT3 genes (from the same strain), which showed higher similarity to either the A or B isoform (*Drosophila* and mouse enzymes) in the blastp search were used.

The only identified tick STT3 shows high similarity to insect STT3 B isoforms as well as to vertebrate B isoforms. It is clearly more closely related to insects as to other animals (Fig. 22).

The obtained alignments were used for construction of the phylogenetic tree describing the relationship of the STT3 M-regions of the different organisms. Two STT3 subfamilies are clearly distinguished – the STT3 A and STT3 B subfamilies (Fig. 23). The A subfamily is formed by STT3 A isoforms from vertebrates and insects. Amoeban (*Dictyostelium*) and the *Schistosoma* STT3 CAX74505 are also included in this subfamily. In this regard it is noteworthy to underline the *Schistosoma* CAX74505 gene in this subfamily as it is annotated as the B isoform in the NCBI database. Overall higher conservation of the STT3 A M-region in all organisms is demonstrated by lower branch length values in the STT3 A subfamily compared to the B subfamily (not shown). Overall, evolution of the two isoforms early in the evolution of Eukaryotes is observable (Fig. 23).

The STT3 B subfamily is again represented by the vertebrate and insect B isoform enzymes, the fungal enzymes, and the *Schistosoma* XP_0025777965 protein. The *Ixodes* STT3 is also included in this subfamily – therefore, the STT3 B name will be used further on for this enzyme. The Ixodes STT3 B M-region seems to be closely related to the protein from insects.

The bacterial STT3 M-regions form a clearly distinguished group. Furthermore, two *Leishmania* enzymes form the same strain are both grouped together with the *Trypanosoma* enzyme and do not seem to be evolutionary related to the separation of the isoforms A and B in higher Eukaryotes (Fig. 23).

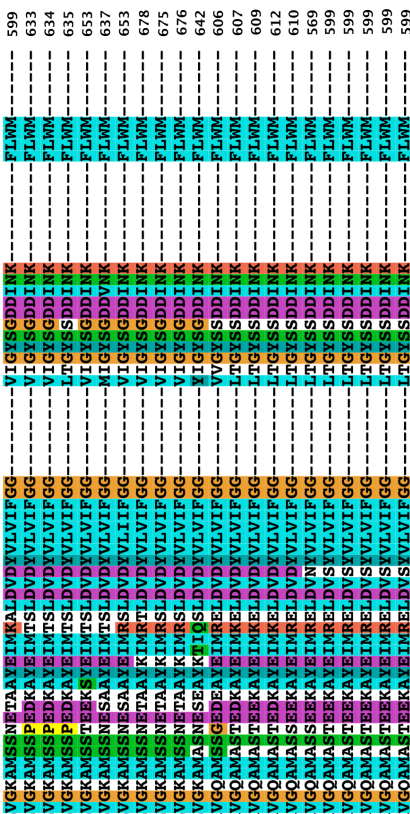

Figure 22. Alignment of the middle (catalytic) domain of the STT3 proteins from different organisms. Note the highly conserved catalytic site WWDYG.

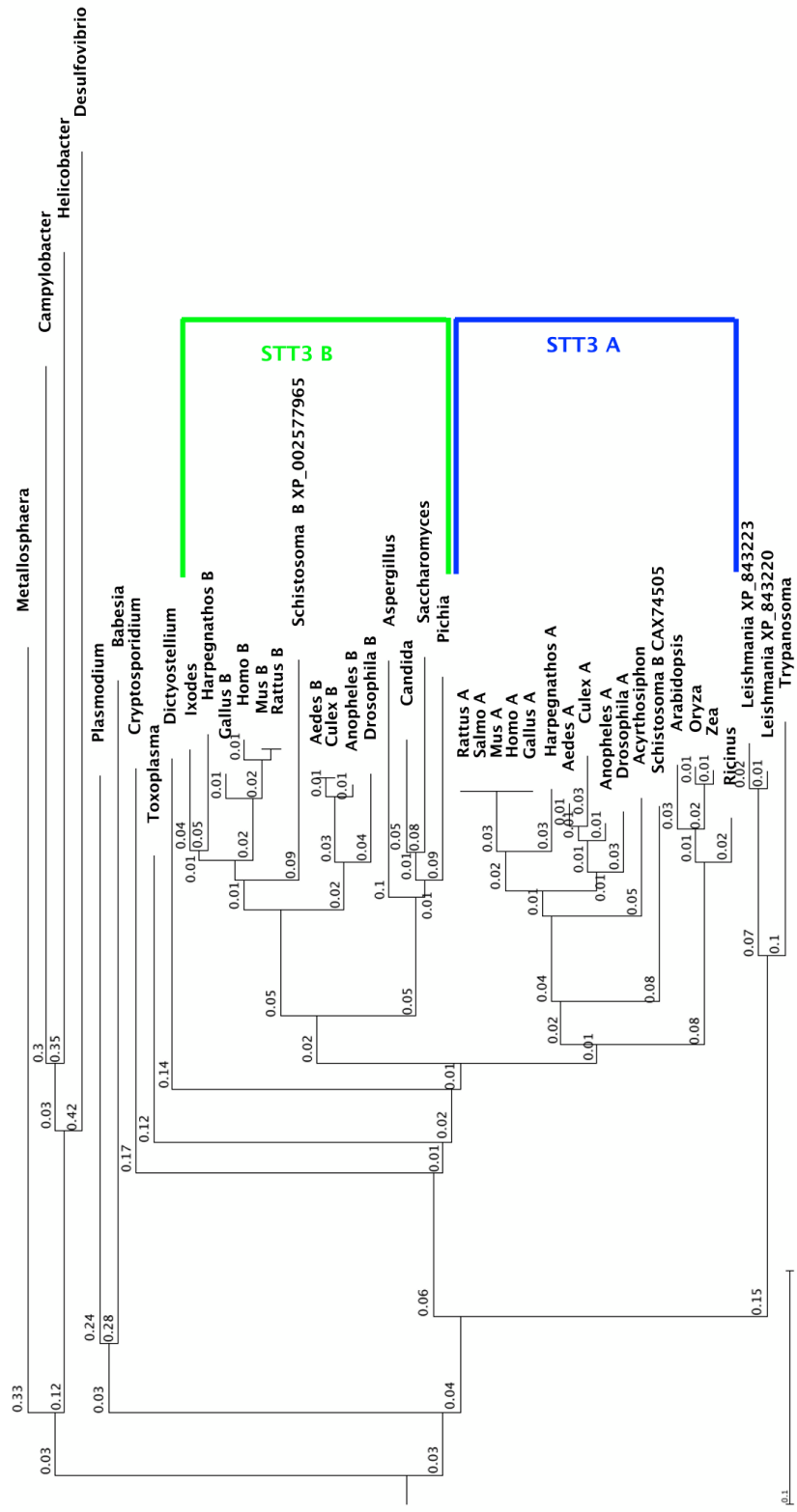

Figure 23. Evolutionary tree of the M-region of the STT3 proteins from different organisms. The animal STT3 A (blue) and B (green) isoforms are marked. Branch length values are shown for each branch. In the cases, where the branch length is not shown, its value is below 0.01.

Tick N-glycans modifying glycosyltransferases

MGAT1

Several tick MGAT1 proteins were annotated in databases: EEC11859 (432 amino acids), EEC16808 (423 amino acids), EEC05957 (593 amino acids), and EEC04392 (394 amino acids, does not contain the N-terminus of the protein); all these proteins were identified in the *I. scapularis* tick. Human α1,3-mannosyl-glycoprotein-β1,2-*N*-acetylglucosaminyltransferase catalytic domain [388] was used for search of other not annotated tick MGAT1 proteins. However, these searches did not reveal any other MGAT1 enzymes. .

MGAT2

Two correctly annotated *I. scapularis* proteins can be found in databases, namely EEC18519 (263 amino acids) and EEC15707 (178 amino acids, does not contain the N-terminus). Human α1,6-mannosyl-glycoprotein-β1,2-*N*-acetylglucosaminyltransferase enzyme catalytic domain [388] was used for search of other tick MGAT2 proteins with no further results.

MGAT3

Three correctly annotated MGAT3 proteins from *I. scapularis* were found in databases: EEC12692 (450 amino acids), EEC14328 (451 amino acids), and EEC01072 (377 amino acids). Further searches using the catalytic domain from the human β1,4-mannosyl-glycoprotein-β1,4-*N*-acetylglucosaminyltransferase [396] did not reveal novel MGAT3 proteins in other tick species.

MGAT4

The databases contain one MGAT4 from *I. scapularis*, EEC19486 (427 amino acids). No further enzymes were found using the blastp and tblastn searches using the catalytic domain of the bovine α1,3-mannosyl-glycoprotein-β1,4-*N*-acetylglucosaminyltransferase [389].

MGAT5

Databases do not contain an MGAT5 tick enzyme. The blastp and tblastn searches were performed using the catalytic domain of α1,6-mannosyl-glycoprotein-β1,6-*N*-acetylglucosaminyltransferase from rat [390]. The protein XP_002415819 was identified as the only MGAT5 enzyme present in databases (however, it is annotated as *N*-acetylglucosaminyltransferase VI).

Galactosyltransferases

Databases contain a number of identified or putative galactosyltransferases. Altogether 30 entries can be found which are assigned as galactosyltransferases (EEC01137, EEC01937, EEC01974, EEC02567, EEC03594, EEC04002, EEC04837, EEC04861, EEC05002, EEC05674, EEC06975, EEC07893, EEC09539, EEC10461, EEC10678, EEC11126, EEC12031, EEC12774, EEC13720, EEC14416, EEC14521, EEC14655, EEC15673, EEC16731, EEC18814, EEC19022, EEC19298, EEC19299, EEC19350, EEC19754), seven as β1,3-galactosyltransferases (EEC03890, EEC09950, EEC13701, EEC15036, EEC16000, EEC16656, EEC20348), four as β1,4-galactosyltransferases (EEC01356, EEC06029, EEC06755, EEC10831), and one is annotated as xylosyl-β1,4-galactosyltransferase (EEC05368). All of these proteins were identified in *I. scapularis*.

For searching of databases for other not annotated galactosyltransferases, the amino acid sequences of these proteins were used as well as of the *Drosophila* protein Brainiac (AAF45918) which was identified as the member of galactosyltransferase family [391]. Four hypothetical proteins from *I. scapularis* were found: EEC00965, EEC06028, EEC09359, EEC12600.

Sialyltransferases

Two tick sialyltransferase (ST) proteins can be found in databases: putative α2,3-sialyltransferase (EEC07371) and α2,6-sialyltransferase (ADO17789). Furthermore, a conserved hypothetical protein EEC07911 contains the sialyltransferase family conserved domain. All three proteins come from the *I.*

scapularis tick. The conserved amino acid sequence of sialyltransferase family catalytic domain was used in search of other ST genes [392] with negative results.

Fucosyltransferases

Similarly to galactosyltransferases, a number of tick fucosyltransferases can be found in databases. All the below mentioned proteins were identified in *I. scapularis*. Three of the proteins were designated also with their function: α1,3-fucosyltransferase (EEC05695, homologue of the human FUT6) and two α1,6-fucosyltransferases (EEC04286, EEC04076, homologues of the human FUT8). Furthermore, two O-fucosyltransferases are present (EEC20385, EEC01803), both similar to the human α1,2-fucosyltransferase FUT1. Other proteins are named as fucosyltransferases; these are similar to human α1,3/4-fucosyltransferase FUT3 (EEC05777), human α1,3-fucosyltransferase FUT4 (EEC10765, EEC20141), human α1,3-fucosyltransferase FUT6 (EEC04854, EEC06499), human α1,3-fucosyltransferase FUT7 (EEC05232, EEC06309, EEC16923), human α1,3-fucosyltransferase FUT9 (EEC06971, EEC19232), and human α1,3-fucosyltransferase FUT10 (EEC17289, EEC20251).

Blastp and tblastn searches using the sequences of known tick and mammalian fucosyltransferases as well as the conserved motifs of fucosyltransferases [213] revealed further hypothetical proteins, which are similar to the human α1,2-*O*-fucosyltransferase FUT2 (EEC12110, EEC17162, EEC17623), human α1,6-fucosyltransferase FUT8 (EEC04309, EEC07543, EEC07876), human α1,3-fucosyltransferase FUT9 (EEC18134), and human α1,3-fucosyltransferase FUT11 (EEC01068). Database searches did not reveal any fucosyltransferase in the other tick species.

Other glycosyltransferases

Other glycosyltransferases with not clear function are present in the tick genome. For example, there are the glycosyltransferase domain-containing

proteins EEC08312 and EEC10418 which bears similarity to glucoside xylosyltransferase (bee XP_624895 and mouse BAE27879) and xylosyltransferases EEC20592 and EEC05368, glycosyltransferase EEC03387 similar to β1,3-*N*-acetylglucosaminyltransferase (*Xenopus*, AAI58333) or the *N*-acetylgalactosaminyltransferase EEC08781.

5.1.1.2 Expression of α2,3-sialyltransferase in I. ricinus cell line

The presence of sialyltransferases in *I. ricinus* genome suggests possible expression of the enzymes and consequently the production of sialylated glycans by ticks. Therefore, α2,3-sialyltransferase (EEC07371) specific primers were designed for both RT-PCR and real-time RT-PCR detection of the sialyltransferase DNA.

Oligo-dT18 primer was used for preparation of cDNA from IRE19 cell line. Next, sialyltransferase was detected using PCR. Amplification of the gene fragments performed well using PPP premix, GreenTaq Polymerase, Taq Polymerase, and Accu Polymerase (data not shown).

The reaction worked well at temperatures from 49°C to 53°C (Fig. 24) as well as 56°C (data not shown). Specificity of the reaction was confirmed by sequencing of the PCR product.

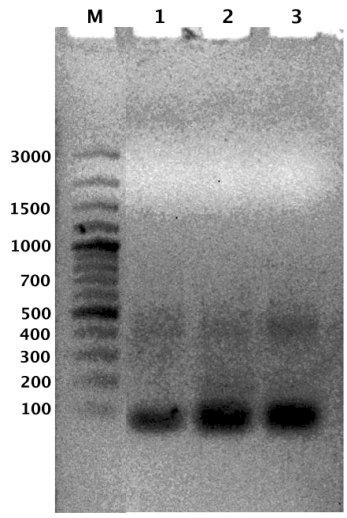

Figure 24. PCR amplification of sialyltransferase gene from IRE19 cDNA. Annealing temperatures used were 49°C (1), 51°C (2), and 53°C (3).

5.2 Glycosylation in ticks

The presence of glycosyltransferases, which allow the *N*-glycosylation of tick proteins lead to an attempt to identify the different *N*-glycans, present in ticks. The *N*-glycans of two tick proteins, isolated in native form from ticks, were

analyzed – the lectin Dorin M from the soft tick *O. moubata* (Chapter 6.2.1.1) and hemelipoglycoprotein (also a possible lectin) from *D. marginatus* (Chapter 6.5.1.1).

Regarding the host immunity against tick feeding, tick tissues and molecules in contact with host's blood are the most interesting – it is possible, that insect/plant type of glycans can start the immune reaction, and host type glycans can on the other hand hinder it. Therefore, tick saliva and salivary gland extracts (SGEs) have been chosen for glycomic analysis (Chapter 5.2.2.1).

More specifically, the presence of sialylated glycoproteins in tick saliva and salivary proteins was studied as well (Chapter 5.2.2.2). A combination of mass spectrometry and electron microscopy was studied in this study.

Furthermore, MS analysis of *N*-glycans from tissues of the *I. ricinus* is currently under way (MSc. thesis of Zuzana Šimonová, supervisor: Prof. Libor Grubhoffer, co-supervisor: Ján Štěrba). *N*-glycomic profiles of the *I. ricinus* life-stages (eggs, larvae, nymphs, adult males and females) is also analyzed (Ph.D. thesis of Jarmila Dupejová, supervisor: Prof. Libor Grubhoffer, co-supervisor: Dr. Marie Vancová).

5.2.1 Published results

5.2.1.1 Deciphering Dorin M glycosylation by mass spectrometry

A 640 kDa lectin (37 kDa subunits) was isolated from *O. moubata* hemolymph and named Dorin M. This lectin was recognized by lectins ConA, LCA, and weakly by WGA suggesting the presence of *N*-glycans with terminal mannose, core-fucose and possibly terminal GlcNAc or sialic acid. Chemical deglycosylation reduced the mass of subunits by approximately 5 kDa. Three classical (Asn-Xxx-Ser/Thr) sequons and non-classical (Asn-Xxx-Cys) *N*-glycosylation sequon are present in its amino acid sequence [276, 277, 279].

All three classical sequons are modified. Two glycans (Asn41, Asn129) are high-mannose type with nine mannose residues and the third (Asn171) contains

four to five mannoses and a core-fucose. The non-classical site (Asn187) was not found to be glycosylated.

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Deciphering Dorin M glycosylation by mass spectrometry

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The soft tick, *Ornithodoros moubata*, is a vector of several bacterial and viral pathogens including *Borrelia duttoni*, a causative agent of relapsing fever and African swine fever virus. Previously, a sialic acid-specific lectin Dorin M was isolated from its hemolymph. Here, we report on the complete characterization of the primary sequence of Dorin M. Using liquid chromatography coupled to mass spectrometry, we identified three different glycopeptides in the tryptic digest of Dorin M. The peptide, as well as the glycan part of all glycopeptides, were further fully sequenced by means of tandem mass spectrometry (MS^2) and multiple-stage mass spectrometry (MS^3). Two classical N-glycosylation sites were modified by high-mannose-type glycans containing up to nine mannose residues. The third site bore a glycan with four to five mannose residues and a deoxyhexose (fucose) attached to the proximal *N*-acetylglycosamine. The microheterogeneity at each site was estimated based on chromatographic behavior of different glycoforms. The fourth, a non-classical N-glycosylation site (Asn–Asn–Cys), was not glycosylated, probably due to the involvement of the cysteine residue in a disulfide bridge.

Keywords: glycosylation, tandem mass spectrometry, liquid chromatography, lectin, soft tick

5.2.2 Submitted manuscripts and manuscripts in preparation

5.2.2.1 N-glycan core fucosylation is a common structural feature in tick salivary glycoproteins

Mass spectrometric analysis of *I. ricinus* glycans was performed using MALDI-TOF/TOF. *N*-glycan samples from fed female saliva and salivary gland extracts (SGEs), unfed female SGEs, (unfed) male SGEs were prepared using a

combination of PNGase A and PNGase F enzymes ensuring the release of all types of *N*-glycans. Furthermore, MS analysis of PNGase F-released glycans was performed. Complementary experiments with lectinoblotting, western blotting using anti-α1,3-core-fucose antibodies were performed. Finally, the glycans were localized in tick salivary glands using fluorescence microscopy.

Complex glycans with terminal HexNAcs were found in all samples in high amounts as well as abundant core-fucosylated structures. Core-fucose seems to be present in both α1,3- and α1,6-linkages. Moreover, changes in the abundance of different glycan types have been observed between the fed female SGEs and unfed female or male SGEs, and between the fed female saliva and fed female SGEs. These results are prepared for submission to a peer-reviewed scientific journal.

5.2.2.2 Uptake and incorporation of sialic acid by the tick Ixodes ricinus

Sialic acid was detected in salivary gland extracts and gut of fed and partially fed *I. ricinus* females using MALDI-TOF/TOF mass spectrometry. Glycoproteins containing NeuGc were localized in salivary gland and gut thin sections using electron microscopy. Furthermore, differences in the sample structure and glycan labeling were observed in electron microscopic experiments in dependence of the sample preparation methods. These results were submitted to Parasitology Research.

5.3 Glycosylation in other Arthropods

5.3.1 Unpublished results

The presence of complex glycans in tick saliva in high abundance brought the question if they originated from the parasite or are the remnants of the glycoproteins from the blood meal. To further address this question, *N*-glycans

from another blood-feeding arthropod, the kissing bug *Triatoma infestans* (insect), were analyzed.

Freshly fed *T. infestans* adults were homogenized and *N*-glycans were released and prepared for MALDI-TOF/TOF analysis as described above. In comparison to MS spectra of *N*-glycans from tick saliva or SGE, the spectra of *T. infestans* glycans (Fig. 25) were much more simple and the high-mannose structures $HexNAc_2Hex_{3\text{-}9}$ predominated (*m/z* 1171.6, 1357.7, 1579.8, 1783.9, 1988.0, 2192.1, 2396.2). Two core-fucosylated high-mannose glycans were also present – $HexNAc_{2\text{-}3}Hex_2dHex$ (*m/z* 1141.6 and 1345.7). Only one hybrid type glycan was observed with composition of $HexNAc_3Hex_3dHex$ (*m/z* 1590.8).

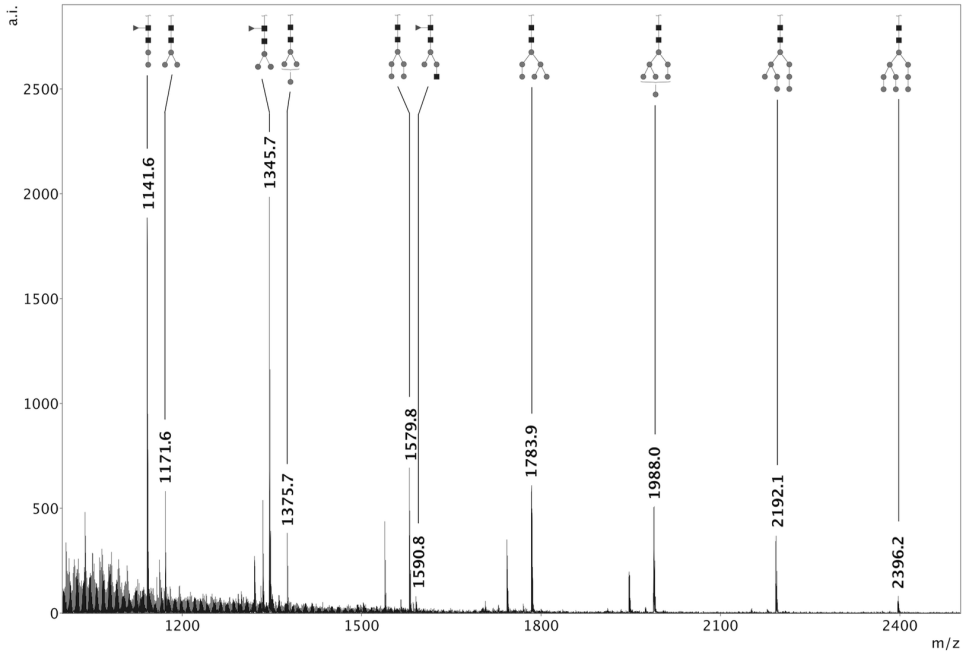

Figure 25. MALDI-TOF/TOF spectrum of N-glycans purified from T. infestans bug. The identified glycan structures are depicted. Peaks corresponding to contamination by a series of hexoses are present.

5.4 Glycosylation in *Borrelia burgdorferi*

The presence of glycosylation was investigated in Lyme disease spirochetes *Borrelia burgdorferi*, mainly concerning the published suggestion of *N*-glycosylation of two surface proteins (OspA and OspB) and of the flagellin FlaA (Chapter 6.3.1.1). Furthermore, lectin affinity purification of glycoproteins and lectinoblotting was performed on *Borrelia* samples (Chapter 6.3.2) including *B. burgdorferi* s.s., *B. afzelii*, and *B. garinii* genospecies.

5.4.1 Published results

5.4.1.1 Flagellins and Outer surface proteins from Borrelia burgdorferi are not glycosylated

The presence of *N*-glycosylation was proposed in *B. burgdorferi* spirochetes in three proteins previously: OspA and OspB [304, 305], and FlaA [307]. Therefore, *B. burgdorferi* B31 cells were fractionated and the resulting outer membrane and periplasmic flagella fractions were analyzed by lectinoblotting with negative results. OspA, OspB, FlaA, and FlaB proteins were isolated from these fractions and digested using trypsin, GluC, and their combination. The resulting peptides were analyzed by nano-LC-MS.

The proteins contain altogether 22 putative *N*-glycosylation sequons; peptides containing 20 of them were identified in MS spectra. However, these peptides did not carry glycans. Complementary staining of borrelia thin sections with lectins and anti-rabbit serum showed strong presence of serum-derived molecules on the surface of spirochetes. Thus, glycosylation of borrelia proteins was not confirmed and tight binding of serum-derived glycoproteins was suggested. The original paper is reproduced with permission.

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Flagellin and Outer Surface Proteins from *Borrelia burgdorferi* Are Not Glycosylated▽

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We investigated the presence of glycoproteins in *Borrelia burgdorferi*. We did not find any evidence for glycosylation of the major outer membrane proteins OspA and OspB or the structural flagellar proteins FlaB and FlaA. We suggest that glycoproteins present on the surface of *B. burgdorferi* may be tightly bound culture medium glycoproteins.

Lyme disease is a tick-borne disease caused by the spirochete *Borrelia burgdorferi* and is a chronic disease characterized by skin, joint, heart, and neurological sequelae. Mammalian receptors which bind Lyme disease spirochetes, as well as bacterial ligands which promote cell interactions, are believed to be critical to the infectious process (6). Sambri and colleagues (15) reported on the glycosylation of two outer membrane (OM) proteins from *B. burgdorferi*, OspA and OspB, which stained following periodate oxidation and were sensitive to enzymatic deglycosylation by peptide *N*-glycosidase F (PNGase F; EC 3.5.1.52; New England Biolabs), which specifically cleaves between the innermost GlcNAc and Asn residues. These outer surface proteins were shown to be lipoproteins (3) which play a role in colonization and survival in the tick (12, 14) and which are implicated in later stages of human disease (1). Evidence for glycosylation has been presented for another borrelial protein, FlaA, which is associated with periplasmic flagella (PF), and posttranslational modification was also suggested for the structural protein of flagella, FlaB (5, 8). This protein was shown to be stained following periodate oxidation and by the digoxigenin (DIG)-labeled lectins *Sambucus nigra* agglutinin and *Galanthus nivalis* agglutinin. The protein was also sensitive

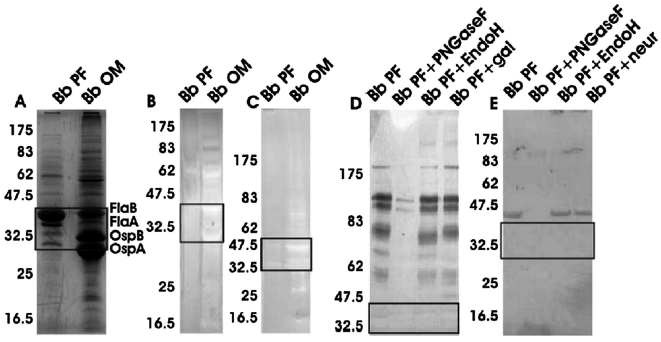

FIG. 1. Detection of glycoproteins in *Borrelia burgdorferi* PF and OM fractions. Crude PF and OM preparations were obtained by modification of the method described by Ge et al. (8). The OM fraction was obtained following Triton X-100 extraction of cells. After removal of bacterial cells by centrifugation, the OM fraction was collected from the supernatant by ultracentrifugation. PF were obtained by shearing of the Triton X-100-extracted cell pellets following their resuspension in PBS and vortexing in the presence of 1-mm-diameter glass beads. Crude PF preparations were obtained from the supernatant fraction by ultracentrifugation. (A) Coomassie brilliant blue-stained SDS-PAGE gel; (B) DIG glycan detection; (C) *G. nivalis* agglutinin lectin reactivity; (D) *S. nigra* agglutinin lectin reactivity; (E) *M. amurensis* agglutinin lectin reactivity. Numbers at the left are molecular masses (in kilodaltons). Bb, *B. burgdorferi*; EndoH, endoglycosidase H; gal, β-galactosidase; neur, neuraminidase.

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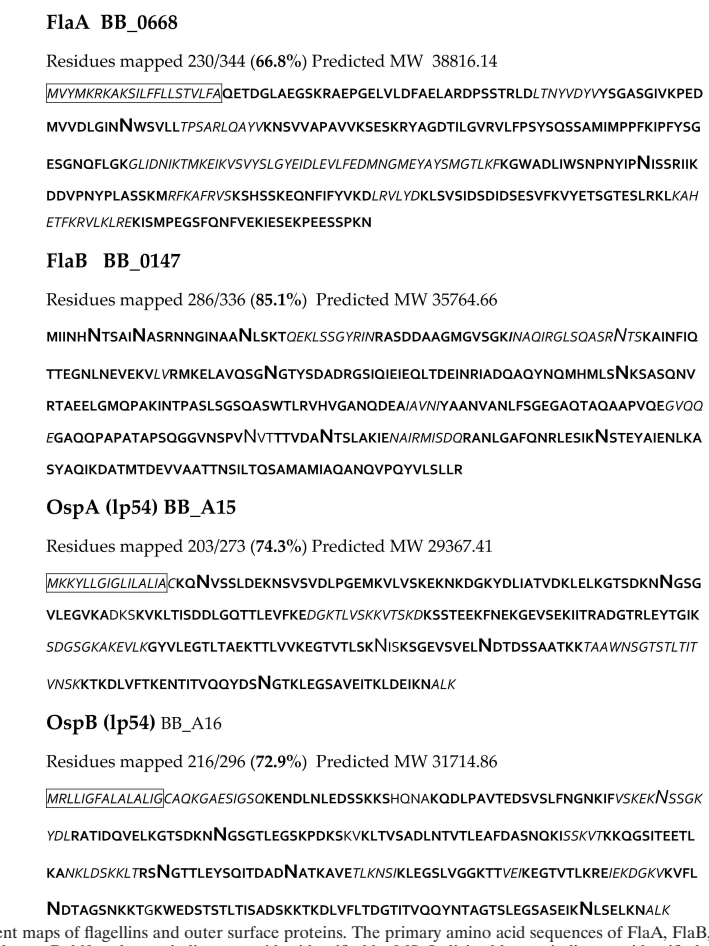

FIG. 2. Assignment maps of flagellins and outer surface proteins. The primary amino acid sequences of FlaA, FlaB, OspA, and OspB from *B. burgdorferi* B31 are shown. Boldface letters indicate peptides identified by MS. Italicized letters indicate unidentified peptides. Sites of potential N-linked glycosylation are indicated by increased font size. Boxed sequences indicate predicted signal sequences of FlaA, OspA, and OspB. MW, molecular weight.

to enzymatic deglycosylation by PNGase F, suggesting the N-linked attachment of glycan (8). Finally, Coleman and Benach (7) demonstrated that the immunodominant 41-kDa antigen recognized by sera of Lyme disease patients is the FlaB protein. Interestingly, the mass observed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels of 41,000 Da is considerably larger than the predicted mass of FlaB (35,742 Da), which may be due to either anomalous migration on SDS-PAGE gels (17) or posttranslational modification. The identification of flagellin structural proteins as glycoproteins has also been described for other spirochete species (2, 11, 21), although the precise structures of these glycans and mode of attachment remain to be determined.

We undertook this study to characterize the OspA, OspB, FlaA, and FlaB proteins from *B. burgdorferi* strain B31. Surprisingly, we were unable to demonstrate N-linked glycosylation by both indirect staining and detailed structural analysis. However, several other proteins from membrane preparations were stained as glycoproteins and were labeled by lectins. We provide preliminary evidence indicating that these proteins may be culture medium components which appear to bind tightly to the surfaces of Lyme borreliosis (LB) spirochetes.

Both crude PF and OMs were prepared from *B. burgdorferi* according to a modified method for isolation of periplasmic flagella using Triton X-100 (8) and analyzed by SDS-PAGE (Fig. 1A). The cells were washed three times with 10 mM phosphate-buffered saline (pH 7.2) and three times with 0.1 M phosphate buffer (pH 7.4) prior to isolation. The identification of OspA, OspB, FlaA, and FlaB from OM and PF preparations was made following gel band extraction, tryptic

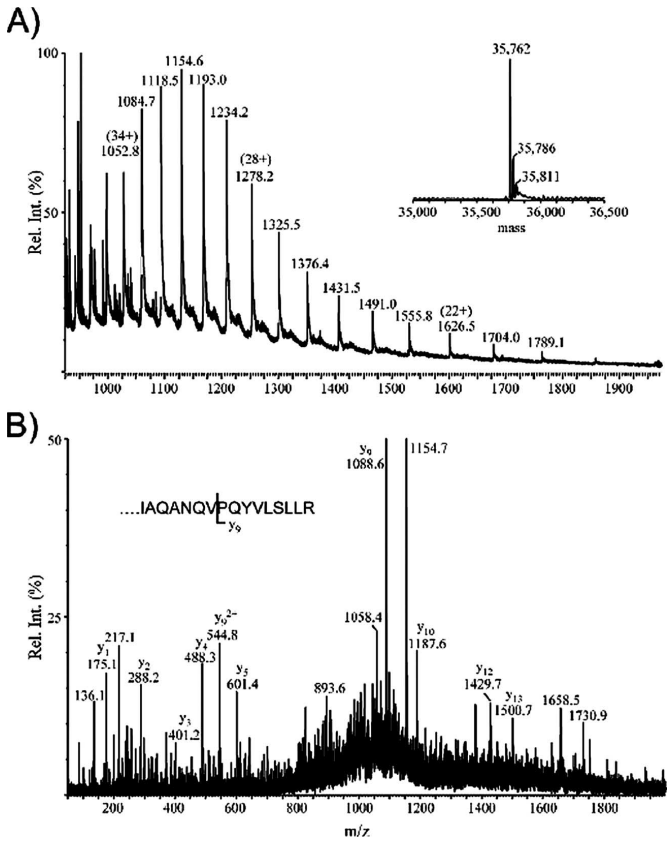

FIG. 3. Electrospray ionization (ESI)-MS analysis of FlaB flagellin protein from *B. burgdorferi*. (A) ESI-MS of the intact protein. The protei solution was infused into the ESI source of a Q-TOF2 mass spectrometer at a flow rate of 1 μl/min. The deconvoluted molecular mass profile c this protein is presented in the inset. (B) MS/MS analysis of the $(M + 31H)31^+$ ion at *m/z* 1,154.6. The collision offset was 30 V. The *y* fragmen ions are indicated in the spectrum, and the corresponding portion of the C-terminal amino acid sequence from FlaB flagellin is presented in th inset. Rel. Int., relative intensity.

digestion, and peptide assignment by mass spectroscopy (MS) analysis (Fig. 1A). Similar results were obtained for *Borrelia afzelii* and *Borrelia garinii* OM and PF preparations (data not shown). To determine if these proteins were glycosylated, we first used periodate oxidation (a Roche DIG glycan detection kit and a Pierce GelCode glycoprotein staining kit according to the manufacturers' instructions) for detection of glycoproteins in PF and OM samples. We did not observe any positive reaction with the proteins of interest (FlaA, FlaB, OspA, OspB) (Fig. 1B), although a number of higher-molecular-weight proteins appeared positive by this reaction. Using a Roche DIG glycan differentiation kit, which utilizes lectin binding, we observed no positive reaction of FlaA, FlaB, OspA, and OspB proteins with the lectins *G. nivalis* agglutinin (Fig. 1C), *S. nigra* agglutinin (Fig. 1D), and *Maackia amurensis* agglutinin (Fig. 1E) or with *Arachis hypogaea* (peanut) agglutinin and *Datura stramonium* agglutinin (data not shown). However, using the *Sambucus nigra* agglutinin lectin, w stained a number of high-molecular-mass protein band (47,000 to 200,000 Da) in both OM and PF preparations (Fig 1D). A single protein with a molecular mass of 45,000 Da i both PF and OM preparations was stained by the *M. amurensi* agglutinin lectin. All samples were subjected to enzymatic deg lycosylation using PNGase F (Fig. 1D), endoglycosidase H (E(3.2.1.96), β-galactosidase, and neuraminidase (all purchase from New England Biolabs) (data not shown), and we failed t observe any shift in the molecular weights of proteins OspA OspB, FlaA, and FlaB. It should be noted that the sensitivity o this approach would detect changes only where the glycosyla tion process contributed a minimum of 5 to 10% of the protei mass. Again, several of the *S. nigra* agglutinin-reactive protein with molecular masses higher than 47,000 Da were sensitiv to enzymatic deglycosylation with PNGase F, as was th 45,000-Da *M. amurensis* agglutinin-reactive protein (Fig. 1I

and E). The specificity of staining reactions was verified in each case by using positive and negative controls as described by the manufacturer (data not shown).

Next, a concerted effort was made to map the OspA, OspB, FlaA, and FlaB proteins. Following excision of each protein band from an SDS-PAGE gel and proteolytic digestion, peptides were analyzed by MS. A number of classic eukaryotic N-linked sequon consensus sequences (*N*-X-S/T) (10) are present in the primary amino acid sequences as indicated in Fig. 2 (FlaA, two sites; FlaB, nine sites; OspA, five sites; and OspB, six sites). Of these, three are bacterial N-glycosylation sequons (D/E-X-*N*-X-S/T) (13) (one in FlaB and two in OspA). Nanovolume liquid chromatography–MS and MS/MS analysis performed by multiple tryptic digestion and GluC and tryptic/GluC double digestion was successful in assigning 79.5% of OspA peptides, 86.8% of OspB peptides, 92.8% of FlaB peptides, and 71.8% of FlaA peptides (Fig. 2). Peptides containing 20 of the 22 N-linked sequons from these proteins were identified and shown not to carry an N-linked glycan modification. Peptides containing the remaining two putative *N*-glycosylation sites (FlaB and OspB) were not identified. No evidence for peptides with masses that were anomalous to those of any of the four proteins was obtained, indicating that these proteins are not glycosylated with either N- or O-linked glycans.

The observed mass of FlaB from SDS-PAGE gels had been shown to be approximately 5,000 Da higher than that predicted by the primary sequence. To confirm that we had not missed any sites of modification by peptide analysis, we next determined the intact mass of FlaB from the PF. Infusion of the PF protein sample into the mass spectrometer resolved a single major protein species with a mass of 35,760 ± 10 Da, which corresponds to the predicted mass of FlaB (35,742 Da). The mass discrepancy of 18 Da is most likely attributable to the oxidation of a methionine residue during sample preparation (Fig. 3). Signals corresponding to the FlaA, OspA, and OspB proteins were not found in the mass spectra, probably due to the low levels of ionization of these proteins.

It has been previously shown that the surface of *B. burgdorferi* is able to bind a number of lectins (9, 18, 20). DIG glycan detection and lectin labeling indicated that OM and PF preparations contained reactive proteins which did not correspond to FlaA, FlaB, OspA, or OspB. Immunogold labeling of *B. burgdorferi* cells grown in BSK-H medium demonstrated that the surfaces of spirochete cells were reactive to both *S. nigra* agglutinin lectin (Fig. 4A) and anti-rabbit serum (Fig. 4B). As complete BSK-H medium contains rabbit serum, we next investigated whether the source of these glycan-positive proteins was from the BSK-H medium. Examination of the medium alone by SDS-PAGE analysis and glycan-*S. nigra* agglutinin detection revealed that some of the OM-positive proteins corresponded to glycan-positive medium components (Fig. 4C). An immunoglobulin heavy-chain protein of approximately 83,000 Da has previously been shown to be coisolated from complete BSK-H medium (16) and may correspond to one of the *S. nigra* agglutinin-positive proteins identified in this study in both BSK-H medium and OM preparations (Fig. 4C).

In conclusion, FlaA, FlaB, OspA, and OspB proteins purified from LB spirochetes grown under standard laboratory conditions in BSK-H medium are not glycosylated. Obviously,

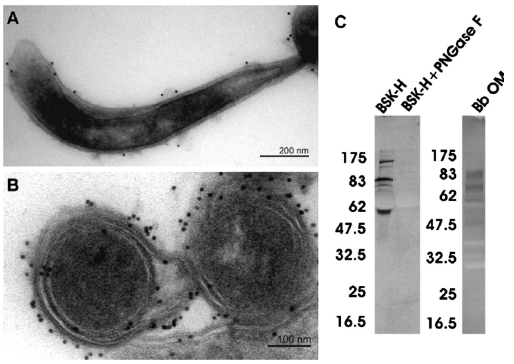

FIG. 4. Surface-associated binding of medium glycoproteins to *B burgdorferi*. (A) Cryosection and *S. nigra* agglutinin lectin labeling of *B burgdorferi* cells. (B) Cryosection of *B. burgdorferi* cells labeled with anti-rabbit serum. (C) *S. nigra* agglutinin lectin staining of diluted BSK medium and *B. burgdorferi* (Bb) OM. Numbers at the left are molecular mass markers (in kilodaltons).

this does not rule out the possibility that the organism may be able to glycosylate proteins while growing in vivo in an arthropod vector or mammalian host, and the recent development of dialysis membrane chamber implants in a rat peritoneum for the in vivo cultivation of LB spirochetes (4) may facilitate such studies. While earlier work indicated that the borrelial OspA, OspB, and FlaA proteins carry N-linked glycans, the extensive structural analysis performed in this study indicates that N-linked glycosylation did not occur. Moreover, bioinformatics analysis of six spirochete genomes revealed no evidence of a conserved STT3 (*pglB*) oligosaccharyltransferase, which is the enzyme required for the transfer of N-linked glycans from a lipid carrier to an asparagine residue of the protein (19). Still, we cannot rule out the possibility of the presence of a novel glycosyltransferase in the genome of *B. burgdorferi* and the possibility of its activation at different stages of the complex life cycle of LB spirochetes.

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5.4.2 Further studies on the presence of glycosylated structures in *Borrelia*

The above mentioned study did analyze in more depth the glycosylation of four *B. burgdorferi* proteins. However, it does not rule out the possibility of protein glycosylation in Lyme disease spirochetes. Therefore, this issue was studied in more depth.

Blastp search did not reveal any similarity to other bacterial STT3 enzymes nor the WWDYG sequence is present was found in the *B. burgdorferi* s.s. strain B31 (with the sequenced genome) or any other known protein sequences in *Borrelia* or spirochetes.

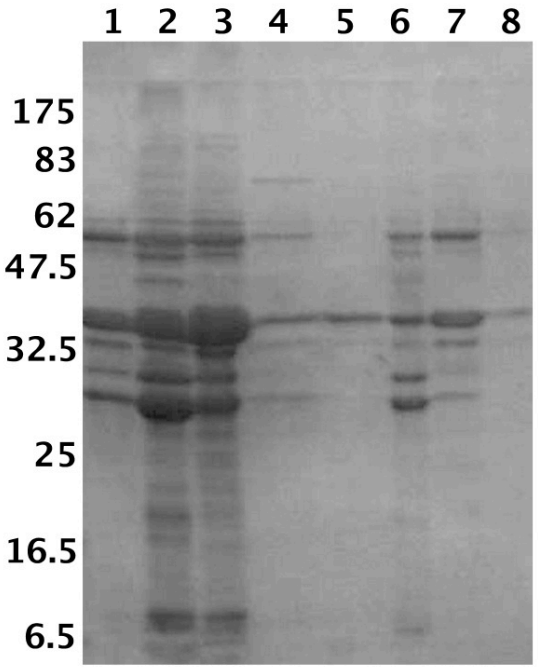

Figure 26. Effect of tunicamycin on molecular weights of borrelial proteins. Proteins from Borrelia burgdorferi s.s. grown in the presence (lanes 5-8) or absence of tunicamycin (1-4) in the growth media were fractionated into four fractions – outer membranes were removed by two subsequent washes with detergent (OM1 – lanes 2, 6, OM2 – lanes 3, 7), next periplasmic flagella were released (lanes 1, 5) from the cell cylinders (lanes 4, 8).

Tunicamycin, which is an inhibitor of *N*-glycosylation, was added to the culture media used for the growth of *Borrelia*. No obvious shift in molecular weight of the isolated proteins was observed in *B. burgdorferi* B31 grown with or without the addition of tunicamycin (Fig. 26). Also, lectin staining persisted in the protein samples from *B. burgdorferi* grown in the presence of tunicamycin (data not shown).

Separation of Bb OM fraction on 15% polyacrylamide gel showed the presence

of two bands in the area corresponding to the FlaA (positively identified by MS). These two protein bands were cut off the gel, electroeluted and analyzed by lectinoblotting (SNA lectin). However, both proteins gave negative lectin staining (data not shown).

The periplasmic flagella fraction (PF) prepared from *B. burgdorferi* s.s. was used for 2D electrophoretic separation. The proteins were first separated in a pI range from 3 to 10. The electrofocused proteins were separated on a 12.5% polyacrylamide gel using SDS-PAGE. Coomassie Brilliant Blue-stained gel

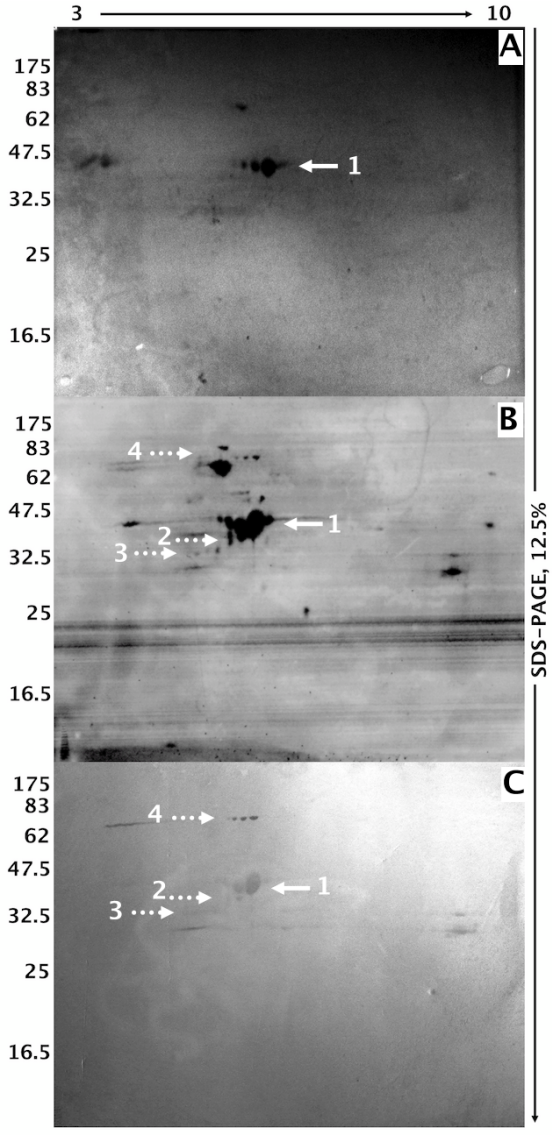

Figure 27. 2D electrophoresis of B. burgdorferi PF fraction. The first dimension (IEF) was performed in the range of pI from 3 to 10, the second dimension separation was performed using 12.5% polyacrylamide gel SDS-PAGE. A – CBB staining of the gel, B – SYPRO Ruby staining of the electroblotted proteins, C – immunoblotting using anti-B31 and anti-CB53 reactive sera. Arrow (1) shows FlaB protein isoforms, dashed arrows show isoforms of other borrelia proteins – FlaA (2), OspB (3). Proteins were marked based on the MS identification from 1D gels.

revealed several isoforms of the FlaB protein (Fig. 27A, arrow 1), while the other proteins were barely visible. The proteins were electroblotted and stained using SYPRO Ruby protein stain (Fig. 27B). Besides the FlaB protein (arrow 1), further proteins were also visible, including isoforms of the FlaA protein (dashed arrow 2) and OspB (dashed arrow 3). Isoforms of an unidentified protein were also visible (dashed arrow 4). The electroblotted proteins were stained with sera from mice immunized using B31 and CB53 spirochetes (Fig. 27C). All four proteins and their isoforms were stained which confirms their borrelia origin.

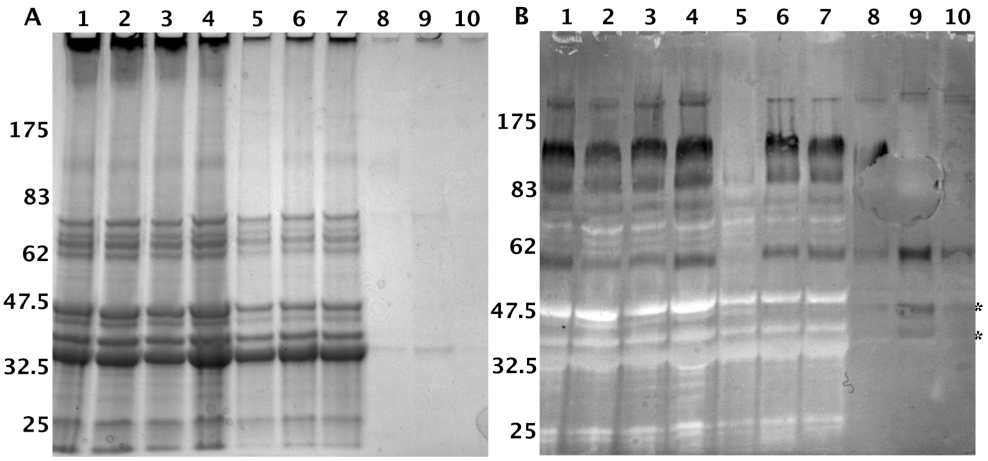

Figure 28. Sialylated structures from B. burgdorferi s.s. OM fraction were purified using QIAGEN Qproteome Sialic Glycoprotein Kit. Lectins SNA (lanes 2, 5, 8), WGA (lanes 3, 6, 9), and MAA II (lanes 4, 7, 10) were used. 1 – OM fraction in bind buffer, 2-4 – flow-through, 5-7– wash, 8-10 – eluate. A – CBB staining of the electrophoretically separated proteins, B – SNA affinity staining.

The sialylated glycoproteins from the outer membrane (OM) fraction of borrelia were purified using lectin-affinity chromatography (Qproteome Sialic Glycoprotein Kit, Qiagen). SNA-positive proteins with Mw in the size of the Fla and Osp proteins have been purified using this kit (Fig. 28); however, the mass spectrometry analysis did not bring sufficient data for their identification.

An SNA-positive band appeared also in the flow-through of the Bb OM sample after the Qproteome Sialic Glycoprotein Kit purification in another experiment, while no such protein appeared in the sample itself or in the eluate

(data not shown). The MS identification of this protein was not successful either and it did not appear in further experiments.

Besides the analysis of glycosylation in *B. burgdorferi* s.s., glycosylation was studied also in other borrelia – *B. afzelii* (strain B023) and *B. garinii* (strain Fuji). Glycoproteins were detected in these bacteria by Schiff staining and lectin staining. Interestingly, some difference have been observed in the glycoprotein staining patterns, for example in the SNA lectin staining (Fig. 29) or in MAA II lectin staining (data not shown).

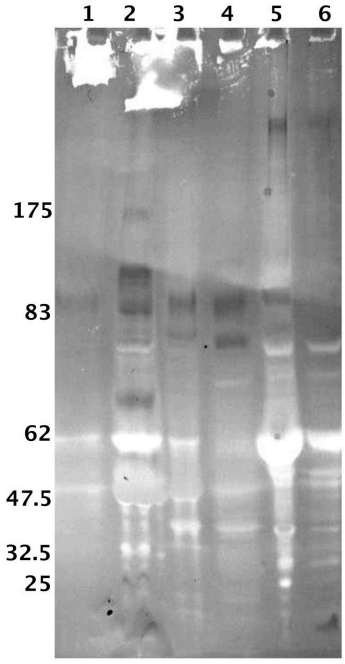

Figure 29. SNA staining of PF (lanes 1, 3, 5) and OM (lanes 2, 4, 6) fractions from B. burgdorferi s.s. (lanes 1, 2), B. afzelii (lanes 3, 4), and B. garinii (lanes 5, 6).

Enzymatic deglycosylation of *B. afzelii* and *B. garinii* PF and OM fractions was performed using three enzymes – PNGase F, and Endo H removing *N*-glycans and neuraminidase (releasing sialic acid). Similarly to *B. burgdorferi* s.s. proteins, enzymatic deglycosylation of samples from these two strains did not result in the shift of proteins molecular weights (deglycosylation of *B. afzelii* proteins shown on Fig. 30).

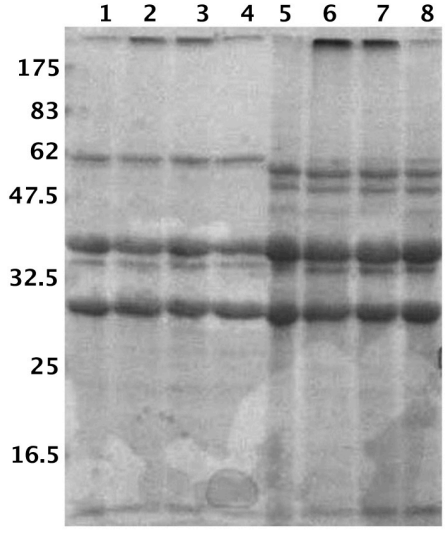

Figure 30. Enzymatic deglycosylation of B. afzelii PF (lanes 1-4) and OM (lanes 5-8) proteins. PNGase F (lanes 2, 6), Endo H (lanes 3, 7), and neuraminidase (lanes 4, 8) were used.

Furthermore, glycan staining was performed on *B. burgdorferi* s.s. (B31), *B. afzelii* (B023), and *B. garinii* (Fuji) deglycosylated samples. Staining using DIG Glycan Detection kit as well as GNA, MAA, and PNA lectins did not reveal any obvious differences in the staining patterns of the deglycosylated samples (data not shown).

Next, *B. burgdorferi* s.s. spirochetes were grown in medium with the addition of 0.1% Triton X-100 (which is used for removing of the outer membrane in the fractionation process). Furthermore, spirochetes grown under normal conditions were treated with Triton X-100 (PBS with 1% detergent) and the resulting cells (cell cylinders with periplasmic flagella) were let to grow in BSK-H (data not shown). In both cases, the lectin and Schiff staining did not reveal glycoproteins in these samples except the common contamination resulting from immunoglobulins from the serum [397].

5.5 FReP proteins/lectins in ticks

The presence of lectins (carbohydrate-binding proteins) was investigated in ticks along with the analysis of glycans and glycosylation. Fibrinogen-related proteins were identified in hemolymph of the ticks *D. marginatus*, *R. appendiculatus*, *R. pulchellus*, and *R. sanguineus* by immunostaining (Chapter 6.5.1.2). RT-PCR analysis of tick cDNAs and subsequent sequencing revealed partial sequences of three FReP proteins sequence in *D. marginatus*, *H. punctata*, and *H. impeltatum*. (Chapter 6.5.2.2). Another tick hemolymph protein showed reactivity with antibodies directed against FRePs event hough its sequence does not contain the fibrinogen domain (Chapter 6.5.1.1). Furthermore, expression of the lectin Dorin was showed in *O. moubata* cell lines (Chapter 6.5.2.1).

5.5.1 Published results

Some of the results describing tick FRePs were published in two papers – Hemelipoglycoprotein from the ornate sheep tick, *Dermacentor marginatus*: structural and functional characterization (Chapter 6.5.1.1) and Fibrinogen-related protein in ixodid ticks (Chapter 6.5.1.2).

5.5.1.1 Hemelipoglycoprotein from the ornate sheep tick, Dermacentor marginatus: structural and functional characterization

Three functions have been attributed to carrier proteins in blood-feeding arthropods – binding, transportation, and storage of heme. Several carrier proteins have been identified in ticks; one of them is hemelipoglycoprotein from *D. marginatus*. This protein was characterized by biochemical methods. The protein has two subunits (95 and 100 kDa), it is glycosylated, and four *N*-glycan structures have been identified $HexNAc_2Hex_9$, $HexNAc_2Hex_{10}$, $HexNAc_4Hex_7$, and $HexNAc_4Hex_8$. The protein was recognized by sera directed against other tick FRePs and the purified protein was able to hemagglutinate red blood cells. Furthermore, the protein forms high-molecular weight complexes with another hemolymph proteins which are also recognized by anti-FReP sera.

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Hemelipoglycoprotein from the ornate sheep tick, *dermacentor marginatus*: structural and functional characterization

Jarmila Dupejova[1], Jan Sterba[1,2*], Marie Vancova[1,2], Libor Grubhoffer[1,2*]

Abstract

Background: Tick carrier proteins are able to bind, transport, and store host-blood heme, and thus they function also as antioxidants. Nevertheless, the role of carrier proteins in ticks is not fully understood. Some of them are found also in tick males which do not feed on hosts to such an extent such as females (there are differences in male feeding in different tick species) and thus they are not dealing with such an excess of heme; some of the carrier proteins were found in salivary glands where the processing of blood and thus release of heme does not occur. Besides, the carrier proteins bind relatively low amounts of heme (in one case only two molecules of heme per protein) compared to their sizes (above 200 kDa).
The main aim of this study is the biochemical characterization of a carrier protein from the ornate sheep tick *Dermacentor marginatus*, hemelipoglycoprotein, with emphasis on its size in native conditions, its glycosylation and identification of its modifying glycans, and examining its carbohydrate-binding specificity.

Results: Hemelipoglycoprotein from *D. marginatus* plasma was purified in native state by immunoprecipitation and denatured using electroelution from SDS-PAGE separated plasma. The protein (290 kDa) contains two subunits with molecular weights 100 and 95 kDa. It is glycosylated by high-mannose and complex *N*-glycans $HexNAc_2Hex_9$, $HexNAc_2Hex_{10}$, $HexNAc_4Hex_7$, and $HexNAc_4Hex_8$. The purified protein is able to agglutinate red blood cells and has galactose- and mannose-binding specificity. The protein is recognized by antibodies directed against plasma proteins with hemagglutination activity and against fibrinogen-related lectin Dorin M from the tick *Ornithodoros moubata*.
It forms high-molecular weight complexes with putative fibrinogen-related proteins and other unknown proteins under native conditions in tick plasma. Feeding does not increase its amounts in male plasma. The hemelipoglycoprotein was detected also in hemocytes, salivary glands, and gut. In salivary glands, the protein was present in both glycosylated and nonglycosylated forms.

Conclusion: A 290 kDa hemelipoglycoprotein from the tick *Dermacentor marginatus*, was characterized. The protein has two subunits with 95 and 100 kDa, and bears high-mannose and complex *N*-linked glycans. In hemolymph, it is present in complexes with putative fibrinogen-related proteins. This, together with its carbohydrate-binding activity, suggests its possible involvement in tick innate immunity. In fed female salivary glands, it was found also in a form corresponding to the deglycosylated protein.

Background

Ticks are obligate ectoparasites of mammals, birds, and reptiles which feed only on blood of their hosts. Blood provides a rich source of nutrients needed for processes associated with development to the next life-stage and egg production. During the digestion of the blood, erythrocytes are lysed and heme is released. As ticks do not possess the ability to synthesize heme [1], they utilize the host-blood heme. For this reason, as well as to neutralize the toxic effects of free heme, ticks use carrier proteins which bind and store it. Their nomenclature is

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not uniform and the proteins are called different names; some of them are described below.

Hemelipoglyco-carrier proteins (CP) are the most abundant proteins in the hemolymph [2,3]. The best studied are the CPs in hard ticks, *Dermacentor variabilis* [4] and *Rhipicephalus microplus* [2]. Hemelipoprotein (HeLp), carrier protein isolated from *R. microplus*, has molecular weight of about 354 kDa and consists of two subunits with 103 kDa and 92 kDa. It occurs mainly in hemolymph of adult tick stages in concentrations of around 50 mg/ml and is one of the most abundant hemolymph proteins. This molecule is able to bind heme in the ratio of two moles of heme to one mole of native HeLp and contains 3% carbohydrates, and 33% lipids [2]. HeLp carbohydrates contain mainly mannose which comprises more than 90% of all carbohydrates present in HeLp which corresponds to glycans found in ticks [[5], unpublished results]. The protein contains also neutral lipids, phospholipids, cholesterol esters, and cholesterol oleate. Labeling of HeLp with ^{55}Fe showed that this protein participates in heme transportation from hemolymph into ovaries during oogenesis [2].

Another CP, hemelipoglyco-carrier protein (DvCP), found in hemolymph of both male and female ticks *D. variabilis*, shows a significant sequence homology with HeLp [4-7]. DvCP has a molecular weight of 210 kDa and has two subunits with molecular weight of 98 kDa and 92 kDa. Similarly to HeLp, DvCP contains lipids and carbohydrates [4]. DvCP was localized to fat body, salivary glands, ovary, and muscles of partially fed females [3].

The role of carrier-proteins in ticks is not fully understood. Some of them are found also in males which engorge a more limited volume of blood and thus are dealing with lower amounts of heme [4]. Moreover, the carrier protein persisted in *D. variabilis* males hemolymph in higher concentrations and for a longer period of time after detachment as compared to tick females [4]. Thus, it is possible that these proteins play also roles other than transport and storage of the heme.

In the current study, hemelipoglycoprotein (HLGP) from the hemolymph of the tick *D. marginatus*, closely related to DvCP, was isolated and characterized by electrophoretic and blotting techniques, surface plasmon resonance (SPR) and MALDI-TOF/TOF (Matrix-assisted laser desorption/ionization-Time-of-flight/Time-of-flight) and ESI-FT-ICR (Electrospray ionization-Fourier-transform ion cyclotron resonance) mass spectrometry analysis. The molecular weight of the protein and its subunits, *N*-linked glycans modifying the protein, and carbohydrate-binding specificity were determined. The protein was also recognized by antibodies directed against Dorin M, a fibrinogen-related lectin from *Ornithodoros moubata* [8], and sera raised against hemagglutination activity of the *D. marginatus* hemolymph.

Methods

Ticks

Unfed and partially-fed (further referred to as "unfed" and "fed") females and males of the tick *Dermacentor marginatus* were obtained from the tick facility of the Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic in České Budějovice. After metamorphosis, males and females were separated and kept in glass vials in wet chambers at 26°C until feeding/plasma and tissue preparation. Females and males were allowed to feed on laboratory guinea pigs for 6 days.

Plasma and tissue preparation

Hemolymph was collected after cutting off a part of tick's anterior leg with fine scissors. The hemolymph from 8 to 10 ticks (approx. 10-15 μl and 5 μl for unfed and fed ticks, respectively) was collected directly into 50 μl of 0.9% NaCl containing protease inhibitors (Pierce, Thermo Fisher, Rockford, IL). The solution was centrifuged at 4°C for 10 min at 100 g to pellet the hemocytes. The resulting supernatant was then clarified at 23000 g for 20 min and both the plasma and hemocyte fractions were stored at -20°C.

Gut and salivary glands were dissected from partially-fed females, thoroughly washed in phosphate-buffered saline, pH 7.4 (PBS) to remove possible contamination, and organs from five ticks were homogenized with 300 μl PBS for 2 minutes at frequency of 30 Hz in TissueLyser II (Qiagen, Hombrechtikon, Switzerland) and stored at -70°C.

SDS-PAGE and Blue Native/SDS-PAGE electrophoresis

For SDS-PAGE, plasma samples were diluted 1:5 in PBS, mixed with loading buffer with or without reducing agent dithiothreitol (Fermentas, Thermo Fisher, Vilnius, Lithuania) and heated for 5 min at 95°C. SDS-PAGE was performed on 4-17.5% gradient gels [9] in Mini-PROTEAN electrophoresis system (Bio-Rad, Hercules, CA). Gels were stained with PageBlue Protein Staining Solution (Fermentas). Approximately 5 μg proteins were loaded in the case of tick plasma and hemocytes, while 15 μg of tick gut and salivary gland proteins were applied.

In the case of BN-PAGE/SDS-PAGE, samples were mixed with 5% glycerol and 0.01% Ponceau S and separated on native gradient gel (3.5% stacking, 6-13% separating gel) [10] together with native protein molecular weight standards (Sigma-Aldrich, St. Louis, MO) in Mini-PROTEAN electrophoresis system. Gels were stained with PageBlue Protein Staining Solution or used

for second dimension of 2 D electrophoresis. For the second dimension, the BN-PAGE separated proteins were used for Tris-Tricine SDS-PAGE [10]. Briefly, 12% separating gel was prepared, gel strips from BN-PAGE were incubated for 1 h in cathode buffer (0.1 M Tris, 0.1 M Tricine, 0.1% SDS, pH 8.25), inserted above the separating gel and overlaid with 4% stacking gel. Electrophoresis was performed for about 2.5 hours at a current limit of 50 mA using anode buffer (0.1 M Tris, 0.0225 M HCl, pH 8.9) and cathode buffer [10]. The separated proteins were stained with PageBlue Protein Staining Solution.

Electroelution of HLGP

Electroelution was performed using ElutaTube™Protein, DNA and RNA Extraction and Dialysis Kit (Fermentas). Bands corresponding to HLGP were cut out of the gel, placed into ElutaTube vial with electrophoresis running buffer (0.025 M Tris, 0.192 M glycine, 0.1% SDS), and placed in the supporting tray in the electrophoresis tank with running buffer. Electroelution ran for 3 hours at 100 V. Samples were precipitated by four volumes of acetone, pelleted, the precipitated proteins were air-dried, and dissolved in water with resulting concentration of approximately 0.5 mg/μl.

Anti-HLGP and anti-HA polyclonal serum preparation

Mice were housed in the Animal facility of the Institute of Parasitology, Biology Center of the ASCR in České Budějovice in plastic cages with sawdust bedding. Pellet diet and water were supplied *ad libitum* and mice were handled in accordance with the Animal Act of the Czech Parliament.

D. marginatus plasma proteins were separated by SDS-PAGE and stained with PageBlue Protein Staining Solution (Fermentas). HLGP bands (corresponding to approximately 50 μg of protein) were cut out and homogenized with 240 μl PBS (1×). Incomplete Freund's adjuvant was added in a 1:1 ratio and 80 μl of this solution was subcutaneously injected to BALB/c mice. Immunization was repeated 3× every 14 days by subcutaneous injection of 80 μl of this solution. Blood sera were collected 14 days after the last immunization. Sera were supplemented with glycerol (1:1), aliquoted and stored at -20°C.

Anti-hemagglutination activity (anti-HA) serum was prepared as described elsewhere [11]. 20 μl of tick hemolymph was added to 50 μl of 2% suspension of mouse red blood cells. After 1 h incubation, the hemagglutinated cells were washed, resuspended in 200 μl of PBS, and 50 μl of the mixture was injected into mice. The immunization was repeated three times every 14 days. Blood sera were collected 14 days after the last immunization. Sera were supplemented with glycerol (1:1), aliquoted and stored at -20°C.

Immunoblotting

The electrophoretically separated proteins were transferred to PVDF membrane [12] for 1 hour at 20 V. The PVDF membrane was washed in PBS, cut into strips, and incubated for 1 hour in 5% skim powdered milk in PBS. Strips were then incubated for 1 hour in mouse anti-HLGP or anti-HA serum, washed with PBS-Tween 20 (0,05% Tween 20 in PBS) and incubated with goat anti-mouse antibody conjugated with alkaline phosphatase (Vector Laboratories, Burlingame, CA) in 5% milk. After incubation, strips were washed with PBS-Tween 20 and PBS. Reaction was developed in alkaline phosphatase-staining solution (Vector Laboratories) and after the development of sufficient signal it was stopped by washing the strips several times in Milli-Q water.

Schiff staining and lectinoblotting of HLGP

Electroeluted HLGP was separated by SDS-PAGE and electroblotted to PVDF membrane. The presence of glycosylation was detected with DIG Glycan Detection Kit (Roche Applied Science, Mannheim, Germany) and the glycan types modifying the HLGP were identified using DIG Glycan Differentiation Kit (Roche Applied Science) utilizing lectins SNA, GNA, DSA, PNA, and MAA II, conjugated with digoxigenin.

Membrane strips for Schiff staining were washed with PBS, the glycoproteins were oxidized by 10 mM sodium periodate in 0.1 M sodium acetate buffer, pH 5.5 at room temperature for 20 minutes, washed in PBS, and incubated with DIG-3-O-succinyl-ε-aminocaproic acid hydrazide in sodium acetate buffer for 1 hour at room temperature. After three washes in Tris-buffered saline, pH 7.5 (TBS, 0.05 M Tris, 0.15 M NaCl) the strips were blocked in blocking solution (provided by the manufacturer and diluted 1:9 in TBS) for 1 hour at room temperature, washed in TBS, incubated with anti-DIG antibodies conjugated with alkaline phosphatase in the blocking solution for 1 hour at room temperature, washed with TBS, and finally, the color reaction was developed in BCIP/NBT solution in 0.1 M Tris, 0.05 M $MgCl_2$, 0.1 M NaCl, pH 9.5. The reaction was stopped by thorough washing in Milli-Q water.

For lectinoblotting, the membrane strips were washed in TBS and blocked in blocking solution for 1 hour at room temperature. Next, the strips were washed in TBS and in TBS supplemented with 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and incubated in the supplemented TBS with the individual DIG-conjugated lectins for minimum 1 hour at room temperature. The strips were washed again in TBS, incubated with anti-DIG antibodies conjugated with alkaline phosphatase in the blocking solution for 1 hour at room temperature, washed with TBS, and the color reaction was developed as described above.

Enzymatic deglycosylation

Electroeluted HLGP was deglycosylated using glycosidases Endo H (New England Biolabs, Ipswich, MA) or PNGase F (New England Biolabs) and PNGase A (Roche Applied Science) under reducing and non-reducing conditions. For deglycosylation using PNGase A/F under reducing conditions, 68 μl of electroeluted HLGP was mixed with 10 μl denaturation buffer (5% SDS, 0.4 M DTT) and heated on 95°C for 10 min. The solution was mixed with 10 μl 0.5 M sodium phosphate, pH 7.4, 10 μl Nonident P-40, 0.5 μl PNGase A, 0.5 μl PNGase F, and 1 μl Milli-Q water.

For deglycosylation using Endo H under reducing conditions, 68 μl of electroeluted HLGP was mixed with 10 μl denaturation buffer (5% SDS, 0.4 M DTT) and heated on 95°C for 10 min. The solution was mixed with 10 μl 0.5 M sodium citrate, pH 5.5, 2 μl Endo H, and 10 μl Milli-Q water. Deglycosylation under non-reducing conditions was performed similarly with the denaturation step to be omitted. Deglycosylation reactions were performed overnight at 37°C. All reactions were performed in duplicates.

N-linked glycans from HLGP and *D. marginatus* hemolymph were prepared from reduced and alkylated electroeluted proteins, which were digested by trypsin (Roche Applied Science) in PBS, overnight at 37°C. The peptides were purified using C18 spin columns (Harvard Apparatus, Holliston, MA), vacuum-dried and deglycosylated as described above using a mixture of PNGase A and PNGase F to ensure the release of all *N*-glycans. The released glycans were purified using a combination of C18 and active-charcoal spin columns (Harvard Apparatus) [13]. *N*-glycans preparation and their subsequent MS analysis were performed in duplicates.

Solid-phase permethylation of *N*-glycans for MS analysis

PNGase A/F-released glycans were vacuum dried, resuspended in 5 μl of water, and 70 μl of dimethylformamide and 25 μl of methyl iodide were added. The mixture was applied onto NaOH beads (Sigma-Aldrich) in spin-columns (Harvard Apparatus), incubated for 15 minutes, and the columns were centrifuged for 1 minute at 1500 × G. 25 μl of methyl iodide was added to the solution, again applied onto NaOH beads, incubated for 15 minutes, and centrifuged [13]. Next, the beads were washed with acetonitrile, centrifuged, and the solutions were pooled. The permethylated glycans were recovered by liquid/liquid extraction with chloroform and washing with 0.5 M NaCl and HPLC-grade water. Finally, the glycans were vacuum dried.

Immunoprecipitation

50 μl of *D. marginatus* hemolymph plasma, diluted 1:5 in 0.9% NaCl, was mixed with 1 μl of 1% Tween 20 in PBS, 5 μl of protease inhibitors (Pierce), and with 444 μl of PBS. The mixture was incubated with 5 μl of anti-HA/anti-HLGP serum at 4°C overnight. 10 μl of magnetic Dynabeads with Protein G (Invitrogen, Carlsbad, CA) were added into the solution and incubated 45 min at room temperature. The beads were then washed six-times in 0.01% Tween 20 in PBS. HLGP was eluted using elution buffer containing primary amines (Micro-Link Protein Coupling Kit, Pierce). Immunoprecipitation experiments were performed three-times with similar results.

Surface plasmon resonance (SPR)

Quantitative measurement of interactions between HLGP and carbohydrates was performed on BiaCore 3000 instrument (GE Healthcare, Buckinghamshire, UK) (National Centre for Biomolecular Research, Michaela Wimmerová and Lenka Malinovská, Masaryk University, Brno, Czech Republic). A chip with immobilized monosaccharides (α-D-galactose on channel 1, α-D-mannose/α-L-fucose on channel 2, α-D-mannose on channel 3, and α-L-fucose on channel 4) was used. 15 μl of samples were injected on the chip and the response of individual channels was monitored at a flow-rate of 5 μl/min. Dilutions of the samples as well as samples containing EDTA (25 mM) were injected for complementary experiments. Both experiments with and without EDTA were performed in duplicates.

Hemmaglutination assay

The determination of hemagglutination activity (HA) was performed in 96-well U-shaped microtitration plates by serial two-fold dilution of 50 μl samples in 50 μl of 0.15 M NaCl [9]. Next, 50 μl of 2% (v/v) suspension of mouse erythrocytes in 0.15 M NaCl was added to each well. The titer of HA was evaluated after 1 h incubation at room temperature and expressed as the reciprocal value of the last sample dilution causing visible agglutination.

Mass spectrometry

Purified HLGP or gel slices containing HLGP were trypsinized according to the manufacturer's instructions (Roche Applied Science) and subjected to LC-MS analysis. Peptides were separated by HPLC on C18 silica (Agilent, Santa Clara, CA) using acetonitrile gradient (5-80%) as a mobile phase and analyzed by FT-ICR mass spectrometer (Thermo Fisher). The obtained data were compared to *Acari* non-redundant and Swiss-Prot databases using Mascot (a proprietary identification program, Matrix Science, Boston, MA) using strict criteria.

The permethylated glycans were spotted on matrix-assisted laser desorption/ionization (MALDI) plate with 2,5-dihydroxybenzoic acid and analyzed on 4800

MALDI TOF/TOF Analyzer (Applied Biosystems, Life Technologies, Carlsbad, CA) in positive-ion mode. The data were converted to mzXML format and further analyzed by mMass [14] and Glycoworkbench [15] software. Mass spectrometric analyses were performed in National Center for Glycomics and Glycoproteomics, Indiana University, Bloomington, IN, USA (Benjamin F. Mann, William R. Alley, Jr., and Milos V. Novotny).

Results

HLGP detection and identification in *D. marginatus* hemolymph

Putative lectin molecules in the hemolymph of the tick *Dermacentor marginatus* were detected using sera and antibodies recognizing similar molecules in tick species. The sera used were directed against HA of the hemolymph of *D. marginatus* tick in addition to antibodies recognizing Dorin M protein, a lectin from the hemolymph of the tick *Ornithodoros moubata*. Several putative carbohydrate-binding proteins with molecular weight around 37 kDa, 79 kDa, 80 kDa, and a high-molecular protein with molecular weight of approximately 290 kDa were identified in non-reduced hemolymph (Figure 1A). The reactivity of antibodies differed for these proteins and the 290 kDa molecule was the only one, recognized by all the antibodies.

The bands containing all four proteins were excised from the SDS-PAGE gel and mass spectrometric analyzes (ESI-FT-ICR MS) were performed. Although the three low molecular weight proteins were not identified, the 290 kDa protein (asterisk, Figure 1A) was identified as hemelipoglycoprotein (HLGP) related to hemelipoglyco-carrier protein (CP) from *D. variabilis*. The amino acid sequence coverage was 10.2% and 4.0% (see Additional File 1; Table S1 and Additional File 2; Table S2, respectively) for hemelipoglycoprotein precursor 1 and 2 of the homologous HLGP from *D. variabilis*, respectively.

Next, polyclonal antibodies against this protein were raised. The following immunoblot analysis showed that the anti-HLGP antibodies recognized the 290 kDa protein under non-reducing conditions and two subunits with molecular weights of 95 and 100 kDa under reducing conditions (data not shown).

Isolation of HLGP

Further, HLGP was isolated from the plasma under both native and denaturing conditions. Native protein was purified using immunoprecipitation with Protein G coupled to magnetic beads and anti-HLGP serum (see Additional File 3; Figure S1; asterisk). The denatured protein was isolated from electrophoretically separated hemolymph by cutting out the HLGP protein from the gel and its subsequent electroelution (see Additional File 3; Figure S1; asterisk). In both cases, the purification of HLGP was successful.

Characterization of HLGP

To assess the molecular weight of the protein under native conditions, blue native-PAGE and subsequent SDS-PAGE of tick plasma were performed. The results show that HLGP (arrow, Figure 2A) is part of a high-molecular weight complex together with some other proteins which have not been identified yet (Figure 2A). Under native conditions, the complex has size of approximately 450 kDa (arrow, Figure 2B). However, some of these proteins are recognized by antibodies directed against tick lectins and HA (data not shown).

Schiff staining of HLGP showed, that the protein is indeed glycosylated (Figure 3A, lane 2). The glycan moieties were further analyzed by lectin-affinity staining. Terminal mannose-specific GNA lectin and Gal-β(1-4)-GlcNAc-specific DSA lectin showed strong binding to HLGP (Figure 3A, lanes 4,5), α(2-6)-linked sialic acid-specific SNA lectin stained HLGP weakly (Figure 3A,

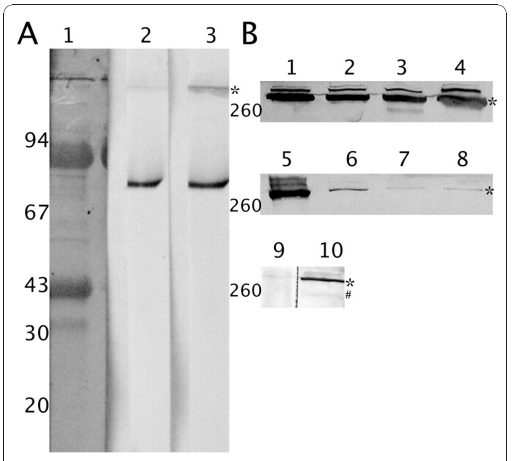

Figure 1 Identification of putative FReD proteins in *D. marginatus* plasma and detection of HLGP in tick tissues. Molecular weights of standard proteins are depicted. A) Immunoblotting of non-reduced electrophoretically separated *D. marginatus* plasma proteins. 1 - *D. marginatus* plasma proteins, stained with Coomassie Brilliant Blue, 2 - immunostaining of plasma proteins using antibodies against HA of *D. marginatus* hemolymph serum, 3 - immunostaining of plasma proteins by anti-Dorin M antibodies. B) Immunoblotting of HLGP in *D. marginatus* tissues using mouse polyclonal anti-HLGP serum. 1 - fed female plasma, 2 - unfed female plasma, 3 - fed male plasma, 4 - unfed male plasma, 5 - fed female hemocytes, 6 - unfed female hemocytes, 7 - fed male hemocytes, 8 - unfed male hemocytes, 9 - fed female gut, 10 - fed female salivary glands. * marks the position of native glycosylated HLGP, # marks the deglycosylated form of HLGP.

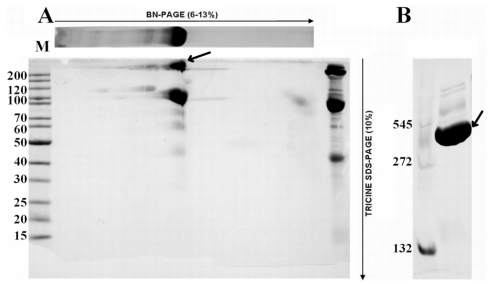

Figure 2 2 D and native electrophoreses of plasma proteins and HLGP. A) Blue native/SDS-PAGE 2 D electrophoretic separation of *D. marginatus* plasma proteins, stained by Coomassie Brilliant Blue (CBB). BN-PAGE separated plasma proteins are showed above the gel and acrylamide gradients are depicted above the gel (native electrophoresis) and on the right side (SDS-PAGE electrophoresis). HLGP is marked with an arrow. B) Blue native PAGE of *D. marginatus* plasma proteins, stained by CBB. 1 - native molecular weight standard, 2 - tick plasma. Complex of HLGP with other proteins is marked with an arrow.

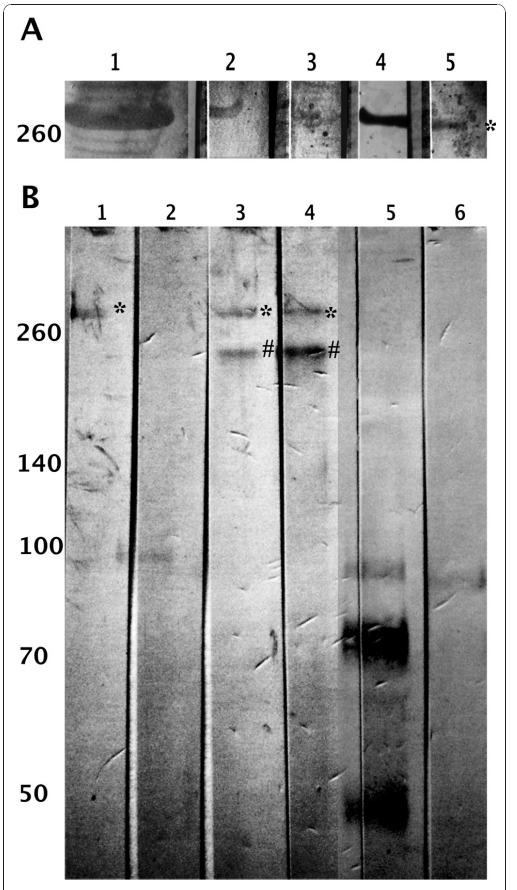

Figure 3 HLGP is a glycosylated protein. Molecular weights of standard proteins are depicted. A) Schiff staining and lectin staining of electrophoretically separated and electroblotted purified HLGP. 1 - tick plasma proteins, stained with Coomassie Brilliant Blue, 2-5 electroblotted HLGP staining. 2 - Schiff staining, 3 - SNA lectin staining, 4 - GNA lectin staining, 5 - DSA lectin staining of purified HLGP. Positive reaction in Schiff staining confirms the presence of glycans on HLGP. Types of glycans identified by positive reactions in lectinoblotting are described in text. B) Immunodetection of deglycosylated purified HLGP using anti-HLGP serum. 1,3,4 - non-reduced electroeluted HLGP and 2,5,6 - reduced electroeluted HLGP stained with anti-HLGP serum. Samples in lanes 3 and 5 were deglycosylated using Endo H, samples in lanes 4 and 6 deglycosylated using PNGase A/F. * - native glycosylated HLGP, # - deglycosylated form of HLGP.

lane 3), while Gal-β(1-3)-GalNAc and α(2-3)-linked sialic acid-specific lectins PNA and MAA did not give positive reaction (data not shown). These results suggest the presence of *N*-glycans of hybrid and complex type with terminal mannose, galactose or sialic acid. DSA lectin binding to HLGP can indicate the presence of *O*-glycans, too.

The proportion of the glycan part of the molecule was determined from deglycosylation reactions of HLGP. Enzymes Endo H and the mixture of PNGase A/F were used to cleave off the *N*- glycans. Anti-HLGP serum detected two proteins with 250 kDa and 290 kDa in both PNGase A/F (Figure 3B, lane 3) and Endo H treated purified native HLGP (Figure 3B, lane 4). As these endoglycosidases work under native conditions with a slower reaction rate, the larger protein (asterisks) presumably represents the glycosylated form and the 250 kDa protein (hashes) the deglycosylated form of the protein. Thus, the size of the *N*-glycan moieties was estimated to approximately 40 kDa.

MALDI-TOF/TOF analysis of PNGase A/F released, permethylated *N*-glycans from purified HLGP provided the information on their masses and composition. The MS spectra showed presence of high-mannose structures $HexNAc_2Hex_9$ and $HexNAc_2Hex_{10}$ (ion m/z 2396.7 and 2600.8, respectively) and complex glycans $HexNAc_4Hex_7$ and $HexNAc_4Hex_8$ (m/z 2477.8 and 2684, respectively) (Figure 4A). Composition of the glycan structures was confirmed by MS/MS analysis (data not shown). Spectra contained also contaminating series of hexose ions and of *O*-glycans (e.g. ion m/z 1707, not marked in the figure), present also in other tick samples such as salivary glands, gut, hemolymph, ovaria, or whole tick homogenates (unpublished results). For comparison, *N*-glycans from *D. marginatus* hemolymph were also analyzed. All four glycan structures, identified in HLGP samples, were present also in hemolymph

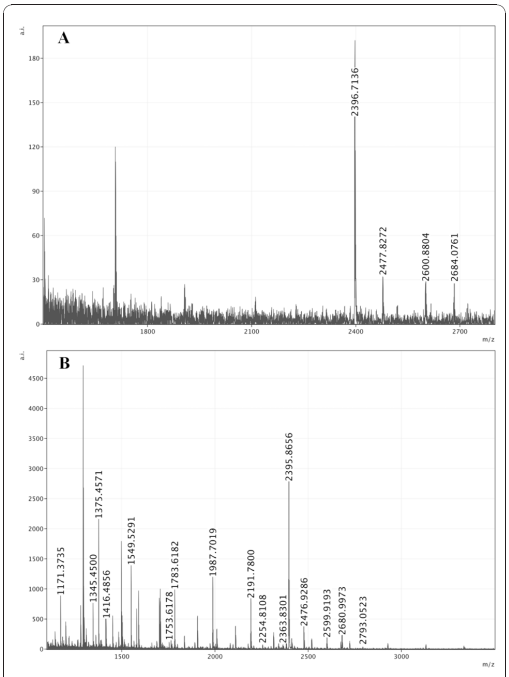

Figure 4 Mass spectra of *N*-glycans from the purified HLGP and from *D. marginatus* hemolymph. A) MALDI-TOF spectrum of enzymatically released *N*-glycans from HLGP. Four peaks corresponding to s $HexNAc_2Hex_9$ (m/z 2396.7), $HexNAc_2Hex_{10}$ (m/z 2600.8), $HexNAc_4Hex_7$ (m/z 2477.8), and $HexNAc_4Hex_8$ (m/z 2684) are marked. Three more peaks are visible corresponding to contaminating series of hexoses and *O*-glycans (not labeled). B) MALDI-TOF spectrum of enzymatically released *N*-glycans from *D. marginatus* hemolymph. A range of glycan structures was identified, namely high-mannose type glycans $HexNAc_2Hex_3$ to $HexNAc_2Hex_{10}$ (m/z 1171.3, 1375.4, 1783.6, 1987.7, 2191.8, 2395.9, 2599.9) and complex fucosylated and non-fucosylated glycans such as $HexNac_3Hex_3$ (m/z 1416.5), $HexNAc_2Hex_4dHex_1$ (m/z 1549.5), $HexNAc_2Hex_5dHex_1$ (m/z 1753.6). Again, contaminants were present in the sample - series of hexoses as well as *O*-glycans (not labeled).

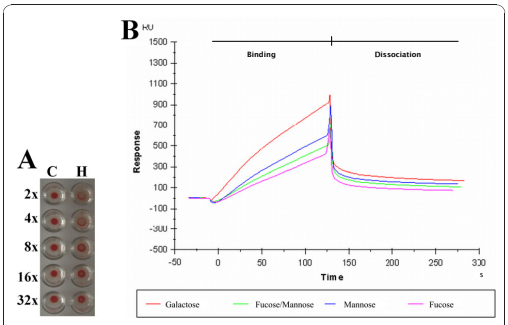

Figure 5 HLGP is a carbohydrate-binding protein - HA and SPR. A) Hemagglutination activity of purified HLGP. C - negative control (no hemagglutination activity), H - serial two-fold dilution of electroeluted HLGP. Hemagglutination activity is visible in dilutions 2× to 16×. B) Carbohydrate-binding specificity of purified HLGP assessed by SPR on a chip with four channels arranged sequentially as galactose-fucose/mannose-mannose-fucose. Red line corresponds to signal from the channel with immobilized galactose, green to channel with immobilized fucose and mannose, blue to channel with immobilized mannose and purple to channel with immobilized fucose. The fucose/mannose channel contained half amount of the corresponding carbohydrates and serves as control. Lines correspond to SPR response in real-time (not normalized for the sequential flow through channels). Galactose showed the strongest binding by HLGP, some binding was observed also for mannose. Fucose was not recognized by the protein.

(Figure 4B). Other hemolymph protein *N*-glycans were identified as high-mannose type ranging from $HexNAc_2Hex_3$ to $HexNAc_2Hex_8$ (m/z 1171.3, 1375.4, 1783.6, 1987.7, 2191.8) and fucosylated and non-fucosylated complex type glycans such as $HexNac_3Hex_3$ (1416.5), $HexNAc_2Hex_4dHex_1$ (1549.5), $HexNAc_2Hex_5dHex_1$ (1753.6). Also in this case, a series of hexoses as well as *O*-glycans were present in the sample (Figure 4B).

In accordance with the presence of hemagglutination activity (HA) in hemolymph (unpublished results), the purified HLGP demonstrated HA (Figure 5A). The inhibition of HA with monosaccharides was not performed due to low amounts of sample available and thus the carbohydrate specificity was addressed by surface plasmon resonance experiments. A chip with channels with immobilized galactose, fucose, and mannose was used. The channel with immobilized fucose and mannose (half amounts) was used as a control of binding-response. The experiment showed binding specificity of the purified HLGP towards galactose (Figure 5B). HLGP showed also a weak binding-specificity towards mannose as seen from comparison of the signal from the mannose channel and the control fucose/mannose channel (Figure 5B). The binding of HLGP to carbohydrates was inhibited by EDTA (data not shown).

Tissue distribution

The presence of HLGP in tick tissues was determined by immunoblotting. In addition to plasma, hemocytes (as the cellular component of hemolymph) from both fed and unfed females and males, fed female salivary glands (to investigate the presence of HLGP in salivary glands and possibly also in saliva), and gut were investigated.

In all samples, anti-HLGP serum detected the same protein with the same molecular weight (Figure 1B). While amount of the protein was similar in both females and males, regardless of blood-feeding, HGLP was present in higher amounts only in hemocytes from

fed female tick. In males, the amount of HLGP did not increase during the blood-feeding.

Interestingly, in the salivary glands (Figure 1B, lane 10), the anti-HLGP serum detected two forms of this protein, corresponding in size to the glycosylated (asterisk) and the deglycosylated (hash) forms (compare with Figure 3B, lanes 3,4).

Discussion

This work was aimed towards the characterization of hemelipoglycoprotein, the most abundant protein of tick hemolymph. To date, only one function of the protein has been identified - the binding and storage of heme [2,4]. Surprisingly, in our work, the protein was recognized by antibodies, which were directed against the first isolated tick lectin Dorin M [8] but also sera against the hemagglutination activity of the hemolymph of ticks *D. marginatus* and *I. ricinus*. Hemagglutination activity (HA) is an ability of some proteins to bind carbohydrates on red blood cells and thus to form clusters. HA is a typical characteristic of lectins, proteins with the ability of specific recognition of specific carbohydrates and their reversible binding. Antibodies raised against HA of tick hemolymph recognize proteins which bind red blood cells in this manner and potentially also proteins, which form tight complexes with them. Anti-Dorin M antibodies, on the other hand, react more specifically with proteins containing FReD domain or lectins (unpublished results).

All the used antibodies detected similar proteins in hemolymph; one of the putative lectin molecules with the molecular weight of 290 kDa (with two subunits of 86 and 90 kDa) was identified as hemelipoglycoprotein (HLGP). The theoretical masses of the subunits for the HLGP from *D. variabilis*, based on convertase cleavage, are 85 and 93 kDa for HLGP precursor (GenBank accession No. EEC1882) and 79 and 92 kDa for HLGP precursor 2 (GenBank accession No. EEC17915) [16] which clearly corresponds with our results for *D. marginatus* protein. The molecular weight of the protein is similar to other tick hemelipoglycoproteins or carrier proteins (another name of the protein) whose size ranges from 200 kDa to 365 kDa [2,4]. The proteins, described by these authors, contained two subunits of 92 and 98 or 92 and 103 kDa.

Hemelipoglycoproteins contain the N-terminal lipoprotein domain, a domain of unknown function, and the von Willebrand domain type D, but they do not contain the fibrinogen-related domain, nor any other known lectin domain; moreover, hemelipoglycoproteins are not phylogenetically related to FReD proteins [17]. On the other hand, the N-terminal lipoprotein domain is proposed to have carbohydrate, lipid, and metal-binding properties [16]. The N-terminal lipoprotein domain shares high similarity with vitellogenins from insects and other arthropods and the carbohydrate-binding specificity of vitellogenin was showed for example in the Colorado beetle hemolymph (*Leptinotarsa decemlineata*) [18]. The lipoprotein domain of the DvCP from *D. variabilis* is similar also to vitellogenin from fish of the genus *Branchiostoma*, which also shows hemagglutination activity and carbohydrate-binding ability [19].

HLGP and the other proteins recognized by the anti-HA antibodies in the *D. marginatus* hemolymph form a high-molecular weight complex under native conditions (Figure 2A, B). Thus, in the process of anti-HA antibodies preparation, HLGP could be co-purified with proteins with lectin activity. This can explain the anti-HA antibodies binding to HLGP; however, this fact does not explain the binding of anti-Dorin M antibodies to the protein. The structure of HLGP, FReD-containing proteins, and Dorin M are not known and HLGP does not show any similarity to FreD proteins on the primary structure level. However, recognition of HLGP by anti-HA and anti-Dorin M antibodies, together with carbohydrate-binding SPR studies suggest, that HLGP shares similar epitopes with the lectin domains of the FReD-containing proteins and that HLGP is structurally, rather than sequentially, similar to lectins/FReD-containing proteins.

The electroeluted and native purified HLGP were able to bind to immobilized monosaccharides in SPR experiments or to hemagglutinate red blood cells (Figure 5A, B). This corresponds to hemagglutination experiments performed on hemolymph from *D. marginatus* (unpublished results) as well as from other ticks [8,17,20]. The diminished carbohydrate-binding activity in the presence of EDTA (data not shown) suggests dependency on calcium (divalent) cations, which corresponds to calcium dependence of HA of crayfish plasma vitellogenin-related protein [21] and sea urchin vitellogenin [22].

FReD-containing proteins are present in vertebrates and in invertebrates. They were described as humoral factors of innate immunity which are able to recognize PAMPs (pathogen-associated molecular patterns); their other functions include participation in regulation of embryo development or in cell adhesion. Donohue and colleagues [16] and Maya-Monteiro and colleagues [2] suggest also other possible roles of carrier proteins in the ticks apart from the heme-storage and prevention of oxidative stress. Our finding of HLGP participation in a high-molecular weight complex with other putative FReD-containing proteins, the presence of two forms of HLGP in tick salivary glands, and its carbohydrate-binding properties point to some other, yet undiscovered, roles of the protein.

HLGP from *D. marginatus* is modified by *N*-glycans as was showed by Schiff staining and lectin affinity staining

(Figure 3A). PNGase A/F and Endo H endoglycosidases decreased the size of HLGP by 40 kDa under native conditions (Figure 3B). The reactivity of HLGP with lectins DSA, GNA, and SNA indicated the presence of *N*-glycans with terminal mannose, terminal α(1-4)-linked galactose and α(2-6)-linked sialic acid. On the other hand, the absence of binding by the MAA II and PNA lectins suggested the absence of α(2-3)-linked sialic acid and of terminal α(1-3)-linked galactose. The subsequent mass spectrometric analysis of permethylated *N*-glycans from the purified HLGP revealed the presence of four different glycan moieties. Two were high-mannose type containing two *N*-acetylhexosamines and nine and ten hexose molecules, and the other two were complex glycans comprising four HexNAcs and seven and eight hexoses, possibly with terminal galactoses. Thus, the mass spectrometric analysis confirmed the results from lectin-affinity staining. Similar glycans were found also in other tick glycoprotein with identified glycans, Dorin M from *Ornithodoros moubata* [5]. In Dorin M, two high-mannose glycans and a core-fucosylated structure were identified.

Surprisingly, core-fucosylated *N*-glycans were not found in HLGP, even though their presence was confirmed in *D. marginatus* hemolymph (Figure 4B) and they are the most abundant type of glycans found in tick saliva and salivary glands (*Ixodes ricinus*, unpublished results). The role of core-fucosylation and protein-specific fucosylation in ticks will be one of the exciting problems of tick glycobiology and physiology.

HLGP has been detected in several tissues of the tick - hemolymph, hemocytes, gut, and the protein in both glycosylated and non-glycosylated form also in salivary glands. Previously, HLGP was immunolocalized inside the gut cells, on their surface, as well as inside the salivary duct and epithelium cells of salivary glands (unpublished results). Its presence was shown in these tissues also in other ticks and in different life stages of ticks [3,4]. Furthermore, HLGP is present in salivary glands of *D. marginatus* in two forms, one of them corresponding in size to the non-glycosylated form of the hemolymph (glycosylated) protein, the other to the hemolymph protein. While Gudderra and colleagues [3] showed, that DvCP in the salivary glands of *D. variabilis* does not contain heme in its molecule, the presence of two forms of the protein was not described to date elsewhere. The role of HLGP in tick saliva is not clear nor is the importance of the glycosylation of HLGP or the other tick salivary proteins for the tick feeding.

Conclusion

A heme-carrier protein from the hemolymph of the ornate sheep tick *Dermacentor marginatus*, hemelipoglycoprotein, was characterized. The protein is *N*-glycosylated, bearing high-mannose and complex type glycans without core-fucose. Nevertheless, core-fucosylated glycans are abundant in hemolymph. Hemelipoglycoprotein forms a complex with fibrinogen-related proteins in hemolymph. It shows carbohydrate-binding activity, which is divalent cations-dependent. Moreover, the protein is present in two forms in fed female salivary glands and one of these forms corresponds to the deglycosylated protein. Altogether, these findings suggest also other roles of the protein in ticks different from its heme-binding function. Due to the carbohydrate-binding activity, one of the possibilities is the involvement of hemelipoglycoprotein in innate immunity processes.

Additional material

Additional File 1: Tryptic fragments identified in hemelipoglycoprotein precursor [*Dermacentor variabilis*], coverage 10.2%. Mass:178646, Score:12286, Queries matched: 244.

Additional File 2: Tryptic fragments identified in hemelipoglycoprotein precursor 2 [*Dermacentor variabilis*], coverage 4%. Mass:171972, Score: 707, Queries matched: 12.

Additional File 3: Purification of HLGP. Molecular weights of standard proteins are depicted. A) Immunoprecipitation of HLGP from *D. marginatus* hemolymph. 1 - Immunostaining of non-reduced HLGP in tick plasma, 2,3 - immunostaining of immunoprecipitated non-reduced HLGP. B) Electroelution of electrophoretically separated HLGP from *D. marginatus* plasma. SDS-PAGE separated proteins stained with Coomassie Brilliant Blue. 1 - non-reduced plasma proteins, 2 - non-reduced electroeluted HLGP. * marks the position of native glycosylated HLGP, # marks the deglycosylated form of HLGP.

Abbreviations

AP: alkaline phosphatase; CP: carrier proteins; dHex: deoxyhexose; DSA: *Datura stramonium* agglutinin; FReD: fibrinogen-related domain; FReP: fibrinogen-related protein; GNA: *Galanthus nivalis* agglutinin; HA: hemagglutination activity; Hex: hexose; HexNAc: N-acetyl hexosamine; HLGP: hemelipoglycoprotein; MAA: *Maackia amurensis* agglutinin; PAMP: pathogen-associated molecular pattern; SNA: *Sambucus nigr*a agglutinin; SPR: surface plasmon resonance.

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Authors' contributions

JD conducted electrophoreses, blotting experiments, HA assay, and participated in SPR experiments, JD and JS participated in anti-HLGP and anti-tick HA sera preparation and in HLGP purification, JS and MV performed mass spectrometry and analysis of data, MV performed tick tissue

preparation and participated in their analysis, LG co-ordinated the experiments, all authors participated in the design of experiments, manuscript preparation, and they approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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5.5.1.2 Fibrinogen-related proteins in ixodid ticks

Besides a "true" lectin galectin from *O. moubata*, the other lectins identified in ticks belong to fibrinogen-related proteins. Immune sera reactive with these proteins were used for identification of FRePs in several tick species. These proteins showed also reactivity with antibodies directed against the fibrinogen domain of human ficolin. They were shown to be glycosylated and were localized in tick salivary glands, midguts, and hemocytes.

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Fibrinogen-related proteins in ixodid ticks

Jan Sterba[1*], Jarmila Dupejova[1], Miroslav Fiser[1], Marie Vancova[1,2] and Libor Grubhoffer[1,2*]

Abstract

Background: Fibrinogen-related proteins with lectin activity are believed to be part of the tick innate immune system. Several fibrinogen-related proteins have been described and characterised mainly on the basis of their cDNA sequences while direct biochemical evidence is missing. One of them, the haemolymph lectin Dorin M from the tick *Ornithodoros moubata* was isolated and characterised in more depth.

Results: Several fibrinogen-related proteins were detected in the haemolymph of ixodid ticks *Dermacentor marginatus*, *Rhipicephalus appendiculatus*, *R. pulchellus*, and *R. sanguineus*. These proteins were recognised by sera directed against the tick lectin Dorin M and the haemagglutination activity of the ticks *R. appendiculatus* and *D. marginatus*. Cross-reactivity of the identified proteins with antibodies against the fibrinogen domain of the human ficolin was also shown. The carbohydrate-binding ability of tick haemolymph was confirmed by haemagglutination activity assays, and this activity was shown to be inhibited by neuraminic acid and sialylated glycoproteins as well as by N-acetylated hexosamines. The fibrinogen-related proteins were shown to be glycosylated and they were localised in salivary glands, midguts, and haemocytes of *D. marginatus*. Hemelipoglycoprotein was also recognised by sera directed against the fibrinogen-related proteins in all three *Rhipicephalus* species as well as in *D. marginatus*. However, this protein does not contain the fibrinogen domain and thus, the binding possibly results from the structure similarity between hemelipoglycoprotein and the fibrinogen domain.

Conclusions: The presence of fibrinogen-related proteins was shown in the haemolymph of four tick species in high abundance. Reactivity of antibodies directed against ficolin or fibrinogen-related proteins with proteins which do not contain the fibrinogen domain points out the importance of sequence analysis of the identified proteins in further studies. Previously observed expression of fibrinogen-related proteins in haemocytes together with the results of this study suggest involvement of fibrinogen-related proteins in tick immunity processes. Thus, they have potential as targets for anti-tick vaccines and as antimicrobial proteins in pharmacology. Research on fibrinogen-related proteins could reveal further details of tick innate immunity processes.

Background

Together with mosquitoes, ticks are the primary vectors of a broad-range of dangerous human and animal pathogens. Some of the well-known infections transmitted by hard ticks (Ixodidae) are Rocky Mountain spotted fever, ehrlichiosis, tick-borne encephalitis, Lyme borreliosis, theileriosis or babesiosis while soft ticks (Argasidae) are vectors of African swine fever and tick-borne relapsing fever [1,2].

Tick haemolymph is a complex fluid composed of plasma and haemocytes. It serves several functions such as transportation of nutrients but it contains also the components of the immune system and helps the tick to fight injuries. Until now, haeme-storage proteins [3,4], defensins [5-7], a tick haemolymph lectin Dorin M from *Ornithodoros moubata* [8,9], and several others have been described.

Tick defence against pathogenic microorganisms is based on recognition of pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides or peptidoglycans with lectins [10]. Invertebrate/arthropod lectins are believed to be functional analogues of immunoglobulins due to their specific binding to surface carbohydrate structures of pathogens [11]. In arthropods, fibrinogen-related proteins (FRePs) are described as humoral factors of the innate immune system with the ability to recognise PAMPs. FRePs are molecules containing fibrinogen-related domain in the

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C-terminus; some of these domains exhibit carbohydrate-binding activity [12,13]. FRePs from *Tachypleus tridentatus* (Tachylectin 5A and 5B) with high-sequence homology to human ficolins selectively bind terminal *N*-acetyl group of sugars [14] and this lectin-activity is important in the innate immunity processes [12].

Lectins/FRePs have been found in several ticks [8,15-17] in the haemolymph, the midgut, and salivary glands (SGs) [reviewed in [11]]. Generally, tick lectins display affinity towards sialic acid [8] and *N*-acetyl-D-glucosamine (GlcNAc) [15] as well as galactose [17]. Several FReP-encoding sequences were also described to date: Ixoderin A, Ixoderin B, Ixoderin A-like (*Ixoderin ricinus*), Dorin M, and OMFREP (*O. moubata*) [18]. The OMFREP mRNA is detected in haemocytes and SGs. Ixoderin A is expressed in haemocytes, SGs, and the midgut; Ixoderin B is expressed mainly in SGs and weakly in haemocytes [18]. Sequence similarity of the identified tick lectins with Tachylectins suggests their involvement in innate immunity [9]. Dorin M is still the only purified tick lectin. It is a 640 kDa homomer composed of subunits with 37 kDa [8] and exhibits haemagglutination activity, which is inhibited by *N*-acetylated hexoses, *N*-acetyl neuraminic acid, and sialylated glycoproteins [8,9]. The protein is glycosylated and as such it is recognised by several lectins specific for high-mannose and complex glycans [8]. Three *N*-glycosylated sites described in the protein bear two high-mannose glycans with up to nine mannose residues and a core-fucosylated paucimannosic structure [19].

In *I. ricinus*, 85 kDa lectin was partially characterised with specificity for *N*-acetyl neuraminic acid, *N*-acetyl glucosamine, and D-galactose; however, its sequence is not known [15]. The protein was localised to tick haemocytes and other tissues [16]. Haemagglutination activity of the *Rhipicephalus appendiculatus* haemolymph, the midgut, and SGs have been also characterised. This activity was higher in blood-fed ticks compared with unfed ticks [20].

Furthermore, hemelipoglycoprotein was recognised by serum directed against the Dorin M and against the haemagglutination activity of the *D. marginatus* haemolymph [4]. However, the protein does not show similarity to fibrinogen or fibrinogen-related proteins [3]. Structural similarity of the protein to the fibrinogen domain was therefore suggested [4].

Herein, we describe reactivity of the haemolymph of several ticks with sera against the previously identified tick lectin Dorin M and against haemagglutination activity of the tick haemolymph as well as with antibodies recognising the fibrinogen domain of the human ficolin. The observed fibrinogen-related proteins were further characterised in regards of their glycosylation and they were localised in tissues of the tick *D. marginatus*.

Results

Tick haemolymph haemagglutination

Haemagglutination activity (HA) analyses of tick haemolymph samples isolated from *Dermacentor marginatus*, *Rhipicephalus appendiculatus*, *R. pulchellus*, and *R. sanguineus* were performed using 2% suspension of rabbit erythrocytes. In all studied ticks, the haemolymph exhibited haemagglutination activity; HA titre was 512 for *D. marginatus* haemolymph and 192 in the case of the three *Rhipicephalus* haemolymphs (not shown).

Next, haemagglutination inhibition was studied using monosaccharides and glycoproteins (see Table 1). The most potent inhibitors of HA for each tick species were *N*-acetyl neuraminic acid (NeuAc) and sialylated glycoproteins. Moreover, *N*-acetylated hexosamines showed inhibition of the agglutination. However, differences in HA inhibition were detected among tick species and the highest inhibition was observed in *R. sanguineus*.

Reactivity of the tick haemolymph with antibodies to putative FRePs

We used different rabbit/mouse sera directed against potential FRePs: the tick lectin Dorin M (closely related to fibrinogen domain of ficolins); haemolymph lectin molecules with HA activity of ticks *D. marginatus* (anti-(DM) HA) and *R. appendiculatus* (anti-(RA)HA); and immune sera raised against putative *D. marginatus* FReP proteins from this study with molecular weights of 36 kDa (anti-DMF1) and 290 kDa (anti-DMF3). Moreover, we tested antibodies directed against *I. ricinus* lectin (85 kDa) [16]

Table 1 Haemagglutination inhibition assays on tick haemolymph

Saccharide/glycoprotein	D. m.	R. a.	R. p.	R.s.
NeuAc	0.0625	0.0312	0.0312	0.0312
ManNAc	0.125	0.0624	0.5	0.0312
GalNAc	0.125	0.5	0.5	0.0625
GlcNAc	0.125	0.125	-	0.0625
L-fucose	-	0.5	-	0.25
BSM	0.00244	0.0312	0.0312	0.0312
PSM	5	0.25	0.5	0.25
fetuin	1.67	0.5	-	0.0625
Polysialic acid	0.208	nd	nd	nd
asialofetuin	3.33	nd	nd	nd

The following saccharides and glycoproteins were used: *N*-acetyl neuraminic (sialic) acid (NeuAc), N-acetyl mannosamine (ManNac), *N*-acetyl galactosamine (GalNAc), *N*-acetyl glucosamine (GlcNAc), fucose, polysialic acid, bovine submaxillary mucine (BSM), porcine stomach mucine (PSM), fetuin, asialofetuin. Except for asialofetuin, all glycoproteins are sialylated. Concentration inhibiting HA is depicted in mol/l for monosaccharides and mg/ml for polysialic acid and glycoproteins. nd - not determined.

We studied haemagglutination activity in ticks *D. marginatus* (D.m.), *R. appendiculatus* (R.a.), *R. pulchellus* (R.p.), *R. sanguineus* (R.s.).

with results corresponding to findings obtained using the immune sera against the potential FRePs (data not shown).

When assessing the presence of potential FRePs in the *D. marginatus* haemolymph by immunoblotting, four proteins were identified by both anti-(DM)HA and anti-Dorin M antibodies with molecular weights of approximately 36 kDa, 79/80 kDa, and 290 kDa under non-reducing conditions (Figure 1A). Three proteins were recognised by anti-(RA)HA serum also in *R. appendiculatus*, *R. pulchellus*, and *R. sanguineus* haemolymphs, where the molecular weights of the detected putative FReP proteins were 58 kDa, 75 kDa, and approximately 290 kDa (Figure 1B). Moreover, weak reaction was observed for a 45kDa protein in *R. pulchellus* (Figure 1B, lane 2); however, this protein was not observed in the other *Rhipicephalus* species. The 75 kDa and 290 kDa protein bands were detected also by anti-Dorin M serum (Figure 1C).

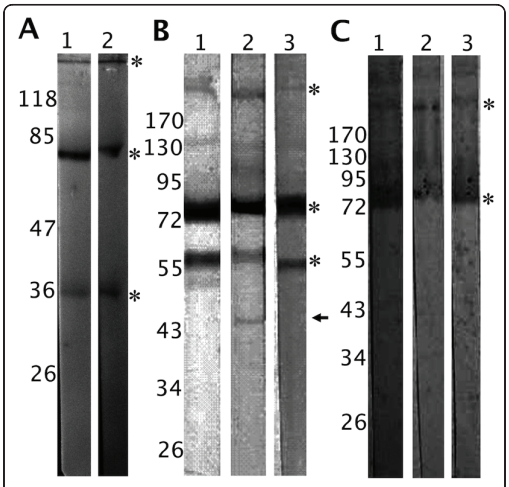

Figure 1 Immunodetection of the putative fibrinogen-related proteins in tick haemolymph. **1A** - Electrophoretically separated and electroblotted non-reduced *D. marginatus* haemolymph proteins immunostained using mouse anti-(DM)HA serum (lane 1) and rabbit anti-Dorin M serum (lane 2). Four proteins were detected with molecular weights of approximately 36, 79/80, and 290 kDa (marked with asterisks; the 79/80 kDa double-band is marked by one asterisk). **1B** - Electrophoretically separated and electroblotted non-reduced *R. appendiculatus* (lane 1), *R. pulchellus* (lane 2), and *R. sanguineus* (lane 3) haemolymph proteins immunostained using mouse anti-(RA)HA serum. In each sample, three proteins were detected with size of 58, 75, and 185 kDa (asterisks). Furthermore, a protein band with molecular weight of 45 kDa was observed in *R. pulchellus* (arrow). **1C** - Electrophoretically separated and electroblotted non-reduced *R. appendiculatus* (lane 1), *R. pulchellus* (lane 2), and *R. sanguineus* (lane 3) haemolymph proteins immunostained using rabbit anti-Dorin M serum. Proteins with molecular weight of 75 and 185 kDa were detected, as in the case of anti-(RA)HA serum (asterisks).

Reactivity of the putative tick FRePs with anti-ficolin antibodies

To confirm the similarity of the identified tick proteins to the fibrinogen domain, we performed immunoblotting of haemolymph proteins from the studied ticks using two commercial antibodies directed against the fibrinogen domain of the human ficolin 1: anti-FCN1 H and anti-FCN S.

All four (36 kDa, 79/80 kDa, and 290 kDa) proteins were recognised in *D. marginatus* haemolymph by the anti-FCN1 H antibodies (Figure 2, lane 1). In the three *Rhipicephalus* species haemolymphs, only the 75 and 290 kDa proteins were recognised (Figure 2, lanes 2, 3, and 4). Furthermore, we performed the immunoblotting on the purified hemelipoglycoprotein from *D. marginatus* [4]; the purified protein was recognised by the anti-FCN1 H antibodies as well (Figure 2, lane 6). Recombinant human ficolin 1 was used as a control (Figure 2, lane 5). Similar results were obtained also using the second antibodies, anti-FCN1 S (data not shown).

Putative FRePs in tick haemolymph are glycosylated

Positive glycoprotein staining (either non-specific Schiff periodate staining or glycan-specific lectinoblotting) of tick haemolymph proteins revealed abundant glycosylation of proteins. Protein bands corresponding to putative FReP proteins were also positively stained (data not shown). To address the glycosylation of these proteins more specifically, we performed enzymatic deglycosylation reactions of the haemolymphs from *D. marginatus*, *R. appendiculatus*, and *R. sanguineus* ticks. After deglycosylation, putative FRePs were detected using anti-(DM)HA, anti-(RA)HA, anti-DMF1 or anti-DMF3 sera. Thus, changes in molecular weights of the putative FRePs could be detected without the interfering staining of the other haemolymph proteins.

In all cases, deglycosylation of haemolymph proteins changed the FRePs immunostaining pattern. In *D. marginatus* haemolymph, anti-DMF1 serum stained the 36 kDa protein under reducing conditions. After deglycosylation with *N*-glycosidase F (cleaves off the whole *N*-glycan except for structures containing core α(1,3)-bound fucose), reactive protein bands with molecular weights of 30, 31, 33, 34, and 36 kDa appeared (Figure 3A). The enduring reaction of the 36 kDa band suggests incomplete deglycosylation reaction. The anti-DMF1 serum detected also the 79/80 kDa proteins, which under reducing conditions migrated as three protein bands with molecular weights of 58, 60, and 66 kDa suggesting the presence of non-covalently bound subunits. The molecular weights of these three bands decreased after *N*-glycosidase F treatment and

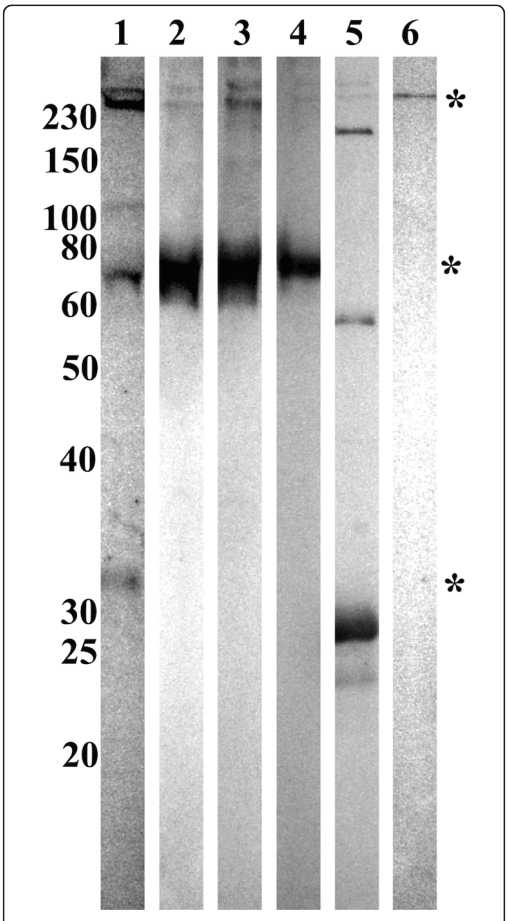

Figure 2 Immunoblotting of tick haemolymph proteins with anti-ficolin antibodies. Electrophoretically separated and electroblotted non-reduced haemolymph proteins from *D. marginatus* (lane 1), *R. appendiculatus* (lane 2), *R. pulchellus* (lane 3), and *R. sanguineus* (lane 4) were immunostained using rabbit anti-FCN1 H antibodies. Recombinant human ficolin 1 was used as a control (lane 5). Purified hemelipoglycoprotein from *D. marginatus* haemolymph, which was identified by MS as one of the recognised proteins was used as a control (lane 6). However, this protein does not contain the fibrinogen domain [3]. The same proteins as in Figure 1A were detected in *D. marginatus* haemolymph (36 kDa, 79/80 kDa, and 290 kDa proteins; marked with asterisks; the 79/80 kDa double-band is marked by one asterisk). In *Rhipicephalus* ticks haemolymphs, the 72 kDa and 290 kDa proteins were detected, but not the 55 kDa protein. Additionally, the purified hemelipoglycoprotein from *D. marginatus* was detected by the anti-FCN1 H antibodies. Non-reduced recombinant human ficolin 1 served as a positive control. Antibodies positively reacted with subunits of the protein (approximately 30 kDa) as well as with higher molecular weight complexes (approximately 60 kDa, 180 kDa, 250 kDa, 280 kDa).

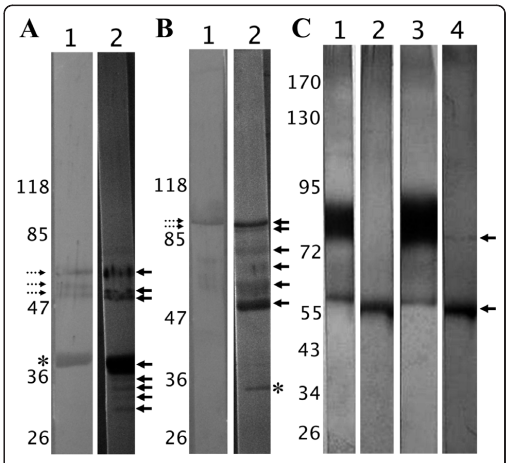

Figure 3 Deglycosylation of the putative fibrinogen-related proteins in tick haemolymph and their immunodetection. **3A** - Reduced *D. marginatus* haemolymph proteins (lane 1) were enzymatically deglycosylated (lane 2). The FReP proteins were detected using anti-DMF1 serum, which was raised against the 36 kDa protein (asterisk). After deglycosylation, additional bands appeared with sizes of 31, 33, and 34 kDa (arrows). Cross-reactivity of the serum with 79/80 kDa proteins was observed (bands with molecular weights of 58, 60, and 66 kDa - lane 1, dotted arrows). The molecular weight of these proteins shifted after deglycosylation and three bands were observed at 54, 58, and 63 kDa (lane 2, arrows). **3B** - Reduced *D. marginatus* haemolymph proteins (lane 1) a were enzymatically deglycosylated (lane 2). The FReP proteins were detected using anti-DMF3 serum, which was raised against the 290 kDa protein. This protein is composed of two subunits which have 95 and 100 kDa (lane 1, dotted arrows). After deglycosylation (lane 2), additional bands appeared at 50 and 74 kDa (arrows). We observed cross-reactivity of the serum also in this case, when the same bands were observed for the 79/80 kDa proteins (lane 2, arrows) as in the case of anti-DMF1 serum (see Figure 2A). The protein band at 34 kDa (lane 2, asterisk) is probably a protein cleavage product. **3C** - Reduced *R. appendiculatus* (lanes 1,2) and *R. sanguineus* (lanes 3,4) haemolymph proteins before (lanes 1,3) and after deglycosylattion (lanes 2,4). The FReP proteins were detected using anti-(RA)HA serum. In both tick samples (lanes 1,3), 58 kDa band was observed and a protein smear from 75 to 90 kDa (75 kDa protein and the subunits for 185 kDa protein). The deglycosylation diminished the reactivity of the protein smear and only the band at 58 kDa remained visible (lanes 2,4; arrows).

they were observed at 54, 58, and 63 kDa (Figure 3A). Same results with lower intensity were obtained for the 79/80 kDa proteins also using anti-DMF3 serum (Figure 3B).

Anti-DMF3 serum stained two bands at 95 and 100 kDa under reducing conditions suggesting non-covalently bound subunits in the 290 kDa protein. After the *N*-glycosidase F treatment, four bands belonging to the protein were observed with molecular weights of 50, 74, 95, and 100 kDa. While the larger bands suggest incomplete deglycosylation, appearance of the 50 and the 74

kDa bands shows cleavage of the glycan moiety from the subunits of the 290 kDa protein (Figure 3B). Another stained protein at Mw of 34 kDa also appeared on the blot.

The reactivity of anti-(RA)HA serum with deglycosylated proteins from the *Rhipicephalus* ticks haemolymphs markedly decreased (Figure 3C). In haemolymph under reducing conditions, the serum recognised the 58 kDa protein and a smear from 75 to 90 kDa, which we speculate represents the 75 kDa protein as well as the subunits of the 290 kDa protein. After deglycosylation using Endo H enzyme, which cleaves between the two innermost GlcNAcs of the *N*-glycans, only one protein remained reactive with molecular weight of 56 kDa in both *R. appendiculatus* and *R. sanguineus* haemolymphs. In *R. sanguineus*, a slight reaction at 75 kDa was visible, representing possibly the incompletely deglycosylated protein (Figure 3C).

The *N*-glycosidase F treatment on *Rhipicephalus* ticks haemolymph as well as deglycosylation of *D. marginatus* haemolymph using Endo H enzyme resulted in disappearance of the anti-(RA)HA or anti-Dorin M sera staining (data not shown).

Localisation of FRePs in *D. marginatus* organs

Taking advantage of specific anti-FReP sera, we performed immunolocalisation of these proteins in the midgut, SGs, and haemocytes dissected from the partially fed *D. marginatus*.

In a type III of SG acini, anti-DMF1 serum labelled structures inside the epithelial cells that surround the secretory cells (Figure 4A). In the acinus type II, positive reaction of this serum was detected inside of the secretory granules located in the cytoplasm of cells occurring near the acinar duct (b cells; Figure 4B). We observed anti-DMF1 labelling inside the midgut cells (Figure 4C) and, surprisingly, in perinuclear region of haemocytes attached to SGs (Figures 4D, E). Only haemocytes attached to SGs showed reactivity with anti-DMF1 serum while free circulating haemocytes did not appear to contain FRePs (data not shown).

Anti-DMF2 serum showed the presence of FRePs in the cytoplasm of midgut cells (Figure 4F) and inside of haemocytes attached to SGs (Figure 4G). Again, circulating haemocytes did not appear to contain FRePs (data not shown).

Anti-DMF3 serum localised FRePs in surface structures above midgut cells as seen in the longitudinal section (Figure 4H) and inside of several surface cells of the midgut as evident from the tangential section (Figure 4I). Anti-DMF3 labelling was observed inside the salivary duct in the cytoplasm of epithelium and in the cuticular layer facing to the lumen of the salivary duct (Figures 4J,K).

Identification of FRePs by mass spectrometry

Coomassie Brilliant Blue-stained protein bands, corresponding to the positively immunostained putative FRePs, were cut off the SDS-PAGE gel, alkylated and reduced, and finally trypsin digested. Next, the tryptic peptides were analysed by mass spectrometry. The 290 kDa proteins from *D. marginatus* and from the three *Rhipicephalus* species were identified as hemelipoglycoprotein from *D. marginatus* (GenBank: ABD83654, Table 2). Identification of the other putative FRePs was not successful. However, examination of the hemelipoglycoprotein amino acid sequence (from *D. variabilis*, [4]) did not show the presence of the fibrinogen domain.

Discussion

These results provide further information about tick lectins/FRePs. In agreement with our previously published results, we detected haemagglutination activity (HA) in haemolymph of ticks *Dermacentor marginatus*, *Rhipicephalus appendiculatus*, *R. pulchellus*, and *R. sanguineus* [15]. HA was previously detected in *R. appendiculatus* and it was inhibited by GalNAc, xylose, and fructose [21,22]. Our results show *N*-acetylated saccharides and sialic acid together with sialylated glycoproteins as the most potent inhibitors of HA in all studied *Rhipicephalus* species and in *D. marginatus*. These observations suggest involvement of NeuAc and the *N*-acetyl groups in saccharide recognition by tick lectins/FRePs. Previously, we observed similar inhibitory pattern also for *Ixodes ricinus*, *Ornithodoros tartakovskyi*, *O. pappillipes*, and *Argas polonicus* [15].

The haemagglutinins of the tick haemolymph seem to belong to the fibrinogen-related protein family, as was shown in the case of the lectin Dorin M from *O. moubata* which exhibited HA [8]. Haemagglutination is commonly mediated by carbohydrate specific binding and thus, by lectins [23]. Immune sera against the haemagglutination activity are therefore used for detection of unknown lectins [20].

Antigenic similarities among all examined lectins/FRePs were confirmed by cross-reactivity of antibodies directed against these proteins among various ixodid and argasid tick species. We used the immune serum raised against the Dorin M (which was purified in native state from the haemolymph of *O. moubata* [8]) to detect the presence of FReP proteins also in ixodid ticks. Anti-Dorin M serum reacted with four haemolymph proteins in *D. marginatus* (molecular weights of 38, 79/80, and 290 kDa) and with three protein in *Rhipicephalus* ticks (molecular weights of 58, 75, and 290 kDa). An additional protein with size of 45 kDa was detected in *R. pulchellus*. Similarity of epitopes was shown for putative FRePs from *D. marginatus*, where the sera raised against the different

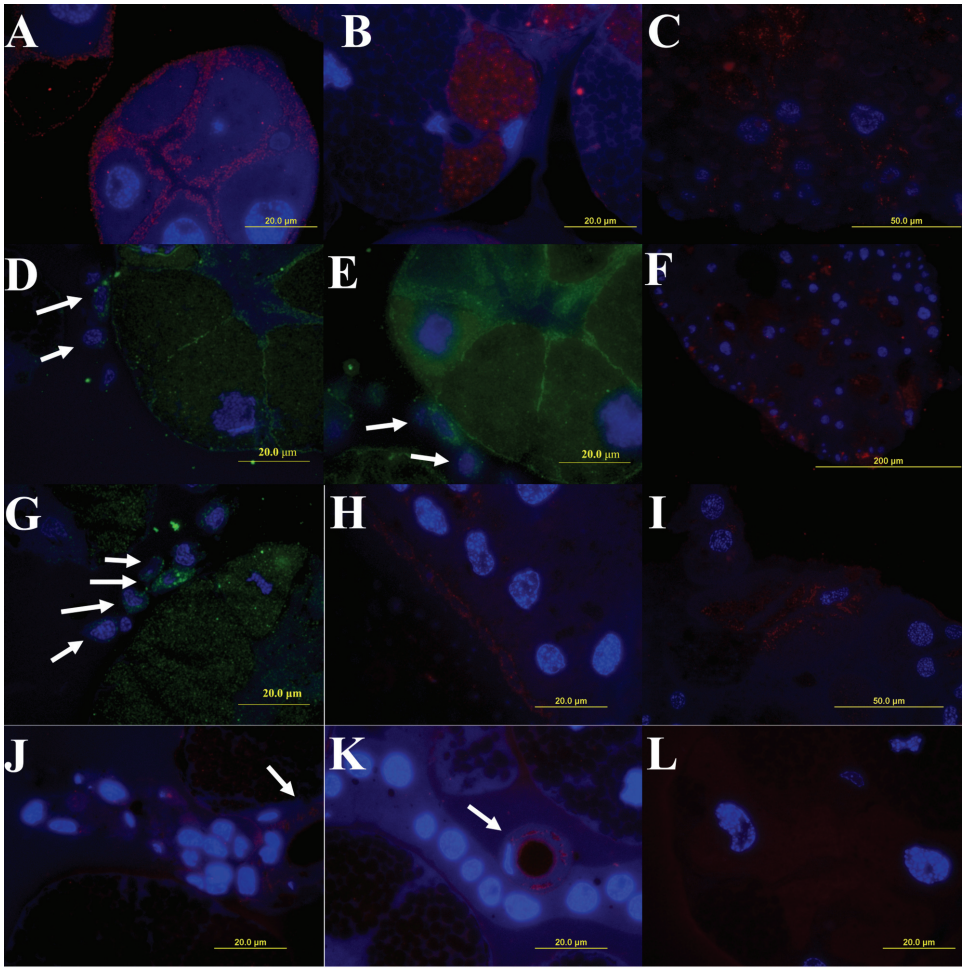

Figure 4 Immunolocalisation of FRePs in *D. marginatus* organs using fluorescence microscopy. Thin sections of midgut (C, F, H, I), salivary glands (A, B, J, K, L), and haemocytes attached to salivary glands (D, E, G) from fed female *D. marginatus* were labelled with sera raised against *D. marginatus* FRePs. Anti-DMF1 (A, B, C, D, E), anti-DMF2 (F, G), and anti-DMF3 (H, I, J, K) antibodies were used. Control reaction were carried out, in which anti-DMF sera were omitted (L). FRePs were localised on the surface and inside epithelial cells surrounding secretory granular cells of the acini type II (A, D, E); in granules of secretory cells located in the acini type III (B) and in haemocytes attached to the surface of salivary glands (D, E, G; arrows). Positive labelling reactions were observed in the cytoplasm of epithelium of salivary duct cells (J, K; arrows) and in thin cuticular layer facing to the lumen of the salivary duct (J, K). Anti-DMF sera detected structures on the surface (H) and inside the midgut cells (C, F, I). A, B, C, F, H, I, J, K, and L - Cy3-conjugated secondary antibodies (red); D, E, and G - FITC conjugated secondary antibodies (green). Cell nuclei were counter-stained using DAPI (blue).

FRePs cross-reacted with all proteins. The same proteins were detected using sera raised against the HA of ticks - *D. marginatus* and *R. appendiculatus* (Figures 1A, B) and *I. ricinus* (data not shown). These antibodies should be directed against lectin-like molecules present in the haemolymph [20]. The same staining patterns obtained using these sera and the anti-Dorin M serum again suggests close relatedness of FReP proteins and the fact that they belong to lectins.

Finally, commercial antibodies directed against the fibrinogen domain of ficolin reacted with the identified putative tick FRePs (Figure 2). Except the biggest

Table 2 Summary of the MS identification experiments performed on the 290 kDa proteins from the studied ticks

	Number of repetitions	Amino Acid sequence coverage
D. marginatus	5	10.2%
R. appendiculatus	3	13.4%
R. pulchellus	3	4.2%
R. sanguineus	3	20.8%

Number of MS experiments performed for identification of each of the 290 kDa proteins from the ticks *D. marginatus*, *R. appendiculatus*, *R. pulchellus*, and *R. sanguineus*. All the proteins were identified as hemelipoglycoprotein (*D. variabilis* hemelipoglycoprotein GenBank:ABD83654) and the amino acid sequence coverage is indicated for each protein.

recognised protein in each species, hemelipoglycoprotein, none of the proteins was identified by mass spectrometry. Most probably this results from the missing information on gene sequences from almost all tick species; the only known tick genome is that of *I. scapularis*. However, a Dorin M-like protein was identified in cDNA libraries from *D. marginatus* using degenerate primers specific for the fibrinogen domain of Dorin M and other FRePs (GenBank:ACI22373) [24]. As *D. marginatus* hemelipoglycoprotein does not contain the fibrinogen domain, we conclude, that this protein could be one of the smaller putative FRePs identified in this study in *D. marginatus* (36 kDa or 79/80 kDa proteins).

The largest of these proteins in each case were identified as hemelipoglycoprotein (HLGP). The protein was recognised by sera directed against the FReP proteins, HA of tick haemolymphs as well as against the fibrinogen domain itself. However, the protein does not contain the fibrinogen domain and therefore, the protein is not related to FRePs [3]. One of the explanation, could be epitope-similarity of the protein to the fibrinogen domain. Structural rather than sequence-similarity to other fibrinogen or other lectins is implicated also by saccharide-binding by HLGP [4]. However, reactivity of anti-fibrinogen antibodies with HLGP show, that the recognition of protein by anti-FReP antibodies does not necessarily mean the presence of the fibrinogen domain in the recognised protein.

The putative FRePs detected in this study seem to be glycosylated. Glycosylation is the common feature of lectins/glycan-binding proteins [25]. Decrease in molecular weights of the detected proteins was observed after enzymatic deglycosylation in the range of several Daltons, which corresponds to the low-mass arthropod/tick glycans [[4,19,26], unpublished results]. The presented results for hemelipoglycoprotein also correspond to our previous findings [4]. In *D. marginatus*, a small molecular weight protein was detected after deglycosylation (34 kDa) using anti-DMF3 serum. However, the molecular weight difference is too big to be related to the glycosylation and we assume this band to represent a protease cleavage product of the 290 kDa protein.

Further, the newly detected putative FRePs and hemelipoglycoprotein were immunolocalised in *D. marginatus* tissues. One of the FReP-specific sera labelled granules of secretory SGs acini type II suggesting secretion of the proteins to the host. The detection of FRePs in surface parts of acini type III and midgut and especially in haemocytes associated with SGs may indicate their putative innate immune functions. Surprisingly, we were not able to detect FReP proteins in freely circulating haemocytes. Tick FRePs/lectins as well as hemelipoglycoprotein are expressed in same organs as shown by detection of the proteins or their mRNA [4,11,18].

Involvement of carbohydrate-binding proteins in the tick immune reaction was shown previously in the case of *Theilleria parva* infection of *R. appendiculatus* [27]. Thus, the detection of new members of fibrinogen-related protein family in the *Dermacentor* and *Rhipicephalus* ticks, and their further characterisation can bring new information about the tick innate immunity processes and open new ways in struggle with ticks and tick-borne diseases. Hemelipoglycoprotein, which does not contain the fibrinogen domain but seems to share structural features with FRePs was shown to bind saccharides as well [4]. In fish, vitellogenin (closely related to hemelipoglycoprotein of ticks) was shown to be directly involved in immune reaction and in recognition of carbohydrate moieties on the surface of invading bacteria [28].

Conclusions

Fibrinogen-related proteins with lectin activities are present in all studied tick species. These proteins involved in recognition of NeuAc and the *N*-acetyl groups are expressed in midgut, salivary glands as well as haemocytes attached to the salivary glands. Information about specific activities of these exemplary molecules could reveal much information on tick innate immunity processes and help in future design of anti-tick vaccines. Furthermore, cross-reactivity of antibodies against the FRePs and the fibrinogen domain itself with proteins which do not contain the fibrinogen domain point out the need for sequence analysis of proteins identified by such antibodies.

Methods

Ticks

Partially-fed females of ticks *Dermacentor marginatus*, *Rhipicephalus appendiculatus*, *R. pulchellus*, and *R. sanguineus* were obtained from the tick rearing facility of the Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic in České Budějovice. Females were allowed to feed on guinea pigs for 6 days.

Haemolymph and tissue preparation

Haemolymph was collected after cutting off a part of anterior leg of partially-fed female by fine scissors. The haemolymph from 8 to 10 females (corresponding to 10-15 μl) was collected into 50 μl PBS containing protease inhibitors (Pierce). The solution was centrifuged at 4°C for 10 minutes at 100 × g to pellet the haemocytes. The resulting supernatant was then clarified at 23000 × g for 20 minutes and both the plasma (further referred to as 'haemolymph') and haemocyte fractions were stored at -20°C. Haemolymph was prepared for ticks *D. marginatus R. appendiculatus*, *R. pulchellus*, and *R. sanguineus*.

Midgut and salivary glands were dissected from partially-fed females of the tick *D. marginatus*, thoroughly washed in PBS to remove possible contamination from gut-content, and put into 0.9% NaCl at 4°C before their processing for the fluorescence microscopy analysis.

Haemagglutination activity (HA) and HA inhibition

Haemagglutination activity assays were performed as described earlier [20] using 2% suspension of rabbit erythrocytes in 0.15 M NaCl for 1 hour at room temperature. Two-fold dilution of haemolymph was prepared in U-type bottom microtitration plates. Haemagglutination buffer (50 mM Tris-HCl, 0.15 M NaCl, 20 mM CaCl2, pH 7.0) was used for the experiments. Reciprocal value of the last dilution of the highest sample dilution still causing visible agglutination was used as the titre of HA and the amount of haemagglutinins in this well is defined as 1 HA unit.

HA inhibition was performed in serial dilution of saccharides and glycoproteins.. Haemolymph diluted to contain 1.5 HA unit and 2% erythrocytes suspension were added. The 50% inhibitory concentration was determined after 1 hour at room temperature as the lowest inhibitor concentration that completely inhibits the binding activity of 1.5 HA unit.

Commercial antibodies and polyclonal serum preparation

Two commercial antibodies produced in rabbits were used for confirmation of epitope similarities of the studied proteins with ficolin. The first, anti-FCN1 H (HPA001295, Sigma-Aldrich), recognise amino acids 199 to 307 from the human ficolin 1 while the second, anti-FCN1 S (SAB2100804, Sigma-Aldrich), is specific for the region 180-229 of the protein. Thus, both antibodies are specific for the fibrinogen domain of the ficolin, which comprises amino acids 115 to 325.

We have described the preparation of anti-haemagglutination activity (anti-HA) serum elsewhere [20]. Briefly, mouse erythrocytes were incubated with *D. marginatus* (serum referred to as 'anti-(DM)HA') or *R. appendiculatus* (serum referred to as 'anti-(RA)HA') haemolymph, washed, mixed with Freund's adjuvant in a 1:1 ratio and injected into mice. Immunisation was repeated 4 × every 14 days. Blood was collected 14 days after the last immunisation. Sera were supplemented with glycerol (1:1), aliquoted and stored at -20°C.

Polyclonal immune sera against the *D. marginatus* FReps were prepared using the respective proteins cut out the SDS-PAGE gel. Anti-DMF1 serum was raised against the 36 kDa protein, anti-DMF2 against the 79/80 kDa double-band, and anti-DMF3 against the 290 kDa protein. Each protein band was cut out and homogenised with 60 μl PBS (1×). Freund's adjuvant was added in a 1:1 ratio and 80 μl of this solution was subcutaneously injected to BALB/c mice. Immunisation was repeated 4 × every 14 days. Blood sera were collected 14 days after the last immunisation. Sera were supplemented with glycerol (1:1), aliquoted and stored at -20°C.

Mice were handled according to internal rules of the Institute of Parasitology, BC ASCR, Ceske Budejovice and the Animal Act of the Parliament of the Czech Republic.

SDS-PAGE and immunoblotting

For SDS-PAGE, haemolymph samples were diluted 1:5 in physiological buffer, mixed with loading buffer (Fermentas) and heated for 5 min at 95°C. SDS-PAGE [29] was performed on 4-17.5% gradient gels. Gels were stained with PageBlue Protein Staining Solution containing Coomassie Brilliant Blue (Fermentas).

The electrophoretically separated proteins were transferred to the PVDF membrane according to [30] for 1 hour at 20 V. The PVDF membrane was washed in PBS (14 mM NaCl, 0.15 mM KH2PO4, 1.8 mM Na2HPO4, 0.27 mM KCl, pH 7.2), cut into strips, and incubated for 1 hour in 5% skim powdered milk in PBS. Strips were then incubated for 1 hour in mouse the appropriate antibodies (see above), washed with PBS-Tween 20 (0.05% Tween 20 in PBS) and incubated with goat anti-mouse/anti-rabbit antibody conjugated with alkaline phosphatase (AP, VectorLabs) in 5% milk. After incubation, strips were washed with PBS-Tween 20 and PBS. Reaction was developed in AP staining solution (VectorLabs) and after the development of sufficient signal was stopped by washing the strips several times in distilled water.

Enzymatic deglycosylation

Haemolymph samples were deglycosylated using glycosidases Endo H (New England Biolabs) or N-glycosidase F (New England Biolabs) in 50 mM sodium phosphate buffer, pH 7.4, overnight at 37°C, according to the instructions of the manufacturer. Denaturation of proteins using SDS and DTT at 95°C for 10 minutes was performed prior the deglycosylation. Deglycosylation reactions were carried out in duplicates.

Fluorescence microscopy

SGs and midguts were fixed in 4% formaldehyde/0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 (PB) for 2 hours at 4°C. Pieces of tissues were embedded in 10% gelatine at 37°C, rinsed in PB at 4°C and dehydrated with a gradient series of ethanol (30% at 0°C, 50%-100% at -15°C for 1 h) in the EM AFS freeze substitution device (Leica). The specimens were gradually infiltrated with 25%, 50%, 75%, and 100% resin LR White (Polysciences) with 0.05 g benzoyl methylether/10 g resin at -15°C. The specimens were embedded to BEEM capsules (Polysciences) and polymerised at -15°C for 24 hours under UV radiation.

After polymerisation, the semithin sections were cut using the ultramicrotome EM UC6 (Leica) and dried on microscopic slides.

The sections were blocked in Tris-buffered saline, pH 7.4 (TBS) with 0.05% Tween-20 containing 3% BSA for 3 hours and incubated with 1:20 anti-DMF1 or anti-DMF2 or anti-DMF3 sera overnight at 4°C. After washing in TBS, the sections were incubated with anti-mouse secondary antibody conjugated with FITC (Sigma-Aldrich) or Cy3 (Jackson Immunoresearch) for 3 hours at ambient temperature, washed thoroughly, and the cell nuclei were stained in 1 μl/ml DAPI (Sigma-Aldrich) solution for 15 minutes at ambient temperature. After washing, the sections were mounted in a solution composed of 2.5% DABCO/95% glycerol containing n-propyl galate (15 mg/ml) (Sigma-Aldrich) and examined using a BX51 fluorescence microscope equipped with a DP70 camera (Olympus).

Mass spectrometry

Putative FRePs were cut out the SDS-PAGE gels and reduced, alkylated, and trypsinised (ROCHE) according the manufacturer's instructions and subjected to LC-MS analysis. Peptides were separated by NanoAcquity UHPLC (Waters) on C18 silica (BEH300 column, Waters) using acetonitrile gradient (5-80%) as a mobile phase and analysed by ESI-QTOF PREMIER mass spectrometer (Waters). The obtained data were compared to *Acari* non-redundant and Swiss-Prot databases using ProteinLynxGlobalServer software (Waters) under strict criteria.

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Authors' contributions

JD, MF, JS conducted electrophoreses, blotting experiments, and HA assay, JD and JS participated in anti-DMF and anti-tick HA sera preparation, MV performed tick tissue preparation and together with JD performed the FM analysis, JS prepared the manuscript, and LG co-ordinated the experiments. All authors participated in the design of experiments and they approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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5.5.2 Further results

5.5.2.1 O. moubata lectin Dorin M is produced by O. moubata OME21 cell line and is released to the culture medium

Dorin M is known to be produced in several *O. moubata* tissues [276, 279]. On contrary, it was not known, if the OME cell lines are able to produce this lectin nor the actual cell types which are present in the culture. Therefore, OME21 [395] cell lysates as well as the medium from the culture (collected after 14 days) were analyzed for the presence of Dorin M using anti-DorinM serum and serum directed against a lectin from *I. ricinus* (which show cross-reactivity towards Dorin M and other tick FRePs) [275, 398, 399].

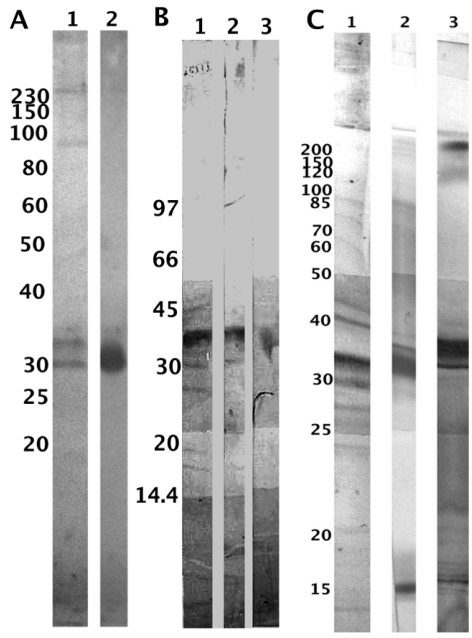

*Figure 31. O. moubata cell line OME21 is producing Dorin M lectin. **A** – immunoblotting using serum directed against the I. ricinus lectin. 1 – OME21 cells, 2 – medium from the OME21 culture. **B** – immunoblotting using anti-Dorin serum (1) and serum against the I. ricinus lectin (2, 3). 1, 2 – OME21 cells, 3 – medium from the OME21 culture. **C** – immunoblotting using anti-Dorin serum. 1 – OME21 cells, 2, 3 – medium from the OME21 culture. In (3), sterile L15 medium was added into the solution of the primary antibody.*

A protein band with molecular weight corresponding to Dorin M (approximately 30-35 kDa) reacted with both sera (Fig. 31). Similarly, a protein band from the culture medium was reactive with both sera. Complementation of anti-Dorin M serum with sterile naive L15 medium did not alter the reactivity towards the protein.

The protein was partially purified using the published isolation method used for purification from tick hemolymph [276]; however, high amounts of contaminating proteins were present (data not shown).

5.5.2.2 Lectin- and FReP-like proteins identification in ticks

mRNA was isolated from several tick species and the corresponding cDNAs were prepared. These were used for analysis of the presence of fibrinogen-related proteins (FRePs).

Several primer pairs were used for this purpose. First, degenerate primers designed based on the knowledge of the fibrinogen domain [276, 277, 279] were used. Three novel proteins were identified in *D. marginatus* (DMFREP, ACI22373), *H. impeltatum* (ixoderin-like protein, ACI22374), and *H. punctata* (Dorin M-like protein, ACI22375) (Table 6).

Further degenerate primers have been designed based on the newly identified tick FReP sequences and were used for further analyses. These experiments did not result in sequences of acceptable quality.

Table 6. Nucleotide sequences of the identified FRePs in three tick species.

DMFREP [*Dermacentor marginatus*] ACI22373
GGGWTVIQRRGQFGNRVYHFYRNWTEYANGFGHPSDEYWIGNHALHT LTSGDEEMALRVVLRNHTEEAVSADYESVRIASEADLFRIEVGKYLGPEGW DAFSVNGQNFSTFDRDNDEYEHNCAHTFDGAWWYE
ixoderin-like protein [*Hyalomma impeltatum*] ACI22374
GGWTVIQRRGQYGNRVFHFYRNWTEYANGFGNPSDEYWIGNQALHYLT SSDEKMAL
dorin M-like protein [*Haemaphysalis punctata*] ACI22375
GGGWTVIQRRGQFGNRVFHFYRNWTEYAHGFGNPTDEYWIGNRALHAL TAGDDQMALRVV

6 Discussion

Still increasing sensitivity and availability of instrumentation such as mass spectrometers brings new and new possibilities into the field of glycobiology. Increasing number of organisms have been studied regarding their ability to perform protein glycosylation, the structure of their glycans, and the function of the glycan moieties. Common glycan structures were identified in higher organisms, in contrast to bacteria, where a range of possible structural units was present. Currently, several main areas of research are being followed in glycobiology – glycome changes associated with human diseases and related organisms, glycans in non-vertebrate organisms, their function, and possible effect on humans (direct or indirect), and applied research directed towards the use of genetically modified organisms for production of recombinant proteins.

This thesis describes some aspects of glycobiology of ticks, interesting and both medically and economically important invertebrate blood-feeding parasite, the tick.

The genome of the only tick species, deer tick *Ixodes scapularis* is known to date. This North American tick is a close relative of the European common tick *I. ricinus*. All the basic enzymes necessary for *N*-glycosylation are present in *I. scapularis* genome as well as glycosidases and glycosyltransferases. However, no such information is available for the other tick species.

Probably the most important step in the process of protein *N*-glycosylation is the attachment of the glycan precursor to the protein catalyzed by oligosaccharyltransferase. In lower organisms, it has only one subunit while in higher organisms it is a multimeric protein. The catalytic unit is always the same, named STT3 in Eukaryotes. The *I. scapularis* genome also contains this protein; however, there is only one STT3 isoform present in the tick genome, while all the other higher Eukaryotes from plants to mammals (including arthropods) contain two isoforms – STT3A and STT3B [136, 400]. The tick STT3 shows high

similarity to STT3B isoforms from Eukaryotes, mainly to insect proteins. This is surprising, as the STT3A seems to be responsible for constitutive glycosylation and is more conserved, while STT3B seems to play a role in regulation of *N*-glycosylation [199]. Other authors suggest the importance of both subunits for the function of the OST complex [401]. However, the absence of the STT3A isoform was not reported to date in other higher Eukaryote. Still, there is a possibility of the presence of both STT3 isoforms in ticks with the A isoform waiting for its discovery.

Ticks possess not only the enzymatic machinery needed for the production of the whole range of high-mannose glycans (glucosidases, mannosidases) but also the enzymes required for further modification of the glycan core. These are *N*-acetylglucosaminyltransferases (MGAT1 – MGAT5), galactosyltransferases (β1,3-, β1,4-, and not annotated), sialyltransferases (α2,3- and α2,6-), number of fucosyltransferases (α1,3- and α1,6-modifying the *N*-glycans as well as general fucosyltransferases), and other glycosyltransferases. The presence of these enzymes suggests the ability of ticks to produce hybrid and complex type glycans as well as core-fucosylated *N*-glycans with fucose α1,3- and α1,6-linked to the core GlcNAc. Similarly, glycosyltransferases responsible for production of hybrid and complex glycans were found in other arthropods such as *Drosophila* flies and some of them were even produced as functional recombinants in *Pichia* [402].

However, the presence of a gene in the genome does not necessarily mean also the expression of the corresponding protein. Therefore, confirmation of the expression of these enzymes will be needed. The presence of sialylated glycans in tick tissues was in focus of our laboratory previously. Therefore, expression of the tick α2,3-sialyltransferase was analyzed and it was confirmed in tick cell cultures as described in this thesis. The expression of sialyltransferase [403] and the presence of sialylated glycans was already confirmed in *Drosophila* [235] and the ability of insect cells to scavenge sialic acid from their environment was also observed [404]. Further evaluation of the sialyltransferase (and other

glycosyltransferases) expression in tick tissues will be needed in this regard. Sialylated (NeuGc) glycans have been directly observed in tick tissues for the first time in the form of mono- and disialylated glycans (submitted manuscript). They were localized to the external surface of the basement membrane covering the salivary glands but not the basement membrane of the gut. Most probably, the observed NeuGc-containing glycans are of host origin and their highly-specific localization suggests particular roles for these structures in the basement membrane of tick salivary glands.

Besides the glycosyltransferases, results of *N*-glycans analysis are described in this thesis as well. *N*-glycans modifying two tick proteins have been analyzed. First, Dorin M lectin from the *O. moubata* tick contains three classical *N*-glycosylation sequons. Two sites are modified by high-mannose type glycans with up to nine hexoses while the third one is a core-fucosylated high-mannose glycan with four to five mannose residues [405]. Second, four different *N*-glycan structures are present in another tick hemolymph protein, hemelipoglycoprotein from *D. marginatus* – two high-mannose glycans with nine and ten hexoses and two complex glycans with four HexNAcs and seven and eight hexoses; no core-fucosylated structure has been identified [398]. In hemelipoglycoprotein, the actual glycans occupying concrete glycosylation sites are not determined. While core-fucosylated glycans are not present in hemelipoglycoprotein, they were found in *D. marginatus* hemolymph [398]. This suggests protein-specific glycosylation of some tick proteins.

In this regard, it is important to mention the detection of hemelipoglycoprotein in two forms in *D. marginatus* salivary glands – one of the forms correspond to the glycosylated protein (from hemolymph), while the other to its deglycosylated form [398]. The presence of the glycosylated and nonglycosylated forms has yet to be confirmed by mass spectrometry. Similarly, diversity in protein glycosylation has been suggested for *T. infestans* salivary apyrases [406] or the human salivary amylase [407]. Furthermore, the presence of

glycosylated and non-glycosylated forms of the human cysteine-rich secretory protein 3 in saliva and other secreted fluids has been demonstrated [408]. The protein shows similarity to pathogenesis-related proteins in plants and thus its involvement in innate immunity has been suggested.

Hemagglutination activity and thus possible lectin activity has been shown for both Dorin M and HLGP. Glycosylation of lectins is quite common and can even increase the lectin-carbohydrate interaction [219]. Examples of glycosylated insect lectins can be also found [409, 410].

The presence of non-fucosylated and core-fucosylated *N*-glycans in *I. ricinus* tick glycoproteins is not surprising as these structures were identified in abundance in other arthropods [242, 244, 245, 411] as well as in other tick species [254, 257]. Hybrid and complex type glycans have been identified in arthropods even though in much lower amounts compared to high-mannose structures [242, 244, 245, 247, 411-413]. Similarly, hybrid and complex glycans were found in ticks, mainly using lectinoblotting [254-256]. The presence of high amounts of the more complex glycans in the hemelipoglycoprotein as well as *D. marginatus* hemolymph was surprising.

These results were nevertheless confirmed by MS analysis of *N*-glycans from *I. ricinus* saliva and salivary glands. Although high-mannose glycans were common in the *I. ricinus* saliva and salivary glands glycome, complex or hybrid glycans represented 49 to 70% of all glycans in some cases. The high-mannose glycans were present from the smallest to the more extensive ones (containing three to ten hexoses). In correspondence with the known enzymatic pathways of *N*-glycan trimming/elongation reactions [153], core-fucosylation was present on paucimannosic structures with the maximum of five hexoses. In contrast to *Drosophila N*-glycans [235], only monofucosylated structures were present. Immunoblotting with anti α1,3-coreFuc antibodies and with fucose-specific lectins in combination with *N*-glycosidase A and *N*-glycosidase F digests revealed the presence of both α1,3- and α1,6-core-fucosylation in tick salivary

glycoproteins. These results correspond with the analyses of *Drosophila* glycans [235]. Furthermore, α1,3-core-fucosylation in *N*-glycans from *I. scapularis* salivary glands was also shown [257]. The presence of α1,3-fucosyltransferase (EEC05695) and α1,6-fucosyltransferases (EEC04076, EEC04286) in the tick (*I. scapularis*) genome shows the ability of tick in the genus *Ixodes* and possibly also of the other ticks to produce core-fucosylated structures.

However, the presence of the α1,3-linked core-fucose in salivary *N*-glycans is very interesting as these structures can trigger sometimes furious immune/allergic reaction [242, 414]. This stands in sharp contrast to the actual effect of tick saliva on the host's immune and defense systems which are markedly inhibited. There are several possible explanations for this contradiction:

– tick immunogenic *N*-glycan structures may not affect the efficiency of the inhibiting salivary proteins and the inhibition is so effective that the host does not launch an effective immune response;

– or the tick *N*-glycans trigger the immune response, but it is much effectively inhibited;

– or the tick *N*-glycans help the host systems inhibition in some way.

Precise description of the role of tick *N*-glycans and especially the possible immunogenic glycan structures in the effects of tick saliva on the host can bring an entirely distinct view of the ongoing processes and new insight into tick-host interactions on the molecular level.

Besides the high abundance of core-fucosylated structures, complex glycans were also common in tick salivary *N*-glycome. High-mannose structures, which are major component of *N*-glycomes in other arthropods, represented only 43% of the total glycans in *I. ricinus* fed female saliva. Yet lower percentages of high-mannose glycans are present in the salivary extracts from fed and unfed females and from males. Here, the explanation could be simply the persistence of the host glycoproteins or their glycan moieties throughout the feeding or even molting into the next stages [415-418]. However, several observations suggest the

tick origin of the identified *N*-glycans – the high abundance of core-fucosylated structures, core-fucosylation of the majority of complex glycans, and the dominance of less extensive structures (less than seven residues) in all the studied materials (and mostly in feeding females).

Localization of core-fucosylated structures in tick salivary glands is another argument indicating the tick origin of these structures. Lectin and antibody staining of α1,3- and α1,6-core-fucosylated glycans revealed their presence in cell membranes or secretory granules in salivary glands which suggests their specific excretion into saliva and the host in the end. Underestimation of the presence of complex *N*-glycans in insects as well as in other arthropods was suggested in relation to low amounts of glycoproteins isolated from the total proteins using GNA (a mannose-specific lectin) affinity chromatography [419].

As mentioned above, differences in different glycan classes have been observed between the fed female saliva and salivary gland extracts as well as between salivary glad extracts (SGEs) of fed females versus the unfed females and the males. Generally, high-mannose structures and less-extensive glycan structures (less than seven residues) dominated the spectra of glycans from fed female saliva. On the other hand, unfed tick (males and females) SGEs contained more core-fucosylated complex glycans and more-extensive structures (more than seven residues). These structures were more abundant in *I. ricinus* males compared to unfed females. Sexual dimorphism in glycan expression in salivary glands has been observed in mosquitoes *A. gambiae* but differences between the glycans present in salivary glands have been observed also between different mosquito species [420]. Tick males contain a special type of acini (type IV acini); the expression of proteins related to acini type IV can partly explain the observed differences [34]. The differences between the glycan content of salivary glands isolated from feeding and unfed tick females can be connected with the digestive processes taking place during the feeding.

Differences in the abundance of glycan types in feeding females saliva and SGEs could demonstrate specific excretion of glycosylated molecules into saliva and subsequently into the host [421]. This is quite logical as lower amounts of core-fucosylated *N*-glycans (containing fucose in the α1,3-linkage) would lower the potential immune reaction of the host [242, 244]. However, the presence of similar glycan structures in both the saliva and SGEs confirms the possibility to use salivary gland extracts as a substitution for saliva (which is more laborious and timely to prepare) not only for immunologic and other studies, for example [417, 422] but also for glycomic analyses.

In the context of highly abundant complex glycans in tick saliva and salivary glands (but also in other tick tissues and whole tick homogenates; preliminary results of Z. Šimonová and J. Dupejová), surprising is the absolute dominance of high-mannose glycans in the blood-suckling bug *T. infestans*. This corresponds to the findings of Volf and colleagues, who observed PSA lectin (mannose and glucose specific) binding to *T. infestan*s saliva [423]. While the differences in *N*-glycans of *Drosophila* and ticks could be explained by the blood-feeding behavior of ticks, absence of complex glycans in *Triatoma* questions such theory. The function of complex glycans in tick saliva is not clear and several possibilities arise:

- they serve some function in interaction with the host defense systems, or
- they are present just because the tick produces them, or
- they are artifacts from the blood meal.;

On the other hand, other blood-feeding arthropods such as *Triatoma* possibly do not need this type of glycans or maybe they are not able to produce them and rely on some other ways how to influence the host.

Analysis of *N*-glycomes of other blood-feeding arthropods is needed to explain such discrepancies.

Besides the tick glycosylation, tick lectins have been also studied within the frame of this thesis. *O. moubata* hemolymph lectin Dorin M was characterized regarding its *N*-glycosylation. This lectin with its specificity towards sialic acid is quite interesting. However, the native protein is formed by dozens of subunits and thus its production as recombinant protein would not be a simple one. Therefore, expression of this lectin was assessed in the *O. moubata* cell lines. Immunoblotting with sera directed against Dorin M as well as another lectin from *I. ricinus* revealed the expression of Dorin M in this cell line. Moreover, the protein was secreted into the cell culture medium. Thus, *O. moubata* cell lines can be used for production of this lectin even without the need to destroy the cell culture, as it can be purified from the culture medium. Similarly, insect lectins were found to be produced in insect cell cultures which were considered useful for their production [424].

Several other lectins were identified in other ixodid ticks. cDNAs from several ticks were analyzed for the presence of fibrinogen-related proteins (FRePs) using degenerate primers and partial sequences of three novel FReP proteins were identified in *D. marginatus*, *H. punctata*, and *H. impeltatus*. Furthermore, immunostaining of hemolymph from *D. marginatus*, *R. appendiculatus*, *R. pulchellus*, and *R. sanguineus* revealed presence of several FRePs in these ticks. One of these proteins has been identified as hemelipoglycoprotein (HLGP) in all these tick species. Hemelipoglycoprotein does not contain the fibrinogen domain [123] and thus the anti-FReP sera reactivity with this protein could suggest similarity of the hemelipoglycoprotein epitopes with the FReP proteins. However, the purified *D. marginatus* hemelipoglycoprotein showed hemagglutination activity as well as weak carbohydrate-binding [135] and it contains von Willebrand factor-like domain [144]. A lectin from *Bombyx mori* has been shown to be similar to von Willebrand factor [483]. Furthermore, the lipoprotein domain of HLGP was suggested to have carbohydrate-binding activity [144].

Under native conditions, *D. marginatus* HLGP formed high-molecular weight complex with other proteins; some of them corresponded to the other FRePs from *D. marginatus*. This finding together with the hemagglutination activity and carbohydrate binding could suggests the involvement of HLGP and the other FRePs in immune processes of the ticks [425]. Moreover, heme binding and storage was suggested as the primary role for HLGP [123]; however, the protein was present even in unfed *D. marginatus* females and in males, which do not feed in such an extent as the females [398].

Tick hemelipoglycoproteins are able to bind heme even though the amount of bound heme per protein molecule is not remarkable when taking into account the size of the proteins. For example, *R. microplus* HeLp binds two mols per one mol of the protein [117]. Whether or not is this ratio acceptable in the means of energy for building such protein is questionable. Bacteria produce 30 kDa proteins that bind two hemes per molecules [426]. In the bloodsucking bug *Rhodnius prolixus*, the heme-binding protein has only 15 kDa [427, 428]. Thus, other roles such as involvement in defense mechanisms could complement the heme storage function of tick hemelipoglycoproteins.

Finally, glycosylation of a tick-borne pathogen, Lyme disease spirochete *Borrelia burgdorferi* s.s. was studied in this thesis. Lectin binding towards the surface of *B. burgdorferi* was reported by several authors [303, 310, 311]. Furthermore, glycosylation of borrelia proteins was described by two groups. Sambri and colleagues described the *N*-glycosylation of the outer membrane proteins A and B using *N*-glycosidase F digestion and Schiff staining [304, 305]. Ge and colleagues on the other hand described glycosylation of the FlaA protein from *B. burgdorferi* s.s [307]. They purified the periplasmic flagella fraction of the borrelia cells, electrophoretically separated the flagellar proteins, and electroeluted the FlaA protein. Next, the Schiff staining and lectinoblotting in combination with *N*-glycosidase F treatment were used to prove the glycosylation

of the purified FlaA. Still another authors showed anomalous migration of FlaB flagellin (estimated Mw 41 kDa compared to the theoretical mass of approximately 36 kDa) [309]. In an attempt to confirm these finding and identify the glycans *N*-linked to these four borrelial proteins, samples from several genospecies of the *B. burgdorferi* s.l. complex were analyzed.

Lectinoblotting, Schiff staining, and *N*-glycosidase F digestion experiments on *B. burgdorferi* s.s. (strain B31) spirochetes whole cell lysates, cell cylinders, OM, and PF fractions did not confirm the previously published results, and furthermore, mass spectrometric analysis of OspA, OspB, FlaA, and FlaB proteins did not reveal glycans attached to these proteins [394]. Similar results were obtained on electroeluted FlaA protein. In addition, the effect of tunicamycin, which is an effective inhibitor of *N*-glycosylation [382], was followed in B31 spirochetes. If a protein is *N*-glycosylated, this modification should not be present in borrelia grown in the presence of tunicamycin and a shift in molecular weight of the naturally glycosylated proteins should be observed. However, in the case of these four proteins (FlaA, FlaB, OspA, and OspB) as well as of other major borrelial proteins, no such shift was observed. All these results taken together suggest the absence of *N*-glycosylation of the above mentioned proteins.

On the other hand, the presence of several isoforms of the FlaA and FlaB proteins, lectin affinity chromatography purification of lectin-positive unidentified proteins with molecular weights similar to Osp and Fla proteins, and the appearance of glycoproteins in some flow-through fractions after lectin affinity chromatography could represent production of glycoproteins in low amounts or by spirochetes under specific conditions. Variation of FlaA protein content can be deduced also from the results of Ge and colleagues [307]; the anti-FlaA antibodies reactivity differed greatly in their samples (strong reactivity in sample containing 10 μg of proteins while barely visible band in a sample containing 50 μg of proteins).

As borrelia are able to bind host proteins from its surroundings in the outer membrane[303, 310, 311, 429-432], it is also possible that the observed glycoproteins originate from the environment – either the growth medium or the infected host. This possibility was proved by growing the borrelia in the presence of Triton X-100. The detergent Triton in the medium solubilized the outer membranes; as the result, only immunoglobulins (from the BSK-H medium complemented with rabbit serum) [397] were detected in the subsequent lectinoblotting of samples from these borrelia.

Moreover, glycosylation of FlaA, FlaB, OspA, and OspB proteins has not been shown in other Lyme disease spirochetes (*B. afzelii* strain B023 and *B. garinii* strain Fuji). Surprisingly, differences in lectin staining patterns were observed between the genospecies which could mean either glycosylation of different proteins or binding of different glycoproteins by different genospecies.

In conclusion, *N*-glycosylation of flagellins with glycans sensitive to *N*-glycosidase F is not likely. Moreover, the responsible oligosaccharyltransferase is not known in borrelia or other spirochetes and these bacteria do not contain proteins with the WWDYG sequence. Bacterial flagellins, if glycosylated, usually contain *O*-glycans and in cases with *N*-glycosylation of flagellar proteins, the *N*-glycans did not contain the *N*-glycan core typical in Eukaryotes [433].

7 Conclusions and future perspectives

Glycobiology of ticks is still an unknown field in both the glycobiology and parasitology; however, further research in tick glycans can substantially broaden the knowledge in both glycobiology and parasitology. Regarding saccharide-binding proteins, several tick lectins have been identified and characterized more thoroughly. Two of them are quite interesting. The first is Dorin M, which is a sialic acid-specific lectin, naturally occurring as a high molecular weight homomer (approximately twenty units in one molecule) with possible uses in practical applications in glycobiology research as well as in research of the role of tick lectins in tick innate immunity. In this regard, production of Dorin M by tick cell lines and its release to the culture medium can greatly help in the purification of the native protein. The other lectin is hemelipoglycoprotein, which was shown to have carbohydrate-binding activity, while it does not contain a known carbohydrate-binding domain. As the protein participates also in heme storage, it will be interesting to find the connection between these two functions and possibly its possible involvement in the tick's immune processes.

However, identification of the precise role of other tick lectins such as fibrinogen-related proteins [276, 277, 279, 425] or galectins [281] in tick innate immunity will be of much importance; it looks like at least some of the tick lectins are part of a complement-like defense mechanism in ticks [134, 434-436].

On the other side, tick glycans can participate in the interaction between the tick salivary proteins and the mammalian defense systems. Furthermore, their importance for transmission of at least some of the tick-borne pathogens (*A. marginale*) has been shown [257]. Tick saliva and salivary gland extracts contain core-fucosylated *N*-glycans with the fucose attached to the glycan core by both the α1,3- and α1,6-linkages. α1,3-linked core-fucose was shown to be immunogenic and even allergenic in some cases in mammals including humans [242, 414]. Therefore, analysis of tick glycomes, identification of the

glycoproteins, and glycosylation changes during the tick development are necessary; next, it will be possible to assess the role of these glycans in the interaction of ticks and their hosts and in the pharmacological effects of tick saliva. Such findings will be highly important and potentially useful in our fight with ticks in future.

Sialic acid was shown to be present in glycans in tick tissues by both lectin staining and mass spectrometry [256, submitted manuscript]. Sialic acid is used by some pathogens and parasites as a mimicry [437-439], hiding them from the vertebrate immune system. Also in this case, understanding of the role of sialylated glycans from tick saliva for the tick feeding can direct future anti-tick protective measures development. Furthermore, the ability of ticks to produce sialylated glycans should be clearly stated in the view of the ability of insects but also bacteria to produce sialic acid [235, 312, 404, 439].

8 List of publications

8.1 Scientific papers

Růžek D., Štěrba J., Kopecký J., Grubhoffer L.: The supposedly attenuated Hy-HK variant of highly virulent Hypr strain of tick-borne encephalitis virus is obviously a stain of Langat virus. Acta Virol. 50(4), 227-278, 2006.

Štěrba J., Vancová M., Rudenko N., Golovchenko M., Tremblay T.-L., Kelly J.F., MacKenzie R.C., Logan S.M., Grubhoffer L.: Flagellin and outer surface proteins from *Borrelia burgdorferi* are not glycosylated. J Bacteriol. 190: 2619-2623, 2008.

Man P., Kovář V., Štěrba J., Strohalm M., Kavan D., Kopáček P., Grubhoffer L., Havlíček V.: Deciphering Dorin M glycosylation by mass spectrometry. Europ J Mass Spectrom. 14: 345-354, 2008.

Dupejova J., Sterba J., Vancova M., Grubhoffer L.: Hemelipoglycoprotein from the ornate sheep tick, *Dermacentor marginatus*: structural and functional characterization. Parasit Vectors, 4:4, 2011.

Sterba J., Dupejova J., Fiser M., Vancova M., Grubhoffer L.: Fibrinogen-related proteins in ixodid ticks. Parasit Vectors, 4:127, 2011.

8.2 Abstracts from conferences, published in peer-reviewed journals

Růžek D., Kopecký J., Štěrba J., Golovchenko M., Rudenko N., Grubhoffer L.: Non-virulent strains of TBE virus circulating in the Czech Republic. J Clin Virol. 36 (Suppl. 2): S41, 2006 (P117).

Štěrba J., Dupejová J., Fišer M., Golovchenko M., Rudenko N., Grubhoffer L.: Identification and characterisation of lectins in several tick species. FEBS J. 276 (Suppl. 1): 88, 2009.

Grubhoffer L., Hajdušek O., Vancová M., Štěrba J., Rudenko N.: Glycobiochemistry of ticks, vectors of infectious diseases: carbohydrate-binding proteins and glycans. FEBS J. 276 (Suppl. 1): 141, 2009.

8.2 Book chapter

Grubhoffer L., Rudenko N., Vancova M., Golovchenko M., Sterba J.: Circulatory system and hemolymph: structure, physiology, molecular biology. In: Biology of Ticks, edited by Sonenshine D.E. and Roe R.M. Oxford University Press, planned publication in 2013.

8.3 Abstracts from conferences

2005, 10th International Conference on Lyme Borreliosis and Other Tick-Borne Pathogens, Vienna, Austria, one poster: "Identification of two potential molecular determinants of an attenuated temperature-sensitive phenotype of tick-borne encephalitis virus".

2006, XX. Joint Congress of the Czech and Slovak Societies for Biochemistry and Molecular Biology, Piešťany, Slovak Republic, one poster: "Questing for the glycosylation of outer membrane and flagellin proteins of *Borrelia burgdorferi*".

2007, Third European Congress of Virology, Nürnberg, Germany, one poster: „Immunopathological features of tick-borne encephalitis".

2007, Structure and Stability of Biomacromolecules 2007 and satellite miniconference Modern Trends in Biomacromolecular Research, Košice, Slovak Republic, one poster: "Questing for the glycosylation of outer membrane and flagellin proteins of *Borrelia burgdorferi*".

2007, 8th Multinational Congress on Microscopy, Prague, Czech Republic, one poster: "Observation of flagella and viruses in low-voltage transmission electron microscope".

2008, INTERLEC23 Edinburgh/Stirling, Scotland, UK, two posters: „Fibrinogen-related proteins in tick *Dermacentor marginatus* hemolymph"; „The presence of sialic acid in the salivary glands of the tick *Ixodes ricinus*".

2008, ICTTD Bioinformatics Course 2008, České Budějovice, Czech Republic, one poster: "Questing for the glycosylation of outer membrane and flagellin proteins of *Borrelia burgdorferi*".

2008, XXI. Joint Congress of the Czech and Slovak Societies for Biochemistry and Molecular Biology, České Budějovice, Czech Republic, one poster: "Fibrinogen-related proteins in tick *Dermacentor marginatus* hemolymph".

2009, CUKRBLIK 2009, Brno, Czech Republic, an oral presentation "Fibrinogen-related proteins/lectins in ticks" and one poster: "The presence of sialic acid in the salivary glands of the tick *Ixodes ricinus*".

2009, 11th Congress of ISCDI (International Society of Developmental & Comparative Immunology), Prague, Czech Republic, one poster: "Detection and partial characterisation of several lectins/FReD proteins in hard-ticks".

2009 FEBS Congress, Prague, Czech Republic, an oral presentation "Fibrinogen-related proteins in ticks. Identification and characterisation of lectins in several tick species" and one poster: "Glycobiochemistry of ticks – vectors of infectious diseases: carbohydrate-binding proteins and glycans".

2010, MicroScale Bioseparations 2010, Prague, Czech Republic, one poster: "Analysis of minute volumes of tick saliva samples using MALDI TOF/TOF mass spectrometry".

2010, XIV. Meeting of Biochemists and Molecular Biologists, Brno, Czech Republic, an oral presentation "*N*-glycans from the saliva and salivary glands of the tick *Ixodes ricinus*" and one poster: "Biochemical characterization of *Dermacentor marginatus* hemelipoglycoprotein".

2011, XI. Sigma-Aldrich Conference of Young Biochemists and Molecular Biologists, Žďár n. Sázavou, Czech Republic, oral presentation "*N*-glycans in saliva and salivary glands of the tick *Ixodes ricinus*".

2011, Ticks and Tick-borne Pathogens Conference 7, Zaragoza, Spain, four posters: "*Ornithodoros moubata* cell culture producing the tick lectin, Dorin M"; "Characterization of the hemelipoglycoprotein from the tick, *Dermacentor marginatus*"; "Mass spectrometric identification and electron microscopy localization of sialic acids in *Ixodes ricinus* salivary glands"; "Glycobiology of ticks".

2011, XV. Meeting of Biochemists and Molecular Biologists, Brno, Czech Republic, one poster: "Core-fucosylation in *Ixodes ricinus* ticks: Life-stage- and structure-specific detection".

9 List of scientific projects

9.1 PI or co-PI

2006: Glycosylation of tick-borne encephalitis virus envelope protein and its influence on virus virulence – Grant Agency of the University of South Bohemia.

2007: Glycosylation of membrane proteins of Lyme borreliosis spirochetes – Grant Agency of the University of South Bohemia.

2009-2011: Glycoprotein analysis of *Ixodes ricinus* salivary glands – Grant Agency of the Academy of Sciences of the Czech Republic (PI: Dr. Marie Vancová, Institute of Parasitology, BC ASCR, České Budějovice, co-PI: Ján Štěrba, Faculty of Science, University of South Bohemia, České Budějovice).

9.2 Participated in

2008-2010: Molecular pathogenesis of tick-borne encephalitis – Grant Agency of the Czech Republic,

2009-2011: Interaction of Lyme borreliosis spirochetes and salivary glands of *Ixodes ricinus* tick: the role of borrelia OspC protein – Grant Agency of the Czech Republic,

2005-2011: Centre of molecular ecology of vectors and pathogens – Ministry of Education,

2010: Distribution and diversity of *B. bissettii* in the southeastern USA and in Europe – U. S. Civilian Research & Development Foundation.

10 Supervised students

10.1 MSc. students

finished theses:

Miroslav Fišer, 2009, Identification and biochemical characterization of lectins in the hemolymph of three species of tick in the genus *Rhipicephalus*, co-supervisor.

Jarmila Dupejová, 2011, Hemelipoglycoprotein from the hemolymph of the tick *Dermacentor marginatus*: purification and biochemical characterization, co-supervisor (together with the defense of her RNDr. „Rerum Naturalium Doctor" degree).

current students:

Zuzana Šimonová, Determination of the *N*-glycome of the tick *Ixodes ricinus*; analysis of *N*-glycans in tick tissues and their comparison, co-supervisor (started in 2010).

10.2 BSc. students

finished theses:

Jarmila Dupejová, 2008, Isolation and biochemical characteristics of FReP proteins in the tick *Dermacentor marginatus*, co-supervisor.

Zuzana Šimonová, 2008, Complete genome sequence analysis of TBE virus, strain Hypr, in different passages in host *in vivo*, co-supervisor.

Karolína Šustrová, 2008, Cultivation of tick-borne encephalitis virus in the presence of inhibitor of glycosylation: Its effect on nucleotide sequence of genes encoding viral proteins, co-supervisor.

Martina Jonáková, 2009, Identification of glycoproteins in samples of the CB53 isolate of the spirochete *Borrelia burgdorferi* s.s., supervisor.

David Pech, 2010, Determination of amino acid sequence of hemelipoglycoprotein from the tick *Dermacentor marginatus* by mass spectrometry, co-supervisor.

Martin Strnad, 2010, Interaction of *Borrelia burgdorferi* spirochetes with the salivary glands of *Ixodes ricinus* and with tick cells *in vitro* observed by immunofluorescence microscopy, co-supervisor.

Adriana Walnerová, 2010, Cloning of the gene coding for Outer surface protein C from the Lyme borreliosis spirochetes, co-supervisor.

current students:

Helena Mondeková, Preparation and analysis of the lipoprotein domain from the Hemelipoglycoprotein of the tick *Dermacentor marginatus*, co-supervisor (started in 2010).

Rita Urbanová, Purification of hemelipoglycoprotein from the hemolymph of the tick *Dermacentor marginatus*, supervisor (started in 2010).

10.3 international students

Ania Janowicz, University of Glasgow, 2008, Tick-borne encephalitis (TBE) virus: recombinant envelope protein E and its putative receptor(s), supervisor.

11 Curiculum vitae

Citizenship: Slovak Republic, date of birth: April 12, 1981, Košice, Slovak Republic

Work experience

2004: Laboratory assistant, Laboratory of Clinical Biochemistry, Policlinic Veľké Kapušany, Slovak Republic.

2004-2007: R&D scientist, Institute of Parasitology, Biology Centre of the Academy of Sciences of the Czech Republic, České Budějovice, Czech Republic.

2006: assistant, Biological Faculty (currently Faculty of Science), University of South Bohemia (USB), České Budějovice, Czech Republic.

2006: work placement, Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada (studies on glycosylation of Lyme disease spirochetes, *Borrelia burgdorferi)*.

2007- : lecturer (assistant professor), Faculty of Science, USB.

2007: work placement, Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada (studies on glycosylation of Lyme disease spirochetes, *Borrelia burgdorferi)*.

2009: work placement, National Center for Glycomics and Glycoproteomics, Department of Chemistry, Indiana University, Bloomington, Indiana, USA (analysis of *N*-glycans from the tick *I. ricinus*).

2010: work placement, National Center for Glycomics and Glycoproteomics, Department of Chemistry, Indiana University, Bloomington, Indiana, USA (analysis of *N*-glycans from the tick *I. ricinus*).

Education

2002: BSc. in Chemistry, Faculty of Science, University of P. J. Šafárik, Košice, Slovak Republic.

2003: Socrates-Erasmus stay at the Laboratory of Biochemistry, Faculty of Chemistry, Aristotle University, Thessaloniki, Greece (Assoc. Prof. Panteleimon Arzoglou; Role of the fVIIa in blood coagulation).

2004: MSc. with honors in Chemistry (specialization Biochemistry), Faculty of Science, University of P. J. Šafárik, Košice, Slovak Republic.

2004- : Ph.D. studies in Molecular and Cell Biology and Genetics, Faculty of Science, USB, České Budějovice, Czech Republic.

Attended courses

2004: Basics of the scientific work, Academy of Sciences of the Czech Republic, Prague, Czech Republic.

2005: Summer School on Vector Molecular Biology, České Budějovice, Czech Republic.

2008: ICTTD Bioinformatics Workshop, České Budějovice, Czech Republic.

2010, 2011: PREFEKT (Systematic complex education of R&D scientists in R&D management and administration; Systematické komplexní vzdělávání pracovníků VaV v oblastech širší problematiky řízení VaV jako nezbytný **PR**vek **EFEKT**ivity výzkumu), Brno, Czech Republic.

Other activities

Grant applications reviewer for the Higher Education Development Fund – boards "Biomedicine" and "Life Sciences" for doctoral and teaching development proposals and for proposals aiming to innovation of laboratories and teaching space.

Opponent of two BSc. theses, reviewer of a paper in Journal of Bacteriology.

Participation in organization of scientific seminars, courses, and conferences:

Summer School on Vector Molecular Biology, České Budějovice, 2005;

ICTTD Bioinformatics Workshop, České Budějovice, 2008;

XXI. Joint Congress of the Czech and Slovak Societies for Biochemistry and Molecular Biology, České Budějovice, 2008;

Student's MERCK Prize in Analytical Chemistry, České Budějovice, 2010;

POSTICK Spring School on Tick Biology and Ecology (Marie-Curie ITN project), České Budějovice, 2011.

Teaching activities

Faculty of Science, USB, in charge of the following courses:

Pharmacology and Toxicology (lectures + laboratory courses; in Czech) – lecturer.

Introduction to Toxicology (lectures + laboratory courses; in Czech) – lecturer.

Basic Laboratory Course in Biochemistry (in Czech) – teacher.

Laboratory Course in Biochemistry (in Czech, English) – teacher.

Advanced Laboratory Course in Biochemistry (in Czech, English) – teacher.

12 Abbreviations

The abbreviations used throughout the text are listed below:
CP – carrier protein
coreFuc – core-fucose in *N*-glycans
dHex – deoxyhexose
ER – endoplasmic reticulum
Fuc – fucose
Gal – galactose
GalNAc – *N*-acetyl galactosamine
Glc – glucose
GlcNAc – *N*-acetyl glucosamine
Hex – hexose
HexNAc – *N*-acetylhexosamine
HLGP – hemelipoglycoprotein
MALDI – matrix-assisted laser desorption/ionization
Man – mannose
SG – salivary glands
SGE – salivary gland extract
TOF – time-of-flight

List of lectin abbreviations:
AAA – *Anguilla anguilla* agglutinin
AAL – *Aleuria aurantia* agglutinin
ConA – Concanavalin A (*Canavalia ensiformis*)
GNA – *Galanthus nivalis* agglutinin
HPA – *Helix pomatia* agglutinin
LCA – *Lens culinaris* agglutinin
LTA – *Lotus tetragonolobus* agglutinin
MAA (MAA II) – *Maackia amurensis* (II) agglutinin
PNA – peanut agglutinin (*Arachis hypogea*)
PSA – *Pisum sativum* agglutinin
SBA – soybean agglutinin (*Glycine max*)
SNA – *Sambucus nigra* agglutinin
UEA – *Ulex europaeus* agglutinin
VVA – *Vicia villosa* agglutinin
WGA (TVA) – wheat germ agglutinin (*Triticum vulgaris*)

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