universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

Production, purification and characterisation of flavivirus antigens

Verfasser

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angestrebter akademischer Grad

Magister der Naturwissenschaften (Mag.rer.nat.)

Wien, 2011

Studienkennzahl lt. Studienblatt:	A 490
Studienrichtung lt. Studienblatt:	Diplomstudium Molekulare Biologie
Betreuerin / Betreuer:	Univ. Prof. Dr. Franz X. Heinz

Danksagung

An dieser Stelle möchte ich mich bei Prof. Dr. Franz X. Heinz bedanken, dass ich die Möglichkeit hatte meine Diplomarbeit am Department für Virologie zu machen, sowie für seine Hilfestellung bei wissenschaftlichen Fragen. Mein Dank gilt auch Dr. Karin Stiasny für produktive Diskussionen in Besprechungen, Dr. Stefan Kiermayr für die Betreuung zu Beginn meiner Arbeit, sowie allen Kollegen für ihre Hilfe und das einmalige Arbeitsklima am Institut, das zu dem Gelingen meiner Diplomarbeit beigetragen hat.

Ein herzliches Dankeschön…

...gilt meiner Familie und besonders meinen Eltern, die mir mein Studium ermöglichten und auf deren Unterstützung ich immer zählen kann. Diese Arbeit möchte ich meiner Oma, die im Jänner 2011 verstorben ist, zum Andenken widmen.

…an meine Freunde ohne die das Studium nur halb so viel Spaß gemacht hätte und ganz besonders an meine liebe Freundin Andi, die immer für mich da ist.

Contents

1. Abstract

Flaviviruses form a genus in the family of *Flaviviridae* and include important human pathogens, like dengue viruses (DENV), Japanese encephalitis virus (JEV), Yellow fever virus (YFV), West Nile virus (WNV) and tick-borne encephalitis virus (TBEV). These lipid-enveloped viruses contain three structural proteins: envelope (E), membrane (M) and capsid (C). The surface of the virions is covered by the large glycoprotein E and the smaller protein M, which is derived by the proteolytical maturation cleavage of its precursor form, the glycoprotein prM. The two surface proteins are anchored in the viral membrane which surrounds the nucleocapsid, consisting of the single stranded, positive-sense RNA genome and the C protein.

As revealed by the combination of x-ray crystallography and cryo-electron microscopy, the E protein is composed of three structural domains (DI, DII, DIII) and forms the major component of the viral surface. Due to its important functions during the flavivirus lifecycle, including receptor binding and membrane fusion, the E protein is the major target for neutralising antibodies. All three structural domains of E can elicit neutralising antibodies, which, however, vary in their potency of neutralisation. Antibodies specific for prM have only low or no neutralising activity, but might form a notable fraction of the polyclonal response after flavivirus infection. Limited information is available about the fine specificity of the polyclonal immune response after flavivirus infection or vaccination and it is largely unknown whether there is an immunodominance of specific structural entities of E.

In this context, the major goal of my thesis was the production and characterisation of several recombinant flavivirus surface proteins and its single domains, which will be used as antigens to dissect the polyclonal immune response after flavivirus infection or vaccination. The specific objectives of this work included the production of a single domain of E – domain I (DI) – of TBEV and WNV, the soluble form of prM of TBEV, WNV and YFV, as well as the pr-peptide of TBEV in correct conformation and high purity. For that purpose, different constructs of E-DI and pr(M) were designed, expressed in *Drosophila* S2 cells and purified by affinity chromatography. The expression and purification procedures for different flavivirus proteins were established and antigens in high yields and purity were obtained. Evidence for proper folding of recombinant proteins was obtained by demonstrating the presence of

disulphide bonds and carbohydrate side chains, as well as by their reactivites with conformation-sensitve monoclonal antibodies (mAbs) and polyclonal sera.

In conclusion, recombinant E-DI of TBEV and WNV, prM of TBEV, WNV and YFV as well as the pr-peptide of TBEV were produced in high purity and proper conformation. These proteins can be used as antigens for different immunological assays to gain more information about the humoral immune response after flavivirus infection and vaccination.

Zusammenfassung

Flaviviren bilden ein Genus in der Familie der *Flaviviridae* und beinhalten wichtige Krankheitserreger wie Dengue, Japanische Enzephalitis, Gelfieber (GF), West Nil (WN) und Frühsommer-Meningoenzephalitis (FSME) Viren. Diese Lipid-umhüllten Viren enthalten drei Strukturproteine: Envelope (E), Membrane (M) und Capsid (C). Die Oberfläche der Viren ist durch das große Glycoprotein E und das kleinere Protein M, das durch proteolytische Spaltung seiner Vorläuferform prM entsteht, bedeckt. Die beiden Oberflächenproteine sind in der Virusmembran verankert, die das Nucleocapsid umgibt, welches aus dem einzelsträngigen RNA-Genom positiver Polarität und dem C Protein besteht.

Die Struktur des E Proteins und seiner drei Domänen (DI, DII, DIII) wurde kristallographisch aufgeklärt und seine Anordnung and der Virusoberfläche durch Kryo-Elektronenmikroskopie bestimmt. Wegen der wichtigen Funktionen von E im Zuge des Lebenszyklus von Flaviviren (Rezeptorbindung und Membranfusion) ist dieses Protein der Hauptangriffspunkt von neutralisierenden Antikörpern. Alle drei strukturellen Domänen von E können neutralisierende Antikörper induzieren, die jedoch in ihrer Neutralisationsaktivität variieren. PrM-spezifische Antikörper haben nur geringe bis keine neutralisierende Aktivität, können aber einen beträchtlichen Anteil der polyklonalen Immunantwort nach Flavivirus Infektionen ausmachen. Tatsächlich gibt es wenig Information über die Feinspezifität der polyklonalen Immunantwort nach Flavivirus Infektionen oder Impfungen, und es ist weitgehend unbekannt, ob es Immundominanz bestimmter struktureller Teile von E gibt.

In diesem Zusammenhang war das Hauptziel meiner Diplomarbeit die Produktion und Charakterisierung mehrerer rekombinanter Flavivirus Oberflächenproteine und deren einzelner Domänen. Diese können als Antigene verwendet werden, um die polyklonale Immunantwort nach Flavivirus Infektionen oder Impfungen zu analysieren. Im Detail sollten eine einzelne Domäne von E – die Domäne I (DI) – von FSMEV und WNV, die lösliche Form von prM von FSMEV, WNV und GFV, sowie das pr-Peptid von FSMEV in hochgereinigter Form und richtiger Konformation produziert werden. Zu diesem Zweck wurden verschiedene Konstrukte von E-DI und pr(M) hergestellt und zur Expression dieser Proteine *Drosophila* S2 Zellen verwendet. Die Expression und Aufreinigung mittels Affinitätschromatographie aller gewünschten

Flavivirus Proteine wurde etabliert und hohe Erträge aller Proteine wurden erzielt. Evidenz für deren korrekte Faltung wurde durch den Nachweis von Disulfid-brücken und Kohlenhydratketten sowie die Reaktivität mit Konformations-sensitiven monoklonalen Antikörpern und polyklonalen Seren erhalten.

Zusammenfassend wurden rekombinante E-DI von FSMEV und WNV, prM von FSMEV, WNV und GFV, sowie das pr-Peptid von FSMEV in hoher Reinheit und richtiger Konformation produziert. Diese Proteine können nun als Antigene für verschiedene immunologische Untersuchungen verwendet werden, um mehr Aufschluss über die humorale Immunantwort nach Flavivirus Infektionen oder Impfungen zu erhalten.

2. Introduction

2.1. Preamble

Flaviviruses comprise a number of worldwide distributed, important human pathogens, including Dengue viruses (DENV), Japanese encephalitis virus (JEV), yellow fever virus (YFV), West nile virus (WNV), and tick borne encephalitis virus (TBEV).

It has been shown by many experimental disease models that the induction of neutralising antibodies is crucial for clearing the virus and for gaining a long-lasting immunity. Upon infection or immunisation with flaviviruses, antibodies are mainly produced against the surface proteins and vary in the potency of neutralisation.

The structure of flavivirus particles is known in great detail. Although, even the structure of the envelope protein, which is the major target for neutralising antibodies, has been determined at atomic resolution, it still remained unclear whether the antibody response after infection or vaccination is dominated by antibodies to specific structural entities, and to which extend the fine-specificity of these responses is subject to individual variation.

In this context, it is the objective of this diploma thesis to produce and characterise recombinant forms of surface proteins and its individual domains to be used in assays, that allow the dissection of the polyclonal antibody response after flavivirus infection or vaccination.

2.2. General introduction

2.2.1. Classification of flaviviruses

Flaviviruses belong to the large family of *Flaviviridae*, which comprise three genera: the Hepacivirus, the Pestivirus and the Flavivirus (Fauquet 2005). All genera of the family *Flaviviridae* show similarities in the organization of their single-stranded positive sense RNA genomes, as well as in replication and in morphology of viral particles (Lindenbach 2007). The genus Flavivirus (from the Latin flavus: yellow) is the largest of the three genera and contains important human pathogens like the dengue viruses (DENV), Japanese encephalitis virus (JEV), yellow fever virus (YFV), West nile virus (WNV), and tick borne encephalitis virus (TBEV).

Flaviviruses can be subdivided into mosquito-, tick-borne and non-vectored clusters and form different serocomplexes, defined by cross-neutralisation tests with polyclonal sera (Fig.2.1), (Calisher, Karabatsos et al. 1989).

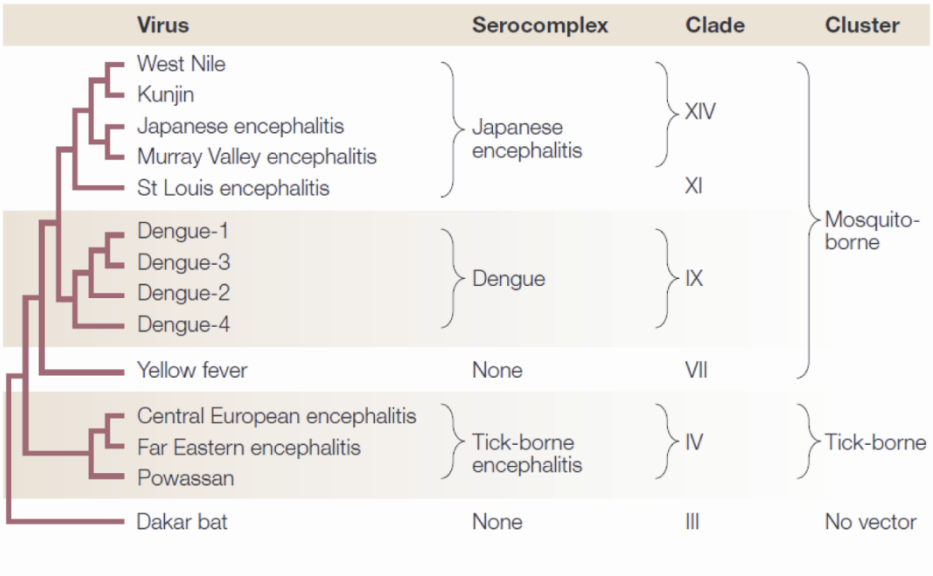


Fig.2.1: Classification of flaviviruses. Relationships between selected flaviviruses are shown in the dendrogram on the left, the serological (serocomplex) and phylogenetical (clade and cluster) classifications of these flaviviruses are shown on the right. Fig. adapted from (Mukhopadhyay, Kuhn et al. 2005).

2.2.2. Epidemiology and pathogenesis

2.2.2.1. Tick borne encephalitis virus (TBEV)

Three subtypes of TBEV have been described: The European, the Far Eastern and the Siberian (Ecker, Allison et al. 1999). TBEV is endemic in regions of Central and Eastern Europe, as well as in large areas of the former Soviet Union, in northern China and northern Japan. The principal vectors are ticks and usually, TBEV circulates between ticks and small vertebrate hosts like rodents. Humans are only incidental hosts and in most cases, infection with TBEV is asymptotic or results in febrile illness. In about one third of the patients infected with TBEV, fever and more severe symptoms including meningoencephalitis can arise after an asymptotic phase of some days. Most severe, long-lasting or permanent neurological sequelae are reported in 10 to 20% of TBEV infections. A formalin inactivated whole virus vaccine protecting from TBEV infection is available and vaccination campaigns in Austria lead to a drastic decline of TBEV caused neuroinfections (Heinz, Holzmann et al. 2007).

2.2.2.2. West Nile virus (WNV)

WNV belongs to the mosquito-borne flaviviruses and can be divided into two genetically different lineages (Lanciotti, Ebel et al. 2002). Usually, WNV cycles between birds and mosquitos, and humans are only incidental hosts. WNV was first isolated in the West Nile district of Uganda, and in 1999 cases of infections were also reported in North America (Brinton 2002). WNV has been considered as an emerging disease agent (Mackenzie, Gubler et al. 2004) and today, it is one of the most widely geographically distributed flaviviruses (Gubler 2007), and can be found in parts of Europe and Africa, the Middle East, Asia, America and Australia. Pathogenesis of WNV infection is similar to TBEV infections. Severe forms of meningoencephaltits are reported especially in elder patients. Currently, there is only a veterinary vaccine for horses available. Human WNV vaccines are in clinical trials (Dauphin and Zientara 2007).

2.2.2.3. Yellow fever virus (YFV)

YFV also belongs to the mosquito-borne flaviviruses and is endemic in tropical and subtropical areas of South America and Africa. Two different cycles of YFV infections are known. In the “jungle yellow fever” the virus is maintained in a cycle between different mosquitos and non-human primates, and people are only sporadically exposed to infected mosquitos. In the so-called “urban yellow fever” the virus is mainly transmitted between the domestic mosquito *Aedes aegypti* and humans (Monath 2001). Most cases of Yellow fever infections cause mild symptoms including headache, nausea, vomiting and fever. In about 15% of YFV infections, symptoms return after a period of some days accompanied by jaundice due to liver damage, giving the virus its name. The vaccine against YFV infection is highly effective and consists of a live-attenuated strain of the virus (17D) (Roukens and Visser 2008).

2.3. Molecular biology of flaviviruses

2.3.1. Genome organization

The viral genome consists of a positive-stranded RNA molecule of about 11kb containing a single long open reading frame (ORF) flanked by non-coding regions, that are important for viral replication (Markoff 2003), (Fig.2.2). A cap structure is present at the 5´ terminal end. The ORF is translated into a polyprotein precursor, which is processed and cleaved by viral and cellular proteases to give rise to the three structural proteins capsid, membrane and envelope (C, prM/M and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5), (Lindenbach 2007).

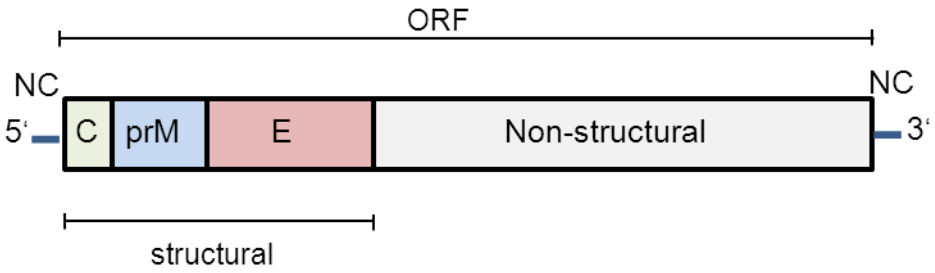


Fig.2.2: Schematic representation of the viral RNA genome. The single open reading frame (ORF) codes for structural and non-structural proteins and is flanked by two non-coding regions (NC).

2.3.2. Virus particle

The spherical flavivirus particle has an approximate diameter of 50 nm (Lindenbach 2007). It is composed of the three structural proteins (C, prM/M, E), a host-derived lipid bilayer, and the genomic RNA. The genome and multiple copies of C compose the nucleocapsid, which is surrounded by a host-derived lipid bilayer with two membrane-anchored glycoproteins, envelope (E) and membrane (M) covering the virion surface.

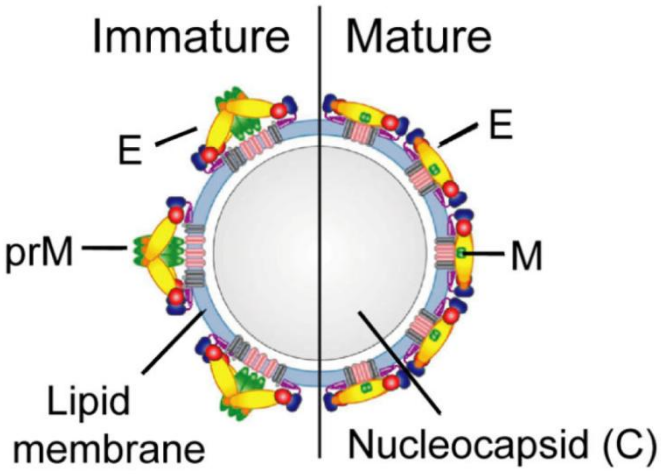


Fig.2.3: Schematic representation of an immature and mature flavivirus particle. The nucleocapsid is enclosed by a host-derived lipid bilayer. Immature virions (left panel) contain trimeric spikes of prM-E heterodimers. In the course of maturation, prM is cleaved into M and in the mature form of the virion, E are arranged in homodimers lying parallel to the viral membrane (right panel). Fig. adapted from (Fritz, Stiasny et al. 2008).

Virions are assembled as immature (non-infectious) particles, where E and the precursor form of M (prM) are associated in heterodimers (Fig.2.3; left). During maturation, the pr-peptide is proteolytically cleaved and structural rearrangements occur. In mature particles, the surface is completely covered by tightly packed E dimers (Fig.2.3; right).

2.3.3. Flavivirus life cycle (Fig.2.4)

Attachment of the virus to the host cell is mediated by the interaction of the E protein with one or more cellular receptors. Several host cell attachment factors have been identified as receptors contributing to flavivirus uptake (Kaufmann and Rossmann 2010). The particles are then internalized by receptor-mediated endocytosis and transported in clathrin coated pits to endosomes. Upon exposure to the mildly acidic pH in this compartment, structural changes in E take place, which mediate fusion of the viral with the endosomal membrane. The nucleocapsid is released into the cytoplasm, where the viral RNA is uncoated. Both, cellular and viral enzymes are responsible for RNA replication and translation. Translation leads to a polyprotein precursor, which is inserted in the membrane of the endoplasmatic reticulum (ER) and processed by host and viral proteases (Fig.2.5). The transmembrane proteins E

and prM form heterodimeric complexes at the lumenal side of the ER, and the viral genomic RNA is recruited to the cytoplasmatic side of the ER via the C-protein. Immature (fusion inactive) virus particles bud into the lumen of the ER (Mackenzie and Westaway 2001) and are transported through the cellular secretory pathway. In immature particles, prM prevents E from undergoing structural rearrangements. In the trans-Golgi network (TGN), the virion is exposed to acidic pH and undergoes conformational rearrangements, rendering the cleavage site of prM accessible to the cellular protease furin. PrM is cleaved by furin into M and the N-terminal pr-peptide (Stadler, Allison et al. 1997). The pr-peptide does not dissociate until the particle is released into the pH-neutral extracellular environment (Yu, Holdaway et al. 2009). The cleavage of prM and the low pH in the TGN triggers the rearrangement of E into fusion-competent dimers. Mature (fusion active) virus particles, but also partially mature virus particles, containing residual uncleaved prM at the surface, can be secreted from infected cells. Also, non-infectious, smaller virus-like particles without nucleocapsids, termed slowly sedimenting hemagglutinin (SHA), are released from the host cell (Fig.2.4).

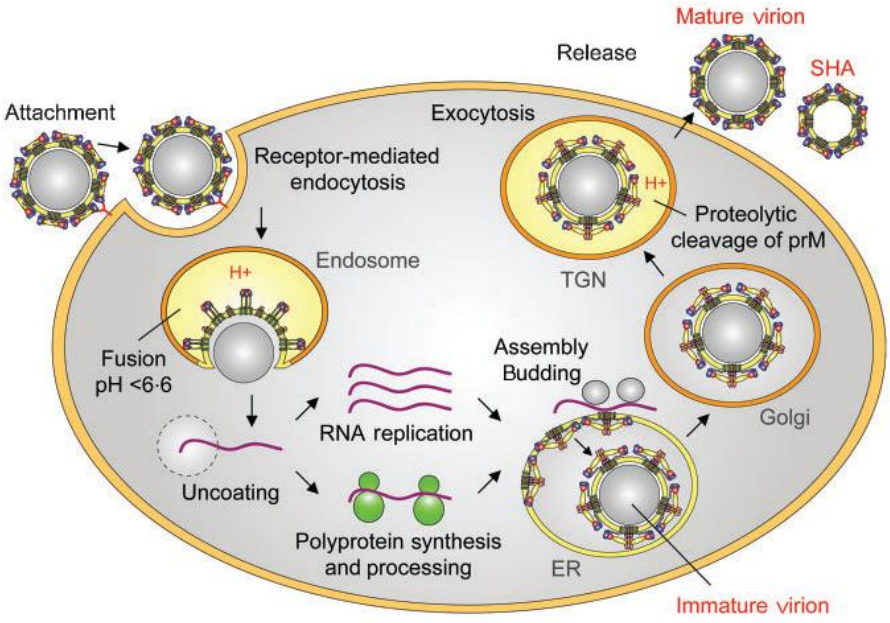


Fig.2.4: The flavivirus life cycle.
For details see text; SHA, slowly sedimenting hemagglutinin; ER, endoplasmic reticulum; TGN, trans-Golgi network. Fig. adapted from (Stiasny and Heinz 2006).

2.3.4. *Viral proteins*

The genomic RNA is translated into one single polyprotein. The three structural proteins (C-prM/M-E) are located at the N-terminal region of the polyprotein followed by the non-structural proteins (NS1-2A-2B-3-4A-4B-5). Parts of the polyprotein are translocated by various signal sequences into the ER membrane and processed by cellular and viral proteases into 10 distinct viral proteins (Fig.2.5).

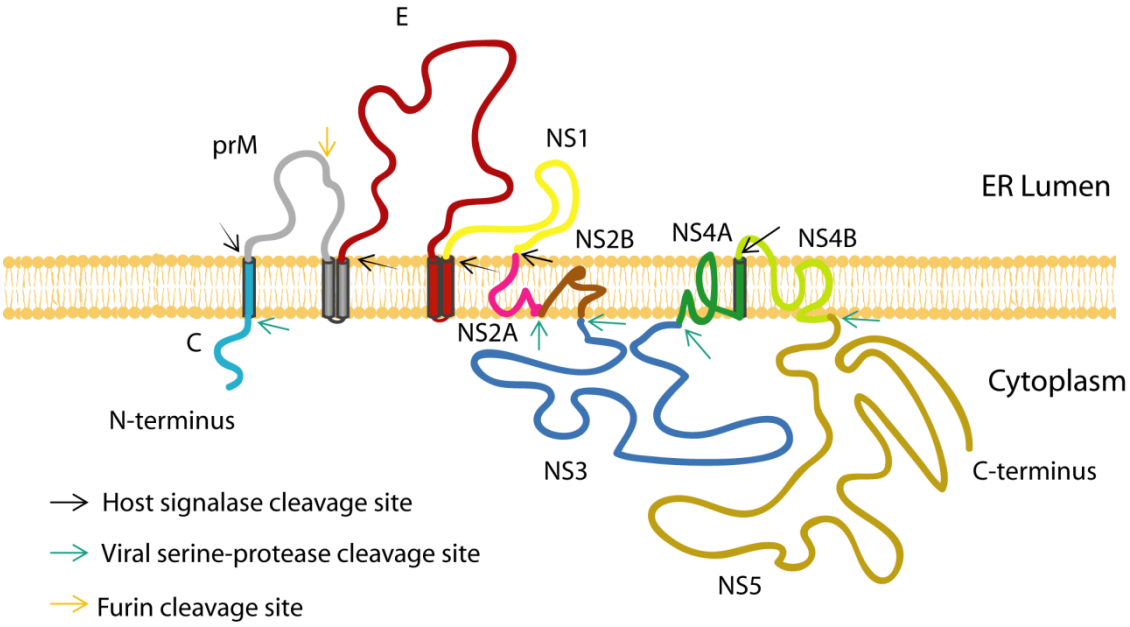


Fig.2.5: Schematic representation of the predicted orientation of the polyprotein across the ER membrane. Transmembrane domains anchoring the proteins in the ER membrane are represented as cylinders, the cleavage sites of host and viral proteases are indicated by arrows. Cleavage of prM is rendered by the cellular protease furin (orange arrow) in the trans-Golgi network. Fig. adapted from (Mukhopadhyay, Kuhn et al. 2005).

2.3.4.1. *Envelope (E) protein*

The envelope (E) glycoprotein is the largest of the three structural proteins (about 53 kDa) and forms dimers on the surface of mature virions at physiological pH (Fig.2.6). E is responsible for receptor binding and membrane fusion and, because of these functions, the major target for neutralising antibodies.

The X-ray crystallographic structures of soluble forms of E (sE; ectodomain) – lacking about 100aa of the C-terminus, including the so-called stem- and anchor region – of several flaviviruses have been determined in their monomeric (WNV) or dimeric form (TBEV, DENV) (Rey, Heinz et al. 1995), (Kanai, Kar et al. 2006) (Fig.2.6B), (Modis, Ogata et al. 2003), (Modis, Ogata et al. 2005), (Nybakken, Nelson et al. 2006), (Zhang, Zhang et al. 2004), (Fig.2.6D). Each E monomer consists of three structural domains, domain I (DI), domain II (DII) and domain III (DIII). The stem region, composed of two amphipathic helices, connects the E ectodomain to its transmembrane region, composed of two helices that anchors E in the viral membrane (Zhang, Chipman et al. 2003).

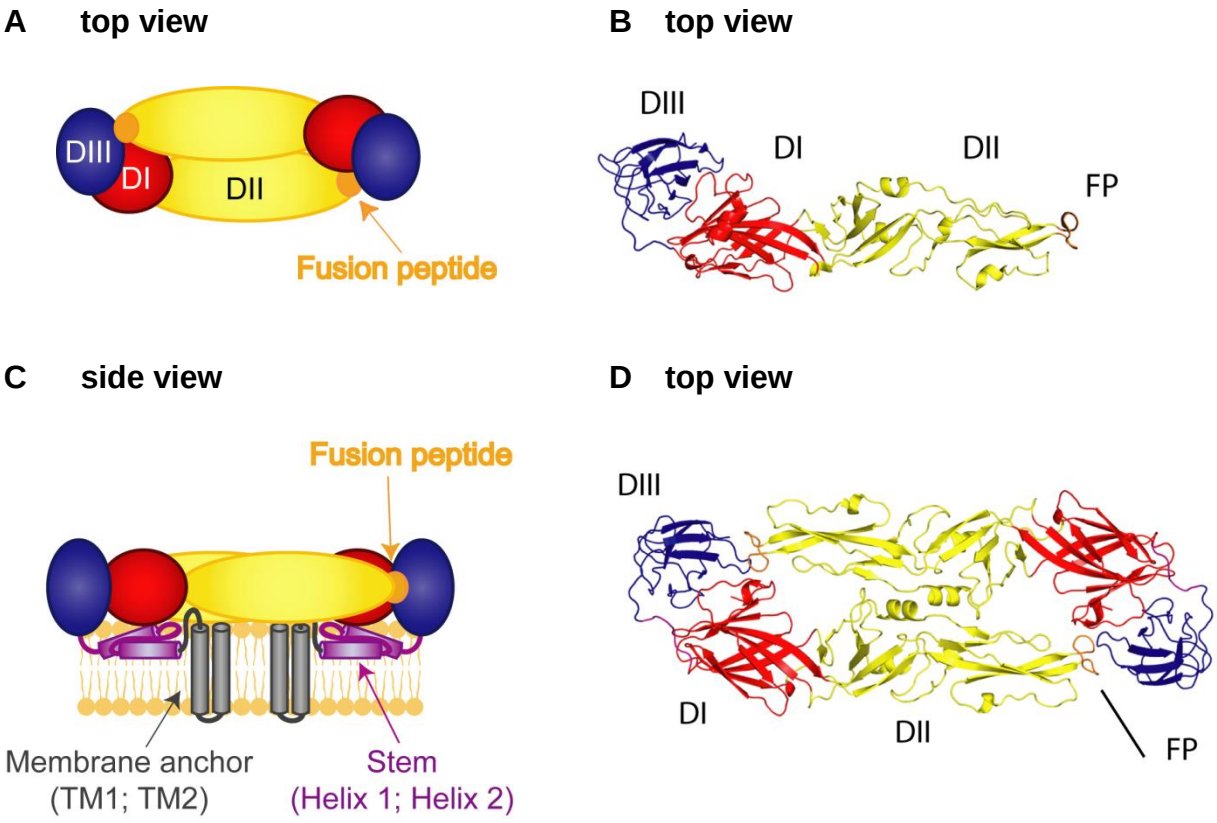


Fig.2.6: Schematics of flavivirus E dimers (A, C) and ribbon diagrams of the crystal structures of the WNV sE monomer (B) and the TBEV sE dimer (D). Domain I is represented in red, domain II in yellow and domain III in blue. In 2.5C the proposed locations of the membrane anchor and the stem region are illustrated. FP, fusion peptide.

The ectodomain of E (Fig.2.7) has 12 conserved cysteins that form six disulphide bonds and in most flaviviruses E is N-glycosylated (Winkler, Heinz et al. 1987).

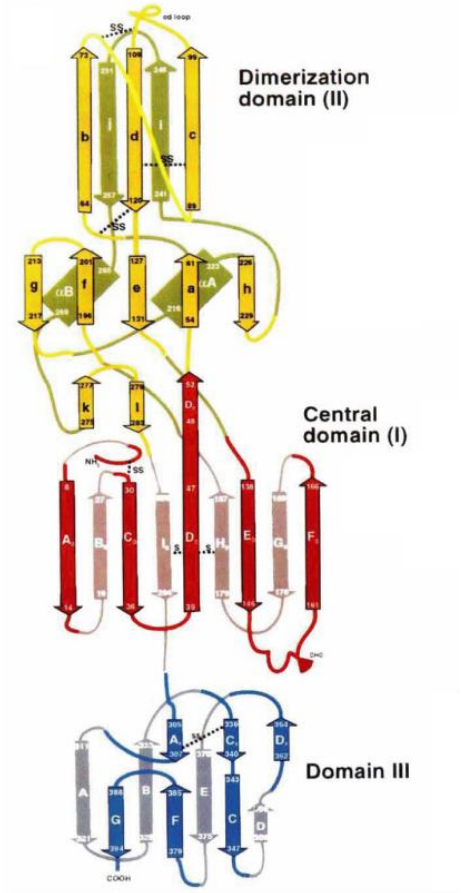


Fig.2.7: Folding diagram of TBEV soluble E. Domain I is represented in red, domain II, which is formed of two loops out of DI, in yellow and domain III in blue. Disulphide bridges are shown as broken black lines, the glycosylation site at the DI is indicated by a red fan. Strands of DI (A_0-I_0) and DIII (A-G) are labelled with upper-case letters, for strands of DII (a-l), lower-case letters are used. Fig adapted from (Rey, Heinz et al. 1995).

Domain I (DI) is the central domain of E with the N-terminus, and is composed of three segments folding into a nine-stranded ß-barrel, that connects DII and DIII via flexible hinges (Fig.2.7). It contains one N-linked oligosaccharide and two disulphide bonds. Domain II (DII; dimerization domain) consists of two large loops which emanate out of DI (Fig.2.7) (Rey, Heinz et al. 1995). At the tip of DII the highly conserved fusion peptide (FP) is present, which is important for membrane fusion (Allison, Schalich et al. 2001). Domain III (DIII) has an immunoglobulin-like fold and is involved in virus attachment to the host cell (Rey, Heinz et al. 1995; Chu, Rajamanonmani et al. 2005).

2.3.4.2. Membrane (prM/M) protein

The prM protein (about 26 kDa) is the precursor of the M protein and present on immature virus particles. Like E, it is anchored with its two C-terminal transmembrane helices in the viral membrane (Mukhopadhyay, Kuhn et al. 2005), (Fig.2.9). During flavivirus maturation, prM is cleaved into the membrane-bound M protein and the N-terminal pr-peptide by the cellular protease furin. The X-ray crystallographic structure of DENV 2 E/pr-peptide complex has been determined (Li, Lok et al. 2008) (Fig.2.8). The pr-peptide consists of seven ß-strands (Li, Lok et al. 2008) and contains six conserved cysteins, which form three disulphide bonds (Nowak, Farber et al. 1989). There are some differences among flaviviruses concerning the number of N-linked glycosylation sites, ranging from one to three (Chambers, Hahn et al. 1990).

Fig.2.8

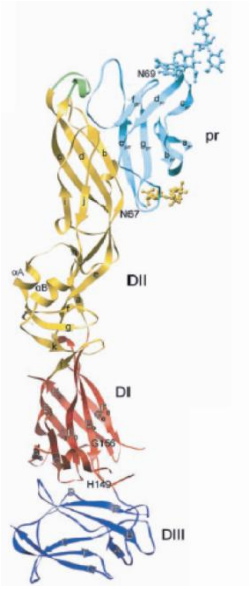


Fig.2.9

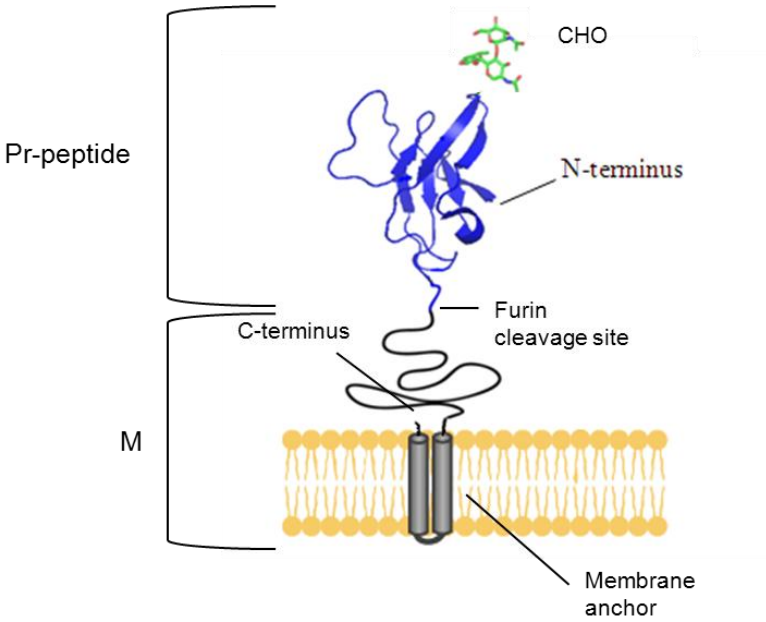


Fig.2.8: Ribbon diagram of the crystal structure of the DENV sE/pr-peptide heterocomplex. The pr-peptide is represented in light blue, the E protein in red, yellow and blue according to its domains. Fig. adapted from (Li, Lok et al. 2008).

Fig.2.9: Schematic representation of the full length prM protein: The crystal structure of DENV pr-peptide with the carbohydrate chain (CHO) is represented as a ribbon diagram in blue, M with its membrane anchor is shown in black.

During translation, prM folds rapidly and probably contributes to proper folding of E (Konishi and Mason 1993), (Lorenz, Allison et al. 2002). As described in 2.2.3, in immature virus particles prM-E heterodimers are present and prM prevents E from undergoing structural changes in the low pH environment of the TGN during exocytosis.

2.3.4.3. Capsid (C) protein

C is the smallest structural protein (about 11 kDa) and is highly basic. It consists of four alpha helices, contains a characteristic internal hydrophobic region, that mediates membrane association and plays a critical role in virion assembly (Kofler, Heinz et al. 2002). The C-terminal amino acid sequence serves as the signal peptide for ER translocation of prM (Lindenbach 2007). Several copies of C protein dimers form the flavivirus capsid.

2.3.5. Recombinant subviral particles

During flavivirus infection, not only whole virions, but also non-infectious subviral particles are secreted from infected cells. These particles are smaller than virions and contain only prM/M and E proteins, but lack the nucleocapsid. Such particles can be produced in recombinant form by coexpressing prM and E proteins (Allison, Stadler et al. 1995; Lorenz, Kartenbeck et al. 2003). These recombinant subviral particles (RSP) exist in two distinct size classes (30 nm and 50 nm) (Allison, Tao et al. 2003), and the smaller one has been characterized by cryo-electron microscopy (Fig.2.10C). RSPs represent a useful, non-infectious tool for the functional analysis of surface proteins in particulate form (Schalich, Allison et al. 1996).

2.3.6. Surface structures of flavivirus particles

Pseudo-atomic structures based on cryo-electron microscopy reconstructions of dengue and West Nile viruses revealed the surface organisation of immature and mature flavivirus particles (Kuhn, Zhang et al. 2002; Mukhopadhyay, Kim et al. 2003; Zhang, Chipman et al. 2003; Mukhopadhyay, Kuhn et al. 2005) (Fig.2.10).

In immature virions, the icosahedral arrangement of 60 trimeric spikes, each composed of E-prM heterodimers, gives the surface a spiky shape (Li, Lok et al. 2008), (Zhang, Corver et al. 2003). During maturation, the pr-peptide of prM is proteolytically removed, leading to structural rearrangements. In mature (infectious) virus, E proteins are arranged as 90 antiparallel homodimers, lying parallel to the viral membrane, thus giving the virus a smooth surface. The 90 dimers are organized in a herringbone-like pattern of 30 rafts of 3 parallel E protein dimers (Lindenbach 2007).

In contrast to virions, in RSPs 30 E protein dimers are arranged in a T=1 symmetry on the surface (Ferlenghi, Clarke et al. 2001), (Fig.2.10C).

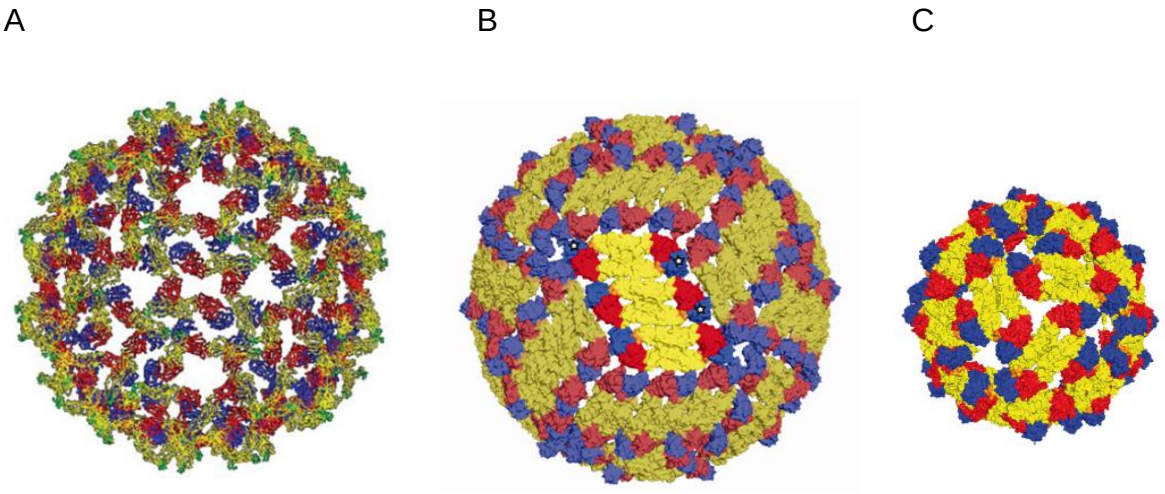


Fig.2.10: Pseudoatomic structures of the surface of immature (A) and mature (B) flavivirus particles as well as RSPs (C), based on cryo-electron microscopy image reconstructions. DI, DII and DIII are shown in red, yellow and blue, respectively. Fig. adapted from (Ferlenghi, Clarke et al. 2001; Zhang, Zhang et al. 2004; Mukhopadhyay, Kuhn et al. 2005; Kiermayr, Stiasny et al. 2009).

2.4. Antibody response to flaviviruses

2.4.1. Humoral immunity against flavivirus infection

B-cells and antibodies play a critical role in immune-mediated protection from flavivirus infections. Antibodies directly neutralise the virus, opsonise it for the complement system or mediate interactions with Fc-receptor-bearing cells. Since E is the major antigen on the flavivirus surface and essential for virus attachment and entry into the host cell, it represents the main target for neutralizing antibodies. These are major determinants for a protective immunity.

The potency of antibodies to neutralise the virus depends on various parameters. The most important are the affinity of the antibody to its epitope and the accessibility of the epitope on the viral particle (Pierson and Diamond 2008). Different mechanisms of neutralisation are possible, depending on the binding site of the antibody to its antigen. In the case of flaviviruses, antibodies can block the attachment of the virus to host receptors, or inhibit conformational changes of E during membrane fusion (Stiasny, Brandler et al. 2007) (Nybakken, Oliphant et al. 2005).

Every domain of the E protein contains epitopes that elicit antibodies with varying neutralizing potency in vitro and efficacy in vivo (Heinz, Berger et al. 1983; Roehrig 2003; Nybakken, Oliphant et al. 2005; Oliphant, Engle et al. 2005; Sanchez, Pierson et al. 2005; Oliphant, Nybakken et al. 2006). Some of the most potent neutralizing antibodies were found to be directed to DIII (Beasley and Barrett 2002) (Oliphant, Engle et al. 2005), (Oliphant, Nybakken et al. 2006), (Sukupolvi-Petty, Austin et al. 2007), (Gromowski, Barrett et al. 2008), (Sukupolvi-Petty, Austin et al. 2010). Also, antibodies specific for the fusion peptide on DII were described. These antibodies do not efficiently neutralise the virus, but as the amino acid sequence of this region is highly conserved among flaviviruses, antibodies are highly cross-reactive also with distantly related members of the genus (Lai, Tsai et al. 2008), (Crill and Chang 2004) (Stiasny, Kiermayr et al. 2006). Also antibodies specific for prM, C or non-structural proteins were observed in post-infection sera (Falconar 1999), (Churdboonchart, Bhamarapravati et al. 1991).

2.4.2. Antibody dependent enhancement (ADE) of flavivirus infection

In ADE, antibodies of non-neutralizing concentration interact with the virus, form complexes and facilitate the entry into Fc-receptor-bearing host cells by Fc-receptor-mediated uptake of the virus (Halstead and O'Rourke 1977), (Pierson and Diamond 2008). This leads to increased replication and virus propagation, enhanced infection and probably – as a consequence – to a more severe course of disease (Vaughn, Green et al. 2000) (Fig.2.11).

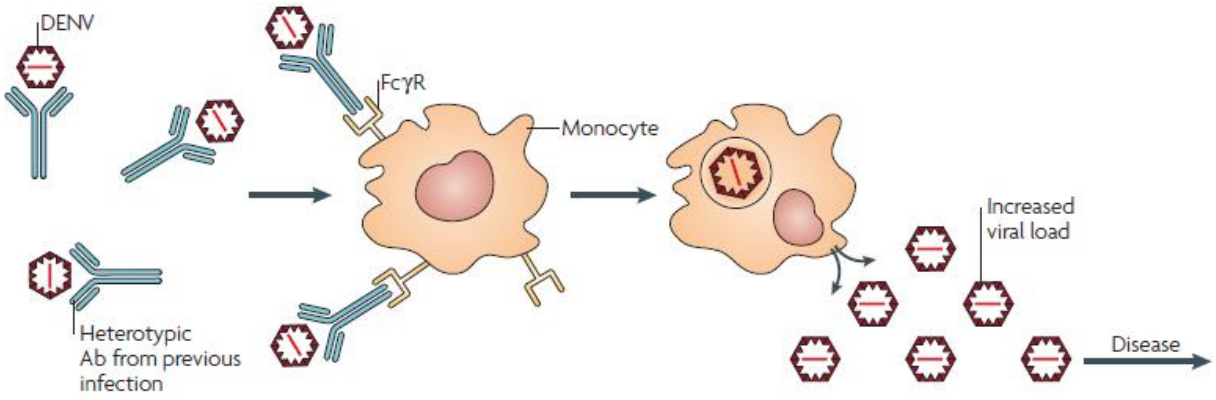


Fig.2.11: Schematics of DENV ADE. A heterotypic, non-neutralising antibody from a previous infection of another dengue serotype binds to the virus and allows its attachment to Fc-receptor-bearing monocytes. This results in uptake into the cell, propagation and an increased viral load responsible for increased severity of the disease. Fig. adapted from (Whitehead, Blaney et al. 2007).

ADE has been studied most extensively with dengue viruses (DENV). DENV can be divided into four different serotypes. Infection with one serotype provides long-lasting immunity to the same serotype, but does not provide protection from infection with other serotypes. A secondary infection with another dengue serotype can lead to a more serious course of disease (Halstead, Udomsakdi et al. 1970), (Vaughn, Green et al. 2000). These observations were explained by ADE. In this case, heterotypic antibodies, elicited by primary infection of one serotype of DENV, are cross-reactive to other serotypes in a secondary infection. Their presence in sub-neutralizing concentrations or reduced avidity does not permit virus neutralisation but can support ADE (Pierson and Diamond 2008).

2.4.3. The role of immature and partially mature virus particles in flavivirus infection

During the flavivirus life cycle, not only fully mature, but a mixed population of mature, immature and partially mature virus particles is secreted from infected cells (Cherrier, Kaufmann et al. 2009) (Junjhon, Lausumpao et al. 2008; Junjhon, Edwards et al. 2010). In partially mature virus particles, varying levels of prM are present on their surface, which is normally cleaved by the cellular protease furin to produce mature particles (Stadler, Allison et al. 1997). It was shown that during DENV and WNV infection relatively high levels of prM containing particles were secreted (Zybert, van der Ende-Metselaar et al. 2008) (Cherrier, Kaufmann et al. 2009).

Monoclonal antibodies directed to prM showed only poor or no neutralising efficiency (Falconar 1999; Beltramello, Williams et al. 2010). However, antibodies specific for prM may play an important role in flavivirus infection, especially after secondary DENV infections. The proportion of antibody responses to prM were shown to be significantly higher in sera from patients with a secondary infection than in sera from patients with a primary infection (Lai, Tsai et al. 2008). High proportions of crossreactive prM antibodies after DENV infections were found, which do not efficiently neutralise the virus but contribute to ADE of infection (Dejnirattisai, Jumnainsong et al. 2010). It was also shown that even fully immature DENV particles become highly infectious upon interaction with prM-specific antibodies, due to facilitated binding to Fc-receptor bearing cells, uptake by receptor-mediated endocytosis and prM-cleavage in the endosome (Rodenhuis-Zybert, van der Schaar et al. 2010).

2.5. Protein expression systems

Expression systems for recombinant proteins include bacteria, yeast, insect and mammalian cells. Expression of viral proteins in their native fold depends on specific characteristics of proteins, like the presence of disulphide bridges and post-translational modifications. Together with the required yield of a protein for different applications, such properties have to be considered for the choice of an appropriate expression system.

2.5.1. Bacterial expression systems for flavivirus proteins

Growth rates of bacteria and the expression levels of proteins are very high and the system is cheap and easy to handle. Post-translational modifications are generally not added and thus, the protein of interest may not be correctly folded and/or funcional. This system is therefore most suitable for the expression of small, self-folding proteins, that do not require post-translational modifications. In previous work it was shown that DIII of different flavivirus E proteins could be produced in its native fold using bacterial expression systems. (Jaiswal, Khanna et al. 2004), (Tripathi, Babu et al. 2008) (Volk, Lee et al. 2007; Volk, May et al. 2009).

2.5.2. Yeast expression system for flavivirus proteins

The advantages using yeast as an expression host are similar to those of bacterial systems. Moreover, yeasts are known to perform many post-translational modifications (Demain and Vaishnav 2009) and can be used to generate glycosylated proteins. Glycans present on proteins expressed in yeast have branched, high mannose structures, but complex type carbohydrate structures are not formed (Tanner and Lehle 1987). Potential problems lie in obtaining the correct conformation of more complex, highly folded proteins. Expression of recombinant DENV E protein in *Pichia pastoris* resulted in proteolytic degradation (Sugrue, Cui et al. 1997). In other work, it was shown that *Pichia pastoris* might be an efficient expression system for the production of DENV full length E (Wei, Jiang et al. 2003) or NS1 (Zhou, Tang et al. 2006).

2.5.3. Insect cell expression systems for flavivirus proteins

Insect cells exhibit relatively slow growth rates and moderate expression of recombinant proteins in comparison to bacterial or yeast expression systems. Since post-translational modifications are similar to mammalian cells, insect cells are suitable for the expression of complex proteins in their native conformation. Usually, N-linked glycans of proteins produced in insect cells contain high mannose structures or are processed into paucimannosidic structures (Hacker, White et al. 2009). Although genomic studies of insects revealed the presence of many glycan transferases and metabolic enzymes, needed for processing glycans into complex glycans at certain developmental stages, proteins expressed in insect cells do not contain significant levels of complex glycans (Kim, Shin et al. 2005), (Tomiya, Narang et al. 2004). The expression of such enzymes is very limited or lost in insect cell-lines derived for cell culture. Complex glycans, such as those containing terminal sialic acid or galactose are predominantly found in mammalian cells.

Flaviviruses naturally replicate in mammalian and insect cells, and successful expression of the envelope protein in insect cells has already been shown for several different members of the genus. Different insect expression systems have been used for this purpose, including the baculovirus expression system with a *Spodoptera frugiperda* cell line (Nybakken, Nelson et al. 2006) or the *Drosophila* Expression System (DES) from Invitrogen. In the DES a stably transfected *Drosophila* melanogaster cell line is established and expression of the protein can be induced. The successful production of WNV- and DENV- sE with the DES has already been shown (Lieberman, Clements et al. 2007), (Modis, Ogata et al. 2005).

2.5.4. Mammalian cell expression systems for flavivirus proteins

Proteins produced in mammalian expression systems have the most complex structural and functional features. The processing machinery of mammalian cells ensures post-translational modifications, native conformation and biological activity of expressed recombinant proteins. Glycans present on proteins are predominantly of complex-type. The expression of TBEV sE protein as well as RSPs is well established in Cos-1 cells (Allison, Stadler et al. 1995) (Heinz, Allison et al. 1995),

(Schalich, Allison et al. 1996; Ferlenghi, Clarke et al. 2001; Lorenz, Kartenbeck et al. 2003) and BHK-21 cells (Liu, Huang et al. 2009). In general, the use of mammalian cells such as Cos-1, CHO or HEK293 is well documented, but the process of creating stable mammalian cell lines can often be laborious and time consuming (Wurm and Bernard 1999). Slow growth rates, moderate expression levels and highly demanding media supplements make the system relatively expensive for large scale protein production.

2.6. Protein purification

Different methods and a series of processes can be performed to purify a single type of protein. For purification, different properties of proteins are exploited, including the size, physico-chemical properties and binding affinity to a ligand. In order to facilitate purification, affinity tags can be fused to the recombinant protein of interest, either at the C- or N-terminus. Such tags allow simple purification of the protein by affinity chromatography.

Tags for affinity purification should be small in size and should have high affinity to their ligand. It is important that the tag does not interfere with the protein of interest and that the native conformation of the protein is not disturbed. In order to recover the protein, a gentle elution procedure is necessary. Commonly used small tags are the polyarginine-tag (Arg-tag), the polyhistidine-tag (His-tag), the FLAG-tag or single and double strep-tags.

The strep-tag is a peptide of nine amino acids, that binds to streptavidin. A variant of the strep-tag is termed strep-tag II (eight amino acids), which has a higher affinity to the streptavidin mutant streptactin (Voss and Skerra 1997). The strep-tag II can be fused to the C- or N-terminus of the protein of interest. It binds to the biotin pocket of streptactin and can be eluted gently with biotin derivatives, such as desthiobiotin (Fig.2.11). Biotin or biotinylated proteins are also bound to streptactin but can be masked by avidin.

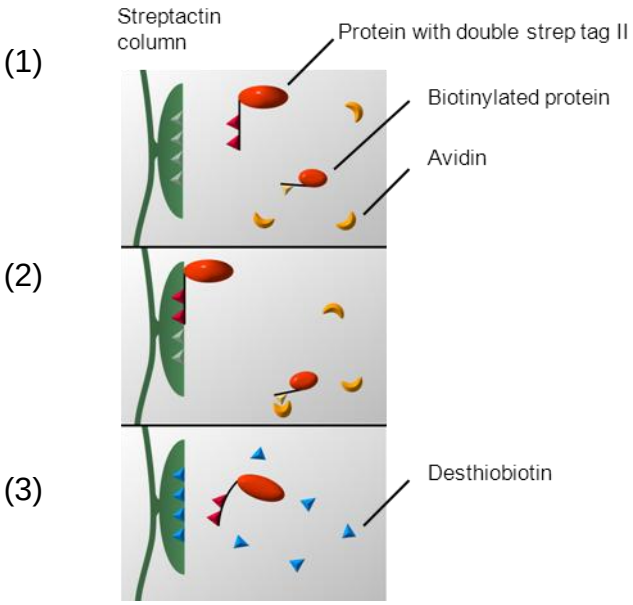


Fig.2.11: Schematics of affinity chromatography based on strep-tag II – streptactin interactions. (1) The protein sample is loaded onto a streptactin column. (2) The protein of interest binds with its double strep-tag II (red triangle) to the streptactin column (green). Avidin (orange half-moon) prevents binding of biotinylated proteins. (3) Elution is performed with desthiobiotin (blue triangle).

3. Objectives

Little is known about the fine-specificity of the polyclonal antibody response to the flavivirus E protein or to structural proteins in general, after flavivirus infection or vaccination. In this context, it was the objective of this thesis to produce specific recombinant antigens to be used in immunoassays for determining the protein- and domain-specificities of antibody populations present in polyclonal sera. Specifically, the goal of my thesis was the production of highly pure and properly folded recombinant flavivirus E protein domain I (TBEV, WNV) and pr(M) protein (TBEV, WNV, YFV) in the *Drosophila* expression system.

The E protein is bifunctional and mediates attachment to host cells and, after virus uptake by receptor-mediated endocytosis, fusion of the viral with the endosomal membrane (Lindenbach 2007). Due to these important functions in the flavivirus lifecycle, E is the major determinant for the induction of virus-neutralizing antibodies and a protective immunity. Epitopes recognised by neutralising antibodies have been identified in all three domains of E (Heinz, Berger et al. 1983; Roehrig 2003; Nybakken, Oliphant et al. 2005; Oliphant, Engle et al. 2005; Sanchez, Pierson et al. 2005; Oliphant, Nybakken et al. 2006). Some of the most potent monoclonal antibodies described so far recognize DIII (Beasley and Barrett 2002; Oliphant, Engle et al. 2005; Oliphant, Nybakken et al. 2006; Sukupolvi-Petty, Austin et al. 2007; Gromowski, Barrett et al. 2008; Sukupolvi-Petty, Austin et al. 2010). Broadly cross-reactive antibodies with low neutralizing potency have been mapped to epitopes containing amino acids of the highly conserved fusion peptide (Crill and Chang 2004; Stiasny, Kiermayr et al. 2006; Lai, Tsai et al. 2008). Although the atomic structure of sE has been determined for several flaviviruses, it is largely unknown whether there is an immunodominance of specific structural entities of E in the course of polyclonal immune responses.

In this context, the first objective of my thesis was the production of a single domain of E (E-DI) of TBEV and WNV in recombinant form. One aspect of this study was the establishment of an expression and purification procedure to obtain high yields of highly purified protein. Another aspect was the quality control of the recombinant protein with respect to the analysis of the correct conformation, including disulphide formation, glycosylation and reactivity with conformation-sensitive antibodies.

PrM is the second glycoprotein present on the surface of flavivirus particles. Monoclonal antibodies specific for prM were described to show no or only poor neutralizing activity (Falconar 1999; Beltramello, Williams et al. 2010). In the case of dengue viruses, prM antibodies have been detected in human polyclonal post infection sera which seemed to be higher in patients with a secondary infection than in patients with a primary infection and have been speculated to contribute to disease severity (Lai, Tsai et al. 2008). So far, prM antibody responses after other flavivirus infections or vaccinations have not been analysed.

Therefore, the second objective of my thesis was the production of prM protein of TBEV, WNV and YFV as well as the pr-peptide of TBEV in recombinant form and the subsequent characterisation of these antigens as described for E-DI.

Purified and characterised proteins will be used as antigens in further studies to dissect the polyclonal antibody response after flavivirus infection or vaccination.

4. Materials and Methods

4.1. Molecular cloning

4.1.1. Media and Buffers

LB medium:	10g Bacto tryptone 5g Yeast extract 10g NaCl ddH_2O to a final volume of 1 l equilibrated to pH 7.0 with NaOH autoclaved
LB agar medium:	LB medium 20 g/l Difco agar autoclaved
Antibiotics:	100 mg/ml Ampicillin in ddH_2O (used as stock)
TE buffer:	10 ml 1M Tris (pH 8.0) 4 ml 0.25M EDTA ddH_2O to a final volume of 1 l
TBE buffer:	10.8 g Tris 5.5 g Boric acid 0.83 g EDTA H_2O to a final volume of 500 ml
DNA loading buffer:	30% (v/v) glycerol 0.25% (w/v) bromphenol blue 0.25% (w/v) xylene cyanol in TE buffer

4.1.2. Plasmids

For the production of recombinant flavivirus proteins in the *Drosophila* expression system (DES) from Invitrogen, the expression plasmid pT389 (provided by the Institut Pasteur, France) was used (Fig.5.4). This plasmid contains a bacterial origin of replication (pUC ori), an ampicillin resistance gene and a metallothionein-promoter for inducible expression of target genes with bivalent cations. PT389 also encodes the *Drosophila* export signal sequence Bip, which allows the secretion of the expressed protein into the cell culture supernatant, an enterokinase cleavage site, as well as a double strep-tag II for purification purposes.

The selection plasmid pCoBlast (provided from Invitrogen) contains a blasticidin resistance gene and was used in this study for the cotransfection of *Drosophila* S2 cells with the plasmid pT389.

Plasmids (designated pMA) containing the different modified flavivirus protein cDNAs, flanked by AvaI and ApaI restriction sites, and an ampicillin resistance gene for selection, were ordered from “Gene Art”.

4.1.3. E. coli transformation

For gene amplification, 1-10 ng of plasmid DNA (plasmids pMA or expression plasmids pT389) were used for the transformation of chemically competent *E. coli* (“One shot MAX Efficiency DH5α competent *E. coli*” from Invitrogen) according to the manufacturer’s manual. Transformed cells were selected on LB plates containing 100 µg/ml ampicilin.

4.1.4. Plasmid preparation

A single colony was picked from LB plates supplemented with 100 µg/ml ampicilin and grown in LB medium containing ampicillin overnight, at 37°C. A Mini Prep kit from Qiagen or a Midi Prep kit from Promega was used for plasmid isolation according to the manufacturer’s instructions. DNA concentrations were determined with the Nano Drop 1000 from peqlab.

4.1.5. DNA restriction enzyme digestion

1-10 µg of plasmid DNA were digested with the FastDigest restriction enzymes *Ava*I and *Apa*I (Fermentas). The DNA was mixed with the enzymes in the proposed buffer in a total volume of 10 µl for analytical or 50 µl for preparative purposes, respectively, and incubated for 30 minutes at 37°C.

*Apa*I restriction site: 5´ ..C^TCGG G..3`
3´ ..G GGCT^C..5`

*Ava*I restriction site: 5´ ..G^GGCCC..3`
3`...CCCGG^G..5´

4.1.6. DNA dephosphorylation

To avoid self-ligation of the digested plasmid, the 5´ends were dephosphorylated with FastAP (Fermentas). 1 unit alkaline phosphatase per µg DNA was added and incubated for 10 minutes at 37°C. The alkaline phosphatase was inactivated by heating the reaction mixture to 65°C for 10 minutes.

4.1.7. Agarose gel electrophoresis

DNA samples were analysed on 0.75% (w/v) agarose gels containing ethidium bromide at a final concentration of 2.5 µg/ml in TBE buffer.

DNA samples were mixed with 1/5 loading buffer and loaded into the slots. As a length marker, *Lambda* phage DNA digested with *Hind*III (fragment sizes in bp: 23130, 9416, 6557, 4361, 2322, 2027, 564 and 125) was used. Separation of DNA fragments according to their size was performed by application of 100 V for 20-30 minutes. Due to the intercalation of ethidium bromide into DNA, DNA bands were visualized using a transilluminator at the wavelength of 302 nm.

4.1.8. Preparative agarose gel electrophoresis

To purify DNA fragments after digestion, the whole reaction mix was loaded onto an agarose gel and fragments were separated as described in 4.1.7. The desired DNA bands were excised with a scalpel from the gel under UV-light and DNA extraction was performed with the Wizard SV Gel and PCR Clean-up system from Promega, according to the manufacturer's manual.

4.1.9. Ligation

Ligation of the expression plasmid pT389 and insert DNA was carried out with T4 DNA ligase from Fermentas. To test maximal ligation efficiency, insert and plasmid were mixed in molar ratios of 3:1 and 6:1. As a negative control, a reaction mix without insert DNA was prepared. The ligation mixture was incubated overnight at room temperature. The ligation mixture was then directly used for *E.coli* transformation, described in 4.3.1.

4.1.10. DNA sequencing

DNA sequencing reaction was carried out using the ABI Prism Big Dye Terminator Cycle Sequencing Kit according to the manufacturer's instructions. The sequence reaction was performed in a thermal cycler (Perkin Elmer) under the following conditions:

	temperature	time	cycle
initial denaturation	96°C	20 sec	1 cycle
denaturation	96°C	30 sec	35 cycles
annealing	50°C	15 sec	
extension	60°C	4 min	
hold	4°C	hold	1 cycle

The DNA products were purified by centrifugation through sephadex matrix plates. Then, the samples were sequenced with an automatical capillary sequencer (Applied

Biosystems, GA 3100) by employing fluorescence-labelled dideoxynucleotides as first described in (Sanger, Nicklen et al. 1977). Obtained sequences were aligned with the virtually cloned plasmids using the software DNAStar or Geneious.

4.2. Protein expression and purification

4.2.1. Media and buffers

Schneider´s S2 complete medium from Fisher Scientific

Insect-XPRESS™ Protein-free Insect Cell Medium with L-glutamine from Lonza

Buffer W (pH 8.0):	100mM Tris/HCl 150mM NaCl 1mM EDTA
Buffer E (pH 8.0):	100mM Tris/HCl 150mM NaCl 1mM EDTA 10mM Desthiobiotin,
TAN buffer (pH 8.0):	0.05M Triethanolamine 0.1M NaCl

4.2.2. Drosophila expression system

The DES was developed by the SmithKline Beecham Corporation and is proprietary to this company. The DES is licensed to Invitrogen Corporation. Materials of the kit are the subject of U.S. Patents No. 5,5500,043, 5,681,713 and 5,705,359.

For the expression of flavivirus proteins, the *Drosophila* Expression System (DES) from Invitrogen was used. Schneider 2 (S2) cells from *Drosophila melanogaster* were easily maintained in Schneider´s complete *Drosophila* medium supplemented with 10% fetal calf serum (FCS) and 1% antibiotics and antimycotics (penecillin, streptamycin and amphyteracin B) from Gibco. The cells were grown in loosely adherent or in shaking suspension cultures at 28°C or room temperature without CO_2.

4.2.2.1. Transfection of S2 cells

Transfection of S2 cells was performed in 6 well plates, seeding $3x10^6$ cells in 3 ml complete growth medium and incubating them for 6-16 hours at 28°C until they reached the logarithmic growth phase. The transfection mix with following components was prepared in a microcentrifuge tube. Solution A: 36µl 2M $CaCl_2$, 19µg expression vector and 1 µg selection vector pCoBlast. DdH_2O was added to a final volume of 300 µl. Solution B: 300 µl 2x HEPES

Solution A was added slowly and dropwise to solution B under continuous vortexing. The transfection mixture was incubated for 30-40 minutes at room temperature until a fine precipitate was formed and then added dropwise to the S2 cells under continuous shaking. After incubation for ~24 hours at 28°C, the cells were washed by centrifuging them for 7 minutes at 100 g, removing the transfection medium and replacing it with complete growth medium. Stably transfected cells were selected 48 hours post transfection by replacing the complete medium with selection medium containing 25 µg/ml blasticidin. The selection medium was exchanged every three days for two weeks until resistant cells started growing and the cells of the negative control – without the selection vector – were dead.

S2 cell cultures were expanded and grown in shaking cultures at 28°C. As a backup, 5-10 aliquots of each transfection were frozen in liquid nitrogen. The cells were split when they reached a cell density of $1\text{-}2x10^7$ cells/ml and diluted to $\sim 2x10^6$ cells/ml. Adaption to protein-free medium was performed by splitting the cells into media containing first 50%, then 75% and 100% Lonza serum-free medium.

4.2.3. Optimisation of induction of protein expression

In order to optimize protein expression, transfected cells were seeded at a density of $2x10^6$ cells/ml in 3 ml complete medium into 6 well plates. Different concentrations of $CuSO_4$ were used for induction, and the course of protein expression until cell density reached $1\text{-}2x10^7$ cells/ml was analysed in a 4-layer ELISA using strep-tactin coated plates, described in 4.3.6.2.

4.2.4. Small scale expression and purification

Transfected cells not adapted to serum-free medium were seeded into 6 well plates as described in 4.2.3. Protein expression was induced by the addition of $CuSO_4$ to the medium to a final concentration of 1 mM. The cell culture was incubated for 7 – 12 days until cell density reached 1-2x10^7 cells/ml. Each day a small aliquot of cell culture supernatant was taken for ELISA analysis described in 4.3.6.2. The cell culture supernatant was cleared by centrifugation for 5 minutes at 16000 g, adjusted to pH>7,5 with Buffer W and was then loaded onto strep-tactin spin columns (Biotag). Washing and elution of the protein was performed according to the manufacturer's manual.

4.2.5. Large scale expression and purification

In order to express high amounts of recombinant proteins, stably transfected cells were adapted to serum-free medium as described in 4.2.2.1 and were scaled up in volumes up to 500 ml under shaking conditions. After induction with 1 mM $CuSO_4$, cells were further propagated for 7-10 days until cells reached a density of about 2x10^7 cells/ml.

4.2.5.1. Ultrafiltration of cell culture supernatant

The cell culture supernatant was clarified by centrifugation for 30 minutes at 4000 g and filtration through a 0.22 µM membrane using a vacuum driven filtration system (Steritop; VWR). Concentration of the cleared cell culture supernatant to 100ml was performed with the Vivaflow 200 concentrator (Sartorius) according to manufacturer's manual.

4.2.5.2. Affinity chromatography

The concentrated cell culture supernatant was supplemented with Avidin to a final concentration of 15 µg/ml to mask biotinylated proteins. Then ~100 ml of the concentrated cell culture supernatant was applied onto an equilibrated 5 ml strep-

tactin superflow column H-PR from IBA (2ml/min) using the chromatographic ÄKTA fast protein liquid chromatography (FPLC) system from GE Healthcare. Recombinant proteins were bound to the column via their double strep-tag II. The column was washed with 25 ml buffer W until a constant baseline was reached and recombinant proteins were eluted with buffer E containing 10 mM desthiobiotin and samples of 1ml were fractionated. PD-10 desalting columns were used to remove low molecular weight compounds such as desthiobiotin, from affinity-purified proteins. The columns were first equilibrated with 25 ml TAN buffer, then 2.5 ml of the sample was applied and the desalted protein was eluted with 3.5 ml TAN buffer.

4.3. Protein characterisation

4.3.1. Media and buffers

Carbonate buffer:
1.59 g Na_2CO_3
2.93 g $NaHCO_3$
ddH_2O to a final volume of 1000 ml (pH 9.6)

PBS:
8 g NaCl
0.2 g KH_2PO_4
0.29 g $NaH_2PO_4x12\ H_2O$
0.2 g KCl
ddH_2O to a final volume of 1000 ml (pH 7.4)

ELISA buffer (EB):
2% sheep serum
2% Tween-20
In PBS (pH 7.4)

ELISA wash buffer:
0.05% Tween-20 in PBS (pH 7.4)

SDS-PAGE gel:

15% separation gel:
1.88 ml 40% acrylamide
1.25 ml 1,5M Tris (pH 8.8)
1.8 ml ddH_2O
50 µl 10% SDS
25 µl Ammonium persulfate (APS)
5 µl TEMED (N,N,N´,N´-tetramethyl-ethylendiamine)

3% stacking gel:	385 µl 40% acrylamide 625 µl 1M Tris (pH 6.8) 3.92 ml ddH_2O 50 µl 10% SDS 25 µl APS 5 µl TEMED
Laemmli sample buffer:	0.125M Tris (pH6.8) 2% SDS 10% glycine 0.0025% bromphenol blue 5% 2-mercaptoethanol prior to use
5x Running buffer:	60 g Tris 288 g glycine ddH_2O to a final volume of 2000 ml 0,1% SDS prior to use
Coomassie solution:	0.1% (w/v) Coomassie blue R350 20% (v/v) methanol 10% (v/v) acetic acid
Fixation solution:	50% (v/v) ethanol 10% (v/v) acetic acid
Destaining solution:	50% methanol 10% acetic acid
Blotting buffer:	5.82 g Tris 2.93 g glycine 3.75 ml 10% SDS 200 ml methanol ddH_2O to a final volume of 1000 ml
Blocking buffer:	1% BSA 0.2% Tween-20 in PBS (pH 7.4)

4.3.2. Protein quantification

For the quantification of purified proteins, *BCA (*bicinchoninic acid) *Protein Assay Kit, from Thermo Scientific Pierce was used according to the manufacturer's manual.*

4.3.3. Protein precipitation with deoxycholic acid (DOC) and tricholoracetic acid (TCA)

Protein solutions were incubated with 0.0015% DOC for 30 minutes at room temperature and then precipitated with 5% TCA overnight at 4°C, followed by centrifugation (16000 g, 10 minutes, 4°C) The pellet was washed three times with ice cold acetone (16000 g, 10 minutes, 4°C) and dissolved in 20 µl sample buffer according to Laemmli with or without ß-mercaptoethanol.

4.3.4. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli

The electrophoresis was performed at 20 mA/gel using 0.75 mm thick gels with a 15% separating gel and a 3% stacking gel. Following protein molecular weight markers were used in this study: Low molecular weight marker (97; 66; 45; 30; 20,1; 14,4 kDa) or prestained rainbow molecular weight marker (high range RN756E – 225; 76; 52; 38; 31; 24; 17; 12 kDa) from GE Healthcare.

For staining, the gel was first shaken for 30 minutes in the fixation solution and then for 30 minutes in the Coomassie solution. After destaining, the gel was dried using a gel dryer (Model 543, BioRad) according to the manufacturer's instruction.

4.3.5. Semidry Western blotting

0,5 µg of purified recombinant protein were subjected to 15% SDS-PAGE as described above. The polyvinyldifluoride (PVDF, BioRad) membrane was soaked in methanol (Merck) for 5 minutes and then – together with the gel and blotting filter paper – equilibrated in blotting buffer. The protein was transferred for 90 minutes at 18V onto the PVDF membrane with the Semidry Transfer Cell from BioRad. The membrane was blocked overnight at 4°C with 1% bovine serum albumin (BSA) in PBS pH 7.4 containing 0.1% Tween 20. The primary antibody, diluted in blocking solution, was added for 2 hours at room temperature. The membrane was washed and incubated with the peroxidase labelled lgG-specific secondary antibody in

blocking buffer dilution for 90 minutes at room temperature. The substrate reaction was carried out using the SIGMAFAST^{TM} DAB tablets.

4.3.6. Enzyme linked immunosorbent assay (ELISA)

4.3.6.1. 3-layer ELISA

Nunc Maxisorp Microtiter plates were coated with TBE virus (1 µg/ml) or recombinant proteins (0,4-1 µg/ml) in carbonate buffer, pH 9.4, overnight at 4°C. Serial dilutions of mouse monoclonal antibodies in ELISA buffer were added to the wells and incubated for 1 hour at 37°C in a humid chamber. After washing the plates, bound antibodies were detected with a peroxidase-conjugated rabbit anti-mouse immunoglobulin G and o-phenylenediamine dihydrochloride (ODP, Sigma) in phosphate-citrate buffer, pH 5.0 (1mg/ml) + 1µl/ml 30% H_2O_2 as a substrate. The reaction was stopped with 2N H_2SO_4 and the absorbance was measured at 490 nm. Data were analysed with the KCjunior software (BioTek) and GraphPad Prism 5.

4.3.6.2. Analysis of protein expression (4-layer ELISA)

Samples of cell culture supernatants, obtained from small scale expression experiments described in 4.2.3 and 4.2.4, were diluted in EB 1:100, applied onto strep-tactin coated microtiter plates (IBA - BioTAGnology) and incubated for 90 minutes at 37°C in a humid chamber. The plates were washed and depending on the samples, the primary antibody (a rabbit serum TBEV-Kα2 or a rabbit serum WNV-KIS2) was added and incubated for 60 minutes at 37°C in a humid chamber. After washing the plates, bound antibodies were detected with a peroxidase-conjugated donkey anti-rabbit immunoglobulin G and o-phenylenediamine dihydrochloride (ODP, Sigma) in phosphate-citrate buffer, pH 5.0 (1mg/ml) + 1µl/ml 30% H_2O_2 as a substrate. The reaction was stopped with 2N H_2SO_4 and the absorbance was measured at 490 nm. Data were analysed with the KCjunior software (BioTek) and GraphPad Prism 5.

4.3.6.3. Quantitative 4-layer ELISA

Samples from large scale protein expressions described in 4.2.5 and an internal standard (10 µg/ml) of recombinant protein were serially diluted and applied onto strep-tactin coated microtiter plates (IBA - BioTAGnology) and incubated for 90 minutes at 37°C in a humid chamber. Depending on the sample, the primary antibody (a rabbit serum TBEV-Kα2 or a rabbit serum WNV-KIS2) was used and the ELISA was accomplished as described above.

4.3.6.4. Analysis of the conformational state of TBEV proteins (4-layer ELISA)

The conformational state of TBEV-E-DI proteins was controlled using mouse monoclonal antibodies in a 4-layer ELISA. Equimolar concentrations of TBEV-E-DI proteins and TBEV sE (10nM) were applied onto strep-tactin coated microtiter plates (IBA - BioTAGnology) and incubated for 90 minutes at 37°C in a humid chamber. After washing the plate, serial dilutions of different mouse mAbs were prepared and applied onto the plates. The ELISA was accomplished as described above, using a peroxidase-conjugated rabbit anti-mouse immunoglobulin G as a secondary antibody.

4.3.6.5. Analysis of the conformational state of TBEV proteins (Blocking ELISA)

The Blocking ELISA was in principle performed as described by Stiasny, et al. (Stiasny, Kiermayr et al. 2006). Equimolar concentrations of TBEV-E-DI proteins and TBEV sE were serial diluted (starting with 100nM). Then the serial dilutions were preincubated with each mAb in a single dilution, determined in a 3-layer ELISA using TBEV as an antigen (corresponding to OD=1). After an incubation period of 90 minutes, the whole preincubation mixture was transferred onto TBEV coated microtiter plates and residual antibodies, which were not blocked by the antigen, reacted with the TBE virus and were then detected by using a peroxidase-conjugated rabbit anti-mouse immunoglobulin G, as described above.

4.3.7. Detection of Glycosylation

4.3.7.1. PNGaseF digestion

In order to investigate the glycosylation state of recombinant proteins, the carbohydrate chain was digested using PNGaseF (New England Biolabs). 2 µg of the expressed protein was mixed with 1 µl of the 10x denaturing buffer and ddH_2O was added to a final volume of 10 µl. The protein was denatured at 95°C for 10 minutes. Then 2 µl of G7 Reaction buffer, 2 µl of the detergent NP-40 and 60 units of PNGaseF were added and the sample volume was adjusted to 20 µl with ddH_2O. The reaction was incubated at 37°C for 60 minutes and after protein precipitation (see 4.3.3.) the protein was analysed by SDS-PAGE, as described in 4.3.4.

4.3.7.2. Glycosylation detection blot

Another method to detect glycans was performed using the Amersham ECL glycoprotein detection system. For this purpose, 0,5-2 µg of proteins were blotted onto a PVDF membrane as described in 4.3.5. Detection of glycosylation was performed as described in the manufacturer's instructions, using the protocol for total carbohydrate labelling on nitrocellulose membranes. The membrane was exposed to a photofilm for 10, 30 and 60 minutes.

4.3.8. Enterokinase digestion

The double strep-tag II of produced proteins was cleaved off by Enterokinase (EK) digestion. 2 µg of the purified, tagged protein were mixed with 25 units of EK and 1 µl of 10xEK-buffer from Invitrogen. The reaction mix was adjusted to 10 µl with ddH_2O and incubated at 37°C for 60 minutes. Successful cleavage was shown by performing SDS-PAGE as described in 4.3.4.

5. Results

5.1. Design of flavivirus antigens

5.1.1. TBEV- and WNV-E-DI

To express DI of TBEV- and WNV-E in its native conformation, constructs were designed on the basis of the crystal structure of sE, considering the following aspects: DI is the central domain of the E protein, flanked by DII and DIII, and contains the N-terminus and the glycosylation site. For expressing an isolated form of E-DI, the two long loops protruding out of DI, representing DII, had to be deleted and replaced by short linkers (Fig.5.1).

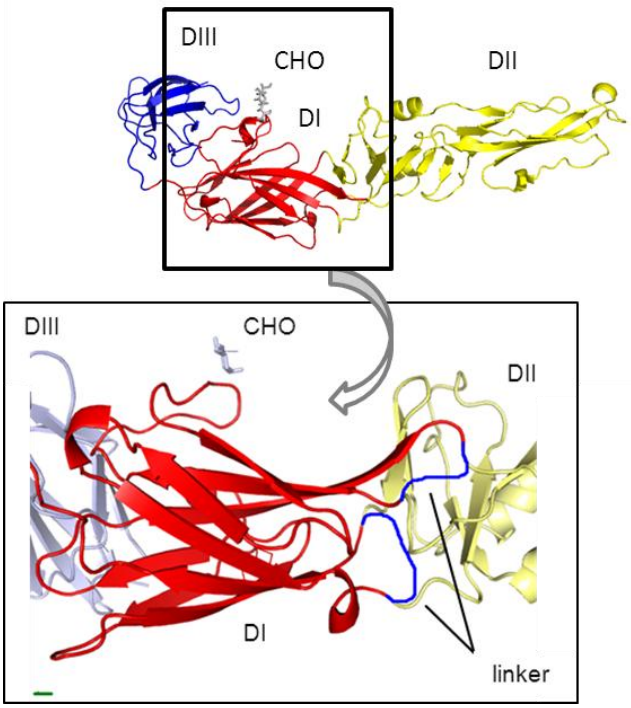


Fig.5.1: Ribbon diagram of sE and the DI-DII and DI-DIII interface of TBE E protein. DI is represented in red with the carbohydrate chain (CHO) in grey, DII is indicated in pale yellow and DIII in light blue. Introduced linkers for the substitution of DII are shown in blue.

In the crystal structure of TBEV sE, the termini of strand D_0 and E_0 of DI are ~10Å and of H_0 and I_0 ~8Å apart. Since an amino acid bond Cα-Cα is about 4.5 Å long, theoretically three amino acids could bridge these gaps *(Fig.5.2A)*. We tested three different linkers for their suitability to produce recombinant, properly folded E-DI. In each construct, the same linker was used to bridge strand D_0 and E_0, and strand H_0

and I_0, respectively *(Fig.5.2).* These linkers contained either four or eight glycines (a small, uncharged amino acid, which should have no effect on the structure of the protein). In addition, a linker composed of glycine-alanine-glycine (GAG) was used *(Table 5.1).* The WNV-E-DI constructs were designed analogous to the TBEV-E-DI constructs.

According to the crystal structures of sE of TBEV and WNV (Rey, Heinz et al. 1995; Kanai, Kar et al. 2006), amino acid (aa) 302 (TBEV) and aa 299 (WNV) constituted the C-terminus of the E-DI constructs (Fig.5.2).

Construct 1: glycine-alanine-glycine linkers	TBEV-E-DI-GAG WNV-E-DI-GAG
Construct 2: 4 glycine linkers	TBEV-E-DI-4G WNV-E-DI-4G
Construct 3: 8 glycine linkers	TBEV-E-DI-8G WNV-E-DI-8G
Construct 4: 8 glycine linkers	TBEV-prM-E-DI-8G

Table 5.1: Amino acid linkers used for the substitution of DII, in the E-DI constructs.

It has been reported that proper folding and stabilisation of the E protein may be dependent on the coexpression of prM (Konishi and Mason 1993), (Lindenbach 2007), and DII is the main interaction site with prM. However, WNV sE was successfully produced for X-ray crystallography without prM (Kanai, Kar et al. 2006) (Nybakken, Nelson et al. 2006). Therefore, all E-DI constructs were designed without prM. Only one construct (TBEV-E-DI with the 8G linkers) was co-expressed with prM, to be able to compare E-DI expressed with and without prM.

In total, four TBEV-E-DI constructs with three different linkers and three WNV-E-DI constructs with three different linkers were cloned into the respective *Drosophila* expression plasmid (described in 5.2.), which contains the sequence for the double strep-tag II and an enterokinase cleavage site. The protein is thus expressed as a fusion protein containing the enterokinase cleavage site and the double strep-tag II (Fig.5.2).

(A) TBEV-E-DI **(B) WNV-E-DI**

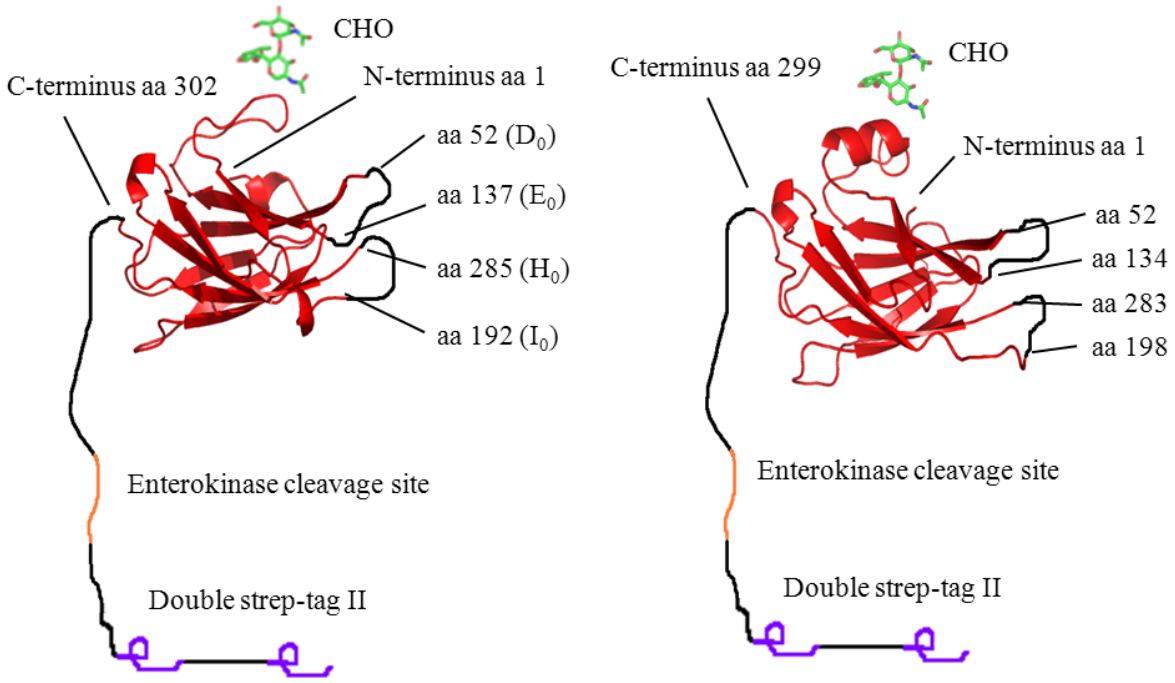


Fig.5.2: Schematics of recombinant TBEV-E-DI (A) and WNV-E-DI (B).

E-DI is shown in red, the carbohydrate chain (CHO) is indicated in green. The two linkers inserted instead of DII are shown in black and the numbers of the amino acids linked together are labelled (aa). Upper letters (D_0, E_0, H_0, I_0) entitle strands of DI as described in (Rey, Heinz et al. 1995). The cloning site of 4 amino acids after the DI-C-terminus is represented in black, the enterokinase cleavage in orange, the double strep-tag II in purple.

5.1.2. TBEV-pr-peptide, TBEV-, WNV- and YFV-prM

The second aim of this study was the expression and purification of the prM proteins of TBEV, WNV and YFV and the pr-peptide of TBEV. In order to produce flavivirus prM in soluble form in *Drosophila* S2 cells, constructs were designed without the C-terminal membrane anchor (Fig.2.9). The furin cleavage site was mutated to produce unprocessed prM. The furin cleavage site mutations used for the different flaviviruses are listed in Tab.5.2.

furin cleavage site	
	Optimal: Arg-X-(Lys/Arg)-Arg Minimal: Arg-X-X-Arg
TBEV furin cleavage site	
prM furin cleavage site in TBEV: Introduced mutations in prM protein:	(aa80) GKQEGSRTRRSVLIPS (aa95) (aa80) GKQEGSRTΔRSVLIPS (aa95)
WNV furin cleavage site	
prM furin cleavage site in WNV: Introduced mutations in prM protein:	(aa80) RCTKTRHSRRSRRSLT (aa95) (aa80) RCTKTRHSSTSSRSLT (aa95)
YFV furin cleavage site	
prM furin cleavage site in YFV: Introduced mutations in prM protein:	(aa80) SAGRSRRSRRAIDLPT (aa95) (aa80) SAGRSSTSSRAIDLPT (aa95)

Table 5.2: prM furin cleavage site mutation strategies. Furin cleavage sites are indicated in green, introduced mutations in red.

In TBEV, the furin cleavage site was mutated by deleting the arginine 88 (Elshuber, Allison et al. 2003). WNV and YFV have two potential furin cleavage sites, and the deletion of one or two arginines would still result in one functional furin cleavage site. Therefore, the three arginines were replaced by two serines and one threonine to mutate the furin cleavage site and to retain the hydrophilic properties of this region, as described previously for DENV (Li, Lok et al. 2008).

These constructs were also cloned into the respective *Drosophila* expression plasmid (described in 5.2.) and the proteins were expressed containing the enterokinase cleavage site and the double strep-tag II (Fig.5.3).

(B)

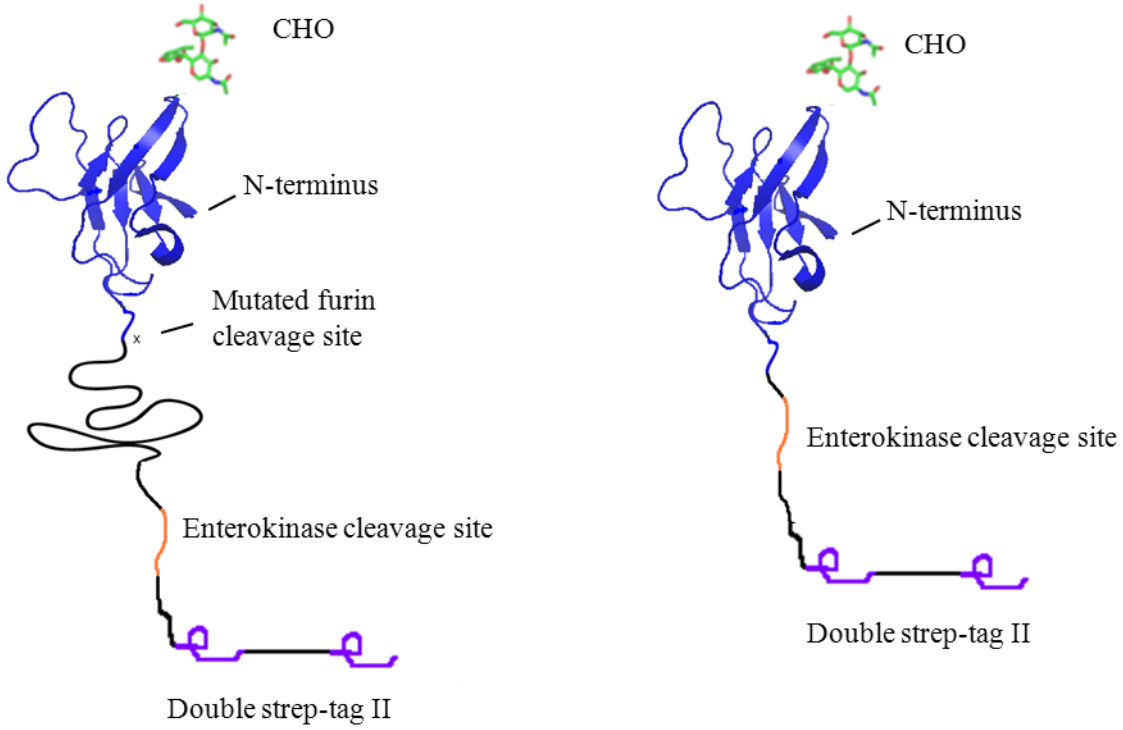


Fig.5.3: Schematics of the recombinant prM (A) and the pr-peptide (B). The crystal structure of DENV pr-peptide containing the carbohydrate chain (CHO), is represented as a ribbon diagram in blue (Li, Lok et al. 2008), the M-part of prM in black. The enterokinase cleavage site is shown in orange, the double strep-tag II in purple.

5.2. Generation of expression plasmids coding for flavivirus antigens

11 different expression plasmids (Table 5.3) for the recombinant expression of E-DI and pr(M) proteins in *Drosophila* S2 cells were generated. The plasmid pT389 used in this study contains an inducible metallothionein-promotor and codes for the Drosophila Bip export sequence, the enterokinase cleavage site and the double strep-tag II (Fig.5.4). The Bip sequence encodes a signal peptide for the export of the protein into the extracellular medium, which is proteolytically removed during exocytosis. The double strep-tag II is used for purification of the protein and can be cleaved off in vitro with enterokinase.

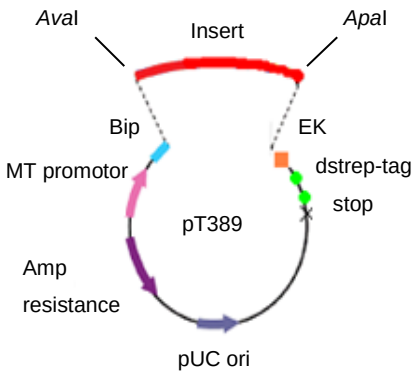


Fig.5.4: Schematic of the expression plasmid pT389 (provided by the Institut Pasteur, France): The plasmid includes an origin of replication (pUC ori), an ampicillin resistance gene, a metallothionein(MT)-promotor, AvaI and ApaI restriction enzyme sites and codes for an export signal sequence (BiP), an enterokinase cleavage site (EK) and a double strep-tag II (dstrp-tag) before a stop codon.

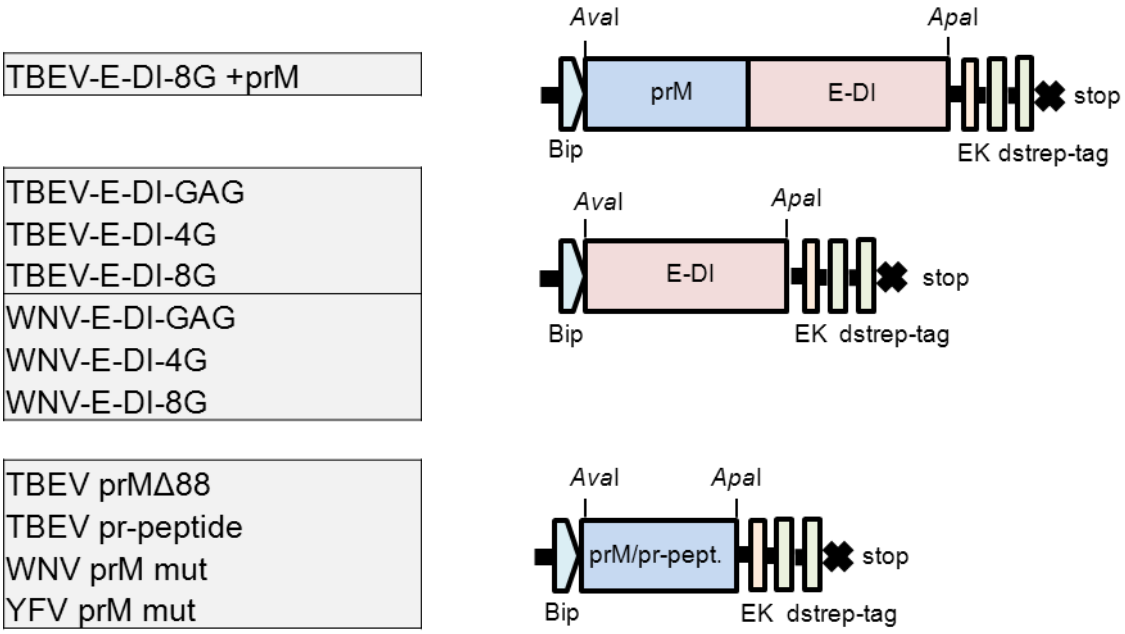


Fig.5.5: Schematic representations of plasmids for the expression of recombinant forms of TBEV-E-DI, WNV-E-DI, as well as TBEV pr(M), WNV prM and YFV prM. The cDNAs of modified DI and prM sequences were cloned with AvaI and ApaI restriction enzyme sites into the expression plasmid, which encodes an export signal sequence (BiP), an enterokinase cleavage site (EK) and a double strep-tag II (dstrep-tag).

For the generation of the respective expression plasmids, synthesized cDNA fragments containing the designed E-DI and pr(M) sequences were cloned into pT389 as described in Materials and Methods. To verify the nucleotide sequence of all generated expression plasmids, the Bip, the insert and the double strep-tag II coding sequences, were sequenced as described in Materials and Methods. Alignment of the generated plasmids matched completely with the virtually designed constructs and no additional mutations were found.

5.3. Expression of flavivirus antigens

5.3.1. Transfection and selection of Drosophila S2 cells

Drosophila S2 cells were transfected with the respective pT389 expression plasmid together with the selection plasmid pCoBlast, as described in Materials and Methods. Transfection of cells with each construct was performed in triplicate and transfected cells were selected in the presence of blasticidin for two weeks.

5.3.2. Optimization of induction

As soon as stably transfected cells were obtained, protein expression was induced by the addition of $CuSO_4$ and the expression rate of recombinant proteins was measured after eight days in a 4-layer ELISA as described in Materials and Methods. Transfected cells from triplicate preparations with the highest expression rate were propagated and used for further experiments.

To optimize the induction and the expression of the desired proteins, different $CuSO_4$ concentrations were compared and the course of protein expression over 8 days was monitored by the detection of the secreted protein in a 4-layer ELISA, as described in Materials and Methods. As an example, the expression of WNV-prM is shown (Fig.5.6).

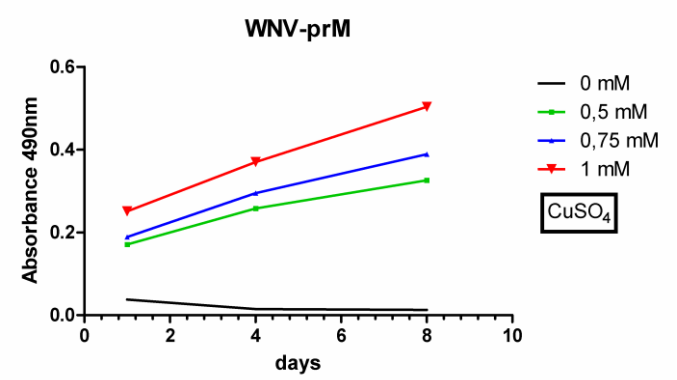


Fig.5.6: Time course of WNV prM secretion into the cell culture supernatant as measured by a 4-layer ELISA. Different concentrations of $CuSO_4$ were used for the induction of protein expression. Day 0 is the timepoint of induction.

As shown in Fig.5.6, the best protein expression was obtained eight days after induction with 1mM $CuSO_4$. Highest cell density (~$2x10^7$ cells/ml) was usually reached after 7-10 days. Induction of protein expression with higher concentrations than 1mM $CuSO_4$ led to a decreased growth of *Drosophila* S2 cells (data not shown).

5.3.3. Upscaling and large scale purification

In order to express high amounts of recombinant proteins, stably transfected S2 cells were adapted to serum-free medium (see Materials and Methods), and scaled up to volumes of 500 ml under shaking conditions. After induction with 1 mM $CuSO_4$ cells were further propagated for 7-10 days. When cells reached a density of about $2x10^7$ cells/ml, the cell culture supernatant was clarified, concentrated by ultrafiltration and the protein was purified by affinity chromatography. The desired protein was bound to streptactin columns, the column was washed to remove unspecifically bound proteins and elution was performed with 10 mM desthiobiotin. As a representative example, the elution profile of recombinant double strep-tagged WNV-prM protein from a streptactin column is shown (Fig.5.7).

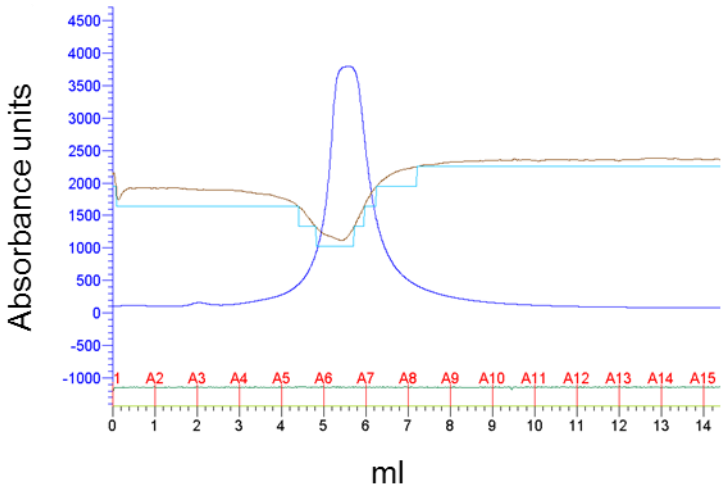


Fig.5.7: Elution profile of WNV prM from streptactin columns. Recombinant WNV-prM was purified from concentrated cell culture supernatant using strep-tactin columns and the chromatographic ÄKTA FPLC system. Samples were fractionated (A1-A15) and eluted WNV prM was collected in fractions A5-A7.

The peak fractions containing the protein and the elution agent desthiobiotin were pooled. Desthiobiotin was removed with PD10 desalting columns and buffer exchange to TAN buffer was performed as described in Materials and Methods. Aliquots were taken from the different steps of the purification procedure and were subjected to SDS-PAGE and ELISA to determine recovery and purity of the protein of interest, as described in Materials and Methods. An example of the analysis of a representative purification procedure (WNV prM) is shown in Fig.5.8 and Fig.5.9.

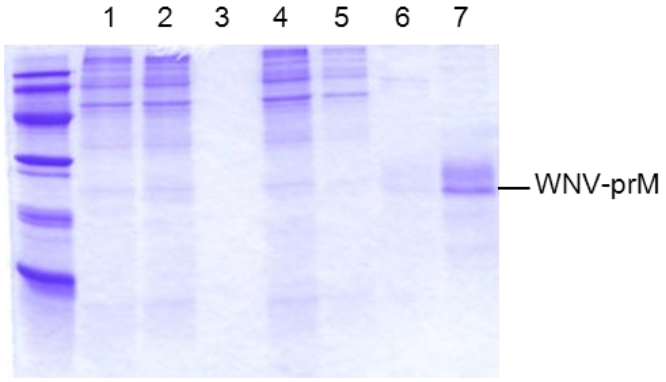
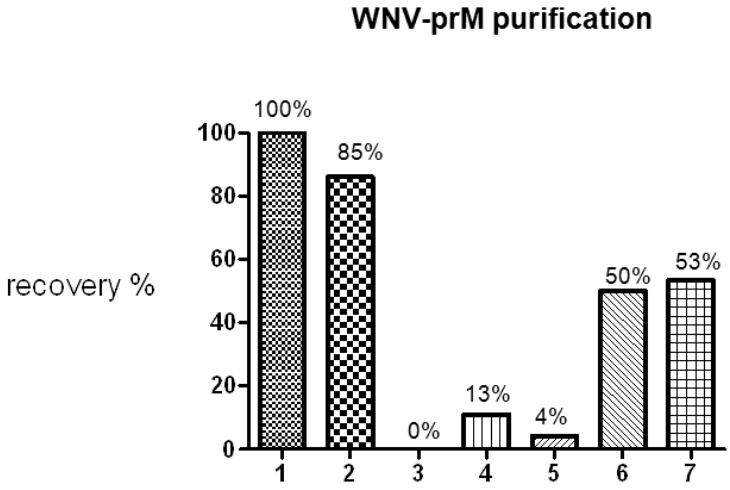


Fig.5.8: Coomassie stained SDS-PAGE gel: Samples of protein purification steps of WNV-prM

***(1)** cell culture supernatant, **(2)** concentrated supernatant after ultrafiltration,*
***(3)** filtrate of ultrafiltration, **(4)** column flow through of affinity chromatography,*
***(5)** wash fraction of affinity chromatography, **(6)** eluted protein*
***(7)** eluted protein (double aliquot)*



***1.** cell culture supernatant*
***2.** concentrated cell culture supernatant after ultrafiltration*
***3.** filtrate of ultrafiltration*
***4.** column flow through of affinity chromatography*
***5.** wash fraction of affinity chromatography*
***6.** eluted protein*
***7.** desalted protein*

Fig.5.9: Recovery diagram of WNV-prM purification. Samples were analysed in a 4-layer ELISA using strep-tactin coated plates.

5.3.4. Yield and purity of recombinant proteins

In summary, 11 recombinant proteins were purified by the procedure described above. The recovery of the expressed proteins after purification and the total yield are listed in table 5.3.

Recombinant protein	Rec. protein in SN [µg/ml]	Yield in high purity [µg]	Recovery [%]
TBEV-E-DI-GAG	0,69	190	55,1
TBEV-E-DI-4G	1,61	430	53,4
TBEV-E-DI-8G	3,75	950	50,7
TBEV-E-DI +prM	4,35	1200	55,2
WNV-E-DI-GAG	n.d.	11320	n.d.
WNV-E-DI-4G	4,19	1190	56,8
WNV-E-DI-8G	70,60	22920	64,9
TBEV pr-peptide	59,85	17060	57,0
TBEV prM	34,60	10410	60,2
WNV prM	14,15	3750	53,0
YFV prM	n.d.	5820	n.d.

Tab.5.3: Summary of total yield and recovery of recombinant proteins after purification from cell culture supernatant (SN); (n.d. – not determined).

Typically, as shown in Fig.5.9 and Tab.5.3, the recovery of the final purified product was in the range of 50-65% of total protein detected in the cell culture supernatant.

In order to analyse the purity of these recombinant proteins, SDS-PAGE was performed (Fig.5.10).

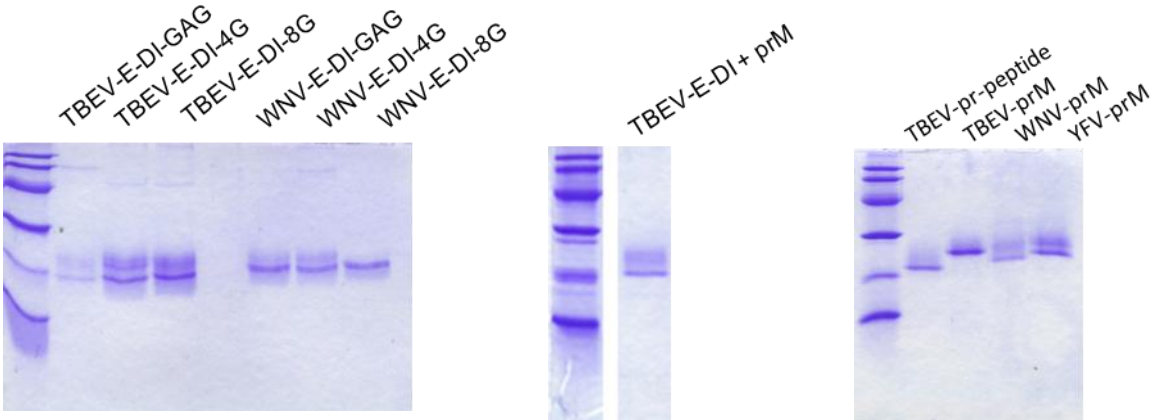


Fig.5.10: Coomassie stained SDS-PAGE gel: Analysis of all purified recombinant E-DI and prM antigens.

All produced proteins were recovered in high purity, but migrated as heterogenous bands in SDS-PAGE. This heterogeneity is caused by the double strep-tag II and is discussed later in 5.4.3. To determine total yield of purified proteins, protein concentrations were calculated as described in Materials and Methods. Despite of some protein lost during the purification procedure, protein quantification resulted in high, but variable protein yields from 190 µg up to 23 mg of highly purified protein from 500 ml cell culture supernatant.

5.4. Characterisation of recombinant antigens

5.4.1. Reactivity with flavivirus monoclonal and polyclonal antibodies

To assess the correct folding and the native antigenic structure of the produced proteins, the different antigens were analysed with polyclonal and/or monoclonal antibodies.

5.4.1.1. TBEV-E-DI

In order to reveal properly folded TBEV-E-DI, the different TBEV-E-DI proteins with varying lengths of their linkers replacing DII were compared with TBEV sE. For this purpose, two non-neutralising TBEV-E-DI specific monoclonal antibodies (mAbs), C4 and C5 (Guirakhoo, Heinz et al. 1989), and two neutralising mAbs, i2 and iC3 (Mandl, Guirakhoo et al. 1989), (Holzmann, Stiasny et al. 1997), were tested in a 4-layer ELISA using streptactin coated plates (see Materials and Methods) (Fig.5.11).

4-layer ELISA

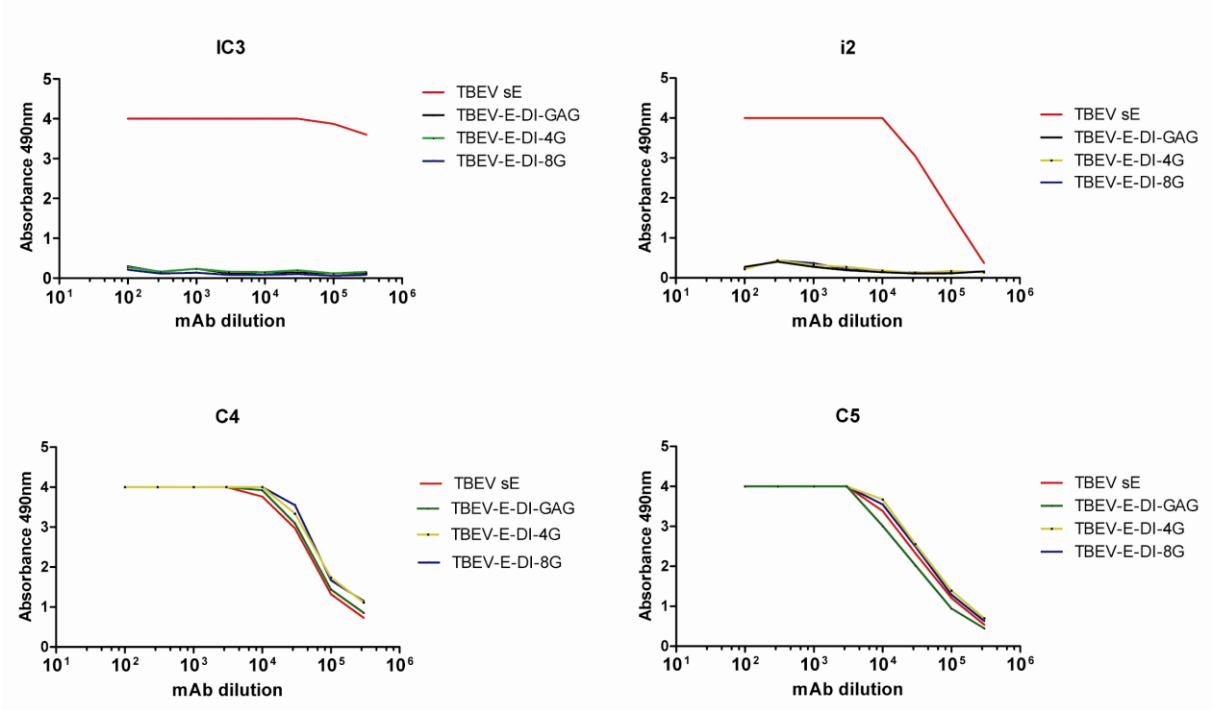


Fig.5.11: 4-layer ELISA with strep-tactin coated plates: ELISA titration curves of TBEV-DI-specific mAbs using equimolar concentrations of TBEV-DI and TBEV sE.

As shown in Fig.5.11, mAbs iC3 and i2 reacted with TBEV sE but did not react with the different E-DI antigens in this ELISA format. MAbs C4 and C5 exhibited the same reactivities with all antigens.

A possible explanation for the lacking reactivity of mAbs i2 and IC3 with E-DI is that the catching of the antigens via their double strep-tag II might cause steric hindrance to the mAbs or that the tag masks the binding epitope. To investigate the reactivity of mAbs i2 and iC3 to TBEV-E-DI antigens in a more native ELISA format, a blocking ELISA was carried out (Fig.5.12), in which the antigen and mAbs were pre-incubated in solution, as described in Materials and Methods. For this purpose, seven TBEV-E-DI-specific mAbs (C2, C3, C4, C5, C6, i2, IC3) and – as a negative control – one TBEV-E-DII-specific mAb (A2) were tested (Guirakhoo, Heinz et al. 1989).

Blocking ELISA

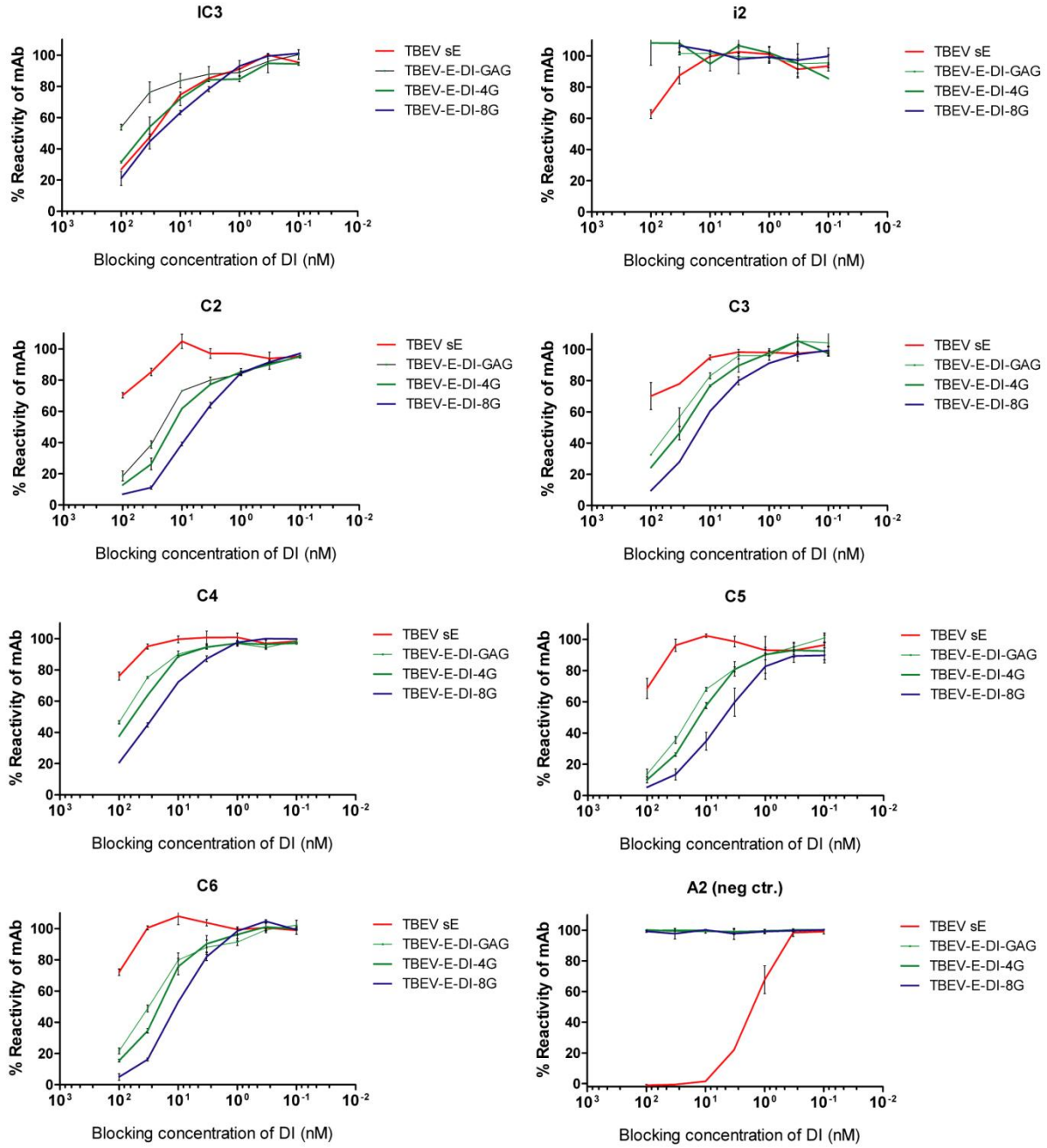


Fig.5.12: Blocking ELISA with TBEV sE and different TBEV-E-DI antigens (standardised to equimolar concentrations) and 8 mAbs. Serial dilutions of antigens were incubated with mAbs and the proportion of mAbs not blocked in this reaction was detected in a 3-layer ELISA with TBE-virus coated plates. Data are represented as means from duplicate measurements, error bars represent standard deviation.

In contrast to the results of the 4-layer ELISA (Fig.5.11), mAb IC3 could be blocked by all TBEV-E-DI antigens. Only the TBEV-E-DI antigen with the GAG linkers reacted to a lesser extent than other TBEV-E-DI antigens and the TBEV sE control with IC3.

In the case of mAb i2, only TBEV sE, but none of the TBEV-E-DI antigens were able to block the mAb. In general, with mAbs C2-C6 the blocking capacity of different TBEV-E-DI antigens was higher than that of TBEV sE. The highest reactivities were obtained with the antigen TBEV-E-DI-8G which was therefore used for further studies.

5.4.1.1.1. TBEV-E-DI coexpressed with prM

A possible explanation for the lack of reactivity could be incorrect folding of recombinant E-DI in the absence of prM, which could function as a chaperone. Since the TBEV-E-DI antigens did not react with the mAbs i2 and IC3 in a 4-layer ELISA, an additional TBEV-E-DI-8G antigen was produced, which was coexpressed with prM (TBEV-E-DI+prM). To investigate whether the coexpression of TBEV-E-DI-8G with prM is necessary to produce E-DI, which is able to react with the mAbs i2 and IC3, a 4-layer ELISA was performed (data not shown). As a control, the reactivity of the mAb C5 to TBEV-E-DI-8G produced with and without coexpressed prM was compared (Fig.5.13).

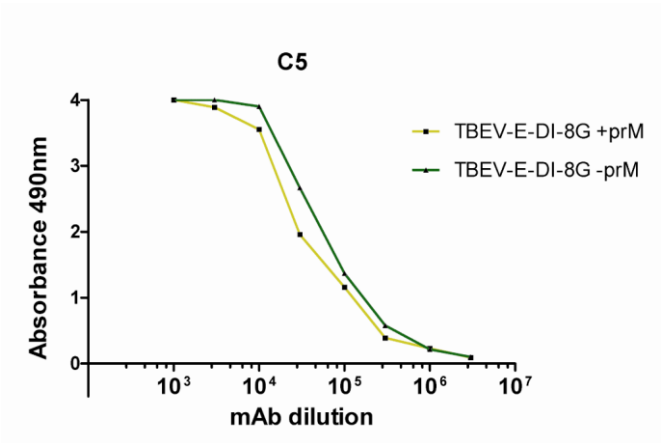


Fig.5.13.: 4-layer ELISA: ELISA titration curves of the TBEV-E-DI-specific mAb C5 using TBEV-E-DI expressed with and without prM.

TBEV-E-DI-8G produced with and without coexpressed prM yielded nearly identical results with the mAb C5 (Fig.5.13), but did not react with the mAbs i2 and IC3 (data not shown). These results demonstrate that the coexpression of TBEV-E-DI with prM

has no influence on the folding of TBEV-E-DI concerning the reactivity of the mAbs i2, IC3 and C5.

TBEV-E-DI antigens with different linkers showed reactivity with E-DI-specific mAbs, except with i2. These results are strong indications that the proteins are properly folded and present in their native conformation.

5.4.1.2. WNV-E-DI

Since there were no mAbs available for WNV-E-DI, the reactivity of WNV-E-DI antigens with different linkers was controlled by Western blot analysis with a polyclonal rabbit serum (WN-KIS2), specific for structural proteins of WNV (Fig.5.14).

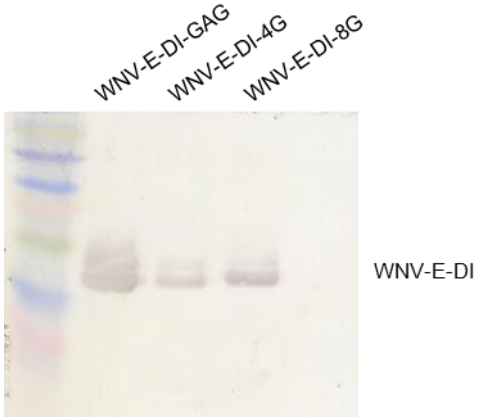


Fig.5.14: Western blot analysis of different WNV-E-DI antigens using polyclonal rabbit serum WN-KIS2.

All WNV-E-DI proteins reacted with the polyclonal serum. Referring to the results obtained with TBEV-E-DI, WNV-E-DI proteins were assumed to have native conformation and the antigen WNV-E-DI-8G was used for further experiments.

5.4.1.3. TBEV pr(M)

To investigate the proper folding of recombinant TBEV prM and the TBEV pr-peptide, the reactivity of the TBEV prM-specific mAb 8H1 (Iacono-Connors, Smith et al. 1996) was tested in a 3-layer ELISA, using immature RSPs (TBEV RSP Δ88) as a control (Allison, Tao et al. 2003), (Fig.5.15), (Materials and Methods). In addition, TBEV prM

was subjected to Western Blot analysis using polyclonal rabbit sera specific for prM (K4prM) or for the structural proteins (KPM-2) of TBEV (Fig.5.16).

Fig.5.15:

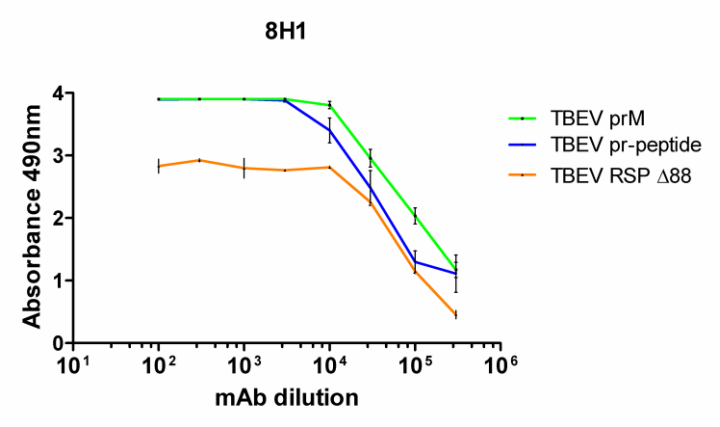


Fig.5.16:

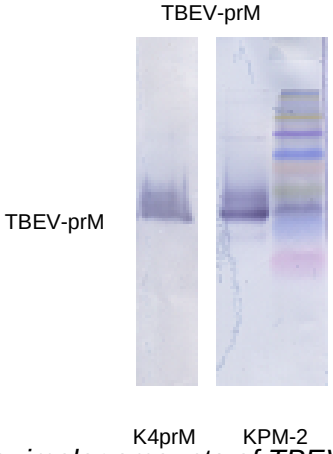


Fig.5.15: 3-layer ELISA: Titration curves of mAb 8H1 using equimolar amounts of TBEV prM, pr-peptide as well as TBEV RSP Δ88 as a control. Data are represented as means from duplicate measurements, error bars represent standard deviation.

Fig.5.16: Western Blot analysis of recombinant TBEV prM using different rabbit sera specific for prM or structural proteins of TBEV, respectively.

The recombinant TBEV pr(M) antigens reacted with the mAb 8H1 in the 3-layer ELISA, shown in Fig.5.15, and TBEV prM reacted also with polyclonal sera (Fig.5.16).

5.4.1.4. WNV prM and YFV prM

For WNV prM and YFV prM no specific mAbs were available. Since WNV- and YFV-prM antigens were produced analogous to TBEV prM, correct folding of WNV- and YFV-prM was assumed and other quality controls, described in 5.4.2 and 5.4.3, were performed.

5.4.2. Formation of disulphide bonds

E-DI has two disulphide bonds, prM and the pr-peptide contain three disulphide bonds. The formation of disulphide bonds is essential for acquiring the native conformation of the proteins. In order to determine the presence of these disulphide bonds, the proteins were treated with or without the reducing agent ß-mercaptoethanol prior to SDS-PAGE as described in Materials and Methods (Fig.5.17).

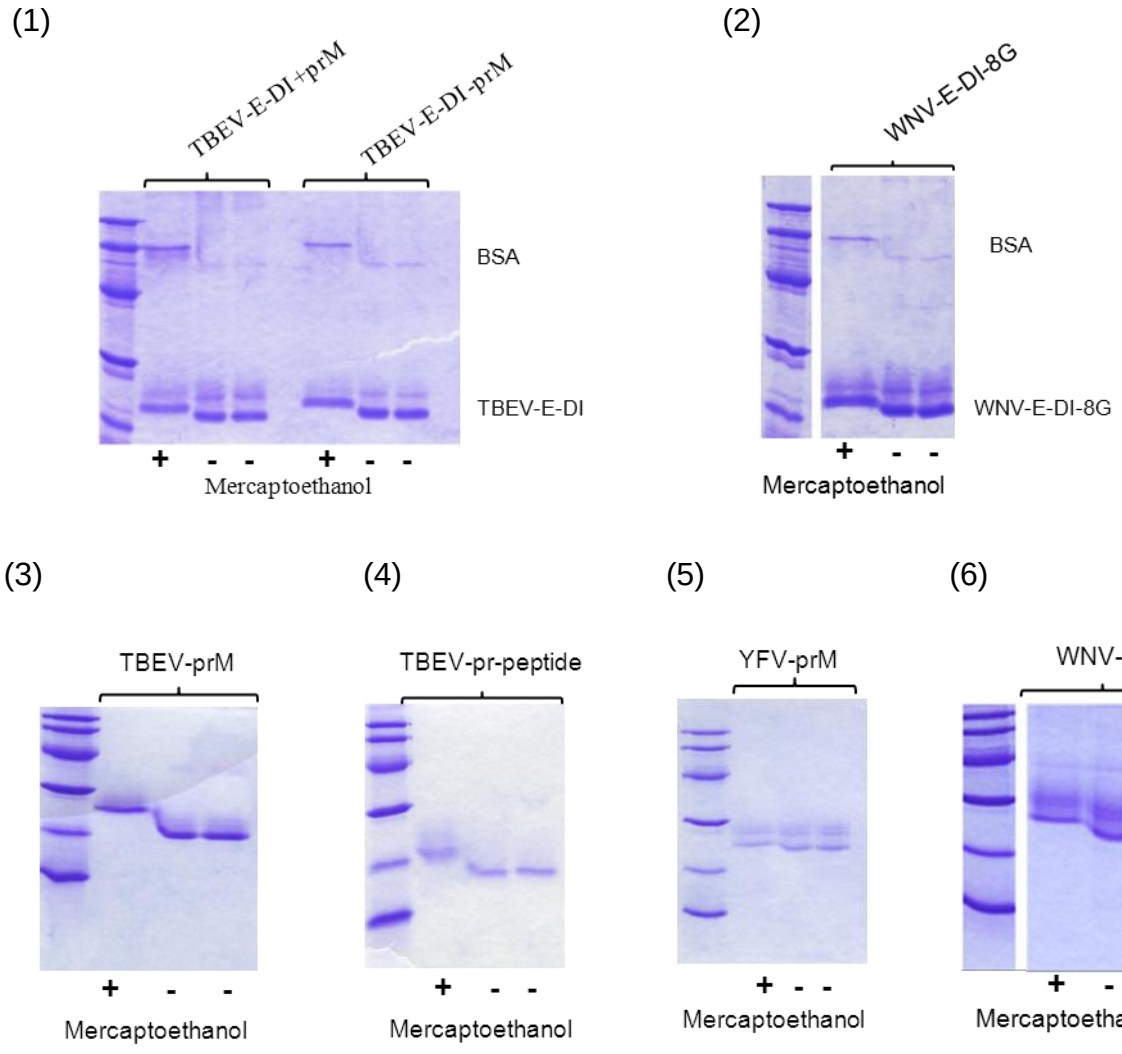


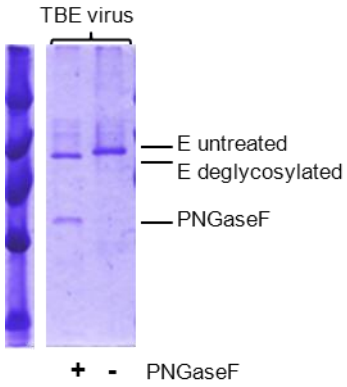
Fig.5.17: Coomassie stained SDS-PAGE gels: (1) TBEV-E-DI-8G produced with and without coexpressed prM, (2) WNV-E-DI-8G, (3) TBEV-prM, (4) TBEV-pr-peptide, (5) YFV-prM and (6) WNV-prM in reduced and non-reduced form.

In all instances, the reduced, completely unfolded protein migrated more slowly than the more compact, non-reduced form of the protein, indicating the presence of disulphide bonds. The lanes with E-DI revealed an additional protein band of bovine serum albumin (BSA), because the samples were derived from a preliminary small scale purification of cell culture supernatant containing FCS, and were therefore not highly purified.

5.4.3. Analysis of the glycosylation state of recombinant flavivirus antigens

E-DI and pr(M) contain one asparagine-linked (N-linked) glycosylation site. The presence of the carbohydrate chain on the protein is important for the correct folding of the protein. In order to verify the glycosylation state of recombinant proteins, digestion with Peptide N-glycosidase F (PNGaseF) was performed. For this purpose, the recombinant proteins and the whole TBE virus (control) were incubated with PNGaseF, an amidase that cleaves between the innermost acetylglucosamine (GlcNAc) and asparagine residues of high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins (Maley, Trimble et al. 1989) and subjected to SDS-PAGE, as described in Materials and Methods.

(1)



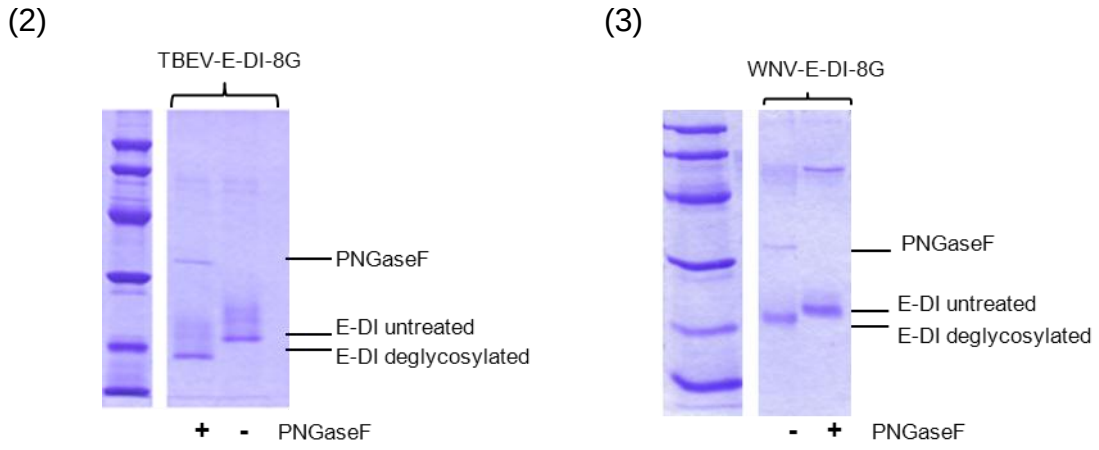


Fig.5.18: Coomassie stained SDS-PAGE gel of (1) TBE virus, (2) TBEV-E-DI-8G and (3) WNV-DI-8G antigens untreated and treated with PNGaseF.

As shown in Fig.5.18, after the PNGaseF-treatment, E proteins of TBE virus (control) and E-DI of TBEV and WNV migrated faster in SDS-PAGE than the untreated controls, indicating the presence of a carbohydrate chain. Since the strep-tagged proteins migrated in both forms (glycosylated and deglycosylated) as heterogenous bands, it was concluded that heterogeneity in the glycosylation patterns were not responsible for this behaviour.

To clarify whether the double strep-tag II causes the heterogeneity of the recombinant protein bands in SDS-PAGE, the effect of tag-removal by enterokinase (EK) from the protein was analysed.

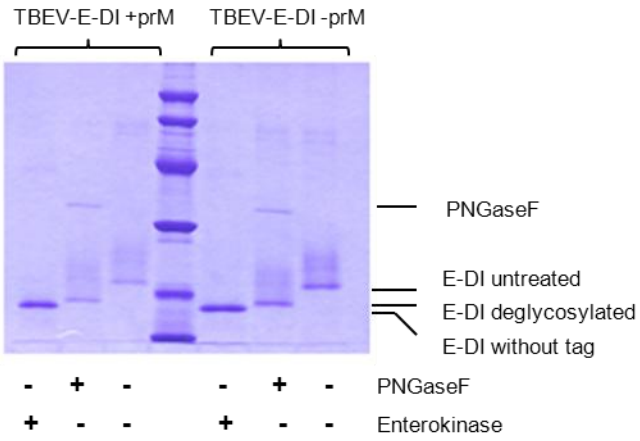


Fig.5.19: Coomassie stained SDS-PAGE. TBEV-E-DI produced with and without coexpressed prM, untreated, treated with PNGaseF and treated with Enterokinase.

As shown in Fig.5.19, the deglycosylated form of TBEV-E-DI coexpressed with and without prM migrated faster than the untreated protein, revealing the presence of a carbohydrate chain. Furthermore, it was shown that the double strep-tag II can be completely removed by treatment with EK, and EK-treated proteins migrated as a single, sharp band.

The same experiments (treatment with PNGaseF or EK) as described for E-DI were also performed with the recombinant prM proteins of TBEV, WNV and YFV (Fig.5.20).

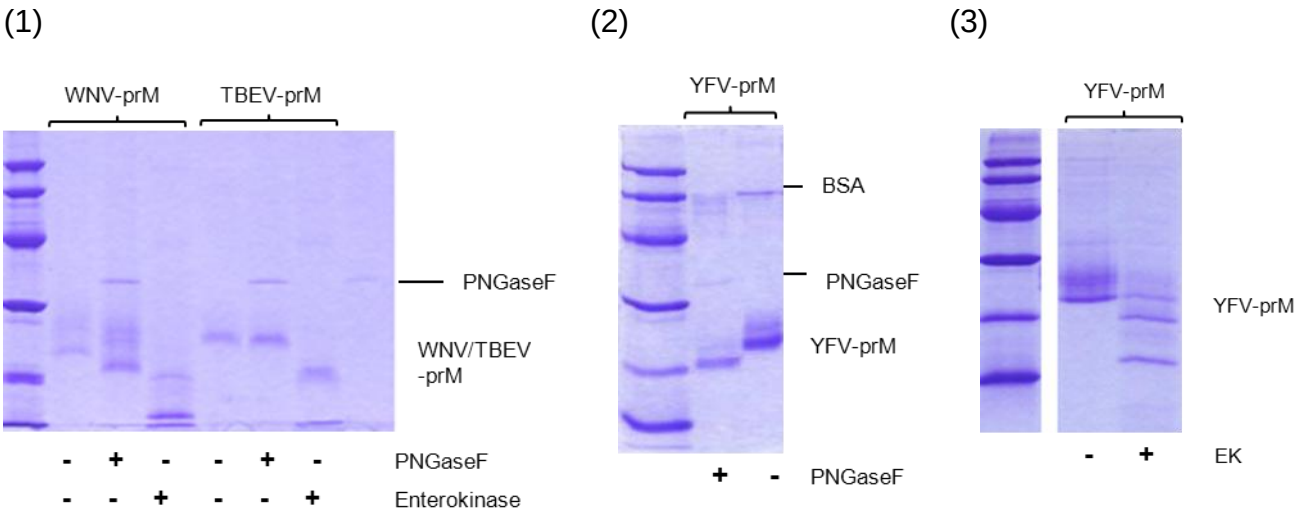


Fig.5.20: Coomassie stained SDS-PAGE: WNV prM (1), TBEV prM (1) and YFV prM (2,3) untreated and treated with PNGaseF or Enterokinase.

In WNV prM the carbohydrate chain was removed by treatment with PNGaseF, but treatment with enterokinase resulted in partial digestion of the whole antigen into small fragments (Fig.5.20). In TBEV prM no migration differences were observed after PNGaseF digestion (Fig.5.20), indicating that the carbohydrate chain is resistant to PNGaseF treatment or that there is no carbohydrate chain present at all. Like WNV prM, the recombinant YFV prM was also degraded by EK.

In order to further analyse the glycosylation state of TBEV-prM, the Amersham ECL (enhanced chemiluminescence) glycoprotein detection system was used, as described in Materials and Methods. In this assay, transferrin and TBEV-E-DI as a

positive control, WNV-DIII as a negative control, as well as TBEV prM were subjected to Western blot analysis and glycosylated proteins were detected on the membrane. For that purpose, the diol-groups of the carbohydrate residues were oxidized and a biotin hydrazide was covalently linked to the carbohydrate residue. The protein was then labelled with a streptavidin-HRP conjugate and the chemiluminescence of ECL detection reagents were visualised on a photofilm (Fig.5.21).

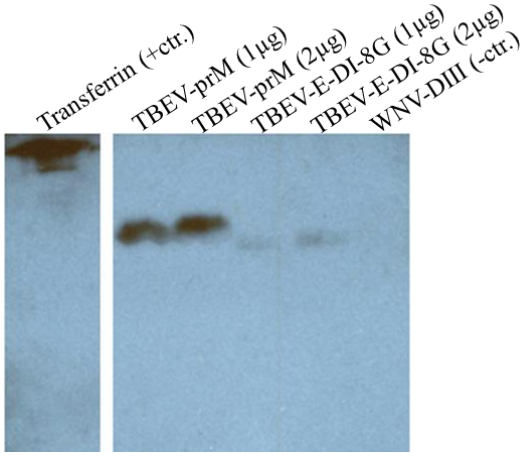


Fig.5.21: Photofilm of the Western Blot detecting chemiluminescence of labelled glycosylated proteins.

As shown in Fig.5.21, transferrin as well as TBEV prM produced a strong signal, indicating the presence of the carbohydrate chain. A weaker signal was obtained with TBEV-E-DI, and as expected, no signal was obtained with the negative control, WNV-DIII.

6. Discussion

Flavivirus particles contain three structural proteins, E, prM/M and C. E is the major target for neutralising antibodies, but also antibodies directed to prM and C have been found in post-infection sera. Little is known about the fine specificity of the humoral immune response after flavivirus infection/vaccination to the E protein or to the structural proteins in general. The major goal of this diploma thesis was the production and quality control of E-protein DI of TBEV and WNV, as well as soluble prM protein of TBEV, WNV and YFV, and the pr-peptide of TBEV in recombinant form. These proteins will then be used as antigens for different immunological assays in further projects to dissect the specificities of polyclonal antibody responses.

Previous studies have shown that the *Drosophila* expression system (DES) could be successfully used for the expression of different flavivirus sE proteins, which were used for crystallisation (Modis, Ogata et al. 2003; Modis, Ogata et al. 2004; Zhang, Zhang et al. 2004; Modis, Ogata et al. 2005; Lieberman, Clements et al. 2007). Therefore this expression system was chosen for our purpose.

The DES is a two-vector based expression system, including a selection vector, which contains a blasticidin resistance gene and an expression vector, coding for the protein of interest. Cotransfection with both plasmids is necessary and was performed in a ratio of 1:19, as proposed in the manufacturer's manual, to ensure the cointegration of the expression cassette with the blasticidin resistance gene into the host cell genome. Nevertheless, it is still possible that cells can be transfected only with the selection plasmid, which are then resistant to blasticidin but do not express the protein of interest. In all cases, the transfection strategy used in this study resulted in high transfection and expression rates of the different flavivirus proteins.

One aspect of the study was the optimisation of the expression of different flavivirus proteins in *Drosophila* S2 cells. We were able to stably transfect S2 cells and the expression rates of different proteins were optimised by testing different concentration of $CuSO_4$ for the induction of protein expression. An inducing concentration of 1mM $CuSO_4$ yielded the highest protein expression levels (Fig.5.6). Higher concentrations of $CuSO_4$ resulted in decreased proliferation of S2 cells and thus in lower protein expression. We achieved high but variable expression rates for the different flavivirus proteins ranging from 0.7-70 µg/ml in the cell culture

supernatant. Variable expression rates can be explained by varying success of the transfection of *Drosophila* S2 cells with the expression plasmid and varying integration of the expression cassette into the host cell genome.

E-DI and pr(M) proteins were expressed with the N-terminal secretion signal BiP, which is cleaved proteolytically in the exocytic pathway. Proteins were secreted into the cell culture supernatant and purified from the concentrated cell culture supernatant by affinity chromatography via their C-terminal double strep-tag II. An end product of 50-65% of the total protein initially expressed and detected in the cell culture supernatant was recovered after affinity chromatography in high purity (Fig.5.9, Tab.5.3).

Losses of protein was observed during concentration of the cell culture supernatant (~15%) and affinity chromatography (~30%) (Fig.5.9, Tab.5.3). Since the analysis of the filtrate of ultrafiltration never revealed any protein, recombinant protein was probably lost due to binding to the membrane of the concentrator. Recombinant protein was also detected in the flow through after loading the concentrated cell culture supernatant onto a strep-tactin column (9-18%). This relatively high proportion of protein in the flow through suggests that there is a limited capacity of the column to bind proteins. After elution of the protein from the column, a proportion of 5-15% was not detected in any of the fractions, indicating that some protein might be bound to the column so tightly via the double strep-tag II, that it cannot be eluted with high concentrations of desthiobiotin. A possible solution would be the elution of recombinant protein with biotin, which has a higher affinity to streptactin. With this strategy the recovery could probably be increased, but it would not be possible to regenerate the column.

Despite these losses of recombinant protein during purification, we were able to produce high yields of highly purified proteins with the procedure established in this study. The purity of recombinant proteins was shown in SDS-PAGE, but strep-tagged proteins migrated as heterogenous bands (Fig.5.10). This heterogeneity of protein bands was shown to be caused by the double strep-tag II. As shown in Fig.5.19 and Fig.5.20, cleavage of the tag from the protein with enterokinase resulted in a defined protein band. A possible explanation for this behaviour is an inhomogeneous binding of SDS to the tag.

Another aspect of the study was the quality control and the characterisation of purified antigens. Since TBEV-E-DI was produced with different linkers, the different proteins were analysed in ELISA with different mAbs using TBEV sE as a control, to identify which linkers are the most suitable to produce E-DI in its native fold.

The TBEV-E-DI-specific mAbs C2-C6 (Guirakhoo, Heinz et al. 1989) and the neutralising, conformation-sensitive mAbs i2 and IC3 were used in this study. Specific residues on E-DI necessary for the binding of i2 and IC3 have been identified by neutralization escape mutants (Holzmann, Mandl et al. 1989; Mandl, Guirakhoo et al. 1989; Holzmann, Stiasny et al. 1997) and RSP mutagenesis (Kiermayr, Stiasny et al. 2009) (Fig.6.1). These results provide important information about the binding epitope of the mAbs i2 and IC3.

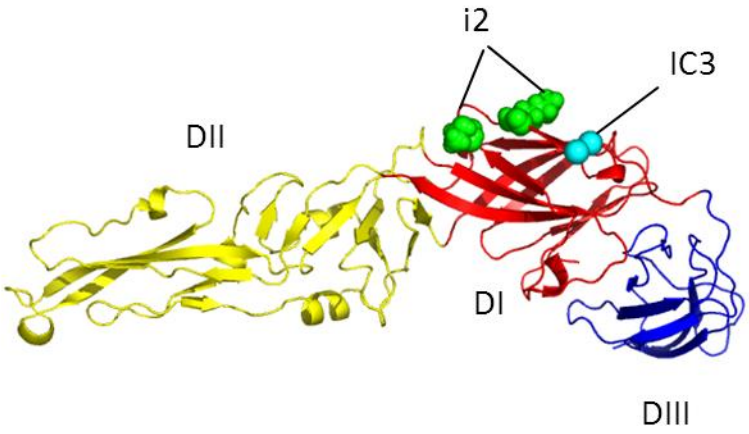


Fig.6.1: Crystal structure of TBEV sE (red: DI, yellow: DII, blue: DIII) with binding sites of monoclonal antibodies i2 (green) and IC3 (cyan).

In a 4-layer ELISA, all TBEV-E-DI antigens – independent of their linkers – recognized the TBEV-E-DI-specific, non-neutralizing mAbs C4 and C5 like the sE control. In contrast, neutralizing, conformation-sensitive mAbs i2 and IC3 showed no reactivity with TBEV-E-DI antigens in the 4-layer ELISA.

These results allow two possible conclusions: Either the E-DI-antigens are not properly folded, or the catching format of the ELISA influences the binding of the mAbs. Therefore, a blocking ELISA was performed, in which mAbs were blocked by antigens in solution. As shown in Fig.5.12, in this ELISA format different TBEV-E-DI antigens, except TBEV-E-DI-GAG, were able to block mAb IC3 to the same extent as TBEV sE. Moreover, in the Blocking ELISA, mAbs C2-C6 exhibited an even higher reactivity with TBEV-E-DI antigens than TBEV sE. One hypothesis to explain this phenomenon is that the epitopes of these mAbs are more accessible in the isolated E-DI than on the whole sE. Comparison of C2-C6 reactivity with different TBEV-E-DI antigens varying in the length of their linkers, revealed TBEV-E-DI-8G to yield the best results. In the case of the mAb i2, none of the different TBEV-E-DI antigens, but only TBEV sE was able to block the mAb. This could be due to the location of its epitope (see Fig.6.1), mapped to the DI/DII interface, which has been described to be a flexible region (Zhang, Zhang et al. 2004). It is possible that the epitope of i2 requires amino acids of DII, or that the flexibility of this region influences the binding of the mAb. Furthermore, prior experiments showed that i2 also exhibited lower reactivities to TBEV sE, than to TBE virions (Kiermayr, Stiasny et al. 2009). Therefore, structural differences between sE and E in particulate form were assumed (Kiermayr, Stiasny et al. 2009), which were probably caused by the flexibility of E at its domain interfaces (Bressanelli, Stiasny et al. 2004; Modis, Ogata et al. 2004; Zhang, Zhang et al. 2004). It is likely, that DI alone does not provide all structural entities for the effective binding of i2.

Since the TBEV-E-DI antigen with 8G linkers and with coexpressed prM also did not react with mAbs i2 and IC3 in a 4-layer ELISA (data not shown), but yielded nearly identical results as the TBEV-E-DI antigen with 8G linkers expressed without prM with the mAb C5 (Fig.5.13), it was concluded that the coexpression of prM is not necessary to obtain E-DI in correct conformation.

In summary, by comparing all TBEV-E-DI antigens to each other, the E-DI with 8G linkers was chosen to be used for further studies. Since WNV-E-DI antigens were produced analogous to TBEV-E-DI antigens, the WNV-E-DI with 8G linkers was also used for further experiments.

Reactivity of the TBEV prM-specific mAb 8H1 was tested with TBEV prM, the TBEV pr-peptide and TBEV Δ88 RSPs as a control in a 3-layer ELISA (Fig.5.15). Both

recombinant proteins reacted with the mAb 8H1, suggesting that the binding epitope is located on the pr-peptide of the prM protein. Reactivity of the mAb 8H1 and also polyclonal sera to recombinant TBEV prM and TBEV pr-peptide are compatible with the correct conformation of these proteins.

To further analyse the proper folding of recombinant proteins, the presence of disulphide bonds was determined by treating the protein with the reducing agent ß-mercaptoethanol. Disulphide bonds, important for protein folding, are reduced by ß-mercaptoethanol and the protein is unfolded. This usually results in slower migration in SDS-PAGE in comparison to the untreated, non-reduced form of the protein, that has a more globular structure. Since prM and the pr-peptide contain three disulphide bonds, the difference was higher in comparison to E-DI proteins, which have only two disulphide bonds.

As another quality control, the glycosylation state of the proteins was analysed. Depending on the host cell, the carbohydrate chain has high-mannose or pauci-mannose structures in insect cells, or it is processed into complex glycans in mammalian cells, respectively. Each of the recombinant proteins expressed in this study contains a single N-linked carbohydrate chain. This was proven by PNGaseF digestion, which cleaves residues of high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins or with the the Amersham ECL glycoprotein detection system, detecting diol structures. Interestingly, TBEV prM seemed to be resistant to PNGaseF digestion, but was found to be glycosylated with the Amersham ECL glycoprotein detection system. A possible explanation could be that the carbohydrate chain of TBEV prM contains a fucose (1-3)-linked to the asparagine-linked N-acetylglucosamine, which is found in glycoproteins from insects and plants and is resistant to PNGaseF (Tretter, Altmann et al. 1991). Indeed, in previous studies, with a *Drosophila* neural cell line (but not with *Drosophila* S2 cells), oligosaccharides containing a fucose (1-3)-linked to the asparagine-linked N-acetylglucosamine were found (Rendic, Linder et al. 2006).

In summary, in this study the procedure for the expression and purification of different recombinant flaviviral proteins was established. High expression rates as well as efficient recovery and high yields of proteins after purification were obtained. It was demonstrated that disulphide bonds were formed and that the proteins were glycosylated. Correct folding of produced TBEV proteins was confirmed with

monoclonal antibodies. These proteins can now be used in further work to dissect the polyclonal antibody response after flavivirus infection or vaccination. They will be valuable tools for different immunological assays in order to learn more about the fine specificity of the humoral immune response against flaviviruses.

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