Folding, assembly and secretion of tick-borne encephalitis virus envelope proteins in mammalian cells

Author(s): Lorenz, Ivo Christian

Publication Date: 2002

Permanent Link: https://doi.org/10.3929/ethz-a-004339046

Rights / License: In Copyright - Non-Commercial Use Permitted
Folding, Assembly and Secretion of Tick-Borne Encephalitis Virus Envelope Proteins in Mammalian Cells

for the Degree of
Doctor of Natural Sciences

presented by
Ivo Christian Lorenz
Dipl. Natw. ETH
born February 24, 1973
citizen of Lichtensteig SG

accepted on the recommendation of

Prof. Dr. Ari Helenius, examiner
Prof. Dr. Franz X. Heinz, co-examiner
Prof. Dr. Josef Brunner, co-examiner

2002
Table of Contents

Abstract .......................................................................................................................... 1

Zusammenfassung ........................................................................................................... 3

1. Introduction ............................................................................................................... 5
  1.1 Folding and Oligomerization of Viral Envelope Proteins ...................................... 5
    1.1.1 Viral Envelope Glycoproteins ........................................................................... 5
    1.1.2 Folding of Viral Glycoproteins in the Endoplasmic Reticulum ......................... 7
    1.1.3 Viral Glycoprotein Oligomerization .................................................................. 10
    1.1.4 Quality Control ............................................................................................... 10

  1.2 Intracellular Virus Assembly and Transport ......................................................... 13
    1.2.1 Virus Budding .................................................................................................. 13
    1.2.2 Assembly of Viruses in Intracellular Compartments .......................................... 14
    1.2.3 Transport of Virions via the Secretory Pathway ................................................ 15

  1.3 Cellular and Molecular Biology of Flaviviruses .................................................... 17
    1.3.1 Classification .................................................................................................... 17
    1.3.2 Pathology ......................................................................................................... 17
    1.3.3 Replication Cycle .............................................................................................. 19
    1.3.4 Structure of the Virions .................................................................................... 20
    1.3.5 Binding and Entry ............................................................................................. 20
    1.3.6 Flavivirus Genome ............................................................................................ 21
    1.3.7 Translation and Polyprotein Processing ........................................................... 21
    1.3.8 Features of the Individual Proteins ................................................................... 23
    1.3.9 Virus Assembly and Transport ......................................................................... 28
    1.3.10 Recombinant Subviral Particles ...................................................................... 29
    1.3.11 Experimental Systems ..................................................................................... 32

  1.4 Goals of this Project ............................................................................................... 32

  1.5 Experimental Strategy ............................................................................................ 33

2. Folding and Dimerization of Tick-Borne Encephalitis Virus Envelope Proteins prM and E in the Endoplasmic Reticulum ......................................................... 35

  Abstract ....................................................................................................................... 37
  Introduction .................................................................................................................. 38
3. Intracellular Assembly and Secretion of Recombinant Subviral Particles from Tick-Borne Encephalitis Virus

Abstract

Introduction

Materials and Methods

Results

Discussion

Acknowledgments

4. Discussion and Outlook

4.1 Discussion

4.1.1 Folding and Maturation of the TBE Virus Envelope Proteins prM and E

4.1.2 Assembly of TBE virus envelope proteins prM and E into Immature Virions

4.1.3 Transport and Secretion of TBE Virus Particles

4.1.4 Green Fluorescent Protein as Marker for Flavivirus Assembly and Transport

4.2 Outlook

4.2.1 Folding Experiments

4.2.2 Assembly Experiments

5. References

Acknowledgments

Curriculum Vitae
Abstract

The folding of a newly synthesized protein from a linear amino acid sequence into a three-dimensional functional conformation is a highly coordinated process that involves stringent quality control mechanisms, ensuring that only properly folded proteins are transported to their target destinations inside or outside the cell. Viral envelope proteins are useful models to analyze the principles of protein folding in vivo. Moreover, analyzing the folding of these proteins may also reveal new insights into viral replication strategies.

The envelope of tick-borne encephalitis (TBE) virus, a flavivirus, contains two proteins, the envelope protein E and the small membrane protein M, the latter being synthesized as a precursor form, prM. In contrast to many other viral envelope proteins that form orthogonal projections to the viral surface, E forms head-to-tail homodimers lying parallel to the viral membrane. By analyzing the folding of TBE virus prM and E expressed recombinantly and in virus-infected cells, we found that both proteins formed disulfide bonds and acquired their native structure within a few minutes after synthesis. Heterodimerization of newly synthesized prM and E occurred rapidly, and E required prM as a chaperone-like factor to fold properly. Both proteins were continuously localized to the endoplasmic reticulum (ER). This was consistent with the finding that TBE virions assemble intracellularly by budding into the ER lumen, followed by transport to the cell surface via the secretory pathway.

Expression of prM and E without other viral proteins leads to the generation of recombinant subviral particles (RSPs), capsidless membrane vesicles containing the envelope proteins, but no nucleocapsid. Secretion of RSPs started one hour after prM and E had been synthesized. Inhibition of glycosylation of E or blockage of glucose trimming on the carbohydrate side chain of E abolished secretion of RSPs. Thus, the carbohydrate side chain of the E protein plays an important role in at least one step of flavivirus oligomerization, assembly and transport.

Taken together, TBE virus envelope proteins prM and E do not require other viral elements to fold and to form the viral envelope, but expression of both proteins as well as the presence and proper glucose trimming of the carbohydrate side chain of E is required for virion assembly. Whether cellular factors are involved in virion formation remains to be elucidated.
Zusammenfassung


mindestens einem Schritt während der Oligomerisierung, des Zusammenbaus oder des Transports der Flaviviren.

Zusammenfassend benötigen die Proteine prM und E des Zeckenenzephalitis-Virus keine weiteren viralen Elemente, um die Virenhülle zu bilden; die Expression beider Proteine und die Zucker-Seitenkette von E sind dafür jedoch erforderlich. Weitere Studien sollen zeigen, ob zelluläre Faktoren am Zusammenbau der Virenpartikel beteiligt sind.
1. Introduction

1.1 Folding and Oligomerization of Viral Envelope Proteins

To exert its function inside or outside a cell, a protein has to acquire a native conformation. The successive transition of a protein from a linear polypeptide into a three-dimensional spatial structure is a process generally termed **protein folding**. Eukaryotic cells have evolved sophisticated mechanisms to govern and control the proper folding of newly synthesized polypeptide chains in various compartments, such as the cytoplasm, the endoplasmic reticulum (ER), mitochondria, or chloroplasts. Protein folding is not a spontaneous event in the cell, but depends on the presence of a variety of folding factors and molecular chaperones that assist nascent polypeptides in acquiring their native conformation.

The lumen of the ER provides a highly specialized folding environment containing a characteristic set of folding enzymes and molecular chaperones (37, 49). Proteins synthesized in the ER undergo numerous co- and posttranslational modifications that are unique to this compartment, such as the formation of disulfide bonds or the addition of Asparagine-linked (N-linked) oligosaccharide chains. Moreover, the ER comprises a quality control system that ensures that only proteins with a fully native structure can leave the compartment for transport along the secretory pathway (76).

Enveloped viruses contain one or more types of membrane glycoproteins that are synthesized in the ER, where they make use of the host cellular machinery to acquire their functional conformation and to form oligomeric complexes. They undergo the same covalent modifications, they depend on the same folding machinery, and they are subjected to the same quality control processes as endogenous proteins. Thus, viral envelope proteins have proven useful models to analyze the principles of protein folding and maturation in the ER of living cells.

1.1.1 Viral Envelope Glycoproteins

Viral envelope proteins are transmembrane proteins whose ectodomains form projections on the virus surface. They confer many functions of the virus, such as binding to cellular receptors, induction of membrane fusion, penetration of the virus
into the cell, and virion assembly at the budding site (84). Many viral membrane proteins form oligomeric complexes that are held together by either noncovalent interactions or by covalent disulfide linkages (31). While some are homooligomers consisting of two to four subunits, others are heterooligomers that contain two or three different types of subunits. Table 1 lists some examples of envelope protein oligomers of different virus families.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Proteins</th>
<th>Oligomer</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alphaviruses</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Semliki Forest Virus</td>
<td>E1, E2, E3</td>
<td>(ABC)₃</td>
<td>(46)</td>
</tr>
<tr>
<td>Bunyaviruses</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Uukuniemi Virus</td>
<td>G1, G2</td>
<td>AB</td>
<td>(121)</td>
</tr>
<tr>
<td>Flaviviruses</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Tick-Borne Encephalitis Virus</td>
<td>M, E</td>
<td>(AB)₂</td>
<td>(66)</td>
</tr>
<tr>
<td>Hepaciviruses</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Hepatitis C Virus</td>
<td>E1, E2</td>
<td>AB</td>
<td>(33)</td>
</tr>
<tr>
<td>Lentiviruses</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Human Immunodeficiency Virus 1</td>
<td>gp120, gp41</td>
<td>(AB)₃</td>
<td>(162)</td>
</tr>
<tr>
<td>Orthomyxoviruses</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Influenza Virus</td>
<td>HA</td>
<td>AAA</td>
<td>(24)</td>
</tr>
<tr>
<td></td>
<td>NA</td>
<td>AAAA</td>
<td>(150)</td>
</tr>
<tr>
<td>Rhabdoviruses</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Vesicular Stomatitis Virus</td>
<td>G</td>
<td>AAA</td>
<td>(88)</td>
</tr>
</tbody>
</table>

**Table 1 Viral envelope glycoprotein oligomers.** The column ‘oligomer’ indicates the composition of a basic unit of the viral envelope protein complex.

An increasing number of X-ray structures of the ectodomains from viral envelope proteins has been published over the past years (101, 126, 155, 159). As an example, influenza hemagglutinin (HA), contains a globular top domain and a stalk involved in trimerization of the molecule by forming a triple coiled-coil structure consisting of three long α-helices (20). The N-linked oligosaccharides are located on the external face of the trimer, while disulfide bonds and hydrophobic stretches are buried inside the folded subunits or subunit interfaces.
1.1.2 Folding of Viral Glycoproteins in the Endoplasmic Reticulum

Protein Translocation and Cotranslational Folding

Viral envelope proteins are translated on membrane-bound ribosomes and inserted cotranslationally into the ER. They contain similar signal sequences like cellular glycoproteins to be translocated (106). Folding of viral glycoproteins can start cotranslationally, as reported for influenza HA (21), and Semliki Forest virus p62 and E1 (110). Cotranslational folding events include the formation of secondary structures, acquisition of single disulfide bonds, and the folding of subdomains within a polypeptide chain.

Disulfide Bond Formation

The redox potential of the ER lumen is sufficiently oxidizing to allow the formation of disulfide bonds between thiol groups of cysteine residues (77). Viral envelope proteins contain numerous disulfide bonds that are usually highly conserved. Moreover, their formation is often crucial for proper folding. Disulfide bonds stabilize the three-dimensional structure of a protein by forming cross-links within the domains, but also by interconnecting domains or subunits.

Mutagenesis of cysteine residues usually leads to a high degree of misfolding and aggregation of the newly synthesized proteins (136). When the redox conditions of the ER are manipulated by addition of dithiothreitol (DTT) to the medium of cells that express influenza HA or the vesicular stomatitis virus (VSV) G protein, disulfide bond formation is inhibited, which leads to the retention of the unfolded glycopolypeptides in the ER (13, 28).

The acquisition of disulfide bonds, which can be either co- or post-translational, is catalyzed by a group of lumenal redox enzymes of the protein disulfide isomerase (PDI) family. Well-characterized members among these are protein disulfide isomerase (PDI) and ERp57 (41). Recently, PDI and ERp57 were shown to transiently form mixed disulfides with cysteines of newly synthesized proteins as intermediates during oxidation in vivo (111). In addition to its redox potential, PDI and its homologs have direct chaperone activity on their substrates (124). Their ability to distinguish between native and non-native disulfide links enables the proteins of the PDI family to catalyze the unscrambling of wrong cysteine bridges into native ones.
This process may be required to dissolve aggregates of newly synthesized proteins in the ER that were generated by the formation of non-native interchain disulfide bonds.

### Table 2 Folding of Viral Envelope Proteins

<table>
<thead>
<tr>
<th>Virus</th>
<th>Protein</th>
<th>$t_{1/2}$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alphaviruses</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Semliki Forest Virus</td>
<td>p62</td>
<td>5 min</td>
<td>(110)</td>
</tr>
<tr>
<td></td>
<td>E1</td>
<td>20 min</td>
<td></td>
</tr>
<tr>
<td>Bunyaviruses</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Uukuniemi Virus</td>
<td>G1</td>
<td>5 min</td>
<td>(120)</td>
</tr>
<tr>
<td></td>
<td>G2</td>
<td>30 min</td>
<td></td>
</tr>
<tr>
<td>Hepaciviruses</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Hepatitis C Virus</td>
<td>E1</td>
<td>&gt; 60 min</td>
<td>(34)</td>
</tr>
<tr>
<td></td>
<td>E2</td>
<td>10 min</td>
<td></td>
</tr>
<tr>
<td>Lentiviruses</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Human Immunodeficiency Virus 1</td>
<td>gp160</td>
<td>&gt; 60 min</td>
<td>(90)</td>
</tr>
<tr>
<td>Orthomyxoviruses</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Influenza Virus</td>
<td>HA</td>
<td>3 min</td>
<td>(14)</td>
</tr>
<tr>
<td>Rhabdoviruses</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Vesicular Stomatitis Virus</td>
<td>G</td>
<td>3 min</td>
<td>(61)</td>
</tr>
</tbody>
</table>

The half-time of oxidation for some viral envelope proteins is listed in Table 2. As can be seen, there are remarkable differences in the kinetics of disulfide bond formation. Oxidation of influenza HA subunits, which sequentially acquire six disulfide bridges passing trough at least two distinct intermediate forms, IT1 and IT2, is completed within 10 min after synthesis (14), while HIV gp160 requires two to four hours to form its 10 disulfide links (90). Thus, the length of the amino acid sequence and the number of disulfide bonds of a protein do not necessarily correlate with the rate of oxidation. The reasons why some proteins require more time to form disulfides than others thus remain unknown.
**N-linked Glycosylation**

One of the major biosynthetic functions of the ER is the covalent addition of core glycans to proteins that are synthesized into the lumenal space leading to the formation of glycoproteins. A preformed precursor "core" oligosaccharide composed of two N-acetylglucosamine, nine mannose and three glucose residues is transferred en bloc to asparagine side chains of newly synthesized proteins ([Figure 1](#)). This process occurs in close proximity to the translocon complex and is a cotranslational process (11).

![Figure 1](#) **Figure 1 The N-linked Core Oligosaccharide.** A core oligosaccharide consisting of 14 sugar moieties is added en bloc to nascent polypeptides at the side chain of an asparagine residue within the consensus sequence Asn-X-Ser/Thr (where X is any amino acid except proline) in the ER lumen. Glucosidases I and II and α-Mannosidase are ER enzymes that selectively remove terminal glucose and mannose residues in the ER as depicted.

Glycosylation has many functions in the biosynthesis of proteins (reviewed in (71)). Located on the outside surface of a newly synthesized protein, N-linked glycans may confer large hydrophilic areas necessary to render a hydrophobic folding intermediate more soluble. Moreover, they have a direct effect on folding in that they influence the structural preferences of a protein close to the glycosylation site, thereby generating a more compact conformation. However, some proteins are able to fold without the need for proper glycosylation, and N-linked oligosaccharides can usually be removed from mature proteins without affecting their folded structure.

One of the most important roles of glycosylation on folding involves the interaction of newly synthesized glycoproteins with the lectin-like chaperones calnexin and calreticulin via their monoglucosylated core oligosaccharide unit (148). As discussed below, binding to calnexin and calreticulin is an essential step in the quality control of many viral as well as cellular glycoproteins.
1.1.3 Viral Glycoprotein Oligomerization

As mentioned above, most viral glycoproteins form oligomeric complexes by noncovalent interactions or by formation of interchain disulfide links between their subunits. These contacts can be established in any of the topological domains of the protein, i.e. the lumenal, the transmembrane, or the cytoplasmic domain (31). Association of viral membrane proteins into homo- or heterooligomers can take place during or after folding of the protein subunits, but usually occurs in a pre-Golgi compartment. In the case of some viruses, oligomerization is required for proper folding of the viral envelope proteins, as reported for semliki forest virus p62 and E1 (9), or hepatitis C virus E1 and E2 (33). Thus, these proteins are likely to oligomerize in the ER.

However, the site of viral envelope protein oligomerization does not necessarily correspond to the site of incorporation into virus particles. Rather, oligomerization may be necessary for the folding, maturation or transport of viral envelope proteins, as well as to hold proteins in a certain conformation to prevent induction of the fusion activity before release of the virus from the host cell.

1.1.4 Quality Control

Quality control was originally described as the process by which export of incompletely folded or assembled proteins from the ER is selectively inhibited (76). The cell has evolved a stringent quality control system to ensure that newly synthesized proteins have acquired their native conformation before transported to their final destination. Misfolded and incompletely folded proteins are common side products of protein synthesis in the ER that have to be separated from correctly folded proteins. Quality control in the ER includes a variety of mechanisms that are partially redundant. The main strategies include retention of incompletely folded proteins in the ER by interaction with molecular chaperones, ER-associated degradation (ERAD), and retrieval to the ER from downstream compartments of the secretory pathway (reviewed in (37)). Viral glycoproteins undergo the same quality control as cellular proteins do and have often been used to elucidate the molecular mechanisms of these processes.
**Molecular Chaperones**

The term “molecular chaperone” was first assigned to a variety of ubiquitous proteins that assist newly synthesized proteins in their folding and assembly (39). Molecular chaperones do not impart any folding or assembly information but rather help the proteins acquire their native conformations and prevent them from undergoing nonproductive pathways leading to misfolding and aggregation (49). Moreover, they serve as retention factors that selectively hold back immature proteins in the ER. Molecular chaperones recognize incompletely folded proteins by a variety of means, such as a general lack of compactness, the presence of mobile loops, or the exposure of hydrophobic peptides (37).

A variety of ER chaperones exists that promote the folding and oligomerization of newly synthesized proteins, such as BiP, calnexin and calreticulin, GRP94, as well as the family of protein disulfide isomerase described above. The properties of these chaperones have been elucidated by analyzing their binding to viral envelope proteins. VSV G protein and Rabies virus glycoprotein G were shown to sequentially interact with BiP and calnexin, indicating that different chaperones may selectively bind to nascent polypeptides at a certain folding state (48, 61). Another study revealed that the binding of calnexin to VSV G protein appeared to depend on the number of glycans, showing that efficient interaction required the presence of both core oligosaccharides (15).

In contrast, influenza HA does not interact with BiP, but extensive binding to calnexin and calreticulin has been demonstrated (60). Many other viral glycoproteins were shown to interact with calnexin and calreticulin, such as E1 and E2 of hepatitis C virus (34), as well as p62 and E1 of Semliki Forest virus (111). This indicates that recognition of glycoproteins by calnexin and calreticulin is likely to occur for most or all proteins that carry one or more N-linked glycans. However, the binding specificity may depend on the position of the core oligosaccharide in the primary sequence. It has been demonstrated that if an N-linked glycan is located within the first 50 amino acids of the primary sequence, the protein cotranslationally binds to calnexin and calreticulin, while proteins carrying their first N-linked glycan downstream of amino acid residue 50 first bind to BiP followed by binding to calnexin and calreticulin (110).
**The Calnexin/Calreticulin Cycle**

Calnexin and its soluble homolog calreticulin are parts of a glycoprotein-specific chaperone system called the calnexin-calreticulin cycle (37). This cycle plays a central role in folding and quality control in the ER lumen. Studies on the association of viral glycoproteins with elements of the calnexin/calreticulin cycle have been particularly helpful in understanding the mechanisms involved in quality control.

Recently, the NMR structure of the calreticulin P-domain and the crystal structure of the entire calnexin ectodomain were published (38, 134). The P-domains of both calnexin and calreticulin fold as an extended arm with proline-rich tandem sequence repeats, which may be involved in interaction with other folding enzymes. The calnexin core corresponds to a lectin domain that binds the monoglucosylated oligosaccharide unit on the nascent glycoprotein.

**Figure 2** The Calnexin Cycle. The ER-resident lectin calnexin (CNX) and its soluble homolog calreticulin (CRT, not shown for simplicity) binds to monoglucosylated oligosaccharides of a newly synthesized protein after trimming of the two outermost glucose residues by glucosidase I and II. ERp57, a thiol oxidoreductase bound to CNX and CRT, can thereby catalyze disulfide bond formation on the glycoprotein. The third glucose residue is removed by glucosidase II, which leads to the dissociation of the complex. If the protein is correctly folded, it can exit the ER. However, if the protein is not correctly folded, the enzyme UDP-glucose:glycoprotein glucosyltransferase (GT) re-adds a glucose residue to the N-linked glycan, and the protein enters the cycle again. Continuously misfolded proteins are retrotranslocated into the cytosol and degraded by a process called ER-associated degradation (ERAD).

**Figure 2** schematically depicts the processing of a newly synthesized glycoprotein in the calnexin/calreticulin cycle. After trimming of two of the three glucose residues from the N-linked glycan by glucosidases I and II (119), nascent glycopolypeptides
bind to calnexin and calreticulin, which are specific lectins for monoglucosylated core oligosaccharides. Calnexin and calreticulin interact with ERp57 (118), which catalyzes the formation of disulfide bonds in the protein bound to the lectin-like chaperones. The complex dissociates upon removal of the remaining third glucose residue by glucosidase II. The enzyme uridine diphosphate (UDP) glucose:glycoprotein glucosyltransferase (GT), which acts as a folding sensor, selectively re-adds one glucose residue to incorrectly folded proteins (139, 140). The proteins can thus re-interact with calnexin and calreticulin and thereby undergo another round of maturation. This cycle continues until the protein is correctly folded and therefore released for exit from the ER.

If a glycoprotein does not reach its native conformation, it is degraded. Typically, the degradation mechanism involves the recognition of a permanently misfolded protein by the loss of one mannose residue in the middle branch of the core glycan. It is thought that the slow-acting ER-resident α-1,2 mannosidase (mannosidase I) acts as a timer for degradation (96). Recognition leads to the retrotranslocation of the misfolded protein into the cytosol where it is deglycosylated by N-glycanase, followed by ubiquitination and degradation in the 26S proteasome (123).

1.2 Intracellular Virus Assembly and Transport

1.2.1 Virus Budding

Enveloped viruses acquire a lipid bilayer through a membrane fission event termed virus budding. This process can occur either at the plasma membrane or in intracellular compartments of the secretory pathway (see below).

It is not clear in every case which viral or cellular proteins are the driving forces behind virus budding, as many different mechanisms of budding appear to exist (reviewed in (45)). An early model of alphavirus budding suggested that the cytoplasmic tail of a viral envelope protein interacted with the nucleocapsid, thereby triggering the fusion process (47). The nucleocapsid would become surrounded by a lipid bilayer containing the spike proteins, eventually leading to the budding of a viral particle. It is now recognized that lateral interactions between spike proteins also play a role. In fact, the envelope proteins of both coronaviruses and flaviviruses were
shown to be incorporated into virus-like particles in the absence of the nucleocapsid (5, 151). However, other virus families, such as retroviruses, do not require the envelope proteins for budding (29). Instead, budding depends on the Gag protein attached to the membrane on the cytoplasmic surface. The Rhabdoviruses are able to form virus-like particles driven by the matrix protein without the spike glycoprotein and the ribonucleoprotein (RNP), although at low efficiency (108). Thus, four classes of budding strategies have been proposed (45):

I. Budding dependent on both capsid and spike proteins (example: alphaviruses)

II. Budding driven by capsid or core protein alone (retroviruses)

III. Budding mediated by envelope proteins only (coronaviruses, flaviviruses)

IV. Budding triggered by matrix protein with the assistance of spikes and RNP (rhabdoviruses)

Whether cellular proteins and factors are involved in these budding mechanisms remains to be elucidated.

1.2.2 Assembly of Viruses in Intracellular Compartments

Budding of virions can occur at different membranes of the cell. Many viruses bud from the plasma membrane, whereas others acquire a lipid bilayer by budding into a compartment of the secretory pathway, such as the ER, the ERGIC, or the Golgi complex (reviewed in (121)). Table 3 gives an overview of the virus families currently known to assemble at intracellular compartments.

The site of assembly of a certain virus is most likely determined mainly by the accumulation of the viral spike proteins in the budding compartment (53). This implies that the envelope proteins must contain targeting signals that confer localization or retention to the particular intracellular compartment where budding takes place. The transmembrane domains of the E1 and E2 envelope proteins of hepatitis C virus (HCV) both contain a signal that mediates retention in the ER (22, 23), and an additional retention signal has been mapped to the juxtamembrane region of the ectodomain of HCV E1 (113). Rubella virus glycoprotein E1 contains an ER retention signal that is masked by forming heterodimers with E2 (74). The transmembrane domain of E2 then confers retention in the Golgi complex where
virus assembly takes place. Thus, Rubella virus has evolved a controlled mechanism of delivering envelope proteins to the budding compartment: the envelope protein oligomer is competent for transport to the Golgi only after heterodimerization of E1 with E2. Bunyaviruses are assembled in the Golgi as well (121). A short sequence in the cytoplasmic tail of the G1 glycoprotein was shown to confer localization to this compartment (8).

<table>
<thead>
<tr>
<th>Membrane Compartment</th>
<th>Virus Genus</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endoplasmic Reticulum</td>
<td>Flavivirus</td>
<td>Dengue</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yellow Fever</td>
</tr>
<tr>
<td></td>
<td>Hepacivirus</td>
<td>Hepatitis C</td>
</tr>
<tr>
<td></td>
<td>Rotavirus</td>
<td>Simian SA11</td>
</tr>
<tr>
<td>ER-to-Golgi Intermediate Compartment</td>
<td>Coronavirus</td>
<td>Avian Infectious Bronchitis</td>
</tr>
<tr>
<td></td>
<td>Poxvirus</td>
<td>Vaccinia</td>
</tr>
<tr>
<td>Golgi Complex</td>
<td>Bunyavirus</td>
<td>Uukuniemi</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Punta Toro</td>
</tr>
<tr>
<td></td>
<td>Togavirus</td>
<td>Rubella</td>
</tr>
</tbody>
</table>

Table 3 Virus Assembly at Intracellular Compartments. Adapted from references (121) and (53).

These findings suggest that viral envelope proteins do indeed contain targeting signals that are sufficient for localization to a certain compartment. It remains to be investigated whether these signals share common features in their amino acid sequence, their position within the protein, and their secondary structure.

1.2.3 Transport of Virions via the Secretory Pathway

Viruses that acquire their lipid envelope by budding at the plasma membrane are released directly into the extracellular space. Viruses that mature intracellularly are released into the lumen of the compartment in which budding takes place. Subsequently, the virions have to be transported to the cell surface for release the virus particles into the extracellular space. This probably involves the compartments of the secretory pathway, because the envelope proteins of many viruses undergo posttranslational modifications and proteolytic processing typically associated with
the Golgi and the trans-Golgi network (35). However, transport of virus particles along the secretory pathway has rarely been observed, indicating that this process is fast and efficient. Secretion of virus particles is likely to occur by the same or similar mechanisms as for cellular proteins, involving transport of virions in membrane vesicles that bud from the donor membrane and fuse with target membranes of compartments along the secretory pathway (reviewed in (129)). A schematic overview of the transport along the secretory pathway is given in Figure 3.

Figure 3 Transport along the Secretory Pathway. Mature proteins leave the ER at “exit sites”, where they are incorporated into COPII-coated vesicles that bud off from the ER membrane. These anterograde vesicles are transported along microtubules and fuse with the ER-to-Golgi Intermediate Compartment (ERGIC). New vesicles budding off from the ERGIC are transported further to the Golgi complex, where the proteins undergo post-translational modifications during their passage through the Golgi cisternae. ER-resident proteins are retrieved to the ER by retrograde transport from the ERGIC and the Golgi in COPI-coated vesicles. Proteins destined for lysosomes, the plasma membrane, or the extracellular matrix are sorted in the trans-Golgi network, where they are selectively packed into various types of vesicles and transported to their final target compartment. Anterograde transport is shown with black arrows, whereas retrograde transport is depicted by gray arrows.
1.3 Cellular and Molecular Biology of Flaviviruses

1.3.1 Classification

Flaviviruses are small enveloped animal viruses containing a single positive-stranded RNA genome. Their name is derived from the Latin word *flavus* (yellow), which refers to the prototype flavivirus, Yellow Fever virus. The flaviviruses are a genus within the *Flaviviridae* family, which also comprises the pestiviruses (from the Latin *pestis*, plague) and the hepaciviruses (from the Greek *hepar*, liver). Another group of unassigned viruses, the GB agents, needs to be formally classified within the family.

Currently, there are nearly 80 members of the flavivirus genus known, about 50% of them being human or animal pathogens. The majority of them are arthropod-borne, transmitted to vertebrates by chronically infected mosquito or tick vectors. However, isolates from bats and rodents without known insect vectors were also identified. The life cycle of the arthropod-borne flaviviruses is very complex, involving relationships between insect vectors, vertebrate reservoirs, humans, and the environment.

1.3.2 Pathology

Flaviviruses cause a variety of diseases including fevers, encephalitis, and hemorrhagic fevers (reviewed in (112)). Entities of major global concern are yellow fever, dengue fever with associated dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), and various forms of encephalitis or meningoencephalitis in Western and Eastern Europe, in Asia, and, to a smaller extent, in America, Australia, and Africa. Based on cross-neutralization studies as well as on classical serological criteria, and according to molecular phylogenetics, the flaviviruses are categorized into several antigenic complexes and subcomplexes. Table 4 summarizes some prevalent members of these complexes and lists their arthropod vectors.

Tick-borne encephalitis (TBE) virus is transmitted to vertebrates by hard ticks, particularly of the *Ixodes* species, mostly *I. ricinus* in European countries. They are most active during the spring and summer months, hence the disease became known as spring-summer encephalitis (Frühsommer-Meningoencephalitis, FSME). Specific strains of the virus are endemic in many Central European countries, but also in Russia, and China. The Central European form is milder than its Russian and
Asian counterparts and typically takes a biphasic course. The first phase occurs 3 to 7 days after infection, with symptoms such as uncharacteristic fever, headache, malaise and muscular pain, usually lasting for 5 to 7 days. After one week of recovery, 20 to 30% of the infected develop the second phase of the disease, which involves the central nervous system (CNS). The patients suffer from severe meningitis or meningoencephalitis, with a case fatality rate of 1 to 2%.

Thus far, vaccination is available for Yellow Fever using the live-attenuated 17D strain, and for TBE and Japanese encephalitis (JE) using inactivated virus. Major efforts are undertaken to develop a vaccine against Dengue virus, with an estimated 100 million cases and over 500'000 cases of DHF/DSS occurring annually in the tropics. One promising candidate live-attenuated vaccine is a chimeric flavivirus generated by replacing the structural glycoproteins of YF-17D with those of Dengue (57). Other viruses within the flavivirus genus may rapidly evolve within the next years or decades and are therefore major challenges for the emerging 21st century.

<table>
<thead>
<tr>
<th>Antigenic Group</th>
<th>Vector</th>
<th>Type Members</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tick-Borne Encephalitis</td>
<td>Tick</td>
<td>Central European Encephalitis (TBE-W)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Far Eastern Encephalitis (TBE-FE)</td>
</tr>
<tr>
<td>Rio Bravo</td>
<td>Tick</td>
<td>Rio Bravo</td>
</tr>
<tr>
<td>Japanese Encephalitis</td>
<td>Mosquito</td>
<td>Japanese Encephalitis (JE)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Murray Valley Encephalitis (MVE)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>St. Louis Encephalitis (SLE)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>West Nile (WN)</td>
</tr>
<tr>
<td>Dengue</td>
<td>Mosquito</td>
<td>Dengue type I (DEN-1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dengue type II (DEN-2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dengue type III (DEN-3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dengue type IV (DEN-4)</td>
</tr>
<tr>
<td>unassigned</td>
<td>Mosquito</td>
<td>Yellow Fever (YF)</td>
</tr>
</tbody>
</table>

Table 4 Members of the Flaviviruses.
1.3.3 Replication Cycle

Many processes within the flavivirus replication cycle are poorly understood, but it seems that all genera of the *Flaviviridae* family share common strategies for viral replication. A very basic scheme (as reviewed in (94)) of the flavivirus life cycle is depicted in Figure 4.

Binding of virus to the host cellular surface occurs via interaction of the major envelope protein E with one or more as yet unidentified receptors. Uptake of the virus occurs via receptor-mediated endocytosis, followed by accumulation of virions in endosomes. The low pH triggers fusion of the viral with the cellular membranes, leading to the release of the nucleocapsid into the cytoplasm. After uncoating, translation of the positive-stranded genome takes place, in parallel with synthesis of minus-strand RNA that serves as template for RNA replication. Assembly of immature virions is believed to occur by budding into intracellular membranes, probably the ER, leading to the formation of virus-containing vesicles. During their transport via the host secretory pathway, the viral envelope undergoes additional processing steps. At the cell surface, the vesicles fuse with the plasma membrane, thereby releasing mature virions into the extracellular compartment.

![Figure 4 The Flavivirus Life Cycle](image)

*Figure 4 The Flavivirus Life Cycle.* Flaviviruses bind to specific receptors on the host cell surface, followed by internalization by receptor-mediated endocytosis. Low pH-induced membrane fusion in endosomes leads to the release of the nucleocapsids from the viral envelope into the cytosol. The ribonucleoprotein complex disassembles, thereby releasing the viral RNA. The viral proteins are synthesized by direct translation of the positive-stranded genome. The flavivirus genome is transcribed into a minus-strand that serves as template for the synthesis of positive-stranded RNAs. After packaging of the viral genome into capsids, virus assembly occurs by budding of the nucleocapsid into the lumen of the ER. During vesicular transport of the immature virions along the compartments of the secretory pathway, the viral envelope proteins are modified, followed by the secretion of fully mature virus particles from the host cell.
1.3.4 Structure of the Virions

Flaviviruses are spherical particles, with a diameter of 40 to 60 nm, consisting of an isometric nucleocapsid surrounded by a lipid bilayer. Mature flaviviruses contain three proteins: a capsid protein (C), and two integral membrane proteins designated M (membrane) and E (envelope). As schematically depicted in Figure 5, E forms homodimers on the viral surface. Mature virions are composed of 6% RNA, 66% protein, 9% carbohydrate, and 17% lipid. Immature virions, which represent precursor forms of progeny virus in infected cells, contain heterodimers consisting of the E protein and a precursor form of the M protein, prM (157). The prM protein is cleaved by a cellular protease during transport of the virus via the secretory pathway, leading to mature virions containing M and E proteins on their surface.

![Immature Virion Mature Virion](Image)

**Figure 5 Mature and Immature Flaviviruses.** The envelope of immature virions consists of multiple copies of prM and E, which associate with each other to form heterodimers. PrM is cleaved by furin into a soluble pr fragment and the membrane-anchored M shortly before release from the host cell. Mature virions contain E and M proteins on their surface. E is present as homodimeric complexes, while the arrangement of the M protein is not known. (adapted from (63), with permission)

1.3.5 Binding and Entry

Many attempts have been made to identify putative cellular receptors for flaviviruses, but our understanding of flavivirus binding and entry is still far from complete. Studies with tissue culture systems have revealed that binding of flaviviruses to host cells may depend on glycosaminoglycans (GAGs). Cell-surface expression of the proteoglycan heparan sulfate (HS) is required for efficient infection of mammalian cells with Dengue virus (21, 75). It has been proposed that the affinity of the viral surface for HS may be an important determinant of tissue tropism and pathogenicity (73). However, the use of laboratory-passaged virus strains may also reflect the adaptation of the virus to the surface properties of a particular host cell line, as
reported for TBE virus. Several proteins on the surface of various host cells were reported to represent candidate flavivirus receptors (12, 105, 131), but none of them have been characterized in detail. An additional mechanism of flavivirus attachment may involve cells expressing Fc receptors on surface. These confer increased binding of viruses under subneutralizing concentrations of flavivirus-reactive antibodies (59).

Flaviviruses enter cells by receptor-mediated endocytosis (50, 79). After binding, virions and virion aggregates are loaded into clathrin-coated pits, followed by uptake of virus particles into coated vesicles. Later on, viruses are found in uncoated lysosomal vesicles, where an acid-induced fusion of the viral and the host-cellular membrane occurs, thereby releasing the nucleocapsid into the cytoplasm (51). Subsequently, the nucleocapsid is thought to be disassembled, followed by translation of genomic RNA and initiation of RNA replication. However, these processes have not been studied in detail for flaviviruses.

1.3.6 Flavivirus Genome
The flavivirus genome comprises a single-stranded RNA of about 11 kilobases (kb) (reviewed in (17)). The RNA is capped at its 5' end and lacks a polyadenylate tail at its 3' end. The genomic RNA has a single long open reading frame (ORF), which serves as template for the translation of a large polyprotein containing three structural and seven non-structural proteins, as described below.

Noncoding regions of around 100 nucleotides and 400 to 700 nucleotides at its 5' and 3' end surround the single ORF, respectively. Conserved sequences and secondary structures within this region may be involved in replication and are likely to regulate the processes of genome amplification, translation, or packaging.

1.3.7 Translation and Polyprotein Processing
Translation of the flavivirus genome leads to the production of a large polyprotein precursor that is co- and posttranslationally cleaved into individual protein chains by host-cell and viral proteases. The structural proteins at the N-terminus include the capsid (C), the membrane protein (M), which is synthesized as a precursor (prM), and the envelope protein (E). The C-terminus of the polyprotein contains the nonstructural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5.
Internal signal sequences and stop-transfer sequences lead to the membrane topology of the flavivirus polyprotein as depicted in Figure 6 (reviewed in (94)). The C-terminus of the capsid is membrane-anchored by a hydrophobic domain that acts as a signal sequence for translocation of prM into the ER lumen. While the first transmembrane segment at the C-terminus of prM acts as a stop-transfer sequence, the second one serves as an internal signal sequence to direct E into the lumen of the ER. The C-terminus of E also contains a stop-transfer sequence followed by a signal sequence, leading to the translocation of NS1 into the ER lumen. NS2A is a transmembrane protein, while NS2B is synthesized into the cytoplasm. However, the protein is highly hydrophobic and may therefore be membrane-associated. NS3 is a cytoplasmic protein, while the C-terminus of NS4A acts as another internal signal sequence leading to the translocation of NS4B into the ER lumen. As in the case of the capsid protein, this C-terminal membrane anchor is removed from the protein by the viral serine protease. NS4B is a membrane-spanning protein followed by NS5, which is entirely cytoplasmic with no direct association with membranes.

Figure 6  Flavivirus Polyprotein and Membrane Topology. (A) The flavivirus genome consists of a positive-stranded RNA of ca. 10.5 kilobases in length with one single open reading frame leading to the production of a polyprotein containing three structural proteins (C, prM, and E) and seven nonstructural proteins (NS1 through NS5). (B) Start- and stop-transfer sequences lead to the membrane topology of the flavivirus polyprotein as depicted. The two envelope proteins prM and E as well as the nonstructural protein NS1 contain consensus sequences for the addition of N-linked oligosaccharides. Cleavages at the ER luminal side are occur by action of a host-cellular protease (black arrowheads), while the viral NS2B/3 protease mediates most cleavages within nonstructural region as well as the cleavage at the C-prM junction (open arrowheads). An as yet unknown protease cleaves between NS1 and NS2A. PrM is cleaved into pr and M by furin or a furin-like protease in the trans-Golgi network (arrow).
The cleavages within the polyprotein are achieved by action of both host proteases and the viral serine protease as shown in Figure 6. Processing at the NS1-NS2A junction occurs by an as yet unidentified host or viral protease in the ER lumen.

1.3.8 Features of the Individual Proteins

**C protein.** The C (capsid) protein (112 aa, MW 11 to 14 kDa) is a highly basic protein that forms a nucleocapsid with genomic RNA. Basic residues that are concentrated at the N- and C-termini of C specifically bind the RNA (83). The hydrophobic central domain of C interacts with cellular membranes, and may be involved in virion assembly (104). C contains a carboxyterminal membrane anchor that serves as a signal sequence for the prM protein (115). Cleavage of this transmembrane segment by the viral serine protease that generates mature C, is a prerequisite for the host signalase to cleave at the N-terminus of prM (7, 144). Therefore, coordinated processing occurs at the C-prM junction of the flavivirus polyprotein, which may have a role in regulation of the viral replication cycle.

**prM/M protein.** The M protein (78 aa, MW 7 to 8 kDa) is a small membrane protein synthesized as a glycosylated precursor, prM (168 aa, MW 25 kDa). PrM contains seven cysteine residues, six of which form three disulfide bonds. These are fully conserved among flaviviruses (17). Shortly before the immature virions are released from the cell, prM is cleaved by the cellular protease furin into the N-terminal soluble "pr" segment and the C-terminal structural protein M (141). M is present in mature virions, and contains a shortened ectodomain and two membrane-spanning segments. In immature virions, prM forms heterodimeric complexes with the major envelope protein E, and the protein has an important role in the transport of the immature virions to the cell surface (70).

**E protein.** The E protein (492 aa, MW 52 kDa) is a type I membrane protein containing 12 highly conserved cysteine residues that form six intramolecular disulfide bonds. E probably interacts with host-cell receptors, and mediates fusion with the host cell membrane. It also carries the major antigenic epitopes leading to a protective immune response.
Treatment of purified TBE virions with trypsin releases a dimeric, soluble fragment of E that contains the N-terminal 80% of E (aa 1-392), i.e. most of the ectodomain except the stem-anchor and the two transmembrane regions at its C-terminus (69). The three-dimensional structure of this fragment has been solved by X-ray crystallography at 2Å resolution (126). The data revealed that the molecular architecture of the flavivirus envelope was distinct from that of other viral glycoproteins for which the three-dimensional structure is known. In contrast to influenza hemagglutinin or human immunodeficiency virus (HIV) gp41, which form spikes projecting away from the virion (155, 159), the flavivirus E protein forms head-to-tail dimers that lie parallel to the viral membrane as shown in Figure 7.

![Figure 7 The E protein Dimer of Tick-Borne Encephalitis Virus.](image)

The overall dimensions of the dimer are ca. 150Å x 55 Å x 30 Å. Each subunit is divided into three structural domains: a central β-barrel (domain I), an elongated dimerization region (domain II), and an immunoglobulin-like module (domain III). All three domains contain mostly β-sheets with a single short α-helix present in domain II. The protein is stabilized by six disulfide bridges, distributed all over the molecule. The single carbohydrate side chain located in domain I may interact with domain II of the neighboring E molecule. The tip of domain II consists of a stretch of
hydrophobic amino acids (the "cd loop"; aa 98-110) that is highly conserved among flaviviruses. A recent study has revealed that this sequence probably corresponds to the fusion peptide of E that interacts with the host cell membrane and mediates fusion (3). Putative receptor-binding sites have been mapped to domain III (91).

Upon low-pH treatment, the E-E dimers on the viral surface dissociate and rearrange irreversibly and quantitatively into E-E-E homotrimers (4). This transition is rapid and involves the dissociation of the original dimer and the association of each E with two E molecules from two adjacent dimers. Upon trimerization, the fusion peptide in the cd loop of domain II is no longer buried by the glycan and domain I of its partner in the dimer, but probably becomes exposed on the viral surface. The E proteins now may form spike-like projections that drive the fusion of the viral with the host cell membrane. The fusion machinery of flaviviruses has, however, structural features that are distinct from the typical coiled-coils seen for influenza hemagglutinin, HIV gp41, and Ebola virus glycoproteins, i.e. so-called class I viral fusion proteins (154). Moreover, these proteins usually contain an N-terminal or an amino-proximal fusion peptide rather than an internal amino acid sequence for this purpose. Thus, the envelope proteins of flaviviruses have been designated class II viral fusion proteins (63).

Newly synthesized E protein associates with prM to form heterodimers. This interaction is a prerequisite for the proper folding and maturation of the E protein (85). E remains associated with prM during virion assembly and transport to the cell surface. The biological function of prM in these processes is probably to protect E from undergoing low pH-triggered conformational changes when transported across the acidic compartments of the secretory pathway.

Although the three-dimensional structure of the stem-anchor at the C-terminus of E is not known, sequence predictions suggested the presence of several important structural elements within this region (aa 393-492). It contains two putative short $\alpha$-helices flanking a highly conserved sequence, and two transmembrane segments constituting the C-terminal membrane anchor of E. Studies with C-terminal deletion mutants have revealed that the first of the two short $\alpha$-helices ($H1^{\text{pred}}$, aa 400-413) is necessary for the transition from dimers to trimers, while the second $\alpha$-helix ($H2^{\text{pred}}$,
Folding, Assembly and Secretion of TBE Virus Envelope Proteins

aa 430-449) is involved in the stabilization of the interactions between E and prM (6). The function of the highly conserved sequence element within the stem-anchor remains unknown, but the high degree of conservation implies an important role during the viral replication cycle. The first of the two transmembrane segments (TM1, aa 449-471) constitutes the actual membrane anchor of E, which further stabilizes the prM-E heterodimer interface (6). The second transmembrane element (TM2, aa 473-496) serves as a signal sequence for the nonstructural protein NS1 during flavivirus polyprotein synthesis. It probably has no further functional role in mature protein E. It may even slip out of the membrane after completion of translocation of NS1 into the ER lumen.

**NS1 protein.** The NS1 protein (351 aa, MW 46 kDa) is an ER-soluble glycoprotein containing 12 highly conserved cysteine residues, which form intramolecular disulfide bonds. NS1 forms homodimers and is secreted from mammalian cells in a hexameric form consisting of three dimers (42, 161). One of the N-linked glycans on NS1 gets modified into a complex-type sugar, while the other remains of the high-mannose type.

The biological function of NS1 is not known in detail, although it has been implicated in various processes during the viral life cycle. The protein was first characterized as the soluble (non-virion-associated) complement fixing (SCF) antigen that strongly elicits humoral responses, and immunization with purified or recombinant NS1 can be protective. Other reports have suggested a role for NS1 in the process of RNA replication (98). However, this is difficult to rationalize given its extracytosolic location.

**NS2A protein.** NS2A (229 aa, MW 22 kDa) is a type I membrane protein that is cleaved off from NS1 by an as yet unknown protease in the ER lumen. NS2A is thought to have a role in the recruitment of RNA templates to the membrane-bound replicase complex (99).

**NS2B protein.** NS2B (130 aa, MW 14 kDa) is a membrane-associated protein containing a conserved hydrophilic region flanked by two hydrophobic domains. The protein forms a complex with NS3, thereby serving as a cofactor for the serine
protease function of NS3 (16). The central conserved domain of NS2B mediates this interaction.

**NS3 protein.** NS3 is a large cytoplasmic protein (621 aa, MW 70 kDa) that associates with membranes through its interaction with NS2B. It is a bifunctional protein, containing both protease and nucleotide triphosphatase/helicase activities (19, 156). The N-terminal portion of the protein shows homology to trypsin-like serine proteases, containing the corresponding amino acid residues for the catalytic triad and the substrate-binding pocket. The solution of the three-dimensional structure of the NS3 protease domain by X-ray crystallography confirmed that the protein exhibits structural similarities to other viral and cellular serine proteases (114).

As mentioned above, the NS2B-3 protease mediates the majority of the cleavages in the non-structural region of the viral polyprotein as well as the cleavage that liberates the capsid from its C-terminal membrane anchor. Comparative analyses of these cleavage sites within the flaviviruses revealed that the NS2B-3 protease preferentially cleaves after pairs of basic residues, which lie before an amino acid with a small side chain (93).

The C-terminal two thirds of NS3 show homology to RNA helicases that utilize the energy of nucleoside triphosphate (NTPs) hydrolysis for unwinding RNA (80). Therefore, the protein may dissociate nascent RNA strands from their template RNA during RNA replication, or it may help to unwind secondary structures that are formed for template recognition or replication initiation. NS3 also has an RNA triphosphatase activity, which may be involved in the modification of the 5' end of the genome for addition of a 5' cap structure.

**NS4A protein** NS4A is a small hydrophobic protein (149 aa, MW 16 kDa) that interacts with NS1. This membrane anchor at the C-terminus of the protein is cleaved off from by the viral NS2B-3 protease, but the protein probably remains membrane-associated. NS4A may have a role in RNA replication, by anchoring components of the replicase complex to cellular membranes (99).

**NS4B protein** NS4B is a hydrophobic protein (252 aa, 27 kDa), which associates with cellular membranes. Its N-terminus is synthesized into the lumen of the ER, but
the correct membrane topology remains unclear. The protein was reported to translocate into the nucleus, and it may be involved in viral RNA replication (158).

**NS5 protein** NS5 (902 aa, MW 103 kDa) is the largest and most highly conserved of the flavivirus proteins. It shares sequence homologies to RNA-dependent RNA polymerases of other positive-stranded RNA viruses, and it contains the Gly-Asp-Asp motif, which is common among these enzymes (127). NS5 locates to the cytoplasm, but it is possibly recruited to replicase complexes at cellular membranes by one of the small membrane-associated viral proteins (NS2A, NS4A, or NS4B) to exert its function as a viral RNA polymerase (94). The protein also shows homology to methyltransferases involved in RNA cap formation (87). Moreover, NS5 can be phosphorylated by an unknown cellular serine/threonine kinase, a process that may regulate the interaction of NS5 with NS3, or redistribute the protein to the nucleus (82).

1.3.9 Virus Assembly and Transport

Flavivirus assembly occurs intracellularly. Ultrastructural studies of flavivirus-infected cells have revealed morphologically mature virions in the lumen of the rough ER (reviewed in (94)). Nascent virions have also been shown to accumulate within smooth membrane-bound vesicles. This is consistent with a dramatic proliferation of intracellular membranes upon flavivirus infection. However, budding intermediates or nucleocapsids without a lipid bilayer were not frequently detected, suggesting that the assembly process must be rapid.

Current ultrastructural and biochemical data suggest the following model for flavivirus assembly (reviewed in (94)): The highly basic C protein binds the viral genomic RNA in the cytoplasm to form a nucleocapsid. This structure acquires a lipid bilayer containing prM and E by budding into the ER lumen. The immature virions are transported in vesicles across the secretory pathway. Further modifications of the envelope proteins occur during this process. The carbohydrate side chains are modified into complex-type sugars by enzymes in the medial Golgi, and maturation cleavage of prM is mediated in the trans-Golgi network (TGN) by furin, followed by secretion of mature virions from the host cell.
1.3.10 Recombinant Subviral Particles

Recombinant expression of the flavivirus prM and E proteins in the absence of the C protein leads to the formation and secretion of a capsidless, membrane-bound non-infectious virus-like particles. No capsid or genomic viral RNA is needed for the formation of these so-called recombinant subviral particles (RSPs). They have been described for a variety of flaviviruses, including tick-borne encephalitis, dengue, yellow fever, and Japanese encephalitis viruses (5, 107, 122, 125). A detailed characterization of TBE virus RSPs has revealed that they are smaller than virions (30 nm vs. 50 nm in diameter), but the structural and functional properties of their envelope proteins are very similar to those in the virion envelope (133). The envelope proteins of RSPs exhibit a native antigenic and oligomeric structure compared with virions, and they are able to undergo the same structural changes as virions upon exposure to low pH, including the irreversible quantitative rearrangement of dimers into trimers. Moreover, the RSPs are able to induce fusion with a host cell membrane with almost identical kinetics to viruses (25).

Due to their non-infectivity, their particulate nature, and native antigenic structure, flavivirus RSPs are excellent candidates for the development of vaccines. Their immunogenicity has been shown for several flaviviruses including TBE virus (64), Japanese encephalitis virus (86), and yellow fever virus (122).

Recently, the structure of TBE virus RSP was analyzed by cryo-electron microscopy (cryo-EM) and icosahedral reconstruction (40). Fitting the high-resolution structure of the E protein ectodomain into the RSP density revealed that 60 copies of E were packed in a T=1 icosahedral surface lattice with an outer diameter of 315Å (Figure 8A). The model displays 2-fold symmetry axes at the dimer interfaces, 3-fold symmetry in the middle of three dimers, and 5-fold axes at the distal ends of each dimer. Given that the overall structure of a TBE virion is similar to the one seen for RSPs, but has a diameter of ca. 500Å, the structure of a virion was modeled with a T=3 icosahedral lattice consisting of 180 copies of the E protein (Figure 8B).
A closer view of the arrangement of E at the dimeric and trimeric interface reveals some more details about the interactions between the proteins (Figure 9). Beside the dimer contacts that have already been characterized when the three-dimensional structure of E was solved (126), there are trimer contacts in which domain III or the C-terminal stem anchor of one E molecule interacts with domain II of a neighboring E protein from another E-E dimer. Upon low-pH activation, domain II may undergo a hinge-like shift outward, thereby projecting away from the viral surface. This outward movement of domain II probably exposes the fusion peptide at the tip of domain II, which then can bind to the host-cell membrane and trigger fusion.
Two distinct transmembrane densities were found in the cryo-EM reconstruction of the TBE virus RSPs (purple spots in Figure 9). The one at the 5-fold axis could clearly be assigned to the transmembrane domain of the E protein. Another transmembrane region was detected in the middle of an E trimer, which is likely to represent the membrane anchor of the M protein. The presence of the precursor protein prM at this position would restrict the lateral freedom of E. This is consistent with the current model that prM locks E in its dimeric form and prevents the protein from undergoing low pH-triggered rearrangement into trimers. After prM cleavage in the TGN, acidic pH would loosen the dimeric contacts leading to monomers, which in turn induces the strengthening the more stable trimeric interactions. Thus, the structural model further supports the biochemical data on the structural changes of the flavivirus envelope (142, 143).
1.3.11 Experimental Systems

Flaviviruses can be replicated in whole animals, e.g. suckling mouse brain, chick embryos, and mosquitoes, as well as in primary or established mammalian, avian, or insect cell lines. Infection of vertebrate cells is usually cytopathic, although chronic infection of certain cell types is possible. Even during the peak of virus infection, host protein synthesis is not inhibited. Infection of mosquito cells is often non-cytopathic, leading to persistent infection and production of extremely high levels of infectious virus particles in the salivary glands.

Several recombinant expression systems were applied to examine the function of flavivirus proteins. In the case of TBE virus, a Simian Virus (SV) 40-derived vector was used to express different portions of the TBE virus envelope proteins. Plasmids included constructs coding for prM or E individually, for prM and full-length E, or for prM and C-terminally truncated variants of E lacking one or two transmembrane segments (2, 6). A large panel of poly- and monoclonal antibodies was raised against various domains and regions of the E protein, which were used as tools for studies on the antigenic and structural properties of TBE virus envelope proteins (55).

1.4 Goals of this Project

In the first part of the project, the folding and maturation of the TBE virus envelope proteins prM and E in living cells was studied. This involved the kinetics of folding, including disulfide bond formation, resistance against reducing agents and acquisition of conformational epitopes. Moreover, the heterodimerization between prM and E was analyzed, as well as the dependence of each other to reach a native structure. The effect of glycosylation on the maturation of prM and E was assessed, and possible binding to ER chaperones, which may promote the folding of the two proteins, was investigated.

The second part focused on the assembly, transport and secretion of TBE virus envelope proteins. The intracellular localization and the site of incorporation of prM and E into immature virions were assessed, as well as the molecular requirements and mechanisms that drive virus budding at the membrane of the ER.
1.5 Experimental Strategy

To analyze the folding, assembly and secretion of TBE virus envelope proteins, the SV40-based vector system described above containing the genes coding for prM or E, or for a polyprotein consisting of prM and full-length E, or for prM and C-terminally truncated E was used. COS-1 cells were transiently transfected with these vectors followed by various assays such as in vivo pulse-chase experiments, indirect immunofluorescence, and electron microscopy. Details on the plasmid constructs as well as on the experimental approaches are explained in the materials and methods sections of papers I and II.

The entire project was done in close collaboration with the group of Prof. Franz Heinz from the Institute of Virology, University of Vienna, Austria. He and his collaborators provided us with the plasmid expression system described above and a panel of poly- and monoclonal antibodies against prM and E.

To verify whether the results obtained with the recombinant expression system did indeed reflect the real processes after virus infection, a series of experiments was carried out by infecting mammalian cells with TBE virus. This work was done at the Institute of Virology in Vienna, Austria, which has the laboratory facilities and the experience required to do experiments with this highly pathogenic biosafety level 3 virus.
2. Folding and Dimerization of Tick-Borne Encephalitis Virus Envelope Proteins prM and E in the Endoplasmic Reticulum

(Paper I)

Ivo C. Lorenz\textsuperscript{1}, Steven L. Allison\textsuperscript{2},
Franz X. Heinz\textsuperscript{2} and Ari Helenius\textsuperscript{1}

\textsuperscript{1}Institute of Biochemistry, Swiss Federal Institute of Technology, CH-8093 Zürich, Switzerland
\textsuperscript{2}Institute of Virology, University of Vienna, A-1095 Vienna, Austria
Abstract

Flavivirus envelope proteins are synthesized as part of large polyproteins that are co- and posttranslationally cleaved into their individual chains. To investigate whether the interaction of neighboring proteins within the precursor protein is required to ensure proper maturation of the individual components, we have analyzed the folding of the flavivirus tick-borne encephalitis (TBE) virus envelope glycoproteins prM and E using a recombinant plasmid expression system as well as virus-infected cells. When expressed in their polyprotein context, prM and E acquired their native folded structure with a t<sub>1/2</sub> of approximately 4 min for prM and about 15 min for E. They formed heterodimeric complexes within a few minutes after synthesis that were required for the final folding of E but not prM. Heterodimers could also be formed in trans when these proteins were coexpressed from separate constructs. When expressed without prM, E could form disulfide bonds, but it did not express a specific conformational epitope, and it remained sensitive to reduction by dithiothreitol. This is consistent with a chaperone-like role for prM in the folding of E. PrM was able to acquire its native folded structure without coexpression of E, but signal sequence cleavage at the N-terminus was delayed. Our results show that prM is an especially rapidly folding viral glycoprotein, that polyprotein cleavage and folding of the TBE virus envelope proteins occurs in a coordinated sequence of processing steps, and that proper and efficient maturation of prM and E can only be achieved by cosynthesis of these two proteins.
Introduction

Flaviviruses belong to those virus genera that encode large polyprotein precursors from which the individual proteins are proteolytically cleaved. Some of the cleavages occur co- or posttranslationally, while others take place later during transit through the secretory pathway. Internal signal sequences and signal peptidase cleavage sites ensure the correct topology of the polyprotein and its cleaved products at the membrane of the endoplasmic reticulum (ER). The use of polyproteins for the generation of structural and non-structural proteins is dictated (in part) by viral replication strategies that do not allow generation of multiple mRNAs, and by the limited size of the genome. Moreover, in the case of low pH-activated viral fusion proteins, proteolysis is often needed to render the proteins fusion competent once they have passed the acidic compartments of the late secretory pathway. The individual glycopolypeptide chains are normally found as subunits of multimeric complexes in which they are closely associated with each other.

From a folding point of view, the early cleavages are especially interesting because they usually occur cotranslationally before folding of the individual proteins has been completed. This means that the individual polypeptide chains have the opportunity to undergo folding as part of a larger complex of polypeptide chains derived from the same ribosome. The folding of the two envelope glycoproteins of Semliki Forest virus (p62 and E1), although dependent on different sets of molecular chaperones, seems, for example, to be intimately coordinated (9). Similarly, the folding of Hepatitis C virus envelope protein E1 appears to be dependent on the second envelope protein, E2 (34, 109). Also, in the case of Uukuniemi virus, a bunyavirus, the two envelope proteins G1 and G2 fold together, although at very different rates (120).

In this study, we have examined the folding of the prM and E envelope proteins of tick-borne encephalitis (TBE) virus, two glycoproteins that follow each other in the polyprotein sequence. TBE virus is a member of the Flavivirus genus of the Flaviviridae, a family of small enveloped RNA viruses that include important arthropod-borne human pathogens such as yellow fever, West Nile, dengue, and Japanese encephalitis viruses. Their positive-stranded RNA genome (ca. 11 kbases) contains a single long open reading frame encoding three structural proteins (capsid
(C), prM/M, and E) and at least seven nonstructural proteins (reviewed in (17)). The polyprotein is co- and posttranslationally cleaved into distinct protein products by host and virus-encoded proteases (94).

The envelope of mature TBE virus contains the major envelope glycoprotein E (MW 52 kDa, one N-linked glycan) and the small membrane protein M (MW 7 to 8 kDa). The E protein mediates virus entry into the cell through receptor binding and low pH-induced fusion in endosomes after receptor-mediated endocytosis, and it also carries the major antigenic epitopes leading to a protective immune response (reviewed in (68)). M is synthesized as a precursor protein, prM (MW 25 kDa, one N-linked glycan), that forms a 1:1 complex with E intracellularly and probably serves as a 'lock' to prevent E from being prematurely activated by low pH during transport through the acidic compartments and vesicles of the trans-Golgi network (54, 56). Shortly before the virus is released from the cell, prM is cleaved, apparently by the cellular protease furin, leading to mature virions (141). Since TBE virus, like most flaviviruses, probably buds into the ER lumen, the envelope glycoproteins are transported through the Golgi complex to the plasma membrane as part of immature virus particles (94).

The solution of the X-ray crystal structure of the E ectodomain purified from mature virions (126) revealed that protein E differs from many other well-characterized viral glycoproteins in that it forms head-to-tail dimers that lie parallel rather than perpendicular to the viral membrane. E is a type I membrane protein with two transmembrane segments at its C-terminus. The ectodomain of the TBE virus E protein is composed of three major parts: a central domain (domain I), a dimerization domain (domain II), and a C-terminal immunoglobulin-like domain (domain III). All three domains are rich in ß-sheets with two short α-helices in the dimerization domain. There are 12 cysteines forming six intrachain disulfide bonds, and the single glycosylation site is located at the dimerization interface. That this type of overall fold is not unique to flaviviruses was recently demonstrated by the X-ray structure of the E1 protein of Semliki Forest virus, an alphavirus (92). PrM is also a type I membrane protein. It contains six cysteines in the ectodomain, forming three disulfide bonds (116).
A few years ago, a recombinant Simian Virus 40-based plasmid vector system was developed to express the TBE virus prM and E proteins as well as C-terminally truncated variants of E (2, 6). This system was used here to analyze the folding of E, as well as prM when expressed individually, in combination with each other, and in cells infected with TBE virus. We found that the maturation of TBE virus envelope proteins prM and E is coordinated and that early interactions between the proteins are needed for complete folding of E but not prM, suggesting that prM serves as a chaperone for the folding of E.
Materials and Methods

Plasmids and virus. The production of recombinant plasmids expressing prM and full-length or truncated forms of E derived from TBE virus strain Neudoerfl (GenBank accession no. U27495) was described earlier (2, 6). In particular, we used vectors expressing prM and the full-length E (SV-PEwt), prM and E truncated after the first transmembrane domain (SV-PE472, i.e. the first 472 amino acids of E), prM and E truncated right after the ectodomain, lacking the stem-anchor elements (SV-PE400), prM alone (SV-prM), or full-length E alone (SV-Ewt). The vector included an SV40 early promoter and an SV40 origin of replication for amplification in COS-1 cells.

TBE virus strain Neudoerfl (102) was grown in primary chicken embryo cells and purified by two cycles of sucrose density gradient centrifugation as described by Heinz and Kunz (67).

Infection of mammalian cells with TBE virus. COS-1 cells (ATCC CRL 1659) were grown in Dulbecco’s Modified Eagle Medium (DMEM, Life Technologies) supplemented with 10% Fetal Calf Serum (FCS), 4 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in 5% CO₂. At 60 to 70% confluency, cells were infected with TBE virus at an m.o.i. of ca. 1 in DMEM containing 2% FCS, glutamine, and antibiotics. The virus-containing medium was replaced by fresh medium 1h after infection. 24 h post-infection, the cells were used for further experiments.

Expression of TBE virus envelope proteins in mammalian cells. COS-1 cells were transiently transfected with plasmid DNA complexed to LipofectAMINE (Life Technologies) in DMEM containing no serum or antibiotics. 5 h after transfection, an equal amount of DMEM supplemented with 20% FCS, 8 mM glutamine 200 U/ml penicillin and 200 µg/ml streptomycin was added to the cells without removing the medium containing the DNA-lipid complexes. 24 h post-transfection, the cells reached an optimal expression level of the incorporated DNA and were used for pulse-chase experiments.

Pulse-Chase Analysis of TBE virus envelope protein folding. Subconfluent 60 mm dishes of COS-1 cells expressing TBE virus envelope proteins were washed once with PBS containing 1 mM Mg²⁺ and 0.5 mM Ca²⁺ (PBS⁺⁺), followed by starvation in
Cys/Met-free medium for 30 min. The cells were pulse-labeled at 37°C by adding 200 μCi of \(^{35}\)S-Met/Cys (ProMix, Amersham) in cysteine/methionine-free medium supplemented with 20 mM HEPES pH 7.4. The pulse was stopped by washing the cells twice with 5 mM unlabeled cysteine/methionine in DMEM containing 10% FCS, glutamine, antibiotics and 20 mM HEPES pH 7.4, followed by incubation with this medium at 37°C for chase times varying between 0 and 60 min. For the dithiothreitol (DTT) resistance experiments, the regular chase was followed by a 4 min incubation at 37°C in a chase medium supplemented with 5 mM DTT. DTT washout experiments were carried out in a similar way, using pulse and chase media containing 5 mM DTT followed by incubation in normal chase medium. After chase, the cells were flooded with ice-cold PBS++ containing 20 mM N-ethylmaleimide (NEM), an alkylating reagent that prevents post-lysis oxidation effects on the newly synthesized proteins (14). After 2 min incubation, the cells were lysed in 800 μl of ice-cold lysis buffer (2% CHAPS in HEPES-buffered saline (HBS, pH 7.4) supplemented with 20 mM NEM, 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml each of chymostatin, leupeptin, antipain, and pepstatin). The nuclei and cellular debris were pelleted by centrifugation, and the post-nuclear supernatants were used for immunoprecipitation.

Lysis of cells for sedimentation analysis was carried out in 1% Triton X-100 in 50 mM triethanolamine pH 8.0 containing 20 mM NEM and protease inhibitors followed by removal of nuclei and cellular debris as described above.

**Immunoprecipitation and SDS-PAGE.** The post-nuclear supernatants were precleared on protein A coupled to sepharose CL-4B beads for 1 to 2 hours at 4°C. 60 to 120 μl of the precleared lysates were subsequently incubated with protein A-Sepharose CL-4B beads (10 μl bead volume) and 1 μl of rabbit polyclonal or mouse monoclonal antibodies directed against the prM or E proteins at 4°C overnight. Immune complexes were pelleted at 8500 x g for 2 min, followed by washing with agitation three times for 10 min each. The first and second washing step were done with 0.5% CHAPS in HBS (pH 7.4), the third one with HBS (pH 7.4). The washed immune complexes were solubilized by adding 40 μl sample buffer and incubated at 95°C for 5 min. After pelleting of the protein A-Sepharose beads the supernatant was divided in two aliquots. For reducing conditions, DTT was added to one of the
aliquots to a final concentration of 100 mM. The proteins were analyzed by 7.5% and 12% SDS-PAGE on minigels followed by autoradiography.

**Antibodies.** For the majority of the precipitations, a polyclonal antiserum (pAb) recognizing both prM and E was used (k-PM(2), (5)). Mouse monoclonal antibodies (mAb) specific for E (A5, B1, C6 (55)), prM (8H1, (78)) or NS1 (LGT 3-509, (78)) were used to detect various epitopes on the proteins and for co-precipitation experiments.

**Determination of half-times for folding.** The half-times for folding of prM, E, and NS1 were determined by densitometric analysis of the protein bands from SDS gels after immunoprecipitation with conformation-specific antibodies (monoclonal antibodies 8H1, A5, and LGT 3-509; see above) from at least three independent experiments. The band intensities (arbitrary units) were plotted against the chase time for each protein. The t₁/₂ value represents the time point at which 50% of the maximal intensity was achieved.

**Sedimentation analysis.** To monitor the heterodimer formation between prM and E, post-nuclear supernatants from cell lysates solubilized in 1% Triton X-100 were loaded on 5 to 20% (w/w) sucrose gradients made with 50 mM triethanolamine (pH 8.0), 100 mM NaCl, and 0.5% Triton X-100. After ultracentrifugation in a Beckman SW41 rotor at 38,000 rpm at 15°C for 22 h, fractions of 800 μl were collected by upward displacement and immunoprecipitated with a polyclonal antiserum recognizing both prM and E as described above. The immune complexes were analyzed by reducing SDS-PAGE followed by autoradiography.

**Endoglycosidase H₉ digestion.** TBE virus envelope proteins were immunoprecipitated and washed three times as described above. The immune complexes were resuspended in 0.5% SDS and 1% β-mercaptoethanol and heated for 10 min at 95°C. After pelleting the protein A-Sepharose beads, the supernatant was divided in two equal aliquots. 50 mM sodium citrate (pH 5.5) was added to both samples, plus 20 units of endoglycosidase H₉ (New England Biolabs) to one of them. The samples were incubated at 37°C for 90 min, followed by addition of reducing sample buffer, separation of the proteins on 12% SDS-PAGE and autoradiography.
Results

Figure 1A summarizes the biogenesis of the TBE virus envelope proteins, from the polyprotein being cleaved into distinct protein chains (pictures 1 and 2), heterodimerization of prM and E (picture 3), and the final processing steps leading to mature virions (picture 4).

A schematic overview of the plasmid constructs used is shown in Figure 1B. Both SV-PEwt and SV-Ewt contained the first 30 codons of the NS1 nonstructural protein, which lies downstream of the E sequence in the polyprotein precursor. These nucleotides were included in the constructs to ensure proper translocation and membrane insertion at the C-terminus of E.

**Figure 1** A) Schematic diagram of the polyprotein cleavage, folding, and maturation of TBE virus envelope proteins prM and E and the first of the nonstructural proteins, NS1. The most N-terminal of the proteins, the capsid protein, is cytosolic and is cleaved by the viral NS2B/3 protease (arrow). It is followed by a signal sequence cleaved by signal peptidase on the lumenal side (filled arrowhead). The signal peptidase also cleaves prM from the E protein and the E protein from the NS1 protein, leading to the translation products shown in picture 2. PrM and E fold in the ER and associate with each other forming heterodimers in immature virions (picture 3). Cleavage of the N-terminal portion of the prM protein by the cellular protease furin (open arrowhead) after transport to the trans-Golgi network leads to mature virions and formation of E-E dimers on the virus surface (picture 4). B) Overview of the plasmids coding for various parts of the TBE virus polyprotein. The two transmembrane segments of the E protein are shown as white boxes. A few amino acids from the carboxyterminus of the capsid protein and the internal signal sequence (S) for proper translocation into the ER lumen were included at the N-terminus of each construct. SV-PE472 and SV-PE400 lack one or both transmembrane regions at the C-terminus of E, respectively. ER, endoplasmic reticulum; Cyt, cytosol; N, N-terminus, C, C-terminus.
**Folding of TBE virus envelope proteins in infected cells.** To study the folding of TBE virus envelope proteins prM and E when expressed as part of the intact viral polyprotein, COS-1 cells infected with TBE virus were analyzed by a pulse-chase approach. Twenty-four hours after infection the cells were pulse labeled for 2 min with $^{35}$S-methionine/cysteine and chased for various times up to 60 min. Before lysis with 2% CHAPS, the cells were treated with 20 mM N-ethylmaleimide to alkylate any remaining free sulfhydryl groups, thus preventing further oxidative folding (14). The viral proteins were immunoprecipitated from post-nuclear supernatants using a polyclonal antiserum (k-PM(2)) that recognizes prM, E, and NS1, or using monoclonal antibodies specific for these proteins individually. The immunoprecipitates were analyzed by SDS-PAGE under reducing and non-reducing conditions, and the bands visualized by autoradiography.

As shown in Figure 2A, three major virus-derived bands could be detected in the reduced samples; prM, E, and NS1, all of which migrated with the expected mobilities (25 kDa, 52 kDa and 45 kDa, respectively). The identification of the E and NS1 bands was confirmed by immunoprecipitation using specific mouse monoclonal antibodies (Figure 2B). In addition, a band at 45 kDa was seen, probably corresponding to labeled actin, which is often seen as a nonspecific contaminant in immunoprecipitations. E and prM were clearly visible after 4 min chase (lanes 2 and 8), and NS1 after 16 min (lanes 4 and 10). Since no major bands of lower mobility were observed, we concluded that all four cleavages needed to generate prM, E, and NS1 occurred rapidly, perhaps even cotranslationally. Furthermore, no cross-linked aggregated forms were detected at the top of the non-reducing lanes.

Analysis of the more weakly labeled bands in the lower part of the gel in Figure 2A revealed that the cleavages and oxidative folding of prM were rapid. prM underwent a large mobility shift when reduced, suggesting that one or more of the three disulfide bonds in this protein form large loops. Since we were not able to detect any oxidation intermediates of prM even after overexposing the gels for autoradiography, these disulfide bonds must have been formed during the 2 min of pulse time.
The higher separation of the molecular weight region of E and NS1 obtained using a 7.5% gel (Figure 2B) revealed further details about the folding. First, it was clear that the non-reduced E band migrated faster than its reduced counterpart (e.g., lanes 3 and 9), consistent with formation of intrachain disulfide bonds. The mature E contains six disulfide bridges, which are located in each of the three domains (126). That their combined effect on the migration of the protein in SDS-PAGE was relatively minor (but consistently observed) is most likely due to the rather small size of the covalent loops that they form in the polypeptide chain. We also observed that the polyclonal antiserum reacted well with E already after 4 min of chase (Figure 2A, lane 2), whereas a monoclonal antibody against domain II of E (A5) yielded a strong band only after 30 min (Figure 2B, lanes 1 to 3). The intensity of the E band increased significantly between 16 and 30 min of chase, and less strongly between
30 and 60 min of chase (Figure 2B, lanes 1 to 3). Taken together, our results suggested that the half-time for formation of the conformational epitope in domain II of E recognized by the monoclonal antibody was about 15 to 20 min, and that oxidation preceded acquisition of the final native structure of the protein.

The NS1 protein was detected by a monoclonal antibody recognizing a conformational epitope after 16 min (Figure 2B, lane 4). No NS1 was precipitated from samples with shorter chase times (data not shown). A slight increase in the electrophoretic mobility of NS1 was observed between 16 and 60 min of chase in both the non-reducing and reducing gels (lanes 4 to 6 and 10 to 12, respectively). This small shift was probably due to glucose trimming on the carbohydrate side chains of NS1. Recognition of the protein by a monoclonal antibody started only after 16 min of chase (lane 4 and data not shown), and folding appeared to be complete after 30 min (lane 5), since no further increase in the amount of precipitated NS1 was observed after 60 min (lane 6).

Our results suggest that the oxidative folding of TBE virus envelope proteins prM and E as well as of the non-structural protein NS1 occurs rapidly. However, judging by the appearance of epitopes recognized by the monoclonal antibodies used against E and NS1, conformational alterations continue to occur in these proteins until 16 to 30 min of chase.

**Folding of recombinant prM and E expressed individually.** To examine the folding and maturation of prM or E alone, we used recombinant plasmids expressing various parts of the TBE virus polyprotein as schematically depicted in Figure 1B. They all lacked the capsid protein at the N-terminus, but two of them (SV-PEwt and SV-Ewt) contained a short 30 amino acid stretch of the NS1 protein (called NS1*). This short sequence of NS1* was included to ensure the correct topology of the C-terminal end of the full-length E protein.

The constructs were expressed in COS-1 cells using a transient expression system. A pulse-chase protocol similar to that employed above was used. When prM and E were expressed individually, relatively simple patterns were obtained (Figure 3A and B, upper panels). In the reduced gel, the E protein gave a single band (56 kDa) immediately after the pulse, labeled E-NS1* in Figure 3A. The E-NS1* band
disappeared with a half-time of 4 to 6 min and was replaced by a faster migrating band (52 kDa) corresponding to the mobility of mature wild-type E protein. Since the shift was not seen for the constructs SV-PE472 and SV-PE400, which produced C-terminally truncated forms of E and therefore also lacked the additional NS1 sequence (see below), we concluded that this shift was due to removal of the C-terminal NS1* sequence. Evidently, the signal peptidase-mediated cleavage was delayed compared to the corresponding cleavage in the full-length polyprotein (Figure 2A). As described below, this delay was similar whether prM was expressed together with E or not.

Figure 3 Folding of recombinant TBE virus envelope proteins expressed individually. COS-1 cells were transfected with plasmids encoding either E (A) or prM (B). A schematic diagram of the topology of the constructs at the ER membrane is shown at the top of the Figures. 24 h post-transfection, the cells were pulse-labeled with 35S-Met/Cys for 2 min and chased for 0 to 16 min. The post-nuclear supernatants were immunoprecipitated either with a polyclonal antiserum recognizing both prM and E (upper panel) or with monoclonal antibodies against prM or E (lower panels). An immunoprecipitation of E with a monoclonal anti-prM antibody and vice versa (lower panels, lane 13) confirmed that the antibodies were specific. The proteins were separated by non-reducing and reducing SDS-PAGE and visualized as described above. Positions of the individual proteins are marked at the side, molecular weight standards are indicated on the right.

Under non-reducing conditions, only a single band was observed in the SDS-PAGE. We therefore believe that this band represents the E protein and that the majority of the E-NS1* was present in the large disulfide-linked aggregates that remained at the top of the gel (Fig. 3A, lanes 1 to 3). If this is indeed the case, the gradual increase
in electrophoretic mobility of the putative E band that occurs in the first 8 min of chase can be attributed to oxidative folding.

A difference was also observed for prM expressed separately compared to the full-length polyprotein in infected cells. As shown in Figure 3B, a shift from a 29 kDa to a 26 kDa form could be observed under reducing conditions, with the latter corresponding to the authentic molecular weight found in wild-type prM. Given the construct, this shift was clearly due to delayed cleavage at the N-terminus of prM, which consists of a few C-terminal residues of the capsid protein and the internal signal sequence for prM. The half-time of this cleavage was about 4 to 8 min. Interestingly, the delayed cleavage was not seen when the prM sequence was followed by E (see Figure 4, below), indicating that the timing of this cleavage depended on the downstream sequences.

We have also analyzed the prM and E proteins expressed individually by immunoprecipitation with anti-prM or anti-E mouse monoclonal antibodies. Although recognized by monoclonal antibodies against domains I and III (B1 and C6, respectively; data not shown), E synthesized alone was hardly precipitated by the monoclonal antibody against domain II (A5). Only faint bands were visible after 30 to 60 min chase (Figure 3A, lower panel). This was a clear difference compared to E expressed as part of the full-length polyprotein (Figure 2B), suggesting that E is not able to acquire its fully mature form when synthesized alone. On the other hand, prM was precipitated using a monoclonal anti-prM antibody, indicating that at least by this criterion prM was able to fold independently of other viral components even though the signal sequence cleavage at its N-terminus was delayed.

Thus, the two TBE virus envelope proteins matured to different extents when produced outside of their usual context within the polyprotein. The E protein expressed alone could not acquire its final conformation, while prM synthesized individually appeared to be completely folded, but proteolytic cleavage at its N-terminus was delayed.

**Folding of prM and E expressed from the same polyprotein precursor.** To test the cleavage processes and folding of prM and E when they were expressed together, but without other viral proteins, we performed pulse-chase experiments
using the SV-PEwt plasmid (Figure 1B), which encodes a polyprotein consisting of a few amino acid residues from the C-terminus of the capsid, followed by the internal prM signal sequence, prM, E, and the NS1* 30 amino acid segment. Figure 4A shows the autoradiography of an immunoprecipitation with an antiseraum recognizing both prM and E. Both proteins oxidized rapidly and with the same kinetics as if expressed individually. Under reducing conditions, the bandshift from E-NS1* to E was observable again, and aggregates were again observed that the top of the lanes under non-reducing conditions. This, together with the experiment shown in Figure 3A, indicated that the cleavage of NS1* from the C-terminus of E occurred independently of prM.

PrM folded properly within 2 min after synthesis and displayed a clear shift between non-reducing and reducing conditions, as shown for prM expressed alone. However, there was no double band visible for prM, as was the case for prM expressed alone. This suggests that signal sequence cleavage at the N-terminus of prM is accelerated when the downstream E protein is present on the polyprotein. The transiently expressed band around 70 kDa detected under both non-reducing and reducing conditions between 0 and 16 min chase probably corresponded to non-cleaved prM-E precursor because it was precipitated both with antibodies to prM and E (Figures 4B and 4C, respectively). Small amounts of this oxidized precursor were seen for all constructs expressing prM and E together.
Figure 4  Folding of TBE virus envelope proteins expressed as a polyprotein precursor. COS-1 cells were transfected with a recombinant plasmid encoding prM and E. 24 h post-transfection, the folding of prM and E was assayed by a pulse-chase approach, followed by immunoprecipitation of the post-nuclear supernatants using a polyclonal antiserum recognizing both prM and E (A) or monoclonal antibodies specific for prM and E as indicated (B and C). The immunoprecipitates were analyzed by non-reducing and reducing SDS-PAGE. Positions of the proteins are marked at the side; molecular weight standards are indicated on the right.
Moreover, in contrast to the individually expressed E protein (Figure 3A, lower panel), E from the SV-PEwt construct was efficiently precipitated by the monoclonal antibody A5 when cosynthesized with prM (Figure 4B). This indicated that E was able to reach a conformation detectable by this antibody only when prM was present.

The half-times of folding for prM and E were determined by densitometric analysis of the bands shown in figs. 4C and 4B, respectively. We obtained t_{1/2} values of 3 to 4 min for prM, and ca. 15 min for E.

The interaction between prM and E involved formation of complexes, since the two proteins could be co-precipitated after 4 min of chase using either anti-E or anti-prM monoclonal antibodies (Figure 4B and 4C, respectively). A small fraction of E and E-NS1* was precipitated with the anti-prM antibody after 0 min chase (Figure 4C, lanes 1 and 7).

Our results indicated that when synthesized together prM and E begin to form stable complexes within a few minutes after synthesis and that this association is needed for E to acquire its final conformation. The presence of E also results in faster signal sequence cleavage at the N-terminus of prM.

**Folding of C-terminally truncated variants of E.** Earlier studies have revealed several functional elements the C-terminal stem-anchor region of the E protein (6), including a small helix involved in trimerization of E under acidic conditions, a conserved region of unknown function, and a second helix required for interaction with prM. To test whether the transmembrane domain at the C-terminus of E has an effect on its folding, we analyzed the folding of prM and C-terminally truncated variants of E lacking the second transmembrane segment (SV-PE472, i.e. prM and the first 472 amino acids of protein E) or both transmembrane segments and stem region (SV-PE400) of E in a pulse-chase experiment.
Figure 5 Folding of C-terminally truncated forms of TBE virus E proteins. COS-1 cells were transfected with recombinant plasmids encoding prM and a full-length E (SV-PEwt), prM, and E lacking the second transmembrane domain (SV-PE472) or prM and stem- and membrane anchor-free E (SV-PE400). A schematic view of the topology of the proteins at the ER membrane is depicted in (A). The degree of interaction of the E protein with prM is diminished when both transmembrane domains are deleted. Folding of the E variants and prM was assessed by a pulse-chase experiment followed by anti-prM/E immunoprecipitation as described above. For each construct, the proteins were resolved by 7.5% and 12% SDS-PAGE (upper and lower panels, respectively), followed by autoradiography (B, C, and D). A folding intermediate for E400 (Ei) can be seen under non-reducing conditions in (D). Positions of the proteins are marked at the side; molecular weight standards are indicated on the right.

Figure 5A depicts the predicted ER membrane topology of these proteins, and Figures 5B, C, and D show the SDS-PAGE of immunoprecipitates using an anti-prM/E polyclonal antiserum. To maximize the resolution for each of the proteins, 7.5% acrylamide SDS gels were used for E and 12% gels for prM. The shift from the 56 kDa to the 52 kDa band under reducing conditions was present only for the SV-PEwt construct, confirming as discussed above that this shift was indeed due to C-terminal cleavage of NS1* from E. Moreover, an additional faint band, migrating slightly slower than E, was detected under non-reducing conditions, probably corresponding to E-NS1*. E472 showed a folding pattern similar to that of Ewt (Figure 5C), but it acquired its fully oxidized state a little earlier than Ewt. E400 oxidized more rapidly.
with some completely oxidized protein form being visible after 0 to 1 min chase (Figure 5D) and also showed a higher degree of aggregation at the top of the non-reducing gel (data not shown). An additional transient band with a slightly lower mobility than the fully oxidized form of E400 was visible under non-reducing conditions (Figure 5D, lanes 1 to 5). This band probably corresponded to incompletely oxidized folding intermediates, which were further oxidized by 16 min chase. For all three constructs, some prM-E precursor was detected that became cleaved after 8 to 16 min chase. PrM displayed identical folding kinetics for all three constructs and was fully oxidized after 2 min pulse and 0 to 4 min chase. In a separate experiment, both E472 and E400 could be precipitated by the A5 mAb, indicating that E can fold without the stem-anchor region (data not shown). Taken together, the data suggest that truncations at the C-terminal end of E had a minor effect on its folding kinetics, and no influence on the folding of prM.

**Folding of prM and E expressed in trans.** The results shown so far suggested that the E protein needs prM to achieve its final native conformation. To test whether this effect depends on expression of prM and E on the same polyprotein, a pulse-chase experiment was carried out with COS-1 cells transfected with the SV-PEwt plasmid (expression of prM and E in cis), with SV-prM and SV-Ewt (expression in trans), or with SV-Ewt alone. The cells were pulsed for 2 min and chased for 60 min. Aliquots of the cellular extracts were immunoprecipitated with three different antibodies: a rabbit polyclonal anti-prM/E antiserum, a mouse monoclonal anti-E antibody, and a mouse monoclonal anti-prM antibody.

The autoradiogram of a reducing SDS-PAGE with the immunoprecipitates is shown in Figure 6. PrM and E were coprecipitated with both the monoclonal anti-E antibody when coexpressed either in cis (lanes 4 and 7) or in trans (lanes 5 and 8). This indicates that prM and E can form complexes whether synthesized on the same polyprotein or not.
**Figure 6** Folding of TBE virus envelope proteins expressed in trans. COS-1 cells transfected with a plasmid coding for prM and E (SV-PEwt) as a polyprotein (expression in cis; PE), or with plasmids encoding prM (SV-prM) and E (SV-Ewt) separately (expression in trans, P+E), or with a plasmid coding for E alone (SV-Ewt; E) were pulsed for 2 min and chased for 60 min. Total cellular extracts were immunoprecipitated using either a rabbit polyclonal anti-prM/E antiserum, a monoclonal anti-E antibody, or a mouse monoclonal anti-prM antibody as indicated above the lanes, followed by analysis of the proteins under reducing SDS-PAGE. Positions of the proteins are marked at the side, molecular weight standards are indicated on the right.

**DTT sensitivity of the E protein.** One parameter that has been used to assess the folding state of a protein is its resistance to reducing agents such as dithiothreitol (DTT) added to the medium (81, 145). When proteins are incompletely folded, their disulfide bonds are more exposed and therefore much more sensitive to such treatment than when the proteins have acquired their native conformation. Earlier studies on influenza hemagglutinin have shown that the subunits become resistant soon after reaching the fully oxidized state but before trimer formation (145, 146). We measured the DTT sensitivity of E expressed from SV-PEwt, SV-PE472, SV-PE400, or SV-Ewt after different times of chase. The fraction of each variant resistant to 5 mM DTT is plotted in Figure 7. When E was expressed from the SV-PE472 construct, 80% became resistant after 60 min of chase. By that time, 50% of full-length E (SV-PEwt) acquired DTT resistance. In contrast, only 15% of the membrane anchor-free form of E (SV-PE400) was resistant to reduction 60 min after synthesis. Interestingly, E expressed without prM (SV-Ewt) did not become resistant to DTT treatment at all. We conclude that prM was required for E to reach the DTT resistant conformation. This result is consistent with the observation described above that the final stages of E folding depend on association with prM.
Figure 7  DTT resistance of C-terminally truncated TBE virus E proteins. Cells were transfected with recombinant plasmids as indicated and subjected to a pulse-chase experiment with 2 min pulses and chases between 0 and 60 min. After chase in a normal non-reducing medium, the cells were chased in a DTT-containing medium for an additional 4 min. The ratio of DTT-resistant E protein vs. total E protein was plotted for the Ewt, E472, and E400 forms.

We also tested the effect of DTT on the folding of prM and E by a DTT washout pulse-chase experiment (data not shown). When DTT is washed out from cells, the oxidative environment in the ER is rapidly restored, and reduced proteins can resume their oxidative folding program (13). When DTT was washed out after 2 min pulse and 4 min chase, both prM and E resumed correct oxidation, but aggregation was strongly increased as seen by analysis of the proteins under non-reducing SDS-PAGE. Reuptake of the folding process combined with increased aggregation was also observed in DTT washout experiments for the SV-PE472 and SV-PE400 constructs (data not shown).

These results suggested that the proteins were able to fold posttranslationally upon removal of the reducing milieu, but this led to a decrease in folding efficiency as well as to a significantly higher degree of aggregation, probably due to the formation of intermolecular disulfide bonds.

**Heterodimer formation between the TBE virus envelope proteins.** Our results from co-immunoprecipitations of prM and E suggested that stable complexes between the two envelope proteins formed early during folding. To further characterize the prM-E heterodimer formation, we subjected the post-nuclear supernatants from a pulse-chase experiment to sucrose velocity gradient centrifugation in gradients containing 0.5% Triton X-100.
Figure 8 Cosedimentation analysis of prM and E at various chase times. Cells transfected with recombinant plasmids encoding prM and E wt (A to C) or prM and the membrane anchor-free E400 (D) were pulsed for 2 min and chased for the indicated times. The post-nuclear supernatants were subjected to velocity sedimentation on 5 to 20% (w/w) sucrose gradients. Subsequently, the proteins were immunoprecipitated from the gradient fractions using a polyclonal antiserum recognizing both prM and E, analyzed on reducing SDS-PAGE, and visualized by fluorography. The relative amount of prM and E protein from each fraction (out of a total of 100% over all 14 fractions) was quantitated. The arrows above fractions 4 to 6 in (C) indicate the S20 sedimentation coefficient standards, as determined by quantification of a velocity sedimentation gradient run with a protein molecular weight marker.

As shown in Figure 8A, the peaks of prM and E overlapped partially at 0 min chase. The prM peak was seen in fractions 5 and 6. The E protein peaked in fraction 6, but it was also detected in faster sedimenting fractions. This indicated that weak interactions between some prM and E molecules occurred, while a larger portion of E formed higher molecular weight clusters or aggregates. Consistent with the results obtained by co-immunoprecipitations (cf. Figures 4B and 4C), interaction between prM and E was further increased after 4 min of chase. (Figure 8B). After 60 min of chase, prM and E co-sedimented almost completely in fractions 5 and 6 at an
average sedimentation coefficient of 4.0, indicating that most of the newly synthesized prM and E molecules had dimerized (Figure 8C).

Earlier studies have shown that the membrane anchor-free E400 protein does not form a stable complex with the prM protein (5). We confirmed this result by analyzing the sedimentation behavior of E400 and prM (Figure 8D). In this case, the proteins peaked at different fractions, and E400 and prM showed average sedimentation coefficients of 3.6 and 2.8, respectively. Thus, prM and E form heterodimers at very early stages of folding, but stable association does not occur without the stem region.

**Endoglycosidase Hf digestion of TBE virus envelope proteins.** Finally, to check the further processing of the newly synthesized TBE virus envelope proteins, the glycosylation state of prM and E was examined by an endoglycosidase Hf (Endo Hf) digestion assay of the immunoprecipitated TBE virus envelope proteins expressed from different DNA plasmids and virus-infected cells. Both prM and E remained fully sensitive to Endo Hf treatment after 60 min chase for all constructs tested in this study as well as in virus-infected cells (Figure 9). The proteins did not acquire resistance against digestion with Endo Hf after 24 hours of chase (data not shown). This indicated that both prM and E were retained in a pre-Golgi compartment.

![Figure 9](image)

*Figure 9* Endoglycosidase Hf sensitivity of TBE virus envelope proteins. 2 min pulse and 60 min chase samples were immunoprecipitated with a polyclonal antiserum recognizing both prM and E, followed by digestion of the immune complexes with endoglycosidase Hf. Subsequently, the proteins were analyzed by reducing SDS-PAGE and visualized by autoradiography. All recombinant constructs plus a sample from virus-infected cells were tested, as indicated on top of the lanes.
Discussion

The generation of proteins in the form of a polyprotein in the ER involves a variety of complexities that are not encountered when proteins are synthesized individually. The cleavage reactions must be orchestrated correctly on both sides of the membrane, and folding must be organized in such a way that the protein components support rather than interfere with each other during maturation. Our results indicate that the TBE virus envelope proteins associate with each other shortly after synthesis for efficient cleavage of prM from the polyprotein precursor and for proper folding of E. However, expression of prM and E without other viral components is sufficient for the acquisition of their fully mature form.

The three signal peptidase cleavages involved in the synthesis of the two TBE virus envelope proteins were found to occur immediately after synthesis in infected cells, and judging by our assays, the folding process was efficiently completed within 30 min after chain termination. At this time, the proteins were already heterodimers, a prerequisite for virus production and secretion. Although complete folding of prM appeared to be possible in the absence of E, interaction with prM and the formation of a heterooligomeric complex was required for E to attain its final conformation. prM and E had to form heterooligomeric complexes before they could reach their final conformations, but it was not necessary for them to be synthesized from the same polyprotein precursor.

It can be estimated that translation takes about 35 sec for prM and about 110 sec for E (14). As a rule, both prM and E were visible as cleaved products already after 2 min pulse and 0 to 4 min chase in recombinant constructs and virus-infected cells. This suggests that the amino- and the carboxyterminal cleavages occurred as soon as synthesis of each segment was completed.

Other studies have shown that cleavage at the C-prM junction of flaviviruses involves two coordinated cleavages (7, 144). Cleavage of the N-terminal signal sequence of prM by a host-cellular peptidase requires prior removal of the capsid protein from this signal sequence by the viral NS2B/3 protease. Our experiments with virus-infected cells suggest that the cleavages in the C-prM region occurred fast and efficiently, because (I) no uncleaved precursor forms were detected, (II) the ratio of
prM to E did not increase with longer chase times, and (III) the amounts of prM and E found in virus-infected cells were similar to those obtained with recombinant constructs where the capsid was not present.

Based on our data, we further suggest that processing of the C-prM junction by NS2B/3 occurs in trans rather than in cis: The NS2B/3 protease sequence ranges from amino acids 1359 to 2111 in the polyprotein (103). Thus, approximately 7 min are required for complete translation of NS2B/3 after C and prM have been synthesized. However, in our experiments the capsid protein has already been cleaved from prM by that time, essentially ruling out an in cis cleavage.

Elimination of the E sequence following the prM protein in one of the recombinant constructs resulted in slower cleavage at the N-terminus of prM, suggesting that the timing of this cleavage is dependent on the presence of downstream E sequences. This implies that processing of the prM signal sequence occurs only after at least part of the E protein has been synthesized.

In all the constructs that contained both prM and full-length or truncated forms of E, a distinct uncleaved prM-E side product could be observed as a band that disappeared with a half time of 30 min, suggesting that if the cleavage between prM and E did not occur cotranslationally immediately after passing the translocon, it was delayed. This phenomenon may be explained by loss of easy access to the signal peptidase after the newly synthesized protein has left the translocon complexes.

When cleaved, the prM protein was already in a form that migrated fast in non-reducing SDS-PAGE. This means that of the three potential disulfide bonds, at least one that forms a large loop must have been in place. It is possible that the protein was, in fact, completely oxidized by the time it was released from the translocon because no further changes in mobility were seen. That disulfide bond formation can take place cotranslationally is well known from previous studies with influenza HA and SFV glycoproteins (21, 111). To be oxidized, the prM protein did not have to separate from its signal peptide nor did it need to be associated with E. The prM protein thus emerged as rapid and robust folder.

The oxidative folding of E was much slower than that of prM, extending over a time period of 8 to 16 min after chain termination. Although the X-ray crystal structure
shows the presence of six disulfide bonds in the mature ectodomain, the shifts in mobility of the protein remained modest. The reason was most likely the small relative size of the loops formed. Although oxidation did take place in the absence of prM, it was apparent that full maturation of E and expression of a conformational epitope could only take place once a stable heterodimer with prM had been formed. The data thus indicate that the folding of E takes place in two phases; one before formation of a stable heterodimer, and one that proceeds slowly after formation of the dimer. The need for co-synthesis for folding and secretion has been previously demonstrated for other flaviviruses (85, 117). Based on those reports and the results presented in this study, prM may considered a chaperone for the folding and dimerization of flavivirus envelope proteins.

By measuring the appearance of a conformational epitope on each of the individual proteins, we obtained folding half-times of 3.5 to 4 min for prM, 15 to 20 min for E, and ca. 18 min for NS1, respectively. The folding of another member of the flavivirus genus has recently been analyzed (26). In the case of dengue virus, the half-times for folding were 15 to 20 min for prM and 60 min for E. Thus, the dengue glycoproteins fold considerably more slowly than their TBE counterparts. The reason for the differences in the observed folding rates is not clear, but since the methods used in the two studies were similar, it is likely that different flaviviruses exhibit different glycoprotein folding efficiencies, a phenomenon that has also been observed with influenza virus HA from different strains (14).

Compared with envelope proteins from other virus families that are synthesized as polyproteins, TBE virus E folds at comparable rates, while prM is an unusually rapid and highly efficient folder. The Semliki Forest virus envelope proteins E1 and p62 are folded after 10 and 20 min, respectively (110). The envelope protein E1 of Hepatitis C virus (HCV) is a slow folder, getting oxidized only after 60 min, whereas HCV E2 can form its disulfide bonds within 10 min of synthesis (34). In the case of Uukuniemi virus from the Bunyamwera family, G1 folds rapidly within 10 min, while G2 reaches its folded form after 60 min only (120).

Heterodimer formation of TBE virus envelope proteins starts within a few minutes after translation, probably as soon as prM is fully folded. In the case of dengue virus,
the two envelope proteins also dimerize within 15 min after synthesis, but the precise half-time of dimerization was not determined (153). Our results showed that interaction between prM and E also can occur when the two proteins are expressed from different messengers, i.e. they can interact productively without being synthesized as part of a common polyprotein. Whether such dimerization in trans occurs in infected cells where the proteins emerge from the same polyprotein is not clear from our data. However, we believe that, in a natural infection, in cis dimerization is more likely because the sucrose gradient data show the prM protein associating with E already within 4 min of synthesis. Moreover, it does so with the simultaneously labeled E protein. If dimerization would occur in trans, one would expect dimers to form between the newly synthesized prM and previously synthesized, unlabeled E proteins that are already further matured. This has been reported for Uukuniemi virus G1 and G2 (120). Furthermore, dimerization in cis is probable because the ratio between labeled prM and E proteins remained identical whether E was co-precipitated with an anti-prM antibody or vice versa.

A recent study has shown striking structural similarities between the TBE virus E protein and the E1 protein of the alphavirus Semliki Forest virus (92). Although alphavirus envelope proteins make use of distinct chaperones, they also interact with each other a few minutes after synthesis by forming heterodimers in cis (10). For Hepatitis C virus, another member of the Flaviviridae, interactions between E1 and E2 occur early, but these proteins seem to be aggregated, and it takes much longer for them to form native, stable heterodimeric complexes (30, 33). In the case of Uukuniemi virus, a bunyavirus, the envelope glycoproteins G1 and G2 form heterodimers rapidly after synthesis, although dimerization occurs in trans rather than in cis (120).

Our results confirmed that the ectodomain of E without membrane anchor cannot stably associate with prM (6). Although the protein appeared to be folded as judged by mAb reactivity (data not shown), most of it remained DTT sensitive. Since E expressed without prM did not acquire DTT resistance, we concluded that interaction of prM with E was required for the latter to reach its final folded state.
We also observed that deleting the stem and membrane anchor of E caused oxidation of the protein to proceed more rapidly. This generated a partially oxidized intermediate that was prominent during folding of the E400 form, but could not be detected for the membrane-anchored forms of E.

The single N-linked glycans on prM and E expressed individually or together remained fully sensitive to digestion with endoglycosidase Hf several hours after synthesis. The secreted forms of E, however, acquire resistance against treatment with Endo Hf (133). Influenza hemagglutinin, which has similar folding kinetics as E, becomes resistant to Endo Hf digestion with a half-time of 15 min, indicating that it had reached the Golgi by that time (137). Therefore, it is likely that prM and E are retained in the ER unless they are incorporated into virus particles, which is consistent with the observation that flavivirus assembly occurs at the ER membrane (reviewed in (94)).

In summary, we have provided evidence for a coordinated maturation of the glycoproteins of TBE virus. The results indicate that E requires prM to reach the native conformation efficiently, suggesting a chaperone-like role for prM. On the other hand, prM needs E for rapid signal sequence cleavage at its N-terminus. PrM can exert its assisting function on E when co-expressed either in cis or in trans. Dimerization of the two proteins occurs rapidly and is required for the final folding steps. However, the proteins flanking prM and E in the TBE virus polyprotein did not have a major influence on the folding process.

**Acknowledgments**

The authors thank Connie Schmaljohn (USAMRIID, Fort Detrick, MD) for providing anti-prM and anti-NS1 antibodies; Karin Mench, Silvia Röhnke, and Walter Holzer for excellent technical assistance, and Karin Stiasny, Lars Ellgaard, and Srinivas Venkitraman for critical reading of the manuscript.

This work was supported by a grant from the Swiss Federal Institute of Technology to A.H. I.C.L. was a recipient of a Short-Term Fellowship from the European Molecular Biology Organization (EMBO).
3. Intracellular Assembly and Secretion of Recombinant Subviral Particles from Tick-Borne Encephalitis Virus

(Paper II)

Ivo C. Lorenz\textsuperscript{1}, Jürgen Kartenbeck\textsuperscript{2}, Steven L. Allison\textsuperscript{3}, Franz X. Heinz\textsuperscript{3} and Ari Helenius\textsuperscript{1}

\textsuperscript{1}Institute of Biochemistry, Swiss Federal Institute of Technology, CH-8093 Zürich, Switzerland
\textsuperscript{2}Deutsches Krebsforschungszentrum, D-69120 Heidelberg, Germany
\textsuperscript{3}Institute of Virology, University of Vienna, A-1095 Vienna, Austria
Abstract

It is generally assumed that flavivirus assembly occurs by intracellular budding of the nucleocapsid into the lumen of the endoplasmic reticulum (ER). Expression of Tick-Borne Encephalitis (TBE) virus envelope proteins prM and E in a plasmid vector system leads to their incorporation into enveloped Recombinant Subviral Particles (RSPs) without the capsid being required. In this study, we analyzed the formation and secretion of TBE virus RSPs and of a soluble E-E homodimer in mammalian cells. Immunofluorescence microscopy showed that E was accumulated in the lumen of the ER. RSPs were detected by electron microscopy in the rough and smooth ER and in downstream compartments of the secretory pathway. Two major particle sizes were detected, indicating that assembly does not necessarily follow one particular symmetry rule. While secretion of sE dimers started 30 min after synthesis, RSPs were detected in the cell culture medium one to two hours after synthesis of prM and E. We also found that the presence of the single N-linked oligosaccharide side chain on the E protein and its trimming by glucosidases was necessary for RSP and sE dimer secretion. Our results suggest that incorporation of prM and E into RSPs occurs at the ER membrane without other viral elements being required, followed by rapid transport along the compartments of the secretory pathway and secretion. Moreover, the carbohydrate side chain of E is involved in at least one maturation or transport step of TBE virions.
Introduction

Before enveloped viruses leave the host cell after replication, they acquire a lipid bilayer by budding at the plasma membrane or at the membrane of an intracellular organelle such as the endoplasmic reticulum (ER), the intermediate compartment, or the Golgi complex. This implies that the viral envelope proteins as well as the viral genome have to be transported to the site of virion formation. Moreover, molecular signals or determinants are usually required for coordinated virus assembly.

Flaviviruses, a genus of small enveloped RNA viruses within the family Flaviviridae, are assembled intracellularly by budding into the ER of the infected cell (reviewed in (94)). Virus particles are detected by electron microscopy in the lumen of the rough ER, and in the lumen of the smooth ER or the ER-to-Golgi intermediate compartment (ERGIC) (79, 153). However, budding intermediates at the ER membrane have not been observed, and the molecular mechanisms of flavivirus assembly are largely unknown.

During flavivirus infections, non-infectious subviral particles that contain the viral envelope proteins but lack the nucleocapsid are released in addition to infectious virions (130). Similar particles are also produced when the flavivirus envelope proteins are expressed without any other viral proteins (5, 107). These recombinant subviral particles (RSPs) have been observed for many flaviviruses, and they were shown to be excellent immunogens (43, 64, 86, 122).

The envelope of Tick-Borne Encephalitis (TBE) virus, the member of the Flavivirus genus used in this study, consists of a regular lattice formed by two viral membrane proteins, the major envelope glycoprotein E (MW 52 kDa), and the small membrane protein M (MW 7 to 8 kDa). They are synthesized as part of a polyprotein precursor that is co- and posttranslationally cleaved into the individual chains (reviewed in (94)).

E mediates virus entry into the cell via receptor-mediated endocytosis, and it carries the major antigenic epitopes leading to a protective immune response (reviewed in (65)). Recently, the protein was shown to contain an internal peptide loop involved in fusion of the viral envelope with the host cellular membrane (3). E is a type I
membrane glycoprotein (carrying one N-linked oligosaccharide) with two transmembrane segments at its carboxyterminus linked by a short cytoplasmic loop. The solution of the X-ray structure of the ectodomain of E showed that the protein forms head-to-tail homodimers on the viral surface (126). When exposed to low pH, the E proteins undergo an irreversible rearrangement leading to dissociation of the dimers followed by formation of trimers that correspond to the fusion-active form of the protein (4).

The N-linked glycosylation consensus sequence of E at position 154 is present in other flaviviruses as well (17). It has been suggested that the carbohydrate side chain may stabilize the dimer contacts between two E molecules (126). Another study has revealed that the N-linked glycan does not play a major role in the antigenic structure of the TBE virus E protein (160). In general, it is not clear whether the carbohydrate side chain of E is involved in flavivirus maturation or not, since the protein is not glycosylated in other flaviviruses, such as some Kunjin or West Nile strains (1, 18).

M is synthesized as a precursor protein, prM (MW 25 kDa) containing one carbohydrate side chain. We and others have shown that prM has a chaperone-like role in the folding and maturation of E (85, 97). Heterodimer formation between prM and E starts soon after synthesis, a process that seems to be mandatory for E to reach its final native conformation. The interaction between prM and E is also important for later processing steps. It has been suggested that prM holds E in an inactive conformation to prevent low-pH rearrangements during transport through the acidic compartments of the trans-Golgi network (70). Shortly before the virus is released from the cell, the pr portion is cleaved from prM by the cellular protease furin, leading to mature virions consisting of E and M molecules (141).

A few years ago, a plasmid vector system was developed that allows expression of a prM-E polyprotein that is co- and posttranslationally cleaved into the individual chains (2, 97). It was shown that prM and E synthesized recombinantly could be incorporated into subviral particles that were secreted into the culture medium (5). These RSPs were smaller in size compared to virions (30 nm in diameter instead of 50 nm) (133). Further studies revealed that they had similar surface properties and
fusion activity as infectious viruses (25, 133). Recently, the structure and protein composition of TBE virus RSPs were resolved by cryo-electron microscopy (40). This study revealed that 60 molecules each of M and E were arranged in a T=1 symmetry on the surface of the particle.

Since RSPs were produced by expression of the two TBE virus envelope proteins only, the capsid cannot be the driving force for their formation at the ER membrane. Studies using plasmids expressing prM and C-terminally truncated variants have shown that the first of the two transmembrane segments of E was sufficient for incorporation of E into RSPs, and that deletion of the entire transmembrane region led to the secretion of soluble homodimeric E proteins (5, 6). When exposed to low pH, the dimers reversibly dissociate into monomers (143).

In this study, we have investigated the assembly and secretion of RSPs as well as soluble E (sE) dimers by recombinant expression of the TBE virus envelope proteins prM and E. We found that the major portion of E was localized to the lumen of the ER. RSPs were detected in the rough and smooth ER, the ER-to-Golgi Intermediate Compartment (ERGIC), and the Golgi complex. RSPs were secreted from the cell within a few hours after synthesis, whereas secretion of sE dimers started already 30 min after synthesis. Inhibition of N-linked glycosylation of E or glucose trimming of the carbohydrate side chain of E lead to a significant decrease in the secretion of RSPs and sE dimers suggesting a critical role for this glycan in E dimer formation.
Materials and Methods

Plasmids and Virus. We used an SV40-based recombinant plasmid vector system derived from TBE virus strain Neudoerfl (GenBank accession no. U27495) expressing prM and full-length E (SV-PEwt, (2)), prM and a membrane anchor-free form of E (SV-PE400, (6)), or prM and full-length E containing a single amino acid mutation within its glycosylation consensus sequence (SV-PEwt*; Ser156Ala). The vector included the SV40 early promoter and an SV40 origin of replication for amplification in COS cells.

TBE virus strain Neudoerfl (102) grown in primary chicken embryo cells was purified in two cycles by sucrose gradient centrifugation as described in (67).

Infection of mammalian cells with TBE virus. COS-1 cells (ATCC CRL 1659) were grown in Dulbecco’s Modified Eagle Medium supplemented with 10% Fetal Calf Serum (FCS), 4 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO2. At 60 to 70% confluency, the cells were infected with TBE virus at an m.o.i. of ca. 1 in DMEM containing 2% FCS, glutamine, and antibiotics. The virus-containing medium was replaced by fresh medium one hour after infection. 24 hours post-infection, the cells were used for further experiments.

Transfection of mammalian cells with recombinant plasmids. COS-1 cells grown to 50 to 60% confluency were transiently transfected with SV-PEwt, SV-PE400, or SV-PEwt* plasmid DNA complexed to LipofectAMINE (Life Technologies) in DMEM without serum or antibiotics. 5 hours after transfection, an equal amount of DMEM containing 20% FCS, 200 U/ml penicillin and 200 μg streptomycin was added to the cells without removing the transfection mixture. 24 hours post-transfection, the cells expressing recombinant TBE virus envelope proteins were used for further experiments.

Immunofluorescence. COS-1 cells grown on 15 mm coverslips were transfected with SV-PEwt plasmid DNA as described above. 24 post-transfection, the cells were fixed with 2.5% formaldehyde in serum-free medium supplemented with 20 mM HEPES pH 7.4 for 20 min at room temperature. The cells were washed twice with serum-free medium, followed by incubation with PBS++ at 4°C for 10 min.
Subsequently, the coverslips carrying the cells were transferred to a humid chamber and permeabilized in PBS++ containing 20% goat serum, 15 mM glycine, and 0.05% saponin for 15 min at room temperature. All following incubating and washing steps were carried out with this buffer. The cells were incubated with an antibody directed against TBE virus envelope proteins and an antibody that recognizes marker proteins of various cellular organelles at dilution rates between 1:100 and 1:500 at room temperature for 45 min. After extensive washing, the cells were incubated with fluorescence-label conjugated secondary antibodies diluted 1:200 at room temperature in the dark for 30 min. The cells were washed five times with permeabilization buffer and twice with water, followed by mounting of the coverslips on glass slides with Mowiol. Fluorescence microscopy was done with a Zeiss Axiovert microscope, and image processing was done with a Hamamatsu CCD camera and the OpenLab software (InVision).

**Electron Microscopy.** COS-1 cells were transfected with SV-PEwt plasmid DNA as described above. 24 h post-transfection, the cells were fixed with 2.5% glutaraldehyde in 50 mM cacodylate buffer (pH 7.2, 50 mM KCl and 2.5 mM MgCl2) for 30 min at room temperature. After washing the cells five times with cacodylate buffer, they were incubated in 2% OsO₄ in cacodylate buffer for 2h on ice, followed by washing with H₂O. The cells were incubated overnight in a 0.5% aqueous solution of uranyl acetate. Subsequently, the cells were dehydrated with increasing concentrations of ethanol, treated twice with propylene oxide for 5 min, and incubated in a 1:1 propylene oxide-Epon mixture overnight. The cells were transferred into pure Epon and incubated for 6h at room temperature followed by embedding in fresh Epon at 63°C for 36 h. The Epon blocks were cut with a Reichert-Jung microtome (Ultracut). Electron microscopy was carried out using a Zeiss EM 910 electron microscope (Zeiss Oberkochen, Germany).

**Pulse-chase analysis of TBE virus envelope protein transport and secretion.** Subconfluent 60 mm dishes of virus-infected or plasmid-transfected COS-1 cells were washed twice with PBS containing 1 mM Mg²⁺ and 0.5 mM Ca²⁺ (PBS++). After starvation in Cys/Met-free medium at 37°C for 30 min, the cells were pulse-labeled with 0.2 mCi ³⁵S-Cys/Met (ProMix, Amersham Pharmacia) in Cys/Met-free medium containing 20 mM HEPES pH 7.4 at 37°C for 2 or 5 min. After removal of the pulse
medium, the cells were washed twice with 5 mM unlabeled Cys/Met in DMEM supplemented with 10% FCS, glutamine, antibiotics, and 20 mM HEPES pH 7.4 followed by incubation with this medium at 37°C for chase times between 30 min and 4 hours.

The reaction was stopped by flooding the cells with ice-cold PBS++ containing 20 mM N-ethylmaleimide (NEM). After incubation at 4°C for 5 min, the cells were lysed with 2% CHAPS in HEPES-buffered saline (HBS, pH 7.4) containing 20 mM NEM, 1 mM phenylmethylsulfonyl fluoride, and 10 µg/ml each of chymostatin, leupeptin, antipain and pepstatin to inhibit proteolysis. The nuclei and the cellular debris were pelleted by centrifugation, and further experiments using the post-nuclear supernatants were done.

Chase media were collected and supplemented with NEM to a final concentration of 20 mM. After pelleting detached cells or debris, the chase media were subjected to immunoprecipitation to detect prM/M and E.

**Temperature shift pulse-chase analysis.** COS-1 cells expressing recombinant prM and E were starved and pulse-labeled as described above. After incubation in chase medium at 37°C for 45 min to allow the proteins to fold completely, the dishes were transferred to a 15 or 20°C water bath, followed by incubation at this temperature for 75 min. Alkylation of the reaction, cell lysis and collection of the chase media were done as described above.

**Pulse-chase experiments with inhibitory drugs.** To block protein transport across the organelles of the secretory pathway, brefeldin A was added to starvation, pulse and chase media to a final concentration of 5 µg/ml. Glycosylation of newly synthesized proteins was prevented by supplementing the starvation, pulse and chase media with 5 µg/ml tunicamycin. Glucose trimming of carbohydrate side chains was inhibited in the presence of 50 mM N-butyl-deoxynojirimycin in starvation, pulse and chase media.

**Immunoprecipitation and SDS-PAGE.** Post-nuclear supernatants and chase media from pulse-chase experiments were preloaded on protein A coupled to sepharose CL-4B beads (Sigma) for 1 to 2 hours at 4°C. 80 to 200 µl of preloaded lysate or 1 ml of preloaded chase medium were loaded on protein A-sepharose CL-4B
beads (10 μl bead volume), and 1 μl antibody was added. The proteins were precipitated by incubation with end-over rotation at 4°C overnight. Immune complexes were pelleted at 8500 x g for 4 min, followed by two washes with 0.5% CHAPS in HBS and one wash with HBS alone with intermittent shaking at 4°C for 10 min. Subsequently, the precipitated material was solubilized by adding 40 μl sample buffer and heated to 95°C for 5 min. After pelleting the protein A-sepharose beads, the supernatant was divided in two equal aliquots, and the reducing agent dithiothreitol (DTT) was added to one of them to give a final concentration of 100 mM. The proteins were analyzed by either reducing or both non-reducing and reducing SDS-PAGE on minigels followed by autoradiography.

**Antibodies.** To detect TBE virus envelope proteins in immunoprecipitation and immunofluorescence experiments, a rabbit polyclonal antiserum recognizing both E and prM as well as the non-structural protein NS1 (k-PM(2), (5)) and a mouse monoclonal anti-E antibody (B1, (55)) were used. For the immunofluorescence co-localization studies, anti-calnexin polyclonal antiserum (62), anti-protein disulfide isomerase mouse monoclonal antibody (StressGen), anti-ERGIC53 and anti-GM130 mouse monoclonal antibodies (both kindly provided by H.-P. Hauri, Basel) were applied. Alexa Dyes (Molecular Probes) were used as fluorescence label-conjugated secondary antibodies in immunofluorescence experiments.
Results

Intracellular localization of TBE virus envelope proteins expressed recombinantly. To determine the intracellular distribution of prM and E, COS-1 cells were transfected with the SV-PEwt plasmid, which codes for both of the glycoproteins (2). The cells were fixed, permeabilized, and double-stained for one or both of the envelope proteins, and for cellular marker antigens. Anti-calnexin (152) and anti-protein disulfide isomerase (PDI, (44)) were used as markers for the ER, anti-ERGIC-53 for the intermediate compartment (135), and anti-Giantin for the Golgi complex (95). A mouse monoclonal anti-E (Figs. 1A and D), a mouse monoclonal anti-prM (not shown), and a rabbit polyclonal anti-E/prM antiserum (Figs. 1G and K) were all found to stain the nuclear envelope and a reticular network extending throughout the cytoplasm. That prM and E had the same distribution was consistent with our observation that they occur as tight heterodimeric complex (97). The distribution overlapped almost completely with the two ER markers (Figs. 1C and 1F). In addition, prM and E showed some overlap with ERGIC-53 (Fig. 1I), and to a minor extent with giantin (Fig. 1L). No staining was observed at the plasma membrane.

We concluded that the majority of prM and E was located in the ER. A small fraction was present in the intermediate compartment and possibly in the Golgi complex. A similar overall distribution was seen when the SV-PE400 construct was expressed (data not shown). This construct encodes prM and a truncated, soluble E protein that contains the ectodomain but lacks the C-terminal membrane anchor (6).
Figure 1 Intracellular localization of TBE virus envelope proteins. COS-1 cells transfected with SV-PEwt plasmid DNA were fixed and subjected to indirect immunofluorescent co-staining with primary antibodies that recognize TBE virus envelope proteins and antibodies against a marker protein of a cellular organelle. The proteins were then labeled with secondary antibodies conjugated to red or green light-emitting fluorophores. A and D refer to cells stained with a monoclonal anti-E antibody, while G and J show cells labeled with a polyclonal antiserum recognizing both prM and E. Immunofluorescent staining of the ER with polyclonal anti-calnexin (CNX) and anti-protein disulfide isomerase (PDI) antisera is presented in B and E, respectively. In panel H a monoclonal antibody against a protein in the ER-to-Golgi Intermediate Compartment (ERGIC) was used, and panel K shows immunostaining of the Golgi with a monoclonal anti-Giantin antibody. Colocalization of viral envelope proteins with the organelle markers is represented by the yellow regions within each cell in the merged images (C, F, I, and L).
Assembly and secretion of recombinant subviral particles visualized by electron microscopy. The transfected cells were also analysed by thin-section electron microscopy as shown in figure 2. In contrast to non-transfected cells (not shown), the transfected COS-1 cells contained virus-like particles in the lumen of various compartments of the secretory pathway. They were most frequently observed within the lumen of the rough ER (Figs. 2A and B). Although less frequently, they were also present in the smooth ER (Figs. 2C, D, and E), in transitional elements (TE) or ERGIC (Figs. 2F and G), in the rims of the Golgi cisternae (Figs. 2H and I), in the trans-Golgi network (TGN, Fig. 2J), and in secretory vesicles (Fig. 2K).

Using microtubules as size marker, 75% of the particles were calculated to have had a diameter of 25 to 30 nm consistent with the size observed for Recombinant Subviral Particles (RSPs), previously shown to be secreted from cells transfected with SV-PEwt (5). In their secreted form, these have been shown to have T=1 symmetry and to contain a lipid envelope, and 60 copies each of E and M (40). The remaining 25% of the particles had a diameter of 40 to 45 nm corresponding to the size of intact TBE virus. These also displayed low electron density at the center indicating that there was an ‘empty’ space were normally the nucleocapsid would be located. Particles of both sizes were found within the same cisterna (Fig. 2G).

In addition, the EM analysis revealed the occasional presence in the ER of long tubular structures (see inlet in Fig. 2G). The diameter (50 nm) and overall appearance suggested that they were related to the larger of the RSPs except that they had grown into extended tubes instead of spherical particles. The longest tubular particle observed was 3.5 μm in length. Since such particles have not been seen in cells infected with TBE virus, they most likely represented products of abnormal budding. That they were not observed in the Golgi complex, suggesting that they may not undergo secretion.
Figure 2  Electron micrographs of COS-1 cells transfected with SV-PEwt. RSPs of two different sizes (ranging from 25 to 31 nm and from 45 to 51 nm) are seen in the lumen of the rough and smooth ER (A to G), in transitional elements (TE) (F and G), the Golgi complex (H and I), the trans-Golgi network (TGN) (J) and in large-sized vesicles, probably representing a secretory vesicle (SV) (K). Insert in (G) shows a long tubular structure (diameter 50 nm) found inside a cisterna of the rough ER. Inserts in (K) show magnifications of RSPs of the small and large size. CI, clathrin-coated vesicle; Co, COP-coated vesicle; MT, microtubule. Short arrows point to a small-sized, long arrows to a large-sized particle. Magnification in (A), (B), (D) to (J) and insert in (G) is the same. Bars: 0.2 μm; inserts in (K): 1 μm.
Taken together, the morphological studies indicated that prM and E were located mainly in the ER but was also present in other compartments of the secretory pathway. In the ER, they induced budding into the lumen of three different forms of recombinant subviral particles of which the most common corresponded to the previously analysed 30 nm RSPs. That more than one particle size was produced was consistent with earlier cryo-electron microscopy observation indicating the secretion of a mixture of 30 and 40 nm particles (40), and with our unpublished results indicating two particle sizes using sucrose gradient velocity centrifugation (ICL and AH, unpublished).

**Secretion Block of TBE virus envelope proteins expressed recombinantly.**

Passage through the secretory pathway can be inhibited at different levels by temperature blocks and by drug treatments. To determine their effect on the secretion of RSPs, COS-1 cells transfected with SV-PEwt were pulse-labeled with $^{35}$S-Cys/Met for 5 min, and chased for 45 min at 37°C to allow folding and dimerization of prM and E (97). To induce a block between the intermediate compartment and the cis-Golgi, the cells were cooled to 15°C for 75 min, and to block exit from the TGN, 20°C incubation was similarly used (132). Moreover, to block exit form the ER, brefeldin A was added to one sample at 37°C during starvation, pulse and chase (32). The post-nuclear supernatants of the cells and the chase media were subjected to immunoprecipitation with a polyclonal anti-E/prM antiserum, followed by non-reducing and reducing SDS-PAGE and autoradiography.

PrM and E were present in cell lysates under all conditions, with no significant differences in the expression level (Fig. 3, upper panel). Some aggregates detected at the top of the non-reducing lanes could be dissolved by reduction, suggesting that they were caused by intermolecular disulfide cross-linking (97). PrM was less efficiently detected in the autoradiograms because the protein does not contain the same number or Cys and Met residues as the E protein and thus incorporates a lower amount of $^{35}$S-Cys/Met. For prM, a clear difference in the electrophoretic mobility between non-reducing and reducing conditions was visible, indicating that the protein had formed disulfide bonds. The corresponding shift in the E protein band was much smaller as previously reported (97).
Figure 3 Blockage of the secretory pathway by temperature shifts and brefeldin A. COS-1 cells expressing recombinant E and prM were pulse-labeled for 5 min and chased for 2 hours. For temperature shift experiments, the cells were chased at 37°C for 45 min to ensure proper protein folding, followed by a 75 min chase at 15 or 20°C to block transport at the ERGIC or Golgi level, respectively. Brefeldin A (BFA) was added to another sample during starvation, pulse and chase, and the cells were kept at 37°C during the entire pulse and chase period. TBE virus envelope proteins from total cellular extracts (upper panel) and chase media (lower panel) were immunoprecipitated with a polyclonal antiserum recognizing prM and E, analyzed by SDS-PAGE and detected by autoradiography. Positions of the individual proteins are marked on the side.

In the sample treated with brefeldin A (lanes 4 and 8), a slight shift in the mobility of E was observed. It may be caused by the brefeldin A-induced fusion of the ER with the Golgi, which exposes ER proteins to the ER to Golgi mannosidases and other modifying enzymes (72). Moreover, the E protein migrated as a double band under reducing conditions (lane 8). Since the SV-PEwt construct contains the first 30 amino acid residues of NS1 (NS1*) at its C-terminus, the upper band might correspond to the non-cleaved E-NS1* form (97). Processing of this short amino acid stretch may be delayed or less efficient in cells treated with brefeldin A.
In each case, the secretion of E decreased dramatically when the secretory pathway was blocked (Fig. 3, lower panel). Thus, the secretion of the RSPs was efficiently blocked when transport through the organelles of the secretory pathway was inhibited.

**Kinetics of RSP and sE dimer secretion.** To compare the secretion kinetics of infectious virions, RSPs, and soluble E (sE) homodimers, we pulse-labeled cells infected with TBE virus or transfected either with SV-PEwt or SV-PE400 plasmid DNA for 5 min. After chase times between 30 min and 4 hours, cell-associated and secreted TBE virus envelope proteins were immunoprecipitated and analyzed as described above. In addition to prM and E, the polyclonal antiserum used recognized the non-structural protein NS1, which is expressed in infected cells and secreted as multimers independently of the virion (27).

As shown in figure 4A, post-nuclear supernatants from virus-infected cells showed three bands corresponding to prM, E, and NS1 (upper panel). The amount of prM and E in the cells decreased between 2 and 4 hours (lanes 3 and 4), with a parallel increase in the extracellular medium (lower panel, lanes 3 and 4). Like the virus, NS1 was efficiently secreted although with faster kinetics. A non-specific band at 45 kDa was visible in the cell-associated samples throughout, probably corresponding to labeled actin (upper panel, lanes 1 to 4).

When expressed from a recombinant construct (SV-PEwt), prM and E were still visible in the cell lysate after 4 hours of chase (Fig. 4B, upper panel), the amount decreasing slightly after 2 to 4 hours (lanes 3 and 4). A faint band of secreted E was detected after 1 hour chase (lower panel, lane 2), with a gradual increase after 2 and 4 hours (lanes 3 and 4, respectively). Small fractions of prM appeared in the chase medium after 2 to 4 hours, probably due to incomplete cleavage by furin (data not shown).
When prM and membrane anchor-free E were pulse-labeled (SV-PE400), the labelled prM and E could be detected in decreasing amounts beyond 4 hours of chase (Fig. 4C, upper panel). Secretion of soluble sE dimers was detected as early as 30 min of chase, increasing steadily until 2 hours of chase (lower panel).

Taken together, the pulse-chase analysis indicated that the viruses, the RSPs, and the soluble E protein dimers were all efficiently secreted. However, there were clear differences in the kinetics. The sE dimers were the fastest, and the intact virus the slowest. Our results indicated that it took the labelled viral envelope proteins 2 to 4 hours to fold, assemble into virus particles in the ER, and to traverse the secretory pathway.
Role of the N-linked glycan in E. As already mentioned, the TBE virus E protein contains a single N-linked glycan located close to the dimerization interface (126). We tested whether this carbohydrate side chain was critical for assembly and secretion of RSPs and sE dimers by interfering with oligosaccharide addition and processing. To inhibit glycosylation, we made use of tunicamycin an inhibitor of core glycan synthesis (36), and we generated an SV-PEwt construct (called SV-PEwt*) in which the glycosylation consensus sequence in E was mutated (Ser156Ala), so that it would not be recognized by the oligosaccharyltransferase. In the latter case, glycosylation of prM, which also contains single glycan, was expected to remain unaltered.

Figure 5 Effect of glycosylation inhibition on secretion of TBE virus envelope proteins. COS-1 cells transfected with SV-PEwt, SV-PE400, or SV-PEwt* (Ser156Ala; i.e. prM and E carrying an amino acid point mutation which abolishes glycosylation of E) were pulse-labeled for 5 min and chased for 2 hours in the presence (+) or absence (-) of Tunicamycin (Tun). The intracellular and secreted fractions of E were immunoprecipitated and analyzed by reducing SDS-PAGE as described above, followed by autoradiography.
Cells transfected with SV-PEwt, SV-PE400 or SV-PEwt* were pulse-labeled for 5 min and chased for 4 hours. The viral glycoproteins were immunoprecipitated with a polyclonal anti-E/prM antisemur and analysed by reducing SDS PAGE. As shown in figure 5, the expression was normal for each construct in the presence and absence of tunicamycin (upper panel), but secretion of the non-glycosylated E was dramatically reduced (lower panel). Quantitation by phosphorimager analysis showed that it was reduced by 90% (compare lanes 1 and 2). Being secreted at a level of 30%, the soluble sE dimer was less affected (lanes 3 and 4).

Figure 6 Secretion of TBE virus envelope proteins after inhibition of glucose trimming with bDNJ. COS-1 cells transfected with SV-PEwt (A) or SV-PE400 (B) were pulse-labeled for 5 min and chased for 2 hours. To prevent glucose trimming on the carbohydrate side chains, the glucosidase inhibitor N-butyl-deoxynojirimycin (bDNJ) was added to starvation, pulse and chase media (+), or to the chase medium after 15 or 30 min as indicated. Immunoprecipitates of post-nuclear supernatants and chase media were analyzed by reducing SDS-PAGE, followed by autoradiography.

To analyze whether the effect was caused by inefficient folding, we precipitated the lysates with conformation-specific anti E and anti-prM monoclonal antibodies previously used assess the antigenic structure of prM and E (55, 78) and to monitor the folding process (97). No differences between glycosylated and non-glycosylated
E and prM could be observed (data not shown). The results also indicated that intrachain disulfide bond formation in E and prM was normal, and that heterodimerization between them occurred normally. N-linked glycans undergo glucose trimming in the ER as a prerequisite for interaction with the calnexin/calreticulin chaperone cycle (37). To test whether inhibition of the glucosidases involved in this process had an effect on secretion, cells transfected with SV-PEwt or SV-PE400 were pulse-labeled for 5 min and chased for 4 h in the presence of N-butyl-deoxynojirimycin (bDNJ), a alpha-glucosidase inhibitor (36), which was added during starvation, pulse and chase, or after 15 min of chase, or after 30 min chase. Cell-associated and secreted viral envelope proteins were immunoprecipitated and analyzed on reducing SDS-PAGE.

As shown in figure 6, again a dramatic decrease in E protein secretion was observed when glucose trimming was blocked. Quantitation indicated that less than 10% of the E protein was secreted when bDNJ was present throughout starvation, pulse and chase (Fig. 6A, lane 2). In the case of soluble E dimers, the effect was again somewhat less (30%; Fig. 6B, lane 2). When bDNJ was added after 15 min of chase, secretion of Ewt and E400 was reduced to 30 and 50%, respectively (Figs. 6A and 6B, lanes 3). After 30 min of chase, secretion was still reduced to 45% and 60%, respectively (Figs. 6A and 6B, lanes 4). This suggested that the effect of bDNJ was likely to involve late assembly steps because according to our previous studies prM and E fold and associate within 20 min (97). Consistent with this interpretation, we found that bDNJ like tunicamycin did not inhibit oxidative folding of prM and E nor the formation of prM/E heterodimers (data not shown).

Our results thus indicated that the single N-linked glycan in the TBE virus E glycoprotein, and its trimming by ER glucosidases, is essential for efficient secretion of RSPs and sE dimers. The glycan is not required for oxidative folding and heterooligomeric assembly of prM/E dimers.
Discussion

In this study, we have analyzed the assembly and secretion of TBE virus envelope proteins prM and E in the form of recombinant subviral particles (RSPs) and as membrane anchor-free, soluble sE homodimers. We found that prM and full-length E were localized to the ER where assembly of virus-like particles occurred. The small amount of viral proteins seen in the intermediate compartment and Golgi complex was probably due to RSPs in transit to the extracellular space.

PrM and E are the only viral proteins needed for production of RSPs (5). Within a few min after synthesis, translocation and cleavage from the polyprotein, they reach their fully oxidized state, heterodimerize, and complete their folding program (97). It is evident from the known crystal structure of isolated, mature E glycoprotein, and from image reconstruction analysis of RSPs after cryo-electron microscopy, that further assembly beyond the prM/E heterodimers involves the formation of homodimers and larger assemblies between prM/E complexes (40, 126). The formation of direct interactions between E proteins is likely to play a crucial role. These interactions most likely arise before or during particle budding in the ER, but they are unfortunately difficult to demonstrate experimentally in cell lysates. When anchor-free E is expressed together with prM, assembly does not proceed beyond the formation of sE dimers (5).

We found that the particles formed by recombinant expression of prM and E were of two distinct size classes with diameters of 30 and 45 to 50 nm. The smaller corresponded to the already well-characterized RSPs with T=1 symmetry (40), while the larger ones had the dimensions of native virus particles (50 nm in diameter). More detailed characterization of these particles will be reported elsewhere (SLA and FXH, unpublished data). That both of these discrete, spherical particles are secreted is suggested by the bimodal size distribution of particles found in the extracellular space (40). In addition, we observed some rod-like particles in the ER of transfected cells. They most likely represented products of abnormal budding in which the vesicle failed to pinch off allowing the bud to grow into an empty, inflexible, tubular structure with a diameter similar to the virus.
Soluble, anchor-free sE dimers were secreted quite rapidly. Pulse chase experiments indicated that they started to appear in the medium already 30 min after synthesis. This rate was similar to that previously reported for anchor free forms of influenza hemagglutinin (137), vesicular stomatitis virus G protein (52), and human immunodeficiency virus gp160 (58). In all these cases, the rate-limiting step is probably folding (31). In comparison, the release of RSPs started one to two hours after glycoprotein synthesis, suggesting that budding and particle assembly were rate limiting. Appearing in the medium only 2 to 4 hours after a radioactive pulse, intact virus particles released from infected cells were even more slowly secreted suggesting that the formation of the larger capsid-containing particles needed more time than formation of the smaller T=1 RSPs. Since budding intermediates could be rarely observed in the ER by electron microscopy, it seemed likely that budding itself is a rapid process. Thus, the time-consuming part of the process was probably the assembly of a pre-budding lattice.

Ultrastructural studies, temperature shift experiments, and the effects of brefeldin A treatment showed that the RSPs are transported through the normal secretory pathway. This is consistent with recent findings on the assembly and secretion of Kunjin virus (100). Immunofluorescence and electron microscopy studies showed that assembly of this virus occurs in the rough ER followed by transport and maturation along the secretory pathway.

The ER membrane is not normally known to form vesicles that bud into the lumen. The formation of RSPs and native viruses is therefore an unusual, non-physiological process. It is possible that once folded, the prM/E heterodimers may alone possess the intrinsic capacity to assemble laterally into an isometric lattice in the ER membrane and its luminal surface. Like elements of the clathrin coat, they may be able to introduce the membrane curvature needed to form a bud, and to trigger the fusion reaction needed to pinch off the membrane vesicle (45). Alternatively, they may rely on additional host cell-derived factors, such as luminal chaperones and folding enzymes. No major host cell proteins are, however, detected in the mature virus or in RSPs (133).
Our immunofluorescence studies, together with earlier biochemical studies (97), showed that the majority intracellular prM and E were localized to the ER as stable heterodimers. It is evident that they have the properties of resident ER membrane proteins. Neither prM nor E have typical dibasic signals in their cytoplasmic domains for ER retrieval (reviewed in (147)). In fact, they have cytoplasmic loops consisting of only a few amino acid residues between their two transmembrane segments. That they can leave the ER when assembled into virions or RSPs means that the ER localization signals are either masked, modified, or prevented from further interaction with retention factors. One possibility is that signals are located in the ectodomain or the short spacer on the lumenal side that connects the ectodomain to the transmembrane domain. Another possibility is that retention is mediated by the transmembrane segments.

Although E contains only a single N-linked glycan, glycosylation and carbohydrate trimming was found to play an important role in the maturation and secretion of RSPs and sE dimers. Preventing glycosylation by tunicamycin, or by mutating the glycosylation consensus sequence in E, drastically reduced the amount of secreted RSPs and sE dimers. A similar drop in secretion was seen when glucose trimming of N-linked glycans was inhibited. Our analysis suggested that unlike the effects of such treatments on most other viral glycoproteins within the flaviviruses (26) or other virus genera (60, 110), the reason for lack of secretion in the case of TBE virus products was not defective folding. Oxidative folding and generation of conformational epitopes on prM and E were in fact normal by all available criteria, and the prM/E heterodimers were formed normally. The step at which the oligosaccharide was needed was therefore downstream from initial folding. Our guess is that it involves formation of E-E dimers because sE, which is normally secreted as dimers, was poorly secreted unless properly glycosylated and glucose- trimmed.

The Asn154 residue that carries the oligosaccharide moiety is located in the central domain of E. The glycan itself could not be visualized in the crystal structure, however, it was suggested that it might interact with the ij loop (aa 248 to 251) in the dimerization domain of the other E subunit and thus contribute to the stability of the dimeric structure (126). However, the function of the sugar moiety on E in the
ER may be linked to prM because a recent image reconstruction study of Dengue virus places M in a pocket between the two E subunits exactly where the glycan in TBE virus is located (89). Thus, it could be that the glycan is essential for forming the proper interface between the two E subunits and the prM subunit in this region.

However, in either case, it is difficult to see why not only the presence of the oligosaccharide but also its glucose-trimming status is essential. The trimming requirement suggests that the calnexin/calreticulin cycle may be involved. Calnexin and calreticulin are ER lectins that interact with partially glucose-trimmed glycoproteins in the ER, and serve as important molecular chaperones (37). They promote correct folding of their substrate proteins and they are involved in quality control and ER retention of incompletely folded and assembled proteins.

Although calnexin co-localized with E in the ER of cells, we were not able to detect any interaction between the TBE virus prM and E and calnexin or calreticulin by immunoprecipitation. This does not necessarily mean that such interactions do not occur in situ, because the complexes with glycoproteins that contain only a single glycan are often weak and difficult to demonstrate (15, 128). The E protein from Dengue virus serotype 1, which carries two carbohydrate side chains, did not interact with calnexin either (26). However, our results from the experiments in which glucose trimming was inhibited clearly suggest an interaction with calnexin and/or calreticulin. If these do indeed bind to the TBE virus E protein in the ER, they may participate as cellular co-factors during RSP and sE dimer assembly. Without them, one might speculate that E dimers do not form properly and that the envelope proteins fail to form the lattice interactions needed for particle budding.

In summary, we have provided evidence that TBE virus envelope proteins are incorporated into RSPs at the ER membrane followed by transport along the secretory pathway. Assembly of prM/E heterodimers into larger assemblies seems to be the rate limiting for secretion of RSPs and the native virus. The presence of the N-linked oligosaccharide in E is required for secretion, as well as the proper glucose trimming of the carbohydrate side chain. It remains to be elucidated whether any cellular factors participate in flavivirus assembly.
Acknowledgments

The authors thank Connie Schmaljohn (USAMRIID, Fort Detrick, MD) for kindly providing anti-prM antibody; and Karin Mench, Silvia Röhnke, and Walter Holzer for excellent technical assistance.

This work was supported by a grant from the Swiss Federal Institute of Technology to A.H. I.C.L. was a recipient of a Short-Term Fellowship from the European Molecular Biology Organization (EMBO).
4. Discussion and Outlook

4.1 Discussion

Viral glycoproteins have often been used as models to investigate the molecular mechanisms of protein folding, maturation and transport in living cells. They are synthesized in the endoplasmic reticulum (ER), where they make use of the cellular folding machinery to acquire their native conformation. Post-translational modifications, oligomeric assembly, and transport of the viral proteins are also carried out by host cell factors. Therefore, while the analysis of viral glycoprotein folding and transport gives an insight into the viral replication strategies, it may also reveal details about the basic principles of protein folding and intracellular targeting in vivo.

In general, our data obtained from experiments using cells transfected with the recombinant constructs expressing prM and E or infection of cells with full-length virus were mainly identical, although the kinetics of folding and secretion slightly differ from each other. This indicates that folding as well as assembly of flavivirus envelope proteins probably does not require other viral factors, e.g. non-structural proteins. The structural proteins may therefore represent an independent functional unit within the viral genome.

4.1.1 Folding and Maturation of the TBE Virus Envelope Proteins prM and E

The three-dimensional structure of the envelope protein E of tick-borne encephalitis (TBE) virus differs from many other envelope proteins of the orthomyxoviruses, paramyxoviruses, retroviruses and filoviruses, which form characteristic coiled coil structures (138, 154). Instead of forming three-stranded coiled coils of \(\alpha\)-helices projecting away from the virus surface, TBE virus E protein forms head-to-tail homodimers lying parallel to the viral membrane. It was therefore assigned to class II viral fusion proteins. The analysis of the folding of this protein thus expands the current knowledge about viral glycoprotein folding towards a new class of viral envelope proteins.
In this context, another class II viral fusion protein is of particular interest. The recently published crystal structure of the E1 protein of semliki forest virus (SFV), an alphavirus, revealed striking structural similarities compared to the TBE virus E protein (92). Although the disulfide bonds lie at different regions within the two proteins, they are the same in number (six) and are distributed over all three domains. This indicates that formation of disulfide bonds contributes to the overall stability of the proteins, but their position within the primary amino acid sequence can vary. As expected, the folding kinetics of SFV E1 and TBE virus E are very similar. Their half-times for oxidation correspond to the ones of envelope proteins from other virus families, such as influenza hemagglutinin (HA) (14) or vesicular stomatitis virus glycoprotein (VSV G) (61). All of them form disulfide bonds within a few minutes after synthesis, and they acquire their native conformation with a $t_{1/2}$ of 5 to 10 min. Therefore, viral glycoproteins generally use similar strategies to acquire their three-dimensional conformation, which are mostly independent of their secondary and tertiary structures. However, there are also examples of viral glycoproteins that fold more slowly, such as human immunodeficiency virus gp160 (90) or hepatitis C virus E1 (34).

The precursor of the small membrane protein M of flaviviruses, prM, is an unusually rapid folder. PrM is able to form its three disulfide bonds within less than two minutes after synthesis, and acquires an epitope recognized by a monoclonal antibody within four minutes. Expression of prM without the E protein following immediately downstream in the polyprotein leads to a delayed signal sequence cleavage at the N-terminus of prM, but it does not block or delay the formation of disulfide bonds. This implies that the protein is able to acquire its disulfide bonds while being anchored into the membrane at both the amino- and the carboxyterminus. PrM may therefore be an interesting model to investigate how a polypeptide chain forms disulfide-linked loops.

The TBE virus envelope proteins interact with each other to form heterodimers within a few minutes after synthesis. The finding that E cannot acquire its native conformation without prM, and the early onset of interaction between the two proteins indicates that prM has a major role in the proper maturation of E. Thus, prM may act as a chaperone-like factor for the E protein to fold correctly. PrM can exert
this function both in cis and in trans. The concept of viral chaperones applies for a
growing number of virus families, including flaviviruses, alphaviruses, bunyaviruses,
and hepaciviruses (9, 85, 109, 120). Thus, viral envelope proteins may have evolved
a simple internal quality control check to ensure the proper maturation of their
surface elements.

Nevertheless, the folding of most viral glycoproteins in the ER depends on folding
enzymes and molecular chaperones that assist the newly synthesized proteins to
acquire their native conformation and to undergo post-translational modifications.
Studies on the folding of viral glycoproteins have also helped in studying the
mechanism of the calnexin/calreticulin cycle that serves as a quality control element
for newly synthesized glycoproteins (37). Interestingly, neither prM nor E of TBE
virus seemed to associate with the lectins calnexin or calreticulin during folding.
Interaction with other common ER chaperones, such as BiP, ERp57 or PDI, was not
detected either. These negative results were obtained by experiments with both the
recombinant expression system and the virus.

There are many reasons why an interaction between the TBE virus envelope proteins
and ER chaperones could not be shown; (i) a highly transient, non-detectable
association, (ii) a low affinity of prM and E for lectins because they carry only one N-
linked oligosaccharide each, (iii) technical limitations imposed by the experimental
methods, or (iv) that the chaperones tested do indeed not bind to prM and E.

A highly transient binding is unlikely because other viral envelope proteins that have
similar folding kinetics do interact with chaperones for several minutes, such as VSV
G (61) or SFV E1 (111). Although SFV E1, as TBE virus E, contains one single N-
linked glycan, it interacts with the lectin-like chaperones calnexin and calreticulin.
However, this association may be indirect, occurring through binding of E1 to p62
that is complexed to calnexin and calreticulin. SFV, like all alphaviruses, shuts of the
synthesis of host cellular proteins after infection, whereas flaviviruses do not.
Therefore, the binding of TBE virus prM and E to chaperones cannot simply be
shown by an anti-chaperone immunoprecipitation as for SFV due to the high
background caused by other newly synthesized proteins that associate with the
chaperone. Sequential immunoprecipitations with anti-chaperone antibodies followed
by anti-E/prM antibodies did not reveal any binding. This may be due to the
destruction of the epitopes of prM and E upon boiling in SDS buffer after the first
immunoprecipitation. Other techniques, such as immunoprecipitation followed by an
immunoblot or intracellular crosslinking were not successful either. Thus, it is
possible that the failure to detect a possible association of prM and E with ER
chaperones was caused by technical limitations, and the question whether the TBE
virus envelope proteins do indeed bind to ER chaperones or not remains to be
elucidated.

The single glycosylation consensus sequence at Asn154 in the E protein of TBE virus
is present in other flaviviruses as well, such as Japanese Encephalitis, Murray Valley
Encephalitis, and some Dengue serotypes (17). The E protein of Dengue contains
additional glycosylation sites, while the E protein of some West Nile or Kunjin virus
strains is not glycosylated at all. Thus, it cannot be concluded from sequence
homologies whether glycosylation has a general important role in the folding and
maturation of flavivirus envelope proteins.

We found that the state of glycosylation had a minor influence on the folding and
maturation of TBE virus prM and E when N-linked glycosylation was inhibited with
tunicamycin or by using a point-mutation of E that abolishes N-linked glycosylation.
The proteins were able to form disulfide bonds, and they were detected by a panel of
monoclonal antibodies. However, the absence of the N-linked glycan on the E protein
or inhibition of glucose trimming lead to a dramatic reduction in the secretion of
recombinant subviral particles (RSPs) as well as of sE dimers, indicating that
glycosylation and glucose trimming on the E protein was required for proper
secretion of TBE virus envelope proteins in both soluble and particulate form. Given
that both the soluble sE dimer and the RSPs were not secreted when glycosylation
was inhibited, it is possible that the carbohydrate side chain is involved in
dimerization of the E subunits. The exact role of the carbohydrate side chain around
position 154 of the E protein in flavivirus assembly and secretion remains to be
established.
4.1.2 Assembly of TBE virus envelope proteins prM and E into Immature Virions

While heterodimer formation between prM and E was readily detectable by co-immunoprecipitations with anti-E or anti-prM monoclonal antibodies as well as by sucrose velocity gradient centrifugation, the trapping of higher-order structures turned out to be much more difficult. Intracellular crosslinking lead to the aggregation of most of the prM and E proteins (data not shown). Upon crosslinking of total cellular extracts prM-E heterodimers, but no E-E homodimers could be detected in SDS-PAGE (data not shown). However, the clustering of TBE virus envelope proteins may be part of the retention mechanism that holds prM and E in the ER for assembly. Whether cellular factors or retrieval processes are involved in the ER localization of prM and E remains to be established.

Given that exactly 60 molecules each of prM and E are needed to form an RSP (40), it is likely that an arrangement or prM and E into a regular lattice occurs prior to incorporation into RSPs, or that this process takes place in parallel to the budding event. On the other hand, budding seems to be rapid since hardly any intermediate budding structures were visible by electron microscopy. Moreover, only a few RSPs were detected in each cell, and data from immunofluorescence experiments and Endo Hf digestions showed that the major portion of E was continuously localized to the ER. Thus, the rate-limiting step in the secretion of RSPs may be the assembly process or the lattice formation at the ER membrane rather than transport to the cell surface. This also seems to apply for the virus because the secretion kinetics are similar as for the proteins expressed recombinantly.

Expression of the TBE virus envelope proteins without other viral elements is sufficient to produce RSPs. This implies that prM and E are able to induce their budding at the ER membrane without the need for a capsid that drives budding from the cytoplasmic side of the membrane. Figure 1 shows two possible models for the incorporation of prM and E into RSPs. In model I, the prM-E proteins interact laterally with each other, which can spontaneously lead to the curvature of the membrane and eventually to the pinching-off of a virion into the lumen of the ER. Model II involves one or several cellular proteins that assist in the assembly process. These proteins may act either on the lumenal or on the cytoplasmic side of the ER.
membrane. However, it is more likely that such factors are located on the luminal side because they would be able to form a coat around the virion that can lead to the formation of a virus particle.

**Figure 1** Two Models for RSP Assembly. In model I, lateral interactions between the TBE virus envelope proteins themselves can act as driving force for the curvature of the ER membrane, leading to the budding of an RSP into the ER lumen. In model II, budding of RSPs into the ER lumen is mediated by a cellular factor (X). X, which can be either ER-soluble or membrane-anchored, would interact with the TBE virus envelope proteins to form a COP- or clathrin-like coat that is disassembled once the particle has formed completely.

The formation of big ‘clusters’ of prM and E in the ER would speak in favor of model I, but this implies that the proteins themselves contain determinants that drive assembly and budding, and that lateral interactions must be sufficient to trigger membrane fission, as suggested for coronaviruses (151). Model II requires an as yet unidentified cellular protein or protein complex that may wrap the virion in analogy to the formation of a COPII coat to produce a transport vesicle. This coat may then rapidly disassemble in the ER and lead to the release of an immature virion into the ER lumen. Given that the single N-linked glycan of E is involved in the secretion of TBE virus envelope proteins, calnexin or calreticulin are candidate molecules for this function. A proteomics screen for ER-resident proteins that interact with prM and E in vitro could reveal whether the virus-alone model or the cellular factor model apply for flavivirus assembly in the ER.
4.1.3 Transport and Secretion of TBE Virus Particles

Our biochemical and ultrastructural data show that once the immature virus particle is assembled, it is transported along the secretory pathway. This probably involves the same functions and mechanisms as for cellular secretory proteins, except that an entire particle has to be transported rather than a soluble or a transmembrane protein monomer or oligomer. As a consequence, the secretion kinetics of sE dimers, RSPs and viruses showed that transport is gradually slowed down. Endo Hf digestions also revealed that processing is incomplete if the sugar processing system has to work on an entire particle (data not shown). This is probably due to the size of the virion itself or due to spatial limitations when the sugar-modifying enzymes have to process a virion with multiple copies of prM and E.

4.1.4 Green Fluorescent Protein as Marker for Flavivirus Assembly and Transport

The rapid development of sophisticated microscopy techniques in the past years has enabled us to follow the intracellular movement of proteins in three dimensions, and to show interactions between different elements involved in a transport process. The green fluorescent protein (GFP) has become a universal molecule to study the intracellular localization and transport of proteins (149). Usually, a fusion protein between GFP and the protein of interest is generated by a relatively simple cloning procedure in which the two genes are inserted in frame next to each other into a plasmid vector. Thus, the protein of interest is "tagged" with a GFP molecule, and its intracellular localization and transport can be followed by green fluorescence under a light microscope.

In the case of flaviviruses, fusion of an envelope protein to GFP may lead to the formation of green-fluorescent RSPs, which may be a useful tool to study assembly and secretion as well as virus entry processes. However, the construction of a TBE virus envelope protein fused to GFP involved some topological constraints. To ensure proper translocation of prM and E into the ER lumen, we chose the C-terminal end of the E protein to fuse GFP with. Since GFP is a cytosolic protein, it is not sure whether it folds correctly in the ER. Moreover, if both GFP and the ectodomain of E were in the ER lumen connected by two transmembrane domains lying next to each other,
this could lead to steric hindrances that may prevent one or both proteins from folding properly. Therefore, we used the C-terminally truncated E472 protein from the SV-PE472 vector, which lacks the second transmembrane domain of E, to create an E472-GFP fusion protein. A schematic view of the fusion protein and its expected topology is shown in Figure 2.

Figure 2 TBE Virus Envelope Protein E fused to GFP. (A) Schematic representation of the polyprotein consisting of an N-terminal signal sequence, prM, E lacking the second transmembrane segment (E472, where 472 is the number of the C-terminal amino acid), a linker of 13 amino acids and the green fluorescent protein (GFP). (B) Expected membrane topology of the E472-GFP fusion protein. The E protein is expressed in the ER lumen, while GFP is located on the cytosolic side, connected to E via the membrane anchor and the linker. (C) Possible formation of GFP-containing RSPs that can be studied by live microscopy experiments.

Preliminary results from mammalian cells transfected with the newly created plasmid pPE472-GFP showed that both prM and E were able to fold correctly, forming disulfide bonds and acquiring their native conformation with the same kinetics in the presence or absence of a cytosolic GFP domain. Heterodimerization of prM and E was not impaired. Fluorescence microscopy studies demonstrated that E472-GFP localized to the ER membrane, while the GFP control experiment showed cytosolic staining. Thus, both E472 and GFP were expressed properly when fused to each other.

Unfortunately, we were not able to observe secretion of E472-GFP molecules in form of RSPs. This can be explained by a simple geometrical consideration: Based on the cryo-EM reconstruction of the TBE RSP (40), five GFP molecules would lie very close to each other at the inside of a five-fold axis where five E proteins are inserted into the lipid bilayer. The size of the GFP molecules may prevent the E proteins from laterally interacting with each other to form a pre-budding structure. Another reason for the failure of RSP formation may be that the presence of GFP on the cytosolic side of the ER membrane may block the assembly process or the interaction of prM or E with a cellular factor required for budding.
One possibility to overcome these problems would be the co-transfection of cells with both the plasmid coding for prM and E472-GFP fusion proteins and the wild-type plasmid coding for prM and E alone, or to generate a vector that contains prM-E and prM-E472-GFP. Expression of both wild-type E and E472-GFP may avoid the steric problems discussed above and lead to the formation of "hybrid RSPs" consisting of E molecules with and without fused GFP.

4.2 Outlook

Taken together, the results presented in this thesis gave an insight into the folding, assembly and secretion of flavivirus envelope proteins in mammalian cells. The first part focused on the folding and dimerization of TBE virus prM and E, while in the second part the formation of higher-order structures, the assembly into virions and their secretion was investigated.

Although this work revealed various new aspects of the biosynthesis and processing of flavivirus envelope proteins, many questions remain unanswered and await further investigation. To achieve this, TBE virus is an optimal model because the biochemical data can be directly correlated with the structural information that is available for the E protein as well as for the RSPs. In the following sections, possible experiments are suggested to continue with the studies presented in this thesis.

4.2.1 Folding Experiments

The experiments on the folding presented here showed that TBE virus prM and E fold at kinetics that were similar to envelope proteins from other virus families, and that prM was required for proper maturation of E. This study could be expanded by the analysis of folding intermediates that contain partially folded domains. This could be achieved by lowering the incubation temperature in pulse-chase experiments to slow down the folding process. However, this may also influence other cellular processes that are involved in the folding process and lead to misfolded or unfolded proteins. The order of disulfide bond formation may be assessed by the generation of cysteine mutants of prM and E. With the additional information on the three-dimensional structure of E, this may lead to a detailed model of the timing of E protein folding.
Another set of experiments could focus on the cleavages at the junctions between the structural proteins of flaviviruses. The study of these processes may be facilitated by use of microsomes rather than living cells. They would provide a more isolated environment with less interfering factors, but the proper folding and maturation of prM and E would first have to be assessed in this system.

The question whether and which cellular chaperones are required for the folding of prM and E is probably one of the most interesting aspects that remain to be elucidated on the biosynthesis of flavivirus envelope proteins. Since our experiments showed that the epitopes on the proteins are highly sensitive to denaturation and heat treating, milder conditions should be tested for the dissociation step between the two immunoprecipitations. Alternative techniques, such as crosslinking, immunoblotting or immunoaffinity columns should also be applied. Moreover, a screen for more ER chaperones that bind to prM and E may help to identify new components involved in glycoprotein folding.

4.2.2 Assembly Experiments

The actual process of assembly, which probably involves homodimerization between two E molecules at a certain stage, and the acquisition of higher-order structures could not be shown in this study, possibly due to technical limitations. Biochemical studies, such as further crosslinking experiments and sucrose velocity gradient centrifugation to assess the sedimentation coefficient of the intracellular structures would be required to gain further insights into the oligomerization processes. The development of new microscopy techniques, such as fluorescence resonance energy transfer (FRET), may also be applied to show the oligomerization of E. Alternatively, a genetic approach by site-directed mutagenesis could reveal amino acids or regions that are important for oligomerization.

The molecular mechanisms of flavivirus budding at the ER membrane remain to be elucidated. It would be highly interesting to find out what the driving force for budding is. The two models presented here could be verified to check whether a cellular factor assists the budding process, or if the envelope proteins themselves are able to trigger the fission of the membrane. The experimental approaches to address this question probably require large screens for cellular proteins (as discussed above)
or the setup of an *in vitro* system, in which the budding process can be reconstituted under well-defined conditions.
5. References


Acknowledgments

It’s done – at last! After three and a half years, I successfully defended my PhD thesis in an atmosphere that was actually quite relaxed and enjoyable. Being a PhD student means a lot of work, but my fascination for viruses and cell biology was always strong enough to keep me going, not to mention all the fun. I would like to acknowledge some people without whom this thesis would never have been possible:

First of all, I would like to thank Ari Helenius for all his guidance and his continuous support during my PhD studies. I have never met a scientist before who combined deep thinking about scientific problems and the creativity to solve them with such a personality and a good sense of humor. Although busy throughout the day, it was always possible to walk into his office with a question or an interesting result. I would also like to thank him for giving me the freedom to pursue my hobby – playing the piano – even at times when I actually was supposed to be in the lab. And I will never forget the legendary lab parties at his home!

Next, I am highly thankful to Franz Heinz, who made my thesis project possible in the first place. His thorough knowledge of the flaviviruses, as well as his advice for working with the TBE virus system were indispensable. My stay at his lab in Vienna to work with the infectious virus was one of the highlights of my PhD studies. It is also where I realized that top-level science could be combined with great hospitality and cordiality.

I am grateful to Josef Brunner for taking the duty as a co-examiner and for giving me some insights into the biochemical aspects of virology. I greatly appreciated his support of my present work, as well as his interest in my future plans.

Next, I would like to say a big ‘thanks!’ to Steve Allison whose competence and helpfulness are unbeatable! Whatever question or problem arose – he always had an answer or a solution. While working in Vienna, I had the privilege to have a kind of ‘personal assistant’ who was able to anticipate my every wish...

I want to thank all the members of the Helenius group for the pleasant atmosphere we had inside and outside the lab. There was always somebody around to share the ups and downs that arise during a PhD. I particularly would like to thank Roberta,
Anna, Paola, Marianne, Andrea, Kowi, Lucas, Vondi and Srini for their support and advice. I am also thankful to Mauri for acquainting me with the pulse-chase techniques at the beginning of my thesis.

The next ones to mention are Toni, Rolf and Tomas: a thousand thanks for your invaluable help (almost 24 hours per day) with technical stuff or computer troubles!

I also would like to thank the people from Franz Heinz’ group in Vienna for having integrated me in their group for five weeks as if I had worked with them for years. I had such a great time there that I really would like to go back one day. I am particularly grateful to Karin, Silvia, Angela, Walter, and Stefan for their help and their friendship.

I am also thankful to Jürgen Kartenbeck who spared no effort to come to Zurich to work personally on my samples. His work complemented my project ideally, and I thank him for the many hours he spent at the microscope searching for budding virus particles at the ER membrane!

Beat Amstutz was the first student who worked under my supervision. I thank him for the E-GFP fusion protein he generated during his semester work, and for his creativity in producing these green fluorescent particles...

My band ‘Chili Drive’ had a great influence on the success of this thesis. The many hours we spent in our cold and humid rehearsal room, and of course the concerts we gave were very important to keep me in a good mood. Thanx, Nadja, Sämi, Luc, Stephan, Marc, Marco, Flo, and Claude – keep on groovin’!

I would like to thank Samuel Nef, Oliver Horváth and Thomas Schubiger for their long-standing friendship, for their advice and help whenever it was needed.

Nathalie Constantin came into my life towards the end of my thesis. With her charme and her smile, she was able to make me laugh even when everything else was going wrong. Merci, Nathalie – je t’aime.

Last, I would like to thank my parents and my whole family for their continuous support and love.
Curriculum Vitae

Name: Ivo Lorenz
Date of birth: 24 February 1973
Nationality: Swiss
Marital status: Single
Address: Hochstrasse 35
8044 Zürich
Parents: Fredy Lorenz, Civil Engineer ETH
Christine Lorenz-Frey, Secretary

Education

7/1998-12/2001 PhD at the Institute of Biochemistry, ETH Zürich, Switzerland
Group of Prof. Ari Helenius
Degree: Dr. sc. nat. ETH

10-11/2000 Stay at the Institute of Virology, University of Vienna, Austria
Group of Prof. Franz X. Heinz
EMBO Short-Term Fellowship Awardee

10/1993-3/1998 Studies in Biology at the ETH Zürich, Switzerland
Degree: Dipl. Natw. ETH
Topics: Biochemistry, Molecular Biology, Cell Biology, Immunology, Anatomy and Physiology

1996 - 1997 Semester Work and Diploma Thesis at the Institute of Medical Virology, University of Zürich, Switzerland
Group of Prof. Karin Mölling

1996 Semester at the Université Paris VII Denis Diderot, France
Scholarship from the ERASMUS European student exchange program

1995 - 1998 Part-time job at CREDIT SUISSE, Zürich
Department `Process and Information Management`

1986 - 1993 Gymnasium Kantonsschule St. Gallen, Switzerland
Matura Typus B (Realgymnasium)

1980 - 1986 Primary School St. Gallen, Switzerland