Interaction between molecules of hantavirus nucleocapsid protein

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Intermolecular interactions of Tula hantavirus N (nucleocapsid) protein were detected in the yeast two-hybrid system, prompting further attempts to study this phenomenon. Using chemical cross-linking and immunoblotting it was shown that the N protein from purified virus and from infected cell lysates as well as recombinant protein produced in a baculovirus expression system are capable of forming dimers, trimers and multimers, thus confirming the capacity of the protein molecules to interact with each other. An ELISA format was developed in which molecules of the recombinant N protein were shown to associate non-covalently, via electrostatic interactions. Divalent cations (Ca$^{2+}$, Mn$^{2+}$, Mg$^{2+}$, Ba$^{2+}$) enhanced the process 3- to 8-fold suggesting that adequate folding of the N protein is crucial for the association. Based on these data a model for hantavirus nucleocapsid assembly is proposed, in which N molecules first trimerize around the viral RNA molecule, and then the trimers gradually assemble forming longer multimers.

Introduction

Hantaviruses (genus Hantavirus, family Bunyaviridae) are enveloped viruses with a segmented, single-stranded, negative-sense RNA genome. S (small), M (medium) and L (large) genome segments encode, respectively, the nucleocapsid (N) protein, two surface glycoproteins (G1 and G2), and the viral polymerase (L) (for review, see Plyusnin et al., 1996). At present, 23 different hantavirus types are known, each associated with a specific rodent host (Elliott et al., 2000). When transmitted to humans, some hantaviruses cause haemorrhagic fever with renal syndrome or hantavirus pulmonary syndrome; others seem to be apathogenic.

Similar to other Bunyaviridae, hantaviruses replicate in the cytoplasm of host cells; the Golgi complex is the site of assembly for most hantaviruses (Plyusnin et al., 1996) although Sin Nombre virus (SNV) and Black Creek Canal virus (BCCV) may also assemble at the plasma membrane (Goldsmith et al., 1995; Ravkov et al., 1997). The L protein of hantaviruses is thought to be responsible for all steps of viral RNA transcription and replication. G1 and G2 glycoproteins are thought to take part in the recognition of receptor(s) on host cell surfaces. Although the nature of receptor(s) remains unknown, it has recently been shown that $\beta_3$ and $\beta_1$ integrins facilitate the entry of hantaviruses into endothelial cells (Gavrilovskaya et al., 1998, 1999). The N protein wraps genomic RNA segments into three viral chromosomes and creates a protective shield for the viral genome. Recombinant N proteins of Hantaan virus (HTNV) and Puuma virus (PUUV) have been shown to bind specifically in vitro transcribed viral RNA (Severson et al., 1999). The carboxy-terminal 100 amino acids (aa) of the recombinant N protein of PUUV were shown to be involved in the RNA binding (Gött et al., 1993). In hantavirus-infected cells N protein is expressed in excess and forms aggregates and filamentous structures in the cytoplasm (Vapalahti et al., 1995; Ravkov et al., 1998). Recently, BCCV N protein was shown to be a peripheral membrane protein (Ravkov & Compans, 2001). The antigenic epitopes are distributed along the N protein molecule; and the main B-cell epitopes are located in the amino-terminal part of the protein (Lundkvist et al., 1996; Vapalahti et al., 1996b).

The aim of this work was to study interaction(s) between molecules of hantavirus N protein. TULV and PUUV (Vapalahti et al., 1992; Plyusnin et al., 1994) were used as model agents. Complete genomes of these two hantaviruses have been cloned and sequenced (Vapalahti et al., 1992, 1996a; Piiparinen et al., 1997; Kukkonen et al., 1998) and several monoclonal antibodies (MAb) have been raised against their N protein antigens (Lundkvist et al., 1991, 1996), providing useful tools for molecular studies.
Methods

**Yeast two-hybrid assays.** The yeast two-hybrid system contained a GAL4 DNA binding domain vector (pGBT8) and a GAL4 transcription activator domain vector (pGAD-GH) (Pharmingen). The cDNA fragment representing the N proteins of PUUV, TULV and DOBV (Dobrava hantavirus) was generated by PCR from corresponding cDNA clones (Vapalahti et al., 1992, 1996a; Nemirov et al., 1999). The primers used included the 5’ and 3’ ends of the N ORF and restriction sites for BamHI and XhoI (TULV N) or NcoI and SacI (PUUV N, DOBV N) as follows. TULV N forward, ttgacattgatgcaaccactgaagat; reverse, aagctgtaagattttaaggtt. PUUV N forward, tctcatggtgactgacagact; reverse, aagactctatcatatgaagctctg. DOBV N forward, ttcatggcaacactagaggaact; reverse, aagagcttaacagcttgagggctcgt. The ORF encoding N protein of TULV was amplified with primers containing BamHI and XhoI restriction sites and was cloned into pGAD-GH. The N protein hybrid of TULV and PUUV (TULV/PUUV N) was created by replacing the NcoI–SacI fragment encoding aa 308–410 of plasmid TULV N/pGBT8 with the corresponding DNA fragment from plasmid PUUV N/pGBT8.

*Saccharomyces cerevisiae* strain Y166 was cotransformed with pGAL1 and pGAD GH constructs by the lithium acetate method (Gietz et al., 1992) and selected for tryptophan and leucine prototrophy on appropriate minimal media. Expression of GAL4 fusions was controlled by immunoblotting with DBD and AD Mabs (Clontech). β-Galactosidase activity was assayed by transferring colonies on selective media containing 200 µM 5-bromo-4-chloro-3-indolyl β-d-galactopyranoside (X-Gal) and growing them at 30 °C for 7 days. Interaction between proteins were recorded by eye when blue colonies appeared on the plate.

**Virus purification.** PUUV and TULV were purified from infected Vero E6 cells by centrifugation through a 10–60% (w/v) sucrose density gradient (Vapalahti et al., 1996a). n-Octyl β-d-glucopyranoside (Sigma) (final conc. 0.5%) was used for permeabilization of virus envelopes.

**Cross-linking of N protein samples.** This was done by using bis(sulfo)succinimidyl suberate (BS) 4 (Pierce). A 10 µl sample of N protein (approx. 1 mg/ml) was incubated with 0.25–0.4 mM BS 4 in PBS (30 min, room temperature). The samples were treated with reducing sample buffer (0.125 M Tris pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.01% bromphenol blue) and after heating (95 °C, 5 min) the proteins were separated on SDS–10% acrylamide gels. Immunoblotting through a 25 gauge needle; the lysate was centrifuged at 16000 × g for 1 h, room temperature) and with TULV and 14 days with PUUV, cells were collected in 1 ml of 25 mM Tris pH 8, 50 mM NaCl, 1 mM EDTA, 1% NP-40 with an EDTA-free cocktail of protease inhibitors and 1.5 ml aliquots were stored at −20 °C.

**Recombinant N (recN) protein purification.** Recombinant PUUV and TULV N proteins were expressed by using the baculovirus system in Sf9 insect cells (Vapalahti et al., 1996b; Lundkvist et al., 1996). Sf9 cells were suspended (50%, v/v) in PBS with an EDTA-free cocktail of protease inhibitors and 1.5 ml aliquots were stored at −70 °C. Cells were thawed (1.5 ml) and collected by centrifugation at 9000 g for 5 min. Cells were washed three times with 1 ml 3 M urea–TEN and resuspended by pipetting. After the final wash the cell lysate was resuspended with 1 ml 8.5 M urea–TEN and incubated 30 min at room temperature. Then 200 µl TEN was added to the suspension and the sample was pelleted at 9000 g for 10 min. The supernatant was recovered. The cell pellet was re-extracted with two pellet volumes of 6 M urea–TEN and centrifuged as above. The supernatant was combined with the previous one. The obtained N protein extract was stored at −20 °C and used in the ELISA format. For further purification the N protein extract was diluted 10-fold with 10% ethyleneglycol–PBS and incubated by rotation with 3 ml of PBS-washed heparin–Sepharose CL-4B (Pharmacia) (50%, v/v) slurry for 30 min at 4 °C. Heparin–Sepharose was packed on a column, allowed to settle and then rinsed with 5 ml 10% ethyleneglycol–PBS. Bound N protein was eluted with 1 M NaCl–TEN by collecting 500 µl fractions. Protein-containing fractions were pooled and total protein concentration was determined by BCA-200 protein assay kit (Pierce). The purity of the N protein preparations was checked by SDS–PAGE.

**Cross-linking of the recN protein.** RecN proteins of TULV and PUUV (1 mg/ml) were incubated in 8 M urea, 20 mM dithiothreitol (DTT), TEN buffer (1 h, room temperature). Lodoacetamide (IAA) was added to a final concentration of 50 mM and incubation was continued for 30 min in the dark. These N protein preparations were dialysed in PBS overnight and concentrated to about 1 mg/ml with Ultrafree-0.5 centrifugal filters (Millipore). Cross-linking of RecN protein was carried out as described above. The cross-linked proteins were treated with reducing sample buffer and analysed by immunoblotting and by Coomassie staining.

**The ELISA format.** The urea extract of PUUV recN protein was diluted to a concentration of 2 mg/ml in PBS pH 7.5 and 100 µl of the solution was added to wells of a 96-well plate (Polysorp; Nunc). The plate was incubated at 4 °C overnight. Control wells were coated with 1% BSA–PBS pH 7.5. To obtain one layer of N proteins wells were washed with 6 M urea–PBS pH 7.5 and rinsed three times with 1% BSA–PBS pH 7.5. PUUV N-coated wells were blocked with 1% BSA–PBS for 1 h at 37 °C and washed three times with incubation buffer (25 mM Tris pH 8, 50 mM NaCl, 1% BSA). The urea extract of TULV recN protein was diluted to 1 mg/ml with incubation buffer containing the indicated amounts of NaCl, CaCl2, MnCl2, MgCl2, BaCl2 or EDTA. TULV N protein solution (100 µl) was added to the PUUV N- or BSA-coated wells and incubated for 1 h at room temperature. Then wells were washed three times with incubation buffer and incubated with 100 µl TULV N-specific MAb 3D3 (0.4 mg/ml) (Lundkvist et al., 1996) (diluted 1/1000) for 1 h at room temperature. After a washing step incubation was continued with 100 µl of HRP rabbit anti-mouse antibody (Dako) (diluted 1/2000) for 1 h at room temperature. After final washings 100 µl of TMB substrate solution (Sigma) was added to wells and incubated for 15 min at room temperature in the dark. The reaction was stopped by adding 100 µl 0.5 M sulphuric acid and A405 values were determined during the next 30 min. Four parallels were done for each experiment.

**Results**

**Study of hantavirus protein interactions in the yeast two-hybrid system.** We started with the yeast two-hybrid system (Fields & Song, 1989), which has been used successfully to discover numerous protein–protein interactions. TULV N protein fused to the GAL4 DNA-binding domain (DBD) was found to interact with TULV N fused to the GAL4 activation domain.
Table 1. Hantavirus protein interactions in the yeast two-hybrid system

The indicated hantavirus proteins were coexpressed in yeast cells and positive interaction was recorded by eye when blue colonies appeared on X-Gal plates.

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<th>GAL4–DBD</th>
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(AD) indicating homotypic interaction (Table 1). The same was seen when another yeast two-hybrid assay, LexA–VP16, was used (data not shown), indicating that the N–N interaction is not dependent on the experimental system selected. Immuno-blotting was used to check that the DBD–N protein and the AD–N protein were properly expressed in yeast.

Further, the interaction in the GAL4 system was observed between the N protein of TULV and a chimeric TULV/PUUV protein, in which the region comprising aa 304–406 of the TULV N protein was replaced with the corresponding region from the PUUV N protein (this replacement was equivalent to an introduction of only seven substitutions at aa positions 308, 311, 362, 390, 391, 392 and 394). Moreover, when the whole molecule of PUUV N protein was used as another ‘partner’ for the TULV N protein, binding of these two molecules occurred also. Finally (and unexpectedly), the N protein of TULV was shown to be capable of interacting with the N protein of DOBV, which is more distant from TULV than is PUUV. According to the colour intensity of positive yeast colonies on the X-Gal plates, the strength of all these interactions was almost equal. These results suggested that interaction(s) between the hantavirus N protein molecules might occur via either highly conserved stretches of aa residues or conserved domain(s) of secondary or tertiary structure (see Discussion).

Study of N protein assembly

To study assembly of the N protein molecules, a chemical cross-linking technique was applied. Purified PUUV and TULV were treated with increasing concentrations of BS<sup>3</sup> (a non-cleavable, homobifunctional cross-linker, which reacts with amino groups in the target protein and is suitable for intermolecular cross-linking, having a spacer arm length of 11±4Α); the products were analysed by SDS–PAGE, and the cross-linked N protein was visualized by immunoblotting (Fig. 1A). In the absence of cross-linker, N protein from the purified virus appeared to be in a monomeric form (when analysed by SDS–PAGE without 2-mercaptoethanol reduction) (Fig. 1A, lane 1). When the cross-linker was applied, the N protein...
assembled into dimers and trimers, and further into higher molecular mass products; the effect seemed to increase with increasing concentration of BS$^3$ (Fig. 1A, lanes 2–7). The same was seen on purified TULV (data not shown) and on cytoplasmic fractions of infected cells (Fig. 1B, C). While without the cross-linker only the N protein monomer was seen, in the presence of BS$^3$ new bands corresponding to dimers, trimers and aggregates appeared. These results suggested that in the viral particles as well as in the infected cells the N protein molecules are kept in close proximity and, therefore, can be cross-linked. Separation by 8% SDS–PAGE clearly showed that the cross-linking products were dimers, trimers and higher multimers; tetramers were not found (data not shown). The monomer and trimer bands seem to appear frequently as doublets; the lower band may be a degraded form of the N protein. A stronger trimer band than dimer band suggests trimers as assembly intermediates during the encapsidation process.

**Characterization of the N–N interaction(s) using an ELISA format**

To gain more insight into the factors that influence interaction(s) between the N protein molecules, the ELISA format was developed. We took advantage of the fact that the N proteins of TULV and PUUV, being closely related and thus capable of reacting with each other, can be specifically recognized by non-cross-reactive MAbs (Lundkvist et al., 1991, 1996). Control experiments showed no reactivity of PUUV recN with TULV-specific MAb 3D3 in the ELISA format used. Because of the relatively low growth titres, preparation of the hantavirus N protein either from virions or infected cells was not feasible. Thus, for the ELISA format TULV and PUUV recN proteins were produced in a baculovirus expression system, and purified by urea extraction. A similar protocol has previously been used to purify HTNV and SNV N proteins for RNA binding experiments (Severson et al., 1999). Although TULV and PUUV recN proteins preserved all the epitopes recognized by two panels of MAbs (Lundkvist et al., 1996; Vapalahti et al., 1996b; our unpublished data) additional characterization of the recN preparations was performed.

Under non-reducing conditions the purified recN was seen as monomers (50 kDa), but also as dimers (100 kDa), trimers (150 kDa) and higher molecular size aggregates (> 200 kDa) (Fig. 2A, lanes 1 and 2). When recN was treated with 2-mercaptoethanol, it was seen only as monomers (data not shown). This indicates that recN molecules are linked together by covalent intermolecular S–S bridges between cysteine residues. When treated with DTT to break the disulphide bridges and subsequently with IAA to prevent oxidation of the free -SH groups, the recN molecules were converted into the monomeric form seen on the non-reducing gel (Fig. 2A, lanes 3 and 4). The treated (i.e. the monomeric) recN proteins are supposed to be in the same conformation as the viral N protein...
under normal (i.e. reducing) conditions in cell cytoplasm. In the cross-linking assay performed with the monomeric TULV and PUUV recN they behaved similarly to the N proteins from purified virus or infected cells (Fig. 2B, C). These results plus ELISA experiments suggest that the majority of recN proteins are folded correctly. Indeed, when some of the ELISA experiments described below were repeated with DTT- and IAA-treated forms of TULV and PUUV recN proteins, the treated and untreated preparations reacted in the same way thus confirming that the observed capacity of the recN for N–N interactions is equal to that of its monomeric form.

First, to study the nature of the N protein interactions the effect of ionic strength was evaluated. In the presence of increasing concentrations of NaCl the attachment of TULV recN to PUUV recN decreased drastically, and 1 M NaCl prevented it almost totally (Fig. 3A), suggesting non-covalent electrostatic interaction(s) between the molecules. Homotypic interaction between N protein molecules was further confirmed using 125I-labelled PUUV recN protein in the ELISA assay: increasing the ionic strength decreased the N–N interaction (data not shown).

Next, the effect of Ca$^{2+}$ on the hantaviral N–N interaction was studied, since the hantavirus N protein contains near the carboxy terminus an aa stretch which resembles the Ca$^{2+}$-binding domain of some viral proteins (see Discussion), and since Ca$^{2+}$ has been shown to be important for maintaining the structural stability of several viruses, including bovine papillomavirus type 1 (Paintsil et al., 1998) and simian virus 40 (Sandalon & Oppenheim, 1997). In our ELISA format the attachment of TULV recN to PUUV recN was enhanced 4-fold

Fig. 3. Effect of NaCl and divalent cations on N–N interaction in the ELISA format. TULV recN was incubated with various concentrations of NaCl (A), or Ca$^{2+}$, Mn$^{2+}$, Mg$^{2+}$ or Ba$^{2+}$ ions (B) in PUUV recN-coated wells. Binding of TULV recN was recorded as $A_{450}$ values.
Fig. 4. Effect of divalent cations and EDTA treatment on N–N interaction. TULV recN was incubated simultaneously with the indicated amounts of Ca$^{2+}$ ions and EDTA in PUUV recN-coated wells. A similar effect of the EDTA was observed with other divalent cations (A). TULV N protein was first allowed to bind to the PUUV N protein in the presence of 20 mM Ca$^{2+}$, Mn$^{2+}$, Mg$^{2+}$ or Ba$^{2+}$ ions and then EDTA was added to the final concentration of 0–2, 2 or 20 mM (B). Bound TULV recN was recorded as $A_{450}$ values. Variation between the control signals in the separate ELISA assays might be due to different batches of N protein preparations, which had undergone repeated freezing–melting cycles.

by 20 mM Ca$^{2+}$. However, other divalent cations (Mn$^{2+}$, Mg$^{2+}$ and Ba$^{2+}$) had a similar effect (Fig. 3B). At 20 mM, Mn$^{2+}$ and Ba$^{2+}$ enhanced binding at least 4-fold and Mg$^{2+}$ by 3-fold compared to the basal level. The interaction started to decrease when the cation (Ca$^{2+}$, Mn$^{2+}$, Mg$^{2+}$ or Ba$^{2+}$) concentration exceeded 20 mM and fell to background level at 75 mM (data not shown). Thus, divalent cations facilitated the N–N interactions, suggesting that their binding can either stabilize or induce the N protein conformation favourable for homotypic interaction. Another possibility might be that the divalent cations directly link N molecules together.

The stimulatory effect of the divalent cations was abolished by EDTA when added together with TULV recN (Fig. 4A). While 0–2 mM EDTA had no effect, higher concentrations (2 and 5 mM) reduced the interaction to the basal level seen without the cation. However, when added to the preformed complex of PUUV recN/TULV recN, the EDTA, even at higher concentrations, had no apparent effect (Fig. 4B).
suggested that a cation in the N–N complex is inaccessible to EDTA, or its presence is crucial only during the initial dimer/oligomer formation, and that as soon as the interaction has occurred, removal of the cation has no effect.

**Discussion**

**Interactions between hantavirus proteins studied in the yeast two-hybrid system**

In this paper evidence for homotypic interaction between N proteins of hantaviruses is presented. This is in line with observations made on the dimerization and oligomerization capacity of capsid proteins of hepatitis C virus (HCV) (Matsumoto et al., 1996), bovine respiratory syncytial virus (BRSV) (Krishnamurthy & Samal, 1998), Marburg virus (Becker et al., 1998) and Sendai virus (Myers et al., 1997). The study of hantavirus N protein interactions was initiated by using the GAL4-based yeast two-hybrid system in which self-association of TULV N protein was observed. The homotypic interaction was verified independently using another yeast two-hybrid system (LexA–VP16) and a mammalian two-hybrid system in HeLa cells (our unpublished observations), suggesting that the observed phenomenon reflects real interactions occurring in viral particles and/or in infected cells.

Furthermore, it was shown that TULV N protein interacted with PUUV and DOBV N proteins in the GAL4-based yeast two-hybrid system. The overall identity at the aa level, when TULV N protein is compared to PUUV and DOBV N proteins, is 79% and 62%, respectively. However, some regions of the three sequences are highly conserved: e.g., the stretch that includes aa 150–200 is 92% identical for TULV N/PUUV N and 74% identical for the pair TULV N/DOBV N. Considering homologous aa substitutions the corresponding similarity values are even higher: 100% and 96%. One could assume that putative interacting domain(s) in the N protein molecule are formed by such highly conserved regions.

**Hantavirus N protein molecules interact with each other electrostatically, and divalent cations enhance the interaction**

Protein interactions detected in the yeast two-hybrid system can be either non-covalent or covalent. To characterize the N–N interaction further, BS3 cross-linking was applied. This cross-linker has been used to investigate, for example, the architecture of the human immunodeficiency virus transmembrane protein gp41 (McNerney et al., 1998) and herpes simplex virus glycoproteins (Handler et al., 1996). Using BS3-directed cross-linking it was shown that the N protein from purified virus and from virus-infected cell lysate, present initially in a monomeric form, was able to assemble into dimers and trimers and, to some extent, also to high molecular mass multimers. These results suggest that the interaction between the N protein molecules keeps them in close vicinity, which is required for efficient cross-linking.

It cannot be completely ruled out that the observed dimers and trimers might be N protein adducts with other cellular or viral proteins (Figs 1 and 2). Coomassie staining clearly showed that other proteins in the recN preparation were almost undetectable (Fig. 2B, lanes 1 and 5) and immunoblotting of the cross-linked virus or cell lysate proteins (Fig. 1) shows a pattern similar to that of cross-linked recN protein. Thus, the 100 and 150 kDa bands most likely represent products of N–N cross-linking.

Similarly, HCV core protein was shown to form multimers in vivo: when the molecules were cross-linked with glutaraldehyde, dimers and trimers were observed; when the treatment time was longer, the amount of monomeric protein decreased and the formation of larger protein complexes increased (Matsumoto et al., 1996). Thus, with respect to multimerization capacity, the TULV and PUUV N proteins resemble HCV core proteins.

It was observed that the recN protein, produced in insect cells infected with recombinant baculovirus, spontaneously formed trimers and oligomers via covalent disulfide bonds, in contrast to the N protein from purified virus or infected cells, which is seen solely in a monomeric form. However, this feature of the recN (which might be explained, e.g., by overproduction in the baculovirus-based system) did not seem to interfere with its capacity for the N–N interactions exploited in the ELISA format, as both recN and a monomeric form of recN proteins reacted equally in that system. It also did not reduce the ability of recN to be recognized by two panels of MAbs (Lundkvist et al., 1996; Vapalahti et al., 1996 b; our unpublished data). We therefore believe that the results obtained with recN are relevant to properties of the native N protein.

Using the ELISA format two important features of the N–N interactions were found. First, the observed interaction was electrostatic, i.e. non-covalent. Second, the interaction was enhanced in the presence of the divalent cations. The most plausible explanation is that intermolecular interactions are facilitated by proper folding of a cation-binding domain in the hantavirus N protein in a way similar to what was observed for Rauscher murine leukemia virus, in which the binding of Co$^{2+}$ and Zn$^{2+}$ induced folding of the unique metal-binding domain in the nucleocapsid protein (Green & Berg, 1990).

One may hypothesize that the interactions between recN molecules could somehow be mediated by traces of cellular RNA from the protein preparations. Our preliminary results, however, show no enhancement of the N–N interactions by heterologous RNA.

It is worth mentioning that near the carboxy terminus of the hantavirus N protein there is a stretch of aa residues which resembles the sequence of the Ca$^{2+}$-binding domain in the VP1 protein which maintains the structural integrity of the viral capsids of avian and murine polyomaviruses (Haynes et al., 1993; Rodgers & Consigli, 1996). This region in the hantavirus N protein (aa 405–416 in PUUV, and aa 401–412 in TULV)
Model for hantavirus N–N interaction

In summarizing our observations so far, it can be hypothesized that during formation of a viral nucleocapsid the N molecules first trimerize (via electrostatic interactions) around the viral RNA molecule, and then these trimers gradually assemble forming longer multimers. One possibility is that three N molecules form a trimmer directly; this then attaches to the viral RNA, the next trimer interacts with the first one, etc. Alternatively, two molecules of the N protein first form a dimer, which then associates with the third molecule available in a monomeric form. Electrostatic interactions might play a role at all steps, and interaction(s) with the viral RNA might assist in correctly orienting the N protein monomers and/or dimers as well as trimers. Divalent cation(s) induce proper folding of the N protein molecules thus facilitating their interactions. We favour the first model as, in our cross-linking experiments, mostly N protein trimers were formed while dimers were in the minority.

During preparation of this manuscript we became aware of a study on SNV N protein oligomerization (Alfadhlí et al., 2001). In that paper the yeast two-hybrid system, sucrose gradient centrifugation and chemical cross-linking technique were used to study N protein assembly. By using the yeast two-hybrid system the SNV N protein homotypic interaction domains were mapped to the N-terminal 40 aa and to the C-terminal half of the N protein. Furthermore, the SNV N protein, after bis-maleimidohexane cross-linking, was found to associate as dimers, trimers and large multimers. These data are in perfect agreement with our findings about TULV and PUUV N protein dimerization and trimerization as well as with our data obtained with a mammalian two-hybrid system, suggesting that the interacting domains of TULV N protein are located within the last 125 aa and in the amino terminus.

We thank Dr Åke Lundkvist for the monoclonal antibodies and for helpful comments. The Haartman Institute and Biocentrum Helsinki yeast two-hybrid core facility, especially Dr Tomi Makela and Ms Kirs Mänttäri, are acknowledged for help with yeast two-hybrid assays. This work was supported by EU contract BMH4-CT97-2499 and Sigrid Jusélius Foundation, Helsinki.

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Received 31 January 2001; Accepted 25 April 2001