Tula hantavirus L protein is a 250 kDa perinuclear membrane-associated protein

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INTRODUCTION

Tula virus (TULV) (genus Hantavirus, family Bunyaviridae) is carried by the European common vole, Microtus arvalis (Plyusnin et al., 1994). The hantavirus genome consists of three negative-strand RNAs: S (small, 1844 nt in TULV), M (medium, 3644 nt in TULV) (Vapalahti et al., 1996) and L (large, 6541 nt in TULV) (Kukkonen et al., 1998). The reverse complements of the genomic RNAs encode the viral proteins: S cRNA encodes the nucleocapsid protein (N), M cRNA the glycoproteins G1 and G2 and based on studies done with other Bunyaviridae, L cRNA is thought to encode a single L protein (Elliott et al., 2000).

Of the four hantavirus structural proteins, the localization of the two glycoproteins and the N protein has been studied. G1 has been found to have a Golgi retention signal that directs both glycoproteins G1 and G2 to the virus assembly site on the membranes of the Golgi into which the virus buds (Shi & Elliott, 2002). The Seoul hantavirus (SEOV) G2 accumulates in the Golgi from 8 h post-infection (p.i.) onwards (Kariwa et al., 2003). The budding of the virus requires the presence of the two glycoproteins and the N protein at the virus assembly site, since these are the viral proteins required for formation of virus-like particles (Bettenbaugh et al., 1995).

The N protein, which encapsidates RNA, is required for RNA synthesis together with the L protein in a Hantaan virus minireplicon system (Flick et al., 2003), thus being the only viral protein required for both virus budding and RNA synthesis. The N protein of Black Creek Canal (BCCV), Seoul (SEOV) and Puumala (PUUV) hantaviruses is expressed in the perinuclear region, and BCCV N protein was further reported to co-localize with Golgi markers (Ravkov & Compans, 2001). In another study, the N protein of SEOV was reported not to co-localize with the Golgi markers but to surround the Golgi (Kariwa et al., 2003). The BCCV N protein was found to be associated with membranes in an assay in which microsomal membranes were separated from cytosolic proteins (Ravkov & Compans, 2001). Of other Bunyaviridae, the N protein of Crimean–Congo haemorrhagic fever virus (CCHFV), genus Nairovirus, was found to be perinuclear but it did not localize in the Golgi (Andersson et al., 2004). The CCHFV N protein interacts with actin, and the disruption of actin filaments abolished the perinuclear localization of the protein (Andersson et al., 2004). The BCCV N protein was also found to interact with actin (Ravkov et al., 1998).

L protein is the putative RNA transcriptase and replicase of the virus. In contrast to the N protein and the glycoproteins, very little is known about it. Hantaan virus L protein has been detected only in virions purified from the growth medium of metabolically labelled Vero E6 cells and the size of the protein was reported to be 200 kDa (Elliott et al., 1984). However, the protein has not been detected in hantavirus-infected cells. Therefore, it is not known whether the whole L ORF is used to encode a protein and whether there is post-translational cleavage of the protein product.
It is known that RNA replication of all positive-strand viruses is associated with membranes (den Boon et al., 2001). Positive-strand RNA viruses cause the formation of vesicles and appressed membranes that sequester viral RNA replication intermediates; this parallels the genome replication of double-stranded RNA viruses and retroviruses (Schwartz et al., 2002). The poliovirus RNA polymerase forms oligomeric arrays on membrane surfaces (Lyle et al., 2002). Of negative-strand viruses, the L RNA polymerase protein of Sendai virus has also recently been found to form oligomers (Smallwood et al., 2002; Cevik et al., 2003) but whether these are associated with membranes remains to be determined. The localization of influenza A virus RNA polymerase subunits of negative-strand viruses has been studied (Akkina et al., 1987) and for La Crosse virus, genus Bunyavirus, family Bunyaviridae, the site of RNA synthesis has been localized to the cytoplasm (Rossier et al., 1986), but there have been no studies on the localization of any hantavirus L protein or the possible membrane association of the protein.

In this report, we describe the development of antibodies against TULV L protein and the detection of the protein in virions, infected cells and transfected cells expressing recombinant L protein. These antibodies enabled the monitoring of the protein expression levels, determination of its size, and they were used to study the membrane association of the L protein. The localization of the L protein in cells was further characterized using expression of autofluorescent fusion proteins.

**METHODS**

**Viruses and cells.** The TULV strain used was Tula/Moravia/ Ma5302V, which originates from *Microtus arvalis* (Vapalahti et al., 1996). A inoculum of 0.1 f.u. per cell was used for infections. Recombinant vaccinia virus expressing T7 RNA polymerase, vTF7-3 (Fuerst et al., 1987), was a gift from Dr Bernard Moss (Laboratory of Viral Diseases, NIAID, National Institutes of Health, Bethesda, USA). Vero E6 and HeLa cells were used for transfection studies. For the concentration of TULV and PUUV, growth medium of infected cells was collected, filtered through a 0.22 μm filter, the viruses were pelleted by centrifugation (80000 g, 3.5 h, 5 °C) and the pellets were dissolved in a buffer containing 50 mM Tris/HCl pH 7-5, 100 mM NaCl, 1 mM EDTA.

**PCR and cloning.** For antigen production, the L ORF was PCR amplified from the vector pET15b (Novagen) under a T7 promoter. This created the three constructs: pET15b/La (nt 37–2190, aa 1–718), pET15b/TLb (nt 2191–4344, aa 719–1436) and pET15b/Lc (nt 4345–6495, aa 1437–2153). For localization studies, the constructs pTM1/L, pTM1/EGFP–L, pTM1/L–EGFP, pTM1/EGFP and pcDNA3/S were made. The L ORF was cloned into the vector pTM1 (Moss et al., 1990) to create pTM1/L. The EGFP sequence was PCR amplified from the vector pEGFP–C1 (Clontech) and inserted into the *NcoI* site of pTM1 to generate pTM1/EGFP and to the 5’ and 3’ ends of the L ORF of pTM1/L to create pTM1/EGFP–L and pTM1/L–EGFP, respectively. The ORF of S TULV was PCR amplified and cloned into the vector pcDNA3 to create pcDNA3/S. The pET15b/L construct used for in vitro synthesis of the L protein and was generated by transferring the L ORF from pTM1/L to pET15b using restriction sites NcoI and XhoI. All cloned sequences were verified by automatic sequencing with ABI PRISM (Perkin–Elmer) carried out by the sequencing facility of the Haartman Institute, University of Helsinki, Finland. All primer sequences are available upon request.

**Antigen expression and immunization.** Protein expression and inclusion body purification was done according to the manufacturer’s instructions (Novagen). Briefly, *E. coli* strain BL21(DE3)C4Ss transformed with pET15b/TLb was propagated in LB medium (containing 50 μg ampicillin ml−1 and 34 μg chloramphenicol ml−1) at 37 °C. IPTG was added at 1 mM to induce T7 RNA polymerase expression and subsequent target protein expression. The bacteria were pelleted (15 min, 4 °C, 6000 g), resuspended in washing buffer (20 mM Tris/HCl pH 7-5, 10 mM EDTA, 1% Triton X-100) and lysed by sonication on ice. The lysate was washed twice with washing buffer, resuspended in PBS, pelleted and then resuspended in PBS containing 30 mM HEPES. The purified inclusion bodies were used for immunization of two rabbits, which was carried out by Davids Biotechnologie (Regensburg, Germany) according to their immunization protocol.

**Affinity purification of antibodies.** The antigen used to immunize rabbits, TLb, was coupled to CNBr-activated Sepharose 4B according to the manufacturer’s instructions (Amersham–Pharmacia). The immune serum was allowed to bind to a TLb/ Sepharose 4B column in TTBS buffer [0.5 M NaCl, 20 mM Tris/HCl pH 7-8, 0.1% (v/v) Tween 20, 0.01% Na2EDTA] and was washed with the same buffer, and the bound antibodies were eluted in fractions with 0.2 M glycine pH 2-8, containing 0.02% NaCl and neutralized with 1 M Tris/HCl pH 8-5. The fractions with the highest protein content were pooled. The antibody solutions were dialyzed against PBS with 0.02% Na2EDTA and stored at −70 °C in 50% glycerol.

**Immunoblotting.** Protein samples were resuspended in Laemmli buffer (Laemmli, 1970), denatured by heating for 3 min at 70 °C, run in denaturing 6% and 10% SDS-PAGE gels, and transferred onto PROTRAN nitrocellulose filters (Schleicher and Schuell). Unspecific binding was blocked by preincubating the filters for 24 h at 4 °C in 3% non-fat milk in TEN-Tween 20 buffer [20 mM Tris/HCl pH 8-0, 1 mM EDTA, 50 mM NaCl, 0.05% (v/v) Tween 20]. The filters were stained with primary antibodies, washed and stained with horseradish peroxidase conjugated secondary antibodies (Dako). Detection of the protein bands was done with enhanced chemical luminescence. To determine copy numbers of L and N proteins, protein bands of TULV infected cell lysates were compared to serial dilutions of known amounts of TLb and purified TULV N protein, respectively. Protein amounts were determined using an image analysis program (U.S. National Institutes of Health, http://rsb.info.nih.gov/ij/). Anti-L serum was used for the detection of the L protein and the monoclonal antibody 6A6 (Lundkvist et al., 1986), for La Crosse virus, genus Bunyavirus, family Bunyaviridae, the site of RNA synthesis was localized to the cytoplasm (Rossier et al., 1986), but there have been no studies on the localization of any hantavirus L protein or the possible membrane association of the protein.

**Purification of microsomal membranes.** Microsomal membranes were purified as described by Rakov & Compans (2001). Briefly, Vero E6 cells infected with TULV or HeLa cells in 75 cm2 flasks infected with vTF7-3 and then transfected with pTM1/L or pTM1/EGFP–L were washed and scraped into PBS 7 days p.i. (for TULV) or 12 h post-transfection (for vTF7-3). Cells were pelleted and resuspended in hypotonic buffer containing 5 mM Tris/HCl pH 7-4, 0.5 mM MgCl2, 25 μM ZnCl2, 0.1 mM DTT, 5 μM ATP, 1 μM GTP and 1 μM GTP. Complete protease inhibitor cocktail (Roche) and incubated on ice for 30 min. The cell debris and nuclei were removed by centrifugation (4 °C, 15 min, 4000 g), the supernatants were centrifuged (4 °C, 100 000 g, 2 h, 0.1 M KCl, 1% deoxycholate, 0.1% Triton X-100, 2 mM EDTA, 1% protease inhibitors), washed once with 100 mM KCl, 20 mM Tris/HCl pH 7-4, 10% glycerol, and resuspended in hypotonic buffer containing 50 mM Tris/HCl pH 8-0, 100 mM KCl, 1% (v/v) Tween 20.

**Immunoblotting.** Protein samples were resuspended in Laemmli buffer (Laemmli, 1970), denatured by heating for 3 min at 70 °C, run in denaturing 6% and 10% SDS-PAGE gels, and transferred onto PROTRAN nitrocellulose filters (Schleicher and Schuell). Unspecific binding was blocked by preincubating the filters for 24 h at 4 °C in 3% non-fat milk in TEN-Tween 20 buffer [20 mM Tris/HCl pH 8-0, 1 mM EDTA, 50 mM NaCl, 0.05% (v/v) Tween 20]. The filters were stained with primary antibodies, washed and stained with horseradish peroxidase conjugated secondary antibodies (Dako). Detection of the protein bands was done with enhanced chemical luminescence. To determine copy numbers of L and N proteins, protein bands of TULV infected cell lysates were compared to serial dilutions of known amounts of TLb and purified TULV N protein, respectively. Protein amounts were determined using an image analysis program (U.S. National Institutes of Health, http://rsb.info.nih.gov/ij/). Anti-L serum was used for the detection of the L protein and the monoclonal antibody 6A6 (Lundkvist et al., 1996) for the N protein detection. The other antibodies used in immunoblotting were anti-GFP (Covance), anti-β-tubulin (Sigma), anti-β-actin (Sigma) and anti-BAP31 (Määtä et al., 2000).

**Purification of microsomal membranes.** Microsomal membranes were purified as described by Rakov & Compans (2001). Briefly, Vero E6 cells infected with TULV or HeLa cells in 75 cm2 flasks infected with vTF7-3 and then transfected with pTM1/L or pTM1/EGFP–L were washed and scraped into PBS 7 days p.i. (for TULV) or 12 h post-transfection (for vTF7-3). Cells were pelleted and resuspended in hypotonic buffer containing 5 mM Tris/HCl pH 7-4, 0.5 mM MgCl2, 25 μM ZnCl2, 0.1 mM DTT, 5 μM ATP, 1 μM GTP and 1 μM GTP. Complete protease inhibitor cocktail (Roche) and incubated on ice for 10 min. The cells were homogenized with a Dounce homogenizer. Soluble was added to 0.25 M and cell debris and nuclei were removed by centrifugation (2000 g, 10 min, 4 °C, twice). Following ultracentrifugation (100 000 g, 2 h,
4 °C), the supernatant (cytosolic fraction) was collected. The pellet (membrane fraction) was washed with the hypotonic buffer and dissolved overnight at 4 °C in SDS buffer containing 10 mM Tris/HCl pH 6-8, Complete, 1% SDS.

**Membrane flotation.** Five days p.i., Tula-infected Vero E6 cells were washed and scraped into PBS. The cells were pelleted and resuspended in hypotonic lysis buffer (10 mM Tris/HCl pH 7-5, 10 mM KCl, 5 mM MgCl2, 20 μg RNase A ml–1, Complete). Following Dounce homogenization, the nuclei were removed by centrifugation. The post-nuclear supernatant was treated on ice for 30 min with 1% Triton X-100, 1% Nonidet P-40, 1 M NaCl, 15 mM Na2CO3, pH 11-5, or left untreated. The cell lysate (500 μl) was then mixed with 85% sucrose (1-2 ml, final 60%) or 57% OptiPrep (final 40%), and layered with 2-5 ml 55% and 1 ml 10% sucrose or 1-5 ml 30%, 1-5 ml 25% and 1 ml 5% OptiPrep. Following ultracentrifugation (100,000 g, 18 h, 4 °C), eight fractions were collected from the top; the proteins were precipitated with methanol–chloroform and analysed by immunoblotting.

**Localization of L protein in cells.** Vero E6 cells were transfected with the autogene pCMV/T7-T7pol (Brisson et al., 1999) that produces T7 RNA polymerase. The cells were then transfected with either pTM1/EGFP or pTM1/L–EGFP. HeLa cells in a 24-well plate were infected with 10 p.f.u. per cell of vTF7-3 and then transfected with either pTM1/EGFP or pTM1/EGFP–L constructs using the transfection reagent Eugene 6 (Boehringer Mannheim). After 48 h (12 h for the vTF7-3-infected cells), the cells were fixed with 3% paraformaldehyde at room temperature, permeabilized with 0-5% Triton X-100, blocked with 1% BSA/PBS and incubated with antibodies diluted in 1% BSA/PBS for 1 h at 37 °C. The primary antibodies used were a 1:100 dilution of polyclonal anti-Puumala hantavirus L antiserum for TULV nucleocapsid protein and a 1:500 dilution of monoclonal anti-GM130 (BD Biosciences) for Golgi staining. These antibodies were detected with Texas red-conjugated secondary antibodies. Hoechst stain was used to stain the nucleus. An Axioplan 2 optical microscope (Carl Zeiss) was used for fluorescence microscopy and a Leica SP2 (Leica Microsystems) for confocal microscopy.

**RESULTS**

**Polyclonal antibodies against L protein.**

The reverse complement of the L RNA segment of TULV has a single large ORF of 6459 nt [nt 37–6495 of 6541 (Kukkonen et al., 1998)]. This was thought to encode the L protein. The expression of proteins larger than 200 kDa is difficult in bacteria and the predicted molecular mass of Tula L is 247 kDa. Therefore, we cloned and tried to express the whole ORF of Tula L in three parts: TLa (nt 37–2190, aa 1–718), TLb (nt 2191–4344, aa 719–1436) and TLc (nt 4345–6495, aa 1437–2153). Of these, only the middle third of the L ORF, TLb, could be expressed in E. coli; no expression of TLa and TLc was detected in Coomassie-blue-stained protein gels (data not shown).

TLb could not be expressed in the soluble form in E. coli and, therefore, we decided to use purified inclusion bodies to immunize two rabbits. Immune and pre-immune sera were first tested for their ability to recognize the antigen itself. Both rabbits produced immune serum that recognized the antigen, TLb, in immunoblotting (data not shown). However, as the immune serum from rabbit number 2 had higher affinity for the antigen, it was used for all subsequent experiments.

**Detection of L protein in virions, infected cells and in vitro translation products.**

We tested the anti-L antiserum we had raised for its ability to recognize native L protein in TULV virions and TULV-infected Vero E6 cells. TULV was concentrated from the growth medium of infected Vero E6 cells. Growth medium of mock-infected control cells was handled in a similar way to get a control for immunoblotting. A single protein of approximately 250 kDa could be detected in concentrated TULV (Fig. 1a). Total cell lysates of TULV- and mock-infected Vero E6 cells were also immunoblotted and the 250 kDa protein band was the one band detected only in the TULV-infected cells (Fig. 1b). This corresponds to the deduced size of the protein encoded by the single large ORFs in the L RNA segment of TULV (Kukkonen et al., 1998) and is larger than the size reported for Hantaan virus L protein (Elliott et al., 1984). The nature of the band of approximately 55 kDa (Fig. 1b) remains unknown. We cannot exclude that it is a degradation product of TULV L.

The antibodies could also detect the L protein of the nephropathia epidemica-causing Puumala hantavirus (data not shown), which has 85% amino acid identity with the L protein of TULV (Kukkonen et al., 1998). 35S-labelled L protein was produced by coupled RNA transcription and translation of an L protein expression vector.
The 250 kDa protein band could be visualized directly by autoradiography of the rabbit reticulocyte cell lysate and it could be immunoprecipitated with the anti-L antiserum (data not shown).

**Amount of viral proteins in TULV-infected cells**

In order to determine the expression levels of TULV proteins in infected Vero E6 cells, cells were infected with TULV and collected 1–8 days p.i. Actin was used as a control and the amount of actin in the cells remained stable (Fig. 2). The glycoproteins G1 and G2 are translated from the same M mRNA; G2 levels, therefore, reflect expression levels of both proteins. The expression patterns of all three viral proteins are similar with expression increasing until peaking at 4–6 days p.i. (Fig. 2).

We determined the mass and copy number of the L and N proteins 6 days p.i. The cells were counted and then lysed in Laemmli sample buffer. The cell lysates were run in a denaturing SDS-PAGE gel together with serial dilutions of standards of known mass. As a standard for the L protein we used the antigen TLb and for the N protein, purified recombinant TULV N protein. The proteins in the gels were immunoblotted with antiserum against the L protein and the monoclonal antibody 6A6 for L and N protein detection, respectively. The intensity of the bands was analysed by the image analysis program IMAGEJ. Standard curves were drawn based on the band intensities of the known amounts of the TLb antigen and purified nucleocapsid protein. The L and N protein amounts in the TULV-infected cells were determined by comparing the L and N protein band intensities to the standard curves. Four independent measurements were made for the L protein and three for the N protein. The L protein copy number in TULV-infected cells was determined to be approximately $10^4$ per cell (mean $1.4 \times 10^4$, SD $4 \times 10^3$) and the N protein copy number was $3 \times 10^7$ per cell (mean $3.4 \times 10^7$, SD $5 \times 10^6$). Thus, there are over 2000 times more copies of nucleocapsid protein than L protein in TULV-infected Vero E6 cells.

**L protein pellets with microsomal membranes**

The RNA synthesis of all positive-strand RNA viruses is associated with membranes (see Schwartz *et al.*, 2002, and references therein). However, little is known of the possible membrane association of RNA synthesis in negative-strand viruses. The new antiserum against TULV L protein allowed us to determine whether the protein is membrane-associated.

In the first method, microsomal membranes and associated proteins were separated from cytosolic proteins by pelleting the membranes by ultracentrifugation. Briefly, Vero E6 cells were infected with TULV, the cells were lysed by Dounce homogenization, nuclei were removed, microsomal membranes were separated from the cytosolic fraction by ultracentrifugation and proteins were detected by immunoblotting. As controls for the purity of the membrane and cytosolic fractions, we used BAP31 as a marker for membrane proteins and $\beta$-tubulin as a marker for cytosolic proteins. BAP31 is an integral membrane protein of the endoplasmic reticulum (Ng *et al.*, 1997) and $\beta$-tubulin is a cytosolic protein (Taverna *et al.*, 2002) that forms tubulin filaments with $\alpha$-tubulin. The controls behaved as expected, with BAP31 found exclusively in the membrane and $\beta$-tubulin in the cytosolic fraction (Fig. 3). We then determined how TULV L and N proteins fractionate. All of the detected L and N proteins were found in the membrane fraction (Fig. 3). For the N protein this was expected, as the N protein of the hantavirus Black Creek Canal virus has previously been found to be membrane-associated (Ravkov & Compans, 2001).

It is not known whether the L protein interacts directly with the N protein but both proteins must bind viral RNA which, thus, mediates the interaction of the proteins. In TULV-infected cells the membrane association of the...
L protein could, therefore, be caused by the membrane association of the N protein. To study whether the L protein expressed without other viral proteins and viral RNA is also membrane-associated, we expressed recombinant L protein in HeLa cells using a vaccinia virus-driven T7 expression system. In the system, cells are first infected with a recombinant vaccinia virus, vTF7-3 (Fuerst et al., 1987), that produces T7 RNA polymerase, and then the cells are transfected with plasmids that have the desired cDNAs under a T7 promoter.

We infected HeLa cells with the recombinant vaccinia virus, vTF7-3, transfected the cells with a construct expressing L protein (pTM1/L), and then detected the proteins from the microsomal and cytosolic fractions by immunoblotting. Recombinant L protein was detected exclusively in the microsomal fraction (Fig. 3). Therefore, the L protein is, in itself, a membrane-associated protein and interaction with the N protein is not required for the association. We also tested the membrane association of the protein constructs used to localize the L protein in cells, EGFP-L and EGFP (see below), and found the EGFP-L in the membrane fraction and the EGFP in the cytosolic fraction (Fig. 3).

The membrane association of the L and N proteins was further confirmed by membrane flotation experiments, in which membrane-associated proteins in cell lysates float to less dense fractions of discontinuous sucrose or OptiPrep gradients, both of which were used. The L protein of TULV-infected cell lysates was dissociated from membranes following 1 M NaCl or 15 mM Na₂CO₃ pH 11.5 treatments. Both L and N proteins were dissociated from membranes by 1% Triton X-100 and 1% Nonidet P-40 treatments, whereas the glycoproteins remained membrane-associated (data not shown). In our membrane flotation experiments, a portion of L and N co-fractionated with cytosolic proteins. In membrane flotation assays, fractions of membrane proteins are commonly seen to fractionate with cytosolic proteins for reasons discussed by Simons & Toomre (2000). However, cytosolic proteins do not float.

### Perinuclear localization of L protein

We next tried to use the antiserum to localize the L protein in TULV-infected cells. However, the antibodies could not detect the L protein in indirect immunofluorescence assays (IFA) and could not, therefore, be used to localize the L protein in cells. One possibility is that the antiserum only recognizes denatured linear epitopes. However, denaturing proteins with guanidine hydrochloride prior to IFA according to the protocol of Peränen et al. (1993), did not enable L protein detection.

To study the localization of the L protein, we decided to make a fusion protein of L with enhanced green fluorescent protein (EGFP). Using EGFP as a tag has the advantage of enabling the detection of the fusion protein without antibodies and, in addition, EGFP-tagged proteins can be detected without fixing the cells, i.e. in live cells. We made constructs in which EGFP was in the C terminus, pTM1/L–EGFP, but also a construct in which EGFP was in the N terminus of the fusion protein, pTM1/EGFP–L. (Fig. 4a). Both constructs were cloned in the T7 expression vector, pTM1, which has a T7 promoter followed by the 5′ untranslated region of encephalomyocarditis virus, which enhances the translation of uncapped mRNAs. As a control we had a construct with EGFP alone in the vector pTM1, pTM1/EGFP (Fig. 4a).

We used two approaches for the expression: (i) Vero E6 cells were co-transfected with either pTM1/L–EGFP or pTM1/EGFP, and the autogene pCMV/T7-T7pol, which has T7 RNA polymerase under both CMV and T7 promoters; (ii) in the other approach we infected HeLa cells with the recombinant vaccinia virus vTF7-3 that expresses T7 RNA polymerase and then transfected the cells with either pTM1/EGFP–L or pTM1/EGFP. The vaccinia virus-driven T7 expression system has been used extensively in the study of viruses of the family Bunyaviridae (Rusasala et al., 1992; Betenbaugh et al., 1995; Dunn et al., 1995; Shi & Elliott, 2002) enabling even the rescue of infectious Bunyamwera virus from cloned cDNAs (Bridge & Elliott, 1996). Furthermore, we did a double infection experiment in which Vero E6 cells were first infected with Tula virus (m.o.i. 0.1) and then 1 h p.i. with the recombinant vaccinia virus vTF7-3 (m.o.i. 10). By 2 days p.i. all the cells were lysed by the lytic vTF7-3 infection, yet we saw the normal Tula N protein expression pattern in the dead cells (data not shown), indicating that vaccinia virus infection does not inhibit Tula virus protein expression.
We first tested by immunoblotting whether we could express the full-length EGFP–L construct in cells. The vTF7-3-infected HeLa cells expressing EGFP or EGFP–L were lysed 12 h after transfection and the cell lysates were immunoblotted using anti-L antiserum and anti-GFP antibodies. The 275 kDa L–EGFP-fusion protein and 27 kDa EGFP were detected (Fig. 4b); the fusion protein had the expected size when compared to unmodified L protein (Fig. 1a, b). The autogene pCMV/T7-T7pol-driven T7 expression system in Vero E6 cells produced less of the fusion L protein products and the background reactivity of the anti-GFP antibodies was high (data not shown).

We then used indirect immunofluorescence to study the localization of L–EGFP and EGFP in Vero E6 cells using the plasmid-driven T7 expression system and EGFP–L and EGFP in HeLa cells using the vaccinia-driven T7 expression system. We studied co-localization with TULV N protein and various markers for cellular organelles. The N protein was expressed by co-transfection with pcDNA3/S. Cells were fixed, permeabilized and the proteins were visualized by indirect immunofluorescence. Transfection efficiencies were low (<10% of cells transfected).

EGFP had a diffuse expression pattern in the cytoplasm and the nucleus as is characteristic for this protein (Fig. 5a). In contrast, L–EGFP and EGFP–L had a more punctated expression pattern characteristic of membrane-associated proteins. The EGFP-tagged L proteins were localized in the perinuclear region. The only cellular marker with which we found any co-localization was the Golgi protein GM130, but the distribution of the proteins differed, with GM130 having wider distribution in the Vero E6 cells (Fig. 5b). L–EGFP had the same localization when expressed in TULV-infected Vero E6 cells (data not shown), indicating that the presence of the other viral proteins does not alter the localization. Confocal microscopy of the EGFP–L construct expressed in HeLa cells also showed partial co-localization with the GM130 marker and also in HeLa cells GM130 had wider distribution than the EGFP–L fusion construct (Fig. 5c). In HeLa cells expressing both EGFP-tagged L protein and TULV N protein, confocal microscopy showed that the proteins had distinct expression patterns, but there was also partial co-localization of the two proteins in the perinuclear region (Fig. 5d).

DISCUSSION

There have been no antibodies available against the L proteins of hantaviruses, which has hampered their study. In our previous attempts, we tried to raise antibodies against TULV L protein by expressing 200–300 aa fragments of the L ORF as glutathione S-transferase (GST) fusion proteins. These were used to immunize both rabbits and mice but no antiserum could be produced (our unpublished results). We were now able to express the middle third (aa 719–1436) of TULV L ORF as an approximately 80 kDa protein in E. coli. The middle third contains the regions conserved in RNA-dependent RNA polymerases, from pre-motif A to motif E (Kukkonen et al., 1998). Using inclusion bodies as antigen, we were able to raise polyclonal antibodies that can detect TULV L protein in immunoblotting. To our knowledge, these are currently the only available antibodies shown to bind a hantavirus L protein.

A single 250 kDa protein was detectable in TULV-infected cells and concentrated virus particles (Fig. 1). The antiserum
also detected PUUV L protein, which has 85% aa sequence identity with TULV L protein (Kukkonen et al., 1998), and it was found to be of identical size. The hantavirus L RNA large ORF of more than 6400 bases is, therefore, used in its entirety to encode a single L protein as has been previously shown for other Bunyaviridae (Jin & Elliott, 1992; Adkins et al., 1995; Lukashevich et al., 1997). The antiserum also recognized recombinant L protein expressed by using in vitro translation and by vTF7-3-driven transient expression in transfected cells (Fig. 4b).

Hantaviruses grow to low titres in cell culture. We typically observe a peak level of only 0.1 f.f.u. per cell. ImmunobLOTS of nucleocapsid protein and glycoproteins (Pensiero et al., 1988) and Northern hybridization blots of vRNAs (Vapalahti et al., 1996) indicate that all of these virion components are abundant in infected cells. We show that the L protein, with a copy number of 10^4, is also abundant enough not to explain the low virus titres. The 3' termini of genomic RNAs contain deletions (Kukkonen et al., 1998; Meyer & Schmaljohn, 2000; Padula et al., 2002) but vRNAs with deletions can be packaged into virions (Kukkonen et al., 1998). Taken together, this points to inefficient assembly of the viral particles as the most probable cause of low virus titres.

With the new antiserum we were able to show hantavirus L protein to be membrane-associated in TULV-infected cells (Fig. 3) in addition to the N protein and the glycoproteins. The L protein was shown to be membrane-associated even in the absence of other viral proteins and viral RNA. Therefore, all the hantavirus structural proteins can independently interact with cellular components localizing the proteins on membranes. The L protein domain responsible for the membrane interaction and the cellular component with which it interacts remains to be...
determined. Our results obtained using membrane flotation indicate that the membrane association of the L protein can be inhibited by treatment with both 1 M NaCl and 15 mM Na₂CO₃ pH 11.5, indicating that the L protein is a peripheral membrane protein.

Having a viral RNA polymerase function on membranes could bring about the advantages of surface catalysis (Lyle et al., 2002). The freedom of motion of the RNA polymerase, RNA template and nucleotides is limited to two dimensions, substrate affinity is increased by binding of several RNA polymerases to the same RNA template, and, perhaps most importantly, reaction products, e.g. cRNA, are retained to be used in subsequent reactions, e.g. synthesis of vRNA (Lyle et al., 2002). The membrane association of Tula virus L protein could be a result of viral RNA synthesis being sequestered within membrane structures. This would not only bring about the advantages of surface catalysis but also protect the virus from antiviral responses triggered by double-stranded RNA, as has been suggested for other RNA viruses (Schwartz et al., 2002). Having RNA polymerases concentrated on membranes can also facilitate template switching and, thus, increase events of homologous recombination which have been observed also for hantaviruses (Plyusnin et al., 2002).

To study the localization of the L protein in cells, we used autofluorescent fusion proteins having EGFP in either the N or C terminus (Fig. 4a). With EGFP in the N terminus it is possible to have prematurely terminated translation products having full EGFP and only partial L protein; therefore, we repeated the experiments with the L-EGFP construct, even though the only EGFP-L fusion protein band we could detect by immunoblotting was the full-length EGFP-L (Fig. 4b). Furthermore, by having both types of constructs we tried to minimize the probability of observing a misfolded protein caused by fusion with EGFP. However, it has even been possible to incorporate EGFP within the open reading frame of a viral RNA polymerase without abolishing its activity (Duprex et al., 2002). Both of the fusion protein constructs localized in the perinuclear region (Fig. 5), which has previously been found to be the localization site for the N protein of BCCV, Seoul and Puumala viruses (Ravkov & Compans, 2001). We found that the L-EGFP and EGFP-L fusion proteins did have at least partial co-localization with the Golgi marker GM130 (Fig. 5). However, the distribution of GM130 differed from those of the L-EGFP and EGFP-L fusion proteins. The L fusion protein also had an expression pattern distinct from that of the N protein (Fig. 5), although the site of the L protein localization also had N protein. Based on these data, it is not yet possible to determine on what cellular structures the RNA polymerase complex is located.

It has been difficult to find any co-localization of the N protein with the Golgi-localized glycoproteins, even though these proteins must co-localize during virus assembly. For example, the Seoul virus G2 co-localizes with the Golgi markers, whereas the N protein does not but rather surrounds the Golgi (Kariwa et al., 2003). The glycoproteins are not required for RNA synthesis (Dunn et al., 1995; Lopez et al., 1995; Flick et al., 2003) and our results, obtained by membrane flotation following detergent treatment, indicate that the glycoproteins are localized in lipid rafts whereas the L and N proteins are not. These data point to the site of RNA synthesis being distinct from the site of virus assembly.

Unlike positive-strand viruses, little is known about the localization of negative-strand RNA polymerases. Here, we provide the first evidence for the membrane association of an RNA polymerase of a negative-strand virus replicating in the cytoplasm. Thus, the membrane association of RNA synthesis can be a universal feature of RNA viruses. This awaits further investigation.

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