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Synthesis and Secretion of Recombinant Tick-Borne Encephalitis Virus Protein E in Soluble and Particulate Form

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A quantitative study was performed to investigate the requirements for secretion of recombinant soluble and particulate forms of the envelope glycoprotein E of tick-borne encephalitis (TBE) virus. Full-length E and a carboxy terminally truncated anchor-free form were expressed in COS cells in the presence and absence of prM, the precursor of the viral membrane protein M. Formation of a heteromeric complex with prM was found to be necessary for efficient secretion of both forms of E, whereas only low levels of anchor-free E were secreted in the absence of prM. The prM-mediated transport function could also be provided by coexpression of prM and E from separate constructs, but a prM-to-E ratio of greater than 1:1 did not further enhance secretion. Full-length E formed stable intracellular heterodimers with prM and was secreted as a subviral particle, whereas anchor-free E was not associated with particles and formed a less stable complex with prM, suggesting that prM interacts with both the ectodomain and anchor region of E.

Mature flavivirus virions contain two transmembrane proteins, the major envelope glycoprotein E (50 to 55 kDa) and the small (7- to 8-kDa) membrane protein M. Protein E mediates functions that are essential for entry of the virus into cells, including receptor binding and low-pH-induced fusion in endosomes, and induces a protective immune response (reviewed in reference 11).

The virion is first assembled as an immature intracellular particle which contains prM, the glycosylated precursor of protein M. It was shown in earlier studies with the flavivirus West Nile virus that prM forms a heterodimeric complex with E in these immature virions (30). Shortly before release from the cell, the prM protein is cleaved by a cellular protease, leaving only the C-terminal portion of this protein associated with the virion (4). It is believed that one important function of prM is to prevent low-pH-induced rearrangements in protein E during transport through acidic compartments of the trans Golgi network which would lead to virus inactivation (10, 12).

Proteins prM and E are encoded in adjacent regions of the flavivirus genome, which contains a single long open reading frame for all of the structural and nonstructural proteins (4). Recent interest in new approaches to flavivirus vaccine development has led to new insights into the requirements for proper processing, assembly, and secretion of recombinant flaviviral envelope proteins (reviewed in references 3 and 28). Secreted E proteins with immunogenic properties have been obtained by expression of vaccinia virus or baculovirus recombinants in eukaryotic cells, either by expressing E alone in a soluble truncated form lacking the membrane anchor region (5–7, 14, 24, 29) or by coexpression of prM and E to yield recombinant subviral particles resembling capsidless viral envelopes (8, 15–19, 23, 25, 27, 31). In the latter case, secretion of full-length E and formation of particles were observed only when E was cosynthesized with a properly processed prM molecule, suggesting that specific molecular interactions between prM and E are important for assembly and transport (15, 21, 27, 31). Although both of these strategies have been shown to give rise to extracellular forms of E, quantitative comparisons of secretion efficiencies are still lacking. Furthermore, the effect of prM on secretion of anchor-free E has not yet been investigated.

Expression and secretion of TBE virus envelope proteins. To study the molecular interactions and mechanisms involved in tick-borne encephalitis (TBE) virus envelope protein transport and secretion, proteins E and prM were produced in COS-1 cells (9) by transient expression from recombinant plasmids containing the viral genes under the control of the simian virus 40 early promoter (1). Two of the constructs contained the gene encoding the full-length wild-type protein E, either alone (SV-Ewt) or together with prM (SV-PEwt). Constructs SV-PEst and SV-Est were identical to SV-PEwt and SV-Ewt, respectively, except that they contained a TAG stop codon at amino acid position 435 in E (1), thereby encoding a truncated form of protein E (Est) lacking the C-terminal 62 amino acids from the membrane anchor region (22). A fifth plasmid construct for expression of prM alone (SV-prM) was made by deleting the portion of SV-PEwt encoding protein E and adding a stop codon immediately after the prM gene. To express the recombinant proteins, COS cells were transfected with CsCl-purified plasmid DNA by electroporation using a Bio-Rad Gene Pulser apparatus.

As shown in Fig. 1A by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), transfection with each of the recombinant plasmids yielded the expected proteins, i.e., full-length E plus prM (SV-PEwt), truncated E plus prM (SV-PEst), full-length E alone (SV-Ewt), truncated E alone (SV-Est), and prM alone (SV-prM), indicating that the processing of the polyprotein occurred normally. An additional band corresponding in molecular weight to the nonstructural protein NS1 was present in the virus-infected controls but not in cells transfected with the recombinant plasmids, all of which lacked the NS1 gene.

When culture medium from the transfected COS cells was analyzed by immunoprecipitation with the same antiserum used for the lysates, it was found that E and Est were secreted when these proteins were expressed together with prM (SV-

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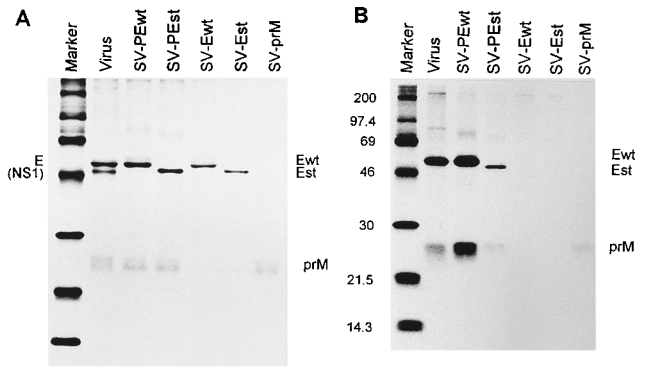

FIG. 1. SDS-PAGE analysis of TBE virus proteins expressed in COS cells. Cells were infected with TBE virus or transfected with recombinant plasmid, labeled for 6 h with [^{35}S]cysteine, and lysed in a buffer containing 1% Triton X-100. TBE virus-specific proteins were then immunoprecipitated from cell lysates and supernatants, separated by electrophoresis (20), and visualized by fluorography. (A) Immunoprecipitation using a polyclonal rabbit serum recognizing both prM and E. The positions of full-length (Ewt) and truncated (Est) E protein as well as prM are indicated. A protein band corresponding to the nonstructural protein NS1, present only in virus-infected cells, is also shown. (B) Immunoprecipitation using the prM-specific monoclonal antibody 8HI. Positions of protein molecular weight markers are shown in kilodaltons on the left.

PEwt and SV-PEst), but very little extracellular protein was detected when E or Est was expressed alone (data not shown).

Kinetics of protein E secretion. To compare the rates and efficiencies of protein E secretion from cells transfected with SV-PEwt, SV-PEst, SV-Ewt, and SV-Est, transfected cells were split into several equal portions, and duplicate supernatant and cell lysate samples were collected at 24-h intervals for 3 days. Protein E in the intracellular and extracellular fractions was quantitated by four-layer enzyme-linked immunosorbent assay (ELISA) (13). Transfection efficiencies were monitored by indirect immunofluorescence (1), and protein E expression was observed in 30 to 40% of the cells, regardless of which construct was used (data not shown).

As shown in Fig. 2A, significant levels of extracellular protein E were obtained with the prM-containing constructs SV-PEwt and SV-PEst, whereas transfection with SV-Ewt did not result in any detectable secretion of E. Anchor-free E expressed in the absence of prM (SV-Est) was secreted only at a very low level.

To compare secretion efficiencies normalized for differences in absolute expression levels, the amount of protein E present in the culture medium at each time point was expressed as a percentage of the total protein E measured in the intracellular and extracellular fractions. The data shown in Fig. 2B reveal strikingly different efficiencies of secretion for each of the four constructs. Wild-type protein E was efficiently secreted when expressed together with prM (SV-PEwt), but even higher efficiency was attained when the membrane anchor was deleted (SV-PEst). On the other hand, the secretion of Est in the absence of prM (SV-Est) was considerably less efficient than that of either wild-type E or Est in the presence of prM. Over 80% of the total E protein from SV-PEst was present in the extracellular fraction 1 day after transfection, compared with 41% at the same time point with SV-PEwt and only 22% with SV-Est. By day 3, the extracellular proportion had reached 94% with SV-PEst and 82% with SV-PEwt but only 41% with SV-Est.

These data suggest the involvement of two independent secretion mechanisms whose effects are additive: one due to the removal of an intracellular retention signal residing in the C-terminal anchor region of E, and the other apparently due to prM-mediated transport. Because the presence of prM strongly affected the secretion efficiency of both wild-type E and Est, it can be concluded that the E anchor itself is not required for prM-dependent transport, implying that important prM-E contact sites lie in the globular ectodomain portion of E.

Interestingly, the cytoplasm of cells transfected with SV-PEst or SV-Est exhibited a uniform reticular staining pattern in the immunofluorescence assay, whereas the constructs containing wild-type E also yielded, in addition to the reticular staining, localized patches of intense fluorescence that were absent in cells expressing anchorless E (data not shown). This finding suggests that the C-terminal portion of E may contain

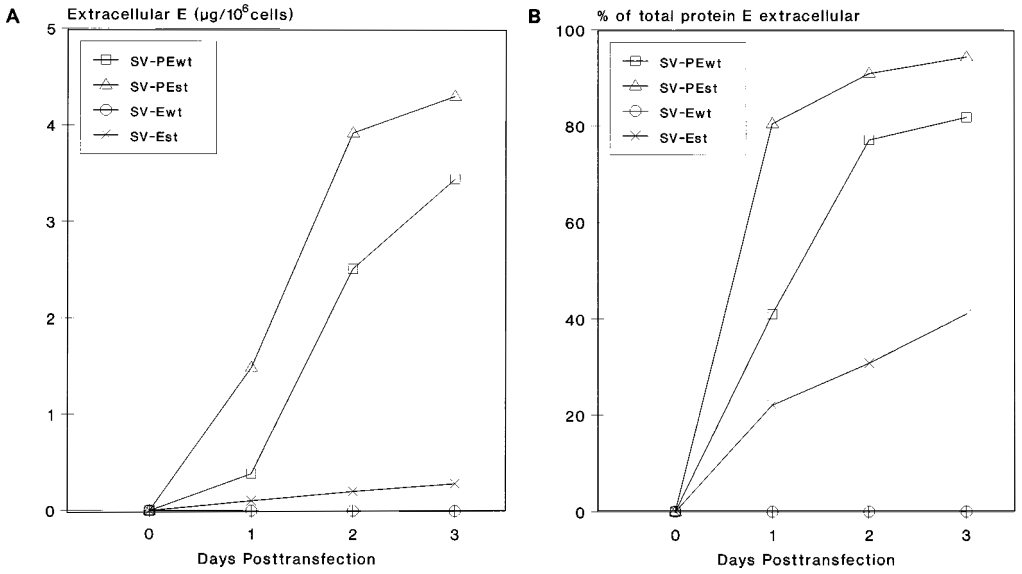

FIG. 2. Time course of protein E expression and secretion. Protein E in intracellular and extracellular fractions was collected at 24-h intervals for 3 days and quantitated by four-layer ELISA (13) using purified TBE virus as a standard. (A) Absolute amount of protein E in the extracellular fraction; (B) percentage of total E protein in the extracellular fraction.

a signal for retention and concentration of this protein in intracellular compartments.

Detection of prM + E heterodimers in infected and transfected cells. The results of the experiments described above imply that molecular interactions with prM are involved in the transport and secretion of E and Est. To demonstrate these interactions directly, Triton X-100 lysates from radiolabeled virus-infected or plasmid-transfected cells were analyzed by immunoprecipitation using the prM-specific monoclonal antibody 8HI (kindly provided by L. Iacono-Connors, U.S. Army Medical Research Institute for Infectious Diseases, Fort Detrick, Md.). Under these conditions, E and Est should be detected only if they are coprecipitated as part of a heteromeric complex with prM.

The ability of monoclonal antibody 8HI to recognize and precipitate prM in the absence of E was demonstrated by using a lysate from SV-prM-transfected cells expressing prM alone (Fig. 1B). Coprecipitation of prM and E was observed with virus-infected cells as well as with cells transfected with SV-PEwt or SV-PEst, indicating the presence of a prM + E complex. Since protein E could be coprecipitated with prM even when the membrane anchor was deleted, it can be concluded that prM binds to the ectodomain of E.

To further characterize the prM + E complex, ^{35}S-labeled lysates from virus-infected, SV-PEwt-transfected, and SV-PEst-transfected COS cells were subjected to sedimentation analysis on a 5 to 20% sucrose gradient containing 0.1% Triton X-100, and gradient fractions were immunoprecipitated with a polyclonal antiserum recognizing prM and E.

Proteins prM and E from TBE virus-infected cells (Fig. 3A) and SV-PEwt-transfected cells (Fig. 3B) cosedimented as a Triton X-100-stable complex at the position predicted for a prM + E heterodimer. The presence of heterodimers in the plasmid-transfected cells shows that formation of this complex is independent of other viral components. Heterodimers of prM and E have also been described for West Nile virus (30), and their involvement in virus assembly appears to be a common feature of mosquito-borne and tick-borne flaviviruses.

In contrast, most of the prM and Est from cells transfected with SV-PEst appeared to sediment as monomers, indicating that prM + Est complexes are unstable compared with wild-type heterodimers. This result, together with the secretion kinetics and coprecipitation results, suggests that prM makes multiple contacts with E. Although contacts between prM and the E ectodomain are evidently sufficient for secretion of E, further contacts within the C-terminal anchor region also appear to be important for the overall stability of the heterodimer.

Protein E secretion by coexpression of prM and E in *trans*. To determine whether protein E secretion could also be induced or enhanced by providing prM in *trans* to E proteins synthesized from a different polypeptide precursor, the protein E-expressing constructs were each cotransfected with a construct expressing prM alone (SV-prM). Cell lysates and supernatants from singly transfected and cotransfected cells were collected on each of 3 days following transfection, and protein E was quantitated by four-layer ELISA (13).

Wild-type E protein expressed from SV-Ewt, as expected, was not secreted when expressed alone, but the secretion defect was overcome by cotransfecting with SV-prM (Fig. 4). Similarly, anchor-free E encoded by SV-Est was secreted much more efficiently when coexpressed with prM in *trans* (Fig. 4). Cosynthesis of prM and E from the same polypeptide, therefore, does not appear to be necessary for proper folding of transport-competent E proteins, and this property should facilitate future studies on prM-E interactions and flavivirus assembly.

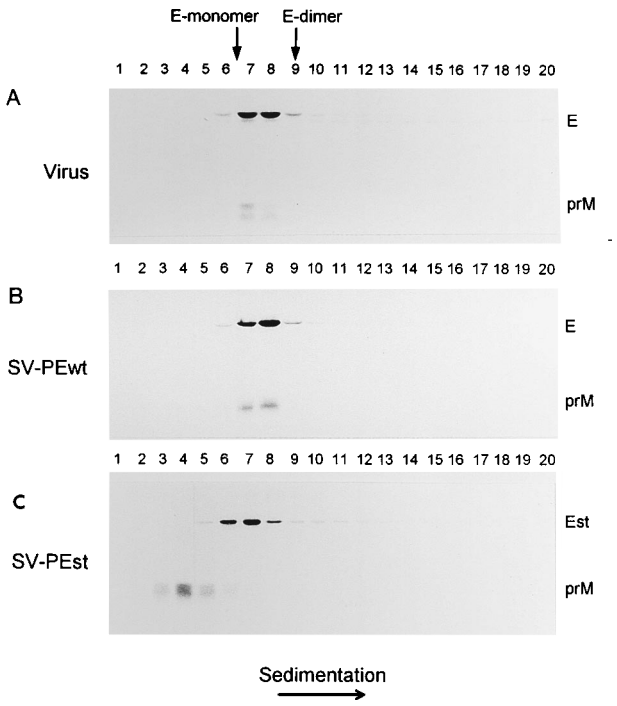

FIG. 3. Sedimentation analysis of heteromeric complexes containing prM and E. Radiolabeled lysates from COS cells infected with TBE virus or transfected with SV-PEwt or SV-PEst were subjected to sedimentation analysis on parallel sucrose gradients containing 0.1% Triton X-100 to prevent aggregation. Material from each gradient fraction was immunoprecipitated with a polyclonal antiserum recognizing both prM and E. The precipitated material was then analyzed by SDS-PAGE and fluorography. Protein E monomers and homodimers (2) were generated by treating purified TBE virus either with 0.5% SDS at 65°C for 15 min (monomers) or with 1% Triton X-100 at room temperature for 1 h (homodimers), and these preparations were run in parallel gradients as sedimentation standards. (A) TBE virus-infected cell lysate; (B) SV-PEwt lysate; (C) SV-PEst lysate. The positions of E, Est, and prM in the gels are shown at the right. The positions of the E monomer and E dimer sedimentation standards in sucrose gradients are indicated by arrows.

Cotransfection of SV-prM with constructs already encoding prM in the same precursor with E (SV-PEwt and SV-PEst) had no effect on the overall efficiency of protein E secretion (Fig. 4), and the relative amounts of protein E in the extracellular fraction were identical on each of the 3 days for the single transfections and SV-prM cotransfections. The failure of excess prM to further enhance secretion of E under these conditions suggests that prM and E probably interact in a 1:1 ratio during transport.

Role of the E anchor region in subviral particle formation. To ascertain whether the recombinant TBE virus E and Est proteins are secreted in a soluble or particulate form, cell culture supernatants from COS cells transfected with SV-PEwt or SV-PEst were analyzed by sedimentation on sucrose gradients, and the sedimentation profiles were compared with that of an unpurified culture medium from TBE virus-infected COS cells. As shown in Fig. 5, the infected cells produced, in addition to the complete virion found in fractions 11 to 13, a more slowly sedimenting protein E-containing particle found in fractions 3 and 4—the noninfectious slowly sedimenting hemagglutinin typical of flavivirus infections (26). The supernatant from SV-PEwt-transfected cells also contained a particulate form of E with a sedimentation velocity similar to that of the

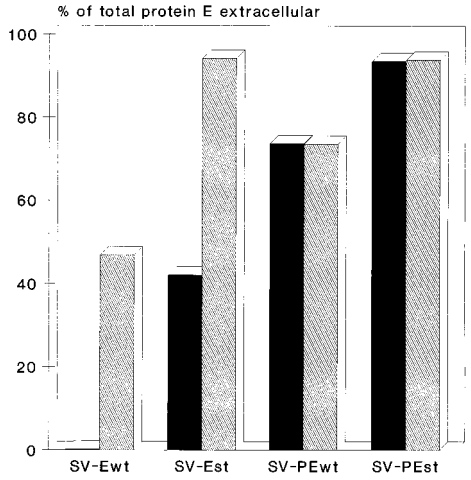

FIG. 4. Changes in protein E secretion efficiency due to coexpression with prM in *trans*. COS cells were transfected with each of the four E-containing constructs, either individually or together with plasmid SV-prM, and protein E in intracellular and extracellular fractions was quantitated as for Fig. 2. The percentage of total protein E in the extracellular fraction 3 days after transfection with the indicated plasmid alone (solid black bars) or after cotransfection with the indicated plasmid and SV-prM (hatched bars) is shown.

viral slowly sedimenting hemagglutinin. In contrast to SV-PEwt, the anchorless form of E expressed from SV-PEst did not sediment as a particle, and all of the Est protein remained at the top of the gradient (Fig. 5). Further sedimentation analysis and chemical cross-linking showed this material to be an Est homodimer (data not shown). The membrane anchor region therefore appears to be required for either the formation or the stability of protein E-containing particles.

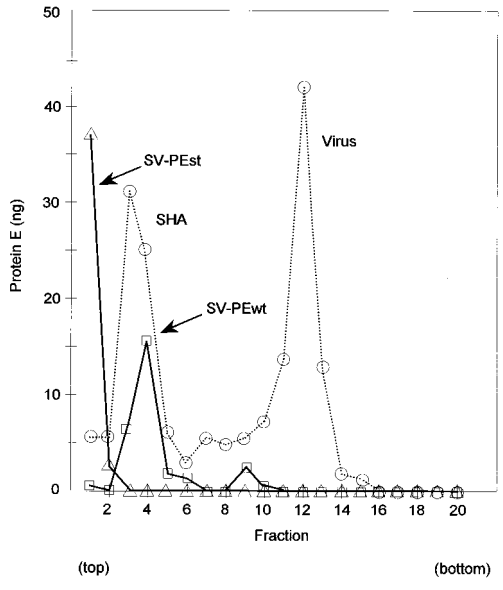

FIG. 5. Sedimentation analysis of secreted forms of protein E. (A) Cleared supernatants from COS cells transfected with SV-PEwt or SV-PEst or infected with TBE virus were applied directly to 5 to 30% sucrose gradients and analyzed by centrifugation. Fractions (0.6 ml) were collected, and protein E in each fraction was quantitated by four-layer ELISA. The sedimentation profile is shown for each supernatant. Circles, virus-infected cells; squares, SV-PEwt-transfected cells; triangles, SV-PEst-transfected cells. Peaks corresponding to virus and slowly sedimenting hemagglutinin (SHA) from infected cells, the particulate form of E from SV-PEwt, and the soluble form from SV-PEst are indicated by arrows.

The recombinant subviral particles (RSPs) produced by the SV-PEwt transfection appear to be analogous to those originally reported by Mason and coworkers (23), who used recombinant vaccinia viruses expressing prM and E proteins from Japanese encephalitis virus, and to particles derived from other mosquito-borne flaviviruses which have since been produced by using recombinant vaccinia virus technology (8, 25, 31). Like the Japanese encephalitis virus-derived particles, those produced by SV-PEwt transfection were readily dissociated by treatment with 0.5% Triton X-100 (data not shown), a property consistent with a lipid membrane-containing vesicle (18). The potential to form RSPs thus appears to be an intrinsic property that is shared by mosquito-borne and tick-borne flavivirus envelope proteins. Their generation by transfection with recombinant plasmids suggests that it should be feasible to produce effective RSP-based vaccines in various eukaryotic cell systems (e.g., stably transfected cell lines) also under conditions not requiring infection with a live virus, an approach which is likely to be applicable to mosquito-borne flaviviruses as well. We have recently observed that RSPs from SV-PEwt-transfected COS cells are antigenically native and induce a protective immune response against lethal TBE virus challenge in mice (10a).

Flavivirus RSPs also represent a potentially valuable tool for investigating envelope protein structure and function. Preliminary experiments indicate that the structural organization of protein E in RSPs is similar to that in virions, and the use of transient expression from plasmids will facilitate the introduction of specific mutations and rapid assessment of their effects. We are currently investigating the molecular characteristics of TBE virus RSPs and evaluating their utility as a model system for studying structure-function relationships in protein E.

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