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RAPID COMMUNICATION

Release of Coronavirus E Protein in Membrane Vesicles from Virus-Infected Cells and E Protein-Expressing Cells

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Coronavirus E protein is a small viral envelope protein that plays an essential role in coronavirus assembly; coexpression of coronavirus M and E proteins results in the production of virus-like particles. The present study demonstrated that mouse hepatitis virus (MHV) E protein was released as an integral membrane protein in lipid vesicles from E-protein-expressing mammalian cells, in the absence of other MHV proteins. Furthermore, our data indicated that the E-protein-containing vesicles, which had a slightly lighter buoyant density than that of MHV, were released from MHV-infected cells. These data implied that E protein alone can drive the production and release of coronavirus envelope in the absence of M protein.

Coronavirus is an enveloped virus containing a large positive-stranded RNA genome. The coronavirus envelope typically contains three virus-specific proteins: S, M, and E. The S protein forms 180- to 90-kDa peplomers, binds receptors on coronavirus-susceptible cells, and induces cell fusion. The M protein, a transmembrane protein, is the most abundant glycoprotein in infected cells as well as in the virus particle. The M protein has three domains; these include a short N-terminal ectodomain, a triple-spanning transmembrane domain, and a C-terminal endodomain. The E protein, previously referred to as the small membrane protein or sM protein, is an acylated protein and is present only in minute amounts in infected cells as well as in the virus envelope. The structure inside the viral envelope is a helical nucleocapsid that consists of viral RNA and N protein. Each coronavirus-specific protein is translated from each of six to eight species of virus-specific mRNAs that have a 3′-coterminal nested set structure (*1*).

E protein is crucial to coronavirus assembly. Coexpression of coronavirus M protein and E protein results in the production of virus-like particles (VLPs), while expression of M protein alone does not produce VLPs (*2–4*). Complementation studies using two defective interfering (DI) RNAs of a prototypic coronavirus, mouse hepatitis virus (MHV), showed that E and M proteins are both required for the production of MHV particles containing the viral nucleocapsid (*5*). E protein also plays a role in coronavirus morphogenesis, because MHV mutants encoding mutated E protein are morphologically aberrant compared to wild-type MHV (*6*). Even though the importance of E protein in coronavirus assembly is known, more detailed characterization of its function has been limited because of its very low abundance in the virus and in infected cells. In infected cells E protein has been shown to be an integral membrane protein (*3*), yet its presence as an integral membrane protein in virus particles has not been demonstrated. The orientation of E protein in the membrane is also unknown.

In the present study we demonstrated that E-protein-containing membrane vesicles were released from E-protein-expressing cells. Furthermore, our data indicated that the E-protein-containing membrane vesicles were released from MHV-infected cells. These data were unexpected, because M protein is also considered essential for coronavirus envelope formation. The data presented here suggest the possibility that E protein alone can drive the production and release of the coronavirus envelope in the absence of M protein.

We used a Sindbis virus expression vector, pSinRep5 (*7*) (Invitrogen, San Diego, CA), to express MHV E protein and M protein at high levels in mammalian cells. This vector contains a promoter for subgenomic transcription and the Sindbis virus nonstructural protein genes 1–4 that are required for replication of vector RNAs. We constructed one Sindbis vector that expressed MHV M

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protein (pSinM) and another that expressed MHV E protein (pSinE) by respectively inserting gene 6 (encoding the M protein) from the JHM strain of MHV (MHV-JHM) and gene 5b (encoding the E protein) from the A59 strain of MHV (MHV-A59) downstream of the promoter for subgenomic transcription. Sindbis vector expressing MHV-JHM E protein was also constructed. However, expression of E protein from this vector was significantly lower than that from pSinE (data not shown). For this reason, pSinE was used for subsequent studies. RNA transcripts from these plasmids were synthesized *in vitro* and each RNA was mixed with the Sindbis helper transcript, DH(26S), which contains the genes for Sindbis virus structural proteins (*8*). When DH(26S) RNA transcripts are cotransfected with the recombinant RNA from pSinRep5, expression of the structural proteins *in trans* from DH(26S) RNA transcripts allows the packaging of recombinant RNA into virions (pseudovirions) (*7*). Because DH(26S) lacks a Sindbis virus packaging signal, DH(26S) RNA is not packaged into pseudovirions.

A mixture of pSinM RNA transcripts and DH(26S) and another of pSinE RNA transcripts and DH(26S) were independently transfected into BHK cells using electroporation (*7*). As a control, DH(26S) and RNA transcripts of pSinRep/LacZ (Invitrogen) encoding the lacZ gene were cotransfected. Culture fluid was collected 30 h after transfection and then inoculated into DBT cells. Cells were metabolically labeled with 50–100 μCi of Tran^{35}S-label (ICN) from 6.5 to 7 h postinoculation for labeling of M protein and from 5 to 7 h postinoculation for labeling of E protein. The cell lysates were prepared with lysis buffer [150 mM NaCl, 50 mM Tris–HCl (pH 7.5), 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and 1 mM phenylmethylsulfonyl fluoride]. Radioimmunoprecipitation analysis of cell extracts from the cells that were inoculated with the culture fluid from cells cotansfected with pSinM transcripts and DH(26S) and from cells cotransfected with pSinE transcripts and DH(26S), respectively, showed excellent expression of M and E proteins (Fig. 1). MHV M protein, N protein, and an unknown protein (shown by an asterisk in Fig. 1) were also coimmunoprecipitated by anti-E protein peptide-2 antibody (*9*) under these experimental conditions. Anti-E protein antibody did not immunoprecipitate expressed MHV M and N proteins (unpublished data), eliminating the possibility that anti-E protein antibody cross-reacts with M and N proteins. Coimmunoprecipitation of M and N proteins in MHV-infected cells by anti-E protein antibody most likely indicated the interaction of viral structural proteins in MHV-infected cells. β-Galactosidase staining of DBT cells at 7 h postinoculation of the culture fluid from the cells cotransfected with pSinRep/LacZ transcripts and DH(26S) demonstrated a high level of expression of β-galactosidase (data not shown). These data demonstrated that pseudovirions carrying pSinE RNA transcripts (SinE pseudovirions), pSinM RNA transcripts (SinM pseudovirions), and pSinRep/LacZ transcripts (SinLacZ pseudovirions) were released from the cells that were cotransfected with DH(26S) and pSinE transcripts, DH(26S) and pSinM transcripts, and DH(26S) and pSinRep/LacZ transcripts, respectively. Infection of pseudovirions and subsequent replication of recombinant vector RNA resulted in a high level of expression of cloned proteins in DBT cells (Fig. 1).

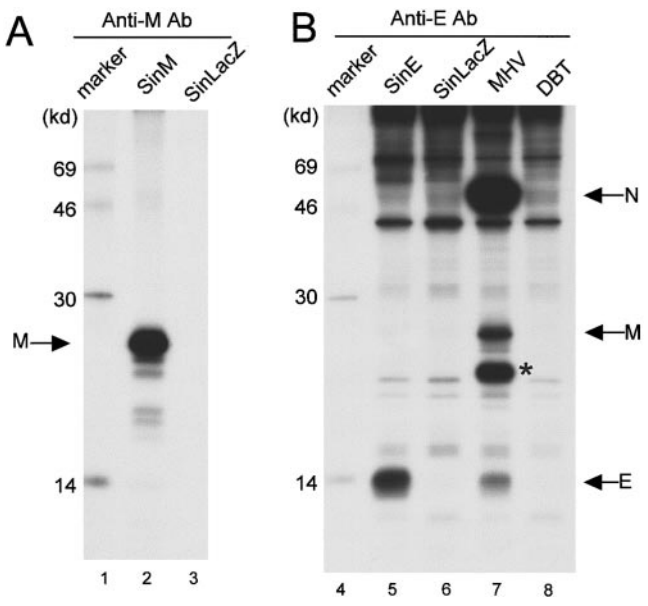

FIG. 1. Analysis of intracellular MHV proteins after infection of SinM and SinE pseudovirions. DBT cells were mock infected or infected with recombinant Sindbis pseudovirions. Intracellular proteins were labeled with Tran^{35}S-label for from 6.5 to 7 h p.i. (A) or from 5 to 7 h p.i. (B). Intracellular proteins were immunoprecipitated with anti-M protein monoclonal antibody J.2.7. (A) or anti-E protein peptide-2 antibody (B). Inoculum: SinM pseudovirion, lane 2; SinLacZ pseudovirion, lanes 3, 6; SinE pseudovirion, lane 5; MHV-A59, lane 7; mock infection, lane 8. Lanes 1 and 4, ^{14}C-labeled protein size marker.

To determine whether E protein is released from the E-protein-expressing cells, SinE pseudovirion-infected DBT cells were radiolabeled with Tran^{35}S-label from 5 to 9 h p.i. As controls, DBT cells were infected with SinM pseudovirions alone or coinfected with SinM pseudovirions and SinE pseudovirions. Culture fluid was harvested at 9 h p.i. and briefly centrifuged to remove cell debris. Culture media were applied onto a discontinuous sucrose gradient consisting of 20 and 60% sucrose in NTE buffer [100 mM NaCl, 10 mM Tris–HCl (pH 7.5), and 1 mM EDTA]. After centrifugation at 26,000 rpm for 15 h at 4°C in a Beckman SW28 rotor, the interface between 20 and 60% sucrose was collected and diluted threefold with NTE buffer. The sample was further purified by the 20–60% continuous sucrose gradient centrifugation at 26,000 rpm for 15 h at 4°C in a Beckman SW28 rotor. Ten fractions were collected from the bottom of the gradient and an equal amount of 2× lysis buffer was added to each fraction. M and E proteins in each of the sucrose fractions were immunoprecipitated with a mixture of

anti-M protein monoclonal antibody J.2.7 (*10*) and anti-E protein peptide-2 antibody (*9*). No M protein signal was detected in the culture fluid from SinM pseudovirion-infected DBT cells (data not shown). From the cells coexpressing M and E proteins, both M and E proteins were detected in three sucrose fractions (Fig. 2A). These results were expected; expression of M protein alone does not produce VLPs, while coexpression of M and E proteins produce VLPs (*2–4*). Characterization of culture fluids from coexpressing cells showed that the distributions of M and E proteins in the gradient were not identical; the major M protein signal was in fractions 6 and 7, while the major E protein signal was in fractions 7 and 8; M protein signal in fractions 6 and 7 was about 100 times higher than that in fraction 8 and the E protein signal in fractions 7 and 8 was about 15 times higher than that in fraction 6. To our surprise, E protein was released into the culture supernatant from E-protein-expressing cells (Fig. 2B). The released E protein sedimented to a sucrose density of about 1.13 g/cm^3, which was slightly lighter than the major signal of M protein (density 1.16–1.14 g/cm^3) released from coinfected cells; the relative intensity of E protein signal in fractions 7–9 was 2, 4.3, and 1, respectively. The presence of E protein in culture fluid was not due to the release of an intracellular form of E protein as a result of cellular disruption, since no cytopathic effects were detected in SinE pseudovirion-infected DBT cells at 9 h p.i. In coexpressing cells, we saw a slightly different distribution of E and M proteins in the sucrose gradient (Fig. 2A), indicating that VLPs, containing M and E proteins and free E protein, not associated with M protein, were released into the culture media.

To know whether the release of E protein from E-protein-expressing cells was an artifact of the expression system, release of E protein from MHV-infected cells was examined. MHV-A59-infected cells were radiolabeled with Tran^{35}S-label from 5 to 9 h p.i. The culture fluid was collected and MHV was purified on a 20–60% continuous sucrose gradient under the same conditions as described above. MHV structural proteins in each fraction were immunoprecipitated with a mixture of anti-MHV serum, anti-M protein monoclonal antibody, and anti-E protein antibody (Fig. 3A). MHV S protein, N protein, and M protein appeared in a single radioactive peak that corresponded to a sucrose density of 1.18 g/cm^3, which is the buoyant density of MHV particles (*11*). E protein had two radioactive peaks, one corresponded to the MHV buoyant density of 1.18g/cm^3 and the other peak had a density of about 1.13 g/cm^3, which was similar to the density of released E protein from E-protein-expressing cells. These data suggested that E protein that was not associated with MHV particles was released from MHV-infected cells. A different centrifugation condition was used to further confirm the release of E protein from MHV-infected cells. ^{35}S-labeled culture fluid from DBT cells, which were infected with MHV-A59 or SinE pseudovirions, was collected 9 h p.i., as described above, and then applied onto a discontinuous sucrose gradient consisting of 20 and 60% sucrose in NTE buffer. After centrifugation at 26,000 rpm for 15 h at 4°C in a

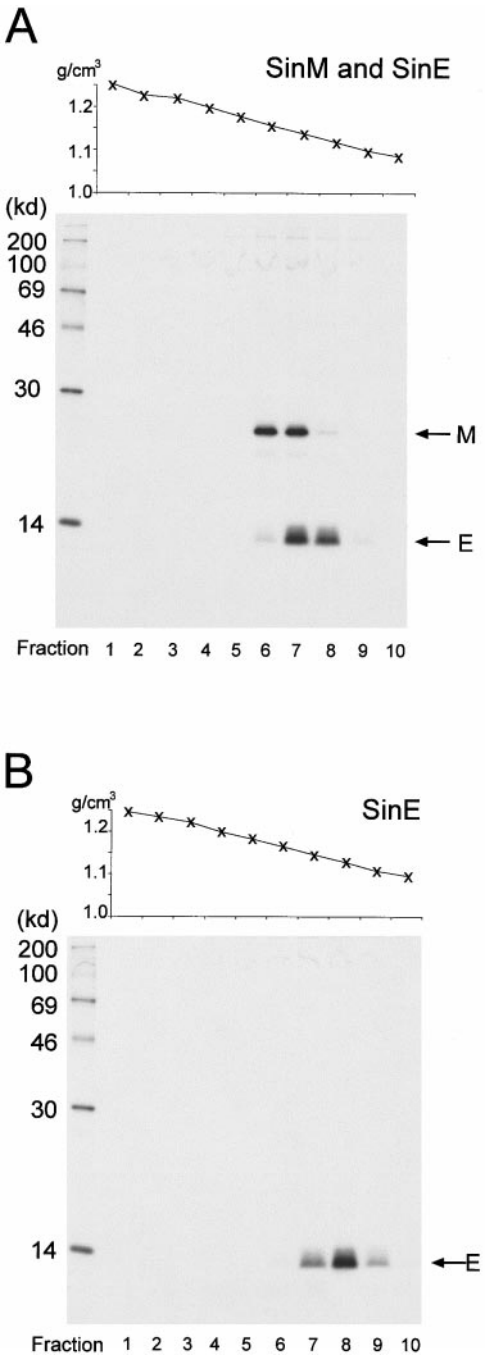

FIG. 2. Release of E protein from E-protein-expressing cells. DBT cells were coinfected with SinM and SinE pseudovirions (A) or infected with SinE pseudovirion (B). Cells were radiolabeled with Tran^{35}S-label from 5 to 9 h p.i. Released VLPs or E-protein-containing vesicles were purified by sucrose gradient centrifugation, as described in the text. M and E proteins in each fraction were immunoprecipitated with a mixture of anti-M protein monoclonal antibody and anti-E protein antibody. The ^{14}C-labeled size marker is shown on the left of each gel.

Beckman SW28 rotor, the interface between 20 and 60% sucrose was collected and diluted threefold with NTE buffer. The sample was layered over a 10–30% continuous sucrose gradient, with a 60% sucrose cushion at the bottom of the gradient. The gradients were centrifuged at 30,000 rpm for 1 h at 4°C in a Beckman SW41 rotor. Twelve fractions were collected from the bottom of the gradient and MHV structural proteins in each fraction were immunoprecipitated with a mixture of anti-MHV serum, anti-M protein monoclonal antibody, and anti-E protein antibody. Densitometric analysis of N protein in the sample from MHV-infected cells showed a single radioactive peak in fraction 4 with a sucrose density of 1.15 g/cm^3 (Fig. 3B). MHV M and S proteins also had a single radioactive peak in fraction 4 (data not shown), demonstrating that fraction 4 represented the major MHV peak. Radioactive signal of E protein, which was broader than that of N protein, had two peaks. One of the peak signals was in the heavier fraction 4; this signal most probably represented E protein in MHV particles. The other peak signal of E protein had a lighter density of about 1.11 g/cm^3. Under the same centrifugation condition, released E protein from the E-protein-expressing cells had a single radioactive peak in fractions 6 and 7; the densities of sucrose in fractions 6 and 7 were 1.12 and 1.11 g/cm^3, respectively (Fig. 3C). E protein released from the E-protein-expressing cells and the E protein signal from MHV-infected cells, corresponding to the lighter density, had very similar sucrose densities. Essentially the same result was obtained in three independent experiments. These results strongly indicated that E protein that was not associated with MHV particles was released from MHV-infected cells.

To establish the buoyant density of released E protein, culture fluids from E-protein-expressing cells were applied onto a discontinuous sucrose gradient and centrifuged as described above. The interface between 20 and 60% sucrose was collected, diluted, and separated again on a 10–30% continuous sucrose gradient at 30,000 rpm in a Beckman SW40 rotor at 4°C for 1, 3, 6, or 12 h. Radioimmunoprecipitation of E protein from the sucrose fractions showed that released E protein sedimented to sucrose densities of 1.10–1.13 g/cm^3 after 1 h centrifugation. Released E protein sedimented to a sucrose density of about 1.13 g/cm^3 after 3 h centrifugation, and longer centrifugation did not affect the sedimentation profile (data not shown); these data demonstrated that the buoyant density of E protein was about 1.13g/cm^3. The released E protein had a narrow distribution in the sucrose gradients under all the described centrifugation conditions (data not shown).

We conducted flotation analysis to determine whether the E protein was released, from the E-protein-expressing cells, as a membrane protein in vesicles or as a secreted protein. The E protein released from SinE pseudovirion-infected cells was partially purified by discontinuous sucrose gradient centrifugation. Sucrose crystals were added to the partially purified E protein to a final concentration of 67% sucrose, and the sample was placed at the bottom of a centrifuge tube. Subsequently, 5 ml of 65% sucrose and 3 ml of 10% sucrose solutions were overlaid onto the 67% sucrose solution. The gradients were centrifuged at 35,000 rpm for 18 h at 4°C, and fractions were collected from the top of the gradient. Radioimmunoprecipitation of E protein by anti-E protein antibody determined that most of the E protein settled at the interface between 65 and 10%

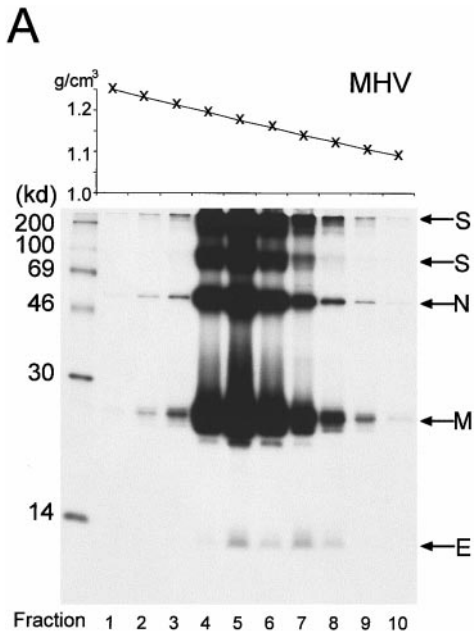

FIG. 3. Release of E protein from MHV-infected cells. DBT cells were infected with MHV (A, B) or SinE pseudovirion (C). The density of the sucrose fractions is shown at the top of each panel. (A) Culture media from MHV-infected cells, which were radiolabeled with Tran^{35}S-label from 5 to 9 h p.i., were applied onto a discontinuous sucrose gradient consisting of 20 and 60% sucrose and centrifuged at 26,000 rpm for 15 h at 4°C in a Beckman SW28 rotor. The interface between 20 and 60% sucrose was collected, diluted, and then applied onto a 20–60% continuous sucrose gradient. After centrifugation at 26,000 rpm for 15 h at 4°C in a Beckman SW28 rotor, fractions were collected and MHV-specific proteins in each fraction were immunoprecipitated with a mixture of anti-MHV serum, anti-M protein monoclonal antibody, and anti-E protein antibody. The ^{14}C-labeled size marker is shown on the left of the gel. (B, C) MHV-infected cells (B) or E-protein-expressing cells (C) were radiolabeled with Tran^{35}S-label from 5 to 9 h p.i. Culture medium was then applied onto a discontinuous sucrose gradient consisting of 20 and 60% sucrose and centrifuged at 26,000 rpm for 15 h at 4°C in a Beckman SW28 rotor. The interface between 20 and 60% sucrose was collected, diluted, and layered over a 10–30% continuous sucrose gradient with a 60% sucrose cushion at the bottom of the gradient. The samples were centrifuged at 30,000 rpm for 1 h at 4°C in a Beckman SW41 rotor. Twelve fractions were collected and MHV structural proteins in each fraction were immunoprecipitated with a mixture of anti-MHV serum, anti-M protein monoclonal antibody, and anti-E protein antibody. Portions of gels showing N and E proteins (B) and E protein (C) are shown. Relative intensities of N protein (triangles) and E protein (open boxes) are also shown.

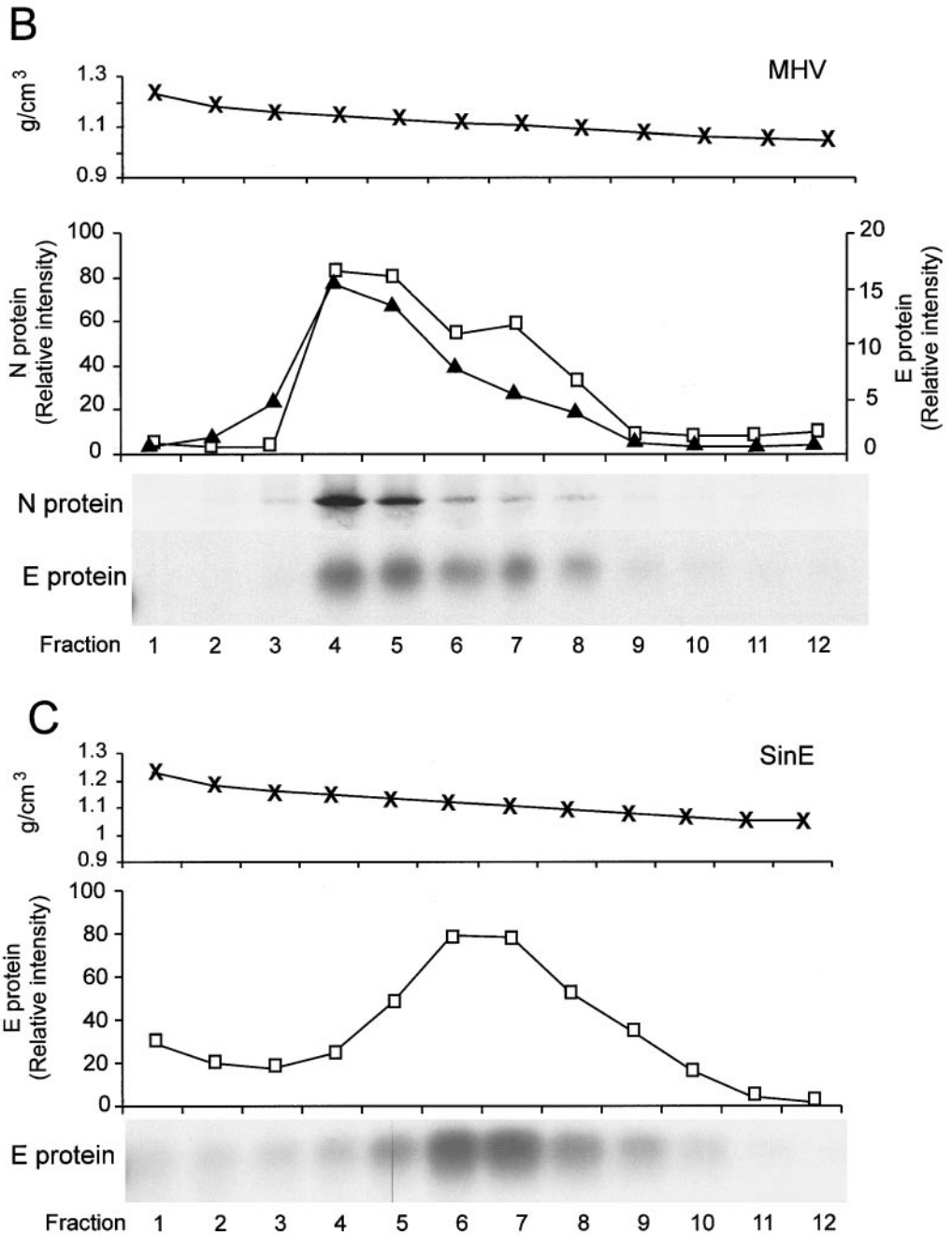

FIG. 3—*Continued*

sucrose, which indicated that the E protein had migrated from the 67% sucrose solution to the interface during centrifugation (Fig. 4A). When NP-40 was added to the 67% sucrose solution at a final concentration of 1% and the same flotation analysis was performed, the majority of E protein was detected in the 65–67% sucrose fractions (Fig. 4A). In flotation analysis, membrane-associated proteins localize at the interface of 65 and 10% sucrose and soluble proteins remain in the heavier sucrose loading zone of the gradient (*12*). Therefore, these data demonstrated that released E protein was associated with lipid membrane vesicles and that NP-40 treatment disrupted this association.

Next we used a sodium carbonate treatment method to determine whether the E protein was released as an integral membrane protein. Culture fluids from E-protein-expressing cells were incubated in 100 mM Na_2CO_3 at pH 11 for 30 min on ice. As a control, the culture fluid was incubated in a buffer containing no Na_2CO_3 at pH 7.0. Sodium carbonate treatment disrupts membrane vesicles, releasing the peripheral membrane proteins from these vesicles, while integral membrane proteins are not

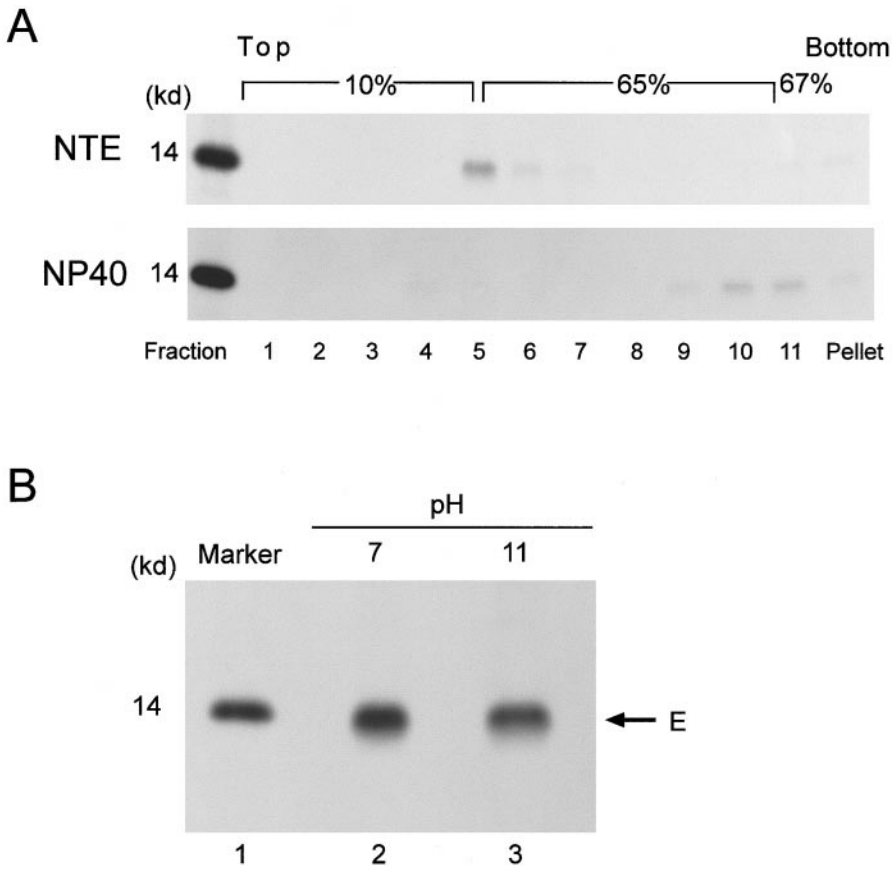

FIG. 4. Released E protein exists as an integral membrane protein in membranous vesicles. (A) Flotation analysis of E-protein-containing vesicles. The sucrose fraction containing partially purified E-protein-containing vesicles was adjusted to 67% sucrose in NTE (NTE) or 67% sucrose in NTE plus 1% NP-40 (NP-40) and overlaid with 65 and 10% sucrose. After centrifugation fractions were collected and E protein was immunoprecipitated using anti-E protein antibody. Fraction 11, fractions 6–10, and fractions 1–4 represent 67, 65, and 10% sucrose, respectively. Fraction 5 represents the interface between 65 and 10% sucrose. The ^{14}C-labeled 14-kDa size marker is shown on the left of each gel. (B) Analysis of E-protein-containing vesicles after sodium carbonate treatment. Culture fluid from E-protein-expressing cells was incubated in 100 mM Na_2CO_3 at pH 11 (lane 3) or pH 7 (lane 2) for 30 min on ice. After incubation, the samples were centrifuged at 50,000 rpm for 1 h at 4°C in a Beckman SW50 rotor. Pelleted protein was immunoprecipitated with anti-E protein antibody. Lane 1, the ^{14}C-labeled 14-kDa size marker.

affected (*3, 13*). After incubation, the samples were centrifuged at 50,000 rpm for 1 h at 4°C in a Beckman SW50 rotor. E protein was present in the pellet after both alkali treatment and the neutral pH treatment (Fig. 4B), leading us to conclude that E protein was released as an integral membrane protein in lipid vesicles.

The release of E-protein-containing membrane vesicles from MHV-infected cells and E-protein-expressing cells further emphasized the pivotal role of E protein in coronavirus assembly. Other examples, where expression of a single viral protein results in production and release of lipid vesicles, exist: the Gag protein of Rous sarcoma virus (RSV) and the M protein of vesicular stomatitis virus (VSV) both have the ability to bud from cells independently of other viral proteins (*14, 15*). Recent studies indicate that a proline-rich motif found within the VSV M protein and the RSV Gag protein interacts with WW domains of cellular proteins (*16, 17, 18*) and this interaction may be important for virus budding (*18, 19*). The WW domain is a highly structured, modular domain of 38–40 amino acids that facilitates protein–protein interactions. The WW domain is present in a wide variety of cellular proteins with diverse functions and consists of a hydrophobic pocket that is flanked by two perfectly conserved tryptophan (W) residues (*16*). Involvement of the WW domain of some host protein in budding of MHV E-protein-containing vesicles seems less likely, as MHV E protein lacks the proline-rich motif found in rhabdoviruses and RSV. Nevertheless, some host protein may possibly interact with E protein and this interaction may drive the budding of coronavirus. VSV and RSV bud from the cytoplasmic membrane, while coronavirus buds from an internal compartment between the endoplasmic reticulum and the Golgi apparatus; cellular protein(s) that localizes at the budding site may affect coronavirus budding.

Although E protein is a major player in coronavirus envelope formation and budding, E protein alone may be insufficient for nucleocapsid incorporation into the virus particles. Earlier we established a complementary DI

system, in which MHV particles are released after coinfection of two DI RNAs; one is a self-replicating DI RNA encoding viral polymerase function and N protein, and the other subgenomically expressing M and E proteins (*5*). Release of MHV particles containing nucleocapsid requires synthesis of both E and M proteins in the complementary DI system (*5*). Presumably, in the complementary DI system E-protein-containing vesicles were released from the cells, in which self-replicating DI RNA and another DI RNA encoding only E protein were replicated. We know that no MHV nucleocapsid was detected in the supernatant (*5*), suggesting that E-protein-containing vesicles may not include nucleocapsid. Furthermore, sucrose gradient centrifugation of MHV particles showed two E protein radioactive peaks, whereas N protein had only one peak corresponding to the MHV buoyant density (Fig. 3), indicating that membrane vesicles containing only E protein do not include nucleocapsid. Another envelope protein, S protein, is not involved in the incorporation of the nucleocapsid, because MHV particles containing nucleocapsid are produced in the absence of S protein (*5*). Most probably, M protein functions to incorporate nucleocapsid into virus particles. Likely, M protein interacts with the nucleocapsid and E protein at the coronavirus budding site, and these intermolecular interactions facilitate the envelopment of the nucleocapsid. Data demonstrating that purified M protein and viral RNA interact *in vitro* (*20*) and that M protein and nucleocapsid interact in MHV-infected cells (Narayanan, Maeda, Maeda, and Makino, unpublished data) support this model. This model is also consistent with the present data that M, N, and E proteins were coimmunoprecipitated by anti-E protein antibody in MHV-infected cells (Fig. 1B).

VLPs that are made of M and E proteins are morphologically similar to coronavirus particles (*3, 4*), whereas the morphology of E-protein-containing vesicles is not known. Poor production of E-protein-containing vesicles in E-protein-expressing cells and a lack of anti-E protein monoclonal antibodies which are suitable for immunogold-labeling studies are the main obstacles in determining the shape of E-protein-containing vesicles. Characterization of E-protein-containing vesicles is important. If E-protein-containing vesicles and MHV particles have similar sizes and morphologies, then E protein alone may determine the size and shape of coronavirus particles. If E-protein-containing vesicles are significantly different from MHV particles in size and shape, then perhaps M protein is important for the determination of the size and morphology of coronavirus particles.

E-protein-containing vesicles were released from MHV-infected cells (Fig. 3), demonstrating that the production of E-protein-containing vesicles was not an artifact of the expression system. Probably E-protein-containing vesicles are also produced in coronavirus-infected humans and animals. Do E-protein-containing vesicles have any biological functions? Recently Baudoux *et al.* (*4*) showed that VLPs that are made of coronavirus transmissible gastroenteritis virus (TGEV) M and E proteins can induce α-interferon in leukocytes. Their study suggests that M protein in VLP is important for α-interferon induction. Further studies will show whether MHV E-protein-containing vesicles have any biological functions.

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