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The Nucleocapsid-Binding Spike Subunit E2 of Semliki Forest Virus Requires Complex Formation with the E1 Subunit for Activity

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Alphaviruses, such as Semliki Forest virus (SFV), mature by budding at the plasma membrane (PM) of infected cells and enter uninfected ones by a membrane fusion process in the endosomes. Both processes are directed by the p62/E2-E1 membrane protein heterodimer of the virus. The p62 protein, or its mature form E2, provides a cytoplasmic protein domain for interaction with the nucleocapsid (NC) of the virus, and the E1 protein functions as a membrane fusogen. We have previously shown that the p62/E2 protein of SFV controls the membrane fusion activity of E1 through its complex formation with the latter (A. Salminen, J. M. Wahlberg, M. Lobigs, P. Liljeström, and H. Garoff, J. Cell Biol. 116:349–357, 1992). In the present work, we show that the E1 protein controls the NC-binding activity of p62/E2. We have studied E1 expression-deficient SFV variants and shown that although the p62/E2 proteins can be transported to the PM they cannot establish stable NC associations.

Semliki Forest virus (SFV) is an alphavirus which has been extensively used as a model to study the assembly, structure, and function of enveloped viruses. Both the nucleocapsid (NC) and the spikes of the membrane are organized according to T=4 icosahedral symmetry (10, 19, 48, 57). There are 80 spikes in the membrane, each of which consists of three copies of a heterodimeric membrane protein complex. The NC contains the viral genome in complex with 240 copies of capsid (C) proteins. The C proteins are arranged into 30 hexameric and 12 pentameric capsomers. The spikes undergo lateral interactions with each other at the outer membrane surface and NC interactions via their internal domains or tails.

All structural proteins of SFV, that is, the C protein and the membrane proteins p62 and E1, are synthesized from a common coding unit on the viral subgenome (23). The order of synthesis is C-p62-6K-E1. The individual chains are generated cotranslationally by the serine protease activity of C and by the signal peptidase activity of the endoplasmic reticulum (ER) membrane (1, 3, 4, 6, 11, 22, 23, 26, 27, 32, 43, 44). The 6K protein represents a spacer-peptide, which functions as a signal sequence for E1. After insertion of the p62 and E1 proteins into the ER membrane, they heterodimerize into p62-E1 complexes in a fast and efficient manner and are then transported to the plasma membrane (PM) to take part in virus budding (3, 46, 64).

Results from genetic, biochemical, and molecular modelling experiments show that it is the p62 protein, or its intracellular cleavage product E2, that mediates NC binding and thereby drives virus budding (2, 8, 20, 28, 31, 40, 45, 53, 56, 62). The E1 protein carries the membrane fusion activity of the virus (13, 21, 47, 50), which is required for virus penetration into uninfected cells (41). A central question about virus assembly and entry is how the budding and membrane fusion functions are controlled so that they are expressed at the right moments in the virus replication cycle.

Results from studies of the control mechanism of the membrane fusion function suggest that the E1 protein obtains a certain "metastable" conformation, with a suppressed fusion activity, through its complex formation with p62 (1a, 38, 51). This conformation is then chaperoned by the p62 protein throughout the assembly phase in the infected cell and by the p62 cleavage product E2 in the virus particle. Activation of the E1 fusogen occurs in two steps. The first is cleavage of p62 into E2. This takes place inside the infected cell just before virus budding (12). The cleavage destabilizes the heterodimer sufficiently to allow the second step, the actual activation of the fusion function of E1 (59). This occurs after the particle has been endocytosed by an uninfected cell and involves low-pH-induced dissociation of the heterodimeric spike unit and subsequent homotrimerization of E1 into a membrane fusion-active conformation (7, 19, 29, 41, 58, 60).

Earlier results on virus budding suggest that the E2 tail-C protein interaction is controlled both by a posttranslational maturation of the tail and by the oligomeric state of the tail (14, 28, 35, 36). In the present work, we have investigated whether E1 plays any role in the control of this interaction. Using an SFV cDNA clone, we have constructed SFV genomes (i) lacking E1 and (ii) lacking both 6K and E1 and analyzed virus assembly in cells transfected with the corresponding replication-competent RNAs.

MATERIALS AND METHODS

Cells and viruses. Baby hamster kidney (BHK-21) cells (American Type Culture Collection, Rockville, Md.) were cultivated as described previously (3). Wild-type (wt) SFV was derived from pSFV4 (34).

Plasmids. Apart from pSFV4, which contains the entire SFV genome, we used (i) pSFV-C-p62-6K, which encodes viral proteins C, p62, and 6K but not E1; (ii) pSFV-C-p62, which encodes viral proteins C and p62 but not 6K or E1; (iii) pSFV-p62-6K, which encodes viral proteins p62 and 6K but not C or E1; and (iv) pSFV-E1, which encodes viral protein E1 but not C, p62, or 6K.

The plasmids pSFV-C-p62-6K and pSFV-C-p62 were engineered by introducing two stop codons (TAG) directly after the coding sequences for 6K and p62, respectively, in pSFV4. For this, we used the oligonucleotides 3′-GGCG GTCTCGAATCATCGTAAGCTGTCATTACGGC-5′ and 3′-GGCGCCCG CGTGCGTATCATCCACCGTCTCTGATACCGG-5′ (the changed bases are

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underlined) and the mutagenesis protocol described in reference 30. Plasmid pSFV-p62-6K was generated by replacing the 2,591-bp *Nsi*I-*Spe*I fragment in pSFV-p62-6K-E1 (56) with the corresponding fragment in pSFV-C-p62-6K. Plasmid pSFV-E1 was created by first introducing a *Bam*HI site in the 6K gene of the SFV genome. The partial 6K gene and the entire E1 gene was then subcloned as a *Bam*HI-*Spe*I fragment into the pSFV-3 expression vector (33). The *Bam*HI mutagenesis primer was 3′-ACGGAGTCTTTCCTAGGCGCAGCGACATT CTCGGAA-5′. The beginning of the translation product of the new subgenome region is NH_2-Met-Asp-Pro-Arg-Arg-Cys, where the last residue corresponds to residue 43 of 6K.

Antibodies. We used mouse acites preparations of anti-E1 monoclonal antibody UM 8.139 (5, 59) and anti-E2 monoclonal antibodies UM 5.1 (5, 59) and K-26/98 (obtained from A. Salmi, Virology Department, University of Turku, Turku, Finland). The latter antibody precipitates specifically glycosylated forms of p62 and E2 from Nonidet P-40 (NP-40) lysates of infected or transfected cells (20a).

Transfection and preparation of recombinant SFV. SFV expression plasmids were used for transcription of replication-competent SFV RNA in vitro as described previously (34). Transfection of cells with RNA has also been described previously (55). The recombinant virus was prepared by cotransfection of cells with recombinant SFV RNA and helper 1 RNA as described previously (33). Infection of cells with wt SFV or recombinant SFV has been described previously (59).

Metabolic labelling of cells, preparation of cell lysate and isolation of virus particles. Transfected or infected cells were labelled with [^{35}S]methionine and chased as described previously (3). The preparation of NP-40 lysates of cells has also been described previously (59). *N*-Ethylmaleimide (20 mM) was included in the lysis buffer to block free SH groups. Virus particles in medium samples were recovered by pellet formation through a 10% (wt/wt) sucrose cushion (54). Pelleted material was taken up in sodium dodecyl sulfate (SDS) gel-loading buffer as described below for immunoprecipitates.

Immunoprecipitation of viral proteins. Immunoprecipitation was performed as described previously with 100 μl of cell lysate (59). The precipitate was taken up into 50 μl of SDS gel-loading buffer. This contained 200 mM Tris-HCl (pH 8.8), 20% glycerol, 5 mM EDTA, 0.02% bromophenol blue, 1 mM methionine, and 4% SDS. The mixture was incubated at 70°C for 3 min and centrifuged. The supernatant was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). When a sample was to be analyzed under reducing and nonreducing conditions, the supernatant was divided in two and dithiothreitol was added to one part to a final concentration of 50 mM. This sample was then incubated at 95°C for 3 min before being subjected to SDS-PAGE.

Digestions with endoglycosidases. Endoglycosidase H (endo H [5 mU/μl]; Boehringer, Mannheim, Germany) treatment of a lysate sample was as follows. A 5-μl volume of lysate was mixed with 20 μl of endo H buffer (50 mM sodium citrate [pH 6.0], 1% SDS) and 1 μl of endo H. Incubation was carried out for 16 h at 37°C. A 10-μl aliquot of the reaction mixture was processed for SDS-PAGE under nonreducing conditions. This was done by adding 5 μl of fourfold-concentrated SDS gel-loading buffer (without SDS), 4 μl of 20% SDS, and 1 μl of H_2O and incubating the mixture at 70°C for 2 min. Lysate samples were treated with *N*-glycosidase F (NGase F [50 mU/μl]; Boehringer) essentially as described for the endo H treatment. NGase F buffer contained 50 mM sodium acetate (pH 5.2), 20 mM EDTA, and 5% 1-*o*-*n*-octyl-β-D-glucopyranoside.

Biotinylation of viral proteins at the PM. Confluent monolayers (10^6 cells/dish) were infected with SFV-C-p62-6K recombinant virus at a multiplicity of infection of 5. After virus adsorption (1 h at 37°C) and further incubation (4 h), the cells were starved for methionine (15 min) and then labelled with [^{35}S]methionine for 15 min. The pulse media were removed, and the cells were chased for 0, 30, 60, 120, or 180 min. The cultures were shifted to 0°C and washed with cold phosphate-buffered saline (PBS). A 500-μl volume of PBS containing sulfo-NHS-SS-biotin (Pierce Chemical Co., Rockford, Ill.) at 0.5 mg/ml was added to each culture, and these were incubated at 0°C for 30 min under gentle shaking. The biotinylation solution was removed, and the label was quenched for 15 min in 50 mM NH_4Cl–PBS. The cultures were washed twice in cold PBS and then lysed in 400 μl of NP-40-containing buffer (59). The lysates were cleared by centrifugation in an Eppendorf benchtop centrifuge at 11,000 rpm. Samples of the total lysates were analyzed by SDS-PAGE under reducing conditions. Then 200-μl volumes of the lysate samples were used for the streptavidin reactions. For this, the samples were incubated overnight with 30 μl of packed streptavidin-agarose (Sigma Chemical Co, St. Louis, Mo.) at 5°C. The protein-biotin-streptavidin-agarose complexes were recovered by pellet formation and then washed once in a buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1% Triton X-100 (TX-100), 0.1% SDS, 0.2% bovine serum albumin (BSA), and 0.5 mM phenylmethylsulfonyl fluoride (PMSF); three times in a buffer containing 200 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% TX-100, 0.1% SDS, 0.2% BSA, and 0.5 mM PMSF; three times in a buffer containing 50 mM Tris-HCl (pH 8.0), 1 M NaCl, 1% TX-100, 0.1% SDS, 0.2% BSA, and 0.5 mM PMSF; and, finally, twice in 10 mM Tris-HCl (pH 8.0). The washed precipitates were suspended in 100 μl of gel-loading buffer containing DTT and boiled at 95°C for 3 min, and 10-μl portions were applied to a polyacrylamide gel.

SDS-PAGE. Samples of immunoprecipitates, streptavidin precipitates, virus pellets, and lysates were run on 10% gels. The electrophoresis system has been described previously (54). After SDS-PAGE, the gel was treated for fluorography with 1 M sodium salicylate for 30 min, and then dried. The radioactivity in the viral protein bands was quantitated with a FUJIX BAS 2000 TR phosphorimager (Fuji). Molar ratios were calculated after normalizing the counts of the respective proteins to their methionine contents.

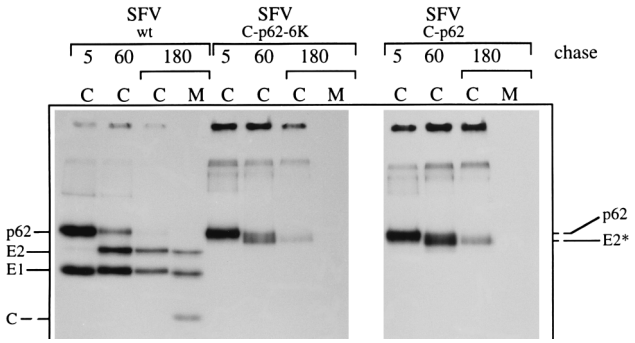

FIG. 1. SDS-PAGE analyses of viral proteins in cells and media of cultures transfected with SFV wt, SFV-C-p62-6K, or SFV-C-p62 RNA. Groups of transfected BHK-21 cell cultures were pulse-labelled with [^{35}S]methionine for 15 min at 6 h posttransfection. The cultures in each group were chased for 5, 60, and 180 min, respectively. The cells were lysed, and the medium samples from the longest chase were processed for particle isolation. Glycosylated viral membrane proteins were recovered from lysates by immunoprecipitation with a mixture of anti-p62/E2 and anti-E1 monoclonal antibodies. Samples of immunoprecipitates (C) and medium pellets (M) were analyzed on 10% gels under nonreducing conditions. The figures represent autoradiographs of gels. Viral proteins are indicated. E2* is a processing product of p62.

Morphological analyses. Immunofluorescence analyses and electron microscopic analyses have been described in references 54 and 59.

Sedimentation analyses. Cell lysates were analyzed for viral membrane protein oligomers by sedimentation in sucrose gradients as described in reference 3. Analysis for NC was done as described in reference 15. The gradients were fractionated from below, and samples (20 μl) of each fraction were processed for SDS-PAGE under nonreducing conditions.

RESULTS

SFV-C-p62 particles cannot be produced. The possibility that an NC-p62 interaction, in the absence of E1, would be sufficient for SFV-like particle production was explored by pulse-chase experiments of cells transfected with the SFV RNA variants SFV-C-p62-6K and SFV-C-p62. SDS-PAGE analyses of radiolabelled viral proteins in cell and medium samples from the transfected cells showed that although the C and p62 proteins were both produced at levels corresponding to those of wt SFV, there was no release of particles into the media of the transfected cells (Fig. 1; see also Fig. 4, lanes 1 and 4). We also noted in the analysis shown in Fig. 1 that part of the pulse-labelled p62 protein expressed from the E1 deletion variants underwent aggregation (see the material at the top of the gel, which is mostly absent in the wt strain); processing into a slightly faster-migrating form, E2* (between 5 and 60 min of chase); and apparent degradation (mostly between 60 and 180 min of chase).

We reasoned that the inhibited particle formation of the SFV deletion variants might be due either to inefficient transport of the p62 protein to the budding site(s) at the PM or to a weakened p62-NC interaction. To find out, we first analyzed whether p62 expressed by the variants underwent normal intracellular transport-dependent processing events, that is, p62 cleavage and sugar modification. Since the p62 protein behaved the same whether synthesized with or without the 6K protein, we will discuss only the experiments that were done with the 6K-containing constructs.

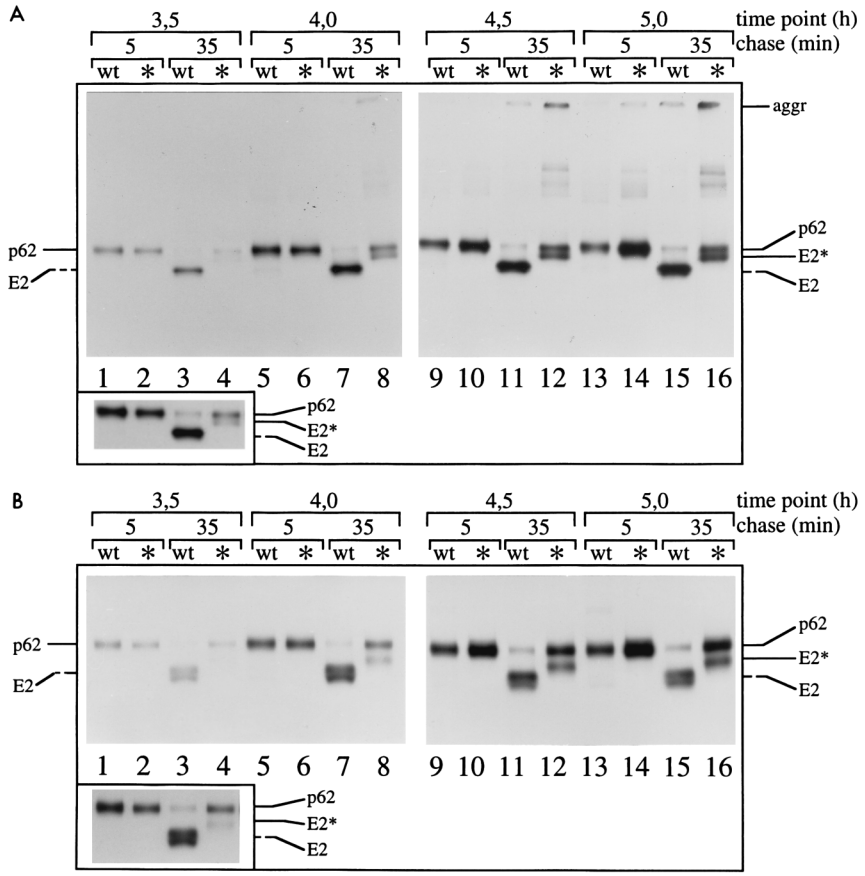

FIG. 2. SDS-PAGE analyses of viral proteins in SFV wt and SFV-C-p62-6K RNA-transfected cells at different time points posttransfection. Pairs of transfected cell cultures were pulse-labelled for 10 min at the indicated time points after transfection. The cultures of each pair were chased for 5 and 35 min, respectively. The cells were lysed, and glycosylated p62/E2 proteins were isolated by immunoprecipitation. Samples of the immunoprecipitates were then prepared for gel electrophoresis. Analyses were done under nonreducing (A) and reducing (B) conditions. Equal amounts of samples were used in each panel. Overexposures of bands in lanes 1 to 4 are shown as small inserts in both panels. Lanes marked * refer to analyses of SFV-C-p62-6K samples.

The p62 protein expressed from SFV-C-p62-6K is cleaved into E2 and becomes endo H resistant. Figure 2 shows a pulse-chase analysis of the p62 protein produced in BHK-21 cells at several different time points after transfection with SFV-C-p62-6K and SFV wt RNA, respectively. The p62 protein of the SFV variant migrated as the p62 protein of wt SFV when analyzed after a 5-min chase. At 35 min of chase, the p62 protein of wt SFV was cleaved into E2, which migrates considerably faster than p62. In the SFV variant, the corresponding p62 cleavage did not seem to take place. Instead, this p62 was processed into a form (E2*) which migrated between p62 and E2. This aberrant processing of p62 was seen in the analyses performed 3.5 h after transfection (overexposed lanes in Fig. 2A). A quantitation of the processed and unprocessed p62 separated under reducing conditions (Fig. 2B) showed that about one-fourth of p62 was processed at 3.5 h after transfection. This increased to a maximum of about two-fifths at 4.5 h after transfection. At the same time, the rate of viral protein synthesis increased about ninefold. When the proteins were analyzed under nonreducing conditions, it became evident that the increased rate of synthesis was accompanied by a significant misfolding of the p62 protein. This was seen in the 4.5- and 5.0-h samples as disulfide-linked protein aggregates in the top part of the separating gel and in the bottom of the sample slot (Fig. 2A, lanes 12 and 16). Comparisons of the amounts of p62 and E2* in the corresponding reduced and nonreduced samples suggested that it was the unprocessed p62, and not E2*, which aggregated. The pulse-chase analyses showed that the level of aggregation increased with time. After a 5-min chase, very few aggregates were found (lanes 10 and 14), whereas the aggregates constituted about half of the unprocessed p62 in the samples chased for 35 min (lanes 12 and 16).

To assess the nature of the aberrant p62 processing, pulse-labelled proteins that had been chased for 5 and 60 min were treated with NGase F and endo H. Figure 3A shows the results of the NGase F analyses. It is evident that this glycosidase, which removes the entire N-linked sugar units from the glycoproteins, converts both E2 of SFV wt RNA-transfected cells and E2* of SFV-C-p62-6K RNA-transfected cells into deglycosylated E2 (lanes 6 and 7). This suggested that p62 expressed from both RNAs is transported to the *trans* Golgi, where it is subjected to the normal host-mediated cleavage (12). The reason why E2* migrates slower in gels than E2 was investigated by an endo H analysis of this protein. It has been shown that the E2 of wt SFV contains two N-linked sugar units, which mostly remain in an endo H-sensitive form when the E2-E1 heterodimer is transported through the Golgi complex (25, 39, 42). This was confirmed by the endo H analysis of control E2 (Fig. 4A). In contrast, the corresponding analyses of E2* showed that this was completely resistant to endo H (Fig. 4B). Therefore, the low mobility of E2* most probably depends on the trimming of its sugar units.

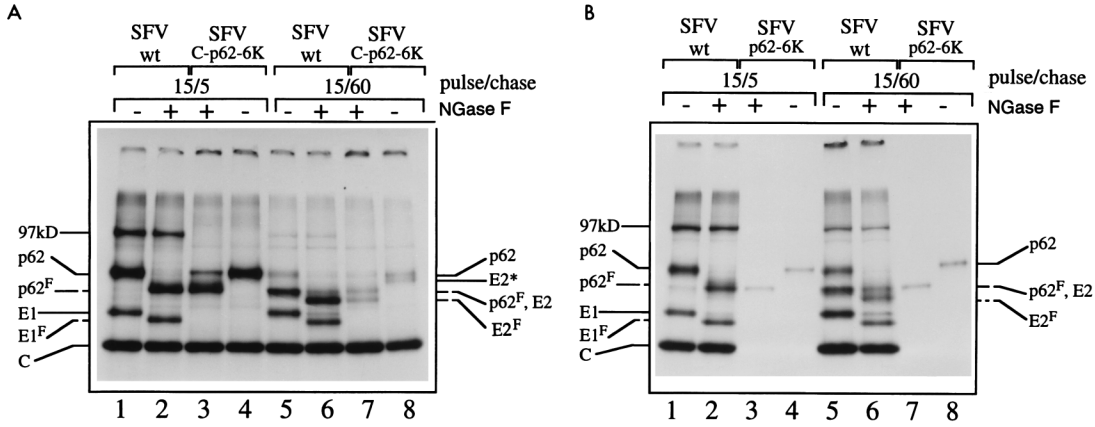

FIG. 3. NGase F analyses of viral proteins. Cells were transfected separately with SFV wt and SFV-C-p62-6K RNA (A) and SFV wt and SFV-p62-6K RNA (B). For each transfection, pairs of cultures were pulse-labelled for 15 min at 6 h posttransfection. The cultures of each pair were then chased for 5 and 60 min, respectively. The cells were lysed, and samples were either incubated with NGase F or mock treated. Equal portions of the incubation mixtures were analyzed by SDS-PAGE under nonreducing conditions. The deglycosylated forms of viral proteins are indicated by F. Note that the deglycosylated p62 ($p62^F$) runs as glycosylated E2. The band denoted 97kD represents untranslocated p62-6K-E1 precursor.

E2* is present at the PM. The possible expression of the p62/E2* protein at the PM of cells was studied by immunofluorescence and cell surface biotinylation of transfected cells. For the former analyses, we used cell cultures that had been transfected with SFV-C-p62-6K RNA. Parallel cultures were processed for internal and surface staining of p62/E2*. The results revealed strong staining of the PM in nonpermeabilized cells and of internal structures in permeabilized cells (Fig. 5). For the biotinylation analyses, cell cultures were infected with SFV-C-p62-6K recombinant virus, pulse-labelled with [^{35}S]methionine, and then treated with a biotin reagent either directly or after different chase times. After detergent lysis, the biotinylated proteins were recovered and analyzed by SDS-PAGE. The results showed that E2* but not p62 became biotinylated at the cell surface (Fig. 6). Maximum labelling of E2* was seen after 120 min of chase. At this time point, the labelled and streptavidin-captured fraction of E2* represented about 30% of the total E2*. We conclude that a significant fraction of E2* reached the PM in SFV-C-p62-6K RNA-transfected cells.

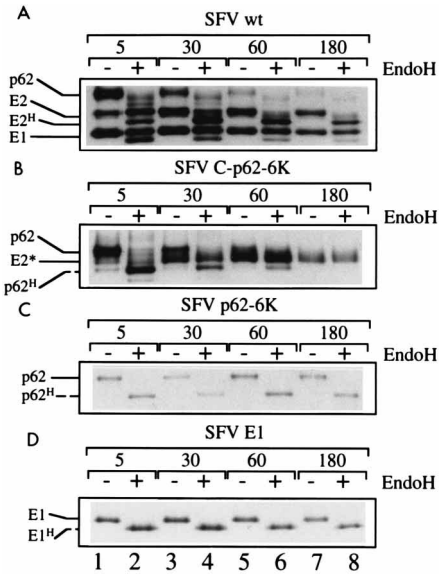

FIG. 4. Endo H analyses of viral proteins. The cells were transfected separately with SFV wt (A), SFV-C-p62-6K (B), SFV-p62-6K (C), and SFV-E1 (D) RNA. For each transfection, a series of four cultures were pulse-labelled for 15 min at 6 h posttransfection. The cultures of each series were then chased for 5, 30, 60, and 180 min. The cells were lysed, and samples were either incubated with endo H or mock treated. Equal portions of the incubation mixtures were analyzed by SDS-PAGE under nonreducing conditions. The endo H-digested forms of the viral proteins are indicated by H. Note that endo H-digested p62 runs as mock-treated E2. Furthermore, note that intermediate digestion products of p62 and E2 are seen in the analyses.

p62 is retained in the ER when expressed at low level. When p62 is expressed from SFV-p62-6K RNA, it is produced at a 10- to 20-fold-lower level than when expressed from SFV wt RNA or SFV-C-p62-6K RNA. This is due to the lack of a translation-enhancing RNA structure which is located in the coding region of the C protein (18, 52). Most interestingly, we found that when p62 was produced at this low level, it was retained in the ER. This is shown by the pulse-chase and glycosidase analyses in Fig. 3B and 4C, respectively. First, the p62 protein is not processed into E2* when expressed from SFV-p62-6K RNA (Fig. 3B, lanes 4 and 8; Fig. 4C, lanes 1, 3, 5, and 7). Instead, all the p62 remained intact in a form that was sensitive to endo H (Fig. 4C, lanes 2, 4, 6, and 8). As a comparison, we show an endo H analysis of pulse-labelled and chased E1 which has been expressed without p62 (Fig. 4D). Under these conditions, E1 is retained in the ER and consequently is completely sensitive to endo H (9, 17, 27, 44). However, an immunofluorescence analysis of cells transfected with SFV-p62-6K RNA showed, in addition to a clear internal staining, a weak surface staining for p62 (Fig. 5). This suggested that the retention of separately expressed p62 is not as complete as that of separately expressed E1. In the latter case, the immunofluorescence analyses showed only internal staining (Fig. 5).

A concentration-dependent retention of p62 was also supported by the analyses of p62 processing in SFV-C-p62-6K RNA-transfected cells shown in Fig. 2. In this experiment, p62 processing increased concomitantly with the elevated rate of synthesis at later time points after transfection. This behavior can be explained by the saturation of a p62 protein retention mechanism.

The p62 and E2* proteins are monomeric, whereas the C forms NCs in NP-40 lysates. We also tested the oligomeric state of p62/E2* and C proteins that have been expressed from SFV-C-p62-6K RNA. For this purpose, samples of NP-40 ly-

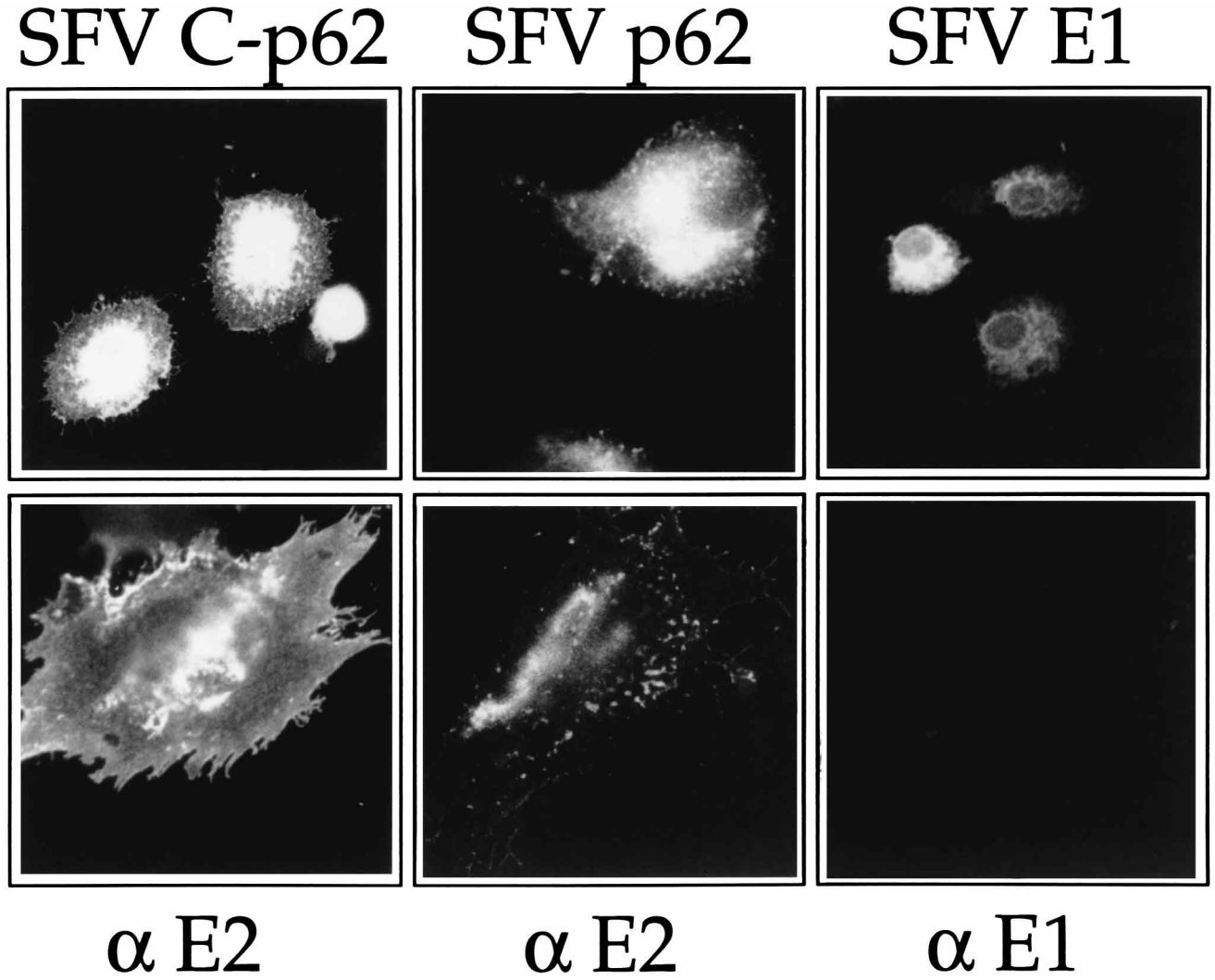

FIG. 5. Immunofluorescence analyses of cells transfected with SFV-C-p62-6K, SFV-p62-6K, and SFV-E1 RNA. Parallel cultures of cells transfected with each kind of RNA were fixed and stained at 6 h posttransfection. One culture of each pair was processed for internal staining (top row), and the other one was processed for surface staining (bottom row). We used monoclonal antibody UM 5.1 for staining of SFV-C-6K and SFV-p62-6K RNA-transfected cells. The SFV-E1 RNA-transfected cells were stained with monoclonal antibody UM 8.139.

sates from transfected and pulse-labelled cells were analyzed by sedimentation in sucrose gradients. In these gradients, both the p62 and the E2* proteins sedimented like monomeric entities. The sedimentation of p62 is shown in Fig. 7B. As a control, viral proteins from low-pH-treated and NP-40-solubilized virus particles were sedimented on an equivalent gradient. Under these conditions, the E1 protein is found both in monomeric and in homotrimeric forms whereas the E2 protein is present only in the monomeric form (58, 60). When the samples were analyzed for NC-like structures as described previously (15), the bulk of the C protein in the SFV-C-p62-6K RNA transfected cells was shown to migrate like wt NCs (data not shown).

The NCs of SFV-C-p62-6K RNA transfected cells cannot bind to E2* at the PM. To see whether the NCs could bind to the E2* at the PM or not we performed an electron microscopic analysis of SFV-C-p62-6K RNA-transfected cells. As a control, cells transfected with SFV wt RNA were analyzed. In the latter case, many NCs were seen both in the cytoplasm and at the PM (Fig. 8A). Many of the PM-associated NCs appeared to be involved in a budding process. In the SFV-C-p62-6K RNA-transfected cells, several NCs were seen in the cytoplasm but none were associated with the PM. A representative picture is shown in Fig. 8B. This suggested that the E2* proteins that were present at the PM were not able to establish stable interactions with the NCs in the cytoplasm.

DISCUSSION

We have shown that p62 which is expressed without E1 can be transported to the PM and also undergo correct maturation cleavage. If one takes into account that the rate of synthesis of the separately expressed p62 corresponds to that of p62 expressed from wt SFV RNA (Fig. 1) and that about one-third of the former p62 matures into E2* (Fig. 2), one can approximate that about one-third as many E2* molecules as E2-E1 heterodimers reach the PM in a wt RNA-transfected cell. If the NC-binding capacity of E2* is intact, this should be enough to support virus budding. wt virus production starts at much lower rates of synthesis of structural proteins (16). However, we were

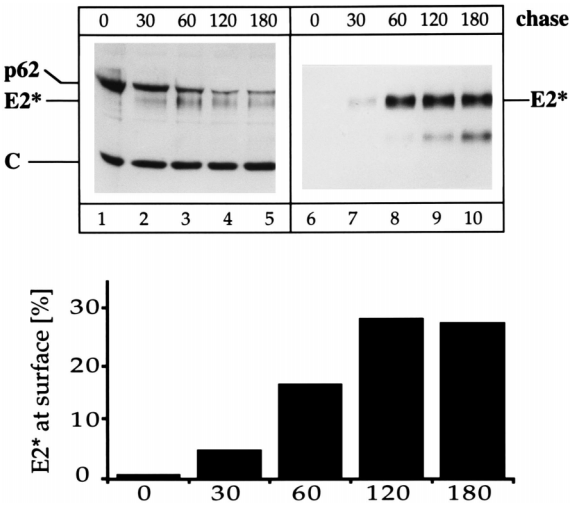

FIG. 6. Biotinylation analyses of E2* at the PM of infected cells. Five cultures were infected with SFV-C-p62-6K recombinant virus. These were pulse-labelled after 5 h and chased as indicated. The cells were then incubated at 4°C with the biotin reagent. The cell cultures were lysed, and biotinylated proteins were isolated by streptavidin precipitation. Precipitates were analyzed by SDS-PAGE (lanes 6 to 10). Note that these analyses showed, in addition to E2*, a small amount of faster-migrating material. This probably represents an E2* degradation product. Unprecipitated samples of lysates were analysed in parallel (lanes 1 to 5). All analyses were done under reducing conditions. The lower panel shows the fraction of total E2* which at each time point has been biotinylated and captured by streptavidin.

unable to find any p62/E2* and C protein-containing particles in the media of SFV-C-p62(-6K) RNA-transfected cells. The detection limit in our SDS gel-based budding assay is about 1/100 of the wt virus release. This suggests that E2* proteins cannot bind to NCs as efficiently as can E2 in E2-E1 heterodimers.

One possible explanation for the budding-negative phenotype of the E1 expression-deficient SFV variants is that the separately expressed p62/E2* cannot, like the wt p62/E2-E1 heterodimers, form spike-like trimeric structures. Such complexes might be necessary to establish stable associations with the NC, for instance by providing polyvalent (trimeric) NC-binding sites for the interaction. Our finding that the separately expressed p62/E2* is monomeric after solubilization with NP-40 is consistent with this explanation. However, it should be noted that the possible p62/E2* oligomers might still exist but in a form which is sensitive to solubilization. This has been shown to be the case with the trimeric heterodimer complex in the SFV spike (49, 63, 64).

If heterodimerization controls the budding reaction by allowing spike formation, it follows that dissociation of the E2-E1 heterodimer, as it occurs during virus uptake in uninfected cells, will weaken the NC-envelope interaction (58). Therefore, the p62/E2-E1 heterodimeric interaction might represent a general regulator of alphavirus assembly and entry processes; it controls both the membrane fusion activity and the NC envelope association and dissociation reactions during virus budding and penetration, respectively. This model is supported by the results of an earlier study of ours in which we coexpressed two kinds of SFV and monitored the virus release (14). One of the viruses was wt-like, whereas the other was unable to form particles because of inactivation of the NC-binding site in the membrane protein heterodimer. Those results showed that the budding-negative virus interfered very efficiently with the budding-competent one. This suggested that spikes with mixed heterodimer compositions were formed and that these could not efficiently support virus formation

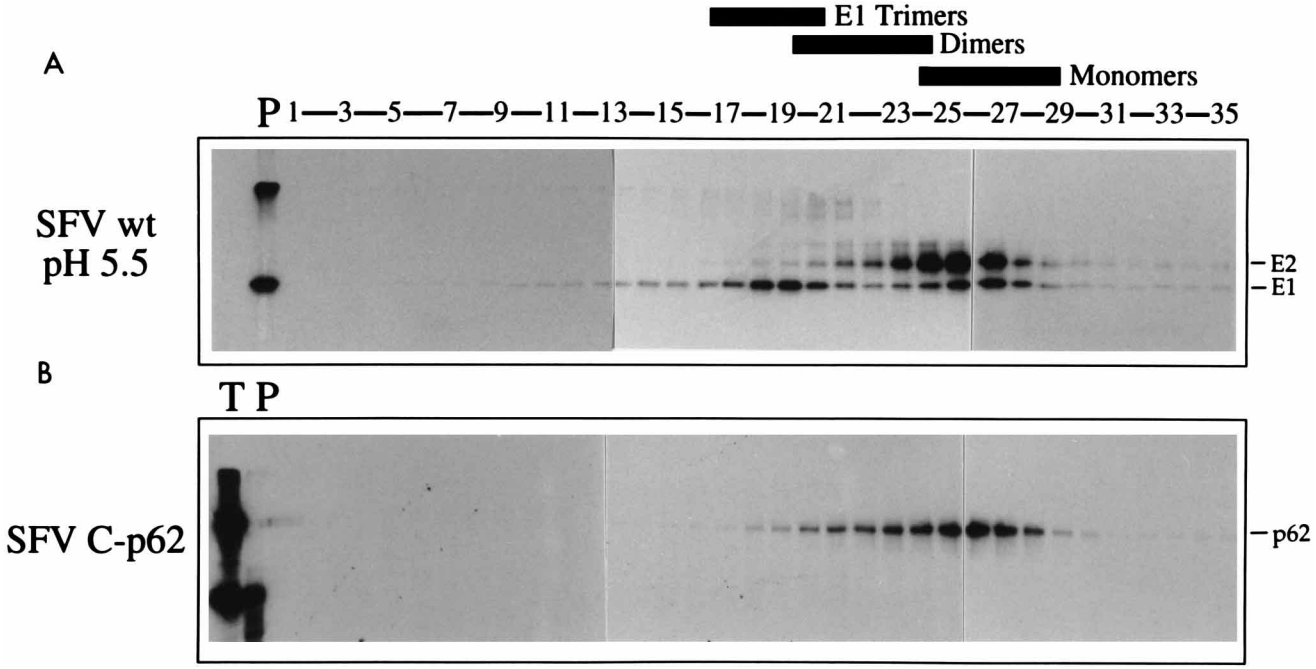

FIG. 7. Sedimentation analyses of p62 from SFV-C-p62-6K RNA-transfected cells. Transfected cells were pulse-labelled at 6 h posttransfection for 10 min and chased for 5 min. A lysate was prepared, and a sample of this was layered on a 5 to 20% (wt/wt) sucrose gradient. This was run at 39,000 rpm in an SW41 rotor (Beckman) for 24 h at 4°C. The gradient was fractionated, and samples of each fraction were analyzed by SDS-PAGE. (B) Autoradiograph of the gel. (A) Similar analysis of spike proteins from [^{35}S]methionine-labelled SFV that has been incubated in a low-pH buffer before solubilization. The acid treatment results in the dissociation of the E1-E2 heterodimers in the viral envelope and the partial association of E1 into homotrimers and higher homooligomers (58, 60). The approximate migration of viral membrane protein trimers, dimers, and monomers in the gradient is indicated. Analyses of top fractions are to the right.

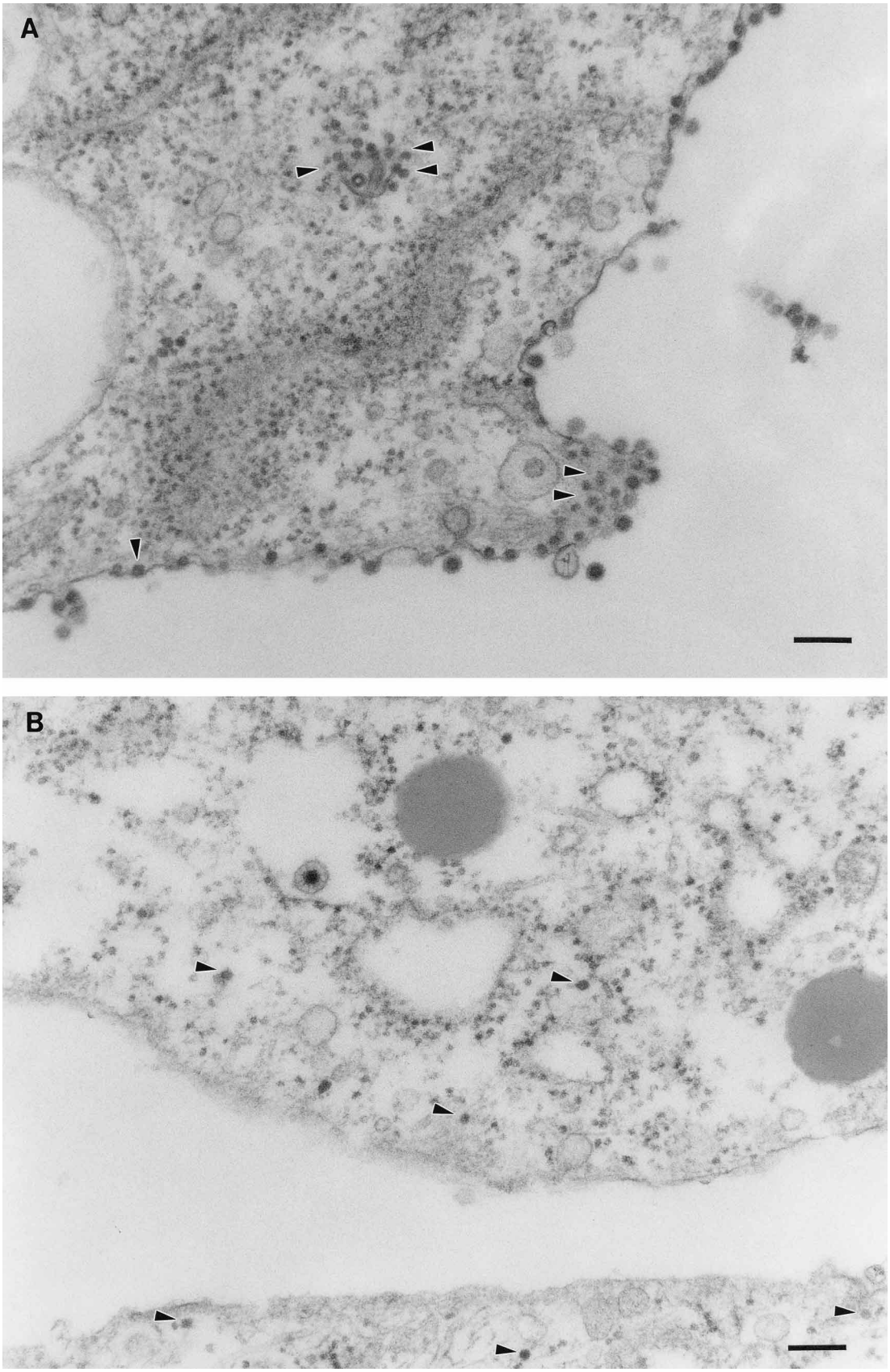

FIG. 8. Electron microscopic analyses of cells transfected with SFV-C-p62-6K RNA. Cells transfected with SFV wt RNA and SFV-C-p62-6K RNA, respectively, were prepared for electron microscopic analyses at 6 h posttransfection. Sections of fixed cells were stained with uranyl acetate and lead citrate. (A) Analyses of SFV wt RNA-transfected cells; (B) SFV-C-p62-6K RNA-transfected cells. Arrowheads point at NCs (spherical structures with a diameter of approximately 40 nm). Bar, 150 nm.

because they did not possess a full complement of active NC-binding sites.

Another explanation for the lack of production of C-p62 particles is that the tail of p62/E2 requires heterodimerization with E1 for maturation into an NC-binding competent conformation. The cytoplasmic tail of PE2/E2 of the Sindbis virus (SIN) has been shown to undergo several modifications in the infected cell which appear to be necessary for virus budding. These include palmitoylation of its two conserved Cys residues and phosphorylation and subsequent dephosphorylation of its conserved Tyr residue (28, 35, 36). The distal portion of the PE2 tail seems, furthermore, to undergo a topological switch from a membrane-buried to a cytoplasmically exposed location during intracellular transport (37). It is possible that these modifications of the PE2 tail are dependent on the formation of a stable complex between PE2 and E1.

The general importance of the p62/E2-E1 heterodimer for virus budding is also supported by the results of a recent study with a SIN variant that expresses a chimeric heterodimer. It was found that the chimeric heterodimer (p62 from SIN and E1 from Ross River virus) was formed and transported to PM like that of the wt SIN but that it was unable to bind NCs at the PM (61). Labelling experiments with biotin indicated that the chimeric heterodimer had an aberrant conformation. Thus, this probably prevented the E2 from interacting with the NC in a proper way.

It was quite surprising in the present study to find that p62 was retained in ER when it was produced at a low level with the SFV-p62-6K vector. This suggests that separately expressed p62 actually represents an ER-retained protein. At present, the mechanism for this retention is unclear. However, it is evident that it involves saturatable receptors.

When we monitored the fate of the separately expressed p62, produced at increasing rates of synthesis in the time course study (Fig. 2), we found that apart from being transported, excess p62 was also misfolded into disulfide-linked aggregates. Therefore, it seems as if the retained and unprocessed p62 is not completely stable but aggregates over time in the ER. In another recent study, we analyzed the behavior of newly synthesized E1 when expressed separately from p62 (1a). We found that this protein was also retained in the ER when expressed at comparatively low levels. However, when the rate of synthesis of this protein was increased, there was no transport out from the ER, but all the excess E1 that was made was immediately misfolded into disulfide-linked aggregates. The differences in the behavior of newly synthesized p62 and E1 membrane proteins probably reflect their different roles during the formation of the p62-E1 heterodimer. In this complex, the p62/E2 protein seems to chaperone an E1 conformation with a suppressed membrane fusion function (1a, 38, 51). Without p62, E1 cannot obtain this conformation but will mostly aggregate. According to our favored heterodimerization model, the p62 protein, which is translated first from the common coding unit, waits inside the ER translocon for the insertion of the subsequently translated E1 (1a, 3). This gives the p62 protein the opportunity to stabilize E1 through complex formation immediately after the latter protein has been made. In this model, it is evident that the p62 protein, which is made first, should, at least initially, be stable as a separate protein.

That separately expressed p62 is partially retained in the ER and that the "escaped" E2* molecules cannot support virus budding at the cell surface provide two additional examples of the tight quality control of the particle formation process of alphaviruses. Other examples are (i) the already mentioned linkage of E1 transport out of the ER and complex formation with p62 (9, 17, 27, 44), (ii) the dependence of NC formation on genome encapsidation (56), and (iii) the spike-NC interaction that drives the budding reaction (56). Altogether, these control steps very efficiently prevent the production of incomplete particles in alphavirus-infected cells. This is in clear contrast to many other viruses, e.g. retroviruses, which lack the corresponding control systems and therefore produce substantial amounts of waste products (24).

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