Expression of the TM Protein of Rous Sarcoma Virus in the Absence of SU Shows that This Domain Is Capable of Oligomerization and Intracellular Transport

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The SU and TM subunits of the Rous sarcoma virus glycoprotein, which are derived from a single polypeptide precursor, have been expressed independently with a simian virus 40 vector. The TM protein retains the ability to form an oligomer which resembles the TM oligomer derived from the wild-type glycoprotein complex present in virions. Oligomerization of the recombinant TM protein is more rapid than that observed for the intact glycoprotein expressed from the simian virus 40 vector and is required for its transport out of the endoplasmic reticulum. Oligomeric TM is terminally glycosylated in the Golgi complex but is less stable than the intact wild-type protein and does not accumulate at the cell surface. The SU protein, in contrast, does not form detectable oligomers but is efficiently secreted into the culture medium. These observations suggest that the oligomerization domain of the Rous sarcoma virus glycoprotein lies in the TM protein and that it can function independently of SU.

Chemical cross-linking and sedimentation gradient analyses indicate that the envelope glycoproteins of retroviruses are oligomeric complexes that contain multiple copies of the extracellular (SU) and transmembrane (TM) subunits (7–9, 25, 28, 30). The envelope glycoprotein is synthesized as a single precursor protein that oligomerizes in the endoplasmic reticulum (ER) prior to transport to the Golgi complex, where it is cleaved by a cellular endoprotease to yield the SU and TM proteins. The oligomeric association is maintained after cleavage, and the oligomers are incorporated into virus after transport to the cell surface (8). The contribution of this apparently general oligomeric structure to the function of these proteins in the viral life cycle has not been elucidated. As has been observed for other viral and cellular membrane proteins (16), formation of the oligomer may be critical for transport of retroviral glycoproteins to the cell surface. The oligomeric structure may also be required in the process of membrane fusion that is mediated by retroviral glycoproteins. This is supported by the observation that a mutant human immunodeficiency virus type 1 (HIV-1) glycoprotein that is defective in fusion can dominantly interfere with fusion when it is coexpressed with the wild-type glycoprotein (10). Characterization of the interactions involved in oligomerization may provide clues for attempts to interfere with virus assembly or infectivity and also addresses a fundamental aspect of intracellular protein maturation.

Disruption of the disulfide bond linking the SU and TM subunits of the Rous sarcoma virus (RSV) glycoprotein dramatically alters the sedimentation behavior of the oligomeric complex on sucrose gradients. Rather than cosedimenting as part of a larger structure, the two subunits appear in distinct fractions following reduction; the SU elements behave as monomers while the TM subunits sediment as a homo-oligomer (8). A similar interaction among TM proteins has been observed in the glycoprotein complex of the Mason-Pfizer monkey virus. On sucrose gradients buffered at pH 6.0, the TM

protein (gp22) migrates together with SU (gp70), while at pH 7.5 gp70 dissociates from the complex, leaving an oligomer of gp22 (9). An oligomeric form of the HIV-1 TM protein (gp41) from disrupted virions has also been reported (26). These observations suggest that interactions among the TM proteins are a major factor stabilizing the glycoprotein oligomer. RSV glycoproteins lacking the cytoplasmic and membrane-spanning sequences readily oligomerize (8), indicating that oligomerization involves the extracellular domain of the glycoproteins.

Since oligomerization occurs in the ER, interactions among the TM proteins that might contribute to formation of the oligomer occur when these are part of the precursor molecule. We have expressed the TM subunit as a separate protein to examine whether it retains the ability to oligomerize. By deleting the coding sequence of gp85 (SU), two TM genes were created; in the first, three of the four N-terminal amino acids of gp37 were altered, while in the second a native TM coding sequence was maintained. In the present study, we have analyzed the fate of the proteins produced from these genes. Expression of these proteins in CV-1 cells was examined by using a late-replacement simian virus 40 (SV40) vector (5). Previous work has shown that expression of the wild-type glycoprotein in this system produces a processed, oligomeric complex indistinguishable from that seen in infected avian cells. The recombinant TM proteins readily oligomerized in a structure that mimics what is seen for the full-length precursor. The SU subunit has also been expressed as a separate protein. In contrast to the TM protein, an oligomeric form of SU was not detected, even though this protein was readily transported through the secretory pathway and released into the culture medium.

MATERIALS AND METHODS

Mutagenesis of *env* genes and expression in an SV40 system. Oligonucleotide-directed mutagenesis was performed with a single-stranded DNA template produced from a pSELECT-1 vector (Promega, Madison, Wis.) containing the *KpnI* to *XbaI* fragment of RSV Prague subgroup C molecular clone pATV8 (18). The recombinant TM gene *rgp37D* was constructed by

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FIG. 1. Construction of the mutant genes for expressing TM as an independent protein.

deletion of nucleotides 5492 to 6514, a deletion that results in replacement of the four N-terminal codons of gp37 with the four N-terminal codons of mature gp85 (Fig. 1). The rgp37W gene was produced by oligonucleotide-directed mutagenesis of the rgp37D sequence inserted into pSELECT as a *KpnI-XbaI* fragment. This gene restores the wild-type N-terminal sequence Ser-Val-Ser-His to the recombinant gp37 protein. After confirmation by sequence analysis that the mutated plasmids contained only the desired mutations, *KpnI-XbaI* fragments carrying the rgp37D and rgp37W mutations were inserted into the late-replacement SV40 vector SV.KX (5) in place of the wild-type *env* sequence to yield SV.37D and SV.37W, respectively. To express the SU subunit independently, the codon for Lys-340 of *env* was mutated to a TAA termination codon, thereby producing a protein terminated after Arg-Arg in the Arg-Arg-Lys-Arg cleavage site. An *EcoRI-XbaI* fragment carrying this mutation was inserted into SV.KX to generate SV.SU. The A(−) RSV glycoprotein which lacks the cytoplasmic and membrane-spanning sequences has been characterized previously (23). Expression of the mutant proteins in CV-1 cells infected with recombinant SV40 stocks from cells cotransfected with the SV40 vectors and helper virus was examined (5).

Cells were metabolically labeled at 48 to 72 h postinfection. Pulse-labeling with [³H]leucine (>140 Ci/mmol; Dupont NEN, Boston, Mass.) was preceded by a 60-min incubation in leucine-free medium, while labeling with [³⁵S]methionine-cysteine (>1,000 Ci/mmol; EXPRE³⁵S protein labeling mix; Dupont NEN) followed a 20-min preincubation in medium lacking cysteine and methionine. Labeled cells were chased by incubation in complete medium after removal of the labeling solution. After lysis in buffer A (25 mM Tris [pH 8.0], 50 mM NaCl, 1.0% Triton X-100, 1.0% deoxycholate), proteins were immunoprecipitated with fixed *Staphylococcus aureus* and a polyclonal antibody to purified RSV envelope glycoprotein or an antiserum raised to a peptide consisting of the C-terminal cytoplasmic domain of gp37 (anti-gp37c) kindly provided by Gary Davis (DuPont-Merck, Wilmington, Del.). Secreted proteins were immunoprecipitated from culture medium after addition of 5× buffer A.

Sedimentation gradient analysis of oligomer formation. For sucrose gradient analyses, 35-mm plates of infected CV-1 cells were labeled with 75 μCi of [³H]leucine. Cell lysates were prepared and fractionated on sucrose gradients (5 to 25%) in the presence of 40 mM octyl glucoside as described previously (8). Fractions were immunoprecipitated with the anti-gp37c

antiserum and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography. Endoglycosidase H (endo H) digestions were performed on immunoprecipitated fractions after solubilization in 1% SDS–25 mM Tris (pH 6.8)–5 mM 2-mercaptoethanol at 100°C for 5 min. An equal volume of 150 mM citrate (pH 5.3) containing 1 mU of endo H (Boehringer Mannheim, Indianapolis, Ind.) was added, and samples were incubated at 37°C for 16 h.

Immunofluorescence. For immunofluorescence analyses, cells were fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min and washed with PBS supplemented with 25 mM glycine, pH 8.0. Surface staining of fixed cells employed a polyclonal chicken antiserum diluted 1:50 in PBS containing 5% bovine serum albumin and then a fluorescein-conjugated goat anti-chicken serum (Southern Biotechnology, Birmingham, Ala.). Fixed cells were permeabilized by a 10-min incubation with 0.5% Triton X-100 in PBS for total cell immunofluorescence. These cells were incubated with a 1:50 dilution of anti-gp37c antiserum, binding of which was then detected with a Texas red-conjugated goat anti-rabbit serum (Southern Biotechnology).

Surface biotinylation. Attached CV-1 cells expressing the RSV glycoprotein were biotinylated after being labeled with [³H]leucine in a 30-min pulse and 2-h chase, following the procedure described by Lisanti et al. (20). After being washed six times on ice with PBS containing 1 mM MgCl₂ and 0.1 mM CaCl₂ (PBS-MC), cells were incubated with sulfo-succinimido-biotin (Pierce, Rockford, Ill.) at 0.5 mg/ml in PBS-MC for 30 min on ice. The cells were then incubated with serum-free medium for 5 min and were washed four times with PBS-MC containing 20 mM glycine (pH 8.0). Cells were lysed in buffer A and immunoprecipitated with the anti-gp37c antiserum. After separation by SDS-PAGE, the immunoprecipitates were transferred to nitrocellulose. Blots were blocked with 5% nonfat dry milk in PBS, washed in PBS containing 0.1% Tween (U.S. Biochemical, Cleveland, Ohio), and incubated with horseradish peroxidase-conjugated streptavidin (Fisher, Pittsburgh, Pa.). Biotinylated proteins were then detected by developing the blot with the ECL Western blotting (immunoblotting) detection system (Amersham, Arlington Heights, Ill.).

RESULTS

Expression of RSV TM proteins. To express the RSV TM protein gp37, a deletion was introduced into the *env* gene that extended from the fifth codon of the mature gp85 protein to the fifth codon of gp37, leaving the signal peptidase cleavage site unaltered (Fig. 1). Cleavage at this site would yield a gp37 molecule having the N-terminal sequence Asp-Val-His-Leu-Leu in place of the Ser-Val-Ser-His-Leu sequence found at the N terminus of TM that arises from cleavage of the *env*-encoded precursor by a Golgi complex endoprotease (15). Thus, the mutant would differ from wild-type gp37 only at three of the four N-terminal residues, and it is designated rgp37D to denote the presence of an N-terminal aspartic acid residue. CV-1 cells infected with SV.37D express an immunoprecipitable protein similar in size to that of the mature gp37 detected in SV.KX-infected cells (Fig. 2). This suggests that the signal peptide had been removed and that the protein was glycosylated in the same manner as wild-type gp37, which has two N-linked oligosaccharides. Signal peptidase cleavage at the expected site was confirmed by N-terminal microsequencing of immunoprecipitated rgp37D (data not shown).

Oligomerization of the RSV TM protein. Sedimentation

FIG. 2. Comparison of the SDS-PAGE mobilities of rgp37D and wild-type gp37. Cells infected with SV.KX or SV.37D were lysed and immunoprecipitated with anti-gp37c following a 20-min pulse (P) or a pulse followed by a 2-h chase (C). Arrows identify the precursor (pr95) and cleavage products (gp85 and gp37) for the wild-type glycoprotein.

gradient fractionation of lysates from cells expressing the rgp37D protein revealed the presence of a specific oligomeric complex of the protein (Fig. 3). Monomeric rgp37D was observed in pulse-labeled (5 min) cells (Fig. 3A, fractions 17 to 19), but within a 40-min chase the majority of these molecules had associated into oligomers (Fig. 3B, fractions 13 to 15). The rgp37D oligomer was detected in the same fractions as the oligomeric gp37 present in viral lysates exposed to reducing conditions (reference 8 and data not shown). The migration of the rgp37D oligomer on sucrose gradients was not affected by

FIG. 3. Sucrose gradient fractionation of rgp37D. Cells lysed in octyl glucoside after a 5-min pulse (A) or an additional 40-min chase (B) were fractionated on 5 to 25% sucrose gradients. A cell lysate was also prepared in OGL containing 50 mM dithiothreitol following a 2-h chase (C). A total of 21 fractions was collected for each gradient. Immunoprecipitates of fractions 6 to 19 were analyzed by SDS-PAGE. Bands corresponding to rgp37D are indicated by arrows.

FIG. 4. endoH digestion of gradient-fractionated rgp37D. Cell lysates obtained at the indicated chase times following a 5-min pulse were fractionated as described in the legend to Fig. 3. Immunoprecipitates were digested with endoH prior to SDS-PAGE. Proteins migrating at ~24 kDa that are endoH sensitive (S) as well as those at ~27 kDa which are partially resistant to endoH (R) are identified.

prior treatment of the lysate with dithiothreitol (Fig. 3C). In addition, the apparent size of immunoprecipitated rgp37D remained unchanged when it was analyzed on SDS-polyacrylamide gels run under nonreducing conditions (data not shown). Thus, despite the fact that one of the five cysteines in the extracellular domain of rgp37D is unpaired, because of the absence of gp85, there are no detectable intermonomer disulfides and, in this regard, the rgp37D oligomer again resembles the oligomer of the full-length glycoprotein.

The rgp37D protein present in the oligomeric fractions of the gradient (Fig. 3B) showed a greater heterogeneity of electrophoretic migration than the monomeric form and included molecules of reduced mobility, suggesting that the oligosaccharide chains of rgp37D were being terminally modified. The coding sequence of gp37 contains two potential sites for N-linked glycosylation, and both of these are utilized in the context of the full-length precursor. endoH digestion of rgp37D was carried out to examine its glycosylation and to assay the transport of this protein beyond the ER. Digestions were performed on immunoprecipitated fractions from sucrose gradients to investigate the connection between oligomerization and transport. Following a 5-min pulse and a 15-min chase, oligomeric rgp37D was detected. However, all of the oligomeric as well as monomeric protein was fully sensitive to endoH, since it was reduced in apparent molecular mass by about 6 kDa following endoH digestion (Fig. 4, S). Protein that was partially resistant to endoH was detected in the 30-min

FIG. 5. Surface biotinylation of RSV glycoproteins. After labeling with [3 H]leucine in a 30-min pulse and a 2-h chase, CV-1 cells expressing wild-type glycoprotein (Env), rgp37D, or rgp37W from an SV40 vector or uninfected cells (–) were biotinylated with sulfo-NHS-biotin. One-half of the immunoprecipitated cell lysates was probed with horseradish peroxidase-conjugated streptavidin after SDS-PAGE and transfer to nitrocellulose to detect biotinylated protein (A). The remainder was subjected to fluorography after SDS-PAGE to detect total biosynthetically labeled protein (B).

chase (Fig. 4, R). These molecules were 2 to 3 kDa smaller in apparent molecular mass than untreated rgp37D, indicating that only one of the N-linked carbohydrates was resistant to endoH. Although a significant amount of monomeric rgp37D was still present at this time, all of the endoH-resistant protein was detected in the oligomeric fractions. An increase in the amount of resistant protein, all of which was oligomeric, was seen in the longer chases (Fig. 4). Thus, only the oligomeric form of the protein was transported out of the ER. Oligomerization of rgp37D, therefore, not only resembles that of the wild-type structure in size and noncovalent association but also remains a requirement for intracellular transport of the protein. However, acquisition of endoH resistance was slow compared to oligomer formation, and this suggests that there are additional requirements beyond oligomerization for rgp37D to exit the ER.

TM protein expressed independently of the SU domain does not accumulate at the cell surface. In the absence of other viral proteins, the glycoprotein complex of RSV is transported to the plasma membrane and stably expressed on the cell surface, where it can be detected by immunofluorescence staining (34). CV-1 cells infected with SV.37D, in contrast, did not exhibit the bright surface immunofluorescence seen on cells expressing the wild-type glycoprotein that were stained with a polyclonal chicken antiserum (data not shown). This difference could reflect a sharp reduction in the amount of glycoprotein expressed on the cell surface or loss of the major epitopes due to deletion of the gp85 coding sequence.

Expression of glycoprotein at the cell surface was therefore examined by treating intact cells with the membrane-impermeable biotinylating agent sulfo-NHS-biotin. Cells were labeled with [3 H]leucine in a 30-min pulse and a 2-h chase and were then washed before incubation with sulfo-NHS-biotin. Both gp85 and gp37 were efficiently biotinylated on cells infected with the wild-type *env*-expressing vector SV.KX (Fig. 5A, Env). Although sulfo-NHS-biotin reacts with primary amines and gp37 has 7 versus 8 lysines for gp85, gp37 was consistently more

heavily biotinylated than gp85. In contrast, cells expressing rgp37D exhibited very little biotinylated TM protein. This difference cannot be explained by the amount of protein synthesized in the infected cells, since the amount of metabolically labeled TM protein following a 2-h chase was actually greater in the rgp37D-expressing cells than in Env-expressing cells and even exceeded the total of cleaved gp37 and pr95 in the latter (Fig. 5B). Cells expressing rgp37W, which has the authentic N terminus of gp37, exhibited a greater amount of biotinylated TM, but these lysates also contained more metabolically labeled protein. Thus, both rgp37D and rgp37W were much less accessible to sulfo-NHS-biotin than was the wild-type glycoprotein complex, consistent with a marked reduction in surface expression.

Immunofluorescence staining of permeabilized cells also indicated a difference in localization of rgp37 relative to that of the wild-type glycoprotein. A prominent feature of cells infected with SV.37D or SV.37W was the intense staining of vesicular structures that were variably distributed throughout the cytoplasm (Fig. 6A and B). This staining pattern was not seen in uninfected cells or cells expressing the full-length Env (Fig. 6C and D). In addition, the rgp37 proteins were detected at an asymmetric perinuclear location also exhibited by the wild-type glycoprotein, which probably represents nontransported proteins. In contrast to rgp37, however, the wild-type glycoprotein was detected at the surfaces of permeabilized cells (Fig. 6C). Since the antiserum reactive with the C terminus of gp37 would not be expected to exhibit any selectivity in binding to rgp37 versus the TM protein in the Env complex, this difference in staining provided further evidence for a significant alteration in the distribution of gp37 when it is expressed independently of SU.

Independently expressed TM protein is degraded more rapidly than the wild-type SU-TM complex. When pulse-labeled rgp37D and wild-type proteins were chased for longer than 2 h, a marked difference in stability was observed (Fig. 7). The wild-type precursor is converted to SU and TM during the chase periods, although some unprocessed precursor is still present at 5 h (Fig. 7B, endoH(–) lanes). However, the total amount of glycoprotein remains virtually constant. Pulse-labeled rgp37D is degraded during chases beyond 1 h (Fig. 7A). Immunoprecipitated rgp37D was quantitated by scanning SDS-polyacrylamide gels with an AMBIS radioanalytic imaging system. Taking the average from experiments performed in quadruplicate, it was observed that the amount of rgp37D decreased by 59% between 1 and 2.5 h of chase and by a further 67% between the 2.5- and 5-h chases. The rgp37W protein was degraded at a rate similar to that of rgp37D (data not shown).

endoH digestion of rgp37D, even after the 5-h chase, resolves the protein into a mixture of fully sensitive molecules (S) and proteins with one of the two oligosaccharides resistant to endo H (R) (Fig. 7A). The endoH-sensitive rgp37D present at 2.5 and 5 h was probably a mixture of monomeric and oligomeric species that are not competent for transport out of the ER (Fig. 4). Both the endoH-sensitive and -resistant rgp37D proteins are lost during the extended chases. Loss of the endoH-sensitive protein may result from its conversion to endoH resistance, degradation without transport beyond the ER, or a combination of the two. Degradation of the endoH-resistant protein could be retarded, although not completely blocked, by inclusion of 100 μ M chloroquine in the labeling and chase media (data not shown). Chloroquine has been shown to inhibit degradation of mutant Env proteins that were rapidly targeted to lysosomes (5).

endoH digestion of the wild-type glycoprotein shows a

FIG. 6. Immunofluorescence of rgp37-expressing cells. CV-1 cells expressing rgp37D (A), rgp37W (B), or wild-type Env (C) and uninfected cells (D) were fixed in paraformaldehyde, permeabilized with 0.5% Triton X-100, and stained with anti-gp37c antiserum. Bound antibody was detected with a Texas red anti-rabbit immunoglobulin G conjugate.

mixture of fully resistant TM proteins and TM proteins that on the basis of their electrophoretic mobilities appear to have one endoH-resistant and one endoH-sensitive oligosaccharide (Fig. 7B). It does not appear that this is the result of incomplete endoH digestion, since in the same samples pr95 is quantitatively converted to a discrete, more rapidly migrating protein band and gp85 is also shifted to a lower position in the gel. The significance of this apparent heterogeneity in the glycosylation of gp37, which differs from the pattern seen with rgp37D, is not clear.

When expressed independently, the SU protein does not oligomerize but is efficiently secreted. Independent expression of the SU protein was achieved by introduction of a premature termination codon following Arg-339, which is a central residue in the Arg-338-Arg-Lys-Arg cleavage recognition site. This rSU protein was detected in pulse-labeled cells that had been chased for 2 h as a cell-associated molecule with a molecular mass of ~68 kDa, consistent with it being a heavily glycosylated protein analogous to wild-type SU (Fig. 8, lane SU C). At this time, a significant fraction of the labeled protein had been secreted into the culture medium as a larger molecule with a heterogeneous size of approximately 85 kDa (lane SU M). This increase in size is consistent with terminal modification of the oligosaccharides as the protein traversed the Golgi complex on its way out of the cell. Cell-associated and secreted forms of the A(-) RSV glycoprotein, which lack the membrane-spanning and cytoplasmic domains, were examined in parallel as a reference for the efficiency of secretion. The cell-associated A(-) protein consisted of the uncleaved precursor (Fig. 8, lane A(-) C), while the secreted protein was cleaved to gp85 and a truncated form of gp37 (Fig. 8, lane

A(-) M). The rSU protein appeared to be secreted with an efficiency similar to that of the A(-) form of Env. However, there appeared to be a greater size heterogeneity in the secreted SU protein.

We have previously shown that while most of the secreted A(-) molecules behave as monomers in gradient analyses, an oligomeric form of this protein can be identified in cell lysates (8). Cell-associated rSU protein was therefore analyzed by sedimentation gradient centrifugation to determine whether this protein could be detected in an oligomeric form (Fig. 9). The rSU present in the pulse lysate (Fig. 9A) formed a peak on the gradient around fractions 10 and 11, and the sedimentation of this protein was not significantly altered in the chase (Fig. 9B).

DISCUSSION

The experiments reported here show that the TM glycoprotein of RSV is able to oligomerize when it is expressed as an independent protein. This is consistent with detection of oligomers of the TM protein derived from wild-type envelope molecules but indicates further that TM itself carries a motif sufficient for formation of these oligomers. As was observed with the wild-type envelope precursor, a discrete oligomer of gp37 (TM) forms during a chase period in intact cells and is not detected in lysates of pulse-labeled cells. Formation of the oligomer presumably requires the context of the ER for expression of the interacting motifs in the correct conformation and at a sufficient concentration. The rgp37D oligomer mimics the TM oligomer derived from virus both in size and in its resistance to the reducing agent dithiothreitol. Although

FIG. 7. Time course assay of the stability of rgp37D. (A) CV-1 cells infected with SV.37D were lysed after a 15-min pulse-labeling with [35 S]methionine-cysteine or after the indicated chase periods and were immunoprecipitated with anti-gp37c. The samples were then divided into two aliquots, one of which was incubated with endoH (+), the other of which was incubated with buffer alone (-), and both were subsequently analyzed by SDS-PAGE. S and R identify the fully endoH-sensitive and partially -resistant forms of the protein, respectively. (B) Wild-type glycoprotein was immunoprecipitated from SV.KX-infected cells. Labeling, immunoprecipitation, and endoH digestions were performed as described for panel A.

rgp37D is expected to have an unpaired cysteine resulting from the absence of SU, this does not play a critical role in the interaction between monomers, and the apparent oligomer is not an artifact of disulfide-mediated aggregation. Since only oligomeric rgp37D acquires endoH resistance, formation of the rgp37D oligomer, like that of wild-type precursor, appears to be required for transport of the protein out of the ER. An oligomeric form of the wild-type Env precursor is detected prior to cleavage into SU and TM, and a mutant RSV Env precursor protein that is defective in oligomer formation is not transported out of the ER (8, 34).

The dependence of protein transport out of the ER on oligomerization could reflect the presence of a retention signal on the monomeric protein that is lost in oligomerization. A region or patch on the monomer that directs stable association with a resident ER component would constitute such a retention signal. An example is the retention of the immunoglobulin heavy chain through interaction with BiP and its release following assembly with the light chain (14). Oligomerization

FIG. 8. Assay of secretion of rSU. CV-1 cells infected with SV.(A-) [A(-)] and SV.SU (SU) were pulse-labeled for 30 min and chased for 2 h. Glycoproteins present in the cell lysate (C) and culture medium (M) were immunoprecipitated and subjected to SDS-PAGE.

either might directly obscure the retention signal, or it could be required for subsequent structural changes which inactivate the retention signal. In cases in which oligomerization directly affects retention, sequences involved in oligomerization and retention may overlap so that interactions between the subunits obscure the retention signal. The susceptibility of the T-cell receptor α chain (TCR α) to retention and degradation in the ER when expressed alone, as well as its ability to assemble with the CD3 δ chain, could be transferred to the Tac protein by a 9-amino-acid sequence (1, 21). Since this sequence, which includes two basic residues, occurs in a putative membrane-spanning domain of TCR α and is functional in this position in Tac, it is presumably constrained to an α helix that is not affected by folding of the protein, although the membrane association of this sequence in the TCR α protein is not clear (13, 29). Overlap of oligomerization and retention sequences might also occur when oligomerization is a late step in maturation of the folded protein, e.g., in the case of influenza virus hemagglutinin (HA), in which trimerization occurs after formation of the proper intrachain disulfide bonds (2, 4). Alternatively, the retention and oligomerization sequences could involve distinct regions of the protein. Oligomerization would allow the protein to be transported on account of

FIG. 9. Gradient analysis of cell-associated rSU. CV-1 cells infected with SV.SU were lysed in octyl glucoside following a 15-min pulse with [3 H]leucine (A) or after a subsequent 2-h chase (B), and the lysates were fractionated on 5 to 25% sucrose gradients. A total of 15 fractions was collected and immunoprecipitated.

conformational effects of assembly that inactivate the retention signal.

Since the TM protein expressed alone resembles the full-length Env precursor in its ability to form an oligomer that is a prerequisite for transport, this subunit presumably bears both the putative retention signal and an oligomerization sequence. Consistent with this interpretation, expression of the SU subunit alone yields a protein that is efficiently transported without forming detectable oligomers. Presumably, the SU protein lacks both the retention signal and the oligomerization sequence and can therefore exit the ER as a monomer. Oligomerization of rgp37D, which occurs with a half-life of approximately 20 min, is significantly faster than that of the full-length precursor which has a half-life of greater than 60 min. It appears, then, that the SU domain actually retards the interactions between TM sequences necessary for oligomers to form. However, transport of rgp37D to the Golgi complex, detected as acquisition of endoH resistance, lags behind oligomerization and is not accelerated as a result of the more rapid oligomerization. This suggests that there are additional requirements beyond oligomer formation for the protein to exit the ER. This lag in transport of oligomeric rgp37D indicates that retention is not restricted to the monomeric protein and cannot be accounted for simply by an overlap of retention and oligomerization sequences. It remains to be seen to what extent this applies to the full-length Env precursor protein or whether this is a peculiarity of independently expressed TM protein.

Although rgp37D is able to oligomerize and exit from the ER, it is defective in surface expression relative to that of the wild-type SU-TM complex and is also degraded more rapidly. Mature protein that traverses the Golgi complex appears to be targeted to lysosomes instead of accumulating at the cell surface, since the stability of the endoH-resistant form of rgp37D is enhanced significantly in the presence of chloroquine. Some of the cytoplasmic vesicles containing rgp37D were also stained by antibody to a lysosomal marker (data not shown). Thus, the low level detected on the surface of cells is consistent with rgp37D being endocytosed after reaching the plasma membrane. This phenotype has been observed for a mutant RSV glycoprotein with a charged amino acid introduced into the membrane-spanning sequence (17). The cytoplasmic domain of the RSV glycoprotein contains a sequence reminiscent of characterized endocytosis signals in other proteins, and this sequence might mediate a more rapid endocytosis of the mutant proteins; however, the function of this sequence has not been characterized. On the other hand, we cannot exclude the possibility that the majority of the mature rgp37D protein never reaches the cell surface. While the feature of the rgp37D protein that causes its instability is unknown, it does not involve the amino acid substitutions at its N terminus, since the rgp37W protein exhibits a similar instability and reduced surface expression. In contrast, deletion of a large portion of the SU-encoding sequence from the HIV-1 *env* gene has been reported to result in expression of TM protein at the cell surface which is also capable of inducing membrane fusion (24).

The TM proteins of retroviruses are functionally analogous to the influenza A virus HA2 subunit, serving as transmembrane anchors for the receptor-binding subunits and bearing fusogenic sequences. The structure of HA2, determined by X-ray crystallography of a soluble HA trimer (35), also has been used as a basis for constructing models of the TM proteins (11). A prominent feature of the ectodomain of the HA2 subunit is a pair of roughly antiparallel α helices composed of amino acids 38 to 53 and 74 to 126 that are separated

by a loop. Interactions among HA monomers include a major contact site that involves residues 74 to 126 of the HA2 subunits. The N-terminal, membrane-distal parts of the helices form a triplex coiled coil, while the more membrane-proximal halves are packed around the hydrophobic fusion sequences present at the N termini of the HA2 subunits (33, 35). The extracellular domain of the TM protein of RSV also contains two potential α -helical sequences at residues 30 to 85 and 105 to 120 (12, 27). The more N-terminal helix contains a heptad repeat sequence with primarily hydrophobic residues recurring at positions 1 and 4 (3, 11). This type of sequence is present also in the N-terminal half of the long α helix in HA2 that forms a coiled coil (3) and has been shown to be directly involved in oligomer formation by the reovirus σ 1 attachment protein (19). However, it remains unclear what role the heptad repeats play in oligomerization of retroviral glycoproteins. Mutations in a similarly positioned heptad repeat in the HIV-1 TM protein interfere with envelope glycoprotein function but do not appear to affect oligomerization of these proteins (6). Peptides representing the wild-type heptad repeat region, on the other hand, form higher-order structures (32) that can be disrupted by these same mutations (31). Another region of the HIV-1 glycoprotein, corresponding to the shorter, more C-terminal potential helix of the RSV TM protein, has been proposed to be involved in oligomerization on the basis of binding of anti-peptide antibodies (22). We are presently analyzing additional mutations in the extracellular domain of RSV TM in an attempt to more precisely identify the sequences involved in oligomerization of this protein.

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