Mutational Analysis of the Oligomer Assembly Domain in the Transmembrane Subunit of the Rous Sarcoma Virus Glycoprotein

DAVID A. EINFELD AND ERIC HUNTER*

*Department of Microbiology, University of Alabama at Birmingham,
Birmingham, Alabama 35294-2170*

Received 29 July 1996/Accepted 9 December 1996

The transmembrane (TM) subunits of retroviral envelope glycoproteins appear to direct the assembly of the glycoprotein precursor into a discrete oligomeric structure. We have examined mutant Rous sarcoma virus envelope proteins with truncations or deletions within the ectodomain of TM for their ability to oligomerize in a functional manner. Envelope proteins containing an intact surface (SU) domain and a TM domain truncated after residue 120 or 129 formed intracellular trimers in a manner similar to that of proteins that had an intact ectodomain and were efficiently secreted. Whereas independent expression of the SU domain yielded an efficiently transported molecule, proteins containing SU and 17, 29, 37, 59, 73, 88, and 105 residues of TM were defective in intracellular transport. With the exception of a protein truncated after residue 88 of TM, the truncated proteins were also defective in formation of stable trimers that could be detected on sucrose gradients. Deletion mutations within the N-terminal 120 amino acids of TM also disrupted transport to the Golgi complex, but a majority of these mutant glycoproteins were still able to assemble trimers. Deletion of residues 60 to 74 of TM caused the protein to remain monomeric, while a deletion C terminal of residue 88 that removed two cysteine residues resulted in nonspecific aggregation. Thus, it appears that amino acids throughout the N-terminal 120 residues of TM contribute to assembly of a transport-competent trimer. This region of TM contains two amino acid domains capable of forming α helices, separated by a potential disulfide-bonded loop. While the N-terminal helical sequence, which extends to residue 85 of TM, may be capable of mediating the formation of Env trimers if C-terminal sequences are deleted, our results show that the putative disulfide-linked loop and C-terminal α -helical sequence play a key role in directing the formation of a stable trimer that is competent for intracellular transport.

Retroviral envelope glycoproteins are synthesized as a glycosylated precursor protein (Env) that is cleaved during intracellular maturation to a transmembrane (TM) subunit and a surface (SU) subunit. The Env complex mediates both receptor binding, via the SU subunit, and cell-virus membrane fusion via TM (16, 26). Shortly after translation individual Env precursors associate into an oligomeric structure that remains stable through the process of intracellular transport and maturation (11)—a feature shared by fusion proteins of several other enveloped viruses (27). Details of the structure of the native Env complex and the contribution of the oligomeric association of Env to its function remain unclear.

The hemagglutinin (HA) of influenza virus is the most thoroughly characterized viral fusion protein and serves as a potential model for retroviral Env proteins because of its similar organization into a TM subunit (HA2) containing a fusion peptide and an SU subunit (HA1) that has receptor binding activity. An α -helical region of HA2 forms a major interface between monomers of HA, and the fusion peptide at the N terminus of HA2 is sequestered in the interior of the trimer (30, 32). Stable interactions among the TM subunits of retroviral Env oligomers have been observed, and independent expression of the Rous sarcoma virus (RSV) TM protein has shown that this domain is capable of forming oligomers on its own (11, 12, 21, 22). The TM proteins of many retroviruses

contain a conserved pair or trio of cysteine residues (19, 23). This sequence feature is flanked by regions that have been predicted to form α helices (14). Consistent with this prediction, the TM protein of human immunodeficiency virus type 1 (HIV-1) appeared to consist largely of α helices, as determined by Fourier transform infrared spectroscopy (8). By analogy to HA, α -helical sequences of TM proteins may form interfaces in oligomeric Env complexes.

A mutant RSV glycoprotein (C3) with an insertion of 20 foreign amino acids followed by a termination codon after residue 102 of TM is defective in oligomerization (11). This protein also is blocked in transport beyond the late endoplasmic reticulum (ER)/early Golgi (31). This contrasts with the oligomerization and efficient secretion of the complete ectodomain of the glycoprotein (11, 20). Expression of just the RSV SU domain, on the other hand, produces a protein that is transported despite the fact that there is no detectable oligomerization (12). The failure of the C3 protein to oligomerize could result from truncation of the RSV sequence or from introduction of foreign amino acids. In this study, Env proteins with truncations or deletions introduced into the TM domain were examined to clarify the basis of the defect of the C3 mutant, to identify the region of TM required for oligomerization, and to investigate the connection between oligomerization and intracellular transport.

The results of these studies indicate that the N-terminal 120 amino acids of TM are required for truncated Env proteins to oligomerize in a transport-competent structure. This region of TM contains the fusion sequence and the two predicted α helices flanking a trio of cysteines. Proteins truncated after

* Corresponding author. Mailing address: 256 Bevill Biomedical Research Building, 845 19th St. South, Birmingham, AL 35294-2170. Phone: (205) 934-4321. Fax: (205) 934-1640. E-mail: ehunter@uab.edu.

FIG. 1. Schematic representation of mutations engineered into the TM domain of the RSV *env* gene. The positions of carboxy-terminal truncations are indicated by vertical arrows, and the truncation mutants are designated according to the number of gp37 (TM) amino acid residues remaining or by the number of the carboxy-terminal TM residue and the one-letter code(s) for the additional residue(s) encoded by the inserted oligonucleotide. Deletion mutations are shown as horizontal bars encompassing the deleted region. Potential α -helical regions are shown as filled rectangles, the positions of cysteine residues are indicated by the letter C, and oligosaccharide addition sites are represented by branched structures. The gray rectangle indicates the location of the hydrophobic membrane-spanning domain of TM.

residue 88, which lies at the end of the more N-terminal predicted helix, also oligomerize but are defective in transport. The ability of this sequence to direct oligomerization, however, is affected by adjacent sequences. Thus, truncation after residue 105 of TM yields a protein that does not form detectable oligomers. A more carboxy-terminal region of TM that includes residues 105 through 120, and which is also predicted to form an α helix, appears to play a key role in native oligomerization region or by forming an integral part of the oligomerization domain. The implications of these findings are discussed in relation to the assembly and biological activity of the Env protein.

MATERIALS AND METHODS

Env expression and mutagenesis. The RSV Env protein was expressed from the recombinant simian virus 40 (SV40) vector pSV.KX (7). The *env* gene used for these studies is from the Prague subgroup C molecular clone, pATV8 (17). Mutants C3 and A(–) have been described (20, 31). The TM105 and TM129 mutants were generated by oligonucleotide-directed conversion of codons 106 and 130 of the TM coding sequence subunit to termination codons. The template for this mutagenesis was a single-stranded DNA from the phagemid pSELECT-1 (Promega, Madison, Wis.) carrying the RSV *env* gene as a *KpnI*-*XbaI* fragment. To construct additional truncation mutants, as well as deletions in *env*, an *EcoRI*-*XbaI* fragment of the *env* gene (nucleotides 5232 to 7094 [15]) was inserted into pTZ18R. An oligonucleotide (5'-TTAACTAGTTAA-3') was used to introduce premature termination codons by ligation into blunt-ended plasmids digested within the *env* fragment at the following restriction sites (nucleotide positions) (24): *PpuMI* (6553), *SmaI* (6585), *AflIII* (6613), *AvaI* (6677), *PstI* (6718), *NcoI* (6766), and *AflIII* (6856). Deletion mutant TM18SM88 was made by digesting this plasmid with *PpuMI* and *NcoI*, filling the blunt ends, and ligating in the presence of an 8-nucleotide *Clal* linker to maintain the open reading frame. TM18SM37 has a *Clal* linker inserted between filled ends from a *PpuMI*/*AflIII* digestion. Ligation of the filled ends of an *AflIII*/*AvaI*-digested plasmid produced TM37I59, while TM72RST88 has an 8-nucleotide *SalI* linker inserted between a filled *NcoI* site and a blunt-ended *PstI* site. TM59/75 was made by oligonucleotide-directed deletion of the sequence between the *AvaI* and *PstI* sites by using the pSELECT vector described above. Digestion with *NcoI* and *AflIII* followed by ligation of the compatible ends yielded TM88/119. All of these mutant *env* genes were expressed by inserting the mutated *EcoRI*-*XbaI* fragment into pSV.KX. Forms of TM18SM37 and TM72RST88 that lacked the membrane-spanning and cytoplasmic domains were constructed by replacing the 3' *NcoI*-*XbaI* segment with that of the mutant A(–) (20). The positions of truncations and deletions relative to other features of the TM domain are depicted in Fig. 1.

Nomenclature. Truncation mutants, which contain all of SU, are designated by their carboxy-terminal residue, such as TM105, which has a termination codon after amino acid 105 of TM, or by their carboxy-terminal Env residue and additional residues encoded by the inserted oligonucleotide, such as TM88VN, which terminates following the Val-Asn inserted after TM residue 88. Deletion mutants are identified by the residues flanking the deletion, so that TM59/75 contains SU plus residues 1 to 59 and 75 to 198 of TM. Where amino acids were introduced by a linker inserted to maintain the open reading frame, these also are included, as in TM72RST88. The designation A(–) is appended in those instances in which the deletion mutant also has a termination codon just prior to the membrane-spanning sequence.

Gradients and immunoprecipitations. Oligomerization was assayed by sucrose gradient fractionation of the Env proteins in cell lysates, because secreted forms of this protein, which form stable intracellular oligomers, are unstable and dissociate to monomers on sucrose gradients (11). CV-1 cells grown on 60-mm plates were infected with recombinant SV40 stocks at 48 to 72 h prior to metabolic labeling. The cells were labeled with 100 μ Ci of [35 S]methionine-cysteine (>1,000 Ci/mmol; EXPRE 35 S 35 S Protein Labeling Mix; Dupont, NEN) in 0.5 ml of methionine-cysteine-free medium after a 20-min preincubation in medium lacking cysteine and methionine. For analysis of oligomerization, labeled cells were lysed in OGL buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 40 mM octyl- β -D-glucoside) following a 10-min pulse-labeling or after a 10-min pulse and a 2-h chase. Supernatants from a 5-min spin in a microcentrifuge were loaded on gradients of 5 to 25% (wt/vol) sucrose in OGL buffer and centrifuged in an SW41 rotor at 40,000 rpm and 17°C for 16 h. The gradients were collected from the bottom in 15 fractions, which were immunoprecipitated with a polyclonal rabbit antiserum to RSV Env. To determine the extent of secretion of proteins into the culture medium, cells were labeled as above except that the pulse period was extended to 20 min. After the pulse or a chase of the indicated length, the culture medium was collected and cells were lysed by addition of lysis buffer A (25 mM Tris [pH 8.0], 50 mM NaCl, 1.0% Triton X-100, and 1.0% deoxycholate). The medium samples were centrifuged to remove cells, supplemented with a 1/4 volume of 5 $\times$ lysis buffer A, and precleared by incubation with fixed Staph A. Both cell lysates and culture medium samples were then immunoprecipitated as above.

RESULTS

Residues 105 to 120 of TM contribute to formation of a transport-competent oligomer. Truncation mutants of the RSV *env* gene, constructed as described in Materials and Methods, contained the entire SU domain and various lengths of the TM domain (Fig. 1). The mutant genes were expressed from an SV40-based vector system in CV-1 cells, which we have shown previously accurately reflects the expression of Env in avian cells (11, 20). The oligomerization and intracellular

FIG. 2. Gradient analysis of oligomer formation by TM105 and TM129. CV-1 cells expressing TM105 or TM129 were labeled in a 10-min pulse with [35 S]methionine-cysteine and lysed immediately or after a 2-h chase in complete medium. Lysates were sedimented on 5 to 25% sucrose gradients. A total of 15 fractions was collected from the bottom of the gradient and immunoprecipitated with an antiserum to the RSV glycoprotein.

transport of truncation mutants TM105 and TM129 were examined in detail, since a mutant with 20 foreign amino acids inserted after residue 102 was defective in both processes (11). Sucrose gradient analysis of pulse-labeled TM105 protein resulted in its immunoprecipitation from fractions 9 to 11 (Fig. 2). The sedimentation of these Env proteins is consistent with a monomeric structure and resembles that seen with wild-type Env (data not shown and reference 11). After a 2-h chase, TM105 protein was still present primarily in fractions 9 to 11. This indicates that no stable oligomers formed during the chase. The TM129 protein also appeared monomeric at the end of the pulse-labeling, since it was detected only in fractions 8 to 10 (Fig. 2). Following the 2-h chase, however, TM129 was present in fractions 4 to 6 as well as fractions 8 to 10 (Fig. 2). The faster-sedimenting material in fractions 4 to 6 indicated formation of a discrete oligomer, which is most likely a trimer, based on the sedimentation behavior of the full-length Env protein on these gradients (data not shown). The labeled TM129 protein consisted of a nearly equal mixture of trimers and monomers after the 2-h chase. Thus, the absence of residues 106 to 129 had a deleterious effect on the formation of oligomers or their stability on the sucrose gradients, while residues carboxy terminal of position 129 appeared to be dispensable for stable oligomer formation.

The intracellular transport of these proteins was assayed by monitoring their secretion into the culture medium. Secretion of the TM129 protein was at least as efficient as that of the complete ectodomain of RSV Env (TM148), judging from the relative amounts of cell-associated and secreted forms of these proteins after a 1-h chase (Fig. 3). For TM105 the amount of secreted protein relative to that of the cell-associated form was markedly reduced. The block to transport of the TM105 protein was less complete than that seen for C3, but it was clearly defective in transport relative to TM148 and TM129. Thus, truncation of the RSV protein, in the absence of the addition of foreign amino acids, can affect its transport.

The N-terminal region of TM can inhibit the intracellular transport of soluble SU. Neither the TM105 protein nor the independently expressed SU protein assembles into a detect-

FIG. 3. Secretion of TM105 and TM129. CV-1 cells expressing TM148, TM129, TM105, or C3 proteins were labeled in a 20-min pulse with [35 S]methionine-cysteine and chased for 1 h in complete medium. Cell lysates (C) and the culture media (M) were then immunoprecipitated with the anti-Env antiserum.

able oligomer, but the latter is efficiently transported (12), while TM105 is defective for transport. A series of truncated Env proteins (Fig. 1) that terminate within the N-terminal 105 residues of TM was examined to characterize the region of TM that interfered with transport. Premature termination codons were introduced by insertion of a 12-nucleotide linker at restriction endonuclease sites within the extracellular domain of TM. These proteins are designated by their carboxy-terminal residue, as in the case of TM29, TM59, and TM120, or by the last Env residue plus the terminal residues introduced by the linker, e.g., TM17LN, TM37IN, TM73N, and TM88VN (Fig. 1).

The TM120 protein resembled TM129 in being efficiently secreted, as can be seen from comparing the amounts of cell-associated (Fig. 4A) and secreted (Fig. 4B) forms of these proteins after a 3-h chase. By contrast, all shorter proteins truncated within the TM domain appeared defective in trans-

FIG. 4. Intracellular transport of truncated Env proteins. Following a 30-min pulse with [35 S]methionine-cysteine and a 3-h chase, cell lysates (A) and culture media (B) were harvested from Env-expressing CV-1 cells. Samples were immunoprecipitated prior to analysis.

FIG. 5. Gradient analysis of oligomer formation by truncated Env proteins. CV-1 cells expressing the mutant proteins were labeled and fractionated as described in the legend to Fig. 2.

port. A comparison of TM17LN and SU shows that appending as few as 19 amino acids to the SU domain interfered with its transport, and this effect appears stronger for proteins truncated between residues 39 and 88. The block to transport, however, is not complete, and the proteins detected in the medium have the heterogeneous appearance of terminally glycosylated proteins.

The N-terminal 88 amino acids of TM can direct oligomer formation. The transport-defective C-terminally truncated proteins were examined to determine if they could form oligomers. Following a 10-min pulse-labeling, all of the proteins sedimented as monomers (Fig. 5). TM17LN was present in fractions 10 to 12; TM29, TM37IN, and TM59 peaked in fractions 10 to 11; and TM73N, TM88VN, and TM120 were detected primarily in fractions 9 to 11 (Fig. 5). Following a 2-h chase the TM120 protein appeared as a mixture of monomers and oligomers. The presence of faster-sedimenting protein in fractions 5 to 7 is consistent with formation of a trimer. TM120, then, resembles TM129 in forming an oligomer and being efficiently secreted. With the exception of TM88VN, all of the

other truncated proteins remained as monomers. Following the chase, TM88VN was detected in fractions 5 to 7 in addition to the monomeric position at fractions 10 to 11. Since TM73N and all of the shorter proteins did not assemble into oligomers, residues 74 to 88 of TM appear to be important for oligomer formation in TM88VN.

Deletions within the N-terminal region of TM can affect oligomerization. To characterize further the elements of TM that are involved in oligomer formation, a series of deletions was introduced within the first 120 amino acids of TM. In contrast to the C-terminal truncations, these mutations were constructed in the context of a full-length, membrane-anchored form of the RSV *env* gene. Deletion of residues 19 to 87 of TM from a membrane-anchored Env precursor completely disrupted oligomer formation. This protein, TM18SM88, was present in a peak at fractions 9 to 11 of the gradient after pulse-labeling and remained in this region of the gradient following the chase (Fig. 6). Smaller deletions in this region of Env were generated by individually deleting each of the sequences that separated successive termination sites in the se-

FIG. 6. Gradient analysis of oligomer formation by deletion mutants of the Env protein. CV-1 cells expressing the mutant proteins were labeled and fractionated as described in the legend to Fig. 2.

ries of truncated proteins. Pulse-labeled TM18SM37 sedimented in fractions 8 to 10, but the majority of this protein shifted to fractions 4 to 6 following the chase, a pattern similar to that seen for the wild-type protein (data not shown), indicating that this protein oligomerized efficiently (Fig. 6). Some of the TM18SM37 protein migrated to near the bottom of the gradient, suggesting that aggregates also formed. TM37I59 proteins are mostly present around fraction 10 after the pulse-label, with lesser amounts as far down in the gradient as fraction 6 (Fig. 6). After the chase two discrete peaks of TM37I59 were present, in fractions 9 to 11 and 5 to 7. The position of the TM37I59 oligomer on the gradient was shifted upward slightly relative to that of TM18SM37. This difference was also seen in the position of the monomers. Although the deletions in both proteins are close in size, TM37I59 has lost an N-linked glycosylation site as well as a cysteine residue that would be expected to form an intrachain disulfide. Since a difference was also seen between the monomers, the difference in sedimentation of the oligomers is unlikely to represent a difference in the number of subunits in the oligomers of the two proteins. Therefore, residues 19 to 36 and 38 to 58 appear to be individually dispensable for oligomer formation.

The TM59/75 protein was centered at fraction 10 following the pulse, and its position on the gradient did not change during the chase, after which it appeared in fractions 9 and 10 (Fig. 6). Although the amount of labeled protein for this mutant was low relative to the amounts for the others, there was no evidence of oligomers lower in the gradient. Thus, removal of residues 60 to 74 resulted in a protein that could no longer oligomerize. Surprisingly, the TM72RST88 protein, in which residues 72 to 87 were replaced with Arg-Ser-Thr, exhibited a shift from primarily monomer (fractions 9 to 10) in the pulse to a mixture of monomer and oligomer (fractions 9 to 10 and 4 to 6, respectively) during the chase (Fig. 6). Some oligomers were detected already after the pulse, but they increased in number relative to monomers during the chase. This result indicated that residues 73 to 88 are not required for oligomer formation in these internally deleted proteins, even though their absence from TM73N is the difference between that oligomerization-defective protein and the TM88VN protein, which forms oligomers. Of the four smaller deletions within residues 19 to 87, only TM59/75 was unable to oligomerize.

Deletion of amino acids 89 to 118 in the TM88/119 protein had a significant effect on the sedimentation of the Env protein. Immediately after the pulse-labeling, the protein behaved as a mixture of monomers and larger complexes (Fig. 6). In addition to peaks representing monomers (fractions 10 to 11) and discrete oligomers (fractions 5 to 7), a significant amount of the protein was present in fractions 1 to 4, which probably contained larger aggregates of the protein. While some aggregates of other mutants were detected following a chase, these generally could not be detected after the pulse (Fig. 6). The sedimentation pattern of TM88/119 did not change significantly during the chase. Thus, the associations formed between monomers of this protein formed very early or during lysis and were most likely aberrant.

Analysis of deletions in a soluble form of Env. All of the deletions in the full-length Env protein described above resulted in proteins that were defective in their ability to be secreted, regardless of their ability to oligomerize. This block in transport to the Golgi complex is evident from the lack of terminal glycosylation of these proteins, which normally causes a distinct increase in the apparent molecular weight of uncleaved Env proteins. Figure 6 shows that, for each of the deletion mutants, only uncleaved precursor and no SU product was detected following chases of pulse-labeled cells; no TM

FIG. 7. Gradient analysis of oligomer formation by TM18SM37/A(–) and TM72RST88/A(–). CV-1 cells expressing the mutant proteins were labeled and fractionated as described in the legend to Fig. 2.

cleavage product was detected in the region of gel that is not shown. To determine if the membrane anchoring of deletion mutants which were able to oligomerize affected their assembly and transport, soluble forms of these proteins were examined by combining the deletions with the TM148 [A(–)] truncation, which removes the membrane-spanning and cytoplasmic domains of Env. TM18SM37/A(–) behaved as a monomer in pulse-labeled lysates, appearing in gradient fractions 8 to 10 (Fig. 7). After a 2-h chase, however, a mixture of monomers and oligomers (fractions 4 to 5) was observed. Similarly, the TM72RST88/A(–) protein also showed a shift from monomers (fractions 8 to 10) to a mixture of monomers and oligomers (fractions 4 to 6) during the chase (Fig. 7). Some larger complexes were present in fractions 2 and 3, a species also observed for TM72RST88 (Fig. 6). Both TM18SM37/A(–) and TM72RST88/A(–) were defective in transport out of the cell (data not shown). Thus, we can conclude that the transport defect of these deletion mutants, which are able to oligomerize, is not due to a constraint placed on the protein by anchorage in the membrane. Oligomerization of these proteins presumably requires a specific conformation, but this conformation is not sufficient for intracellular transport.

DISCUSSION

Oligomers of the RSV glycoprotein are formed during the folding and maturation of the envelope precursor in the ER. Proteins labeled in a short pulse migrate in velocity gradients as monomers, confirming that oligomer formation does not occur following lysis and that these proteins are at an intermediate stage of maturation that precedes stable oligomer formation. Assembly of the glycoproteins into oligomers is likely to be dependent on their folding within the unique environment of the ER, in which they can adopt conformations necessary to expose the motifs that mediate interactions between colocalized monomers. We have attempted to characterize the elements of the RSV glycoprotein that are involved in oligomer formation by monitoring assembly of mutant glycoproteins *in vivo* in CV-1 cells infected with recombinant SV40 vectors. Previous studies have shown that the different steps in Env biosynthesis, oligomerization, and maturation are similar in this expression system and in RSV-infected avian cells (11, 20).

Our results indicate that the presence of the N-terminal 120 amino acids of TM is necessary and sufficient for assembly of a transport-competent oligomer of truncated RSV Env molecules. The C-terminal 28 residues of the ectodomain of TM, together with its membrane-spanning and cytoplasmic sequences, can be deleted without affecting oligomer formation

or transport through the secretory pathway. Although the SU domain is also present in this truncated protein, we have shown previously that this subunit has no independent ability to oligomerize. In contrast, the TM subunit does oligomerize when expressed independently of SU (12). The region of the TM protein present in TM120 includes two potential α helices spanning residues 30 to 85 and 105 to 120. These potential helical regions flank Cys⁹⁰, Cys⁹⁷, and Cys⁹⁸, in addition to Asn¹⁰⁰, which is modified by N-linked glycosylation. This structural motif is highly conserved, with two or three cysteine residues and at least one adjacent N-linked glycosylation site being present at a similar location in a number of retroviral TM proteins (19, 23). While the presence of two α helices in the RSV TM protein has been proposed on the basis of predictive algorithms (14), there is no direct evidence for their presence in the precursor protein that folds in the ER. The results of biophysical and crystallographic analyses have been reported for a 55-amino-acid fragment, which encompasses the N-terminal α -helical domain and the cysteine motif, of the murine leukemia virus TM protein. These studies show that this fragment can form a stable trimeric coiled coil analogous to that seen in the acidified, fusion-active form of the influenza virus HA2, supporting the concept that this domain can assume a helical secondary structure (13). Similar conclusions were drawn from biophysical analyses of HIV and simian immunodeficiency virus TM fragments (2, 18). However, if the analogy with HA2 holds, this N-terminal domain of the retrovirus TM might lack helical structure in the precursor molecule, since this region of the influenza virus HA2 protein is constrained from folding into a helical structure until the process of fusion is triggered by the low pH of the endosome (3, 4, 28, 32).

Truncation after residue 105, or after residue 102 (mutant C3, which has an additional 20 foreign amino acids inserted), of the RSV TM abrogates both oligomer formation and intracellular transport. The C3 protein is nearly identical in length to TM120, and the similar defect in both C3 and TM105 suggests that residues 106 to 120 of TM make a specific contribution to oligomer assembly. Since this sequence could form a short amphipathic helix, this structural motif may mediate interactions essential for stable oligomer formation. The inefficient intracellular transport of TM105 may reflect a requirement for the Env protein ectodomain to oligomerize in order to attain a transport-competent state (12).

Two mutant proteins did form higher-molecular-weight complexes despite the absence of this second potential helix. TM88/119, which has a deletion spanning the region from the end of the first predicted helix to the end of the second predicted helix, formed oligomers, but larger aggregates were also prominent and these complexes were detected in the pulse, indicating that their formation was not dependent on the normal maturation process. TM88VN did form discrete oligomers during the chase period, but these complexes were defective in intracellular transport. Thus, components of the secretory pathway are able to distinguish TM88VN oligomers from transport-competent Env oligomers. As discussed below, we believe that the interactions mediating formation of TM88VN oligomers are not the same as those driving assembly of wild-type Env oligomers but may instead resemble those responsible for an oligomeric form found in the fusion-active state of the molecule. If the N-terminal helix were the primary determinant for oligomerization, one would not expect TM73N and TM72RST88/A(–) to have different phenotypes. The failure of TM73N to oligomerize suggests that in the absence of C-terminal sequences the entire N-terminal helical domain is essential for assembly into oligomers, but in the context of an intact

ectodomain [present in TM72RST88/A(–)] residues 73 to 88 of the N-terminal helix are not required for oligomerization.

Retroviral Env proteins may undergo two discrete oligomerization events during their lifetime. The first of these is the process of assembly oligomerization that occurs within the rough ER and is the subject of this study, while the second is initiated following receptor binding and is a prerequisite for virus-induced membrane fusion. The potential helix corresponding to residues 30 to 85 of the RSV TM can be found in many TM proteins and has been noted to contain a leucine zipper or heptad repeat domain (9, 25). This sequence was initially proposed to be involved in assembly of Env oligomers based on the ability of similar sequences to form coiled coils in other proteins. However, mutations in the proposed leucine zipper of the HIV-1 glycoprotein did not interfere with oligomer assembly or transport of this protein (5, 6, 10), even though they abrogated the biological (fusion) properties of the glycoprotein. This heptad repeat region, however, can mediate oligomerization under certain conditions. Peptides corresponding to the HIV-1 leucine zipper region associate into very stable, higher-order coiled-coil protein structures (28, 29), and fusion of this heptad repeat region from the HIV-1 TM to a monomeric protein induces it to form discrete oligomers (1). Moreover, in murine leukemia virus the involvement of this domain in a triple-stranded coiled coil has been unequivocally demonstrated by X-ray crystallography (13). It is possible, then, that oligomerization of TM88VN involves the formation of a similar coiled coil and that this oligomerization resembles that seen when the heptad repeat of HIV-1 is fused to the C terminus of a foreign monomeric protein. In this case, the TM88VN oligomer would arise from interactions that are made possible by the truncation and are distinct from those in TM120 and the full-length molecule.

Unlike TM88VN and despite the presence of the N-terminal helix, TM105 did not form oligomers. The additional residues present in the latter protein, which includes three cysteines, presumably constrain the heptad repeat motif from inducing oligomerization. These cysteines could interact with Cys⁴⁵, which is internal to the heptad repeat, or with Cys⁹ to stabilize (possibly nonnative) structures that do not oligomerize. Alternatively, residues 89 to 105 may stabilize a more native TM structure which, like the native full-length Env molecules, lacks strong associations between heptad repeat regions and remains monomeric. Extension of the protein to residue 120 could allow formation of transport-competent oligomers, with residues 105 to 120 either causing formation of a more native TM domain or appending a critical interface for oligomerization to an already relatively native but monomeric structure. As a helix, residues 105 to 120 would be amphipathic, with largely hydrophobic residues at positions a and d and lysines at positions f and g (Fig. 8A), and so could mediate intersubunit interactions.

Deletions at the N or C terminus of the potential helix overlapping the heptad repeat sequence (TM37I59 and TM72RST88) do not block assembly of oligomers, consistent with the absence of a role for this entire region in assembly. Nevertheless, these proteins are defective for intracellular transport and are likely to have altered conformations. In contrast, a deletion that removes 15 residues from the center of this domain (TM59/75) does block oligomer assembly. This may reflect a more drastic conformational defect that precludes oligomerization. It is interesting to note, however, that in the context of a predicted helix, TM59/75 is the only mutation that results in the introduction of charged residues (aspartic acid and arginine in faces a and d, respectively) that would disrupt the hydrophobic surface at the C terminus of this heptad repeat (Fig. 8B). Thus, we cannot rule out the possibility that inter-

A.

B.

	a	d
wild type	GLAALVVI	AISTLINL
TM37I59	VIVVI	ALINL
TM72RST88	GLAALVV	AISTLIT
TM59/75	GLAALD	AISTR

FIG. 8. (A) Helical wheel analysis of the predicted C-terminal α -helical domain which encompasses residues 105 to 120 of gp37. Note the predominantly hydrophobic a and d faces of a short amphipathic helix. (B) Amino acid composition of the a and d faces following a helical wheel analysis of the N-terminal helical domain (residues 28 to 85) of wild-type gp37 and of the same, but internally deleted, region in mutants TM37I59, TM72RST88, and TM59/75.

actions between a helical structure formed by this domain and that predicted between residues 105 and 120 might be necessary for oligomer formation. Biophysical studies have pointed to helical interactions between two analogous regions of the HIV and simian immunodeficiency virus TM proteins expressed from bacteria (2, 18).

The mutations described here define a region between residues 105 and 120 of the RSV TM that is critical for the assembly of an oligomeric molecule that can be efficiently transported through the secretory pathway of the cell. It is intriguing to note that a similarly located helical domain of HA2 stabilizes the HA trimer (3, 29, 32), raising the possibility that a conserved structural motif is involved in stabilizing the tertiary structure of these two viral glycoproteins.

ACKNOWLEDGMENTS

We acknowledge Susan Dubay, Geraldine Long, and Tshana Thomas for excellent technical assistance.

We acknowledge the UAB Center for AIDS Research DNA Sequencing Core, supported by core grant AI-27767. This work was supported by grant CA-29884 from the National Institutes of Health.

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