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Uncoupled Expression of Moloney Murine Leukemia Virus Envelope Polypeptides SU and TM: a Functional Analysis of the Role of TM Domains in Viral Entry

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Moloney murine leukemia virus ecotropic envelope expression plasmids were used to demonstrate that the synthesis of the retroviral envelope SU and TM polypeptides can be uncoupled with retention of biologic activity. By substituting a glycosyl-phosphatidylinositol (GPI) membrane anchor for part or all of the retroviral envelope transmembrane protein and creating several deletion variants of the TM subunit, we have begun to dissect the role of the TM protein in envelope function. We show that a GPI-anchored envelope can be incorporated into virions and binds receptor. We found that the envelope cytoplasmic tail, while not required, influences the efficiency of retroviral transduction at some step after membrane fusion (possibly by interacting with core). The membrane-spanning domain of TM is involved in membrane fusion, and this function is distinct from its role as a membrane anchor. As few as eight amino acids of the putative membrane-spanning domain are sufficient to achieve membrane anchoring of envelope but not to mediate membrane fusion. In addition, though not required, the membrane-spanning domain may have some direct role in the incorporation of envelope into virions. Finally, the extracellular domain of TM, besides containing the putative fusion domain and interacting with SU, may influence the synthesis or stability and the glycosylation of envelope, possibly by affecting oligomerization of the complex and proper intracellular transit.

The envelope proteins of retroviruses play a critical role in the viral life cycle (10, 43). In the Moloney murine leukemia virus (MuLV), the envelope complex is an oligomer of heterodimers composed of two polypeptides, gp70 and p15E (25, 36). These proteins are encoded by the *env* gene and processed from a common precursor, gp80, by a cellular protease (49, 50). In the virion, or at the time of viral budding, p15E is processed further by a viral protease that clips a short peptide (R peptide) from the carboxy terminus. Viral tropism is determined in large part by the specific interaction between the viral receptor on the host cell and the gp70 envelope glycoprotein (SU) (33, 37). The p15E protein (TM) is an integral transmembrane protein which associates with SU through noncovalent interactions and possibly a disulfide bond. It serves to anchor the envelope complex to the virion membrane and is also a vital component of the viral entry process (13, 19, 27, 38, 46). Elucidating the determinants of envelope assembly, incorporation into virions, and viral entry into the host cell will be of importance in understanding infectivity and may be useful in developing retrovirus-based vectors that can be targeted to specific cell types.

The function of cellular integral transmembrane proteins and the vesicular stomatitis virus G protein have been studied by removing the membrane-spanning and cytoplasmic domains and substituting sequences which direct the covalent anchoring of the protein to the cell surface through a glycosyl-phosphatidylinositol (GPI) linkage (6, 9, 18, 23). These glycophospholipid-anchoring (GLA) sequences have also been utilized to anchor secreted proteins to the cell surface in a manner that retains their biological activity (4, 5, 26). By substituting GLA sequences for part or all of the retroviral envelope TM subunit, we have begun to dissect the role of this integral transmembrane protein in the viral life cycle. We observe that GLA sequences permit the incorporation of envelope into virions. Such virions bind their target cell receptor and in one case can mediate the post-receptor-binding events necessary for retrovirus-mediated gene transfer. Our results have implications for the nature of the oligomeric envelope structure and suggest a revised model of coupled receptor binding.

MATERIALS AND METHODS

Envelope constructs. Nucleotide positions correspond to the numbering of Shinnick et al. (44). Amino acid (aa) 1 is the first amino acid of the envelope signal peptide. All envelope constructs were expressed from a cytomegalovirus promoter-driven expression vector which contained an simian virus 40 poly(A) termination signal. pCEE, which contains the entire Moloney MuLV ecotropic envelope sequence (5408 to 7847), has been previously described (32). pCE2 was a gift from J. Mason. It was constructed by excising the promoter-envelope-poly(A) cassette from pCEE with *Hin*dIII and *Fsp*I and cloning it between the *Hin*dIII and *Eco*RV sites of a pBR322 derivative in which the *Eco*RI and *Nde*I sites had been destroyed. The Moloney MuLV envelope was subsequently modified by PCR techniques to convert the *Xho*II restriction enzyme site at position 7164 to a *Bgl*II site, while maintaining the native amino acid sequence. pMAD was a gift from R. Morgan. It is a Moloney MuLV subclone in Bluescript (Stratagene, La Jolla, Calif.) of the unique *Nsi*I-*Bss*HII fragment, which spans a region upstream of the gp70 carboxy terminus to within the 3′ long terminal repeat. The GLA sequences from decay-accelerating factor were isolated from plasmid pLUK-ASN as a 126-bp *Bam*HI fragment (26). pCEET was constructed by linearization of pCEE at the unique *Cla*I site, Klenow enzyme

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fill-in of ends, and ligation of a 14-bp *Xba*I STOP linker (New England Biolabs no. 1062). pGLA15E was created by substituting the *Bam*HI fragment from pLUK-ASN, after repair with Klenow enzyme, for the unique *Eco*RV fragment in pCEE between positions 7609 and 7705. pCEET1 was created by substituting a 14-bp *Xba*I STOP linker for the unique *Eco*RV fragment in pCEE. 15EΔ was generated by first creating a deletion between the *Xho*II sites at positions 7164 and 7584 within pMAD to produce pMADΔ. The deletion was then shuttled into pCEE by substituting the *Nsi*I-*Cla*I fragment from pMADΔ for that in pCEE. pGLA70 was created by cloning the *Bam*HI fragment from pLUK-ASN into the *Bgl*II site of pCE2 after Klenow enzyme fill-in of ends. pS70 was constructed by linearization of pCE2 with *Bgl*II, Klenow enzyme fill-in of ends, and ligation of a 14-bp *Xba*I STOP linker. The p15E expression vectors were constructed as follows. For p15EP and p15EL, sequences between the *Sca*I and *Bgl*II sites in pCE2, encoding aa 33 to 462 of gp70, were replaced with a synthetic oligonucleotide encoding gp70 aa 453 to 462 or aa 33 to 40 and aa 462, respectively. p15E10 was a gift from J. Mason. It was constructed by removing the sequences in pCEE between the cytomegalovirus promoter and the *Hpa*I site at position 7198 in p15E and inserting a rat growth hormone leader sequence. All plasmid constructs were confirmed by DNA sequencing (Sequenase 2.0; U.S. Biochemical, Cleveland, Ohio).

Cell lines, DNA transfection, and viral titers. All cell lines were maintained in Dulbecco's modified essential medium (DMEM; Biofluids, Rockville, Md.) except XC cells, which were grown in Eagle's minimal essential medium (Biofluids). All media were supplemented (10%) with heat-inactivated fetal calf serum (HyClone, Logan, Utah). The GPL line has been described elsewhere (32). Briefly, it is a stable 3T3-derived cell line which constitutively expresses MuLV Gag/Pol and contains the retroviral vector genome LNL6 (2). As such, these cells continuously produce so-called bare viral particles that lack an envelope protein. When GPL cells are transfected with an envelope expression vector, complete virions with a retroviral vector genome are produced. The titer of these virions can be determined by measuring the transfer of the LNL6-encoded neomycin resistance (Neo^r) gene to an appropriate target cell. XC cells were a gift from Janet Hartley. The gpLA15ESN line was established by shuttling the GLA15E envelope into the retroviral vector LXSN, producing retroviral vector particles, and transducing the GP8 cell line with them (26, 32). DNA (30 μg of envelope vector) was transfected into GPL or 3T3 cells (5×10^5 cells per 100-mm-diameter dish) by the calcium phosphate precipitation method, using reagents from 5′-3′ Inc. (Boulder, Colo.). In cotransfection experiments, a control plasmid DNA was added as needed to control for the total amount of DNA transfected. At 16 to 20 h posttransfection, the cells were washed and fresh medium was added; 48 h later, the cell supernatant was collected, filtered, and used immediately or stored at −70°C prior to assay. Viral titer as G418^r CFU per milliliter was determined as previously described (32).

Cell and virus labeling and immunoprecipitation. Approximately 48 h after transfected GPL cells had been in complete medium, the tissue culture plates were rinsed twice with phosphate-buffered saline (PBS), and methionine-free DMEM with 10% dialyzed fetal calf serum was added. The cells were incubated at 37°C for 2 h and then labeled overnight in 100 μCi of [^{35}S]methionine (Trans-label; ICN, Irvine, Calif.) per ml. The cell medium was collected, filtered, and fractionated by centrifugation ($500 \times g$) for 30 min at 4°C in a Centriprep-100 ultrafiltration unit (Amicon, Beverly, Mass.). Cell lysis was done as described before (32). For both cell lysates and concentrated virus, equal numbers of trichloroacetic acid-precipitable counts per minute were immunoprecipitated under identical conditions as described previously (32). Polyacrylamide gel electrophoresis (PAGE) and fluorography were performed as described elsewhere (32).

Flow cytometry. The virus binding assay was performed as described previously (20) except that 3.5 ml of viral supernatant was used for 10^6 target cells and all incubations were performed at 4°C. In addition, in some experiments, immunostaining was performed with the MuLV ecotropic envelope-specific monoclonal antibody 273 (AIDS Reagent Repository, Rockville, Md.). Ecotropic (in contrast to amphotropic) virus binding and subsequent steps must be performed at this lower temperature to prevent internalization of the bound virus (32). Medium from GPL cells transfected with a control DNA was used in the virus binding assay to determine the level of background immunostaining. In virus binding assays using fractionated viral preparations, an aliquot of the viral sample was centrifuged in a Centriprep-100 unit as described above. The effluent after centrifugation (i.e., material of <100 kDa) was analyzed in the virus binding assay in parallel with the unfractionated viral sample. This allowed each viral sample to serve as its own negative control and permitted an estimation of what component of the fluorescence signal was due to binding of non-virion-associated envelope in the assay. Detection of cell surface envelope on 10^6 transfected cells was performed as in the virus binding assay after the virus adsorption step. GPL cells transfected with a control DNA were used to determine the level of background immunostaining. The transfected cells were dissociated with enzyme-free dissociation solution (Specialty Media, Inc., Lavallette, N.J.) prior to immunostaining.

PIPLC digestion. gpLA15ESN cells, dissociated in enzyme-free dissociation solution, were washed in DMEM–0.3% bovine serum albumin and resuspended in 0.1 ml of the same. The cells were incubated at 37°C for 1 h in the presence or absence of phosphatidylinositol specific phospholipase C (PIPLC) (Boehringer Mannheim, Indianapolis, Ind.) at 1 U/ml. Following digestion, cells were collected by centrifugation, washed, and immunostained as described above.

Membrane fusion assay. About 1×10^5 to 2×10^5 3T3 cells in a 60-mm-diameter dish were transfected with 30 μg of envelope expression vector DNA as described above; 24 h after fresh medium had been added, nonirradiated XC cells (~10^6) were added to the plate, and the culture was incubated for an additional 24 h. Plates were stained with 1% methylene blue in methanol prior to photography.

RESULTS

Envelope constructs. DNA maps of the envelope constructs and their predicted structures in the membrane are shown schematically in Fig. 1. In Table 1, the amino acid sequences at the molecular junctions are shown. The rationale for the various envelope constructs is presented below.

To test whether the cytoplasmic tail of p15E is required for the proper transport and cell surface expression of the viral envelope, the CEET construct was made. This product terminates at the membrane-cytoplasm interface, resulting in an envelope that lacks a cytoplasmic tail.

In the GLA15E construct, GLA sequences have been substituted for the cytoplasmic domain and all but the N-terminal-most 8 aa of the TM membrane-spanning domain. This permits the membrane-anchoring role of these sequences to be distinguished from other activities that they may have in

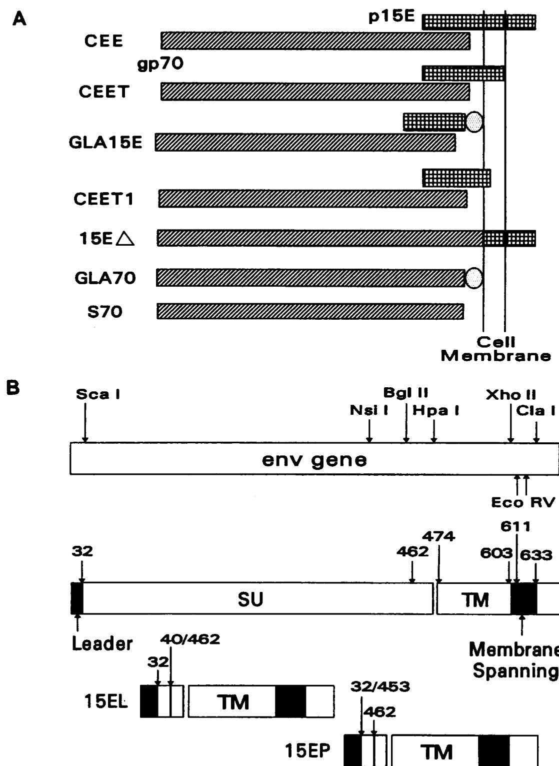

FIG. 1. Schematic of envelope structures. (A) Predicted structures in the cell membrane of the described envelope constructs. The topmost structure is that of the wild-type envelope encoded by the pCEE expression plasmid. The polypeptide sequences are shown with a diagonally hatched box (gp70) and a cross-hatched box (p15E). A GPI anchor is shown with a dotted circle. (B) Alignment of the structure of the gp80 envelope precursor under a limited restriction map of the *env* gene. The gp70/p15E proteolytic processing site is indicated by a break in the open box depicting gp80. The predicted structure of the p15E precursor encoded by the 15EL and 15EP expression vectors is also shown. The SU (gp70) and TM (p15E) polypeptides are indicated, as is the leader signal sequence and membrane-spanning domain of TM. Amino acid positions relevant to the envelope constructs described in the text and Table 1 are indicated. Note that the *Bgl*II site is derived from the *Xho*II site in the native envelope gene.

envelope function. To determine whether the residual 8 aa of the membrane-spanning domain that are present in GLA15E are sufficient for membrane anchoring, the CEET1 envelope was made. This construct terminates in TM at the same point at which the GLA sequences are present in GLA15E.

The role of the extracellular domain of TM was tested by using the 15EΔ construct. In this envelope, 7 aa from the carboxy terminus of SU and the extracellular portion of TM have been deleted, resulting in the fusion of the slightly truncated carboxy terminus of gp70 to the membrane-spanning domain of p15E.

We used the GLA70 envelope to examine whether TM could be dispensed with altogether. In this construct, the GLA sequence was introduced just upstream of the gp70/p15E proteolytic processing site. The envelope thus contains GLA sequences fused to the slightly truncated (5 aa deleted) carboxy terminus of gp70 and no p15E sequences. Finally, to assess the need for a membrane-anchoring domain in GLA70, a termination codon was introduced at the same position that the GLA sequences are present in GLA70 to create S70.

Expression of envelope constructs. To determine whether the various envelope constructs were synthesized, transfected GPL cells were metabolically labeled with [^{35}S]methionine and cell lysates were immunoprecipitated with a polyclonal antiserum against envelope. This antiserum does not recognize the TM protein. Immunospecific bands corresponding to the envelope precursor (gp80) and mature form of SU (gp70) are observed in the wild-type envelope cell lysate (Fig. 2, lane 2).

The CEET, GLA15E, and CEET1 envelopes appear to be as abundant in cell lysates as the wild-type envelope (Fig. 2, lanes 3 to 5). They are present predominantly as the propolypeptide, suggesting that they may not be cleaved as efficiently as the wild-type envelope. The precursor form of CEET, GLA15E, and CEET1 runs ahead of the wild-type gp80, as would be expected of envelopes that are truncated in the carboxyl half of TM (Fig. 1). As the 195-aa TM protein contributes only 10 kDa to the apparent molecular mass of the propolypeptide, it is not clear what the predicted apparent molecular masses of these truncated proteins would be. The CEET1 precursor (611 aa) runs, as expected, ahead of the GLA15E propolypeptide (634 aa). However, the slower mobility of CEET1 relative to CEET (634 aa) and the existence of a GLA15E product with an apparent molecular mass of greater than 80 kDa suggest that there may be an alteration of the normal glycosylation pattern in the GLA15E and CEET1 envelopes.

The 15EΔ, S70, and GLA70 envelopes share the common feature that the carboxy terminus of gp70 and the extracellular domain of p15E have been deleted (Fig. 1). As such, there is no precursor which is cleaved to yield SU and TM envelope products. These envelopes are not as abundant in cell lysates as the wild-type envelope is (Fig. 2, lanes 6 to 8). The major product of all three envelopes has the expected relative mobility. However, if glycosylation of the gp70 sequences had occurred as it does in the wild-type envelope, products with an apparent molecular mass of closer to 70 kDa would have been expected for the S70 and GLA70 envelopes, and products of slightly greater than 70 kDa would have been expected for the 15EΔ envelope. In addition to the major envelope products, 15EΔ has minor products of between 80 and 86 kDa, and S70 and GLA70 may have minor species with apparent molecular masses of between 70 and 75 kDa.

Cell surface-associated envelope. To determine whether the various envelope constructs were properly transported through the Golgi complex to the cell surface, GPL cells, 48 h after being transfected with an envelope expression vector, were stained in an indirect immunofluorescence assay using a primary monoclonal antibody against SU and subjected to flow cytometry.

The CEET envelope is expressed on the cell surface to the same extent as the wild-type envelope (Fig. 3), demonstrating that the cytoplasmic tail of p15E is not required for the proper transport and cell surface expression of the viral envelope. The substitution of GLA sequences for the majority of the TM membrane-spanning domain in the GLA15E construct permits this envelope to be anchored to the cell surface as well as the wild-type envelope is (Fig. 3). This result indicates that the membrane-spanning domain of TM is also not required for the cell surface expression of envelope. Figure 3 shows that CEET1 is also expressed on the cell surface, though to a lesser extent than GLA15E, indicating that as few as 8 aa of the membrane-spanning domain are sufficient to obtain membrane anchoring of envelope.

The role of the extracellular domain of TM was tested by using the 15EΔ construct. This construct is expressed on the cell surface but to a lesser extent than the wild-type envelope,

TABLE 1. Envelope junction sequences

Construct	Amino acid sequence[a]
Envelope	
CEET	604 620 634 S P W F T T L I S T I M G P L I V L L M I L L F G P C I L N R *
GLA15E	604 S P W F T T L m i P
CEET1	604 S P W F T T L I *
15EΔ	450 462/603 620 T Y H S P S Y V Y G L F E / R S P W F T T L I S T I M G P L I V L L M I L L
GLA70	450 464 T Y H S P S Y V Y G L F E R S i P
S70	450 464 T Y H S P S Y V Y G L F E R S *
p15E	
15EP	1 9 32/453 M A R S T L S K P L K N K V N P R G P L I P L I L L M L R G V S / S P S Y V Y
15EL	9 40/462 P L K N K V N P R G P L I P L I L L M L R G V S T A S P G S S P / E R S N R H
15E10	474 500 d a e L T L A L L L G G L T M G G I A A G I G T G T T A L M A T Q Q F Q Q L Q

[a] Substitutions and insertions are in lowercase. *, termination. In GLA constructs, the terminal P shown is the first residue of the GLA sequence.

indicating that the extracellular portion of TM is also not necessary for the surface expression of envelope (Fig. 3).

Since neither the intracellular nor the extracellular portion of TM appears to be individually required for transport of SU to the cell surface, we examined whether TM could be dispensed with altogether. Results with the GLA70 construct (Fig. 3) show that in the complete absence of TM, SU is transported and anchored to the cell surface. To assess the need for a membrane-anchoring domain in GLA70, a termination codon was introduced at the same position that the GLA sequences are present in GLA70 to create S70. The S70 envelope also appears to be anchored to the cell surface despite the absence of a membrane-anchoring moiety (Fig. 3). Data presented below suggest that the signal obtained with S70 is due to anchoring of the protein to the cell surface, rather than the binding of free envelope to the viral receptor.

GLA15E is anchored through a GPI linkage. The ability of the N-terminal-most 8 aa of the membrane-spanning domain to anchor the CEET1 envelope to the cell surface raised the possibility that GLA15E was also anchored in this fashion rather than via a GPI linkage. To determine the nature of the association of GLA15E with the cell membrane, we tested whether GLA15E could be liberated from the cell surface by

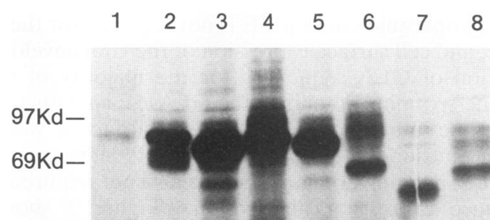

FIG. 2. SDS-PAGE of viral envelope proteins immunoprecipitated from lysates of metabolically labeled cells. GPL cells transfected with an envelope expression vector were labeled with [^{35}S]methionine and lysed. Equal numbers of trichloroacetic acid-precipitable counts per minute from all lysates were immunoprecipitated and run on the gel under reducing conditions. Lanes: 1, negative control; 2, wild type; 3, CEET; 4, GLA15E; 5, CEET1; 6, 15EΔ; 7, S70; 8, GLA70.

treatment with PIPLC (7). This lipase is highly specific for the GPI linkage in GLA-anchored proteins (29). gpLA15ESN is a stable 3T3-derived cell line that expresses the GLA15E envelope on its surface (data not shown). Incubation of these cells with PIPLC results in a loss of two-thirds of the surface associated envelope from the cell, indicating that the GLA15E envelope is anchored to the cell surface via a GPI linkage.

Incorporation into virions. We have recently described a method to measure the specific binding of virions to host cells (20). In the assay, medium from virus-producing cells is incubated with an appropriate target cell, and then the receptor-bound virus is detected on the target cell surface by indirect immunofluorescence and quantitated by flow cytometry. The assay is also capable of detecting the binding of free envelope to target cells. However, because free envelope is monovalent and virions (containing hundreds of envelope polypeptides) are polyvalent with respect to the epitope recognized by the primary monoclonal antibody, the assay largely detects virions. This was illustrated by transfecting 3T3 and GPL cells in parallel with the envelope expression vectors. Since GPL cells (which are derived from 3T3 cells) contain the retroviral *gag/pol* genes and 3T3 cells do not, GPL cell medium will contain assembled enveloped virions after transfection whereas the 3T3 cell medium will contain only envelope that may have dissociated from the membrane surface. The results for wild-type envelope and GLA15E are shown in Fig. 4. Despite comparable amounts of cell surface-associated envelope (data not shown), the supernatant from transfected GPL cells scores much more strongly than medium from transfected 3T3 cells in the virus binding assay. Thus, the assay is a measure of both envelope incorporation into virions and binding of virus to its cellular receptor.

The results of the virus binding assay with the CEET, GLA15E, CEET1, 15EΔ, GLA70, and S70 constructs are shown in Fig. 5. Each of the envelopes appears to be incorporated into virions and binds the viral receptor, with CEET and GLA15E scoring as well as wild-type envelope. The monoclonal antibody (83A25) used in these studies recognizes an epitope in the conserved carboxy terminal one-fourth of gp70. The 15EΔ, GLA70, and S70 envelopes all have a modified

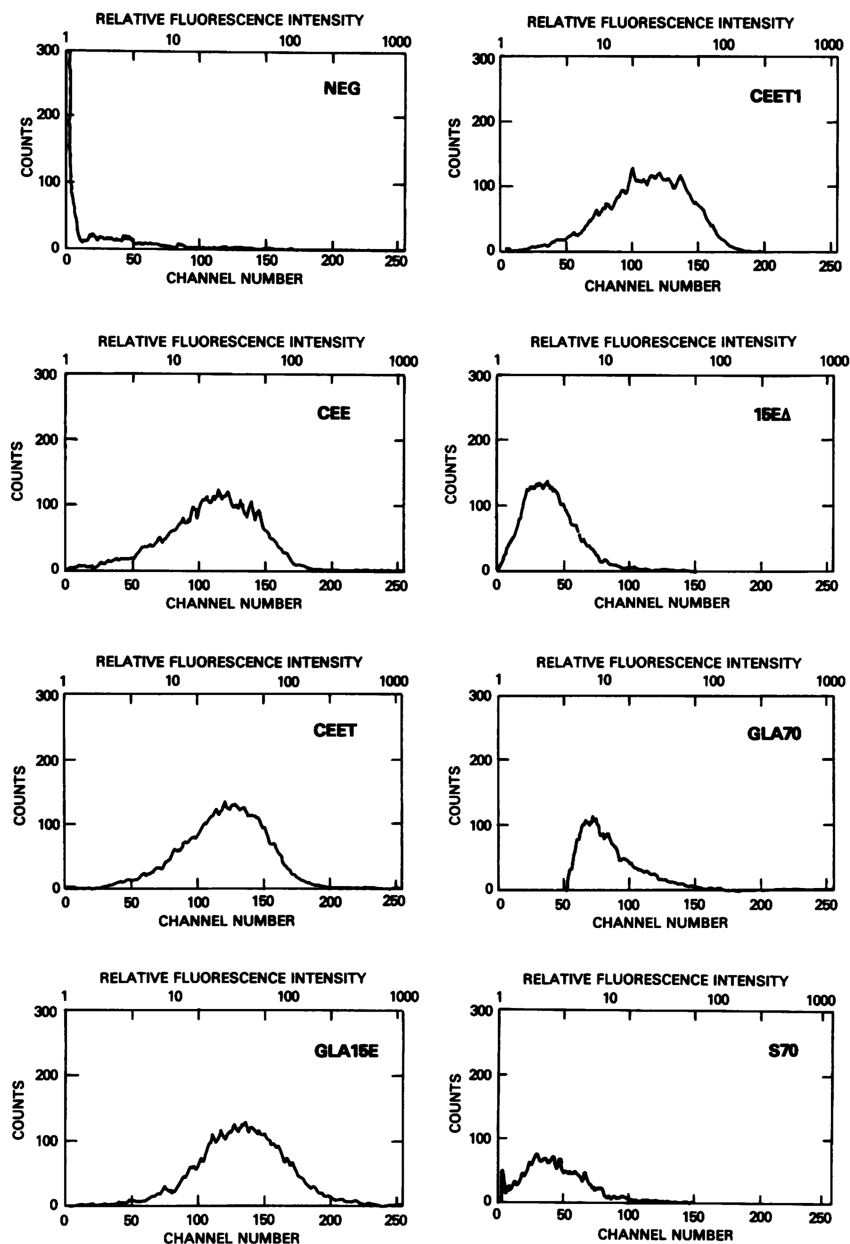

FIG. 3. Cell surface-associated envelope. The indirect immunofluorescence flow cytometry patterns for wild-type envelope encoded by pCEE and the various constructs expressed in GPL cells are shown. Counts along the ordinate reflect the number of stained cells. Background staining was subtracted. All constructs were tested at least three times, and representative patterns are shown. NEG, negative control.

gp70 carboxy terminus, raising the possibility that the diminished signal observed with these envelopes was due to impaired antibody recognition. To address this question, virus binding studies were also performed with monoclonal antibody 273. This monoclonal antibody is specific for ecotropic envelope and thus presumably recognizes an epitope in the amino-terminal one-fifth of gp70. Comparison of the immunofluorescence obtained with these antibodies indicates that 83A25 is the more sensitive of the two and that there is no difference in the relative signal obtained with the two monoclonal antibodies for the various envelopes (data not shown).

It was not expected that the S70 envelope, which lacks a membrane-anchoring moiety, would be associated with the cell surface (Fig. 3) or incorporated into virions (Fig. 5). To help verify that the signal we observed was due to the binding of virions and determine what the contribution of free envelope might be to that signal, we fractionated an aliquot of the viral sample in a Centriprep-100 unit. The use of this ultrafiltration unit allows the separation of material with a molecular mass of less than 100 kDa from virions. We chose the Centriprep method because we have found it to be the least disruptive technique available, from the perspective of retaining both biological activity and physical integrity.

The effluent after filtration (material of <100 kDa) was used as a negative control and analyzed in the virus binding assay in parallel with the unfractionated viral sample. Examples of the

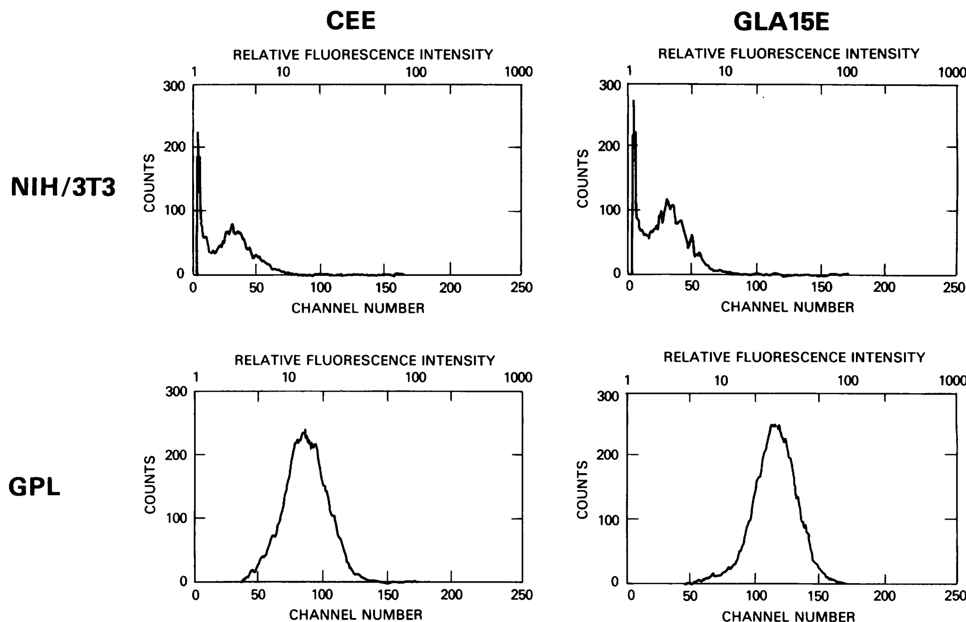

FIG. 4. Virus binding activity in the medium from 3T3 and GPL cells. The indirect immunofluorescence flow cytometry patterns for target cells following virus binding with virions containing the wild-type envelope encoded by pCEE or the GLA15E envelope are shown. Counts along the ordinate reflect the number of cells stained. Background staining was subtracted. The experiment was performed at least three times, and representative patterns are shown.

flow patterns obtained with viral samples and their Centriprep-100 effluent fractions are shown in Fig. 6 for the wild-type, 15EΔ, GLA70, and S70 envelopes. The results indicate that there is a minimal contribution from free envelope to the virus binding signal. The other envelopes were also analyzed in this manner, and the same results were obtained (data not shown). The results obtained with CEE in Fig. 6 reveal that a substantial amount of virus binding activity of <100 kDa molecular mass can pass through the Centriprep-100 filter membrane, thus indicating that aggregation of the envelope and retention in the unit are not responsible for the lack of virus binding in the S70 effluent fraction. While the S70 effluent fraction had no detectable binding activity, immunoprecipitation of the effluent and retained portions of the S70 fractionated supernatant showed that there were comparable amounts of S70 protein present in the two fractions (data not shown). This finding again indicates that envelope can pass through the Centriprep-100 filter membrane and corroborates the results in Fig. 4 and 6 that non-virion-associated envelope does not contribute substantially to the virus binding signal.

Several species of some of the envelope constructs were observed upon immunoprecipitation of cell lysates (Fig. 2). To determine which of these envelope proteins was incorporated into virions, the viral supernatant from metabolically labeled cells, after fractionation and concentration by ultrafiltration, was immunoprecipitated and analyzed by sodium dodecyl sulfate (SDS)-PAGE (Fig. 7). As expected, only the mature processed form of envelope (gp70) is detected in the wild-type envelope and CEET samples. The GLA15E and CEET1 samples each contain, in addition to gp70, a higher-molecular-mass species that corresponds to that observed in the cell lysates. The predominant form of the 15EΔ envelope found in virions corresponds to the higher-molecular-mass form which was the minor species observed in cell lysates. Both the GLA70 and S70 envelopes are much less abundant in virions than in cell lysates, with S70 barely detectable in virions above the background in this gel. Both appear to contain only a gp70 protein, though GLA70 may contain a higher-molecular-weight species as well that comigrates with a nonspecific band. As for 15EΔ, the lower-molecular-mass (<70-kDa) envelope product seen in the S70 and GLA70 cell lysates is not detectable in the corresponding virions.

Transduction capacity. To assess the capacity of the various envelope constructs to mediate post-receptor-binding events, the virions present in the GPL cell medium after transfection were tested for the ability to transfer the retrovirally encoded Neo^r gene present in GPL cells to a target cell. It was anticipated that envelopes which lack the putative fusion domain of TM (15EΔ, S70, and GLA70) would be defective in transduction. Since 15EΔ and GLA15E have converse TM structural deletions, lacking the extracellular TM domain or the membrane-spanning and cytoplasmic TM domains, respectively, we also tested whether these constructs would complement one another when cotransfected. CEET-bearing virions transduced cells at ~10% of the control level, demonstrating that the envelope cytoplasmic tail is not required for viral entry or subsequent steps. The failure of the other envelope constructs to mediate transduction attests to the necessary roles of the membrane-spanning and extracellular TM sequences in mediating post-receptor-binding events. The inability of 15EΔ and GLA15E to complement one another may reflect either a requirement for TM sequences to be present in *cis* or an inability of these envelopes to associate in an oligomeric structure. Evidence presented below suggests that the latter may be the case.

Membrane fusion assay. As the CEET, GLA15E, and CEET1 envelope constructs all retain the putative TM fusion domain, we expected that for these envelopes, defective transduction reflected a block in a step after membrane fusion. To directly examine membrane fusion, we used the XC indicator cell line (19, 22). XC cells fuse with cells that are infected with ecotropic MuLV, forming syncytia that can be quantitated to

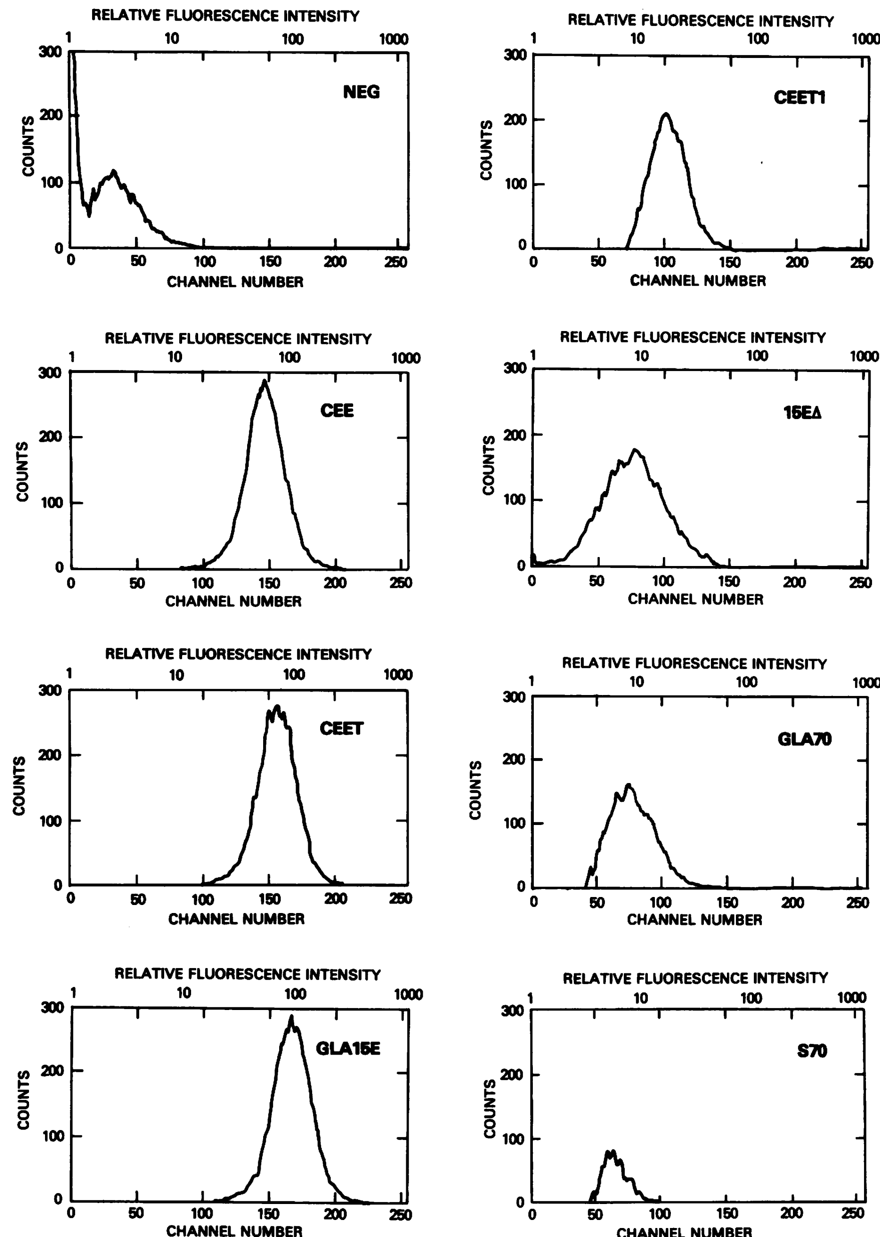

FIG. 5. Virus binding activity of the envelope constructs. Indirect immunofluorescence flow cytometry patterns for target cells following virus binding with virions containing the wild-type envelope encoded by pCEE or various envelope constructs are shown. Counts along the ordinate reflect the number of cells stained. Background staining was subtracted. All constructs were tested at least three times, and representative patterns are shown. NEG, negative control.

obtain titers of infectious virus (41). When 3T3 cells are transfected with a wild-type envelope vector and then cocultivated with XC cells, numerous syncytia are observed. When the same was done with the pCEET, pCEET1, and pGLA15E envelope vectors, pCEET syncytium formation was equivalent to that of wild-type envelope, but pGLA15E and pCEET1 did not produce syncytia above the background level (Fig. 8). These results indicate that sequences within the membrane-spanning domain of TM are necessary for membrane fusion.

Uncoupled expression of SU and TM proteins. Having found that each of the envelope constructs except CEET was incapable of mediating post-receptor-binding events, it was of interest to determine whether their TM defects could be complemented by the coexpression of a wild-type p15E. This question in turn poses another more fundamental question. Since the synthesis of the envelope proteins is coupled, with SU and TM being processed from a common precursor, can their synthesis be uncoupled and can the separate units subsequently associate into a functional complex?

Three different p15E expression vectors were constructed and tested. The 15E10 vector utilizes the rat growth hormone leader directly fused to the N terminus of p15E. As a consequence of the construction, the first 4 aa of the native p15E NH_2 terminus have been deleted and replaced with 3 aa (Table

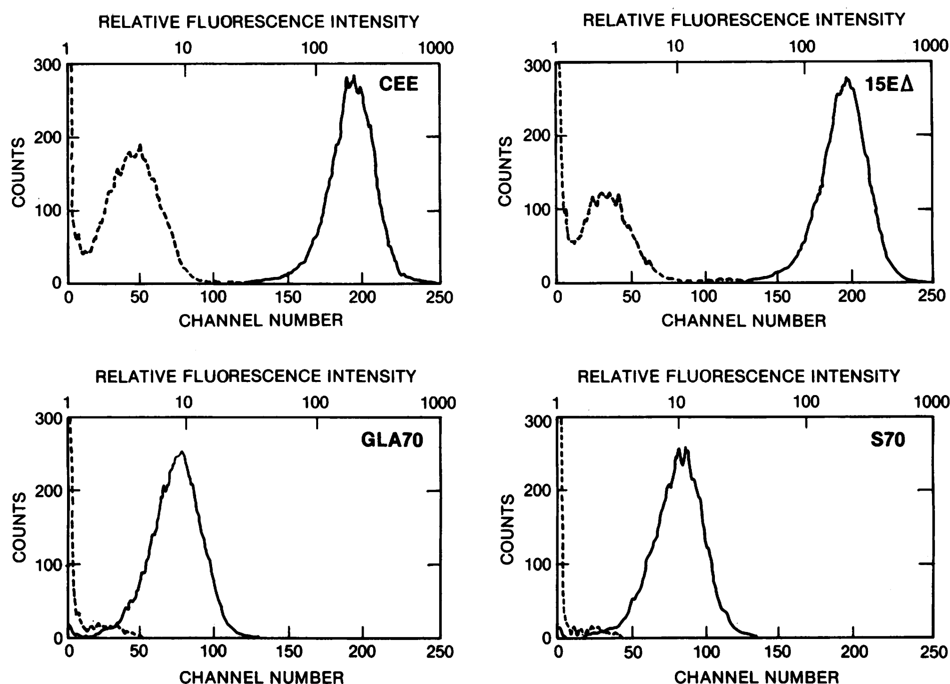

FIG. 6. Virus binding activity of Centriprep-100-fractionated viral supernatants. Indirect immunofluorescence flow cytometry patterns of target cells following binding with unfractionated viral supernatant (solid lines) or the effluent from fractionated supernatant (broken lines) are shown. CEE encodes the wild-type envelope. Counts along the ordinate reflect the number of cells stained. Results shown are from the same experiment.

1). For the 15EL and 15EP constructs, the native envelope gp80 leader is used to direct the transport of the protein. In these constructs, mature p15E is processed from a precursor that contains 15 aa derived from gp70 by cleavage at the gp70/p15E proteolytic processing site.

To determine what effects, if any, coexpression of the p15E vectors would have on the transport or virion incorporation of the envelope constructs, flow cytometry assays for surface expression and virus binding were performed. Cotransfection with a p15E vector resulted in a diminution of cell surface envelope expression and virus binding for all of the constructs except S70 and GLA70, which were unchanged. The virus binding obtained with GLA15E and GLA15E/15EP, which is representative of results obtained with the other constructs, is shown in Fig. 9.

Complementation of the TM defects in the envelope constructs by coexpression with a p15E expression vector was assessed by measuring transduction of the G418^r marker as described above (Table 2). As a negative control, a plasmid (pMAD) encoding p15E sequences but lacking both a promoter and a leader was used. Both the 15EL and 15EP vectors were found to rescue GLA15E but none of the other envelope constructs. The titer of the rescued GLA15E with 15EP was ~1% of the control vector titer. 15E10 did not rescue GLA15E or any of the other constructs.

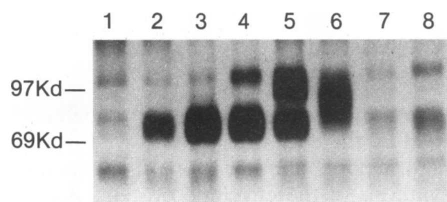

FIG. 7. SDS-PAGE of virion envelope proteins immunoprecipitated from the culture medium of metabolically labeled cells. GPL cells transfected with an envelope expression vector were labeled with [^{35}S]methionine. The culture medium was fractionated by centrifugation, and equal numbers of trichloroacetic acid-precipitable counts per minute from all samples were immunoprecipitated and run on the gel under reducing conditions. 1, negative control; 2, wild type; 3, CEET; 4, GLA15E; 5, CEET1; 6, 15EΔ; 7, S70; 8, GLA70.

DISCUSSION

It has been proposed for some enveloped viruses that receptor binding and membrane fusion are coupled events (30). Upon binding of the viral ligand to its cellular receptor, a conformational change is induced and transmitted to the envelope transmembrane protein (17). This exposes a fusion domain in the envelope protein, enabling the retroviral membrane to fuse to the cellular membrane (48). Our results necessitate at least some modification of this model.

The ability of an independently encoded wild-type p15E to complement the defect in GLA15E demonstrates that the expression of SU and TM can be uncoupled, with subsequent retention of biologic activity. Furthermore, it indicates that the envelope molecule that mediates the receptor-binding event (GLA15E) need not be the same molecule that mediates post-binding events. This uncoupling of receptor binding from membrane fusion leads us to propose that the conformational changes that occur upon receptor binding are capable of being transmitted within the oligomeric envelope complex via p15E-p15E interactions.

Of the three p15E expression vectors tested for the ability to complement the TM defects in our various envelope constructs, only 15E10 did not rescue GLA15E. Immunoprecipitation of ^{35}S-labeled cells indicated that 15E10 was synthesized (30a). Besides having a different leader sequence, 15E10 differs

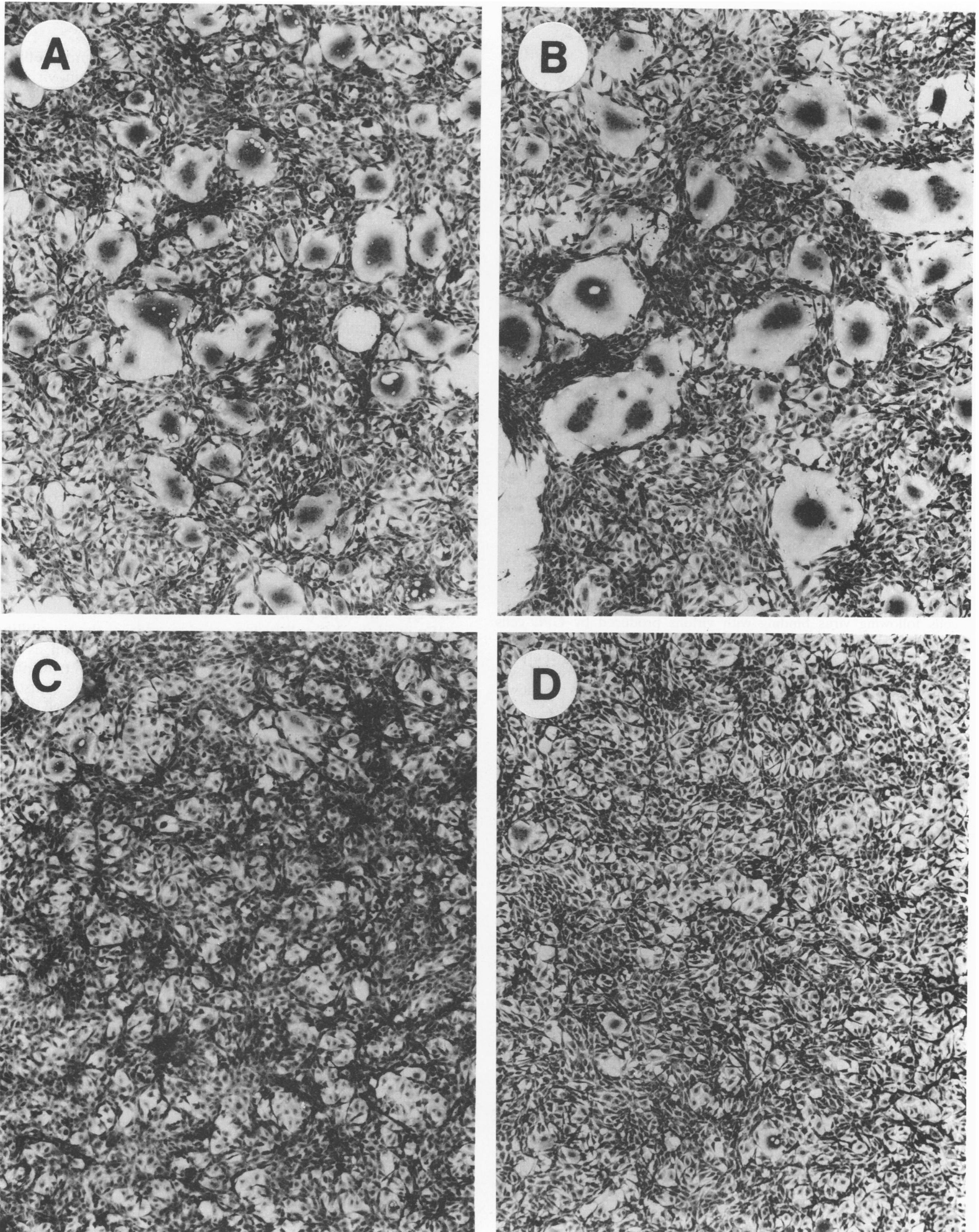

FIG. 8. Membrane fusion. A representative field demonstrating syncytium formation produced by cocultivation of envelope-expressing 3T3 cells with XC cells is shown. All photograph are at 50× objective magnification and from the same experiment. (A) Wild-type envelope; (B) CEET; (C) GLA15E; (D) CEET1.

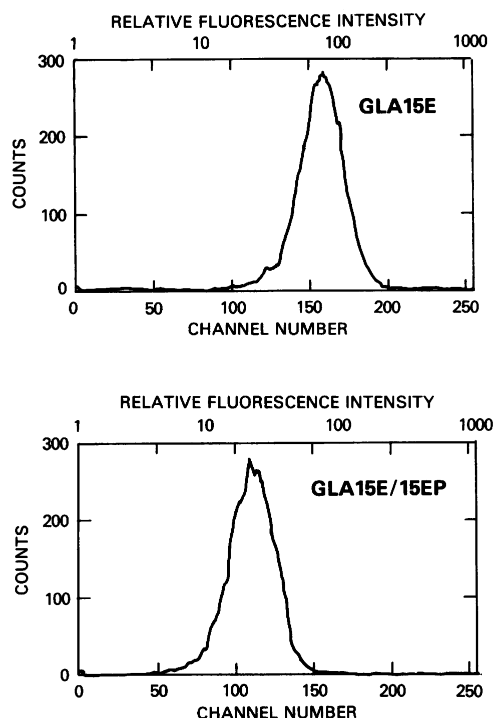

FIG. 9. Virus binding activity of coexpressed GLA15E and 15EP. The indirect immunofluorescence flow cytometry patterns for target cells following virus binding with virions produced by GPL cells transfected with GLA15E alone or cotransfected with 15EP (GLA15E/15EP) are shown. Counts along the ordinate reflect the number of cells stained. Background staining was subtracted. The experiment was performed at least three times, and representative patterns are shown.

from the other two p15E constructs in being mutated at the amino terminus of the encoded TM, which may be important for its proper function (19). Although it may be possible that complementation arose as a consequence of homologous recombination between our cotransfected vectors, the failure of both 15E10 and our promoterless p15E control plasmid to complement the envelope vectors essentially excludes homologous recombination as an explanation for our results.

The 15EL and 15EP expression vectors rescued GLA15E but not the other envelope constructs. In these experiments, complementation was achieved by cotransfection of vector DNAs, and the virions produced had a titer ~1% of the control value. As a cell must take up both plasmids for the virions to be rescued, the titer obtained by cotransfection of GLA15E and 15EP would tend to underestimate the efficiency with which the GLA15E envelope can associate with an independently expressed wild-type p15E.

TABLE 2. Titers of viral supernatants from GPL cells transiently coexpressing p15E and envelope construct

Envelope construct	Titer (G418[r] CFU/ml)			
	pMAD	15EP	15EL	15E10
CEE	2.3×10^4	ND[a]	ND	ND
GLA15E	0	3.0×10^2	8.3×10^1	0
15EΔ	0	0	0	0
GLA70	0	0	0	0

[a] ND, not determined.

The failure to rescue the other constructs may simply reflect the lower level to which they are incorporated in virions. However, there are also major structural differences between GLA15E and the other envelopes which might account for their inability to be rescued by p15E. One difference is that all of the others lack the TM extracellular domain, the implication being that p15E-p15E intermolecular interactions are a component of normal oligomeric envelope structure and occur through the extracellular domain of this protein. Such interactions have been proposed to occur in the Rous sarcoma virus (RSV) retroviral envelope (12). Another structural difference between GLA15E and the other constructs is that they have an altered gp70 carboxy terminus. In the case of GLA70 and 15EΔ, the carboxy terminus of gp70 is anchored to the cell surface and may thus be sterically hindered relative to its conformation in the native heterodimer. Such a constraint might also account for the inability of these envelopes to mediate transduction when coexpressed with p15E.

The inability of GLA15E alone to mediate transduction was not apparent a priori. The few MuLV envelope mutants which contain deletions within the membrane-spanning or cytoplasmic domains of p15E that have been reported in the literature are either not incorporated into virions, defective in receptor binding (as measured by superinfection resistance), or deficient in SU association (15). In contrast, GLA15E is incorporated into virions and binds receptor as well as the wild-type envelope. CEET, like RSV retroviral envelope mutants which lack the cytoplasmic domain of TM, was not defective in virion association but had a titer only 10% of that obtained with the wild-type envelope (34).

We used the XC cell fusion assay to address the possibility that these envelopes were defective in membrane fusion, as has been observed for primate retrovirus envelopes with truncated cytoplasmic tails (45). Cell fusion with CEET was indistinguishable from that with wild-type envelope. Presumably then, the reduced titers seen with CEET reflect some role the cytoplasmic domain (or R peptide) may have in association of core with the virion or in some step after membrane fusion (11, 39). Cell fusion could not be detected when GLA15E or CEET1 was expressed either alone or with wild-type p15E. However, since virions pseudotyped with GLA15E coexpressed with p15E can transduce cells, the XC assay may not be sensitive or specific enough to detect low levels of membrane fusion. Nonetheless, the results indicate that the membrane-spanning domain of TM is a determinant of membrane fusion.

By using flow cytometry to measure the cell surface expression of a series of envelope constructs altered in the TM subunit, we have demonstrated that the TM component of the envelope heterodimer is not required for the proper intracellular trafficking of the SU protein. In the case of the 15EΔ envelope, transport occurs in the absence of proteolytic processing of gp70 from p15E, as is the case for RSV and human immunodeficiency virus (HIV) (31, 35).

It should be noted that performing the analysis of cell surface-associated envelope with GPL cells may result in an enhanced immunofluorescence signal due to binding of released virus and/or envelope to the cell surface. This contribution, however, may be minimal or nonexistent as a result of internalization of the virus-receptor complex at 37°C (39) and/or down regulation of the receptor in cells now expressing the envelope protein. Data obtained with 3T3 cells show the same relative surface expression of envelope as that observed in GPL cells (data not shown).

It has been reported that Thy-1, a murine cell surface molecule that is naturally anchored through a GPI linkage, is

TABLE 3. Summary of envelope properties

Construct	Property[a]					
	Stably synthesized	Present on cell surface	Mediates fusion with XC cells	Present in virions and binds receptor	Mediates retroviral transduction	Transduction defect complemented by 15EP
Wild type	+	+	+	+	+	NA
CEET	+	+	+	+	+	ND
GLA15E	+	+	−	+	−	+
CEET1	+	+	−	+	−	−
15EΔ	+	+	−	+	−	−
GLA70	+	+	−	+	−	−
S70	+	+	−	+	−	−

[a] NA, not applicable; ND, not determined.

found in the envelope membrane of MuLVs (3). We show that substitution of a GLA moiety for most of the membrane-spanning domain permits association of an ecotropic envelope with the cell surface to the same extent as the wild-type envelope. The presence of this GLA moiety results in the GPI linkage of the GLA15E envelope to the cell surface and permits incorporation of the envelope into virions. Though such viral particles bind the viral receptor, they are incapable of entering cells, as they appear to be defective in fusion. Since the submission of this report, a number of reports describing several different GPI-anchored viral envelope constructs have appeared (14, 21, 28, 42, 47). Results with the GLA HIV envelope are remarkably similar to our own (42). This HIV envelope is also anchored to membranes through a GPI linkage and incorporated into virions, but the virions are defective in cell entry because the envelope does not mediate membrane fusion (42).

We have recently shown that the residual 8 aa of the membrane-spanning domain present in GLA15E are not needed for the association of GPI-anchored envelope with the cell surface or incorporation into virions (40). These same 8 aa, however, are required for anchoring of the CEET1 envelope to the cell surface (40). While 8 aa would represent an unusually short transmembrane anchor, it may be that the membrane-spanning domain in CEET1 is longer than what is predicted on the basis of the putative membrane-spanning domain in the wild-type envelope. There is an 8-aa hydrophobic sequence directly upstream from the carboxyl-terminal hydrophobic octapeptide in CEET1. A charged residue occurs between these hydrophobic stretches; however, there is precedence for this in the membrane-spanning domains of the HIV envelope and the glycophorin molecule. A detailed analysis of which sequences in TM actually constitute the membrane-spanning domain is under way.

In contrast to other unanchored envelopes, a low amount of the S70 envelope (which lacks both a TM and a GLA moiety) could be detected on the cell surface by flow cytometry (24, 34, 45, 46). Besides the high sensitivity and distinct signal obtained with flow cytometry, the virus binding results obtained with fractionated S70 viral samples lead us to believe that the S70 signal is due to membrane-anchored envelope and not the binding of free envelope to the viral receptor. Examination of the S70 sequences reveals a stretch of nearly 50 aa at the carboxy terminus with considerable hydrophobicity. We suspect that these sequences may represent a cryptic membrane-spanning domain that serves in S70 to anchor the envelope to the cell surface.

All of the envelope constructs were also incorporated into virions and mediated virus binding to target cells. It has previously been reported that only a fragment of the Friend leukemia virus SU is required for receptor binding as measured by superinfection resistance (16). A number of studies have shown that the requirements for conferring superinfection resistance are not synonymous with those for receptor binding (8, 15). In agreement with the findings of Heard and Danos, results presented here demonstrate directly that receptor binding by virions is not dependent on any portion of the TM subunit (16).

The envelope constructs studied here were not found in virions to an equal degree. Rather, the extent of incorporation into virions appears to parallel their level of cell surface expression. This in turn reflects their abundance in cell lysates, with those envelopes which lack the TM extracellular domain being the least abundant. These correlations suggest that the presence of TM sequences may influence the synthesis, stability, and/or transit of envelope through intracellular compartments (possibly by affecting oligomerization), as opposed to affecting envelope incorporation into virions per se (12). CEET1 may be the exception to this generalization, as the extent of envelope incorporation into virions appears to be disproportionately low relative to the level of cell surface expression. This observation suggests that the membrane-spanning domain of TM, while not required, may influence the incorporation of envelope into virions, as has been suggested for RSV (17, 34).

Immunoprecipitation of metabolically labeled cell lysates demonstrated that the various envelope constructs were present and migrated with the relative mobility expected on the basis of their amino acid composition. However, those envelopes which lack the TM extracellular domain had mobilities slightly greater than that which would be expected based on the apparent molecular mass of gp70, while those with a truncated membrane-spanning domain had mobilities slightly less than expected. These findings suggest that while p15E sequences are not themselves glycosylated, they may influence the glycosylation of gp70 sequences. Analysis of the envelope proteins present in virions demonstrated the presence of the mature gp70 in wild-type, CEET, GLA15E, and CEET1 virions. In addition, GLA15E and CEET1 virions appear to contain the precursor envelope, though this may represent residual free envelope present in the viral sample. The presence of a mature envelope protein with a molecular mass of 70 kDa was not as apparent in immunoprecipitates of S70 and GLA70 virions, though we conclude it is present on the basis of our collective results. Interestingly, the lower-molecular-mass form of these envelopes (present in cell lysates) is not present in virions. Thus, certain glycosylation events may be required for surface expression and/or incorporation into virions, though we cannot exclude the possibility that the lower-molecular-mass species are products of proteolysis. The observation that the higher-

molecular-mass form of the 15EΔ envelope appears to be selectively incorporated into virions may support the foregoing proposal.

Our results are summarized in Table 3. We have shown that the expression of the retroviral envelope SU and TM polypeptides can be uncoupled with retention of biologic activity. This finding has implications for a model of envelope receptor binding and membrane fusion. By constructing GPI-anchored retroviral envelope proteins and several deletion variants of the TM envelope subunit, we have begun to dissect the multiple roles of the envelope transmembrane protein in the viral life cycle. In the absence of TM, a GPI-anchored SU can be incorporated into virions and binds receptor. We found that the envelope cytoplasmic tail, while not required, influences the efficiency of retroviral transduction at some step after membrane fusion (possibly by interacting with core). The membrane-spanning domain of TM is involved in membrane fusion, and this function is distinct from its role as a membrane anchor. As few as 8 aa of the membrane-spanning domain are sufficient to achieve membrane anchoring of envelope but not to mediate membrane fusion. Finally, the extracellular domain of TM, besides containing the putative fusion domain and interacting with SU, may influence the synthesis, stability, and/or glycosylation of envelope, possibly by affecting oligomerization of the complex and proper intracellular transit.

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