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Relationship between SU Subdomains That Regulate the Receptor-Mediated Transition from the Native (Fusion-Inhibited) to the Fusion-Active Conformation of the Murine Leukemia Virus Glycoprotein

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Envelope glycoproteins (Env) of retroviruses are trimers of SU (surface) and TM (transmembrane) heterodimers and are expressed on virions in fusion-competent forms that are likely to be metastable. Activation of the viral receptor-binding domain (RBD) via its interaction with a cell surface receptor is thought to initiate a cascade of events that lead to refolding of the Env glycoprotein into its stable fusion-active conformation. While the fusion-active conformation of the TM subunit has been described in detail for several retroviruses, little is known about the fusion-competent structure of the retroviral glycoproteins or the molecular events that mediate the transition between the two conformations. By characterizing Env chimeras between the ecotropic and amphotropic murine leukemia virus (MLV) SUs as well as a set of point mutants, we show that alterations of the conformation of the SU glycoprotein strongly elevate Env fusogenicity by disrupting the stability of the Env complex. Compensatory mutations that restored both Env stability and fusion control were also identified, allowing definition of interactions within the Env complex that maintain the stability of the native Env complex. We show that, in the receptor-unbound form, structural interactions between the N terminus of the viral RBD (NTR domain), the proline-rich region (PRR), and the distal part of the C-terminal domain of the SU subunit maintain a conformation of the glycoprotein that is fusion inhibitory. Additionally, we identified mutations that disrupt this fusion-inhibitory conformation and allow fusion activation in the absence of viral receptors, provided that receptor-activated RBD fragments are added in *trans* during infection. Other mutations were identified that allow fusion activation in the absence of receptors for both the viral glycoprotein and the *trans*-acting RBD. Finally, we found mutations of the SU that bypass in *cis* the requirement for the NTR domain in fusion activation. All these different mutations call for a critical role of the PRR in mediating conformational changes of the Env glycoprotein during fusion activation. Our results suggest a model of MLV Env fusion activation in which unlocking of the fusion-inhibitory conformation is initiated by receptor binding of the viral RBD, which, upon disruption of the PRR, allows the NTR domain to promote further events in Env fusion activation. This involves a second type of interaction, in *cis* or in *trans*, between the receptor-activated RBD and a median segment of the freed C-terminal domain.

Retroviruses are enveloped viruses that penetrate host cells by a process of fusion between the viral and cell membranes (25). This process is catalyzed by a fusogenic activity expressed by the viral surface glycoproteins. The surface of retroviruses consists of an array of membrane-anchored glycoproteins that are expressed as trimers of two subunits, the SU (surface) and the TM (transmembrane) proteins, which are derived from a single protein precursor. The SU subunit harbors the determinants of interaction with the cell surface receptor, whereas the functions of the TM subunit include anchorage of the trimer complex in the viral membrane and achievement of membrane fusion.

The retroviral TM proteins have many similarities with the fusion subunits of other enveloped viruses, such as Ebola virus, paramyxoviruses, and orthomyxoviruses (29). The structure of the most stable conformations of these proteins has been determined and is thought to represent the fusion-active conformation of the proteins after their fusogenicity has been activated. The C-terminal region from each TM subunit packs into grooves on the outside of the triple-stranded coiled-coil, forming a six-helix bundle, with the fusion peptide and transmembrane domain of TM being positioned at the same end of the molecule. This folding therefore brings the viral membrane close to the cell membrane and hence initiates the merging of the lipid bilayers and the formation of fusion pores.

The transmembrane domains and cytoplasmic tails of several TM proteins harbor determinants that facilitate the fusion process. They may contribute to destabilization of the viral membrane and/or to enlarging the fusion pores (17, 38, 47). Additionally, at least for the *Gammaretroviridae* and *Betaretroviridae* families, cleavage of the cytoplasmic tail by the viral protease at a late step of virion assembly is mandatory to prime the fusogenicity of the glycoprotein, perhaps by inducing a modification in the ectodomain (10, 11, 50, 52).

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The fusion process involves activation of the viral fusion proteins and their subsequent refolding into fusion-active conformations (24). Two distinct pathways of fusion activation have been described. The fusogenicity of pH-dependent viruses, such as orthomyxoviruses, is activated by the acid pH found in the endosomal vesicles into which the virions are routed following receptor binding (57). In contrast, the fusion activation of pH-independent membrane-enveloped viruses, such as paramyxoviruses (31) and most retroviruses (37), is induced by the interaction of their glycoproteins with their receptors and is thought to occur at neutral pH.

The attachment subunits of the viral glycoproteins play an essential role in fusion activation, as they contain residues that can activate the fusion subunits (13, 33, 40). For orthomyxoviruses, ionization of residues that belong to both the attachment and fusion subunits is thought to initiate the structural rearrangements of the glycoprotein (13). In contrast, activation of the fusion proteins of paramyxoviruses and retroviruses involves interactions between the attachment and fusion subunits and is necessarily coupled to receptor binding. Consistently, in addition to the determinants that specify binding to the cell surface receptor, the attachment proteins from these viruses contain determinants that are involved in molecular dialogues with their fusion subunits.

For the *Gammaretroviridae*, such determinants have been found spread throughout the SU protein (3, 14, 32–34, 41, 62). How these determinants are mobilized upon receptor binding, interrelate, and ultimately activate the TM protein is poorly understood. For murine leukemia viruses (MLVs), previous studies have suggested that attachment of the receptor-binding domain (RBD) to the cell surface receptor modifies the conformation of the RBD. The receptor-activated RBD needs to interact with a conserved disulfide loop of the carboxy-terminal domain (C domain) of the SU to promote further events of fusion activation (4, 5, 32, 34). Such an interaction may occur in *cis*, via the proper RBD of the viral glycoprotein itself, or in *trans*, via a distinct RBD expressed by virion-free Env glycoproteins expressed endogenously by the infected cells or provided by neighboring Env trimers (32). This pathway is critically dependent on a fusion activation determinant located at the amino terminus of the RBD and harboring a conserved peptide motif (SPHQV) centering on a histidine residue. Deletion or nonconservative substitution of that histidine has no effect on receptor recognition or virus binding but abolishes fusion triggering in both cell-cell and virus-cell fusion assays (3, 4, 32, 34, 62).

Recent results have indicated that the native MLV SU holds a fusion-inhibitory conformation (4, 33) and that the fusion-activating interaction between the receptor-activated RBD and the C domain may occur only if the native structure of viral SU has been modified upon receptor binding (4). This initial conformation of the Env complex seems to be stabilized by interactions between subdomains of the SU (33, 42) and between the SU and the TM (46). Other results have shown that the proline-rich region (PRR), which connects the RBD and the C domain, is essential in mediating the stability of the viral glycoprotein. Mutations of the proximal region of the PRR, which is conserved within the SUs of the *Gammaretroviridae*, dramatically reduce the stability of the Env complex (22, 33, 61). Additionally, we recently reported that, compared to the wild-type glycoprotein, such PRR-mutated amphotropic glycoproteins exhibit strongly increased fusogenicity, as they induce the formation of syncytia in cell culture (33). Furthermore, they seem to require fewer viral receptors than the parental glycoprotein to trigger membrane fusion (33).

In this study, we sought to further investigate the relationship between the stability of the Env complex and the control of fusogenicity. We analyzed the contribution of individual segments of the MLV SU glycoprotein that modulate fusion activation and/or conformation of the Env complex. We defined some relationships between the determinants that maintain the native MLV Env complex in a fusion-inhibitory conformation, on the one hand, and those that are required to promote fusion activation, on the other.

MATERIALS AND METHODS

Cell lines. The TELCeB6 cell line (16) was derived from the TELac2 line after transfection and clonal selection of a Moloney MLV (MoMLV)-based expression plasmid to produce Gag and Pol proteins. The TELac2 cells were originally derived from TE671 human rhabdomyosarcoma cells (ATCC CRL8805) to express the nlsLacZ reporter retroviral vector (59). Production of infectious retroviral particles by TELCeB6 cells depends on newly introduced envelope expression vectors. CRAV (CHO-hPiT2) (54), Cerd9 (CHO-mCAT1), and Cear13 cells (CHO-rPiT2/mCAT1) (30) are derived from Chinese hamster ovary (CHO) cells (ATCC CCL-61) and express either MLV receptors alone or both ecotropic and amphotropic MLV receptors. XC rat sarcoma cells (ATCC CCL-165) express both ecotropic and amphotropic MLV receptors.

Construction of envelope expression vectors. Plasmid FBASALF, encoding the wild-type MLV-4070A (where the A stands for amphotropic) Env and carrying a phleomycin resistance gene, has been described elsewhere (16). The FBAdelHSALF plasmid (34), derived from FBASALF, was designed to produce a cell entry-defective form of the amphotropic Env by deleting the 36th codon of the 4070A *env* gene (43). The resulting mutant envelope glycoprotein, in which the fifth residue of the SU Env subunit, a histidine, was removed, was named AdelH (34). The FBMOdelHSALF expression plasmid, encoding the fusion-defective MOdelH envelope glycoproteins (34) and harboring the equivalent *del*H mutation, obtained by deleting the eighth residue of the SU, corresponding to the 41st codon of the MoMLV *env* gene (56), was derived from FBMOSALF (16), which encodes the MoMLV ecotropic Env (noted as MO).

The FBASALF, FBAdelHSALF, FBMOSALF, and FBMOdelHSALF plasmids were used as backbones for the construction and expression of envelope mutants.

Expression vectors encoding Env chimeras in which polypeptides corresponding to the proline-rich region (PRO or PRR), the SU carboxy-terminal domain (C domain), or the TM subunit ectodomain (denoted TM) derived from the MoMLV Env were substituted individually (see Fig. 1) or in combinations (see Fig. 2) for the matching domains of the 4070A Env were described previously (32, 33). The resulting Envs were identified according to the substituted ecotropic domain(s) (see Fig. 1 and 2).

For amphotropic and ecotropic Envs, respectively, the boundaries of the various domains were defined as A32-V237 and A34-L262 for RBD; G238 to P297 and G263 to A308 for PRO; G298 to R458 and G309 to R469 for C; and E459 to P654 and E470 to P665 for TM. Residues are numbered starting from the initiation methionine deduced from the amino acid sequences of the 4070A MLV (43) and the MoMLV (56) Envs. The expression vector encoding the A2 and PRR-C2 amphotropic Env mutants, in which P245I and N246Q substitutions were introduced and the 12-amino-acid-long peptide of the PRR was deleted (S284 to P295), respectively, were described previously (33).

All subsequent constructs were generated by PCR-mediated and site-directed oligonucleotide mutagenesis (details and sequences available upon request) and cloned in the FBASALF or FBAdelHSALF Env expression vector. Expression vectors for the C1MO, C2MO, and C3MO and for the C1MO*del*H, C2MO*del*H, and C3MO*del*H amphotropic Env chimeras (32) were derived from the FBASALF and FBAdelHSALF plasmids, respectively, by replacing DNA sequences encoding subregions of the amphotropic Env C domain, defined as G298 to N354, C355 to C409, and S410 to R458 in the 4070A MLV Env sequence, with the homologous subregions of the MoMLV Env, defined as G309 to N365, C366

to C420, and S421 to R469, respectively. For the BDPROMO expression plasmid, the fusion-defective envelope glycoproteins harboring the equivalent H mutation (BDPROMO*del*H) was derived from FBMOdelHSALF.

Plasmids encoding secreted RBDs were derived from the FBASALF and FBMOSALF expression vectors. The carboxy-terminal ends of amphotropic (A-RBD) and ecotropic (E-RBD) RBDs, defined as A32 to G244 and A34 to G269, respectively, were fused in frame to a 9-amino-acid-long RGS-H_6 tag (RGSHHHHHH) (27). Expression vectors encoding either A-RBD*del*H or E-RBD*del*H were generated similarly with the FBAdelHSALF and FBMOdelHSALF plasmids.

Cell-cell fusion assays. TELac2 effector cells were seeded at 5×10^5 cells/well in a six-well plate and transfected with Env-expressing plasmid by calcium phosphate precipitation, as previously described (16). After 24 h, the cells were detached, counted, and reseeded at the 10^5 cells/well in six-well plates. Fresh XC or CHO-PiT2 indicator cells (10^6 cells/well) were added to the transfected cells and cocultivated for 24 to 36 h. The coculture was fixed with 0.5% glutaraldehyde, and the nuclei of the effector cells were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). The cytoplasm and nuclei of all the cells were counterstained by adding May-Grunwald and Giemsa solutions (Merck) according to the manufacturer's recommendations.

Production of retroviral particles. Env expression plasmids were transfected into TELCeB6 cells as reported elsewhere (16). Transfected cells were selected with phleomycin, and phleomycin-resistant colonies were pooled. Virus-containing supernatants were collected after overnight production from confluent Env-transfected cells, filtered through 0.45-μm-pore-size membranes, and stored at 4°C.

Production of soluble RBD fragments. RBD expression vectors were transfected in TE671 cells as reported elsewhere (34). Transfected cells were selected with phleomycin, and phleomycin-resistant colonies were pooled. Expression of RBDs in producer cell supernatants was analyzed by immunoblotting with anti-RGS-H_6 tag antibodies. RBD-containing supernatants were collected after 48 h of production from confluent RBD-transfected cells, filtered through 0.45-μm-pore-size membranes, and stored at 4°C.

Infection assays. Target cells were seeded in 24-well plates at a density of 5×10^4 cells per well and incubated overnight at 37°C. For regular infection without soluble RBD, 400 μl of diluted virus samples containing 4 μg of Polybrene per ml was added to the cells for 4 h at 37°C. For infection in the presence of soluble RBD fragments, target cells were first incubated for 30 min at 37°C with 200 μl of RBD-containing supernatants complemented with 200 μl of fresh regular medium and 5 μg of Polybrene per ml. Then, 100 μl of diluted viruses was added for 4 h at 37°C. After the viral supernatants had been removed, the cells were incubated in regular medium for 48 h at 37°C. X-Gal staining and virus titer determination were performed as previously described and expressed as LacZ infectious units per milliliter of viral supernatant (16).

Immunoblots and antibodies. Cell lysates and virus samples from Env-transfected TELCeB6 cells were prepared and analyzed in Western blots as previously described (33). Virus-containing medium (6 ml) was centrifuged at 30,000 rpm in a Beckman SW-41 rotor for 70 min at 4°C through a 2-ml 20% sucrose cushion. Viral pellets were resuspended in 100 μl of phosphate-buffered saline (PBS). Samples (20 μl) were mixed with 4 μl of loading buffer containing 375 mM Tris-HCl (pH 6.8), 3% sodium dodecyl sulfate (SDS), 30% β-mercaptoethanol, 10% glycerol, and 0.06% bromophenol blue and then analyzed by electrophoresis in 10% polyacrylamide gels in the presence of 0.1% SDS. Samples for Western blot analysis in nonreducing conditions were prepared similarly except that β-mercaptoethanol was omitted from the loading buffer. Proteins were then electrotransferred to nitrocellulose filters and used for immunoblotting.

The blots were blocked in Tris-buffered saline (TBS; pH 7.4) supplemented with 5% milk powder and 0.1% Tween 20 (Sigma). The blots were cut horizontally in two parts at the carbonic anhydrase level (40 kDa) of the Kaleidoscope prestained standards (Bio-Rad, Hercules, Calif.). The part of the blot containing high-molecular-weight proteins was probed with anti-gp70 antibodies (ViroMed Biosafety Laboratories), derived from a goat antiserum produced against the Rauscher leukemia virus gp70, diluted at 1:2,000, and incubated for 1 h. The part of the blot containing proteins of less than 45 kDa was probed with anti-CA antibodies (ViroMed) from a goat antiserum raised against the Rauscher leukemia virus p30 capsid protein (CA), diluted at 1:10,000, and incubated for 1 h. After two washes in TBS, the blots were incubated with horseradish peroxidase-conjugated immunoglobulins raised against goat antibodies. Immunoblots were revealed with an enhanced chemiluminescence kit (SuperSignal West Pico; Pierce).

RESULTS

Loss of Env complex stability correlates with increased fusogenicity. We sought to introduce modifications in the MLV Env complex in order to alter its conformation. Previously described mutations of the MLV SU that destabilize the Env complex have been found in the proline-rich region (PRR) situated in the middle part of the SU (22, 33, 61). Modifications of the PRR of the amphotropic glycoprotein, achieved via introduction of point mutations at its amino terminus (e.g., the A2 mutant, P245I/N246Q [Fig. 1A]) or via its replacement with that of heterologous MLV Envs (e.g., the PROMO mutant [Fig. 1A]), resulted in strongly enhanced fusogenicity and in loss of stability of the Env complex (Fig. 1B and C) (33). The phenotype of these PRR-mutated Envs has been previously described in detail (33).Increased fusogenicity was characterized by the formation of syncytia in up to 25% of the cells, compared to 0.34% with syncytia obtained with the wild-type amphotropic glycoprotein (Fig. 1B). Strong cell-cell fusion occurred in spite of the lack of cleavage of p2R from the cytoplasmic tail (Fig. 1A and C) and the absence of modifications in cell surface expression and receptor-binding properties of the Env chimeras (33) and also occurred when the number of available binding sites on the surface of target cells was reduced, as suggested by the results of receptor interference assays (33). Syncytium formation was observed with several indicator cell types, including XC, CHO-PiT2 (Fig. 1B), and SC-1 cells (data not shown), yet the most dramatic effect was observed with XC rat sarcoma cells (Fig. 1B). Instability was characterized by increased dissociation of the SU from the Env complex and by its consequent accumulation as soluble SU in the cell supernatant (33). In our previous study, we demonstrated that this was due to loosened SU-TM association, as shown by characterization of the Env complexes in virus producer cells (33) and in purified viral particles (Fig. 1C). This led to strongly reduced viral infectivity, most likely because of the very low SU density on virions (Fig. 1B and C), as already discussed (33).

Further modifications of the amphotropic Env conformation were attempted by replacing SU domains located downstream of the RBD with their allelic counterparts derived from a heterologous MLV glycoprotein. These Env chimeras were investigated for Env complex stability and fusion properties (Fig. 1). Among a series of mutants named C1MO, C2MO, and C3MO, in which the first, second, and third parts of the amphotropic SU C domain were replaced, respectively, only the C1MO chimera was found to be highly cell-cell fusogenic. Like the PROMO mutant, the C1MO chimera appeared to be very unstable and showed dramatically reduced viral infectivity as a result of its very low density on viral particles (Fig. 1). In contrast, similar to the wild-type amphotropic glycoproteins, the C2MO and C3MO Env mutants appeared to be stable and exhibited normal SU density of their Env complexes on the virion surface (Fig. 1C). Virions carrying either the C2MO or C3MO mutant glycoprotein were infectious, yielding titers nearly as high as those obtained with wild-type amphotropic glycoproteins (Fig. 1B).

In summary, in the series of mutant Envs characterized here (Fig. 1) as well as in other mutants described elsewhere (A3 and PROFR [33]), those that exhibited high SU instability had

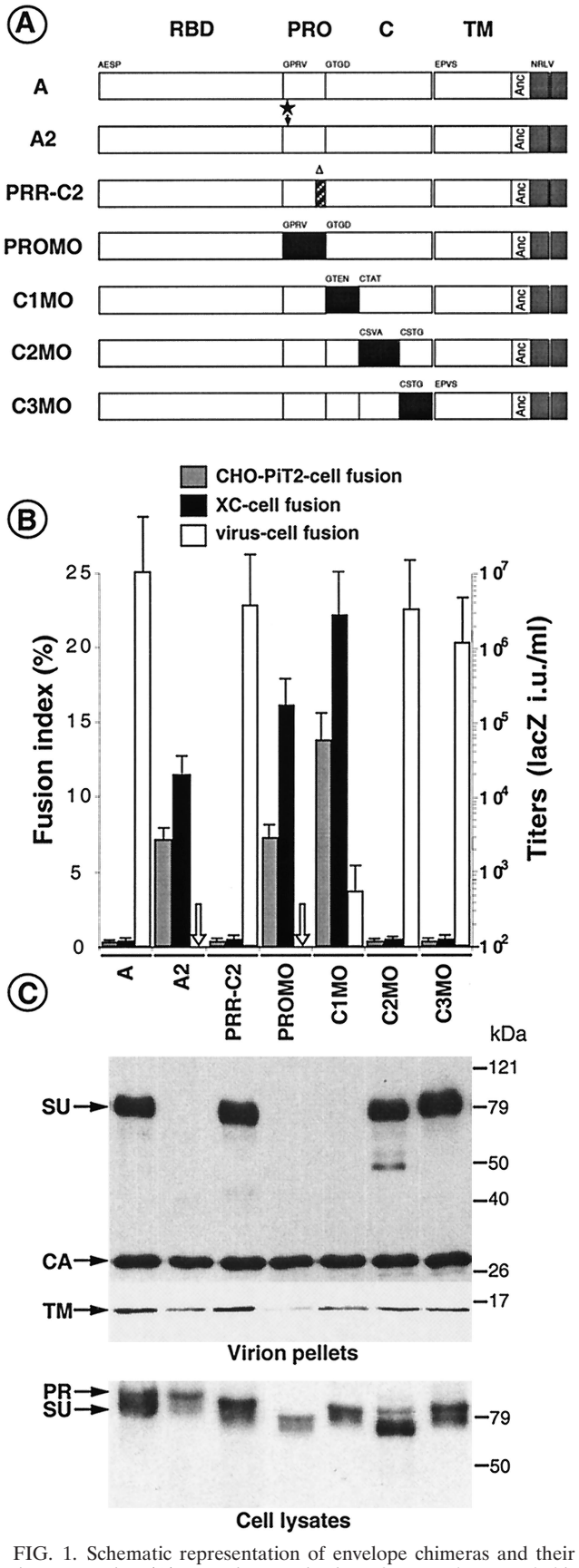

FIG. 1. Schematic representation of envelope chimeras and their fusion properties. (A) Domain organization of parental Env and chimeras. Open and solid boxes represent domains derived from amphotropic 4070A MLV Env (denoted A) and ecotropic MoMLV Env, respectively. The cytoplasmic sequences are shown as grey boxes and consist of the cytoplasmic tails and the p2R peptides. PRO (or PRR), proline-rich region; C, SU carboxy-terminal domain; TM, transmembrane subunit; Anc, anchor domain. The first amino acids of each domain are indicated. The star marks the location of the P245I/N246Q mutation introduced in the proline-rich region of the A2 mutant. The hatched box shows the 12-amino-acid-long deletion introduced in the carboxy-terminal end of the proline-rich region of the PRR-C2 mutant. (B) Results of cell-cell and virus-cell fusion assays. Cell-cell fusion activity was determined after transfection of the envelope expression vectors into TELac2 cells and cocultivation for 24 h with CHO-PiT2 (grey bars) or XC (black bars) indicator cells. The fusion index is defined as $(N - S)/T \times 100$, where N is the number of nuclei in the syncytia, S is the number of syncytia, and T is the total number of nuclei counted. Infectivity was tested with supernatants harvested from stably transfected packaging cells on different target cells (XC, Cear13, NIH 3T3, and TE671 cells) and is expressed as the number of LacZ infectious units per milliliter of viral supernatant. The values show the means ± standard deviations of up to six independent experiments performed on XC target cells (open bars). Identical results were obtained on the other target cells tested. (C) Detection of envelope glycoproteins in pellets of retroviruses generated with the indicated Envs by immunoblotting in reducing and denaturing conditions with anti-SU and anti-TM antisera. Equivalent loading of viral samples was demonstrated by immunoblotting with an anti-capsid (CA) antiserum. The positions of the molecular size markers are shown. Expression of Env glycoproteins in producer cells is shown in the bottom blot by immunoblotting of cell lysates of Env-transfected cells with anti-SU antibodies.

increased cell-cell fusogenicity and vice versa. This pointed to a negative control of fusogenicity in which the native (stable) conformation of the Env complex may inhibit fusion. This native conformation is likely to be stabilized by interactions between subdomains of the SU. A corollary of this notion is that mutations that interfere with the stability of the Env complex may increase its fusogenicity as a result of disruption of interactions between subdomains of the Env complex that are essential to control or refrain fusion activation. Furthermore, these results revealed the presence of Env regions that may directly interact with or at least control interactions between other Env domains to stabilize the Env complex. Thus, the cell-cell fusogenic PROMO chimeric glycoprotein may have become unstable because, as a result of its insertion in an heterologous Env background, its MoMLV-derived PRR may be unable to interact with or to accommodate interactions between other Env domains of amphotropic MLV origins despite the high degree of homology between the two glycoproteins (over 80% identical residues in the SU carboxy-terminal domain).

Thus, as a second corollary to the idea that Env complex conformation controls fusion activation, these assumptions implied that insertion of additional MoMLV Env segments in the PROMO chimera should reconstitute intersubdomain interactions, restore stability of the Env complex, and hence diminish cell-cell fusion. Indeed, insertion of the MoMLV RBD in the PROMO Env (BDPROMO chimera, Fig. 2A) was sufficient to restore Env stability and virus infectivity (33). Similarly, association of the MoMLV SU carboxy-terminal domain with the MoMLV PRR, as in the PROCMO chimera (33), resulted in a stable glycoprotein that had recovered virus-cell fusion and lost cell-cell fusogenicity (Fig. 2B and C). In contrast, associ-

ation of the MoMLV TM ectodomain with the PROMO mutant (PROTMMO chimera; Fig. 2A) was not sufficient to efficiently restore stability of the Env complex.

These data therefore demonstrated the critical role of interactions between subdomains of the SU in mediating Env complex stability and control of fusogenicity. They also indicated that the PRR, in association with the RBD and/or the C domain, is a key partner in the stability of the Env complex. Aiming to further define the relationship between Env structure stability and fusogenicity for the SU C-terminal chimeras, we then sought to delineate the subregions of the C domain that, in association with their homologous PRR, would restore the interactions necessary to control both Env stability and fusogenicity. The C domain was therefore divided into three regions, named C1, C2, and C3. In the context of the PROMO Env background, each of these subdomains, derived from MLV-A, was individually replaced with its counterparts derived from MoMLV (Fig. 2A). Expression of all of these Env chimeras was readily detected by Western blot analysis of lysates of transfected cells (Fig. 2C).

Each of these chimeric amphotropic Env glycoproteins was then analyzed for cell-cell fusion, virus-cell fusion, and Env complex stability (Fig. 2B and C). Insertion of the MoMLV C1 subdomain into the PROMO chimera did not stabilize the Env complex, and the resulting chimera, PROC1MO, was highly cell-cell fusogenic. Retroviruses generated with this chimeric Env were not infectious, most probably owing to the great instability of the PROC1MO glycoprotein on virions (Fig. 2C). In contrast, insertion of the C3 MoMLV subdomain into the PROMO Env backbone (PROC3MO chimera) stabilized the Env complex and resulted in high virus infectivity and loss of cell-cell fusion. Insertion of the MoMLV C2 subdomain resulted in an intermediate phenotype, increased stability in comparison to the PROMO and PROC1MO Env chimeras but less stability than with the PROCMO and PROC3MO Env chimeras. Interestingly, this Env chimera, named PROC2MO, exhibited both cell-cell fusion and infectivity, most likely owing to the partial stability of this envelope glycoprotein (Fig. 2C). Thus, both the C2 and C3 subdomains contained determinants that could confer stability on the Env complex when associated with the homologous PRR.

Altogether, these data established the correlation between cell-cell fusion and Env complex stability. Furthermore, they confirmed the idea that the PRR is a key component of the native Env conformation. Since the RBD and the C domain of the MLV SU are believed to interact before and/or during fusion activation (4, 5, 32, 42, 44), we propose that the PRR regulates their interaction. Disruption of this interaction through alteration of the PRR itself or, alternatively, through modifications of regions of the C domain resulted in Env mutants that were partially or totally unstable and which, consequently, exhibited deregulated fusogenicity. In extreme cases, such as the PROMO and C1MO chimeras, this resulted in highly cell-cell fusogenic glycoproteins. Yet one may predict that other Env chimeras that had apparent Env complex stability, such as the PRR-C2 and PROC3MO glycoproteins, may exhibit more subtle or alternative fusion activation phenotypes. The PRR-mutated Env chimeras will be referred to hereafter as the stabilized PRR-mutated glycoproteins.

Relationships between PRR and NTR region are critical for Env stability and fusogenicity. Several reports have established that the N terminus of the RBD (hereafter named the NTR domain) from the SUs of *Gammaretroviridae* contains a critical fusion activation determinant. Indeed, disruption of the NTR domain either through deletions (e.g., H5*del* or *del*H mutation in amphotropic MLV SU) or through nonconservative substitutions (e.g., H5K mutation in amphotropic MLV SU) of a critical amino-terminal histidine that resides in a conserved SPHQV motif fully abolishes fusion activation (3, 4, 32, 34, 62). Providing RBD polypeptides in the cell culture medium efficiently reverts the fusion-defective phenotype of the mutated viral glycoproteins and allows membrane fusion (4, 32, 34). For wild-type Env glycoproteins, such a rescue in *trans* is dependent on the presence of receptors for the soluble RBD and the viral glycoprotein as well as on the integrity of the NTR domain of the rescuing RBD (4, 34). This indicates that the NTR domain is mobilized following RBD binding to the receptor and triggers further conformational changes of the receptor-bound viral Env complex, in *cis* or in *trans*.

Interestingly, the tridimensional structure of the Friend MLV RBD suggests that the NTR region may interact with the amino terminus of the PRR (19). Moreover, for the MoMLV Env, mutations just before or at the beginning of the PRR can compensate in *cis* for the lethal mutation of the critical histidine of the NTR domain (62), suggesting that the relationship between the two determinants may control fusion activation of the retroviral glycoprotein. We therefore sought to investigate the effects of the association of mutations in the amphotropic SU that individually modulate Env fusogenicity. Thus, the *del*H mutation, which disrupts functions of the NTR domain, was introduced in the MLV Env chimeras with altered fusion properties and/or Env complex stability, which were described in Fig. 1 and 2.

The effect of the *del*H mutation in conformation of the viral Env complex was determined by detecting variations in the proportions of the different forms of SU found in viral particles, as detected in nonreducing and nondenaturing Western blot analyses. Under these conditions, the monomeric form of the SU, the disulfide-linked TM-associated SU form, and the oligomeric (trimeric) SU form (hereafter named mSU, SU-TM, and tSU, respectively) could be readily detected (Fig. 3A). The influence of the *del*H mutation was particularly marked for the PRR-mutated Env chimeras that displayed intense instability. Compared to the parental A2 and PROMO unstable Env chimeras, the A2*del*H and PROMO*del*H mutants had strongly increased Env stability, as judged from the appearance of SU on the viral particles (Fig. 3A). In contrast, the C1MO*del*H chimera was found to be as unstable as its parental C1MO glycoprotein (Fig. 3A). These data therefore indicated that specific relationships between the NTR domain and the PRR modulate Env complex stability. Moreover, stabilization induced by disruption of the NTR region was sufficient to reduce excessive cell-cell fusion (data not shown), further supporting the hypothesis that Env complex stability prevents fusion activation.

Additional evidence for an NTR/PRR structural relationship was provided by examination of the conformation of the other Env chimeras. While disruption of the NTR had no effect on the conformation of the wild-type Envs or of the C2MO and C3MO chimeras, introduction of the *del*H mutation in the

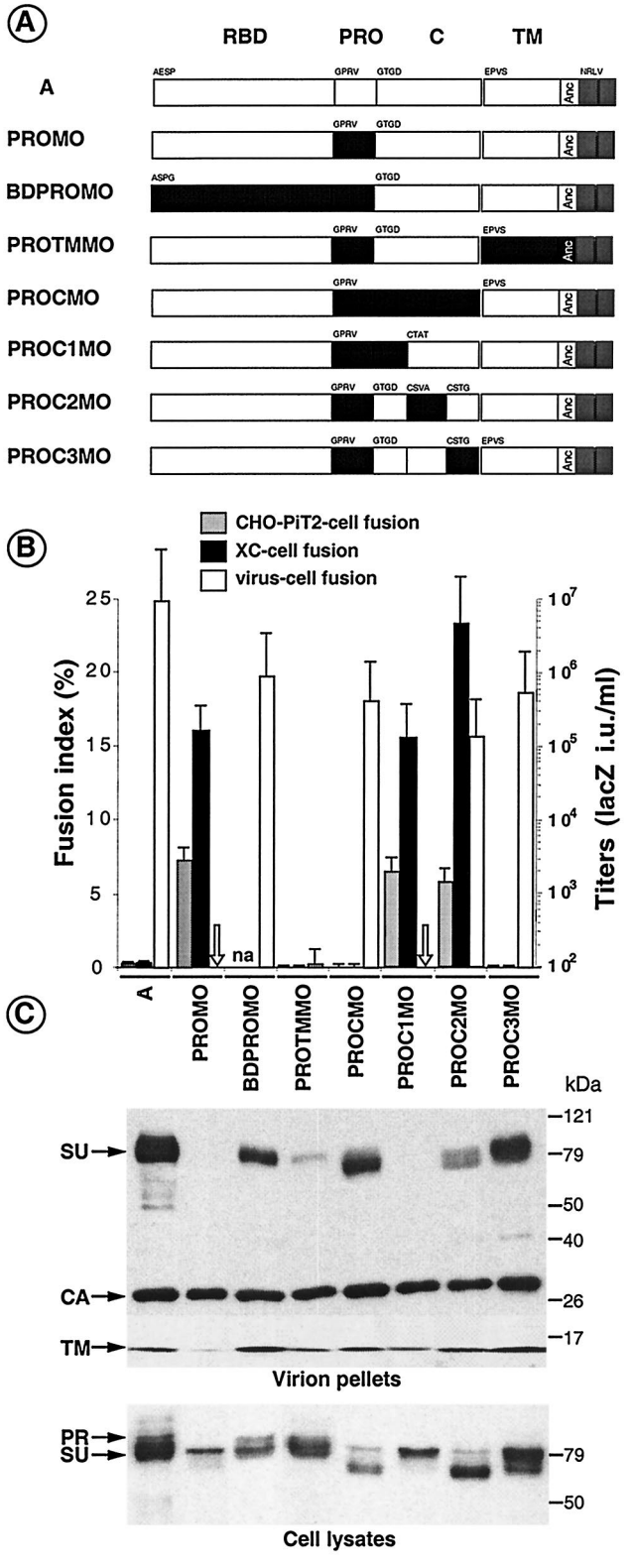

FIG. 2. Schematic representation of envelope chimeras in which multiple Env domains were swapped. (A) Domain organization of parental Envs and chimeras. Open and solid boxes represent domains derived from amphotropic 4070A MLV Env (denoted A) and ecotropic MoMLV Env, respectively. The cytoplasmic sequences are shown as grey boxes and consist of the cytoplasmic tails and the p2R peptides. PRO (or PRR), proline-rich region; C, SU carboxy-terminal domain; TM, transmembrane subunit; Anc, anchor domain. The first amino acids of each domain are indicated. (B) Results of cell-cell and virus-cell fusion assays. Cell-cell fusion activity was determined after transfection of the envelope expression vectors in TELac2 cells and cocultivation for 24 h with CHO-PiT2 (grey bars) or XC (black bars) indicator cells. The fusion index is defined in the legend to Fig. 1. Infectivity was tested with supernatants harvested from stably transfected packaging cells on different target cells (XC, Cear13, NIH 3T3, and TE671 cells) and is expressed as the number of LacZ infectious units per milliliter of viral supernatant. The values show the means ± standard deviations of up to six independent experiments performed on XC target cells (open bars). Identical results were obtained on the other target cells tested. As discussed previously (33, 51), the wild-type MoMLV glycoprotein and the BDPROMO chimera exhibited high cell-cell fusion (data not shown), associated with recognition of the ecotropic receptor, despite the stability of their Env complexes. na, not applicable. (C) Detection of envelope glycoproteins in pellets of retroviruses generated with the indicated Envs by immunoblotting in reducing and denaturing conditions with anti-SU and anti-TM antisera. Equivalent loading of viral samples was demonstrated by immunoblotting with an anti-capsid (CA) antiserum. The positions of the molecular size markers are shown. Expression of Env glycoproteins in producer cells is shown in the bottom blot by immunoblotting of cell lysates of Env-transfected cells with anti-SU antibodies.

PRR-mutated stable or stabilized glycoproteins resulted in alteration of the ratios between SU-TM and mSU and between tSU and mSU. This resulted in significantly increased expression of both SU-TM and tSU. This effect was readily visible for the PRR-C2*del*H, BDPROMO*del*H, and PROC3MO*del*H chimeras and was particularly manifest for the PROMO*del*H chimera, which expressed the monomeric SU and the SU-TM forms at similar levels (Fig. 3A).

Altogether, these data showed that alterations of the NTR domain finely influenced the Env complex conformation of PRR-mutated chimeras and indicated that mutation of the critical amino-terminal histidine prevented the destabilization induced by modifications of the PRR. Thus, in contrast to the PRR, the NTR domain could be negatively involved in stability of the Env complex, since its mutation increased stability. This suggested the possibility that the NTR domain cooperates with the PRR in modulating the stability and conformation of the Env complex. Assuming that the PRR and NTR domains interact and are, respectively, positively and negatively involved in Env stability, it is expected that mutation of either domain will have a more marked effect when the structure of the glycoprotein has been modified by a mutation in the other domain. Thus, considering the possibility that Env stability negatively controls fusion, as discussed above, one possible function of the NTR domain could be that, following its mobilization upon receptor binding, it contributes to destabilization of the Env complex.

Virus-cell fusion was then assayed with retroviral vectors carrying the stable Env chimeras. The A2, PROMO, C1MO, C1MO*del*H, PROC1MO, and PROC1MO*del*H chimeric glycoproteins were indeed discarded from these assays, as no valid conclusions would be made because of their high instability and low density on virions (Fig. 1 to 3). In contrast to wild-type envelope glycoproteins of *Gammaretroviridae* (e.g., MLV-A, MLV-Xeno, MoMLV, Friend MLV, feline leukemia virus B, and gibbon ape leukemia virus [GALV]), for which the *del*H mutation invariably abolished virus-cell fusion by up to

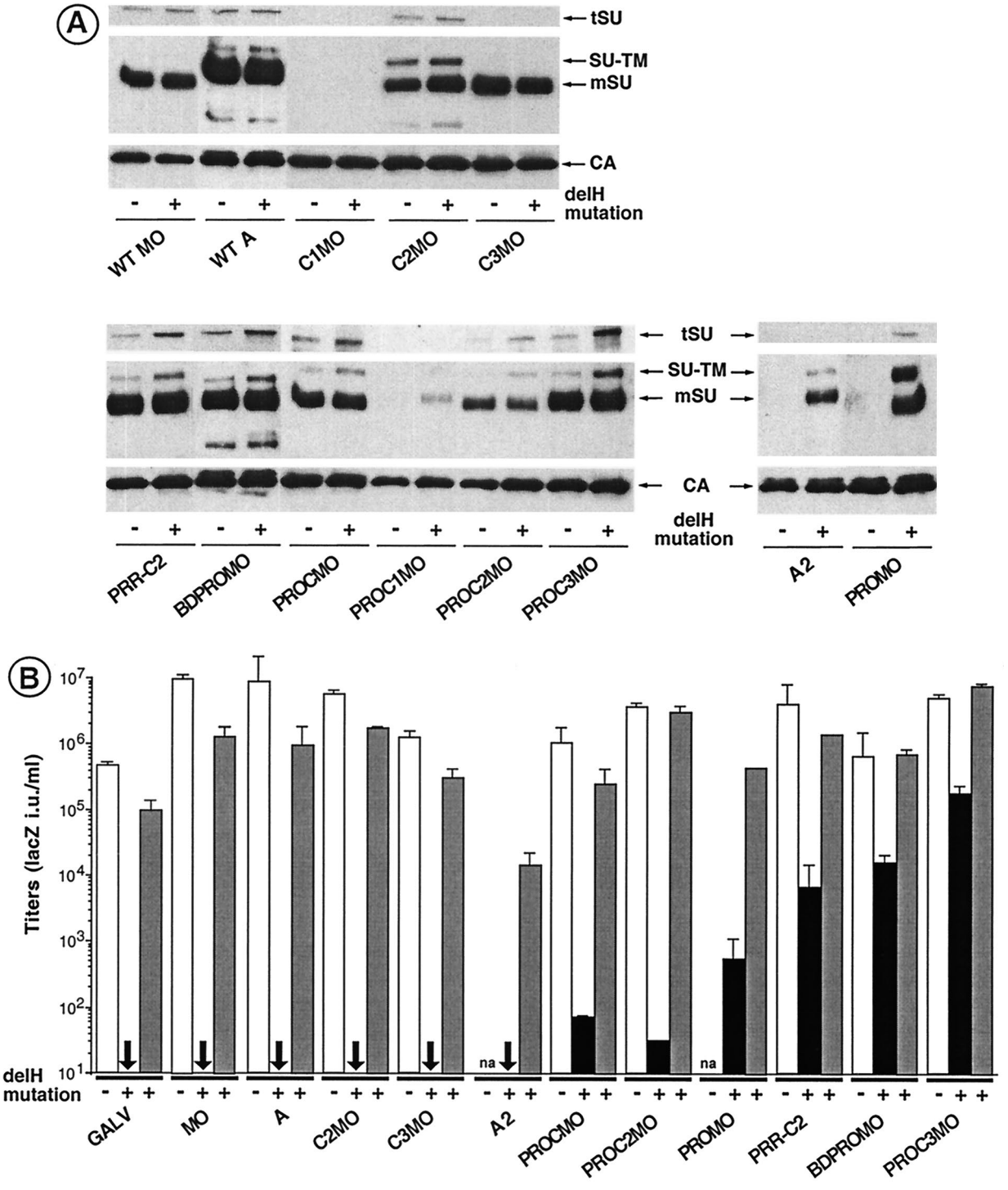

FIG. 3. Characterization of *del*H-mutated envelope chimeras. (A) Detection of envelope glycoproteins in pellets of retroviruses generated with the native (−) and *del*H-mutated (+) Envs by immunoblotting in nonreducing and nondenaturing conditions with an anti-SU antiserum. Equivalent loading of viral samples was demonstrated by immunoblotting with an anti-capsid (CA) antiserum. The positions of the monomeric SU (mSU), disulfide-linked TM-associated SU (SU-TM), and trimeric SU (tSU) forms are shown. (B) Results of virus-cell fusion assays on XC target cells, expressed as the number of LacZ infectious units per milliliter of viral supernatant. Infection assays were performed with virions carrying the native glycoproteins (−) or their *del*H-mutated forms (+), as indicated. For each type of MLV glycoprotein, infections were performed in the absence (open and black bars) or in the presence (grey bars) of ecotropic RBD polypeptides. Activation of retroviruses generated with the *del*H-mutated GALV Env glycoproteins was performed with GALV RBD polypeptides. Infection performed with *del*H-mutated RBD polypeptides did not enhance the infectivity of virions (data not shown). The values show the means ± standard deviations of up to four independent experiments. Identical results were obtained on the other target cells tested (XC, Cear13, NIH 3T3, and TE671 cells). na, not applicable.

1,000,000-fold (3, 4, 32, 34, 62; D. Lavillette and F.-L. Cosset, unpublished data) (Fig. 3B), the *del*H mutation introduced in the Env chimeras described before (Fig. 1 and 2) had variable effects on cell-cell fusion (data not shown) and virus-cell fusion (black bars in Fig. 3B). Like the wild-type glycoproteins from GALV, MoMLV, and MLV-A, the *del*H mutation introduced in the C2MO and C3MO Env chimeras efficiently inactivated fusogenicity. In contrast, the same mutation introduced in the PROCMO, PROC2MO, PROMO, PRR-C2, BDPROMO, and PROC3MO Env chimeras resulted in glycoproteins that allowed infection to proceed at variable efficiencies. Particularly, compared to their parental Env chimeras (open bars in Fig. 3B), the titers of virions carrying the PRR-C2*del*H, BDPROMO*del*H, and PROC3MO*del*H glycoproteins were decreased by only about 1,000-, 100-, and 10-fold, respectively.

Altogether these data indicated that particular SU conformations induced by altered interactions within the Env complex could strongly modulate the functions of the NTR domain and the effect of its mutation. Assuming that receptor binding of the viral RBD initiates conformational changes in the glycoprotein, our results suggest that for glycoproteins bearing modified PRR and/or C domains that subtly altered their conformation, fusion activation was less dependent on receptor interaction of their viral RBD.

Importantly, when RBD polypeptides with intact NTR domains were provided in *trans* during infection with viruses carrying the *del*H-mutated glycoproteins, titers similar to those of parental retroviruses were obtained (grey bars in Fig. 3B). This indicated that, although some mutations in the PRR and/or C domain alleviated in *cis* the need for an intact NTR domain in the viral RBD, full fusion activation required the integrity of the NTR domain in the RBD, in *cis* or in *trans*. These data suggested that, although some Env chimeras seemingly exhibited less dependence on receptor-mediated activation of their own viral RBD for infection, they required additional interactions with receptor-activated RBD to fully activate fusogenicity. Therefore, we sought to investigate further the different functions of the receptor-activated RBD in Env fusion activation.

Modifications of the PRR unlock Env fusion-inhibited conformation. Our data suggest that two consequences of receptor activation of the viral RBD could be to (i) induce destabilization of the Env complex and (ii) activate fusion via interaction with the C domain. It is possible that some of our Env chimeras that have altered stability or fusion properties may reflect intermediate conformations induced during the fusion process, as they seem to require fewer receptor interactions to initiate fusion activation (Fig. 3). If this hypothesis were true, fusion activation of such mutant Envs should be possible in the absence of the viral receptor, simply by provision in *trans* of an RBD fragment bound to its own receptor.

We therefore tested the fusion activation of the mutant Env glycoproteins shown in Fig. 1 to 3 on target cells that expressed the receptors for the *trans*-activating RBD but not for the viral RBD. These cells were derived from CHO cells engineered to express the PiT2 amphotropic receptor (CHO-PiT2), the mCAT1 ecotropic receptor (CHO-mCAT1), or both the PiT2 and mCAT1 receptors (CHO-PiT2/mCAT1). Infection assays were performed in the absence or in the presence of soluble RBD fragments derived from ecotropic and amphotropic MLV SUs (E-RBD and A-RBD, respectively). Viral particles generated with the mutant Envs fell into three groups of phenotypes.

The first group comprised the wild-type ecotropic (noted MO) and amphotropic glycoproteins, the C2MO and C3MO chimeras, and their *del*H mutant counterparts. Viruses carrying these glycoproteins were not infectious on CHO cells (black arrows in Fig. 4) or on transfected CHO cells that did not express viral receptors but those for the soluble RBDs. Likewise, the presence of RBD polypeptides bound to such alternative receptors did not allow infection on the latter target cell types (grey arrows in Fig. 4), yet soluble RBD fragments fully rescued the infectivity of viruses carrying the *del*H Env mutants of this first group of chimeras on cells that expressed the receptors for both the viral and soluble RBD fragments (open bars in Fig. 4). This suggested that these glycoproteins required an initial interaction with the viral receptor to become fusion active and that the effect of this primary interaction cannot be simply overcome by providing receptor-bound RBD fragments in *trans*.

In contrast, mutants of the second group were infectious on viral receptor-negative target cells that expressed receptors for the soluble RBD, provided such RBD polypeptides were added in *trans* during infection (grey bars in Fig. 4). Representatives of this second group included all the stable or stabilized PRR-mutated glycoproteins, i.e., the PRR-C2, PROCMO, and PROC2MO chimeras, their *del*H-mutated counterparts, and the A2*del*H and PROMO*del*H Envs. Indeed, although virions generated with such amphotropic Env chimeras were not infectious on CHO-mCAT1 target cells in the absence of E-RBD fragments (data not shown), titers of up to 10^6 infectious units/ml were obtained when E-RBD polypeptides were added in *trans* during infection (Fig. 4). As expected, soluble A-RBD fragments could not substitute for the E-RBD polypeptides for infection of the same target cells (data not shown), and no infection could be found on CHO cells (black arrows in Fig. 4), indicating that both an RBD fragment and its receptor were required to promote infection in *trans*. Furthermore, no rescue of infectivity was found when *del*H-mutated E-RBD fragments were added during infection of CHO-mCAT1 target cells (data not shown), demonstrating the requirement for an intact RBD in *trans* for the fusion activation of these glycoproteins.

Altogether, these data indicated that particular SU conformations, induced by altered interactions within the Env complex, allowed efficient membrane fusion in the absence of viral receptor-mediated activation of the Env glycoprotein, provided that receptor-bound RBDs supplied in *trans* were present during infection. Importantly, the NTR domain of the viral RBD did not influence this infection pathway. Indeed, whether the viral RBD carried the *del*H mutation or not, similar levels of infectivity were rescued by the receptor-bound *trans*-acting RBD fragments on target cells lacking receptors for the viral particles (Fig. 4).

Finally, the PROC3MO and BDPROMO chimeric glycoproteins and their *del*H-mutated counterparts formed the third group of phenotypes. Like the Env chimeras of the second group, virions carrying these stabilized PRR-mutated Env chimeras were highly infectious in target cells that lacked a receptor for the viral RBD, provided that RBD polypeptides

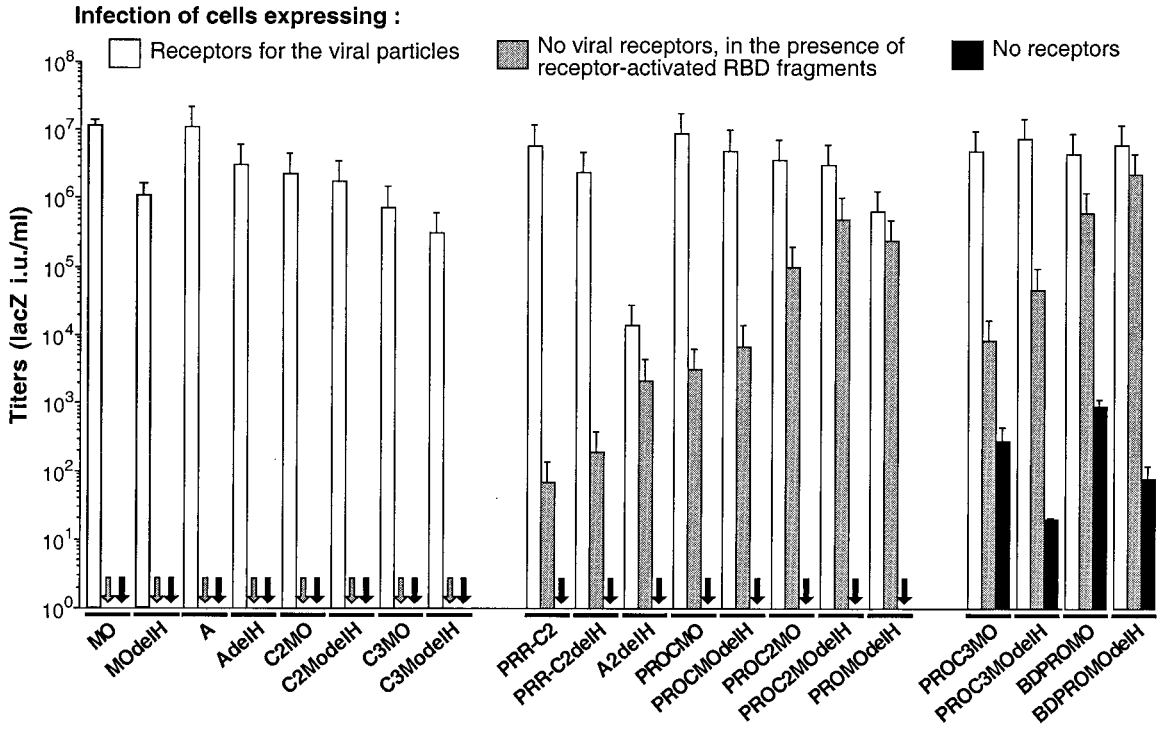

FIG. 4. Infection assays in the absence of viral receptors. Results of virus-cell fusion assays on CHO target cells, which do not express the mCAT1 and PiT2 receptors (black bars) and were engineered to express receptors for the *trans*-acting RBD only (grey bars) or both the mCAT1 and PiT2 receptors (white bars). That the endogenous CHO alleles of these receptors were not able to interact with the viral particles was demonstrated by the absence of detectable binding with tagged ecotropic or amphotropic RBD polypeptides and also with the indicated panel of chimeric Env glycoproteins in binding assays performed as described previously (33). Infectivity is expressed as the number of LacZ infectious units per milliliter of viral supernatant. For each type of glycoprotein, infections were performed in the presence of ecotropic RBD polypeptides except for the MO, MO*del*H, BDPROMO, and BDPROMO*del*H glycoproteins, for which amphotropic RBD polypeptides were used. Infection performed with *del*H-mutated RBD polypeptides did not enhance the infectivity of virions (data not shown). The values show the means ± standard deviations of up to four independent experiments.

targeted to a receptor different of that of the virions were added in *trans*, with titers reaching up to 5×10^6 infectious units/ml (grey bars in Fig. 4). However, mutant Envs of this third group could be distinguished from those of the second group by their capacity to promote infection on cells lacking a viral receptor in the absence of *trans*-acting RBD or of its receptor. Titers of up to 10^3 infectious units/ml could be obtained in CHO cells (black bars in Fig. 4) as well as in CHO-PiT2 cells for BDPROMO and BDPROMO*del*H virions and in CHO-mCAT1 cells for PROC3MO and PROC3MO*del*H virions (data not shown). These results indicated that mutants from this group allowed efficient membrane fusion in the absence of viral receptor-mediated activation of the Env glycoprotein and exhibited a less critical requirement for receptor-bound *trans*-acting RBD polypeptides.

DISCUSSION

Fusion-inhibitory conformation of native MLV Env complex. The MLV Env is expressed in an apparently stable form on virions. Here we found that the stability of the Env complex is provided by interactions between discrete subdomains of the SU. Rupture of these interactions by minimal modifications of the SU results in destabilization of the Env complex, characterized by loss of SU/TM association, and leads to increased fusogenicity. These results imply that the MLV Env complex has a relative stability and can be considered metastable. A second implication is that this native conformation of the Env complex is an inhibitor of fusion. These results are in agreement with the data of others on MLV glycoproteins (4) as well as on fusion glycoproteins of several other viruses (12, 18, 58).

A characteristic feature of the fusion glycoproteins of many membrane-enveloped viruses is that they are synthesized as inactive precursors that undergo several posttranslational modifications in order to be displayed on virions in metastable forms (12). Metastable protein conformations are energy-loaded and are considered biologically active, i.e., fusion competent in the case of the viral glycoproteins, which can be activated to trigger their function. Results have shown that, following their activation, the fusion subunits of glycoproteins from several membrane-enveloped viruses undergo a dramatic refolding of their structure, and the energy released by this conformational rearrangement is thought to be necessary for the fusion process. This leads to the most stable conformation of the glycoprotein, in which the fusion peptides are inserted in the target cell membrane and the C-terminal segments of the ectodomain of the fusion proteins are packed around the central coiled-coil structure (29).

Besides providing the energy required for driving fusion,

metastable Env conformations are essential to ensure the propagation of retroviruses. Indeed, acquisition and control of metastability are highly precisely controlled processes because the fusogenicity of the glycoprotein should not be activated inappropriately or prematurely, i.e., before the virions have reached the target cell surface. Mutant glycoproteins that are too unstable are both highly cell-cell fusogenic and not incorporated on virions. Consequently, not only are such glycoproteins toxic for the virus host cells, they also do not permit infection by progeny virions.

Retroviruses have adopted several solutions to control acquisition and maintenance of metastability. First, Env glycoproteins are expressed as fusion-inactive protein precursors that can block receptors at an early stage of their synthesis. The glycoproteins are proteolytically matured later in the cell secretory pathway, preventing untimely fusion activation (25). Second, interactions between subdomains of the glycoprotein maintain a fusion-inhibitory conformation (4, 33; this report). Additionally, the processed SU and TM Env subunits are held together via a "labile" disulfide bond whose isomerization and disruption might provide more metastability to the envelope complex and increased instability of the SU subunit (46). Finally, for the *Gammaretroviridae* and *Betaretroviridae*, ultimate acquisition of fusion competency will occur only after virion budding and may be promoted by modification of the structure of the Env complex through viral protease-mediated cleavage of its cytoplasmic tail (10, 11, 50, 52).

Fusogenicity of conformation-modified Env glycoproteins. Unblocking of the Env fusion-inhibitory conformation could be achieved by modifications of the glycoprotein that weakened the stability of the Env complex. In the presence of receptor-bound *trans*-acting RBDs, nearly wild-type levels of infectivity were obtained for some of the viruses harboring these conformation-modified glycoproteins despite the absence of receptors for their viral RBD. Thus, the receptor-bound RBD behaved as a complex that triggers the fusogenicity of the viral glycoproteins in *trans* and, although no stable interaction with the virions could be detected (data not shown), can be considered a fusion receptor.

While this extreme uncoupling between virus binding and fusion triggering represents a novel paradigm in the mechanisms of virus entry into cells, this raises questions about the mechanism of cell attachment of the viral particles. Most retroviral receptors that have been cloned to date have been identified on the basis that their ectopic expression allows infection in nonpermissive cells (45). Although these molecules generally bind their cognate retroviral glycoproteins with high affinities, several other factors strongly influence the early events of infection. Results described in the literature have indicated that despite the absence of such "receptors" for their Env glycoproteins, retroviruses can nevertheless efficiently adsorb to the surface of adherent target cells (1, 48, 49, 55) and may be subsequently internalized (15, 35, 36, 39). Such attachment is mediated by components on virus and cell surfaces that can be different from the viral Env and its cognate receptor (21, 49, 60) and that may help virions to reach their specific receptors. Intercellular adhesion/communication molecules or proteins of the extracellular matrix, such as heparan sulfate proteoglycans, play a major role in these initial steps of infection (9, 20, 39) and perhaps are more important in mediating virus-cell attachment than the viral receptors themselves. This indicates that the most important function of the molecules cloned and identified as viral receptors, at least in cell culture, is not to allow attachment of the viral particles but rather to trigger fusion activation of the viral glycoprotein. The results of this work and from other groups (4, 5) obtained with MLVs harboring modified glycoproteins are in agreement with this notion.

The transition between the prefusogenic and fusion-active conformations of Env glycoproteins is likely to proceed in several steps, for which intermediate conformations of the glycoprotein may be identified. Rupture of the initial conformation of the viral glycoprotein is achieved by interaction with the receptor and leads to unlocking of the fusion-inhibitory Env conformation. This first step may induce the dissociation of the C domain of the SU from its RBD, as suggested by the studies of others (4). The second step of fusion activation is conditioned by the achievement of this first step, which has rendered the C domain of the glycoprotein accessible and consists in subsequent interaction in *cis* or in *trans* of a receptor-activated RBD with a conserved disulfide loop of the C domain (4, 5, 32). Modifications of the PRR that do not too greatly impair the stability of the Env complex seem to bring the glycoprotein into a more relaxed conformation that does not need or needs less the primary receptor interaction to disrupt the fusion-inhibitory conformation.

We propose that these glycoproteins (the PROC2MO, BDPROMO, and PROC3MO chimeras) adopt intermediate conformations that mimic those reached by the wild-type glycoprotein following its interaction with the receptor. The chimeric glycoproteins of group II (Fig. 4) may represent fusion intermediates of the first step of fusion activation, since they promote infectivity in the absence of viral receptors, provided that receptor-bound *trans*-acting RBD fragments are present. That disruption of their NTR domains did not change their properties may indicate that molecular transition occurring during this first step requires the NTR domain in wild-type glycoproteins (see below). Interestingly, among the Env mutants that required fewer interactions with receptors to unlock the fusion-inhibitory conformation, the Env chimeras of group III (Fig. 4) could achieve infection even in the absence of the *trans*-activating RBD. Indeed, retroviruses carrying the PROC3MO and BDPROMO chimeras could infect, albeit at low efficiency, cells that lacked receptors. These mutants were also the most insensitive to mutation of the NTR domain, confirming the notion that the structure of these chimeric glycoproteins was intermediate between those of the fusion-inhibitory and fusion-active conformations.

Role of PRR in stability of conformation of the Env complex. It has been proposed that the MLV glycoprotein retains its fusion-inhibitory conformation via interaction of the viral RBD with the C domain and that binding to the receptor leads to disruption of this fusion-inhibited conformation (4). Such an RBD/C domain interaction has also been suggested by the studies of others for the native conformation of the *Gammaretroviridae* Env glycoproteins (23, 42, 44). With SUs that were rendered defective for Env incorporation by changes in either the RBD or the C domain, revertant viruses were isolated after serial passages in cell culture. Characterization of several of these revertants revealed that, in addition to the original mu-

tation, compensatory changes had appeared in the opposite segment of SU, indicating that contacts between RBD and the C domain are required, at least for optimal maturation and viral incorporation of the Env glycoprotein.

Our results are in agreement with these findings and indicate that the PRR acts as a hinge that controls the strength of interaction between the RBD and C domain in the fusion-inhibiting conformation. Alteration of the PRR weakens this stabilizing interaction and mimics the effect of the interaction with the receptor. This explains why viruses carrying such mutant Envs require fewer or no interactions with the receptor for cell entry, as shown in receptor interference assays in the original description of these Env chimeras (33).

The sequence variability of the PRR of the glycoproteins of *Gammaretroviridae* (28) is puzzling in light of its important role in Env conformation. Since the pressure for maintaining its sequence seems less important than that exerted to preserve the structure of the RBD and other critical Env domains, our results indicate that PRR variability may provide a mechanism by which the retroviruses can easily adapt to changes in their environment. For example, in the course of their replication and spread in organisms, retroviruses may encounter conditions in which receptors become less available or less efficient at promoting virus-cell fusion. Intense variability of the PRR would allow, at low cost for the Env structure and function, the selection of mutations that modulate the strength of the blocking conformation of the glycoprotein. The resulting PRR-adjusted retroviruses will therefore adapt to the novel conditions of fusion activation imposed by changes in the number of or affinity with the receptors of the new environment, first by requiring fewer receptor interaction to the Env fusion-inhibitory conformation and second by requiring fewer receptor-bound RBDs to activate fusion. The results obtained with glycoproteins that harbor modifications of the PRR and that consequently allow membrane fusion in conditions where receptors are limiting support this idea (32, 33). Likewise, mutations in the glycoprotein that favor replication of amphotropic retroviruses in avian cells have been described recently and found to reside in the amino-terminal end of the PRR (6), a segment essential for modulating the conformation of the Env complex (22, 33, 61). Importantly, the appearance of mutations that attenuate the fusion-inhibitory conformation may also favor fusion activation in *trans* by glycoproteins of some heterologous *Gammaretroviridae*, whether they are exogenous or endogenously expressed by the host cells. Several endogenous retrovirus loci express Env-derived polypeptides in vertebrates (7, 26, 53), and, although they have been involved in protecting cells from superinfection by receptor blockage (25), they may clearly provide fusion helper functions (2, 32). In addition to providing genetic variability via recombination (8), the interactions of endogenous retroviral sequences with virions from exogenous retroviruses might provide numerous scenarios to favor viral spread and cross-species infections.

Data in this report substantiate the notion that the PRR establishes contacts with other Env segments to maintain the fusion-inhibitory conformation. Important modifications of the PRR (exemplified here by the PROMO mutant) strongly destabilize the Env complex, leading to glycoproteins that are highly cell-cell fusogenic. Introduction of Env segments that belong to the glycoprotein from which the heterologous PRR was derived in the PROMO Env was found to restore Env complex stability (Fig. 2). These results suggest that SU subdomain interactions were reconstituted in these secondary chimeras. This may allow definition of Env segments that act as partners of the PRR in order to ensure the stability of the Env complex. On the one hand, as suggested by the stability of the PROC3MO chimera, the PRR may interact with the distal segment of the C domain. On the other hand, the PRR may interact with the RBD domain, as suggested by the stability of the BDPROMO mutant. A more detailed characterization of the Env segments that need to interact to stabilize the fusion-inhibitory conformation of the glycoprotein will require further investigation of chimeras and point mutants of the MLV SU.

PRR-NTR interactions control initiation of fusion activation. Our results indicate that the NTR domain is involved in conformation of the Env complex and in fusion activation, but in a manner opposite that of the PRR. Indeed, its alteration, through the *del*H mutation, resulted in either stabilization of PRR-mutated Envs (e.g., PROMO*del*H and PROC3MO*del*H Envs) or loss of Env fusogenicity (e.g., A*del*H and C2MO*del*H Envs). Curiously, its effect on Env conformation was particularly marked in the context of Env mutations that reduced the impact of its mutation in fusion activation. Indeed, the disruption of the NTR domain, as in the PROMO*del*H, PRR-C2*del*H, BDPROMO*del*H, and PROC3MO*del*H chimeras, resulted in highly infectious retroviruses and in increased Env complex stability compared to the parental chimeric glycoproteins (Fig. 3). This suggests that a possible function of the NTR domain is to contribute to disruption of the Env fusion-inhibitory conformation.

Indeed, one possibility to explain these results is that the NTR domain and the PRR region interact, as suggested here (Fig. 3) and by the genetic studies of others with second-site revertants of NTR-mutated ecotropic MLVs (62). We propose that such an interaction could prevent an intrinsic ability of the NTR domain to induce destabilization of the Env complex until this interaction has been changed upon receptor binding. Thus, the mutation of the NTR domain of the wild-type MLV glycoproteins would have no apparent effect on conformation (Fig. 3A), owing to their inherent stability. Yet, in the context of PRR-mutated Envs that are unstable or less stable, the NTR domain would lack its partner that preserves the fusion-inhibitory conformation, and its role in destabilization would be dominant, thus explaining why its mutation restored Env complex stability.

In this scenario, a function of the NTR domain would be, in concerted action with the PRR, to control the molecular transitions occurring during the first step of fusion activation, from the fusion-inhibitory to the fusion-activatable conformation of the glycoprotein. Some PRR-mutated glycoproteins, such as the PROC3MO and BDPROMO chimeras, are likely to spontaneously adopt such fusion-activatable conformations. This might explain why viruses carrying such glycoproteins need very few interactions with the receptor to become fusogenic and why the mutation of their NTR domains has limited consequences for fusion.

It is clear that the roles of the NTR domain are not limited to Env stability and, potentially, unlocking of the fusion-inhibitory conformation through its interaction with the PRR. Indeed, optimal fusion activation still requires an intact RBD in

cis or in *trans* for all Env chimeras examined to date, even those that seem to have bypassed the fusion-inhibited conformation (Fig. 3). This suggests that even if the integrity of the NTR domain is dispensable in *cis* in the first step of the fusion activation pathway for some Env chimeras, the presence of the critical histidine of the NTR is mandatory for the second step of fusion activation. Such an interaction is also critically dependent on binding of the *trans*-acting RBD to its receptor. This indicates that receptor binding may modify not only the Env conformation through a pathway that involves both the PRR and the NTR domain but also the NTR domain itself so as to render it active at a later stage of the fusion process. The nature of this later event is unknown at the moment, yet we surmise that interaction of the RBD with the C2 subdomain of the SU carboxy-terminal domain (32) may help to correctly position the NTR in order to allow its optimal interaction with its partner.

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The first two authors contributed equally to this work.

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