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Furin Cleavage Potentiates the Membrane Fusion-Controlling Intersubunit Disulfide Bond Isomerization Activity of Leukemia Virus Env

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The membrane fusion protein of murine leukemia virus is a trimer of a disulfide-linked peripheral-transmembrane (SU-TM) subunit complex. The intersubunit disulfide bond is in SU linked to a disulfide bond isomerization motif, CXXC, with which the virus controls its fusion reaction (M. Wallin, M. Ekström, and H. Garoff, EMBO J. 23:54–65, 2004). Upon receptor binding the isomerase rearranges the intersubunit disulfide bond into a disulfide bond isomer within the motif. This facilitates SU dissociation and fusion activation in the TM subunit. In the present study we have asked whether furin cleavage of the Env precursor potentiates the isomerase to be triggered. To this end we accumulated the late form of the precursor, gp90, in the cell by incubation in the presence of a furin-inhibiting peptide. The isomerization was done by NP-40 incubation or by a heat pulse under alkylation-free conditions. The cells were lysed in the presence of alkylator, and the precursor was immunoprecipitated, gel isolated, deglycosylated, and subjected to complete trypsin digestion. Disulfide-linked peptide complexes were separated by sodium dodecyl sulfate-tricine-polyacrylamide gel electrophoresis under nonreducing conditions. This assay revealed the size of the characteristic major disulfide-linked peptide complex that differentiates the two isomers of the disulfide bond between Cys336 (or Cys339) and Cys563, i.e., the bond corresponding to the intersubunit disulfide bond. The analyses showed that the isomerase was five- to eightfold more resistant to triggering in the precursor than in the mature, cleaved form. This suggests that the isomerase becomes potentiated for triggering by a structural change in Env that is induced by furin cleavage in the cell.

Activation of the membrane fusion proteins of enveloped viruses is a carefully controlled process to ensure that the fusion function is only activated when the virus reaches its preferred entry site in the target cell. The viral fusion proteins are in general made as polypeptide precursors that trimerize while still in the endoplasmic reticulum of the infected cell (16). When the oligomers pass the trans-Golgi complex on their journey to the cell surface, the polypeptide precursors are cleaved into a receptor-binding peripheral and a fusogenic transmembrane subunit by cellular furin or a furin-like enzyme which recognizes an Arg-X-Lys/Arg-Arg motif in the precursor polypeptide (2, 13, 17, 33, 45). The cleavage represents the first step in activating the viral fusion protein (6, 27, 40, 50). It liberates the fusion peptide in the N terminus of the transmembrane subunit and, according to the influenza hemagglutinin model, transforms it into a metastable state (44). In this state the transmembrane subunit has the potential to fuse the viral and the target cell membranes. The fusion is facilitated by refolding of the transmembrane subunit into a stable form. The refolding pathway involves exposure and interaction of the fusion peptide with the target membrane and a subsequent back-folding of the transmembrane polypeptide in a manner which drags the viral and the cell membranes together. However, in the native virus the fusion potential of the transmembrane subunit is suppressed by the interacting peripheral subunit. Therefore, the dissociation or displacement of the peripheral (surface [SU]) from the transmembrane (TM) subunit constitutes the second step of fusion activation. This can be effected by receptor binding on the cell surface, in which case the virus will fuse with the plasma membrane, or after virus uptake into endosomes, in which case the virus fuses internally (16). In the latter case, the acid pH of the endosome constitutes the final trigger for fusion. A variation of this general theme of fusion activation is found in the small and enveloped alpha- and flaviviruses (3, 11). In these icosahedral viruses the chaperoning role of the peripheral protein is taken by a second transmembrane subunit.

It is evident that the strength of the interaction between the peripheral and the transmembrane subunits of the mature fusion protein will constitute a critical point in its activation pathway. If this interaction is too weak, it will promote premature dissociation and fusion activation, resulting in virus inactivation. If it is too strong, it will compromise fusion at the entry site. In this respect it is notable that the fusion protein subunits of many enveloped viruses are disulfide linked (19, 25, 32, 36, 44, 48). Disulfide bonding of fusion protein subunits should efficiently prevent premature subunit dissociation, but it also risks the triggered dissociation at the virus entry site. The relatively harsh triggering condition of low pH in the endosome apparently represents a solution to this problem for some viruses with disulfide-linked subunits, like influenza and avian leukemia viruses (28, 44). In these cases the low pH, or the combined effect of receptor binding and low pH, results in sufficient displacement of the peripheral from the transmembrane subunit so that the latter can be activated, even though the two subunits remain disulfide bonded. An alternative strat-

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egy, which is apparently used by the majority of the leukemia viruses, is to link the intersubunit disulfide bond to an isomerization motif, Cys-X-X-Cys (CXXC) in the peripheral subunit (48). This contains a free thiol, with which it can rearrange the intersubunit disulfide bond into an alternative disulfide isomer within the motif. As the isomerization of the CXXC motif is triggered by receptor binding, the virus gets rid of the intersubunit disulfide bond at the surface of the target cell. This facilitates dissociation of the peripheral subunit and fusion activation.

The fusion activation pathway that is controlled by intersubunit disulfide bond isomerization has been studied in some detail using Moloney murine leukemia virus (Mo-MLV) (48). It was shown that receptor binding induced the exposure of the CXXC thiol in the peripheral subunit for modification by alkylation. This blocked the isomerization reaction in the receptor-bound fraction of the viral fusion proteins (envelope [Env]) and arrested fusion activation. However, the fusion could be rescued by subsequent reduction of the intersubunit disulfide bond with dithiothreitol. Thus, this proved the role of the intersubunit disulfide bond isomerization in controlling the fusion activation process in Mo-MLV. It was possible to trigger the isomerization activity not only by receptor binding but also by treatments of the virus with heat or urea, depleting the virus of Ca^{2+}, and by solubilization of the viral membrane with NP-40 (46, 47). This suggested that the receptor-induced isomerization of the intersubunit disulfide bond was mediated by destabilization of a Ca^{2+}-stabilized structure of the fusion protein.

An interesting question concerns the control of the activity of the CXXC-linked disulfide bond isomerase. As the Env precursor and Env receptor, a basic amino acid transporter, can possibly interact in the biosynthetic transport pathway, this could result in premature isomerization of the disulfide bond between Cys336 (or Cys339) and Cys563 [hereafter Cys336(339)-Cys563], which corresponds to the intersubunit linkage of the mature SU-TM complex and subsequent release of the fusion function directly upon furin cleavage (49). Therefore, it is possible that the isomerase activity remains suppressed in Env until this is cleaved by furin. Alternatively, the Env could be synthesized with the internal CXXC isomer (Cys336-Cys339) of the disulfide bond and rearrange into the SU-TM isomer [Cys336(339)-Cys563] first upon furin cleavage. In the present study we have addressed these questions using Mo-MLV.

Earlier studies have shown that the Mo-MLV Env is made as a gp80 precursor with seven Asn (N)-linked sugar units in the SU portion of the polypeptide (8, 20, 29, 42, 43). The transmembrane and the cytoplasmic domain of gp80 are modified by palmitoylation (15, 31, 52). Upon transport through the Golgi and trans-Golgi complexes, the endoglycosidase H (endo H)-sensitive high-mannose forms of the N-linked sugar units of gp80 are processed into endo H-resistant complex units. In addition, the Env polypeptide is glycosylated at the hydroxyls of several Ser and Thr residues (O-glycosylation) (10, 35). This form of Env is recognized as gp90. In pulse-chase experiments, it is seen only transiently in very small amounts because it is rapidly cleaved into the disulfide-linked SU-TM complex of mature Env (9, 29). During maturation of virus particles by budding at the cell surface, or shortly thereafter, the viral protease releases a piece known as the R peptide from the end of the cytoplasmic (internal) domain or tail of TM (12, 41).

In this study we have used a disulfide-linked tryptic peptide assay to analyze when after synthesis the Cys336(339)-Cys563 disulfide bond is formed in the Env precursor and to test its sensitivity to undergo CXXC-mediated isomerization. For analyses of the late form of the precursor, gp90, this was enriched in cells by inhibiting its cleavage with a furin-inhibiting peptide (FIP). We found that the Cys336(339)-Cys563 disulfide bond was generated posttranslationally in gp80 and that it was significantly more resistant to isomerization induction both in the early gp80 and in the late gp90 forms of the precursor than in the mature, cleaved Env. These results suggest that the disulfide bond isomerase of the Mo-MLV Env precursor is potentiated for receptor-induced activation by a furin cleavage-mediated conformational change.

MATERIALS AND METHODS

Cells and reagents. MOV-3 cells, a gift from G. Schmidt (GSF-National Research Center for Environment and Health, Neuherberg, Germany) was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/liter glucose (Gibco BRL) supplemented with 10% fetal calf serum, 20 mM HEPES, and L-glutamine. HE 699, a polyclonal goat antiserum against the gp69/71 Env glycoprotein (SU) of Rauscher MLV (catalog no. VR-1519AS-Gt) was from ATCC (LGC Promochem, Borås, Sweden). Peptide *N*-glycosidase F (PNGase F; catalog no. 1365193) and O-glycosidase (catalog no. 11347101001) were from Roche Biochemicals (Basel, Switzerland); endo H_f (catalog no. P0703S) was from New England Biolabs (Ipswich, MA), and neuraminidase (catalog no. 480717) was from Calbiochem/Merck (Darmstadt, Germany). ^{14}C-methylated standard proteins (catalog no. CFA 626 and CFA755) were from Amersham Biosciences (Uppsala, Sweden).

Metabolic labeling, furin inhibition, isomerization induction, and cell lysis. Metabolic labeling of proteins in MOV-3 cells was done with [^{35}S]Cys (Amersham Biosciences, Uppsala, Sweden) as described previously (32). Briefly, cells in 3.5- or 6-cm tissue culture dishes were washed twice in phosphate-buffered saline (PBS), incubated in cysteine-free DMEM for 30 min (starvation), labeled for 5 or 15 min (50 μCi of [^{35}S]Cys in 0.5 ml/3.5-cm dish) and chased for up to 2 h in DMEM containing 2 mM Cys. The cells were washed twice in PBS and solubilized in 1% NP-40 in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA; 300 μl/3.5-cm dish) without or with 5 mM *N*-ethyl maleimide (NEM; in both PBS and lysis buffer) to allow or prevent isomerization of disulfide bonds, and incubated for 2 h on ice. Finally, all samples were adjusted to 5 mM NEM and incubated for 10 min at 30°C. To inhibit furin convertase, the cells were incubated for 1.5 h in 5 to 80 μM FIP (decanoyl-Arg-Val-Lys-Arg-chloromethylketone) (catalog no. 260-022-M001; Alexis Biochemicals/Kelab Goteborg, Sweden) prior to labeling, first in DMEM-fetal calf serum (1 h) and then during the Cys starvation (0.5 h). When isomerization was induced by heat instead of NP-40, we incubated the pulse-labeled (15 min) and chased (1 h) cultures at 53°C for 4 to 6 min in 50 mM Tris-HCl (pH 7.45 at 37°C), 150 mM NaCl, and 1.8 mM $CaCl_2$. In control experiments analyzing the mature Env, parallel cultures were treated at 53°C in the presence and absence of 20 mM NEM. To compensate for decomposition of NEM, 10 mM fresh NEM was added every minute during the heat pulse. Lysis of the heat-treated cultures was done in the presence of NEM. In these experiments the FIP treatments were extended to include also the time of chase.

Immunoprecipitation and deglycosylation. Mature and precursor Env proteins were immunoprecipitated by HE699 essentially as previously described (32). Precipitations, typically 150 μl of labeled cell extract (i.e., 50% of a 3.5-cm dish), 6 μl of HE699 anti-SU polyclonal antibody (pAb), and 30 μl of protein A-Sepharose slurry (1:1, vol/vol), all adjusted to 5 mM NEM, were performed overnight at 4°C. The washed immune complexes were either processed directly for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or eluted for further manipulations. To elute proteins, 1 bead volume (15 μl) of 1% SDS in lysis buffer was added, and the samples were incubated for 3 min at 70°C. SDS was inactivated by the addition of 5 bead volumes (PNGase F and endo H_f) or 10 bead volumes (*O*-glycosidase and neuraminidase) of 1.25% NP-40 in lysis buffer. To digest N-linked sugars, aliquots (24 μl) were supplemented with 0.5 μl of PNGase F or 4 μl of 0.5 M Na citrate, pH 5.5, and 0.5 μl of endo H_f. O-linked

sugars were assessed by the addition of 1 μl of *O*-glycosidase, 1 μl of neuraminidase, and 0.5 μl of PNGase F. All deglycosylations were incubated for 16 h at 37°C.

Electrophoresis. Samples were adjusted to 31 g/liter SDS, 0.19 M Tris-HCl, pH 8.0, 93 g/liter sucrose, 14 mM EDTA, 0.6 mg/ml bromophenol blue, and 0.4 mg/ml methionine (1× sample buffer). For reduction of disulfide bonds, 37 mM dithiothreitol was included. The samples were heated for 3 min at 70°C (6 min when the samples were >150 μl) and cooled to room temperature, and a final concentration of 9 mM iodoacetamide (or 10 mM NEM for preparative purposes) was added to reduced and nonreduced samples. SDS-PAGE gels (23), 8 or 10.5 cm long, with 7%, 8%, or 12% total acrylamide, of which 2.6% was bisacrylamide, and 10.5-cm SDS-tricine-PAGE gels (39) with 16.5% total acrylamide, of which 2.6% was bisacrylamide, were run in a Mighty Small II minigel system (Hoefer Scientific, San Francisco, CA).

Gel purification. Metabolically labeled, immunoisolated Env proteins from 720 μl of cell extract prepared under isomerizing or nonisomerizing conditions were separated on 7% SDS-PAGE under nonreducing conditions. The bands of interest were detected by phosphorimaging of the wet gels (typically, a 2-h exposure) and cut out. Gel pieces from 8 to 10 equivalent lanes were crushed in 2 ml of 0.05% SDS in 10 mM Tris-HCl, pH 7.5, extracted at room temperature overnight, and removed by 0.22-μm-pore-size cellulose acetate filters (Schleicher-Shuell, Dassel, Germany). The extracted proteins were concentrated in Microcon YM-30 centrifugal filter devices (Amicon/Millipore, Billerica, MA) to about 150 μl, PNGase F was added (5 μl), and the samples were incubated at 37°C overnight. The samples were adjusted to 1× sample buffer and rerun on 8% SDS-PAGE under nonreducing conditions, and the bands of interest were cut out, extracted, and concentrated to about 15 to 20 μl as above.

Tryptic digestion. Gel-purified and concentrated proteins were incubated for 10 min at 56°C, cooled to room temperature, and treated with 0.15 mg/ml TPCK (*N*-tosyl-L-phenylalanine chloromethyl ketone)-trypsin (catalog no. 37257; Serva, Heidelberg, Germany); 2 mM $CaCl_2$ was added, and the samples were incubated at 37°C for 16 h.

Quantifications. The amount of radioactivity in a protein band was measured using phosphorimage screens (BAS-MS2025) from Fujifilm (Science Imaging Scandinavia, Nacka, Sweden), a Molecular Imager FX, and the QuantityOne program from Bio-Rad Laboratories (Hercules, CA). To estimate the extent of SU-TM isomerization, the amount of SU released from the SU-TM complexes during incubation was measured. This was expressed as a percentage of total SU (i.e., the sum of free and TM-bound SU). The prevalence of the Cys336(339)-Cys563 disulfide bond in Env gp80 and gp90 precursors was determined by measuring the major disulfide-linked tryptic peptide complexes in digests of precursors that were incubated under nonisomerizing conditions. The amounts of 12-kDa complexes [molecules containing the Cys336(339)-Cys563 disulfide bond] and 9.1-kDa complexes (molecules with the internal Cys336-C339 disulfide bond) were quantified from dried SDS-tricine gels. The relative amount (R) of 12-kDa complexes ($R_{12kDa}^{\pm NEM}$) was calculated as a percentage of the sum of the 12-kDa and 9.1-kDa complexes in the sample for each protein precursor under both nonisomerizing (with NEM) and isomerizing (without NEM) conditions. This value was used to express the prevalence of the Cys336(339)-Cys563 disulfide bond. The relative stability (RS) of the Cys336(339)-Cys563 disulfide bond was calculated as relative amount of 12-kDa complex under isomerizing conditions as a percentage of that under nonisomerizing conditions: $RS = (R_{12kDa}^{-NEM}/R_{12kDa}^{+NEM}) \times 100$. In mature Env (SU-TM) the intersubunit Cys336(339)-Cys563 disulfide bond was analyzed in 12% SDS-PAGE under nonreducing conditions. The amount of SU-TM complexes (containing the intersubunit disulfide bond) and free SU subunits (without the intersubunit disulfide bond) was quantified, and the relative amount of intact complexes ($R_{SU\text{-}TM}^{\pm NEM}$) present under nonisomerizing (with NEM) and isomerizing (without NEM) conditions was calculated as a percentage of the sum of SU-TM and free SU. The relative stability of the intersubunit disulfide bond was calculated as the relative amount of SU-TM under isomerizing conditions as a percentage of that under nonisomerizing conditions: $RS_{SU\text{-}TM} = (R_{SU\text{-}TM}^{-NEM}/R_{SU\text{-}TM}^{+NEM}) \times 100$.

RESULTS

Furin inhibition results in the accumulation of gp90, a late form of the Env precursor. We used an FIP to prevent cleavage of the Env precursor into the SU-TM complex in MOV-3 cells. To find optimal conditions for the inhibition, cell cultures were incubated with different concentrations of FIP for 1 h, starved for Cys for 30 min in the presence of FIP, and then pulse labeled with [^{35}S]Cys for 15 min. After a 1-h chase, the cultures were lysed in the presence of NEM, and the viral proteins were captured for nonreducing or reducing 12% SDS-PAGE by an anti-SU pAb (HE699). A control culture that had been labeled in the absence of FIP was processed in parallel. Analysis of this sample under nonreducing conditions revealed the gp80 Env precursor and the slower migrating, cleaved but covalently linked SU-TM complex of mature Env (Fig. 1A, lane 1). The material at the top of the gel represents undissolved Env oligomers (see below). Incubation in the presence of increasing concentrations of FIP up to 20 μM resulted in the accumulation of an increasing amount of Env protein that migrated slightly faster than the mature form (Fig. 1A, lanes 2 to 6).

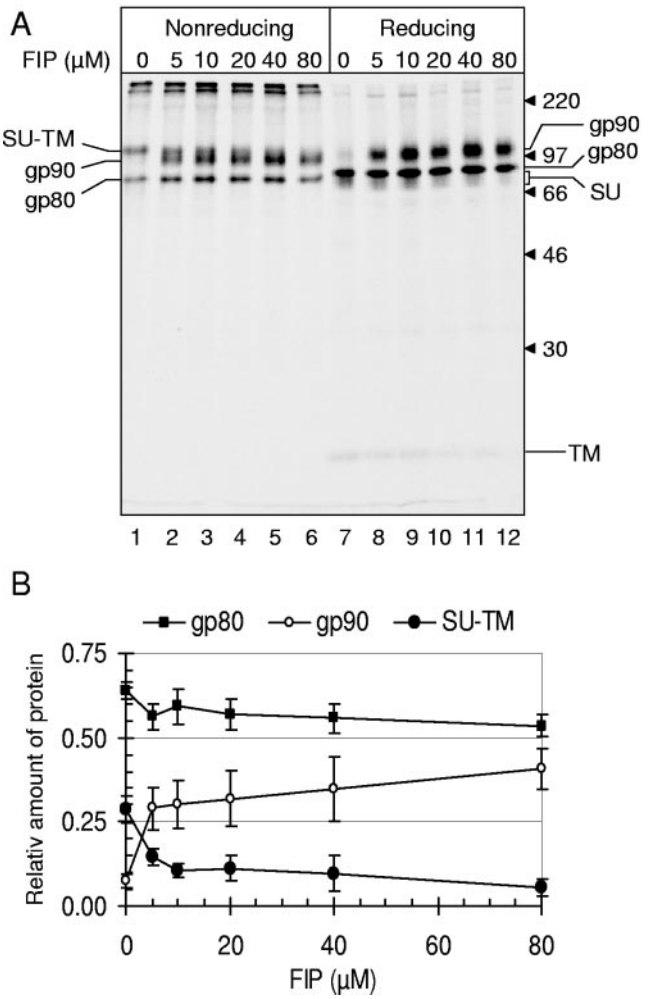


FIG. 1. Suppression of Env precursor cleavage by furin inhibition. MOV-3 cell cultures were incubated in the presence of 0 to 80 μM FIP for 1.5 h and then pulse-labeled with [^{35}S]Cys for 15 min, chased for 1 h, and lysed in the presence of 5 mM NEM. The Env proteins were immunoisolated by an anti-SU pAb and analyzed in nonreducing and reducing 12% SDS PAGE. Panel A represents a phosphorimage of the gel with molecular weight standards (in kDa), the covalently linked SU-TM complexes, the early (gp80) and late (gp90) forms of the Env precursor, and the nonlinked Env subunits SU and TM indicated. Panel B represents the relative amounts of gp80, gp90, and SU-TM complexes ± standard deviation as a function of FIP concentration ($n = 3$).

Analyses by reducing SDS-PAGE showed that, whereas the mature Env in the control and in the FIP-treated sample was released into free SU and TM subunits, the FIP-induced faster migrating form of Env remained as one intact chain (Fig. 1A, lanes 7 to 12). Quantification demonstrated that the FIP-induced protein increased with the FIP concentration at the expense of the mature, but not gp80, Env (Fig. 1B). We concluded that FIP inhibited the cleavage of the Env precursor to the SU-TM complex. The FIP-induced form of Env corresponded most likely to the gp90 form of gp80, which has earlier been observed in small amounts in pulse-chase experiments (29). With the FIP, it was possible to inhibit almost all of the Env that was normally processed by furin. However, incubations at FIP concentrations above 40 μM decreased the yield of all forms of Env, probably as a result of the toxic effects of the drug. Note that the high-molecular-weight material seen under nonreducing conditions was resolved into monomeric forms of the various Env proteins under reducing conditions.

The faster migration of gp90 compared to the SU-TM complex in nonreducing SDS-PAGE has not been reported before. It could be explained by a more extended conformation of the SU-TM complexes than the precursor after SDS denaturation. Alternatively, the migration difference could be due to differences in sugar modifications. Though it is known that the SU subunit of the SU-TM complex and gp90 contain both complex N-linked sugar units and also O-linked units, it is unclear whether the modifications occur to the same extent (10, 35). A detailed knowledge of the glycosylation status of the SU-TM complex and the precursor was regarded as essential for correct interpretation of the mechanisms of a possible late activation of the disulfide isomerase function of Env. Therefore, we compared the gel migration of gp90 and the SU-TM complex after enzymatic deglycosylations.

gp90 and the SU-TM complex are similarly modified by glycosylation. First, the trimming of N-linked sugar units was assessed using digestion with endo-H. This is specific for high-mannose forms of the N-linked sugar units. For this purpose MOV-3-cell cultures were incubated in the presence or absence of 20 μM FIP for 1.5 h, pulse-labeled with [^{35}S]Cys for 15 min, and then chased for 5, 60, 90, or 120 min. The cultures were lysed in the presence of NEM and Env proteins captured by the anti-SU pAb for endo H digestion. The products were analyzed in 7% nonreducing and reducing SDS-PAGE. Control samples were mock treated without endo H. Analyses of the control samples from FIP-treated cultures under nonreducing conditions did not clearly resolve gp90 from the SU-TM complexes, but this appeared as a broadening of the mature Env band (Fig. 2A, lanes 4 to 6) compared to the untreated control (Fig. 2A, lane 2). In this gel the proteins of high molecular weight resolved as apparent Env dimers and trimers. The gp90 was revealed after reduction, which released the subunits of the SU-TM complexes (Fig. 2D, lanes 4 to 6). Note that the SU migrated closely in front of the gp80 band and that the TM was not resolved by the 7% gel. When the samples were treated with endo H and analyzed under nonreducing conditions, the migration of both gp90 and the SU-TM complex were left largely unaffected in contrast to gp80, which increased its gel mobility significantly (Fig. 2B). This is seen as an increased gap between the broad SU-TM complex/gp90 band and the gp80 band compared to the corresponding bands in Fig. 2A. In the reducing gel analyses, the endo H-treated gp90 and the SU subunit, which was released from the SU-TM complex, migrated essentially as in the control, whereas the endo H-treated gp80 moved faster (Fig. 2E). Note that under these conditions the diffuse SU band is well separated from the faster gp80. Thus, these analyses showed that the N-linked sugar units in gp90 have been trimmed beyond the high-mannose stage like those of the mature Env.

The corresponding pulse-labeled and chased samples of viral proteins from FIP-treated and nontreated cells were then subjected to incubation with PNGase F. This treatment removes the entire N-linked sugar unit regardless of its stage of trimming. In this case the gel analyses showed that all Env forms increased their mobility significantly (Fig. 2C and F) compared to the control analyses (Fig. 2A and D). However, neither the mature Env nor the gp90 comigrated with gp80. In the nonreducing gel, the mature Env band migrated noticeably more slowly than the gp80 band, whereas the gp90 closely trailed the gp80 band (Fig. 2C, lanes 2 and 4 to 6). In the reducing gel, gp90 was seen as a distinct band above that of the gp80 (Fig. 2F, lanes 4 to 6). The SU, released from the mature Env by reduction, migrated faster than gp80 (Fig. 2F, lanes 2 and 4 to 6). These analyses showed that both the mature Env and the gp90 carry modifications, in addition to the processed forms of the N-linked sugars, which are not present in gp80.

The presence of O-linked sugars was analyzed by a combined treatment of the 60-min chased virus protein sample from FIP-treated and nontreated cells with PNGase F, neuraminidase and endo-α-*N*-acetyl-D-galactosaminidase (*O*-glycosidase). Neuraminidase was included to remove any terminal sialic acid residues in the O-linked sugars and was found to be required for O-deglycosylation of both gp90 and mature Env as described before (data not shown) (35). The neuraminidase–*O*-glycosidase treatment of the PNGase F-treated gp90 shifted its migration from being slightly slower than similarly treated or solely PNGase F-treated gp80 to be identical with the latter under both reducing (Fig. 2G, lanes 2 and 4) and nonreducing conditions (Fig. 2G, lanes 6 and 8). This confirmed the earlier finding that gp90, but not gp80, is modified by O-glycosylation (35). Similarly, the SU, released from the N- and O-deglycosylated SU-TM complexes by reduction, showed increased mobility compared to SU released from only N-deglycosylated complexes (Fig. 2G, lanes 1 and 3). Note that the PNGase F-treated sample of cells that were not incubated with FIP also revealed some apparent gp90. This is most evident under reducing conditions and probably reflects precursors that have matured into the late form but have not yet been cleaved (Fig. 2G, lane 1) (29). Most importantly, the N- and O-deglycosylated SU-TM complexes still migrated significantly more slowly under nonreducing conditions than the similarly deglycosylated gp90 and gp80 proteins (Fig. 2G, lanes 5 and 7). This shows that differences in the extent of N- and/or O-glycosylation cannot explain the differences in gel migration. Instead, the slower migration of the SU-TM complexes under nonreducing conditions is probably due to a less compact protein structure after SDS denaturation than the precursor.

A tryptic peptide assay to follow the isomerization of the Cys336(339)-Cys563 disulfide bond in newly synthesized Env. Earlier we developed a tryptic peptide assay to follow isomerization of the SU-TM disulfide bond in Env of amphotropic

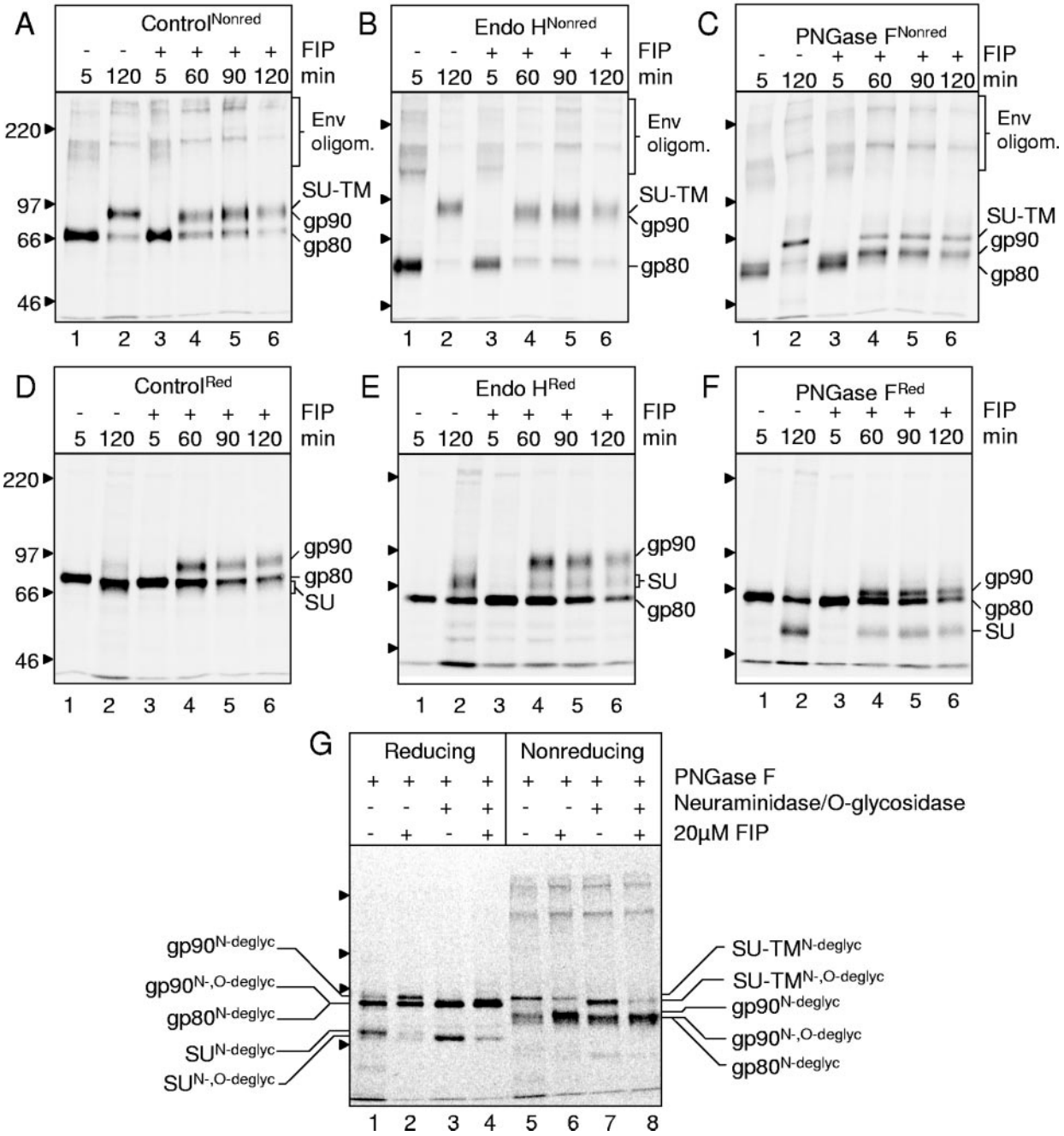
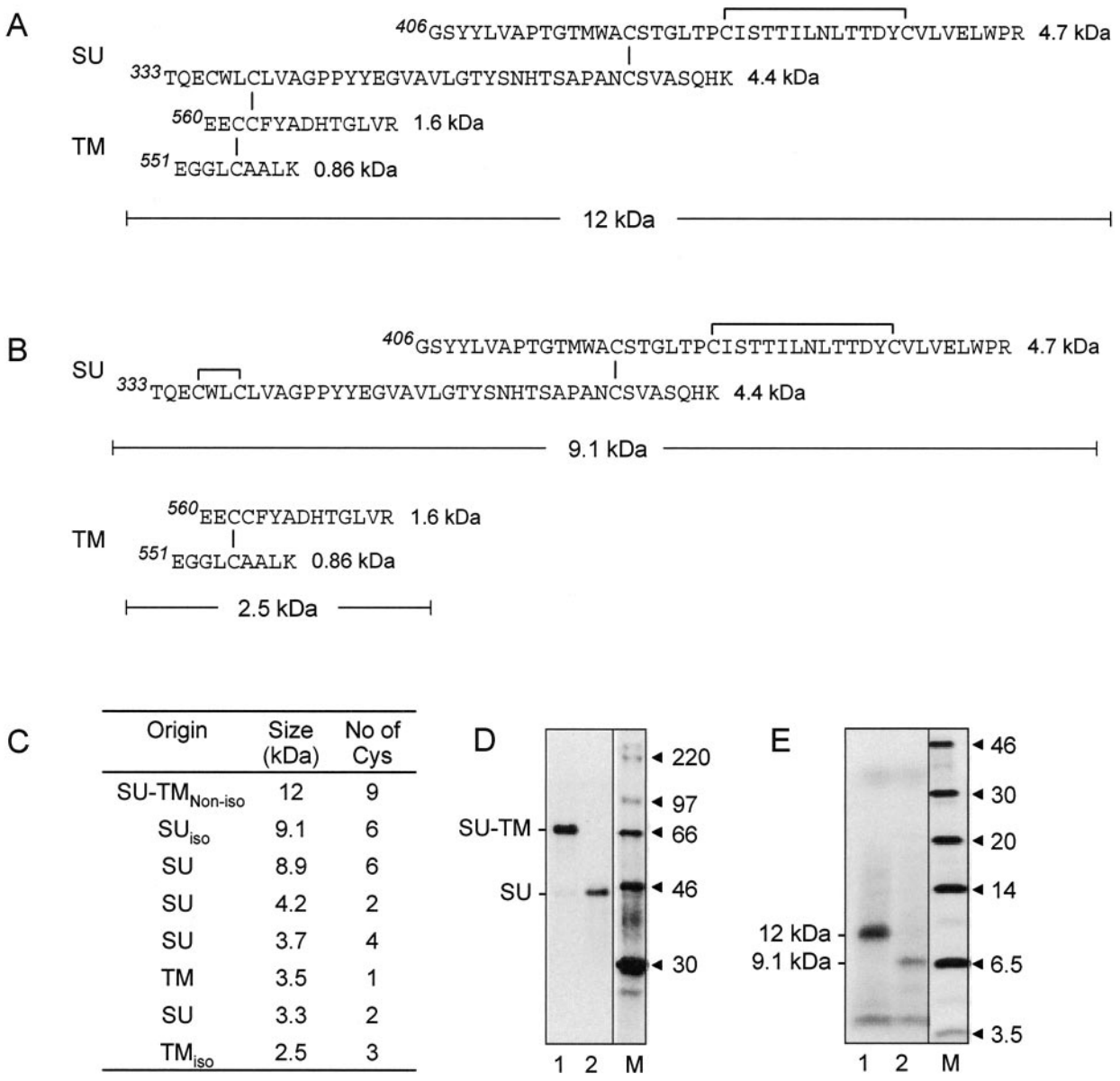


FIG. 2. Modification of Env proteins by glycosylation. MOV-3 cell cultures were treated without or with 20 μM FIP, pulse-labeled for 15 min, chased for 5 to 120 min (A to F) or 60 min (G) and lysed in the presence of NEM. Env proteins were immunoisolated and subjected to mock, endo H or PNGase F treatment (A to F) or treated with a combination of PNGase F, neuraminidase, and *O*-glycosidase (G) and subsequently analyzed by nonreducing (A to C and G, lanes 5 to 8) or reducing (D to F and G, lanes 1 to 4) 7% (A to F) or 8% (G) SDS-PAGE in 8-cm gels. The FIP treatment, time of chase, and deglycosylation conditions are indicated in each panel. The molecular weight standards (arrowheads), the Env proteins (Env oligom), and in panel G the deglycosylation mode (deglyc) of the proteins are indicated.

and Friend MLV (48). This was based on the migration difference in SDS-tricine-PAGE of the major disulfide-linked tryptic peptide complex of Env that either had or had not been subjected to in vitro induced isomerization of the SU-TM disulfide bond. We used a similar assay to study the presence of the corresponding disulfide bond, i.e., the Cys336(339)-Cys563 bond, in gp80 and gp90 of Mo-MLV. The disulfide bond status of the mature Env of Mo-MLV has not been determined, but as the Mo-MLV Env has 84.2% amino acid sequence identity with Friend MLV, including all Cys residues in the signal sequence-cleaved polypeptide chain, this is most likely identical with that of Friend MLV Env (34). In the latter protein all Cys residues but the CXXC thiol and a palmitoylated Cys residue in the transmembrane domain of TM are



C

Origin	Size (kDa)	No of Cys
$SU\text{-}TM_{Non\text{-}iso}$	12	9
SU_{iso}	9.1	6
SU	8.9	6
SU	4.2	2
SU	3.7	4
TM	3.5	1
SU	3.3	2
TM_{iso}	2.5	3

FIG. 3. A tryptic peptide assay to follow isomerization of the Cys336(339)-Cys563 disulfide bond. Panel A shows the primary structure of the predicted disulfide linked tryptic peptide complex, encompassing the intersubunit Cys336(339)-Cys563 disulfide bond, of the native SU-TM complex, and panel B shows the corresponding structures of isomerized Env. The calculated molecular weights of the complexes and included peptides are indicated. The N-terminal amino acid residues of the peptides are numbered. Panel C lists all predicted Cys-containing tryptic peptides and peptide complexes of native SU-TM and isomerization-released SU under nonreducing conditions. Non-iso and iso denote peptide complexes that are specific for the nonisomerized and isomerized states, respectively. (D) Nonreducing 12% SDS-PAGE of gel-purified, N-deglycosylated and [^{35}S]Cys-labeled SU-TM complexes and SU subunits from cell lysates subjected to nonisomerizing and isomerizing conditions, respectively. (E) Analyses of disulfide-linked tryptic peptide complexes from the SU-TM complexes and SU subunits above. The gel-purified, N-deglycosylated proteins were subjected to complete trypsin digestion, and the peptide products were analyzed by SDS-tricine-PAGE.

disulfide linked (15, 26, 36). After isomerization of the intersubunit disulfide bond, the CXXC thiol forms the CXXC disulfide, whereas the TM Cys residue of the prior SU-TM disulfide exposes a free thiol (7, 48). Therefore, mature Env of Mo-MLV is predicted to yield a major deglycosylated disulfide-linked complex of tryptic peptides of 12 kDa, which contains nine Cys residues (Fig. 3A and C). This includes a 4.4-kDa peptide from the SU and a 1.6-kDa peptide from the TM, which participate in the formation of the intersubunit disulfide bond in Env, and the covalently associated 4.7-kDa SU and the 0.86-kDa TM peptides. In contrast, the isomerized Env is predicted to yield a major deglycosylated 9.1-kDa tryptic peptide complex with six Cys residues encompassing only the SU peptides of 4.4 and 4.7 kDa (Fig. 3B and C). In addition, both the isomerized and the nonisomerized Env sample should yield one larger and several smaller peptide complexes. The larger,

an 8.9-kDa peptide complex with six Cys residues, is derived from the tightly disulfide cross-linked N-terminal receptor binding domain. However, as was earlier shown for the amphotropic MLV receptor binding domain, this should form a compact structure that migrates much faster than expected from its molecular weight in nonreducing SDS-tricine-PAGE (48). The predictions were tested using the mature Env of Mo-MLV. To this end, MOV-3 cell cultures were pulse-labeled with [^{35}S]Cys for 15 min, chased for 60 min, and then lysed on ice for 120 min in the presence or absence of NEM to prevent or allow NP-40-induced isomerization of the SU-TM disulfide bond. The Env proteins were immunoisolated and subjected to nonreducing 8% SDS-PAGE. In this analysis the SU-TM complexes from the nonisomerized sample run slightly more slowly than any remaining gp90, whereas the free SU subunits from the isomerized sample run closely in front of gp80 (data not shown). The SU-TM complexes and the SU subunits, with contaminating gp90 and gp80, respectively, were cut out, extracted from the gel, and concentrated by ultrafiltration. The samples were then subjected to PNGase F treatment and a second gel purification. In their N-deglycosylated form, the SU-TM complexes separate from contaminating gp90 (Fig. 2C) and SU from gp80 (data not shown). The isolated N-deglycosylated SU-TM complexes and SU subunits were found to be essentially pure, apart from a minor amount of SU in the former preparation, a result of a limited artificial reduction of SU-TM complexes during sample preparation (Fig. 3D, lanes 1 and 2) (48). The preparations were treated with trypsin, and the mixtures of disulfide-linked tryptic peptide complexes were analyzed by nonreducing SDS-tricine-PAGE. The tryptic digest of nonisomerized Env, i.e., the SU-TM complexes, revealed a major labeled band migrating between the reduced 6.5- and 14-kDa markers (Fig. 3E, lane 1). In contrast, the tryptic peptide complexes derived from isomerized Env, i.e., the SU subunits, showed a major band with faster migration, close to the reduced 6.5-kDa marker (Fig. 3E, lane 2). This suggested that the two major bands indeed corresponded to the predicted major peptide complexes, thus reflecting the isomerization status of Env. In addition both analyses showed the presence of a similar set of faster migrating bands. These probably corresponded to the additional complexes of peptides that were generated by the digestion. Thus, the results were very similar to the corresponding analyses of amphotropic and Friend MLV Env, both of which demonstrated a similar migration shift of their major disulfide-linked tryptic peptide complexes upon isomerization (48). We conclude that we have established an assay by which we should be able to follow the presence or absence of the Cys336(339)-Cys563 disulfide bond in Mo-MLV Env proteins that have not been processed by furin cleavage.

The Cys336(339)-Cys563 disulfide bond isomerization is suppressed in gp80 and gp90. In the absence of alkylator, NP-40 solubilization acts as an efficient trigger of the free CXXC thiol in SU to attack the intersubunit disulfide bond of the SU-TM complex and cause its rearrangement into the CXXC-containing disulfide bond isomer (32, 48). Therefore, we used NP-40 solubilization to study the activity of the isomerase in gp80 and gp90. First we determined the threshold condition to induce isomerization of mature Env with NP-40. To this end, MOV-3 cell cultures were pulsed for 15 min, chased for 60 min, and lysed in the absence of alkylator on ice for increasing times. After NEM addition and a brief incubation at 30°C, Env was captured by immunoprecipitation with an SU-specific pAb, and the degree of isomerization was determined by nonreducing 12% SDS-PAGE. The isomerization was followed as a decrease in covalently linked SU-TM complexes and an increase in free SU (Fig. 4). The analyses showed that only about 15 min of incubation was required for half-maximal isomerization of the intersubunit disulfide bond of mature Env (Fig. 4, lane 6) and that most of it was isomerized by 90 min (Fig. 4, lane 10). Based on these results, we tested the possible isomerization activation in gp80 and gp90 by 120-min incubations in lysis buffer on ice. The isomerization sensitivity of the Cys336(339)-Cys563 disulfide bond was analyzed by nonreducing SDS-tricine-PAGE of respective disulfide-linked tryptic peptide complexes. In the latter assay, isomerization is accompanied by a shift of the 12-kDa tryptic peptide complex into the 9.1-kDa tryptic peptide complex. Thus, gp80 was isolated from MOV-3 cells that had been pulse-labeled for 15 min with [^{35}S]Cys and chased for 60 min. gp90 was from cells that had been treated with 20 μM FIP for 1.5 h before being labeled and chased as the gp80. The cells were lysed in the presence or absence of NEM to prevent or allow NP-40-induced isomerization of the Cys336(339)-Cys653 intersubunit disulfide bond. The Env proteins were captured by immunoprecipitation and separated in nonreducing 7% SDS-PAGE. Regions of the gel containing the respective proteins were cut out, extracted, N-deglycosylated, and subjected to a second gel purification. This separated gp90 from any contaminating SU-TM complexes (Fig. 2C) and gp80 from contaminating SU subunits. The gp80 and gp90 were concentrated, and their purity was verified by 12% SDS-PAGE (Fig. 5A). These analyses also showed that the gel migrations of the precursors were not significantly affected by isomerization promoting or inhibiting treatments. The purified Env precursors were completely digested by trypsin and subsequently analyzed by SDS-tricine-PAGE. Digestion of the precursors that were prepared under

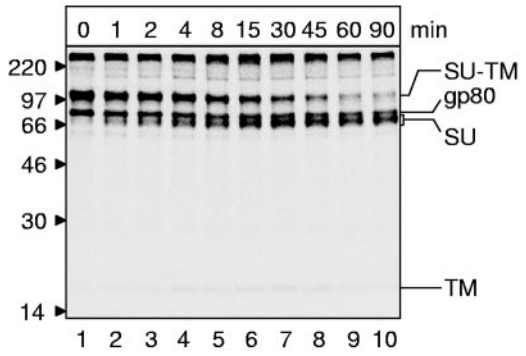


FIG. 4. Kinetics of NP-40-triggered isomerization of the intersubunit disulfide bond in SU-TM complexes. MOV-3 cell cultures were pulse-labeled for 15 min, chased for 1 h, and lysed under isomerizing conditions (in the absence of NEM) at 0°C for 0 to 90 min. NEM was added, the samples were incubated for 10 min at 30°C, and the Env proteins were immunoisolated and analyzed in nonreducing 12% SDS-PAGE. Note that the TM subunit is not recognized by the SU-specific pAb used. The minor amount of free TM present in the gel results from artificial reduction of the intersubunit disulfide bond in a minor portion of the remaining SU-TM complexes.

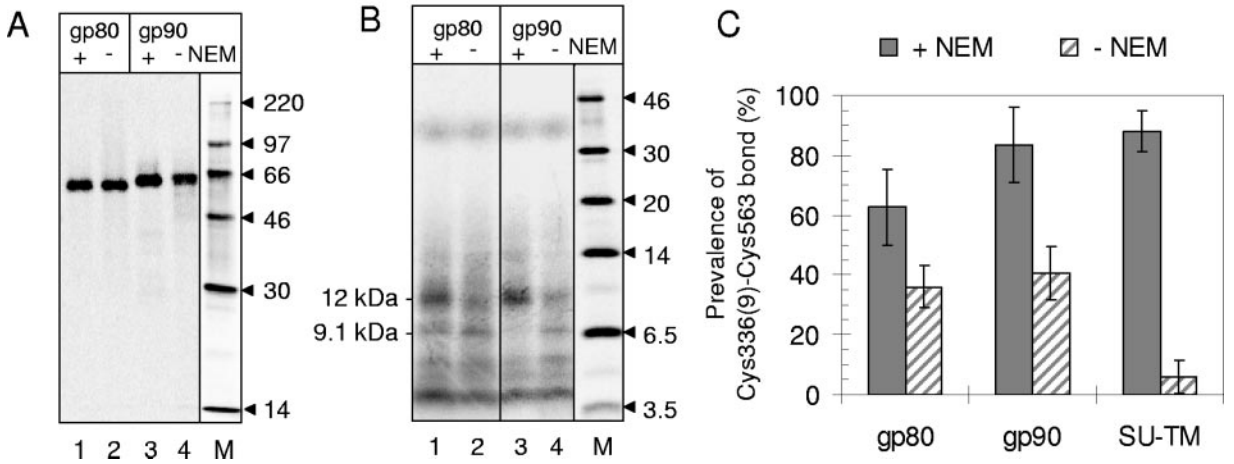


FIG. 5. Suppression of the Cys336(339)-Cys563 disulfide bond isomerase activity in gp80 and gp90. (A) Purified [^{35}S]Cys-labeled gp80 and gp90 from cell lysates subjected to nonisomerizing (+NEM) or isomerizing (−NEM) conditions. The gel-purified and N-deglycosylated precursors were analyzed in 12% SDS-PAGE under nonreducing conditions. (B) Disulfide-linked tryptic peptide complexes from Env precursor proteins, gp80 and gp90, prepared under nonisomerizing (+NEM) or isomerizing (−NEM) conditions. The N-deglycosylated gp80 and gp90, shown in panel A, were subjected to trypsin digestion and analyzed by SDS-tricine-PAGE. Lanes M (A and B), molecular weight marker proteins with their sizes (in kDa). (C) Prevalence and stability of the Cys336(339)-Cys563 disulfide bonds in mature Env and its precursors. The Cys336(339)-Cys563 disulfide bond of gp80 and gp90 was analyzed by the tryptic peptide assay described in panel B. The amounts of the 12-kDa complexes [molecules containing the Cys336(339)-Cys563 disulfide bond] and 9.1-kDa complexes (molecules containing the internal Cys336-Cys339 disulfide bond) were measured. The relative amounts of the 12-kDa complexes in digests of precursors incubated under nonisomerizing (+NEM) or isomerizing (−NEM) conditions, are given as means ± standard deviations ($n = 4$). The intersubunit Cys336(339)-Cys563 disulfide bond in mature Env (SU-TM) prepared under nonisomerizing and isomerizing conditions by solubilization for 120 min on ice was analyzed in 12% SDS-PAGE under nonreducing conditions (compare Fig. 4, lanes 1 and 10). The amounts of intact SU-TM complexes [containing the Cys336(339)-Cys563 disulfide bond] and free SU subunits [without the Cys336(339)-Cys563 disulfide bond] were measured. Shown are the relative amounts of intact complexes obtained under nonisomerizing and isomerizing conditions (mean ± standard deviations; $n = 6$).

nonisomerizing conditions (with NEM) generated mostly the 12-kDa disulfide-linked tryptic peptide complex isomer (63% ± 13% [$n = 4$] with gp80 and 84% ± 13% [$n = 4$] with gp90) (Fig. 5B, lanes 1 and 3, and C, gp80 and gp90 with NEM). Thus, the majority of the precursor molecules contain the Cys336(339)-Cys563 disulfide bond. The low value for gp80 is most likely an underestimate since the gp80 preparation is probably contaminated with some arrested folding intermediates of gp80 (ITgp80; see below) that lack this disulfide. Digestion of precursors prepared under isomerizing conditions (without NEM) yielded a reduced amount of the 12-kDa and an increased amount of the 9.1-kDa complex; the amount of the 12-kDa complex was 36% ± 7% ($n = 4$) for gp80 and 41% ± 9% ($n = 4$) for gp90 (Fig. 5B, lanes 2 and 4, and C, gp80 and gp90 without NEM). Based on this, the relative stability of the Cys336(339)-Cys563 disulfide bond in the precursors was calculated (i.e., the relative amount of 12-kDa complex under isomerizing conditions as a percentage of that under nonisomerizing conditions) and found to be 58% and 49% for the gp80 and gp90, respectively. For comparison we measured the prevalence and stability of the intersubunit disulfide bond in the mature, cleaved Env. To this end pulse-labeled and chased MOV-3 cells were incubated in NP-40 lysis buffer under nonisomerizing (with NEM) or isomerizing (without NEM) conditions, as described. The Env proteins were immunoprecipitated and analyzed in 12% SDS-PAGE, and the amounts of intact SU-TM complexes and free SU in the different samples were measured. Under nonisomerizing conditions, 88% ± 7% of the Env molecules contained an intact SU-TM disulfide bond (Fig. 5C, SU-TM with NEM), while under isomerizing conditions only 6% ± 5% intact SU-TM disulfide bonds remained (Fig. 5C, SU-TM without NEM). The relative stability of the intersubunit disulfide bond was 7%. Consequently, the isomerase is at least about eight times more resistant to NP-40-induction in the precursor than in the mature SU-TM complex. We conclude that the cleavage of the Env precursor by furin potentiates its CXXC-linked disulfide isomerase for activation.

In order to corroborate this major conclusion, we analyzed the isomerization sensitivity in gp90 and SU-TM complexes using heat triggering instead of NP-40. Nonspecific protein perturbation treatments like those of heat, urea, or guanidinium hydrochloride have earlier been shown to trigger isomerization, suggesting that destabilization of Env mediates its triggering (46). To this end, MOV-3 cells were pulse-labeled with [^{35}S]Cys for 15 min and chased for 60 min. The cells were pretreated and also chased in the presence of 20 μM FIP to accumulate Env in the gp90 form. Parallel cultures were processed in the absence of FIP to produce mature labeled SU-TM complexes. The cells were then heated to 53°C for 4 to 6 min to induce isomerization. Controls (i.e., samples at nonisomerizing conditions) were either heated in the presence of NEM (mature Env) or left without heat shock (gp90). After the heat shock the cultures were lysed in the presence of NEM, which prevented NP-40-induced isomerization, and Env immunoprecipitated for nonreducing SDS-PAGE. gp90 was eluted from the gel and further processed for analysis of disulfide-linked tryptic peptide complexes. Isomerization of mature Env was assessed directly from the gel. It showed that expanding the time of heat pulse induced an increasing release of SU

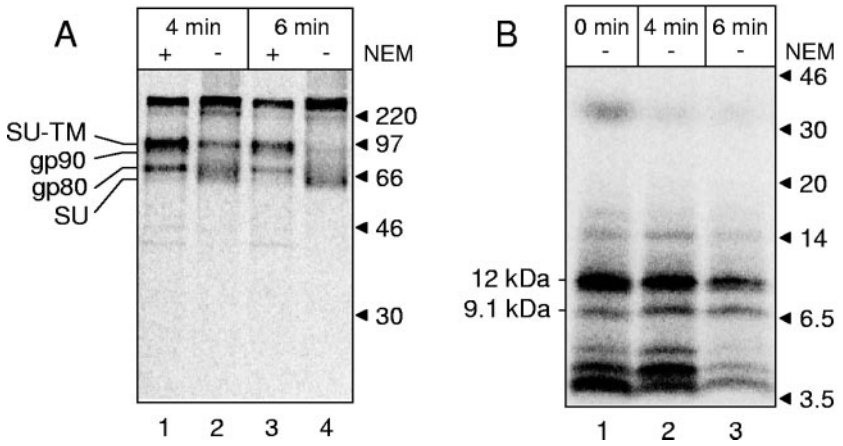


FIG. 6. Suppression of heat-induced isomerization in gp90. MOV-3 cells were treated with 20 μM FIP for 1.5 h, pulse labeled with [^{35}S]Cys for 15 min, and chased for 60 min in the presence of FIP. Parallel cultures were pulse labeled in the absence of FIP. The cells were then heated to 53°C in the presence or absence of NEM as indicated. After this, the cultures were lysed in the presence of NEM, and Env proteins were immunoprecipitated and analyzed by nonreducing SDS-PAGE. The analyses of pulse-labeled Env proteins from cultures incubated without FIP are shown in panel A. Note the efficient induction of isomerization in SU-TM complexes by heat. The gp90 in FIP-treated cultures was eluted from the gel, N-deglycosylated, gel isolated a second time, concentrated, trypsin digested, and analyzed by SDS-tricine-PAGE (B). Note the resistance of the predominant 12-kDa peptide complex to heat-induced isomerization.

from the SU-TM complexes (Fig. 6A). This confirms that the heat-induced isomerization effect observed previously in viral Env is also found in cell-associated Env. After a heat pulse of 4 min, mature Env in the NEM-treated sample had released 8% of the SU (Fig. 6A, lane 1). Under alkylation-free (isomerizing) conditions, the free form was increased to 48% (Fig. 6A, lane 2). After a heat pulse of 6 min, 16% of free SU was found in the presence of NEM. This increased to 84% under isomerizing conditions (Fig. 6A, lane 4). The relative stability of the intersubunit disulfide bond was calculated and found to be 56% at 4 min of heat incubation and 19% at 6 min. In contrast, the tryptic peptide complex analyses of heat-treated gp90 showed predominantly the Cys336(339)-Cys563 disulfide-containing 12-kDa complex (Fig. 6B). Relative to the untreated control (Fig. 6B, lane 1), the stability of the Cys336(339)-Cys563 disulfide bond of gp90 was 90% and 85% after 4 and 6 min of heat incubation, respectively (Fig. 6B, lanes 2 and 3). Thus, the Cys336(339)-Cys563 disulfide bond was about 2 and 5 times more resistant in gp90 than in the mature form of Env after 4- and 6-min heat treatments, respectively. Two experiments with similar results were performed.

The generation of the Cys336(339)-Cys563 disulfide bond coincides with the formation of gp80 from an early folding intermediate. The maturation of the different forms of Env after synthesis was followed in a pulse-chase experiment by analyzing PNGase F-treated viral proteins by reducing and nonreducing 7% SDS-PAGE. The pulse time with [^{35}S]Cys was limited to only 5 min in order to optimize resolution of intermediate forms of Env during the chase. Analysis of the N-deglycosylated gp80 immediately after the pulse and after 5 min of chase showed that the earliest form of gp80 was retarded in its gel migration under nonreducing (Fig. 7A, lanes 1 and 2) but not under reducing conditions (Fig. 7B, lanes 1 and 2). This suggested that the newly synthesized gp80 chains had not yet completed their folding into a correctly disulfide-linked (oxidized) structure but still persisted as folding intermediates (ITgp80). The mature Env, which was cleaved into disulfide-linked SU and TM subunits, appeared in the 30- to 90-min chased samples. This was in the nonreducing SDS-PAGE evidenced by the slowly migrating covalently linked SU-TM complexes (Fig. 7A, lanes 4 to 6) and by the free SU in the reducing gel (Fig. 7B, lanes 4 to 6). The latter analysis also revealed some gp90. This was most likely formed from gp80 by O-glycosylation and transiently revealed before cleavage into mature Env. In the nonreducing SDS-PAGE, gp90 probably constituted a significant part of the material migrating slightly more slowly than gp80 in the 30- to 90-min chased viral protein samples (Fig. 7A, lanes 5 and 6). The various Env forms seen in the nonreducing SDS-PAGE were quantified, and their interrelations at different time points were compared. The comigrating ITgp80 and gp90 were differentiated by calculating the gp80/gp90 ratios under reducing conditions and assuming the same ratio under nonreducing conditions. The results supported an Env maturation pathway from ITgp80 to mature Env via first gp80 and then gp90 (Fig. 7C).

In order to determine whether the Cys336(339)-Cys563 disulfide bond was present or not in ITgp80, we analyzed its disulfide-linked tryptic peptide complexes. ITgp80 was isolated from MOV-3 cells that had been pulse-labeled for 5 min with [^{35}S]Cys. The cells were lysed in the presence of NEM to prevent NP-40-induced isomerization of the Cys336(339)-Cys563 disulfide bond, and ITgp80 was immunoisolated, N-deglycosylated, and purified by a single gel separation. The isolated ITgp80 and the analysis of its disulfide-linked tryptic peptide complexes are shown in Fig. 7D and E. The latter analysis shows that digestion of ITgp80 predominantly generates the 9.1-kDa tryptic-peptide complex. The lack of significant amounts of the 12-kDa peptide complex indicates that the Cys336(339)-Cys563 disulfide bond is not present in most ITgp80 molecules. Consequently, the formation of the intersubunit disulfide bond is a posttranslational event that mediates the transition of ITgp80 into gp80.

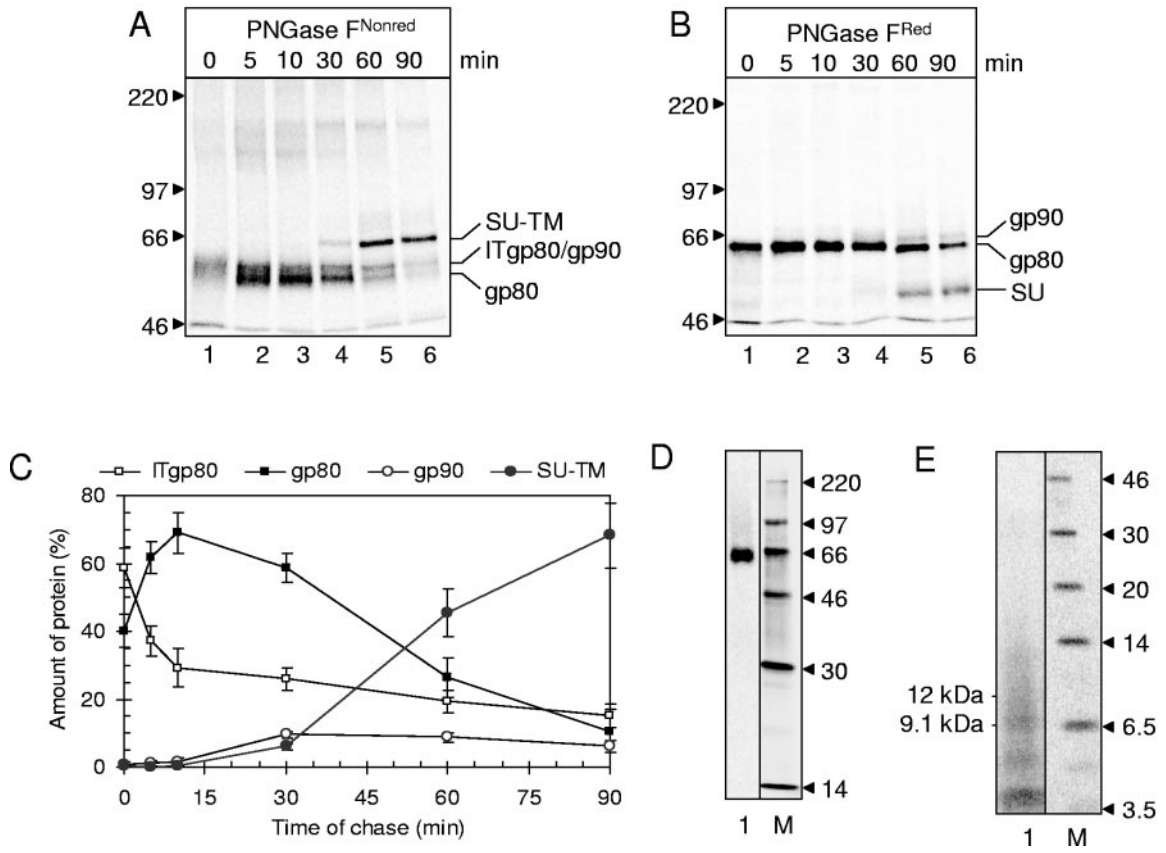


FIG. 7. Posttranslational formation of the Cys336(339)-Cys563 disulfide bond. (A to C) Pulse-chase analyses of newly synthesized Env. MOV-3 cell cultures were pulse labeled for 5 min, chased for 0 to 90 min, and lysed in the presence of 5 mM NEM for 2 h on ice followed by 10 min at 30°C. The Env proteins were immunoisolated and analyzed by 7% SDS-PAGE under nonreducing (A) or reducing (B) conditions. The different forms of Env were quantified, and their relative amounts at different chase times were calculated. Shown are the averages of four separate experiments ± standard deviation (C). (D) Purified [^{35}S]Cys-labeled and N-deglycosylated ITgp80 prepared under nonisomerizing conditions analyzed in 12% SDS-PAGE under nonreducing conditions. (E) Analyses of disulfide-linked tryptic peptide complexes of gel-purified and N-deglycosylated ITgp80. The ITgp80 preparation (D) was subjected to complete trypsin digestion and analyzed by nonreducing SDS-tricine-PAGE.

DISCUSSION

The general strategy by which enveloped viruses control their membrane fusion function is to produce an inactive precursor form of the membrane fusion protein and then use a two-step activation mechanism. In the first one, furin in the infected cell cleaves the precursor into the TM and the peripheral subunits at a late stage during its transport to the plasma membrane. This liberates the fusion peptide at the N-terminal end of TM. Furthermore, the cleavage generates a metastable state in the TM. This subunit likes to adopt a stable conformation via fusion-active intermediates but is hindered in doing so by its interaction with the peripheral subunit. In the second step of fusion activation, receptor binding and/or low pH of the target cell dissociates or displaces the peripheral from the TM subunit so that the latter can express its fusion function. In the present study we show that the furin cleavage of the Env precursor of MLV also potentiates the CXXC-linked intersubunit disulfide bond isomerase in SU, which the virus uses for fusion activation at the cell surface. Thus, although representing a novel control mechanism of membrane fusion, the disulfide isomerase activity of MLV is subjected to the same tight regulation by furin cleavage as other fusion-controlling mechanisms.

The correlation between the isomerase activity and the furin cleavage was not straightforward to prove. As the SU-TM complex was found to migrate more slowly in SDS-PAGE than the late gp90 form of the Env precursor, we had to consider the alternate possibility that the potentiation was brought about by some late modification of the N- or O-linked sugar units of Env or late addition of O-linked sugars and not by the cleavage. It is well known that glycosylation in general affects protein folding, and a recent study shows also that N-linked sugar processing is required for the maturation of the bunyamwera virus (14, 30). However, we demonstrated that the migration difference was not due to glycosylation but most likely to the fact that the cleaved form obtains a more extended structure in complex with SDS under nonreducing conditions than the precursor. Mo-MLV Env is also modified by palmitoylation in its transmembrane peptide and in its cytoplasmic tail (15, 31, 52). However, these modifications occur already in the early gp80 form of the Env precursor and should therefore not be able to influence the change in the activation potential of the isomer-

ase, which occurs later during Env maturation, in the SU-TM complex.

The structural basis for the furin cleavage-mediated potentiation of the isomerase activity is yet unknown. However, it must involve the CXXC motif of the isomerase in the SU subunit. In order for the CXXC thiol to attack the SU-TM disulfide bond and cause the fusion activating disulfide bond rearrangement, it has to be deprotonated. This requires that there is a mechanism in Env that conditionally can lower the pK_a of the CXXC thiol from its uninfluenced value of about 8.3 to about neutral (24). In the case of the endoplasmic reticulum protein disulfide isomerase, structural and molecular dynamics studies have shown that an Arg side chain can temporarily move into the locale of the active site and thereby lower the pK_a of the C-terminal thiol of the CXXC motif. This will facilitate the reoxidation of the reduced protein disulfide isomerase by glutathione. In this view it is possible that the isomerase of Mo-MLV Env is similarly triggered by the approximation of an amino acid side chain that can influence the pK_a of the CXXC thiol. According to our model, this alteration in structure is potentiated by a structural change of Env that is caused by the precursor cleavage and finally triggered by receptor binding. As the final triggering can be done by treatments with heat, urea, guanidinium hydrochloride, Ca^{2+} depletion, and NP-40 solubilization, it is apparent that this step must involve the dissociation of interactions between protein domains in the Env oligomer. This might be necessary to allow a specific restructuring of the locale of the CXXC thiol. The potentiation of the isomerase triggering that is brought about by the precursor cleavage should be represented by a specific alteration of the Env structure. The cleavage liberates the N-terminal end of TM, with the fusion peptide, and the C-terminal end of SU. This should allow for both dissociation of previous interactions at the cleavage site and creation of new ones. In the case of influenza, hemagglutinin (HA) precursor cleavage takes place in a surface loop in the stem of the molecule. The fusion peptide at the end of the HA_2 subunit then seeks its way into a nearby groove where it will bury ionizable amino acid residues (4). This structural alteration of influenza HA completes the formation of the pH-sensitive metastable HA_1-HA_2 complex.

A trivial explanation for the differential NP-40-induced isomerization sensitivity of the precursor and the mature forms of Env would be the different solubilization properties with NP-40. It is reasonable to believe that the triggering of the isomerase by NP-40 must be related to structural effects (destabilization) caused by solubilization of the membrane-bound Env oligomer. Therefore, if the precursor is more difficult to solubilize, for instance due to different intracellular localization, than the mature form, this could explain the apparent isomerase suppression in the precursor. However, the late form of the precursor, the FIP-induced gp90, was almost equally suppressed as the early form although it is expected to largely share the same membranes, i.e., lipid environment, as the mature form of Env. Therefore, this trivial explanation appears unlikely. Furthermore, we confirmed the relative resistance of gp90 to isomerization compared to the mature Env using another isomerization induction treatment, i.e., heat (53°C).

Apart from the intersubunit disulfide isomerase, the MLV contains one additional novel mechanism to control its fusion activity. This is represented by the cleavage of the C-terminal end, the R peptide, of the TM cytoplasmic tail by the viral protease during or soon after virus budding (12, 41). Previous studies have shown that expression of the MLV Env gene in mouse 3T3 cells does not result in any significant cell-cell fusion if the region encoding the end of the cytoplasmic tail is deleted or specifically point mutated (18, 37, 38, 51). Furthermore, it has been demonstrated that MLV or MLV vector mutants with uncleaved R peptide, due to violation of the conserved amino acid sequence at the cleavage site, have greatly reduced capacity to infect target cells (22, 38). Thus, these results suggest that the end of the cytoplasmic tail of TM suppresses the receptor-mediated induction of the fusion function in MLV.

Interestingly, the isomerase of the cell-associated SU-TM complex was sensitive to NP-40 and heat (53°C)-mediated triggering despite retention of its R peptide. However, both incubation at 53°C and solubilization with NP-40 represent powerful isomerase induction treatments, which probably overrides the control mechanism of the R peptide. Indeed, it is possible that the isomerase function is potentiated in two steps, first by the furin cleavage of the late precursor form gp90 and then by viral protease cleavage of the TM cytoplasmic tail. The latter potentiating effect might be possible to study with Env on the cell surface or in virus particles using isomerase induction via Ca^{2+} depletion or receptor binding (47, 48). It should be noted that it was not possible to use these induction treatments in this study because of the intracellular localization of the Env precursor.

The MLV membrane fusion protein appears to be a finely tuned apparatus where the action center is localized around the Cys336(339)-Cys563 (or corresponding) disulfide bond. This is the place for the CXXC thiol catalyzed isomerization of the intersubunit disulfide bond and also the point in TM where the jackknife-like refolding might be initiated (21, 36, 48). It might also be close to the cleavage site as this potentiates the isomerase to become triggered. Therefore, the formation of this part of Env by polypeptide folding and its stabilization by Cys336(339)-Cys563 disulfide bonding should be a demanding task. Notably, it has been demonstrated that of the seven or eight N-linked sugar units in Mo-MLV or Friend MLV, only the conserved one just N-terminal to the CXXC motif in SU is critical for Env maturation (8, 20). It is possible that the sugar unit transferred to this site is a target for calnexin with associated endoplasmic reticulum chaperones, including oxidoreductases. Together these might furnish the action center ready, with the Cys336(339)-Cys563 disulfide bond in place, after the complete Env chain has first been translated. Such a mechanism has previously been demonstrated to be involved in the formation of disulfide bonds of influenza HA, including the Cys14-Cys466 disulfide, which corresponds to the intersubunit disulfide of mature HA (1, 5). Consistent with this view we found in the present study that the Cys336(339)-Cys563 disulfide bond of Mo-MLV Env was formed posttranslationally.

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