trans-Dominant Interference with Virus Infection at Two Different Stages by a Mutant Envelope Protein of Friend Murine Leukemia Virus

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A dominant negative mutant Friend murine leukemia virus (FMLV) env gene was cloned from an immunoselected Friend erythroleukemia cell. The mutant env had a point mutation which resulted in a Cys-to-Arg substitution at the 361st amino acid in the FMLV envelope protein (Env). The mutant Env was retained in the endoplasmic reticulum (ER) and accumulated because of its slow degradation. The NIH 3T3 cells expressing the mutant env were resistant to ecotropic Moloney MLV (MoMLV) penetration, suggesting that the mutant Env traps the ecotropic MLV receptors in the ER. When the mutant env gene was transfected into and expressed in the cells persistently infected with MoMLV, the wild-type Env was trapped in the ER, and the MoMLV production was suppressed. Thus, the mutant Env accumulating in the ER trans-dominantly and efficiently interfered with the ecotropic MLV infection at both the early and the late stages.

The retroviral env gene product (Env) is processed in the endoplasmic reticulum (ER) and transported to the Golgi complex for further modification and proteolytic cleavage into the surface protein (SU) and the transmembrane protein (TM) (15, 23, 32, 36). The Env protein that is correctly processed and expressed on the cell surface is assembled into the virion. Env on the virion mediates its binding to the host cell surface receptor and thereby triggers the infection (15). Thus, Env plays a crucial role in both the early and the late stages of retrovirus replication.

Retroviral Env protein is oligomerized in the ER, and its oligomerization is essential for its transport from the ER to the Golgi complex (8, 37). In proteins which form multimers, some mutants could form abnormal multimers with the wild-type proteins and would disturb normal function as dominant negative mutants (12). For example, a dominant negative Gag mutant of human immunodeficiency virus have previously been reported by Trono et al. (33). They showed that the mutants suppress the wild-type human immunodeficiency virus production and suggested the development of an antiviral strategy based on intracellular immunization (3). Because the surface expression of Env could be cytopathogenic, leading to syncytium formation (19, 31), a dominant negative mutant which inhibits not only the infectious virus formation but also the surface expression of Env is preferable for the antiviral strategy. Here, we present a dominant negative Env mutant of Friend murine leukemia virus (FMLV) which inhibits the processing and the surface expression of the wild-type Env.

Friend erythroleukemia cells produce FMLV, which is a member of the ecotropic MLV (10, 25). The initial FMLV env gene product in the ER is an endoglycosidase H (endo H)-sensitive Env precursor, SU-TM, detected as gPr90env. The SU-TM in the ER is transported to the Golgi complex to form an endo H-resistant modified SU-TM which migrates slightly more slowly than the SU-TM in the ER in gel electrophoresis (9, 17). The SU-TM modified in the Golgi complex is rapidly cleaved into SU (gp70) and TM [p15(E)], which are transported to the cell surface.

We previously obtained a mutant erythroleukemia cell clone, FR-6, by immunoselection with an anti-ecotropic MLV antiserum in the presence of complement (38). The immunoresistant FR-6 cells express a mutant FMLV Env, which is not proteolytically cleaved, and produce no FMLV. For this paper, we cloned the mutant env gene, analyzed the processing of its product, and examined the effect of its expression on ecotropic Moloney MLV (MoMLV) infection. Interestingly, the mutant Env in the ER trans-dominantly and efficiently interfered with MoMLV infection at two different stages.

MATERIALS AND METHODS

Cells and viruses. 745a is a Friend murine erythroleukemia cell line, which harbors FMLV and spleen focus-forming virus (SFFV) (6, 25). FR-6 is an immunoselected mutant cell line which survived the complement-dependent cytopathicity caused by an anti-ecotropic MLV antiserum, anti-FN2 (α-FN2) (38). NIH 3T3 cells were obtained from Y. Kuchino (National Cancer Research Center). The cells were cultured in Dulbecco’s modified minimal essential medium (DMEM) supplemented with 7% fetal bovine serum. MoMLV with an Escherichia coli supF gene in the long terminal repeat (LTR) was provided by R. Jaenisch (Whitehead Institute) (26). Amphotropic MLV was provided by the late W. P. Rowe.

RT-PCR amplification of env gene fragments. Total cellular RNA was subjected to reverse transcription (RT) and polymerase chain reaction (PCR) amplification as described before (28, 29). The primers were synthesized by an oligonucleotide synthesizer (Applied Biosystems Inc.). Positive-strand primers were PA391 (nucleotides [nt] 5391 to 5410 in FMLV [containing an EcoRI site], 5'-GGAGATTCCGCGTC TACCCCATATGAAA-3') and PB6603 (nt 6603 to 6622, 5'-GTCTTGCGAGCACAACCTTTC-3'). Negative-strand primers were NA6693 (nt 6693 to 6764 [containing a SacI
were fixed with volume of the sample amide gel electrophoresis Nitroplus 2000 Trans-blot (Bio-Rad) at 12 oxide nobenzidine-tetrahydrochloride cell surface polyclonal goat globulin G antibody (Cappel) NaCl). Triton X-100, 0.05% SDS, 0.5% sodium mM Tris-HCl, membrane was incubated method (21). Ten micrograms of total RNA cellular RNA was isolated by the extraction, and resuspended with TE (10 mM Tris-HCl, pH 8.0) Triton X-100, 0.05% SDS, 0.5% sodium glycine, 20% in PBS for 5 min at room temperature after fixation.

**Immunoprecipitation analysis.** Cells were plated at a density of 5 × 10^3 cells per 6-cm dish and grown overnight. The cells were incubated in the presence of 100 µCi of [3^2P]methionine (>1,000 Ci:mmol; Amersham) in 2 ml of methionine-free MEM (Nissui Seiyaku) at 37°C for 30 min. After labeling, the cells were washed with PBS and incubated in DMEM containing 7% fetal bovine serum at 37°C for various chase periods. The cells were then lysed with 0.6 ml of RIPA buffer. The lysates were precipitated with 100 µl of 10% protein A (Sigma) and 10 µl of normal rabbit serum at 4°C overnight and centrifuged at 18,500 x g for 3 min. The supernatants were incubated with the polyclonal goat anti-gp70 antibody at 4°C for 2 h and then with 50 µl of 10% protein A at room temperature for 15 min and centrifuged at 2,000 x g for 3 min. The immunoprecipitated pellets were resuspended in the sample buffer and separated through SDS-PAGE, and fluorography was performed. For analysis under nonreducing conditions, the cells were lysed with RIPA buffer containing 40 mM iodoacetamide to prevent disulfide bond formation after cell lysis, and immunoprecipitated pellets were resuspended in the sample buffer without 2-mercaptoethanol. The radioactivity of each band was assayed with an image analyzer (FUJIX, Bas-2000).

**RESULTS**

A Cys-361-to-Arg substitution in FMLV Env impairs its transport from the ER to the Golgi complex. The FMLV genomes including the whole env gene (nt 5391 to 7805 according to Friedrich's numbering [GenBank accession no. X02794]) were cloned from the parental Friend erythroleukemia cell line (745a) and the mutant FR-6. Briefly, two overlapping DNA fragments, nt 5391 to 6693 and 6603 to 7805, were amplified by RT-PCR from the total cellular RNA and ligated at the BspMI site (nt 6659) to reconstruct the FMLV env gene; the reconstructed cDNA from the parental 745a is referred as Fw (Friend wild type), and the cDNA from the mutant FR-6 is fcr (Friend Cys [C]-Arg [R] mutant, see below). Nucleotide sequencing of the cloned cDNA revealed a transition from T (745a) to C (FR-6) at nt 6960 of the FMLV env gene in FR-6, resulting in a substitution from Cys to Arg at the 361st amino acid (aa) in the FMLV Env (Fig. 1A). No other nucleotides, including the cleavage junction between SU and TM, were mutated.

To confirm that the Cys-361-to-Arg mutation in the FMLV Env was responsible for the FR-6 phenotype, the cloned FMLV env genes were expressed in NIH 3T3 cells. The cloned env fragments (nt 5391 to 7706) were introduced into FMLV env expression plasmids containing a selection marker, a hygromycin B-resistant gene, to obtain pHisFW (the wild-type FMLV env expression plasmid) and pHisFcr (the Cys-361-mutated FMLV env expression plasmid) (Fig. 1B). These FMLV env expression plasmids were transfected into NIH 3T3 cells by the calcium-phosphate method (29), and hygromycin B-resistant cell clones FW1 and FCr1 were obtained. FW1 cells express the wild-type FMLV env (fw), and FCr1 cells express the mutant FMLV env (fcr).

The Western blot analysis with the anti-gp70 antibody showed that the wild-type FMLV Env precursor SU-TM (gPr90^w^) in FW1 cells was cleaved into SU (gp70) (Fig. 2, lane 3), while the Cys-361-mutated SU-TM in FCr1 cells remained uncleaved (Fig. 2, lane 4). Therefore, the Cys-to-Arg substitution at the 361st aa in FMLV Env was responsible for the failure of SU-TM cleavage in FR-6.

### Isolation of RNA and Northern (RNA) blot analysis.

Total cellular RNA was isolated by the guanidine-hydrochloride method (21). Ten micrograms of total RNA was electrophoresed through 1% agarose gels, and Southern blot analysis was performed according to the standard procedure (29). The probes used for hybridization were E-XYB0.8, an ecotropic MLV env-specific Xba1-BamHI fragment of MoMLV (nt 5766 to 6537 according to Shinnick's numbering [30]). The probe was labeled with [α-^32P]dCTP by the random-primer method (29).

### Western blot (immunoblot) analysis.

Cellular proteins were extracted with RIPA buffer (20 mM Tris-HCl [pH 7.5], 1% Triton X-100, 0.05% SDS, 0.5% sodium deoxycholate, 150 mM NaCl) and were subjected to Western blot analysis (29). Briefly, the protein samples (20 µg each) mixed with an equal volume of the sample buffer (125 mM Tris-HCl, pH 6.8, 1% SDS, 10% mercaptoethanol, 20% glycerol, 1% bromophenol blue) were separated through 0.1% SDS-10% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a Nitroplus 2000 membrane (Micron Separations Inc.) with a Trans-blot (Bio-Rad) at 12 V for 25 min with transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine, 20% methanol). The membrane was incubated in BLOTTO (5% skimmed milk, 50 mM Tris-HCl, pH 8.0) with the polyclonal goat anti-gp70 (SU) antibody (National Cancer Institute lot 79S000713) overnight at room temperature and then was incubated with the peroxidase-conjugated rabbit anti-goat immunoglobulin G antibody (Cappel) for 90 min at room temperature. Immunoreacting proteins were visualized by 0.03% 3′,3′-diaminobenzidine-tetrahydrochloride and 0.018% hydrogen peroxide in Tris-buffered saline (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl).

### Immunofluorescent staining.

Cells grown on a glass slide were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS). The cells were incubated in BLOTTO with the polyclonal goat anti-gp70 antibody for 60 min at 37°C and then with the fluorescein-conjugated rabbit anti-goat immunoglobulin G antibody (Cappel) for 30 min at 37°C. The glass slide was mounted with 50% glycerol in PBS and inspected under a fluorescence microscope. For permeabilization of cell surface membrane, cells were incubated with 0.1% Triton X-100 in PBS for 5 min at room temperature after fixation.
Subcellular localization of Env protein was studied by immunostaining with the anti-gp70 antibody. Without the detergent treatment, fluorescence appeared on the cell surface in FW1 cells (Fig. 3A) but not in FCR1 cells (Fig. 3C). With the detergent treatment, fluorescence with coarse granular staining was found throughout the cytoplasm in FW1 cells (Fig. 3B). In FCR1 cells, however, fluorescence was localized in the perinuclear space and the granular staining was absent (Fig. 3D). A pattern similar to the latter was previously reported in the mutant Env with a defect in transport from the ER to the Golgi complex (37).

The env gene products were further examined by immunoprecipitation analysis with the anti-gp70 antibody. The transfected cells were labeled with 35S-methionine for 30 min and chased for various periods. The immunoprecipitates were subjected to SDS-PAGE. In both FW1 and FCR1 cells labeled for 30 min without chase, only SU-TM was detected (Fig. 4A, lanes 1 and 2). In FW1 cells expressing the wild-type FMLV Env, a band migrating slightly more slowly than SU-TM appeared after a 30-min chase (Fig. 4A, lane 5). This slightly larger molecule represents the SU-TM modified in the Golgi complex. After a 60-min chase, large numbers of FMLV Env proteins were cleaved into SU (Fig. 4A, lane 9). In FCR1 cells expressing the Cys-361-mutated Env, however, neither modified SU-TM in the Golgi complex nor SU appeared; only SU-TM in the ER was detected, even after a 60-min chase (Fig. 4A, lanes 3, 6, and 10), and the SU-TM was endo H sensitive (data not shown). Similar results were obtained when FW1 cells were treated with brefeldin A (BFA) (5 μg/ml), an inhibitor of protein transport from the ER to the Golgi complex (24, 34) (Fig. 4A, lanes 4, 8, and 12). From these results, we conclude that the intracellular transport of the Cys-361-mutated SU-TM from the ER to the Golgi complex was inhibited.

When the immunoprecipitates were analyzed under nonreducing conditions, the Cys-361-mutated SU-TM migrated slightly more slowly than the wild type (Fig. 4B, lanes 1 and 2). This result suggests that the Cys-361-mutated SU-TM takes an aberrant conformation because of incorrect intermolecular disulfide bond formation. In FW1 cells, a slowly migrating band (indicated as D) was detected in addition to SU, SU-TM, and modified SU-TM after a 30-min chase under nonreducing conditions (Fig. 4B, lane 3). This band might represent the SU homodimer or the heterodimer of SU and TM after proteolytic cleavage. The molecule was absent in both FCR1 cells and BFA-treated FW1 cells (Fig. 4B, lanes 4 and 6). Furthermore, a band with an extremely slow mobility (indicated as P) was detected in FCR1 lysates under nonreducing conditions (Fig. 4B, lane 2). This molecule must be an aggregate of the Cys-361-mutated SU-TM with intermolecular disulfide bonds. It was detected neither in FW1 cells nor in BFA-treated FW1 cells (Fig. 4B, lanes 1, 3, and 6).

The Cys-361-mutated Env accumulates in the ER. The fate of the Cys-361-mutated Env captured in the ER was studied by pulse-chase immunoprecipitation analysis. The analysis

[33-base-deleted env expression plasmid. (C) MoMLV env expression plasmid containing a neomycin-resistant gene. The HindIII (nt 4894-Clal (nt 7674) fragment from pArMLV-48 was introduced into the polylinker sequence in the Mo-2LTR plasmid. Because the Clal site in Mo-2LTR was located in the region encoding TM, the C-terminal 99 bases of env gene were derived from MoMLV, whose amino acid sequence in this region was identical to that of FMLV, except for the 638th amino acid (MoMLV, isoleucine; FMLV, leucine). pHMFW is the wild-type FMLV env expression plasmid, pHmFCr is the Cys-361-mutated env expression plasmid, and pHmFBA is the]
FIG. 2. Western blot analysis of cellular proteins from the transfected cells. Twenty micrograms of cellular proteins extracted from NIH 3T3 cells (lane 1), NIH 3T3 cells infected with Friend leukemia virus (FMLV and SFFV) (lane 2), FW1 cells (lane 3), FCrl cells (lane 4), and FBal cells (lane 5) were separated through SDS-PAGE (10% polyacrylamide) and analyzed with the anti-gp70 antibody. NIH 3T3 cells infected with Friend leukemia virus expressed FMLV Env (SU-TM and SU) and gp55 derived from SFFV (lane 2). Molecular mass markers are indicated in kilodaltons (K) at the right.

of a 30-min labeling without chase showed that similar amounts of FMLV Env proteins were produced in FW1 and FCrl cells and FBal-treated FW1 cells (Fig. 4A, lanes 1, 2, and 4). In FBal-treated FW1 cells, the uncleaved Env proteins in the ER were degraded rapidly, and only about 20% of them were detected after a 6-h chase (Fig. 4C, lanes 2 and 6, 4D). In FCrl cells, however, the Cys-361-mutated Env proteins were retained stably and about 80% of them could still be detected after a 6-h chase (Fig. 4C, lanes 3 and 7), and 4D). Consequently, the Cys-361-mutated Env accumulated because of its extremely slow degradation.

Furthermore, we compared these results with those of another FMLV Env-processing mutant. The mutant env, represented by fba (Friend BamHI site [nt 6547] deletion mutant), was missing 33 bases from nt 6548 to 6580, corresponding to 11 aa (224 to 234 in SU-TM) (Fig. 1A). There is no cysteine residue in the deleted sequence. It was obtained accidentally in another reconstruction experiment. The env gene with the deletion (fba) was ligated to the FMLV env expression plasmid and transfected into NIH 3T3 cells to obtain a hygromycin B-resistant cell clone, FBal. FBal cells expressed unprocessed SU-TM (Fig. 2, lane 5) and showed a

FIG. 3. Immunofluorescent staining of transfected cells with anti-gp70 antibody. In panels B, D, and F, cells were permeabilized by 0.1% Triton X-100 after fixation. (A and B) FW1; (C and D) FCrl; (E and F) FBal.

FIG. 4. Pulse-chase immunoprecipitation analysis of the transfected cells. Cell lysates labeled with [35S]methionine for 30 min were immunoprecipitated with the anti-gp70 antibody and separated through SDS-PAGE (10% polyacrylamide) under reducing (A and C) or nonreducing (B) conditions. (A) Lanes: 1 to 4, no chase; 5 to 8, 30-min chase; 9 to 12, 60-min chase; 1, 5, and 9, FW1; 2, 6, and 10, FCrl; 3, 7, and 11, FBal; 4, 8, and 12, FBal-treated FW1. (B) Lanes: 1 and 2, no chase; 3 to 6, 30-min chase; 1 and 3, FW1; 2 and 4, FCrl; 5, FBal; 6, FBA-treated FW1. The bands indicated as D and P are described in text. Arrowhead indicates modified SU-TM. (C) Lanes: 1 to 4, 3-h chase; 5 to 8, 6-h chase; 1 and 5, FW1; 2 and 6, FBal-treated FW1; 3 and 7, FCrl; 4 and 8, FBal. (D) The change in radioactivity in SU-TM during the pulse-chase period. The radioactivity levels of SU-TMs in panels A, lanes 2 to 4, and C, lanes 2 to 4 and 6 to 8, were counted with an image analyzer.
fluorescence pattern on immunostaining with the anti-gp70 antibody similar to that of FCrl (Fig. 3E and F). The immunoprecipitation analysis of FBa1 cells showed almost the same result as that of BFA-treated FW1; neither SU nor modified SU-TM in the Golgi complex was detected in FBa1 cells (Fig. 4A, lanes 3, 7, and 11). While similar amounts of Env proteins were produced in FCrl, FBa1, and BFA-treated FW1 cells (Fig. 4A, lanes 2, 3, and 4, respectively), the 11-aa-deleted Env proteins were degraded rapidly and only about 10% of them were detected after a 6-h chase (Fig. 4C, lanes 4 and 8, and 4D). These results are compatible with the result that far greater amounts of SU-TMs were detected in FCrl lysates than in FBa1 lysates by Western blotting (Fig. 2) and indicate that the inhibition of Env processing does not necessarily result in its accumulation; the accumulation of SU-TM in the ER is a unique feature of FCrl cells.

The Cys-361-mutated Env accumulating in the ER completely interferes with MoMLV infection. To examine the effect of accumulation of the Cys-361-mutated Env in the ER on ecotropic MLV infection, the transfected cells were superinfected with MoMLV. We used MoMLV with an E. coli supF sequence in the LTR (26) to distinguish the MoMLV-derived genome from the transfected FMLV env gene. NIH 3T3, FW1, FCrl, and FBa1 cells were infected with MoMLV to obtain NIH 3T3-MoMLV, FW1-MoMLV, FCrl-MoMLV, and FBa1-MoMLV cells. On the 7th day after the infection, the 24-h culture supernatants of these cells were harvested. MoMLV titers in these supernatants were assayed by the UV-XC test (27) with NIH 3T3 cells and are (in PFU per milliliter) as follows: NIH 3T3, 3 $\times$ 10$^3$; FCrl, 0; FBa1, 1 $\times$ 10$^3$; and FW1, 0. As shown, the supernatants of the FCrl-MoMLV and FW1-MoMLV cells contained no infectious MoMLV; i.e., the cells harboring the Cys-361-mutated Env in the ER were completely resistant to MoMLV infection, as were those expressing the wild-type Env protein on the cell surface.

As the control, the cells were infected with the amphotropic MLV, which belongs to a different interference group than the ecotropic MLV, and virus titers were examined by focus assay on 3T3-L2–mink cells in the 24-h culture supernatants harvested on the 7th day. The infectious virus titers of NIH 3T3 and FCrl cells infected with amphotropic MLV were $3 \times 10^4$ and $4 \times 10^4$ focus-forming units/ml, respectively. No resistance to the amphotropic MLV was conferred by the Cys-361-mutated Env. Thus, the inhibition of virus infection by the Cys-361-mutated Env is specific to the ecotropic MLV.

The resistance of FW1 cells to MoMLV infection can be explained by interference (35). In order to examine the possibility of interference in FCrl cells, we investigated extrachromosomal proviral DNA in the early phase of the infection. The Hirt’s supernatants (13) of these cells extracted 5 h after the infection were analyzed by Southern blotting with the ecotropic MLV env-specific probe E-XB0.8 (Fig. 5). Extrachromosomal MoMLV proviral DNA was detected in NIH 3T3-MoMLV and FBa1-MoMLV cells but not in FW1-MoMLV or FCrl-MoMLV cells. These results indicate that the Cys-361-mutated Env accumulating in the ER strongly interfered with MoMLV infection before the synthesis of MoMLV proviral DNA.

Expression of the Cys-361-mutated Env in MoMLV-infected cells suppresses virus production by inhibiting Env processing. To investigate the effect of the Cys-361-mutated Env on the wild-type Env processing, the mutant env gene was introduced into the cells persistently infected with MoMLV. Briefly, NIH 3T3-MoMLV cells which persistently produce MoMLV were transfected with fw, fcr, and fba expression plasmids (pHmFW, pHmFCr, and pHmFBA) and selected with hygromycin B to obtain the NIH 3T3-MoMLV cells expressing fw (referred to as NIH 3T3-MoMLV/pHmFW), the NIH 3T3-MoMLV cells expressing fcr (NIH 3T3-MoMLV/pHmFCr), and the NIH 3T3-MoMLV cells expressing fba (NIH 3T3-MoMLV/pHmFBA), respectively. The 24-h culture supernatants were harvested on the 14th day after the transfection, and the infectious virus titers were assayed by the UV-XC test and were as follows: NIH 3T3-MoMLV (NIH 3T3 cells persistently infected with and producing MoMLV), $4 \times 10^3$; NIH 3T3-MoMLV/pHmFCr (NIH 3T3-MoMLV cells transfected with Cys-361-mutated FMLV env), $6 \times 10^3$; NIH 3T3-MoMLV/pHmFBA (NIH 3T3-MoMLV cells transfected with the 33-base-deleted FMLV env), $6 \times 10^3$; and NIH 3T3-MoMLV/pHmFW (NIH 3T3-MoMLV cells transfected with wild-type FMLV env), $1 \times 10^3$. Expression of the wild-type FMLV Env or the 11-aa-deleted Env in MoMLV-infected cells had no significant effect on virus production. Expression of the Cys-361-mutated Env in MoMLV-infected cells, however, reduced virus production to 1/100 of that in the untransfected NIH 3T3-MoMLV cells.

To elucidate the inhibitory step of the MoMLV production in NIH 3T3-MoMLV/pHmFCr cells, we first examined the viral message. Total cellular RNAs were extracted from NIH 3T3-MoMLV, NIH 3T3-MoMLV/pHmFW, NIH 3T3-MoMLV/pHmFCr, and NIH 3T3-MoMLV/pHmFBA cells. Northern blot analysis with the supF-specific probe, which detects mRNAs only from MoMLV, showed the same amounts of MoMLV mRNAs in these cells (Fig. 6). The MoMLV expression was not disturbed at the transcripational level in NIH 3T3-MoMLV/pHmFCr cells.
The translation products of MoMLV env were studied by immunoprecipitation analysis with the anti-gp70 antibody (Fig. 7). MoMLV Env precursor SU-TM, gPr80 env, could be discriminated from the transfected FMLV Env SU-TM, gPr90 env, by the differences in their electrophoretic mobilities (5). Similar amounts of MoMLV SU-TMs were detected in NIH 3T3-MoMLV and NIH 3T3-MoMLV/pHmFCr cells labeled for 30 min (Fig. 7A, lanes 1 and 2). MoMLV env expression was not disturbed at the translational level in NIH 3T3-MoMLV/pHmFCr cells.

The immunoprecipitation analysis after a 30-min chase confirmed that MoMLV Env was cleaved into SU in NIH 3T3-MoMLV cells (Fig. 7A, lane 3). NIH 3T3-MoMLV/pHmFW and NIH 3T3-MoMLV/pHmFBa cell lysates also contained SU (Fig. 7A, lanes 5 and 6). In NIH 3T3-MoMLV/pHmFCr cells expressing both the wild-type MoMLV and the Cys-361-mutated Env, however, neither SU nor modified SU-TM in the Golgi complex was detected (Fig. 7A, lane 4; Fig. 7B, lane 2). Therefore, the wild-type MoMLV env expression was disturbed at the posttranslational level in NIH 3T3-MoMLV/pHmFCr cells; the Cys-361-mutated Env trans-dominantly inhibited the wild-type Env processing to suppress the MoMLV production.

The 11-aa-deleted Env, however, had no significant effect on the virus production. Thus, Env-processing mutants do not necessarily inhibit MoMLV infection; the trans-dominant inhibition is unique to the Cys-361-mutated Env.

The Cys-361-mutated Env trans-dominantly inhibits the transport of the wild-type Env from the ER to the Golgi complex. To confirm the dominant negative effect of the Cys-361-mutated Env on the wild-type Env processing in the absence of the other viral genes, a MoMLV env expression plasmid, pNeMoW, containing a neoresistant marker gene was reconstructed (Fig. 1C) and transfected into NIH 3T3 and FCr1 cells. The transfected cells were selected with G418 to obtain NIH 3T3 cells expressing the wild-type MoMLV env (referred as NIH 3T3/pNeMoW) and FCr1 cells expressing the wild-type MoMLV env (FCr1/pNeMoW).

The results of immunoprecipitation analysis with the anti-gp70 antibody of these cell lysates chased for 30 min after 30 min of labeling are shown in Fig. 7C. MoMLV SU-TM was cleaved into SU in NIH 3T3/pNeMoW cells, while it remained uncleaved in FCr1/pNeMoW cells. Furthermore, the wild-type MoMLV SU-TM-modified in the Golgi complex was detected in NIH 3T3/pNeMoW lysates but not in FCr1/pNeMoW lysates. Thus, the processing of the wild-type Env in FCr1/pNeMoW cells was disturbed before the SU-TM modification in the Golgi complex.

The subcellular localization of Env protein was then analyzed by immunostaining with the anti-gp70 antibody. NIH 3T3/pNeMoW cells (Fig. 8A and B) showed the surface expression of Env as FW1 (Fig. 3A and B), while FCr1/pNeMoW cells (Fig. 8C and D) showed the same fluorescence pattern as FCr1 cells (Fig. 3C and D). Therefore, it is confirmed that the intracellular transport of the wild-type env mutant.
MoMLV Env from the ER to the Golgi complex was trans-dominantly inhibited by the Cys-361-mutated Env accumulating in the ER.

DISCUSSION

Proper folding of the retroviral Env protein in the ER is essential for its oligomerization and its transport from the ER to the Golgi complex, and the disulfide bond plays an important role in the folding (2, 4, 7). The wild-type FMLV Env protein has 20 cysteine residues in SU and is folded correctly with a certain intramolecular disulfide bond pattern in which Cys-361 (the 16th Cys from the N terminal) is disulfide bonded with Cys-373 (the 17th Cys) (20). Because the Cys-361-to-Arg substitution disrupts this disulfide bond, the Cys-361-mutated Env could not be folded correctly. The slightly slower mobility of the mutant SU-TM than the wild-type SU-TM on SDS-PAGE under nonreducing conditions (Fig. 4B, lane 2) gives experimental support to the above notion.

We showed that the intracellular transport of the Cys-361-mutated Env protein from the ER to the Golgi complex was inhibited. One explanation for this inhibition is that the misfolded mutant Env in the ER fails to form an oligomer essential for its transport. Another explanation is that the mutant Env in the ER forms an abnormal multimer which could not be transported into the Golgi complex. The molecule in FCr1 lysates which migrated very slowly on SDS-PAGE under nonreducing conditions (Fig. 4B, lane 2, P) may represent the abnormal multimer, suggesting that the Cys-361-mutated Env aggregates with intermolecular disulfide bonds in the ER.

The cells harboring the Cys-361-mutated Env in the ER were completely resistant to MoMLV infection. The failure of MoMLV infection before the proviral DNA formation must be at the viral penetration, which indicates the ability of the Cys-361-mutated Env to bind to the ecotropic MLV receptors (1, 18) in the ER. It is inferred that the Cys-361-mutated Env traps the ecotropic receptors in the ER, and as a result, the absence of the ecotropic receptors on the cell surface brings about the interference with MoMLV infection. A very weak interference by FMLV SU retained in the ER was previously reported (11). The strong interference by the Cys-361-mutated Env described here is probably due to its accumulation, which may trap almost all of the ecotropic MLV receptors within the ER.

The Cys-361-mutated Env in the ER trans-dominantly inhibited not only the new MoMLV infection but also the production of virus from the established provirus. The suppression of the MoMLV production was caused by the inhibition of the wild-type Env transport from the ER to the Golgi complex. There are two possible mechanisms. First, the mutant Env may consume a factor specific and essential for the retroviral Env transport. Several factors essential for the protein transport, such as BiP, have been reported (2), but no factor specific for retroviral proteins has been described. Second, the Cys-361-mutated Env may bind to the wild-type Env and trap it in the ER; the presence of Env aggregate supports this idea. Probably, the amphotropic Env would not coaggregate with the ecotropic Env, which may also be the reason that the inhibition by the Cys-361-mutated Env is ecotropic MLV-specific.

For antiviral strategy, one of the problems with dominant negative mutants is the leakiness of the wild-type protein function. The inhibitory effect on virus infection by the Cys-361-mutated Env is exerted at two different stages and reinforced because of its accumulation. Therefore, the Cys-361-mutated Env efficiently interferes with MoMLV infection.

It has been argued that dominant negative human immunodeficiency virus Gag and Rev mutants exert their inhibitory effect via formation of nonfunctional multimers with the mature wild-type proteins (14, 22, 33). The Cys-361-mutated Env described here is unique in that it inhibits the maturation of the wild-type precursor and precludes the subcellular Env distribution. The surface expression of the wild-type Env, which could be pathogenic by promoting syncytium formation (19, 31), is inhibited by the mutant Env in the ER.

In conclusion, the Cys-361-mutated Env protein accumulating in the ER efficiently inhibits MoMLV infection at two different stages as a dominant negative mutant (Fig. 9). First, it traps the ecotropic MLV receptors within the ER. Second, it traps the wild-type MoMLV Env within the ER. These downregulations by protein-protein interactions reinforced
by protein accumulation may lead to the development of an antiviral strategy.

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