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Effects of retroviral envelope-protein cleavage upon trafficking, incorporation, and membrane fusion

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ABSTRACT

Retroviral envelope glycoproteins undergo proteolytic processing by cellular subtilisin-like proprotein convertases at a polybasic amino-acid site in order to produce the two functional subunits, SU and TM. Most previous studies have indicated that envelope-protein cleavage is required for rendering the protein competent for promoting membrane fusion and for virus infectivity. We have investigated the role of proteolytic processing of the Moloney murine leukemia virus envelope-protein through site-directed mutagenesis of the residues near the SU–TM cleavage site and have established that uncleaved glycoprotein is unable either to be incorporated into virus particles efficiently or to induce membrane fusion. Additionally, the results suggest that cleavage of the envelope protein plays an important role in intracellular trafficking of protein via the cellular secretory pathway. Based on our results it was concluded that a positively charged residue located at either P2 or P4 along with the arginine at P1 is essential for cleavage.

Introduction

Many cellular and viral proteins are synthesized as proproteins that undergo proteolytic processing by cellular endoproteases to become functionally mature. One of the viral proteins that are proteolytically matured is the retrovirus envelope glycoprotein (Env). Env is cleaved by cellular proprotein convertases (PCs) of the serine-endoproteinase family to produce two subunits—SU, an extracellular glycoprotein responsible for binding to viral receptors, and TM, a transmembrane protein responsible for tethering Env complexes to viral particles and inducing fusion between viral and cellular membranes (Coffin et al., 1997).

The requirement for the proteolytic processing of the envelope protein in retroviruses has previously been studied by several groups. For many years cleavage was speculated to be essential, because it liberates the fusion peptide located at the amino-terminus of the transmembrane subunit (Coffin et al., 1997; McCune et al., 1988). The fusion peptide induces membrane fusion between the target cell membrane and the virus membrane leading to virus entry into the cell.

Via manipulations of the enzyme-recognition sequence, several studies have demonstrated in retroviruses that cleavage is essential for virus infectivity and membrane fusion in murine leukemia viruses (MLVs) (Famulari and Jelalian, 1979; Freed and Risser, 1987; Granowitz et al., 1991; Machida and Kabat, 1982; Zavorotinskaya and Albritton, 1999), in human immunodeficiency virus type 1 (HIV-1) (Bosch and Pawlita, 1990; Dubay et al., 1995; Freed et al., 1989; Guo et al., 1990; Kleny et al., 1988; McCune et al., 1988), in Rous sarcoma virus (RSV) (Dong et al., 1992; Perez and Hunter, 1987) and in mouse mammary tumor virus (MMTV) (Goodman et al., 1993). Some studies have shown that cleavage is not required for the envelope protein export to the cell surface but is required for incorporation of Env in the virus particles (Dubay et al., 1995; Kleny et al., 1988), while others demonstrated that it is a prerequisite neither for transport to the cell surface nor for its incorporation into virus particles (Dong et al., 1992; Famulari and Jelalian, 1979; Guo et al., 1990; Herrera et al., 2005; Machida and Kabat, 1982; Zavorotinskaya and Albritton, 1999) or for virus infectivity (Machida and Kabat, 1982; Zavorotinskaya and Albritton, 1999). Here, as an attempt to resolve these contradictory findings, we have conducted an extensive systematic study of the role of cleavage motif of the Moloney murine leukemia virus (Mo-MuLV) Env in its trafficking, incorporation in the virus particles and membrane fusion.

The envelope glycoprotein of Mo-MuLV is initially synthesized as a 665 amino-acid precursor protein (Shinnick et al., 1981) that is cotranslationally inserted in the membrane of the endoplasmic reticulum (ER). In the ER lumen, high mannose sugars are added to the asparagine residues, intersubunit disulfide bonds are formed, and the protein is trimerized via a coiled-coil located in the TM subunit (Kamps et al., 1991). In the Golgi cisternae, the *N*-linked sugar residues are modified into complex oligosaccharide chains, and *O*-linked sugars are added. Finally, in the trans-Golgi network (TGN) Env is proteolytically cleaved by cellular furin or furin-like proprotein convertases (Basak et al., 2001; Bedgood and Stallcup, 1992; Henderson et al., 1984; Schultz and Rein, 1985; Shinnick et al., 1981; Witte et al., 1977) into two

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subunits, SU and TM, at a sequence highly rich in basic amino-acid residues. The minimum enzyme-recognition sequence for most proprotein convertases is Arg-X-Lys/Arg-Arg↓Y (P_4-P_3-P_2-P_1↓P_1′ respectively, where ↓ represents cleavage, X represents any residue and Y represents any nonhydrophobic residue) (Henrich et al., 2003, 2005; Rockwell and Fuller, 1998; Rockwell and Fuller, 2002; Rockwell et al., 2002; Rockwell and Thorner, 2004; Schechter and Berger, 1968). After cleavage, the two subunits remain attached covalently resulting in the formation of trimers of SU–TM complexes (Opstelten et al., 1998; Pinter and Fleissner, 1977; Pinter et al., 1997; Sanders, 2000). The virus acquires these Env complexes along with the lipid bilayer while budding through cellular membranes. In the budded virus the viral protease cleaves the last 16 amino acids (the R-Peptide) from the C-terminus of Env thereby disrupting a domain that inhibits fusion by Env in the virus-producing cells (Aguilar et al., 2003; Januszeski et al., 1997; Ragheb and Anderson, 1994; Rein et al., 1994; Taylor and Sanders, 2003).

We have investigated the role of proteolytic processing in the function of the envelope protein in Mo-MuLV. The proprotein-convertases recognition site is highly conserved among retroviruses. By engineering a series of substitution mutations in the enzyme recognition sequence we have obtained evidence that cleavage is indeed essential for virus-induced membrane fusion and envelope glycoprotein incorporation into virions. Additionally, our findings suggest that cleavage plays an important role in the intracellular trafficking of the glycoprotein.

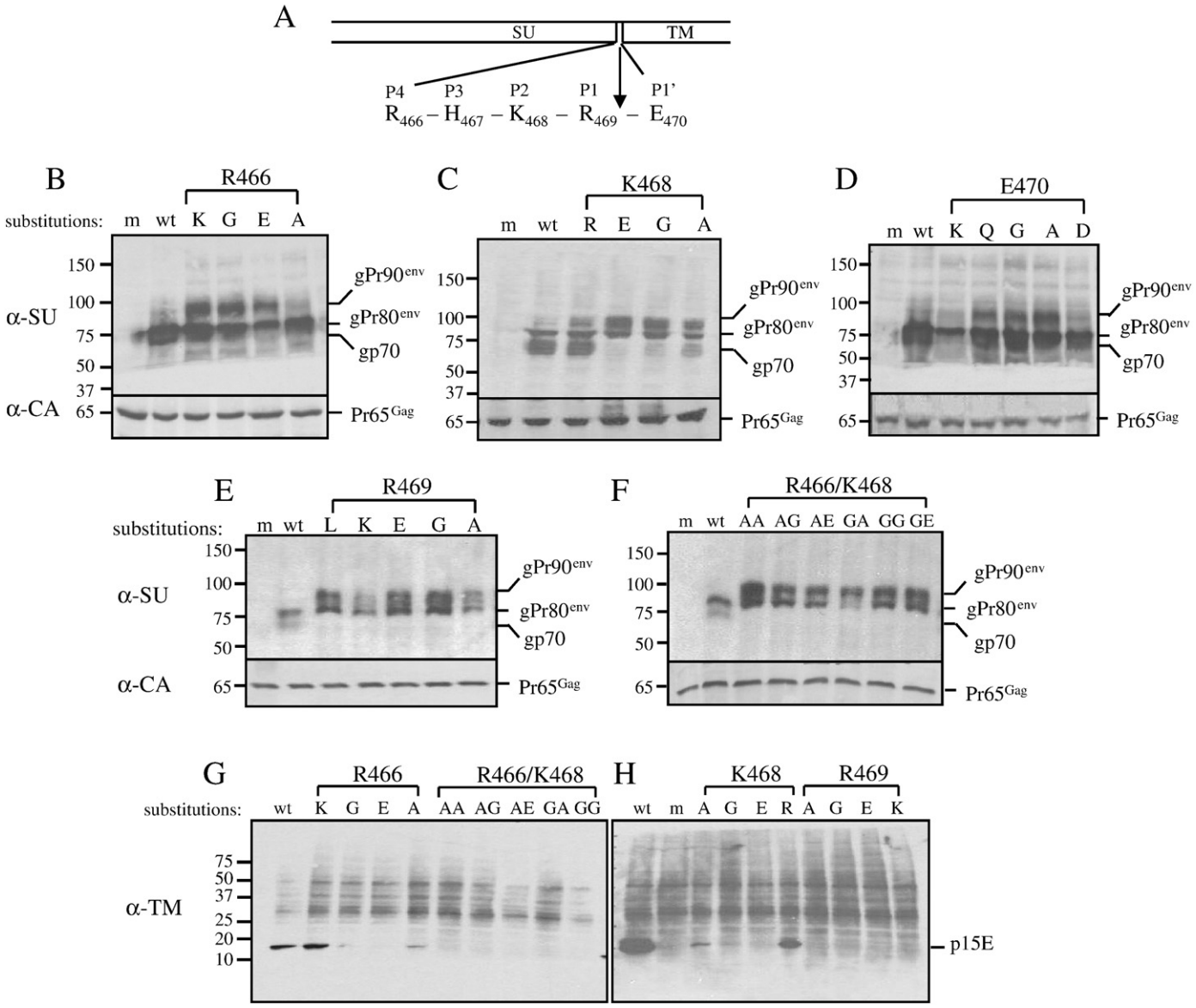

Fig. 1. Mo-MuLV envelope protein cleavage-motif. ↓ represents cleavage site. (A) The positions of the amino-acid residues in the cleavage motif are represented above them and the amino-acid number in the sequence of Moloney murine leukemia virus envelope protein is represented in subscript. (B-F) Analysis of the proteolytic processing of Mo-MuLV envelope proteins: The cell lysates from ΦNXnl*slacZ* cells expressing wild-type or mutant envelope proteins were analyzed by SDS-PAGE (9% acrylamide). The proteins were transferred to the nitrocellulose membranes and probed with α-SU (anti-Raucher gp70 antibody) (B-F upper panels) and α-CA (anti-p30 antibody) antibodies (B-F lower panels) separately. Three species of Env are detected by anti-SU antibody: $gPr90^{env}$ (90 kDa) fully *N*- and *O*-glycosylated and uncleaved Env, $gPr80^{env}$ (80 kDa), an endo-H sensitive, high mannose glycosylated Env, and gp70 (SU) (70 kDa); their positions of migration are indicated on the right. Molecular size marker is represented on the left with the sizes in kDa. Anti-capsid antibody recognizes $Pr65^{Gag}$ protein (65 kDa). (G and H) Immunoblot analysis using α-TM antibody was performed. The cell lysates were analyzed using 10-20% acrylamide gradient gels. A 15 kDa p15E (TM) band is detected by the monoclonal anti-TM antibody. 'wt' represents wild-type envelope protein and 'm' (mock) represents cells transfected with empty vector. In the α-SU panel of B, a small volume of protein from the wt lane has leaked into the mock lane.

Results

In order to study the roles cleavage-motif residues Arg_{466}–His_{467}–Lys_{468}–Arg_{469}↓Glu_{470} (Shinnick et al., 1981) (Fig. 1A) play in substrate recognition by PCs, and the effect of defective processing on Env function, substitution mutations were created for P4, P2, P1 or P1′ codons in *env*. The processing and level of virion incorporation of each mutant Env was investigated, and its capacity to promote membrane fusion and viral transduction was assayed.

The P1 residue is critical along with a P2 or P4 basic residue for efficient cleavage

In order to determine if the mutations created at the cleavage motif affect the proteolytic processing of the envelope protein, ΦNXnls*lacZ* cells (HEK293T cells stably transfected with (Mo-MuLV) *gag* and *pol* genes and a retroviral vector carrying the *lacZ* gene (Taylor and Sanders, 1999) were transfected with the plasmids encoding either wild-type or mutant Env. After 48 h, the cell lysates were harvested and subjected to immunoblot analysis using goat anti-SU polyclonal antibody. Immunoreactive proteins that are present in the lysates of the cells expressing wild-type Env display apparent molecular weights of 80 kDa and 70 kDa representing endoglycosidase H (endo H) sensitive, high-mannose glycans carrying $gPr80^{env}$ and mature SU (gp70) respectively (Figs. 1B–F), as shown previously (Kayman et al., 1991; Witte and Wirth, 1979). Proteins with substitutions for P4 (R466) or P2 (K468) with another basic residue were cleaved at a level close to wild-type (Figs. 1B and C). Cleavage of proteins with substitutions for P2 and P4 with alanine or glycine was reduced as indicated by the presence of immunoreactive uncleaved, endo-H resistant *N*- and *O*-glycosylated Env ($gPr90^{env}$) of ~90 kDa, whereas substitution of either with glutamate reduced cleavage to an almost undetectable level (Figs. 1B and C). Moreover, we examined the effects of substitution mutations created at position P1′ (E470). We found that substitution with an aspartate did not affect the cleavage. An alanine, glycine or glutamine at P1′ made the protein less sensitive to cleavage and a lysine at P1′ completely abolished the cleavage (Fig. 1D). In contrast, in the lysates of the cells expressing Envs with substitutions at P1 (R469), the antibody detected only the 80- and 90-kDa species (Fig. 1E). Further, to investigate whether a basic residue is required at one or the other of the P2 and P4 positions, we created paired mutants where both the residues were simultaneously substituted with alanine, glycine or glutamate. Interestingly, all the paired mutants were found to be uncleaved (Fig. 1F).

These results were confirmed by an immunoblot assay using anti-TM monoclonal antibody. TM (P15E, ~15 kD) was detected in the lysates of cells expressing mutants for which evidence of Env cleavage was observed (Figs. 1G and H), but not in those of cells expressing uncleaved Env (Figs. 1G and H).

In order to verify the identification of the immunoreactive wild-type and mutant proteins in the cell lysates, the proteins present in them were treated with PNGaseF, a deglycosylase that removes all N-linked sugars from proteins. Cell lysates were collected and half of each lysate was treated with PNGaseF. The samples were each analyzed by immunoblot assay using the anti-SU antibody (Fig. 2). After removal of *N*-linked oligosaccharides, the $gPr90^{env}$ and $Pr80^{env}$ migrate with apparent molecular weight of 72 kDa ($Pr72^{env}$) and 68 kDa ($Pr68^{env}$) respectively, as shown previously (Bedgood and Stallcup, 1992; Witte and Wirth, 1979). The cleavage product SU (gp70) when stripped of *N*-linked oligosaccharides has an apparent molecular weight of 50 kDa. Immunoblot analysis of cleavage-defective mutant proteins indicated the presence of the 72 kDa and 68 kDa species (Figs. 2A and B), whereas in the analysis of wild-type protein the 68 kDa and 50 kDa species were found (Figs. 2A and B, wt). This confirms that only $gPr90^{env}$ and $Pr80^{env}$ species are present in the cell lysates expressing cleavage-defective mutants. All three species, 72 kDa, 68 kDa and 50 kDa, were seen in immunoblots of incompletely cleaved mutant proteins such as K468A and R466A indicating the presence of all three forms of the envelope protein (Fig. 2A). These data confirm our analysis of the glycosylated mutant envelope proteins.

Uncleaved envelope protein is unable to promote transduction

To measure the titer of infectious virus particles produced from the cells transfected with plasmids encoding envelope proteins carrying substitutions at the cleavage site, a transduction assay was performed. Plasmids encoding the mutant envelope proteins were transiently transfected into ΦNXnls*lacZ* cells. These cells produce virus particles containing a recombinant β-galactosidase-encoding genome; transduction by the recombinant virus from cells transfected with functional envelope proteins results in β-galactosidase production in the transduced cells. Viruses produced from cells expressing envelope proteins bearing substitutions for P1, except for R469K, were unable to transduce cells (Table 1). Transduction by viruses bearing R466A, R466G, K468A, or K468G Envs were reduced four-fold compared to wild-type Env-bearing virus, whereas transduction by viruses bearing R466E or K468E Envs was reduced by greater than thirty to sixty-fold. Transduction by viruses bearing E470A, E470G or E470Q Envs was reduced approximately 2.5 to 4-fold, whereas the E470K Env was unable to promote transduction. Furthermore, the viruses produced from the cells expressing any of the Envs bearing paired substitutions for P2 and P4 were unable to transduce. In summary, our data provide evidence that the envelope proteins that were found to be uncleaved exhibited significantly reduced efficiency to transduce, whereas the transduction efficiency of efficiently cleaved mutant Envs was not affected by the mutations. Therefore, we can conclude that SU–TM cleavage is required for virus transduction.

Uncleaved glycoprotein is not incorporated into virus particles

Although SU–TM cleavage was clearly required for the promotion of membrane fusion, we also wanted to examine whether the substitutions affected Env incorporation into particles or receptor binding. Previously, it has been demonstrated in MLV (Famulari and Jelalian, 1979; Zavorotinskaya and Albritton, 1999) and other retroviruses such as lentiviruses (Dubay et al., 1995) and avian retroviruses (Perez and Hunter, 1987) that the Env precursor can be incorporated into virions. Here, Env incorporation was investigated through immunoblot assays using anti-SU and anti-TM antibodies along with anti-capsid antibody (to measure the level of production of recombinant virus particles) conducted on virus collected from the supernatant medium of ΦNXnls*lacZ* cells expressing the mutant Envs. There is a general correlation between the levels of cleavage of the mutant Envs (Figs. 1B and D) and the level of incorporation of SU into viral particles (Figs. 3A–C). It is noteworthy, nevertheless that some mutant Envs, such as the R466A, R466G, K468A, and K468G Envs appear to be relatively efficiently incorporated into viral particles after cleavage despite inefficient processing. Uncleaved envelope proteins such as those containing substitutions for P1 (except for R469K) or paired substitutions for both P2 and P4 were not incorporated into virus particles (Figs. 3D and E) indicated by the absence of proteins immunoreactive with anti-SU. In immunoblots using anti-TM antibody, full-length TM (p15E) and TM with the R-peptide removed (p12E) were detected when viruses bearing cleaved Env were analyzed (Fig. 3F). No immunoreactive proteins were detected when virus bearing uncleaved Env proteins was analyzed. Collectively, these results provide strong evidence that proteolytic processing is required for glycoprotein incorporation into the Mo-MuLV virion.

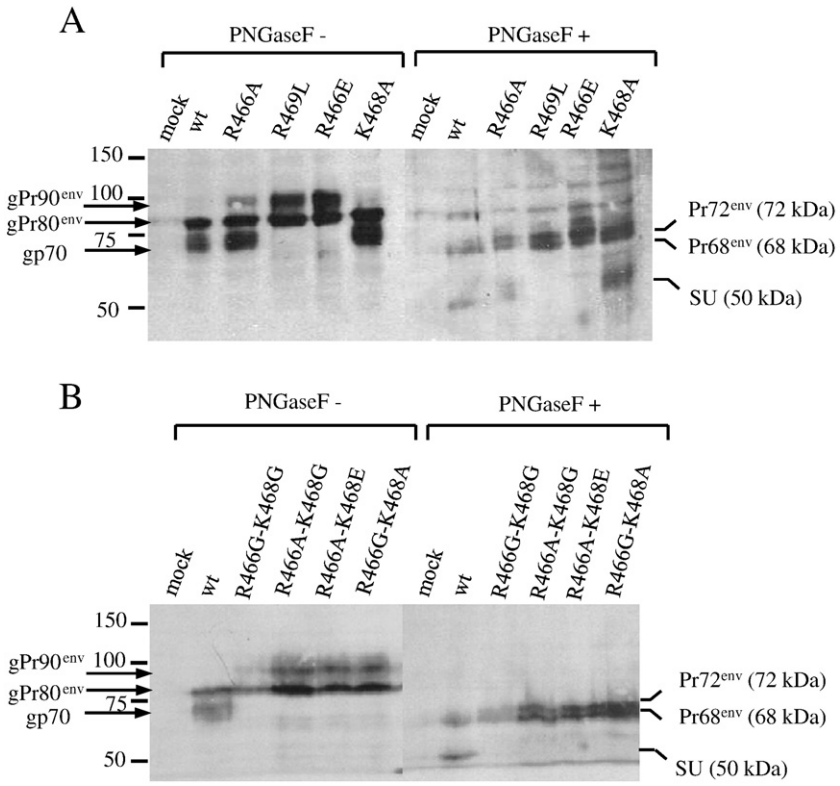

Fig. 2. Deglycosylation assay using PNGaseF. Cell lysates of ΦNXnls*lacZ* expressing wild-type and mutant envelope proteins, treated with PNGaseF (PNGaseF +) and untreated (PNGaseF -) were analyzed by SDS-PAGE (9% acrylamide), transferred onto a nitrocellulose membrane and probed with anti-SU antibody. The positions of migration of PNGaseF untreated proteins are indicated on the left and the positions of deglycosylated $Pr72^{env}$ (72 kDa), $Pr68^{env}$ (68 kDa) and gp50 (SU) (50 kDa) are indicated on the right. Molecular size marker is also indicated on the left. Mock represents cells transfected with empty vector.

Uncleaved envelope protein is unable to induce receptor-mediated membrane fusion

Next, the ability of the mutant envelope proteins to induce membrane fusion was examined. In Mo-MuLV, the envelope protein lacking the R-peptide induces receptor-mediated membrane fusion in NIH 3 T3 cells, forming syncytia (Kubo and Amanuma, 2003; Kubo et al., 2007; Ragheb and Anderson, 1994; Rein et al., 1994; Taylor and Sanders, 2003). To mimic the mature, fusion-inducing envelope protein the last 16 amino-acid residues from the C-terminus of the mutant envelope proteins were deleted (Taylor and Sanders, 1999). To examine the ability of various mutants to promote membrane fusion, a syncytia-formation assay was performed (Table 2). Plasmids encoding the mutant envelope proteins were transiently transfected into NIH 3 T3 cells, and the number of syncytia and the number of nuclei per syncytia were counted to calculate the efficiency of syncytia formation relative to wild-type envelope protein lacking the last 16 residues. Any change at P1 caused complete abrogation of membrane-fusion promotion. R466A/Δ650–665 and K468A/Δ650–665 Envs induced moderately reduced numbers of syncytia, whereas R466K/Δ650–665 and K468R/Δ650–665 Envs induced syncytia formation at levels comparable with that of the wild-type Env. Surprisingly, the R466G/Δ650–665, K468G/Δ650–665, R466E/Δ650–665 and K468E/Δ650–665 Envs induced syncytia formation to an undetectable level, although the virus bearing these mutant proteins mediated substantial levels of transduction.

Table 1
Transduction of NIH 3 T3 cells by virions bearing mutant Mo-MuLV envelope protein.

Env Mutants	Virus Titer (Transducing units per ml TU/ml)
Wild-type	$6.0 \pm 0.4 \times 10^6$
Neg. Cont.	<3
R466A	$1.4 \pm 0.2 \times 10^6$
R466G	$1.5 \pm 0.5 \times 10^6$
R466E	$1.0 \pm 0.2 \times 10^5$
R466K	$6.0 \pm 0.3 \times 10^6$
K468A	$1.8 \pm 0.4 \times 10^6$
K468G	$1.6 \pm 0.2 \times 10^6$
K468E	$2.0 \pm 0.5 \times 10^5$
K468R	$5.8 \pm 0.1 \times 10^6$
R469A	<3
R469G	<3
R469E	<3
R469L	<3
R469K	$2.3 \pm 0.3 \times 10^6$
E470A	$2.7 \pm 1.0 \times 10^6$
E470D	$6.1 \pm 0.8 \times 10^6$
E470G	$2.5 \pm 0.6 \times 10^6$
E470K	<3
E470Q	$1.7 \pm 0.2 \times 10^6$

ΦNXnls*lacZ* cells were transiently transfected with the plasmids encoding the various mutant envelope proteins. After 48 h the supernatant medium was used for transduction of NIH 3 T3 cells. Transduced cells express β-galactosidase that was detected by X-gal staining. Virus titer is given as transducing units per ml (TU/ml). Transduction units per ml was calculated by multiplying the% blue cells by total number of cells divided by total volume of virus-containing media (in ml).

As expected all six paired substitutions completely inhibited syncytia formation (data not shown). E470G/Δ650–665 and E470Q/Δ650–665 Envs exhibited reduced capacity to induce syncytia formation, whereas no syncytia were detected in the case of E470K/Δ650–665. Collectively, these results indicate that cleavage between SU and TM is required for membrane fusion in Mo-MLV.

Efficiency of cleavage of mutant Envs in human and mouse cells

The discrepancy in our results that R466G and K468G Envs did not produce any syncytia but promoted a significant level of transduction was addressed by analyzing the processing of the mutant envelope

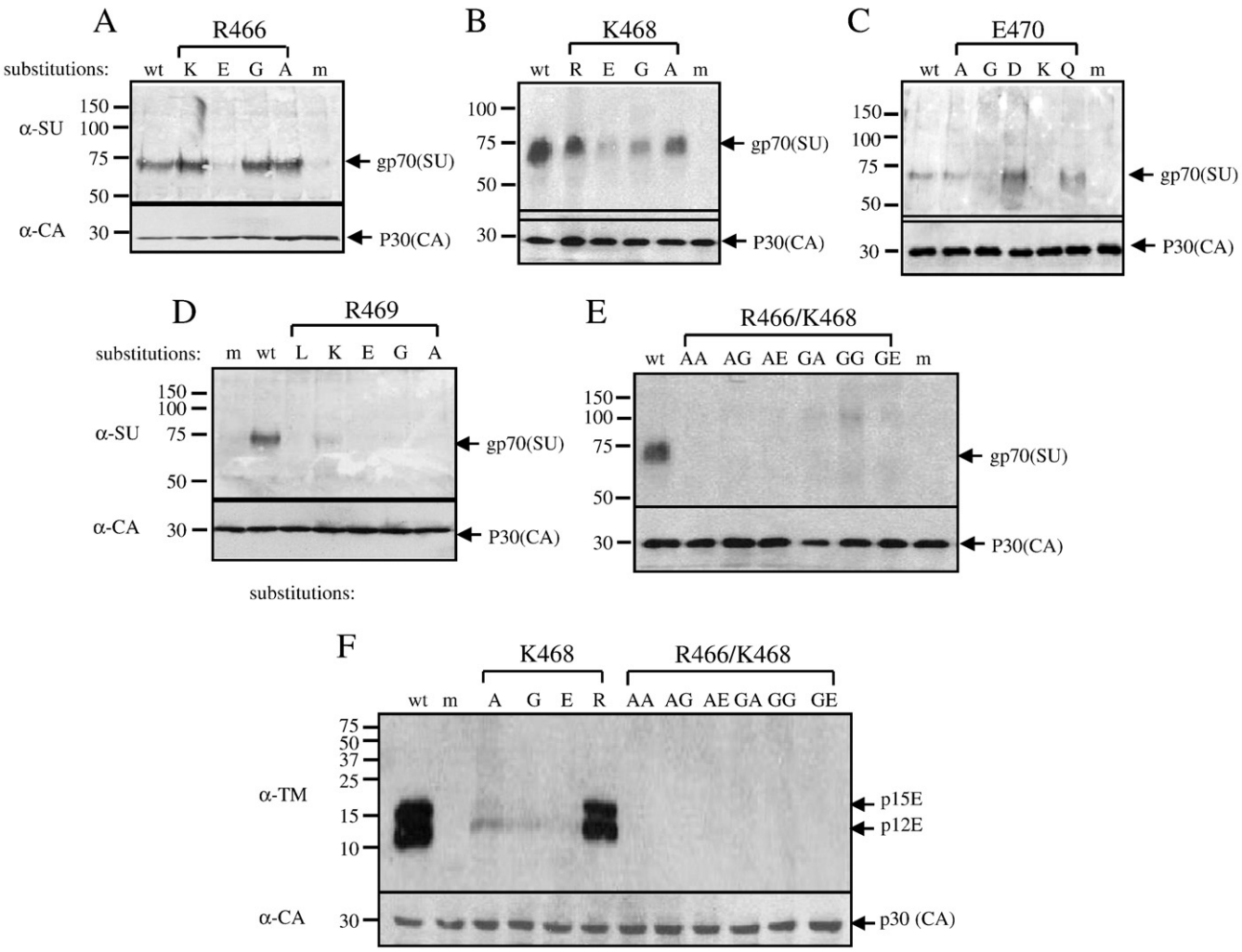

Fig. 3. Analysis of the viral incorporation of mutant envelope proteins bearing substitutions for the proprotein convertase cleavage site. Virus particles budded from ΦNXnl*lacZ* cells expressing wild-type or mutant envelope proteins were collected and analyzed by SDS-PAGE (9% acrylamide) and probed with (A-E) α-SU (anti-Raucher gp70 antibody) and α-CA (anti-p30 antibody) antibodies separately, and (F) α-TM and α-CA antibodies. The migration of the molecular mass markers are indicated on the left and the positions of gp70 (SU) (70 kDa) and p30 (CA) (A-E), and two forms of TM, p15E (full-length TM) and p12E (TM lacking the R-peptide), along with p30 (CA) (30 kDa) (F) are indicated on the right. 'm' (mock) represents cells transfected with empty vector.

proteins in NIH 3T3 cells. Whereas the syncytia-formation assay was performed in mouse NIH 3T3 cells using the envelope proteins lacking the R-peptide, the transduction assay was performed in human 293T cells using full-length envelope proteins. The cell lysates of NIH 3T3 expressing the mutant Envs carrying the full-length C-terminus or lacking the R-peptide were probed with goat anti-SU antibody. It was found that the mature SU (70 kDa) is absent in the lysate of cells expressing R466G/Δ650–665, whereas it is present in the lysate of the cells expression R466G (Fig. 4A). Similar results were obtained for K468G/Δ650–665 and K468G (data not shown). R466E/Δ650–665 and R466E on the other hand are both found to be uncleaved (Fig. 4A). These results indicate that the Δ650–665 envelope proteins with the R466G and K468G substitutions were inefficiently cleaved in the NIH 3 T3 cells.

In order to examine the surface expression of the R466G/Δ650–665 and R466E/Δ650–665 Envs in NIH 3T3 cells a surface immunofluorescence assay was conducted. We found that the proteins were expressed on the cell surface at a slightly reduced level as compared to the Δ650–665 Env and the R466G and R466E full-length proteins (Fig. 4B). These findings suggest that the R466G/Δ650–665 and R466E/Δ650–665 proteins were expressed on the cell surface, but were unable to promote syncytium formation, because of their inefficient processing. It is also possible that the transduction assay is more sensitive than the syncytium-formation assay.

Uncleaved mutant glycoproteins retain capacity to bind to the receptor

Ecotropic murine leukemia viruses, such as Mo-MuLV, use the rodent cationic amino-acid transporter-1 (CAT-1) as their cell surface receptor (Albritton et al., 1989; Wang et al., 1991). It has been demonstrated that endogenously produced MLV envelope protein binds to CAT-1 and reduces its cell surface expression by affecting the interaction between CAT-1 and clathrin adaptor complex-1 (AP-1) (Fujisawa and Masuda, 2007). The reduced surface expression of CAT-1 results in a reduction in superinfection by the same virus or a different virus that uses the same receptor, a phenomenon known as retroviral interference (Steck and Rubin, 1966). To demonstrate that the substitution mutations created at the cleavage site of the envelope protein do not affect the interactions between an endogenously expressed envelope protein and the receptor protein, we performed an interference assay. To perform this assay, stable clones of NIH 3T3 were created that constitutively express the wild-type or the uncleaved mutant envelope protein R469L or R466A/K468A. The transduction of the cells was performed using recombinant ecotropic

Table 2
Syncytia formation promoted by mutant Mo-MuLV envelope proteins.

Mo-MuLV Env mutant	Syncytia Formation (%)*
Wild-type (pen1minΔ650)	100
Neg. Cont.	<0.25
R466A/Δ650-665	45 ± 2
R466G/Δ650-665	<0.25
R466E/Δ650-665	<0.25
R466K/Δ650-665	73 ± 3
K468A/Δ650-665	16 ± 2
K468G/Δ650-665	<0.25
K468E/Δ650-665	<0.25
K468R/Δ650-665	102 ± 7
R469A/Δ650-665	<0.25
R469G/Δ650-665	<0.25
R469E/Δ650-665	<0.25
R469L/Δ650-665	<0.25
R469K/Δ650-665	<0.25
E470A/Δ650-665	114 ± 5
E470D/Δ650-665	128 ± 7
E470G/Δ650-665	67 ± 4
E470K/Δ650-665	<0.25
E470Q/Δ650-665	8 ± 2

NIH 3 T3 cells were transiently transfected with plasmids encoding the mutant envelope proteins lacking the last 16 amino acids at the carboxy-terminus. The number of syncytia and the number of nuclei per syncytia were counted after 36 h of transfection. The percentage of nuclei in syncytia was calculated by multiplying the total number of syncytia by the average number of nuclei per syncytia and dividing by the total number of cells.
*The numbers in the table are normalized to the percentage of nuclei in syncytia formed as a result of expression of wild-type envelope protein lacking the last 16 amino acids (16%).

MLV that uses the mCAT-1 receptor. Amphotropic 4070A MLV, which employs Pit2, a sodium-dependent phosphate transporter (Tailor and Kabat, 1997), as the receptor, was used as a control to demonstrate that the transduction of NIH 3T3 cells was not generally affected by constitutive expression of the envelope proteins. The transduction of cells expressing the wild-type Env by recombinant ecotropic MLV was reduced by approximately 20-fold as compared to NIH 3T3 cells expressing no Env. Transduction of the cells expressing R466A/K468A and R469L was also reduced by ~18–20-fold (Table 3). The transduction of cells expressing mutant proteins remained similar to the cells expressing no envelope protein when they were transduced with the recombinant amphotropic MLV (Table 3) suggesting that amphotropic-virus entry was not inhibited. Reduced transduction of the cells expressing the mutant envelope proteins with the recombinant ecotropic MLV suggests that the mutant Envs are able to bind to the receptor.

The intracellular distribution pattern of uncleaved glycoproteins is altered

The trafficking of the mutant envelope proteins was addressed by studying the intracellular localization of the protein by confocal microscopy. We engineered a clone encoding an EGFP fluorescent marker protein at the C-terminus of the wild-type envelope protein (pMolEnv-EGFP). The fusion protein exhibited normal processing detected by an immunoblot assay using anti-SU (Fig. 5A) and anti-EGFP antibody (data not shown) and virion incorporation (Fig. 5B). Transduction by recombinant virus carrying the MolEnv-EGFP is equivalent to that by virus carrying the wild-type Env, with the titer of the virus carrying the wild-type Env being $6 \pm 0.2 \times 10^6$ and that of the virus carrying TM-EGFP $5.8 \pm 0.2 \times 10^6$. In order to determine the intracellular localization pattern of the incompletely cleaved and uncleaved proteins the P4, P1 and P2–P4 paired substitutions were inserted in the pMolEnv-EGFP. To study their intracellular localization, the fusion proteins were transiently expressed in COS-7 cells. Immunoblot analysis using anti-SU (Fig. 5C) and anti-GFP antibodies (Data not shown) conducted on the cell lysates of mutant protein-EGFP confirmed that the EGFP remained fused to the glycoprotein upon expression in the cells. Forty hours after transfection, confocal microscopy was performed to observe the EGFP fluorescence. Simultaneously, the localization pattern of the wild-type and mutant envelope proteins was detected by an immunofluorescence assay to confirm that EGFP fusion does not interfere with the protein trafficking and its localization. We found that cleaved proteins exhibit a localization pattern similar to the wild-type Env, whereas uncleaved proteins exhibited a localization pattern that was common to all the uncleaved proteins (P1 and P2–P4 paired mutants) in COS-7 cells; therefore we have shown here an example of P1 (R469L-EGFP). The MolEnv-EGFP appeared to be evenly distributed in the cell and was expressed on the cell surface (Fig. 5D). A Similar localization pattern was observed in the case of R466G-EGFP. In contrast R469L-EGFP appeared to be accumulated in the area surrounding the nucleus, in what appeared to be the Golgi complex (Fig. 5D). These results were confirmed by colocalization studies of the fusion-protein with a Golgi complex-specific fluorescent marker-BODIPY (Fig. 5E). The TM-EGFP and R466A-EGFP appeared to be distributed in the cell as well as on the cell surface evenly, whereas, R469L-EGFP and R466A/K468A-EGFP appeared to accumulate in the Golgi compartments, because the Env fusion protein colocalized with BODIPY. These findings suggest that only cleaved protein is transported to the site of assembly, whereas the uncleaved envelope proteins are accumulated in the Golgi compartments. A colocalization study with a late endosomal/lysosome specific antibody (LAMP-1) was also performed to determine the presence of the envelope protein in the late endosomes. Our results suggested that the envelope protein did not colocalize with LAMP-1 (data not shown).

Discussion

Although it has been established that cleavage of the envelope protein into the SU and TM subunits is required for the infectivity of many retroviruses, including MLV, HIV, and RSV, the cellular and biochemical bases of the requirement have not been completely elucidated (Bosch and Pawlita, 1990; Dubay et al., 1995; Famulari and Jelalian, 1979; Freed et al., 1989; Freed and Risser, 1987; Herrera et al., 2005; McCune et al., 1988; Perez and Hunter, 1987; Zavorotinskaya and Albritton, 1999). Our results with the Mo-MuLV Env reinforce the principle that cleavage between SU and TM is essential for function. The sequence requirements at the cleavage site correspond to the known substrate specificity of proprotein convertases (Henrich et al., 2003, 2005; Remacle et al., 2008; Rockwell and Thorner, 2004). We found that a positively charged amino acid, preferably an Arg, is allowed at P1 for cleavage; such a residue can fit well into a flat groove surrounded by acidic amino-acid residues in the S1 binding pocket of furin (Henrich et al., 2003, 2005; Remacle et al., 2008; Rockwell and Fuller, 2002; Rockwell et al., 2002; Rockwell and Thorner, 2004). While a lysine or arginine is preferred at P2 and P4, the presence of Ala, Gly, and Glu at P2 and P4 decrease the susceptibility of the protein to proteolysis, with Glu substitutions being the most deleterious. Our findings also suggest that an acidic residue is preferred for efficient cleavage at P1′, whereas Ala, Gly and Gln moderately decrease the susceptibility. However, a lysine at P1′ completely abrogates cleavage. These data are consistent with the positively charged S1′ subsite of furin (Henrich et al., 2003, 2005; Remacle et al., 2008; Rockwell and Thorner, 2004). Based on the site specific requirements of the enzyme for cleavage indicated by our results, we conclude that furin is the enzyme that cleaves the Mo-MuLV envelope protein. However, out results (data not included) from studies on the cleavage pattern of the wild-type envelope protein in LoVo cells (furin deficient cells) or in the presence of furin inhibitors suggested that other PCs can also cleave Env in the absence of furin, but very inefficiently.

Our results also confirm previous observations (Freed et al., 1989; Freed and Risser, 1987) that substitution of Arg at P1 with a Lys residue reduces the proteolytic processing and virus infectivity and

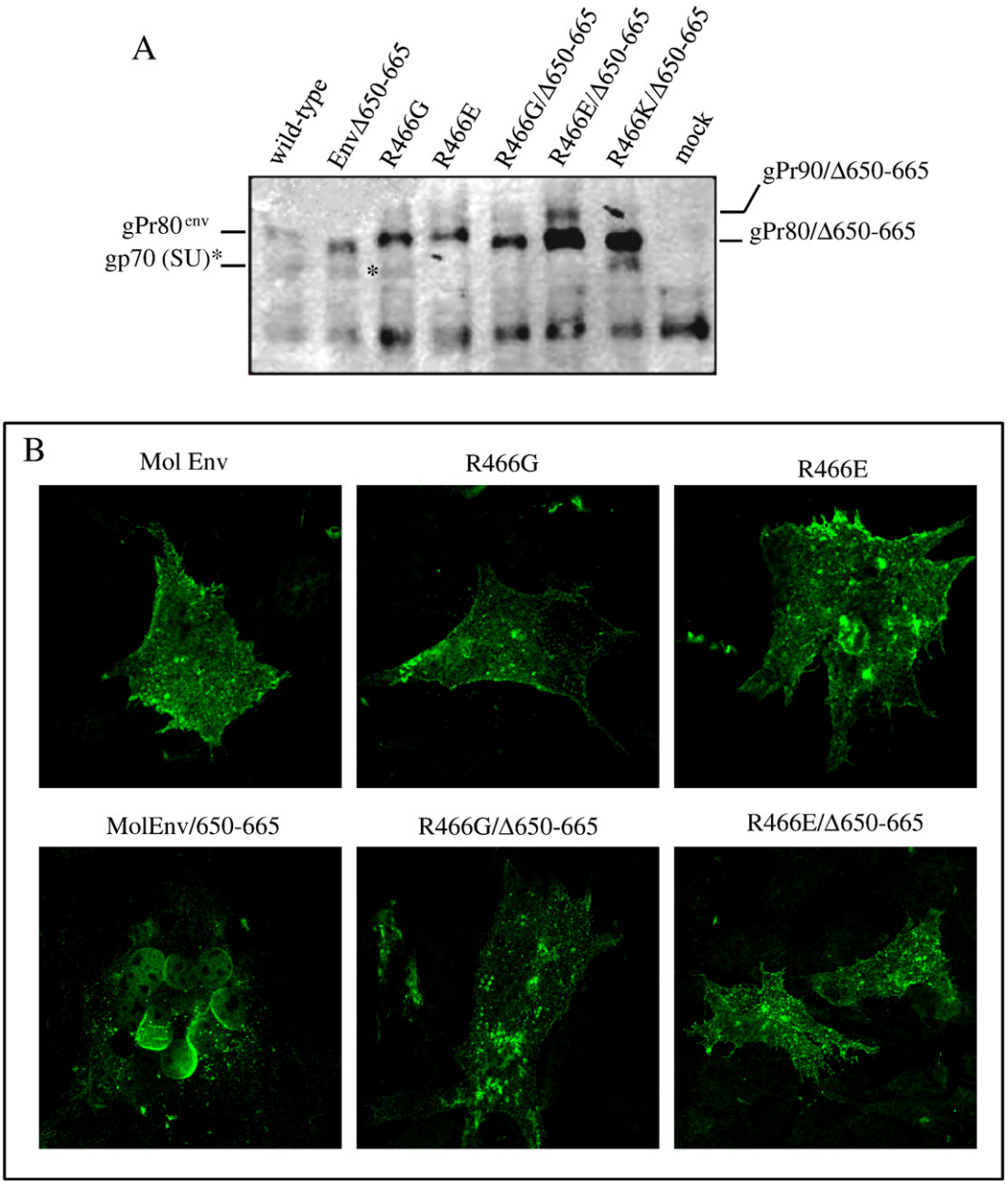

Fig. 4. A) Analysis of the proteolytic processing of the mutant envelope proteins lacking the R-peptide in NIH 3 T3 cells. The cell lysates of NIH 3 T3 cells expressing mutant envelope proteins lacking the R-peptide were analyzed by immunoblot assay using anti-SU antibody and compared with the cell lysates expressing full-length envelope proteins carrying the same substitutions. The positions of migration of $gPr90^{env}$/Δ650-665 and $gPr80^{env}$/Δ650-665, are indicated on the right and the positions of $gPr80^{env}$ and gp70(SU)* are indicated on the left. The wild-type Env lacking the R-peptide (Env/Δ650-665) along with the full-length Env (wild-type) and mock are also shown. B) Cell-surface expression of the mutant envelope proteins lacking the R-peptide in NIH 3 T3 cells. The cells transfected with envelope protein mutants were fixed with 0.4% formaldehyde and probed with anti-SU 83A25 antibody and alexa-488 conjugated secondary antibody. The cell surface expression of the protein was visualized by a confocal microscope.

Table 3
Cellular receptor binding by mutant Mo-MuLV envelope proteins.

Mutant envelope proteins	ΦNX-Eco Titer (TU/ml)	ΦNX-Ampho Titer (TU/ml)
NIH 3 T3 not expressing any envelope protein	$5.1 \pm 0.1 \times 10^6$	$5.1 \pm 0.10 \times 10^6$
NIH 3 T3 expressing wild-type envelope protein	$2.6 \pm 0.11 \times 10^5$	$5.1 \pm 0.14 \times 10^6$
R469L	$2.7 \pm 0.13 \times 10^5$	$5.1 \pm 0.17 \times 10^6$
R466A/K468A	$2.6 \pm 0.12 \times 10^5$	$5.1 \pm 0.15 \times 10^6$

NIH 3 T3 cells were stably transfected with plasmids encoding wild-type, R469L and R466A/K468A envelope protein mutants. The cells were infected by ecotropic-MLV virus produced by ΦNX-Eco and amphotropic-MLV produced by ΦNX-Ampho cells. Forty eight hours after infection the cells were stained with X-gal and the percentage of blue cells was calculated. Transduction units/ml was calculated by multiplying the% blue cells by total number of cells divided by total volume of virus-containing media (in ml).

that a dibasic pair (i.e. two consecutive basic residues) is not crucial for cleavage. Additionally, the requirement of P2 and P4 basic amino acids for cleavage in Mo-MuLV is similar to previous findings for the HIV-1 (Bosch and Pawlita, 1990) and RSV Envs (Perez and Hunter, 1987).

Env protein cleavage appears to be required at two steps necessary for viral infection. We have demonstrated by a syncytia-formation assay that this cleavage is essential for membrane fusion. It was induced only by the envelope proteins bearing an Arg at P1 and at least one basic residue at either the P2 or P4 position. Additionally, it was observed that Δ650–665 Envs with substitutions of the basic residues at P2 or P4 with Gly or Glu (R466G, R466E, K468G and K468E) reduced membrane fusion to an undetectable level, even though the full-length mutant Envs were able to promote transduction. Inefficient processing

caused a reduced level of cell surface expression of the uncleaved mutant Δ650-665 proteins. The combination of reduced cell surface expression, the incapacity of uncleaved Env to promote membrane fusion and a difference in the sensitivity of the transduction and syncytia assays is likely to have contributed to the observed results.

Unexpectedly SU–TM cleavage was also found to be required for incorporation of the envelope protein into virus particles. Uncleaved mutant envelope proteins were not present in the recombinant viral particles. Indeed, the data provide evidence that there is rapid trafficking of cleaved Env proteins into budding virus; mutant Envs that appeared to be inefficiently processed in cells were incorporated at substantial levels into virus. Taking into account the microscopic studies, in which Envs bearing substitutions in the cleavage motif displayed aberrant localization, it can be concluded that there is a reciprocal relationship between processing and trafficking.

Previously it has been demonstrated that substitutions in a putative receptor-binding region of MuLV SU can reduce the efficiency of Env cleavage (Zavorotinskaya and Albritton, 1999). It is of interest that, albeit uncleaved Env molecules were observed to be incorporated into virus particles, cleaved Env was preferentially incorporated. It was concluded that the cleaved Env molecules were responsible for the levels of viral entry determined (Zavorotinskaya and Albritton, 1999). There are two conceivable explanations for these results. Either the uncleaved Env was included in trimers containing a cleaved Env or the identity of the P2 and P4 residues, left unperturbed, is the critical factor in trafficking as indicated by the present study.

It has been demonstrated previously that MLV Env packaging into virus particles requires its interaction with Gag at the site of assembly (Sandrin and Cosset, 2006). The inability of the uncleaved envelope protein to be incorporated into virus particles may result from its absence from membranes at the site of assembly. It can be speculated that Env does not come in contact with Gag and therefore only naked virus-like particles are released from the cells expressing cleavage-defective Envs. Further experiments will be necessary to elucidate the nature of the cellular factors that regulate uncleaved and cleaved Env trafficking to the site of assembly.

Conclusions

Overall, this study provides strong evidence for the proteolytic cleavage of the glycoprotein in its biological role. Many protease-based inhibitors have been investigated and several furin inhibitors

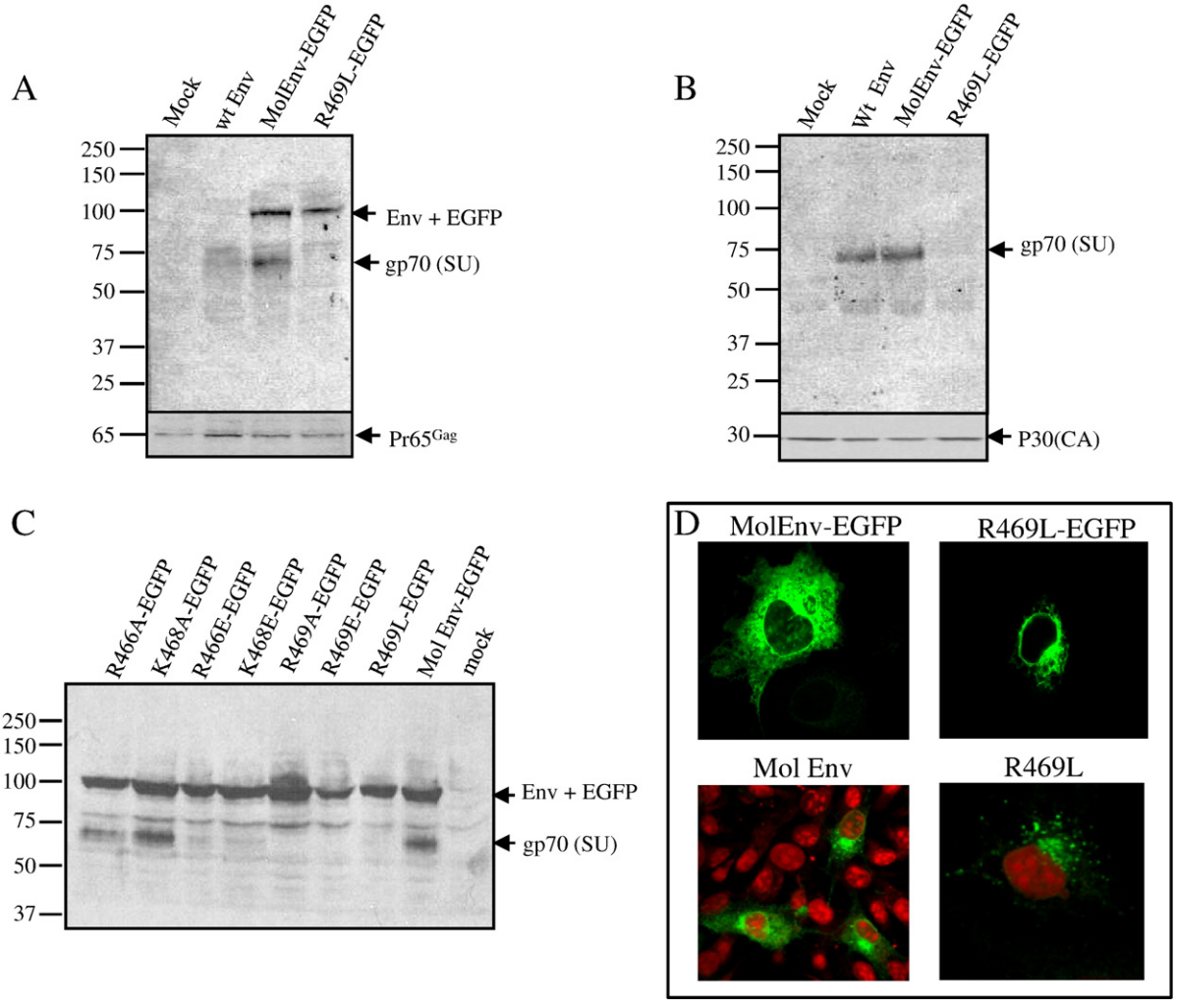

Fig. 5. Intracellular Localization of wild-type and mutant envelope protein: The proteins were expressed in COS-7 cell culture. (A) MolEnv-EGFP protein expression in the cell lysates, (B) virus incorporation of MolEnv-EGFP, and (C) expression of Env-EGFP mutants in cell lysates were detected by immunoblot assay using anti-SU antibody. (D) Fluorescence of MolEnv-EGFP and Env-EGFP mutants was visualized by a fluorescence microscope two days after transfection (upper two panels) or by immunofluorescence of the cells transfected with wt or uncleaved mutant protein expressing plasmid (Lower two panels). The cells were fixed and permeabilized with TritonX-100 and probed with anti-SU 83A25 antibody and Alexa-488 conjugated secondary antibody and visualized by a confocal microscope. (E) COS-7 cells expressing wild-type and mutant Env-EGFP fusion proteins were probed with the trans-Golgi specific marker-BODIPY. Green fluorescence of EGFP and red fluorescence of the Golgi marker was detected using a confocal microscope. 'EGFP' panels represent the green fluorescence from EGFP, 'BODIPY' panels represent the cells stained with Golgi marker, and the 'Merged' panels represent the merge between EGFP and the Golgi-marker. MolEnv-EGFP represents wild-type envelope protein fused with EGFP and mock represents cells transfected with TE buffer and stained with the Golgi marker.

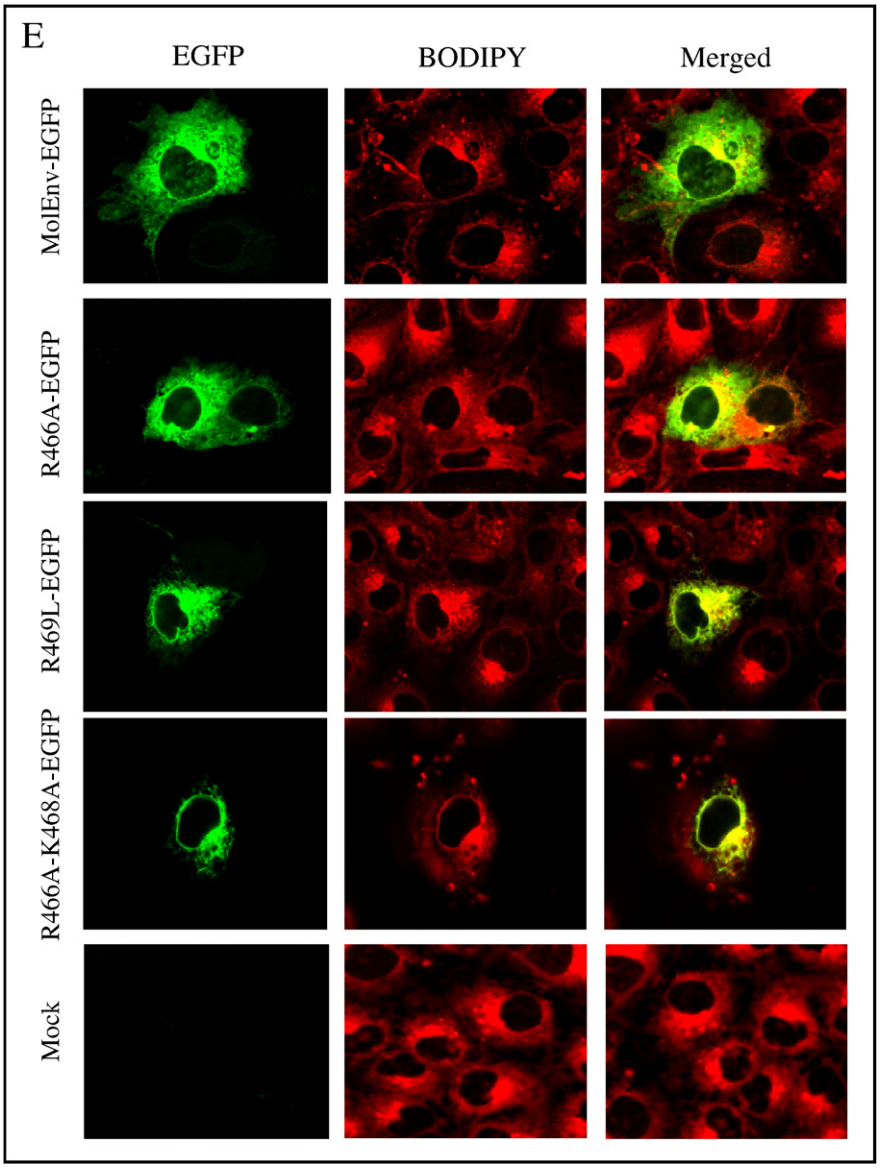

Fig. 5 (*continued*).

have been used to prevent activation of glycoproteins of viruses such as HIV-1 (Kibler et al., 2004), measles virus F_0 (Watanabe et al., 1995), Human Cytomegalovirus (François Jean et al., 2000), respiratory syncytial virus (Basak et al., 2001), and bacterial toxins such as *P. aeruginosa exotoxin* (Sarac et al., 2002) and anthrax (Henrich et al., 2003). Additional research in this direction should lead to further understanding of the virus assembly as well as new ways of blocking Env cleavage and trafficking.

Materials and methods

Cell lines and cell culture

Human embryonic-kidney 293T-based ΦNXgp cells and COS-7 cells were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). ΦNXnls*lacZ* cells, which produce replication-incompetent, envelope protein-deficient, Mo-MuLV particles that contain MFG.S-nls*lacZ*, a retroviral vector encoding a nuclear-localized β-galactosidase (Ory et al., 1996; Sharkey et al., 2001), ΦNX-Ampho, the amphotropic-MLV producing cell line, and ΦNX-Eco, the ecotropic-MLV producing cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 10% fetal bovine serum (FBS; Sigma), penicillin (10 U/ml) and streptomycin (0.1 mg/ml) (PS; Sigma) (DMEM/FBS/PS) at 37 °C in the presence of 5% CO_2. Mouse NIH 3T3 fibroblast cells were cultured in DMEM with 10% calf serum (CS; Gibco-BRL) and 1% PS (DMEM/CS/PS).

Construction of plasmids encoding mutant Mo-MuLV envelope proteins

For the cleavage-site mutants a pMo*env* + 1 min *Bgl*II463 plasmid was created where a TCC codon was changed to TCT to introduce a *Bgl*II site at the amino-acid position 463. The plasmid was digested with *Bgl*II and *Age*I and ligated with double-stranded oligonucleotides encoding the mutations. The *Bst*EII and *Cla*I fragments of the envelope genes were inserted into the Mo-MuLV Env expression plasmids, p*env*1min (encoding wild-type Env) and p*env*1minΔ650–665 (encoding Env lacking the C-terminal 16 residues (Taylor and Sanders, 1999)). pMolEnv-EGFP (encoding an enhanced green fluorescent protein (EGFP) fused to the envelope protein) was created by inserting the EGFP open reading frame at the 3′ end of *env* in p*env*1min using

*Bam*HI and *Eco*RI restriction sites. The *Cla*I/*BstE*II fragments of mutant p*env*1min plasmids were ligated into pMolEnv-EGFP digested with the same restrictions enzymes.

Transfection, viral transduction and syncytia-formation assay

The p*env*1min plasmid encoding the wild-type or a mutant envelope protein was transiently transfected into ΦNXnls*lacZ* cells by the method previously described with the following modifications (Taylor and Sanders, 1999). The plasmid DNA (3 μg) was mixed with 250 μl DMEM and 8 μl Plus reagent (Invitrogen) and incubated for 15 min at 25 °C. In a separate tube 12 μl of LipofectAMINE (Invitrogen) was diluted with 250 μl DMEM. The DNA and LipofectAMINE solutions were mixed, incubated for 15 min at RT and then added to 8 ml of DMEM/FBS/PS. The DNA–LipofectAMINE–culture medium mixture was added to 4×10^6 cells on a 10 cm tissue culture dish. Viral transduction into NIH 3T3 cells and staining for β-galactosidase activity were determined according to the protocol previously described (Taylor and Sanders, 1999). The syncytia-formation assay was performed by transiently transfecting NIH 3T3 cells with the p*env*1minΔ650–665 plasmid encoding the mutant envelope proteins as described (Taylor and Sanders, 1999).

Immunoblotting and PNGaseF analysis

Immunoblot analysis of proteins present in lysates of transfected cells and in virions in the culture medium was conducted with goat anti-Rauscher leukemia virus (RLV) gp71/gp69 polyclonal antibody (1:1000) (ATCC # VR1519AS-Gt) and HRP-conjugated chicken anti-goat secondary antibody (1:5000) (Chemicon), rat anti-TM p15E antibody 10CE11 (1:1000) (kindly provided by A. Pinter (Li et al., 1997)) and goat anti-rat HRP-conjugated antibody (1:5000) as previously described (Taylor and Sanders, 2003). When antibody against capsid protein was used, membranes were probed using goat anti-RLV p30 antiserum (1:1000) (Quality Biotech). Half of the cell lysates were used for PNGaseF enzyme (New England Biolabs) treatment according to the protocol provided by the supplier. The protein samples were subjected to immunoblot analysis using goat anti-RLV gp71/gp69 antibody (1:1000) and chicken anti-goat secondary antibody (1:5000).

Stable cell lines and interference assay

NIH 3T3 cells were stably transfected with the full-length expression vector p*env*1min carrying wild-type, R469L or R466A/K468A mutants, as previously described (Taylor and Sanders, 1999). The viral interference assay was performed according to the protocol described earlier (Taylor and Sanders, 1999). Briefly, ecotropic-MLV producing cells, ΦNX-Eco, and amphotropic-MLV producing cells, ΦNX-Ampho, were transiently transfected with MFG.S-nls*lacZ*, a retroviral vector encoding a nuclear-localized β-galactosidase (Ory et al., 1996; Sharkey et al., 2001). The supernatant was added to 5×10^5 cells and virus titer was calculated 48 h after transduction.

Immunofluorescence assay and microscopy

COS-7 cells (1×10^5 cells) were seeded onto a cover glass #2 (18× 18 mm) in a 6-well plate 24 h before transfection. The cells were transfected with pMolEnv-EGFP encoding either wild-type or mutant Env–EGFP fusion protein. After 40 h, the cells were washed with 1X phosphate-buffered saline (PBS), and the coverslips were mounted in FluoroSafe mounting medium (Calbiochem, San Diego, CA). The fluorescence emission of EGFP from cells was observed using the 60X oil-immersion objective of a Zeiss-1024 confocal laser scanning microscope. Green emission was observed by exciting the fluorophore with a 488-nm laser. For colocalization studies, the cells were incubated with organelle-specific markers for 1 h at 37 °C 40 h after transfection. Golgi-specific marker BODIPY® TR C_5-ceramide complexed to BSA (0.5 mg/ml) that emits red fluorescence (Molecular Probes, OR), and late endosome/lysosome specific marker LAMP-1 conjugated with phycoerythrin was used for live cell imaging.

For the immunofluorescence assay the cells were fixed with 4% formaldehyde for 10 min at room temperature, treated with 50 mM ammonium chloride for 10 min, and then blocked with 1% BSA for 15 min at 37 °C. When permeabilized, the cells were incubated with 0.25% TritonX-100 for 10 min at room temperature after the formaldehyde treatment. Cells were incubated with primary monoclonal antibody, 83A25 (Kind gift of L.H. Evans) in 1% BSA (1:2 dilution) for 1 h and after three washes in PBS, incubated with anti-rat secondary antibody conjugated with alexa-488 (1:500 dilution) for 1 h at 37 °C. The nuclei were stained with propidium iodide for 5 min at room temperature. The coverslips were inverted onto the slides in FluoroSafe solution, and confocal microscopy was performed.

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