Structural criteria for regulation of membrane fusion and virion incorporation by the murine leukemia virus TM cytoplasmic domain

Gwen M. Taylor and David Avram Sanders*

Department of Biological Sciences, Purdue University, West Lafayette, IN 47907-1392, USA

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Abstract

The cytoplasmic domains of viral glycoproteins influence the trafficking and subcellular localization of the glycoproteins and their incorporation into virions. They also promote correct virus morphology and viral budding. The cytoplasmic domains of murine-leukemia-virus envelope-protein TM subunits regulate membrane fusion. During virion maturation the carboxy-terminal 16 amino acid residues of the TM protein are removed by the retroviral protease. Deletion of these residues activates envelope-protein-mediated membrane fusion. Our quantitative analysis of the effects of Moloney murine leukemia virus TM mutations on envelope-protein function support the proposition that a trimeric coiled coil in the TM cytoplasmic domain inhibits fusion. The data demonstrate that cleavage of the TM cytoplasmic domain is not required for viral entry and provide evidence for a model in which fusogenic and nonfusogenic conformations of the envelope protein exists in an equilibrium that is regulated by the cytoplasmic domain. In addition, a conserved tyrosine residue in the TM cytoplasmic domain was shown to play an important role in envelope-protein incorporation into retroviral particles.

Keywords: Retrovirus; Membrane fusion; Envelope; Transmembrane; Murine leukemia virus; Cytoplasmic domain; Protease

Introduction

Viral transmembrane glycoprotein complexes are responsible for the binding of enveloped viruses to cells and the fusion of viral and cellular membranes that leads to viral entry. Although the potential functions of the extravirion domains are conceptually straightforward and have been extensively explored, the roles of the membrane-spanning and intravirion domains are less clear and have been less thoroughly investigated. The membrane-spanning domains may be playing sequence-specific roles in assembly, membrane trafficking and localization, and membrane fusion. The most commonly recognized functions of the intravirion (cytoplasmic in the cell) domains are in glycoprotein trafficking and subcellular localization (Grange et al., 2000; Lodge et al., 1997; Raviprakash et al., 1990; Sauter et al., 1996; Zhang et al., 2000) and incorporation into virions (Christodouloupolos and Cannon, 2001; Lindemann et al., 1997; Owen and Kuhn, 1997; Owens and Rose, 1993; Schnierle et al., 1997; Zhao et al., 1994). Roles in virion morphology (Jin et al., 1997) and membrane-fusion completion and regulation have also been described (Bagai and Lamb, 1996; Brody et al., 1994; Cathomen et al., 1998; Gage et al., 1993; Januszski et al., 1997; Kozerski et al., 2000; Li et al., 2001; Mulligan et al., 1992; Ragheb and Anderson, 1994; Rein et al., 1994; Ritter et al., 1993; Sergel and Morrison, 1995). A role for the cytoplasmic domain of certain retroviral envelope glycoproteins in the activation of cell growth signaling pathways has also been suggested (Beaufils et al., 1993; Palmarelli et al., 2001). In most cases the biochemical and structural bases for the mechanism of action of the intravirion domain have escaped elucidation.

The envelope (Env) proteins of the murine leukemia viruses (MuLVs), γ-retroviruses, provide an interesting model system for investigating the role of viral glycoprotein intravirion domains. During the progress of the Moloney MuLV (Mo-MuLV) Env protein through the secretory sys-
tem, it forms a trimer (Kamps et al., 1991). It is subse-
quently proteolytically processed into two subunits (Hen-
derson et al., 1984; Witte et al., 1977), gp70 (amino acids
34–468) and p15E (amino acids 470–665), that are linked
through a cystine bridge (Opstelten et al., 1998; Pinter et al.,
1997; Pinter et al., 1978; Sanders, 2000). The mature form
of Env is incorporated into a budding C-type retroviral
particle. The gp70 subunit (SU) is on the outside of the
virion, whereas the p15E protein (TM) possesses an extra-
virion domain, a membrane-spanning domain, and a 35-
amino-acid domain that resides within the virion (Pinter and
Honnens, 1983). The TM protein possesses at its amin-
terminal a sequence that encodes the fusion peptide, which,
on exposure, promotes the first steps in the membrane-
fusion process that occurs during viral entry (Jones and
Risser, 1993; Taylor et al., 2001; Zhu et al., 1998). The
membrane-spanning domain of TM participates in the pro-
cess of membrane fusion in a sequence-dependent manner
(Taylor and Sanders, 1999). It has been proposed that the
binding of SU to the cellular receptor results in a thiol–
disulfide exchange reaction that eliminates the disulfide
bond between the SU and TM subunits. This reaction is
hypothesized to result in exposure of the TM fusion peptide
and, consequently, membrane fusion and viral entry (Pinter
et al., 1997; Sanders, 2000).

Mouse fibroblast NIH 3T3 cells are highly susceptible to
entry by Mo-MuLV and therefore bear the receptor to which the
Mo-MuLV Env binds. However, when NIH 3T3 cells
are mixed with cells expressing the Mo-MuLV Env protein,
no syncytia formation takes place. This appears to be a
paradoxical result, because cell–cell fusion is topologically
equivalent to viral envelope–cell membrane fusion, and
cells expressing a fusion protein are being mixed with cells
that possess cell surface receptors. Invoking a requirement
for endocytosis and low pH for the conformational changes
that lead to viral entry does not resolve the problem. Lower-
ing the pH does not promote Mo-MuLV Env-mediated
cell–cell fusion as it does in the case of influenza hemag-
glutinin- or alpha-virus glycoprotein-mediated cell–cell
fusion. A possible resolution to the paradox resides in the
finding that during a late stage of virion maturation the
carboxy-terminal 16 amino-acid residues of the p15E pro-
tein are removed by the retroviral gag–pol-encoded pro-
tease. This modification produces the 12-kDa TM protein
(Crawford and Goff, 1985; Green et al., 1981; Henderson et
al., 1984; Katoh et al., 1985; Schultz and Rein, 1985; Shin-
nick et al., 1981; Sutcliffe et al., 1980). The Env protein
present on the membrane of an infected cell is therefore
structurally different from that found in a mature virion.

Removal of the carboxy-terminal region (the R peptide)
activates Env so that it is capable of fusing membranes in a
receptor-dependent fashion (Ragheb and Anderson, 1994;
Rein et al., 1994). Consequently, the Env is in a precursor
form in the infected cell and is processed to the mature,
membrane-fusion-competent form in the viral particle. Simi-
lar data have recently been obtained concerning the enve-
lope proteins of spleen necrosis virus and porcine endoge-
nous retroviruses (Bobkova et al., 2002). A mutant Mo-
MuLV Env with an arginine residue substituting for
leucine-649 at the TM cytoplasmic domain cleavage site is
not proteolytically processed, and viral particles incorporat-
ing L649R Env have very low infectiousness (Rein et al.,
1994). However, there are some conflicting data. Cells ex-
pressing the envelope protein with the wild-type (full-
length) cytoplasmic domain can fuse with rat XC cells
(Januszeski et al., 1997; Jones and Risser, 1993). There are
conflicting data on the role of endocytosis in MuLV entry
(Katen et al., 2001; Lee et al., 1999; McClure et al., 1990;
Nussbaum et al., 1993; Ragheb and Anderson, 1994). Con-
tradictory data on the subcellular site of expression of the
Mo-MuLV Env also exist (Grange et al., 2000; Yu et al.,
1998). These data have important relevance for the deter-
mination of the site of assembly of Env and Mo-MuLV
cores. Finally, despite previous efforts, there is no under-
standing of the structural basis of the regulation of cell–cell
fusion by the TM cytoplasmic domain.

Through quantitative analysis of the effects of a variety
of modifications of the Mo-MuLV TM cytoplasmic domain
both within and without the context of the expression of
other viral components, we provide additional insight into
this complex phenomenon of fusion regulation. We also
propose a structural basis for the regulation of membrane
fusion by the TM cytoplasmic domain that unifies the avail-
able data.

**Results**

**TM cytoplasmic truncation affects protein expression and
incorporation into virus particles**

To investigate the structural basis of the regulation of
membrane fusion by the TM cytoplasmic domain (TMCD),
we examined the function of a panel of Mo-MuLV Envs
with various TMCD carboxy-terminal deletions (Fig. 1).
We expected that a direct comparison of their capacities
using our quantitative assays would offer insight into the
relative contributions of particular residues versus those of
the overall structure in fusion regulation. Expression and
processing of all mutant Envs with the exception of ∆653–
665 and ∆654–665 were at wild-type levels in gpGFP cells
(Fig. 2). The former had nearly normal levels of SU expres-
sion, but low levels of TM expression, whereas the ∆654–
665 had poor levels of expression of both SU and TM. It is
possible that the ∆653–665 TM is unstable. Nearly all of
the Envs exhibited dramatically reduced levels of incorpo-
ration into viral particles with the exceptions of the ∆639–
665 and ∆658–665 Envs. The R peptide is efficiently re-
moved in the recombinant virus particles in our expression
system, so the increase in migration of TM upon truncation
beyond the natural site of protease cleavage is readily evi-
dent.
Quantitative measurement of syncytia formation and transduction mediated by Envs bearing TMCD truncations

Expression of the wild-type Mo-MuLV Env protein in mouse NIH 3T3 cells, which bear the viral receptor and are susceptible to entry by Mo-MuLV, does not lead to any detectable syncytia formation. We investigated syncytia formation promoted by expression of the various Mo-MuLV Envs bearing TMCD deletions (Fig. 3). The level of syncytia formation is likely to be a complex function of the cell-surface concentration of the Env and of its inherent capacity for fusion promotion. We found that removal of as few as eight residues led to low but detectable syncytia formation. It had previously been observed that deletion of the carboxy-terminal seven residues of the Friend MuLV TMCD, whose sequence is nearly identical to that of the MoMuLV TMCD, did not promote syncytia formation (Yang and Compans, 1997). Decreasing the length of the TMCD increased syncytia formation until the TMCD length was identical to that found in the mature TM intraparticle domain. Further reductions in length progressively inhibited cell–cell fusion.

Transduction by recombinant virus particles produced by gpGFP cells expressing the mutant Mo-MuLV Envs was measured (Fig. 4). Use of the 293T-derived cells, which lack the receptor for Mo-MuLV, eliminates the distorting effect upon quantification of transduction of producer-cell syncytia formation that was present in earlier studies (Januszki et al., 1997). There is a decline in transduction as the size of the carboxy-terminal deletion increases, although when only eight residues remain (Δ639–665), there is only a fourfold reduction in transduction titer. There is not a direct correlation between experimentally determined levels of Env incorporation and transduction. These data indicate that the TMCD is not playing an essential role beyond membrane fusion and viral entry (Januszki et al., 1997) and that there is not a simple relationship between Env incorporation levels and transduction. The TMCD does appear to modulate the efficiency of Env incorporation into virus particles.

Fig. 1. Schematic of analyzed mutant Mo-MuLV Envs bearing mutations in the TM cytoplasmic domain. The amino acid sequence of the Mo-MuLV TM cytoplasmic domain beginning with isoleucine-631 is represented in the one-letter code. The lengths of the cytoplasmic domains remaining in the various deletion mutants are indicated by the solid bars above the Mo-MuLV TMCD sequence. The dashed line represents the site of R-peptide cleavage (between L649 and V650). The L649, L651, and Y655 point mutations are indicated below the Mo-MuLV TMCD sequence.

Fig. 2. Immunoblot analysis of cell lysate and virus bearing Mo-MuLV envelope proteins containing cytoplasmic domain truncations. Proteins in the supernatant medium and cell lysate from gpGFP cells expressing the mutant envelope proteins were separated by SDS–PAGE and immunoblotted with antibody against TM (A) and SU (B) as described under Materials and Methods. Analyses of cell lysate and virus from gpGFP cells that were transfected with the plasmid encoding the wild-type envelope protein (w.t.) or that were mock transfected (−) are also presented. At left are indicated the positions of TM (15 kDa) and SU (70 kDa).
Coiled coils consist of interacting amphipathic α helices whose amino acid sequences have a characteristic seven-residue (heptad) repeat. Hydrophobic residues are generally found at the α and d positions of the repeat. Our hypothetical TMCD coiled coil has predominantly β-branched aliphatic residues at both the α and d interfacial positions (Fig. 5). The β-branched aliphatic residues at these positions have been demonstrated to strongly favor the formation of three-stranded coiled coils (Harbury et al., 1993). In addition, in our predicted TMCD coiled coil a glutamine residue is found at an α position, and, although glutamine residues are rarely found at the α positions of dimeric coiled coils, they are common at those positions in trimeric coiled coils (Woolfson and Alber, 1995). Indeed, the replacement of the conserved asparagine at the interface of the GCN4 dimeric coiled coil with a glutamine residue favors trimeric coiled-coil formation (Gonzalez et al., 1996). It is also noteworthy that the leucine recognized by the viral protease at the R-peptide cleavage site is not buried at the predicted monomer–monomer interface.

Substitutions at the TMCD cleavage site produce effects on cell–cell fusion and transduction

To further explore the roles of R peptide cleavage in membrane-fusion regulation, we examined the effects of substitutions at the R-peptide cleavage site on Env function (Fig. 1). We altered leucine-649 to isoleucine and proline. The viral protease that cleaves the R peptide generally favors uncharged, not β-branched residues at the P1 position (L649 in the TMCD). We anticipated that each alteration should inhibit processing by the viral protease and that the Env bearing the L649P substitution should have an at least partially disrupted coiled coil and should promote

The sequence of the TMCD predicts formation of a three-stranded coiled coil

The data with the Envs bearing the smaller TM truncations (Δ650–665, Δ653–665, Δ654–665, and Δ658–665) indicate that the inherent capacity of the Env for cell–cell fusion promotion increases as the TMCD is shortened. This is consistent with the concept that a structure in the cytoplasmic domain, whose effectiveness is determined by its length, inhibits Env-mediated membrane fusion. The fact that the Δ635–665 Env promotes syncytia formation, albeit at a reduced level, indicates that the role of the TMCD is predominantly to inhibit membrane fusion; there is not a requirement, for example, for TMCD cleavage to occur so that the remaining portion of the TMCD can interact in some manner with the membrane.

Our analysis of the TMCD sequence suggests that it inhibits fusion through formation of a trimeric coiled coil.
syncytia formation in NIH 3T3 cells. We also made substitutions at leucine-651 (L651I and L649I/L651I). The sequence surrounding L651 (648ALVLTQQY655) resembles the cleavage site in the Gag–Pol polyprotein between the Mo-MuLV protease and reverse transcriptase (LQVL/TLNI (Copeland et al., 1985; Yoshinaka et al., 1985)), and we were concerned that the alternative site might be utilized if the primary site following L649 were eliminated.

Examination of the viral incorporation and processing of the mutant envelope proteins produced by gpGFP cells indicates that although the L651I is incorporated and processed similarly to the wild-type, all the other Envs had severe defects in TMCD processing (Fig. 6). The L649P Env was very poorly incorporated into viral particles, and Env incorporation data. It can be concluded that, under our experimental conditions, TMCD cleavage is not required by virus bearing the L649P Env were consistent with the Env incorporation data. It can be concluded that, under our experimental conditions, TMCD cleavage is not required for Mo-MuLV entry. In addition, although very low levels of incorporation of mutant Envs into virus particles leads to defects in viral transduction, it is clear that there is not a

Transduction by recombinant virus bearing the L649I and L651I Envs was little affected, whereas the L649I/L651I Env conveyed transduction at levels that were reduced threefold (Table 1). The lower levels of transduction by virus bearing the L649P Env were consistent with the Env incorporation data. It can be concluded that, under our experimental conditions, TMCD cleavage is not required for Mo-MuLV entry. In addition, although very low levels of incorporation of mutant Envs into virus particles leads to defects in viral transduction, it is clear that there is not a

Table 1

<table>
<thead>
<tr>
<th>Mutant envelope protein</th>
<th>Syncytia formation (NIH 3T3)</th>
<th>Syncytia formation (XC:293T)</th>
<th>Virus titer (TU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L649I</td>
<td>&lt;0.25</td>
<td>9 ± 2</td>
<td>7 ± 2 × 10^4</td>
</tr>
<tr>
<td>L649P</td>
<td>5 ± 1</td>
<td>95 ± 8</td>
<td>6 ± 2 × 10^4</td>
</tr>
<tr>
<td>L649I/L651I</td>
<td>&lt;0.25</td>
<td>0.4 ± 0.6</td>
<td>3 ± 2 × 10^4</td>
</tr>
<tr>
<td>L651I</td>
<td>&lt;0.25</td>
<td>5 ± 2</td>
<td>1 ± 0.2 × 10^5</td>
</tr>
<tr>
<td>Env</td>
<td>&lt;0.25</td>
<td>31 ± 2</td>
<td>1 × 10^8</td>
</tr>
<tr>
<td>Δ650–665</td>
<td>100</td>
<td>100</td>
<td>2 × 10^4</td>
</tr>
<tr>
<td>None</td>
<td>&lt;0.25</td>
<td>&lt;0.25</td>
<td>&lt;3</td>
</tr>
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</table>

The numbers represented here are normalized to the percentage nuclei in syncytia of the wild-type envelope protein lacking the R peptide (Δ650–665), which is 15%.

* Virus titer is given as transducing units per milliliter (TU/ml).


Tyrosine-655 and Env incorporation

To explore further the roles of the TMCD and subcellular localization signals in Env incorporation into virus particles the conserved TMCD tyrosine (AA 655) was substituted with alanine, phenylalanine, serine, or valine (Fig. 1). These mutant Envs had previously been examined with respect to targeting of Env expression and retroviral budding to the basolateral membrane (Lodge et al., 1997). The Y655A, Y655S, and Y655V Envs were completely defective in basolateral targeting, whereas the Y655F Env was partially defective. Although expression of the Y655F Env led to slightly reduced levels of syncytia formation upon incubation with XC cells; expression of the Y655F Env led to slightly reduced levels of cell–cell fusion in this assay. It can be concluded that expression of the Y655A, Y655S, and Y655V Envs at the cell surface is unlikely to be greatly diminished compared to that of the wild-type Env.

Discussion

A number of roles have previously been ascribed to the cytoplasmic domains of viral glycoproteins. These include regulation of the incorporation of viral glycoproteins into virus particles, determination of the site and efficiency of viral budding, governance of the conformation of the glycoprotein extracellular domain and processing, reduction of cell-surface expression of the glycoprotein through promoting endocytosis, and control of virus-particle morphology and of virus envelope–cell membrane fusion (Bagai and Lamb, 1996; Brody et al., 1994; Cathomen et al., 1998; Christodouloupolous and Cannon, 2001; Gage et al., 1993; Grange et al., 2000; Jin et al., 1997; Kozerski et al., 2000; Lodge et al., 1997; Mulligan et al., 1992; Ragheb and Anderson, 1994; Raviprakash et al., 1990; Rein et al., 1994; Ritter et al., 1993; Sauter et al., 1996; Schnerle et al., 1997; Sergel and Morrison, 1995; Zhang et al., 2000; Zhao et al., 1994).

We have made several major findings concerning the Mo-MuLV TMCD. First, the length of the TMCD plays a critical, if complex, role in the regulation of membrane fusion and incorporation of Env into virus particles. The data are consistent with the presence of a structure in the cytoplasmic domain that inhibits and is not required for fusion and whose disruption decreases Env incorporation. We postulate that this structure is a three-stranded coiled coil and provide some evidence in support of this hypothesis. This model is consistent with the analysis of the effects

<table>
<thead>
<tr>
<th>Mutant envelope protein</th>
<th>Viral titer (TU/ml)a</th>
<th>Syncytia formation (XC:293T)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y655A</td>
<td>5 ± 1.0 × 10^4</td>
<td>28 ± 2</td>
</tr>
<tr>
<td>Y655F</td>
<td>1 ± 0.1 × 10^4</td>
<td>13 ± 6</td>
</tr>
<tr>
<td>Y655S</td>
<td>4 ± 0.6 × 10^4</td>
<td>35 ± 7</td>
</tr>
<tr>
<td>Y655V</td>
<td>6 ± 0.2 × 10^4</td>
<td>28 ± 6</td>
</tr>
<tr>
<td>Env</td>
<td>1 × 10^3</td>
<td>25 ± 6</td>
</tr>
<tr>
<td>Δ650–665</td>
<td>2 × 10^4</td>
<td>100</td>
</tr>
<tr>
<td>None</td>
<td>&lt;3</td>
<td>&lt;3</td>
</tr>
</tbody>
</table>

a Virus titer is given as transducing units per milliliter (TU/ml).

b The numbers represented here are normalized to the percentage nuclei in syncytia of the wild-type envelope protein lacking the R peptide (Δ650–665), which is 17%.
of expression of other MuLV Env s bearing TMCD mutations (Januszeski et al., 1997; Li et al., 2001; Yang and Compans, 1997).

Second, detectable cleavage of the TMCD is not required for viral entry. Elimination of the normal site of cleavage by an isoleucine for leucine-649 substitution had little or no effect on viral transduction (Table 1). These data are consistent with those obtained previously that replication-competent Mo-MuLV bearing the L649I substitution could be produced, albeit with slower kinetics, after transfection of mutant genomes into NIH 3T3 cells (Granowitz et al., 1996). Elimination of both the major and a potential alternative cleavage site resulted in only a threefold reduction of transduction.

Third, tyrosine-655, a residue that has been observed previously to target Env expression and retroviral budding to the basolateral membrane (Lodge et al., 1997) participates in enhancing the incorporation of the Mo-MuLV Env into viral particles. Together these results have important implications for the role of the TMCD in Env function.

Regulation of Env function by TMCD cleavage has the hallmarks of the propagation of transmembrane conformational changes that occur during signal transduction. The essential components of the fusion apparatus (the receptor-binding domain, thiol-disulfide exchange motifs, and fusion peptide) are on the extraxirion side of the membrane. The intact TMCD inhibits and is not required for membrane-fusion activation. In this scenario, changes in the conformation of the TMCD somehow alter the conformation of the extraxirion region (Aguiar et al., 2003; Melikyan et al., 2000). Such transmembrane conformational changes are normally conceived as transpiring through the effects that alterations in interactions between monomers on one side of the membrane have upon intermonomer interactions on the other side of the membrane. We have previously proposed that a structure in the TMCD regulates the stability of the oligomeric state of the envelope protein and thereby its capacity for the promotion of membrane fusion (Taylor and Sanders, 1999). We provide support for the concept that it is a three-stranded coiled coil in the intact TMCD that stabilizes the Env trimer. We suggest that there is an equilibrium between a rigid Env trimer, in which exposure of the fusion peptide upon receptor binding is disfavored, and a looser trimer. In the Env with the intact TMCD the rigid trimer is favored, whereas when the TMCD is disrupted through cleavage, truncation, or mutation the trimer is destabilized so that the fusion peptide can be exposed upon receptor binding. This model is plausible in the context of the structure of the mature influenza A virus hemagglutinin in which the fusion peptide is buried at the trimer interface. The postulation of this equilibrium also provides a resolution to the seeming contradiction between TMCD coiled-coil formation and the susceptibility of the TMCD to R-peptide removal. Coiled coils exist in equilibria with unfolded monomers (Zitzewitz et al., 1995) that would be susceptible to cleavage. Our model implies that the TMCD coiled coil is only of moderate stability. It should also be noted that proteolytic processing of the TMCD is inefficient and only has to occur once per trimer to destabilize coiled-coil formation permanently.

Our hypothesis makes a number of predictions. The first is that single substitutions in the cytoplasmic domain that should disrupt the coiled coil may promote cell-cell fusion. This prediction has been fulfilled with the L649P Env and may underlie the activation of cell-cell fusion seen with expression of some other mutant MuLV Envs. It has been demonstrated recently that a single proline for alanine substitution in a predicted α-helix in the cytoplasmic domain of herpes simplex virus type-1 glycoprotein B also promotes syncytia formation, as does deletion of the helix (Foster et al., 2001). These data suggest that the regulation of membrane fusion by viral glycoprotein cytoplasmic domains may have common structural features.

It is noteworthy that the level of syncytia formation with the L649P Env is not as high as that produced by the Δ650–665 Env upon expression in the NIH 3T3 cells, although cell-cell fusion is at comparable levels for the two Envs in the 293T/XC assay (Table 1). Soluble coiled coils normally are at least 28 residues in length and are disrupted by insertions of single proline residues. However, coiled coils attached to membranes have one fewer degree of freedom (they cannot rotate in a plane perpendicular to the membrane) and therefore need not be as thermodynamically stable as they would have to be if they were free in solution. It has been shown with the influenza A virus hemagglutinin that multiple insertions of proline residues may be required for disruption of membrane-bound coiled coils (Qiao et al., 1998).

Our hypothesis also predicts that there should exist Envs bearing substitutions outside of the TMCD that should promote NIH 3T3 cell-cell fusion in the context of the full-length wild-type TMCD. Mo-MuLV Envs bearing a substitution of a serine residue for tryptophan-606 in the juxtamembrane domain possess such a capacity (Taylor and Sanders, 1999). The substitution may counteract the presence of the cytoplasmic domain by reducing trimer stability. One alternative conjecture to the regulation of fusion-peptide exposure by the TMCD is that the regulation of membrane fusion occurs through alterations of the conformation of the membrane-spanning domain, which itself may participate in intermonomer interactions and directly in membrane fusion (Taylor and Sanders, 1999).

The concept that the TMCD is regulating an equilibrium between two forms of Env may explain the susceptibility of XC cells to fusion even in the presence of a full-length TMCD (Jones and Risser, 1993) and the lack of requirement for TMCD cleavage in viral entry seen with the virus bearing the L649I Env (Table 1). XC cells may, for example, have active receptors at higher local concentrations on its surface and, given that there is an equilibrium between fusion-competent and fusion-incompetent forms of Env, there may be sufficient concentrations of active receptor-fusion complexes to lead to some levels of fusion. In the case of the NIH 3T3 cells, there may be insufficient concentrations of such complexes for fu-
sion to occur. However, upon concentration of the Env in virus particles there may be sufficient levels of receptor–Env complexes for the unfavorable equilibrium to be overcome. It is possible furthermore that cell–cell fusion is more sensitive to subtle differences in protein processing or expression at the cell surface than is transduction. This may explain the reduction in fusion between 293T cells expressing the L6491 and L6511 Envs and XC cells.

The data also provide information on the dependence of Env incorporation upon the sequence of the TMCD that can be explained most readily in the context of our structural model. Retroviruses incorporate their glycoproteins preferentially over cellular proteins. They can incorporate productively the glycoproteins of other enveloped viruses, but the structure of the glycoprotein cytoplasmic domain appears to affect the efficiency of incorporation (Christodouloupolos and Cannon, 2001; Lindemann et al., 1997; Lodge et al., 1998; Schnierle et al., 1997). An Mo-MuLV TMCD with an intact coiled coil would have a compact structure that could be accommodated in the virus particle. Once the Env is incorporated the rigidity of the structure would be of lesser importance. As the TMCD is truncated or as the putative TMCD coiled coil is disrupted, the TMCD would become less compact and therefore more difficult to incorporate into viral particles. At a certain point, as exemplified by the Δ639–665 Env, the TMCD is small enough to be incorporated despite the lack of a stable coiled coil. This model can also explain data concerning the incorporation of gibbon ape leukemia virus (GaLV) and GalV/MuLV chimeric Env incorporation into retroviral and lentiviral particles (Christodouloupolos and Cannon, 2001). The presence of a disordered structure in the TMCD may also affect the stability of the mutant protein in the cell (e.g., Δ654–665). These conclusions are not intended to rule out the idea that there is in addition specific recognition of the Mo-MuLV TMCD by the Mo-MuLV Gag polyprotein.

The results on viral incorporation obtained with the mutant Envs bearing substitutions for tyrosine-655 may be interpreted within the same structural context. In the predicted coiled coil, tyrosine-655 is on an exposed surface and therefore available for recognition by cellular and/or viral proteins. Tyrosine-655, which is present within a common subcellular localization motif (YXX-hydrophobic) that in other contexts promotes endocytosis (LaBranche et al., 1995; Sauter et al., 1996), had previously been demonstrated to target Env expression and retroviral budding to the basolateral membrane (Lodge et al., 1997). If incorporation of Env into viral particles occurred at the plasma membrane then one might have anticipated that removal of a potential endocytosis signal would increase cell surface expression of the Env and thereby increase the amount of Env recovered in retroviral particles. In fact, although the Y655A, Y655S, and Y655V Envs are expressed and processed at wild-type levels and are capable of promoting wild-type levels of syncytia formation between 293T and XC cells, they are incorporated at greatly diminished levels. The substitutions do not convert the Env into a form that is fusogenic when expressed in NIH 3T3 cells, so the incorporation defect is not the result of the gross structural alterations that we postulate occur when the TMCD coiled coil is disrupted. These data also are evidence against the hypothesis that the cell–cell fusion observed when the R peptide is removed results from the elimination of the endocytosis signal in the TMCD (Grange et al., 2000).

Two alternatives concerning the role of tyrosine-655 are possible. The first is that tyrosine-655 is directly involved in contact with the Gag polyprotein and that phenylalanine can at least partially substitute for the tyrosine residue. This is similar to the postulated function of a conserved tyrosine residue in the cytoplasmic domain of the alphavirus E2 glycoproteins (Zhao et al., 1994). In this model the Gag polyprotein and the cellular sorting apparatus (e.g., the proteins involved in endocytosis) compete for binding of tyrosine-655. When the Gag polyprotein is not present in high enough concentrations, the virus relies upon the cell to remove the Env protein from the plasma membrane so that immune recognition is inhibited. A similar model has been suggested for the HIV-1 Env (Egan et al., 1996).

Another possibility is that, in the context of the full-length TMCD, tyrosine-655 promotes the trafficking of Env to a subcellular site, such as a lipid raft, where it can promote retroviral budding (Pickl et al., 2001). Remarkably, it has been demonstrated previously that the site of expression of the Mo-MuLV Env can dictate the site of budding of HIV-1 in polarized epithelial cells, despite the lack of sequence similarity between the Mo-MuLV and HIV-1 TM-CDs (Lodge et al., 1997). It was also determined that the localization of budding produced by Env expression is dependent upon tyrosine-655 (Lodge et al., 1997). It is not implausible that tyrosine-655 directs the Env to a site where its incorporation into Mo-MuLV particles takes place most efficiently (Vincent et al., 1999), such as the endosomal multivesicular bodies.

The sequence motif that contains tyrosine-655, Y-X-X-aliphatic, is one that is recognized by certain SH2-domain-containing proteins upon phosphorylation of the tyrosine by tyrosine kinases. It has been proposed that such motifs in the cytoplasmic domains of the bovine leukemia virus and Jaagsiekte sheep retrovirus (JSRV) envelope proteins play roles in the activation of cell-growth signaling pathways (Beaufils et al., 1993; Palmarini et al., 2001). In the case of the JSRV TMCD the tyrosine (tyr-590) that is critical for the ability of the envelope protein to induce the cellular transformation that is likely to lead to ovine pulmonary carcinoma is the equivalent of the Mo-MuLV TM tyrosine-655. It appears plausible that tyrosine-590 of JSRV Env also helps to dictate the subcellular site of expression of Env.

Overall, the main purpose of the regulation of the processing of the TMCD is to ensure that the Env is maximally active only when it is present in the vehicle, the viral particle, which guarantees the maximal transmission of the viral genome concomitant with the fewest deleterious effects to the host. We propose that the Env protein exists in an equilibrium between a form that is competent for fusion promotion and one that is not competent and that it is the
presence of a coiled coil in the TMCD that stabilizes the fusion incompetent form. The existence of the equilibrium allows for the promotion of membrane fusion by EnvS bearing intact TMCDs. We anticipate that secondary and tertiary structures, as opposed to motifs, in the cytoplasmic domains of other viral glycoproteins will be playing important functional roles and that equilibria between conformational states will be recognized as critical regulators of membrane fusion.

Materials and methods

Cell lines and cell culture

Mouse NIH 3T3 fibroblast cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Sigma) with 10% calf serum (CS; Sigma), penicillin (10 U/ml), and streptomycin (0.1 mg/ml) (PS; Sigma) (DMEM CS/PS). 293T and gpGFP cells (which produce envelope protein-deficient replication-incompetent Mo-MuLV particles carrying MFG.S GFP-S65T, a retroviral vector encoding the gag-pol region of human immunodeficiency virus type 1) were grown in DMEM with 10% fetal bovine serum (FBS; Sigma) and PS. Rat XC sarcoma cells were grown in modified Eagle’s medium (MEM; Sigma) with 10% FBS and PS.

Construction of plasmids encoding Mo-MuLV envelope proteins bearing substitutions in the cytoplasmic domain

For the Δ653–665, Δ654–665, Δ656–665, Δ658–665, L649I, and L649P mutations in the cytoplasmic domain, the penv1Δ650–665 plasmid (Taylor and Sanders, 1999) was cut with StuI and NheI and ligated with double-stranded oligonucleotides encoding the mutations. The Δ635–665, Δ639–665, and Δ642–665 mutations were constructed by ligating the doubled-stranded oligonucleotides encoding the mutations between the ClaI and NheI site in the penv1Δ650–665 plasmid. Plasmids encoding the substitutions for tyr-655 (alanine, phenylalanine, serine, or valine) have been used previously (Lodge et al., 1997) and contain double-stranded oligonucleotides encoding for the mutations inserted between the StuI and AflII sites of penv1min (Taylor and Sanders, 1999).

Syncytia formation assays

Syncytia formation assays using NIH 3T3 cells were carried out as described previously (Taylor and Sanders, 1999). NIH 3T3 cells (5×10^5) were transiently transfected with 4 μg of mutant Env-encoding plasmids using LipofectAMINE (Gibco, BRL) and Opti-MEM media (Gibco BRL). Syncytia and the number of nuclei in syncytia were counted 32 h posttransfection. There were approximately 1200 cells per field. Syncytia assays were carried out in triplicate.

When syncytia formation promotion by Mo-MuLV envelope proteins containing the full-length cytoplasmic tail expressed in 293T cells was examined, 2×10^6 293T cells were transfected with 4 μg DNA using LipofectAMINE as above. Twenty four hours posttransfection XC cells and 293T cells were trypsinized and mixed by plating 2×10^6 293T cells and 1×10^6 XC cells on a 60-mm tissue culture dish. After 24 h of growth in DMEM with 10% FBS and PS the syncytia were counted.

Viral transduction assay

Transduction assays were carried out as described previously (Taylor and Sanders, 1999). Plasmids encoding the mutant envelope proteins were transiently transfected into gpGFP cells (Taylor and Sanders, 1999). Medium from the transiently transfected gpGFP cells was incubated with 1×10^6 NIH 3T3 cells in the presence of 8 μg/ml hexadimethrine bromide for 4 h. Forty-eight hours later the cells were removed from the plate and analyzed by flow cytometry with a Coulter XL-MCL flow cytometer, using a 525-nm bandpass filter and a 488-nm air cooled argon laser. Transduction assays were carried out in triplicate.

SDS-PAGE and immunoblot analysis

Virus produced by transfected gpGFP cells was filtered and pelleted through a 25% sucrose cushion at 75,000g and 4°C for 2.5 h in a 50.2 Ti Beckman rotor and suspended in equal volumes of 1×RIPA (140 mM NaCl, 10 mM Tris–HCl; pH 8.0, 5 mM EDTA, 1% Na deoxycholate, 1% Triton X-100, and 0.1% SDS) buffer and SDS–PAGE sample buffer with β-mercaptoethanol. The cells were washed twice with phosphate-buffered saline and lysed in 1 ml of lysis buffer (50 mM Tris–HCl; pH 8.0, 5 mM EDTA, 150 mM NaCl, and 1% Triton X-100). The cell debris was removed by centrifugation at 16,000g. A 15-μl aliquot was used for immunoblot analysis. When antibody against SU was used, the protein samples were run on 7% SDS–PAGE gels, transferred to nitrocellulose as described previously (Sharkey et al., 2001), and probed with goat anti-Rauscher leukemia virus (RLV) gp70 antisera (lot 80S-019; Quality Biotech) (1:1000) and rabbit anti-goat conjugated to horse-radish peroxidase (Chemicon) (1:2500). Proteins were detected by using the ECL detection system (Amersham). When antibody against TM was used, the protein samples were run on 10–20% gradient gels, transferred to PVDF membrane (Millipore) at 100 mA for 1 h in transfer buffer and probed with 2 μg/ml rat monoclonal antibody 10CE11(kindly provided by A. Pinter (Li et al., 1997)) and goat anti-rat conjugated to horseradish peroxidase (Chemicon) (1:10,000). Proteins were detected by using the Pierce Supersignal detection system (Pierce). When antibody against capsid protein was used, membranes were probed using goat anti-RLV p30 antisera (lot 78S000221; Quality Biotech) (1:1000) and rabbit anti-goat conjugated to horse-
radish peroxidase (Chemicon) (1:2500). Proteins were detected by using the ECL detection system (Amersham).

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