# Dissection of a retrovirus envelope protein reveals structural similarity to influenza hemagglutinin

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**Background:** The amino-acid sequences of retroviral envelope proteins contain a '4–3 hydrophobic repeat', with hydrophobic amino acids spaced every four and then every three residues, characteristic of sequences that form coiled coils. The 4–3 hydrophobic repeat is located in the transmembrane subunit (TM) of the retroviral envelope protein, adjacent to the fusion peptide, a region that inserts into the host bilayer during the membrane-fusion process. A 4–3 hydrophobic repeat region in an analogous position of the influenza hemagglutinin protein is recruited to extend a three-stranded coiled coil during the conformational change to the fusion-competent state. To determine the conformation of the retroviral TM subunit and the role of the 4–3 hydrophobic repeat, we constructed soluble peptide models of the envelope protein of Moloney murine leukemia virus (MMLV).

**Results:** The region of the MMLV TM protein external to the lipid envelope (the ectodomain) contains a stably folded, trimeric, protease-resistant core. As predicted, an α-helical segment spans the 4–3 repeat. A cysteine-rich region carboxy-terminal to the 4–3 repeat confers a dramatic increase in stability and displays a unique disulfide bonding pattern.

**Conclusions:** Our results demonstrate that the MMLV TM subunit can fold into a stable and distinct species in the absence of the receptor-binding 'surface' co-subunit (SU) of the envelope complex. As the SU subunit is readily shed from the surface of the virus, we conclude that the TM subunit structure forms the core of the MMLV membrane-fusion machinery, and that this structure, like the fusion-active conformation of influenza hemagglutinin, contains a three-stranded coiled coil adjacent to the fusion peptide.

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## Background

The envelope (Env) proteins of retroviruses bind to cell-surface receptors and promote fusion with the host cell membrane [1]. The mechanism of retrovirus-mediated membrane fusion has remained elusive, because of the lack of detailed structural information about Env proteins. Structural studies have been hindered by the low virus titer in the natural systems studied, and by the large size of the envelope complexes. Furthermore, the association between the two Env subunits, termed the transmembrane (TM) and surface (SU) subunits, is labile, and the protein complexes are membrane-bound.

By contrast, the hemagglutinin (HA) protein of the orthomyxovirus influenza has been characterized by X-ray crystallography in both the native [2] and the low-pH activated forms [3]. These high-resolution HA structures, together with other studies, have led to a model for HA-mediated membrane fusion in which dissociation of the receptor-binding domains from one another [4,5] accompanies extension of a coiled coil to bring the fusion peptides into a position that enables them to interact with the target cell membrane [6]. The fusion-active subunit would then bridge the viral and cellular membranes to form a critical intermediate in the membrane-fusion process.

Although the orthomyxovirus and retrovirus families have no obvious evolutionary relationship, features of influenza HA are similar to those of retroviral Env proteins. First, both HA and Env are composed of two fragments cleaved from a common precursor; in the case of HA, these fragments are referred to as $HA_1$ and $HA_2$. Like $HA_1$, the retroviral SU glycoprotein binds to the host-cell receptor [1]. The retroviral TM subunit contains a hydrophobic sequence at its amino terminus analogous to the fusion peptide of the influenza $HA_2$ subunit [7], which inserts into the target membrane [8,9]. The retroviral TM subunit, like $HA_2$, contains a 4–3 hydrophobic repeat that is predicted to form a coiled coil [10,11]. Finally, the shedding of the SU subunit of retroviruses such as HIV [12] and murine leukemia virus [13] might parallel the displacement of influenza $HA_1$ during the conformational change to the fusogenic state.

The central features of the influenza fusogenic state were preserved in the crystallized fragment of low-pH-converted HA, despite the absence of the fusion peptide, the transmembrane helix and most of the receptor-binding domain [3]. Furthermore, the 'spring' to the fusion-competent conformation was modeled using peptides corresponding to only the coiled-coil regions of HA [6]. We consequently undertook a protein dissection study of a retrovirus Env protein in order to characterize the structure of the retroviral membrane-fusion apparatus.

Moloney murine leukemia virus (MMLV) was chosen for study because its TM subunit is small (15 kDa) and lacks glycosylation sites. Moreover, the disulfide bond between the MMLV SU and TM subunits can be eliminated by an apparent disulfide rearrangement [13]. The requirement

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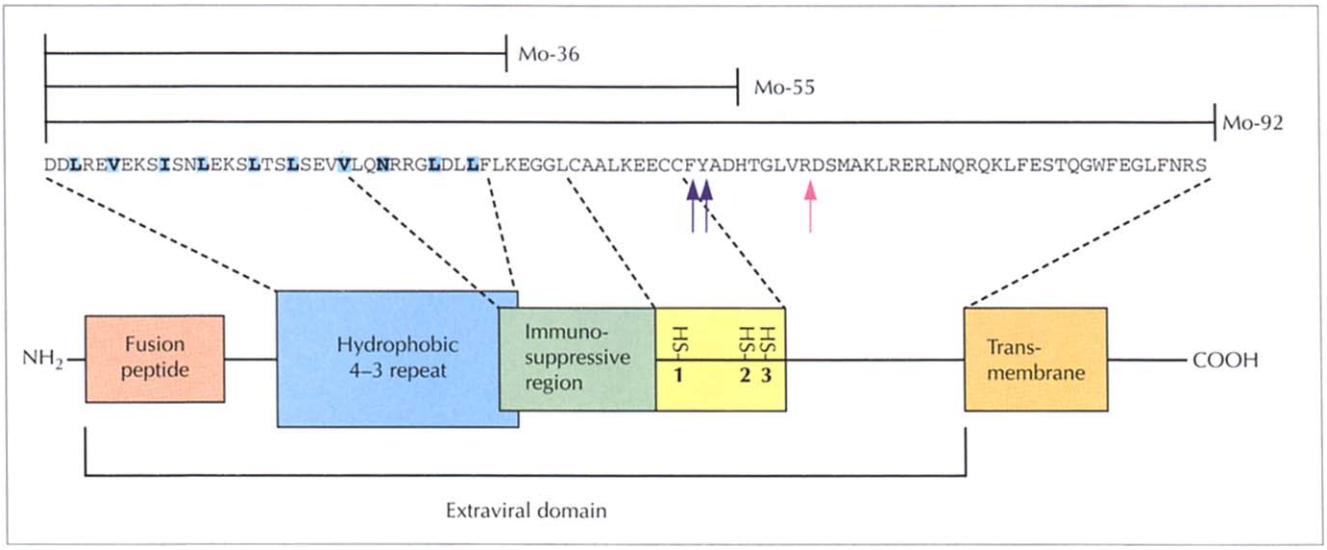


**Fig. 1.** Primary structure of the TM protein of MMLV. A linear map of the protein is shown, with the sequence (single-letter amino-acid code) of Mo-92 specified. Regions modeled by the three peptides, named for the number of residues they contain, are indicated above the sequence. Core residues of the 4–3 hydrophobic repeat are boxed in blue. The three cysteine thiols are numbered 1–3. The trypsin cleavage site is marked by a pink arrow and the two chymotrypsin sites by purple arrows. A description of the immunosuppressive region is found in the Discussion.

for disruption of a covalent bond during shedding of the SU subunit implies a specific mechanism for the disposal of SU. Therefore, the structure of the isolated TM subunit of MMLV may be important in the membrane-fusion mechanism of this retrovirus.

Peptides were designed to determine whether the MMLV TM protein could fold independently of the SU subunit. To avoid the difficulty of working with insoluble peptides, we studied the hydrophilic segment of the transmembrane protein by removing the fusion peptide and the transmembrane region from our model constructs.

## Results

### A peptide model of the TM subunit folds into a stable structure

The primary structure of the MMLV TM protein is shown in Figure 1. We produced a peptide, Mo-92, that extends from Asp45 in the TM sequence to the beginning of the transmembrane region. Asp45 was chosen because it is at an **f** position near the beginning of the 4–3 hydrophobic repeat region, placing the positively charged amino terminus away from the hydrophobic core (Fig. 2), and was predicted by the Paircoil algorithm [14] to be near the start of the coiled coil. To verify this choice, a peptide was constructed starting at Met31 and including the following additional sequence from the TM protein: MATQQFQQLQAAVQ (single-letter amino-acid code). When this peptide was subjected to proteolysis with thermolysin, the amino terminus was trimmed to Val43. Although thermolysin readily cleaves before leucine residues, prolonged incubation did not result in cleavage before Leu47. Therefore, the amino terminus of Mo-92 is within two residues of the start of the protease-resistant, and presumably well-folded, region.

Circular dichroism (CD) spectroscopy (Fig. 3) indicated that our peptide model for the TM protein has a helix content of approximately 59 % (Table 1). The Mo-92

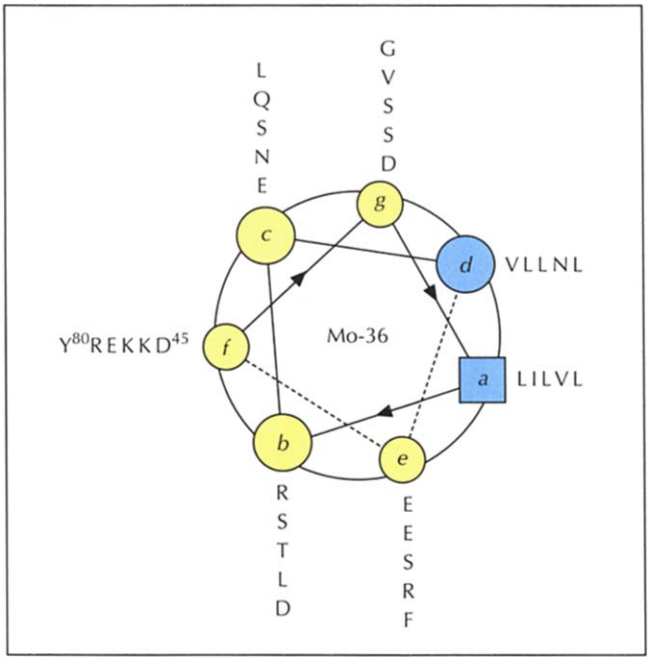


**Fig. 2.** Helical wheel representation of Mo-36. The sequences of coiled-coil proteins consist of 4–3 hydrophobic repeats, where positions of the heptad are labeled with the letters **a–g**. The view is down the helical axis starting at the amino terminus (position **f**) of the peptide, which corresponds to residue Asp45 of the transmembrane protein. Core positions of the 4–3 hydrophobic repeat (a and d) are highlighted in blue, and other positions are shown in yellow.

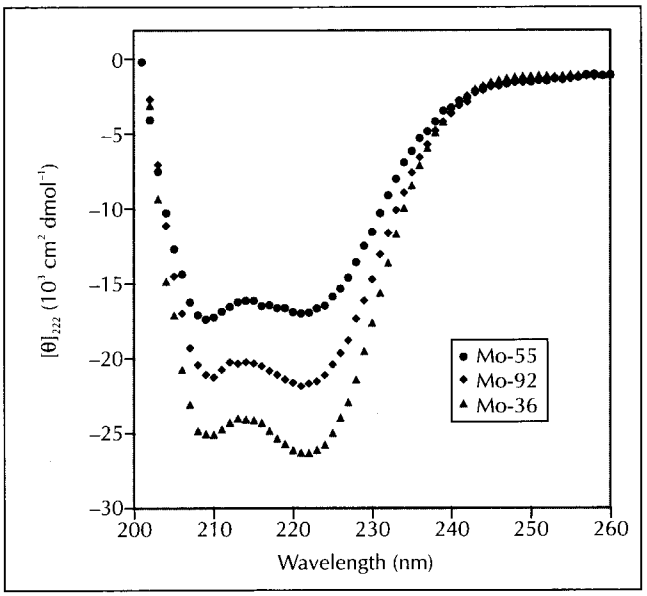


**Fig. 3.** CD spectra of peptide fragments of the TM protein. Each fragment displays a characteristic helical spectrum at 2 °C, with double minima at 222 nm and 208 nm.

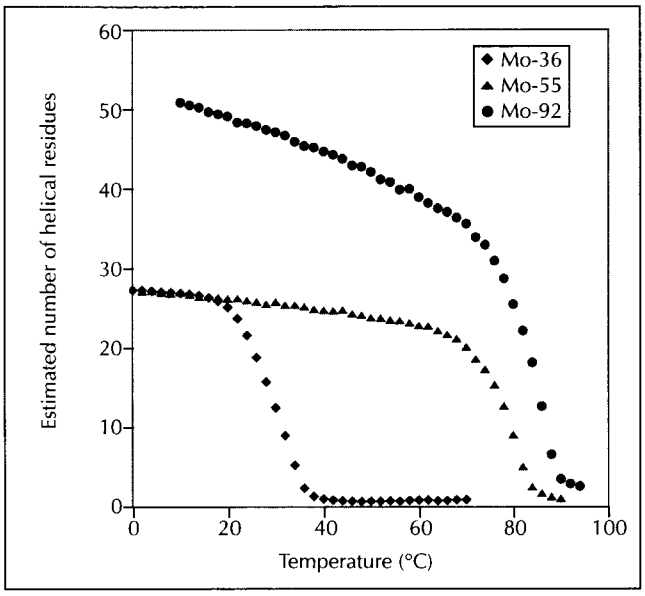


**Fig. 4.** An estimate (see Materials and methods) of the number of helical residues in each fragment plotted as a function of temperature. Mo-36 and Mo-55 lose most of their helicity during the cooperative unfolding transitions. On the other hand, approximately 20 helical residues — almost 40 % of the total helix content — unfold in Mo-92 before the cooperative transition occurs.

structure is remarkably stable, with a temperature midpoint for thermal unfolding ($T_m$) of 86 °C under physiological conditions. Peptides truncated at the carboxyl terminus, denoted Mo-55 and Mo-36 (Fig. 1), were constructed to locate the secondary structure within Mo-92. Mo-36 spans the 4–3 hydrophobic repeat region, and the CD spectrum indicates that approximately 28 residues of this peptide are in a helical conformation; we conclude that the 4–3 repeat region is helical. Because the Mo-55 peptide contains no additional helical residues, as judged by CD analysis, but is substantially more stable than Mo-36 ($T_m$ values of 80 °C and 30 °C, respectively), we conclude that the immunosuppressive and cysteine-rich regions, although folded, are in a non-helical conformation.

Mo-92 has a higher helix content than Mo-55, indicating that additional helical segment(s) are present carboxy- terminal to the cysteine-rich region. However, these segments do not confer a substantial increase in stability. A thermal melting study of Mo-92, in which the CD signal was monitored at 222 nm, revealed a folded baseline with a slope greater than that produced by Mo-55. When the estimated number of helical residues in each peptide is plotted against temperature (Fig. 4), it appears that additional helical region(s) within Mo-92 melt non-cooperatively and independently, prior to the transition for the amino-terminal helical region.

**Table 1.** Helix content and stability of MMLV TM protein model peptides monitored by circular dichroism.

| Peptide | $-[\theta]_{222} \times 10^{-3}$ | Helical residues | $T_m$ (°C) |
|---|---|---|---|
| Mo-36 | 26 | ~ 28 | 30 |
| Mo-55 | 17 | ~ 28 | 80 |
| Mo-92 | 21 | ~ 54 | 86 |

The ellipticity at 222 nm and the temperature midpoint for thermal denaturation are indicated for each MMLV TM protein fragment. Estimates of the number of helical residues in each peptide and determinations of $T_m$ values were made as described in Materials and methods.

### The TM protein forms a trimeric structure

The oligomerization state of the transmembrane protein model peptide, Mo-92, was determined by analytical ultracentrifugation. The data were consistent with a trimer model over a nine-fold concentration range (Fig. 5a). However, a systematic trend is observed in the residuals between the data and the linear fit, becoming more severe with increasing concentration. This behavior is diagnostic of a tendency toward aggregation.

To pare away regions contributing to non-specific aggregation, Mo-92 was subjected to proteolysis, and the products were analyzed by mass spectrometry. Digestion with trypsin (Fig. 1; pink arrow) or chymotrypsin (Fig. 1; blue arrows) removed carboxy-terminal sequences but left the amino terminus intact, yielding fragments similar to Mo-55. Mo-55 thus corresponds to a protease-resistant subdomain within the TM subunit. When Mo-55 was subjected to analytical ultracentrifugation, the resulting data fitted closely to a trimer model, and no systematic residuals were observed (Fig. 5b).

### TM protein contains a specific disulfide bond and a free thiol

The protease-resistant subdomain of the MMLV TM protein contains three cysteine residues, the presence and spacing of which are preserved in C- and D-type retroviruses [15,16]. The formation of disulfide bonds

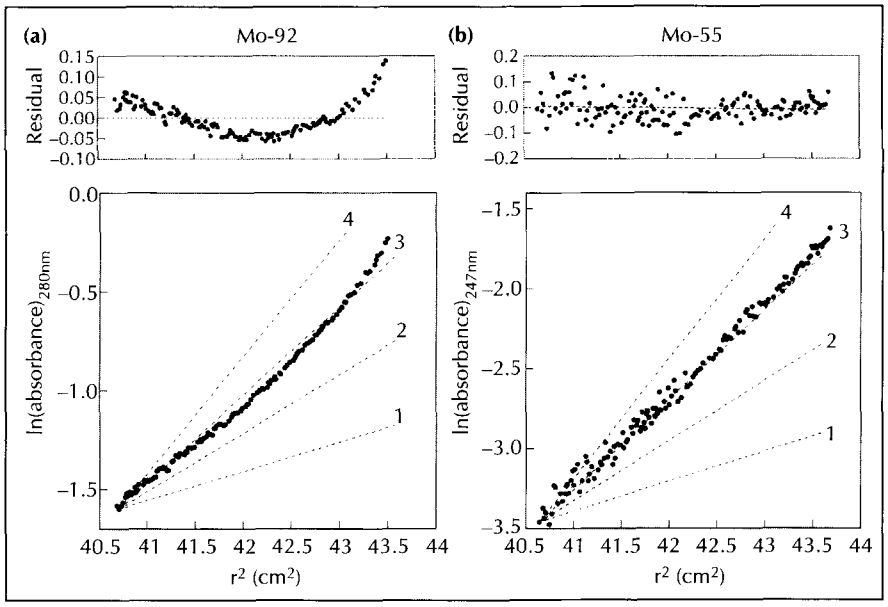


**Fig. 5.** Equilibrium sedimentation of TM protein model peptides. Representative data are plotted as ln(absorbance) against the square of the radius from the axis of rotation. The slope is proportional to molecular mass (see Materials and methods). Dashed lines with increasing slopes indicate calculated values for monomeric (1), dimeric (2), trimeric (3) and tetrameric (4) peptides. **(a)** Data for Mo-92 are consistent with a trimeric model, but with systematic residuals, indicating a tendency to aggregate. **(b)** Data for Mo-55 fit closely to a trimeric model, but leave no systematic residuals.

between these residues is therefore likely to be structurally and/or functionally significant. Mapping of disulfide bonds in Mo-92 was challenging, because there are adjacent cysteine residues that cannot be separated by proteolysis. Instead, oxidized Mo-92 was incubated with 2-nitro-5-thiocyanobenzoic acid (NTCB), which cleaves proteins at free cysteine residues [17].

NTCB-induced cleavage of Mo-92 yielded fragments with molecular masses of 5 597 Da and 5 081 Da, corresponding to the amino- and carboxy-terminal products, respectively, of cleavage at the third cysteine (C3) in the TM sequence (Fig. 1). Control experiments using reduced mutant proteins, in which specific cysteine residues were replaced with alanine residues (used in the disulfide-permutation experiments described below), demonstrated that fragments resulting from cleavage at either of the adjacent cysteine residues could be obtained and readily distinguished from one another by mass spectrometry. Thus, the NTCB cleavage results indicate that only C3 in oxidized Mo-92 is reactive with NTCB, and that C1 and C2 are likely to be protected by an intramolecular disulfide bond. Cleavage of oxidized Mo-55 produced a 5 600 Da amino-terminal fragment, demonstrating that the protease-resistant core is sufficient to specify the unique disulfide-bonding pattern that leaves only C3 reactive with NTCB.

To verify that C3 remains free as a result of the preferential formation of the C1–C2 disulfide bond, we performed a disulfide permutation experiment. Three mutant versions of Mo-92 were constructed by replacing each cysteine individually with alanine. These mutant peptides were oxidized in the presence of 6 M guanidine hydrochloride (GuHCl) to remove structural barriers to formation of non-native disulfide bonds. After refolding by removal of GuHCl, the stability of each oxidized peptide was assayed by thermal denaturation (Table 2). The mutant with a disulfide bond between C1 and C2, denoted Mo-92.3, was most stable, with a $T_m$ of 86 °C. In contrast, the other two mutants (Mo-92.1 and Mo-92.2) are substantially destabilized, with $T_m$ values of 76 °C and 70 °C, respectively. The permutation experiment, repeated using cysteine-to-alanine mutants of Mo-55, gave qualitatively similar results (Table 2). Interestingly, for both Mo-92 and Mo-55 backgrounds, the folded baselines of the three disulfide variants were identical, indicating that alternate disulfide pairings accommodate the helical region completely, albeit with impaired stability. These disulfide permutation experiments are consistent with the NTCB cleavage data and confirm that the most stable form of the isolated TM protein contains a disulfide bond between C1 and C2.

## Discussion

The structure of the retroviral TM protein is central to viral entry into host cells for several reasons. Firstly, this protein contains the fusion-peptide region. In addition,

**Table 2.** Disulfide permutation analysis of MMLV TM protein model peptides.

| Peptide | $T_m$ (°C) | $-[\theta]_{222} \times 10^{-3}$ at 10 °C |
|---|---|---|
| Mo-92.1 | 70 | 20 |
| Mo-92.2 | 76 | 20 |
| Mo-92.3 | 86 | 20 |
| Mo-55.1 | 62 | 17 |
| Mo-55.2 | 72 | 17 |
| Mo-55.3 | 80 | 17 |

The table displays the $T_m$ values and the folded CD signal at 10 °C for the Mo-92 and Mo-55 mutants in the oxidized form. In each set of mutants, the number after the decimal point indicates the position of the alanine. Thus, Mo-92.1 refers to a mutant with a disulfide bond between C2 and C3, and an alanine substitution at C1.

the most highly conserved regions of the retroviral Env proteins are present within the TM subunit. Carboxy-terminal to the 4–3 hydrophobic repeat in C- and D-type retroviruses the TM subunit invariably contains an 'immunosuppressive sequence' [15]. Peptides with the consensus sequence for this region have been shown to inhibit lymphocyte proliferation [18] and the production of interferon γ [19]. Adjacent to the immunosuppressive region are the conserved cysteine residues, at least one of which must be involved in the initial covalent association with the MMLV SU subunit [20]. Furthermore, mutagenesis studies have shown that infectivity of MMLV is particularly sensitive to amino-acid substitutions [21] and insertions [22] in the TM subunit. Finally, a construct expressing the MMLV TM subunit alone partially complements a non-fusogenic Env mutant anchored to the viral membrane by a glycosylphosphatidylinositol linkage [23]. These observations strongly suggest that efforts to elucidate the mechanism of retrovirus-mediated membrane fusion should target the TM subunit.

Conflicting reports have emerged regarding the oligomerization order of retroviral envelope proteins, such as murine and feline leukemia viruses [24,25] and HIV [26–28]. Peptide studies of the HIV TM protein located a helical region in an analogous position to the MMLV 4–3 repeats, but the oligomerization state of these peptides was ambiguous [29]. Our results show that the region of the MMLV TM protein adjacent to the fusion peptide forms a folded, helical and trimeric structure, most likely as a three-stranded α-helical coiled coil.

The immunosuppressive region and the cysteine residues, is also folded in our peptide model of the isolated protein, as shown by the following observations: first, these regions are present in the protease-resistant core; second, the thermal stability of Mo-55 is considerably greater than that of Mo-36; and third, only one of the three potential intramolecular disulfide bonds is found. The observation that a substantial portion of the TM protein is stably folded in the absence of the SU subunit supports our hypothesis that this conformation of the TM protein corresponds to a functional state of the retrovirus envelope.

The TM protein can be isolated on the surface of the virus by a disulfide rearrangement that results in loss of the SU subunit [13]. If shedding of the SU subunit proves to be a required step in membrane fusion by retroviruses, then studying the connectivity of the disulfide bonds in MMLV will provide a novel means of monitoring the progress of a virus envelope protein through the receptor-binding and membrane-fusion events. A disulfide-bonding pattern for the SU subunit released from a murine leukemia virus has been proposed [30]. Our studies show a preference for disulfide-bond formation between the first and second cysteine residues in the isolated TM subunit, leaving the third cysteine with a free thiol. Identification of the disulfide-bonding pattern in the SU–TM protein complex will reveal the nature of the MMLV disulfide rearrangement required for shedding of the SU subunit.

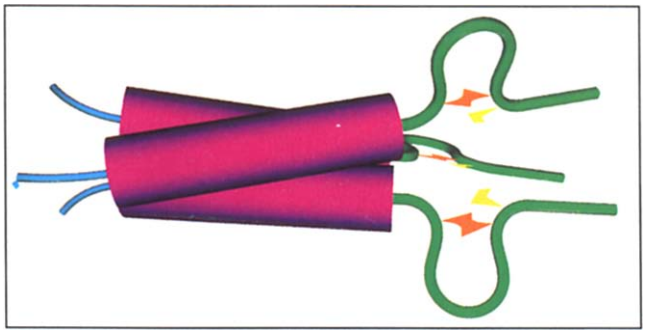

**Fig. 6.** Proposed model for the core of the fusion-active conformation of the MMLV TM protein. Purple cylinders represent the three-stranded coiled coil adjacent to the fusion peptides (blue). For simplicity, supercoiling of the coiled coil is not depicted. The green loops represent the immunosuppressive and the cysteine-rich regions. The free cysteine residues are shown in yellow. The first two cysteine residues readily form a disulfide bond (shown in orange), as judged by NTCB cleavage data and the thermal stabilities of cysteine-to-alanine mutants (see text).

## Conclusions

We propose that shedding of the SU subunit leaves behind a folded, trimeric fusogenic complex (Fig. 6), similar to the low-pH-induced structure of influenza HA [3]. In the native state of influenza, the receptor-binding domains inhibit formation of the extended coiled coil, thereby serving as the basis of the "spring-loaded mechanism" [6] for the HA conformational change. It remains to be seen whether the residues corresponding to the coiled coil identified here are in an alternative conformation in the native SU–TM protein complex. In either case, because the trimeric coiled-coil motif is found in analogous positions in the proposed fusion-competent states of the influenza and Moloney murine leukemia viruses, it is likely that coiled coils will also prove to be central to the membrane-fusion mechanism of retroviruses.

## Materials and methods

### *Production and purification of synthetic peptides*

The Mo-36 peptide and a peptide six residues shorter at the carboxyl terminus, Mo-30, were synthesized using Fmoc chemistry and purified using a method described previously [31]. These peptides contain non-native carboxy-terminal tyrosine residues to facilitate concentration determination [32]. Mo-30 was predominantly unfolded and was not studied further.

### *Production and purification of recombinant peptides*

A DNA fragment encoding Mo-92 was generated by PCR from a plasmid containing the gene encoding the MMLV envelope protein (kindly provided by D. Sanders and R.C. Mulligan), using primers containing 5′ *Nde*I and 3′ *Bam*HI sites. The amplified fragment was subcloned into the pAED4 vector [33]. Plasmid pAED4 containing the sequence encoding Mo-55 was prepared in a similar way, using a different 3′ PCR

primer. Both recombinant peptides included amino-terminal methionine residues for expression. The sequences of the Mo-92 and Mo-55 inserts, as well those of the mutant inserts, were confirmed by dideoxy sequencing (USB). Mo-92 and Mo-55 were expressed in *E. coli* BL21(DE3) pLysS. Colonies were picked directly from the plate, grown in LB until the absorbance at 600 nm was 0.6, and then induced with 1 mM IPTG. Cells were harvested by centrifugation 3 h later.

Mo-92 was purified by lysing cell pellets in glacial acetic acid and centrifuging at 12 000 rpm for 20 min in a Sorvall RC-5B equipped with an SS-34 rotor. The supernatant was collected and diluted to 10 % acetic acid with water. The solution was filtered and purified by reversed-phase high performance liquid chromatography (HPLC) using a Vydac preparative C18 column. A linear water/acetonitrile gradient containing 0.1 % TFA was used at a flow rate of 10 ml $min^{-1}$. Mo-55 was purified by lysing cell pellets in a solution containing 100 mM NaCl, 25 mM Tris (pH 8.0) and 0.1 % (v/v) β-mercaptoethanol. The lysate was centrifuged as described above. The soluble fraction was applied under gravity to a 10 ml bed volume DEAE anion-exchange column. The column was washed with a solution containing 20 ml 100 mM NaCl, 25 mM Tris (pH 8.0) and 0.1 % (v/v) β-mercaptoethanol. Elution was performed with 25 mM Tris (pH 8.0), 0.1 % (v/v) β-mercaptoethanol and a salt gradient from 100–500 mM NaCl. The eluate fractions were analyzed on 18 % SDS–PAGE gels. Fractions containing Mo-55 were pooled and brought to 10 % acetic acid, filtered, and purified by reversed-phase HPLC.

### *Mutagenesis*

DNAs encoding the cysteine-to-alanine mutant peptides were prepared by oligonucleotide-directed mutagenesis of the constructs encoding the parent peptides [34]. Mutant peptides were purified by the identical method used for purification of the corresponding wild-type peptides. Oxidations for the disulfide permutation experiments were performed by incubating 5 mg $ml^{-1}$ mutant peptide in 6 M GuHCl, 0.5 mM oxidized glutathione, 0.5 mM reduced glutathione and 20 mM Tris (pH 8.8), for 24 h at room temperature. Samples were brought to 10 % acetic acid, dialyzed at room temperature against 5 % acetic acid for 48 h with two buffer changes, and lyophilized. The absence of free thiols was confirmed by the lack of reaction with Ellman's reagent in 6 M GuHCl [35]. In each case, disulfide-bond formation was intramolecular as judged by the absence of higher-order species on non-reducing SDS–PAGE gels.

### *Protease digestion*

A solution containing 5 mg $ml^{-1}$ Mo-92 and 0.8 mg $ml^{-1}$ TPCK-treated trypsin (Sigma) in 25 mM Tris (pH 8.0), was left overnight at room temperature. The digestion mixture was then diluted 5-fold into 5 % acetic acid, and the trypsin-resistant fragment was purified by reversed-phase HPLC. The fragment had an observed molecular mass of 6 852 Da. The expected mass for cleavage at Arg104 is 6 856 Da. Mo-92 (5 mg $ml^{-1}$) and chymotrypsin (0.5 mg $ml^{-1}$) (Boehringer Mannheim) were incubated at room temperature in 50 mM $KPO_4$ (pH 7.0). Aliquots were diluted 1:10 in 5 % acetic acid for mass spectrometry. Cleavage occurred after either Phe95 or Tyr96, with observed masses of 5 851 and 6 007 Da, respectively. Calculated masses are 5 843 and 6 007 Da. Longer digestion times favored the shorter fragment. Cleavage of a peptide containing residues from Met31 to Asp98 was carried out with 5 mg $ml^{-1}$ peptide and 0.5 mg $ml^{-1}$ thermolysin in 25 mM Tris (pH 8.0), at 37 °C for 6 h. A mass of 6 290 Da was observed, as compared to the calculated mass of 6 289 Da for a peptide from Val43 to Asp98.

### *CD spectroscopy*

CD spectroscopy was performed using an Aviv 62DS spectrometer equipped with a thermoelectric temperature controller. Peptide concentrations were determined by tyrosine absorbance at 275 nm using $\epsilon$ = 1500 (Mo-30, -36, -55), or by tryptophan and tyrosine absorbance at 280 nm using $\epsilon$ = 6880 (Mo-92) [31]. The Mo-36, Mo-55, and Mo-92 peptide concentrations were 23 μM, 10 μM, and 10 μM, respectively, in 50 mM $NaPO_4$, 150 mM NaCl (pH 7.0), for wavelength scans. Scans were taken at 2 °C, and signal was averaged for 5 sec. All thermal melting experiments were performed at 10 μM in the same buffer. The CD signal was measured in steps of 2 °C, with a 1.5 min equilibration at each temperature. The signal was averaged for 10 sec at each temperature. The $T_m$ for each peptide was determined from the peak in the first derivative of the ellipticity against 1/T curve [36]. All $T_m$ values were determined from thermal unfolding experiments that were > 90 % reversible. The number of helical residues in each peptide was estimated from $[\theta]_{222}$ by assuming that a value of –33 000 deg $cm^2$ $dmol^{-1}$ corresponds to a helix content of 100 % [37]. The unfolded baseline of –4 000 deg $cm^2$ $dmol^{-1}$ was taken as 0 % helical.

### *Analytical ultracentrifugation*

Equilibrium ultracentrifugation studies [38] were carried out using a Beckman XL-A analytical ultracentrifuge, with an An-60 Ti rotor at 20 °C. Mo-92 in 1 ml 50 mM Tris (pH 8.8) was dialyzed overnight against 500 ml 50 mM Tris (pH 8.8), 200 mM NaCl and 0.5 mM DTT. Mo-55 in 1 ml 100 mM Tris (pH 8.8) was dialyzed overnight against 500 ml 50 mM Tris (pH 8.8), 100 mM NaCl and 2 mM DTT. Fractions of the sample were then diluted 1:2 and 1:8 with dialysate to generate protein solutions of approximately 100 μM, 33 μM and 11 μM. Low concentrations of DTT were used as a precaution against intermolecular disulfide bond formation; however, subsequent studies showed reducing agent to be unnecessary. Indeed, similar results were obtained for oxidized Mo-92.3, which does not contain a free thiol, as for Mo-92 in presence or absence of DTT. Mo-92 samples were spun in a six-sector cell at rotor speeds of 15 000 and 17 000 rpm. Mo-55 was spun at 20 000 rpm, 22 000 rpm and 24 000 rpm. Data for Mo-92 were collected for each speed and for each protein concentration at 230 nm, 260 nm, 280 nm and 320 nm. Data for Mo-55 were collected at 229 nm, 247 nm, 280 nm and 300 nm. Data were analyzed using the following equation: $M = [2RT/(1-\bar{\upsilon})\rho\omega^2][d(\ln(c))/dr^2]$, with $\bar{\upsilon}$ as 0.732 $cm^3$ $g^{-1}$ for Mo-92 and 0.730 $cm^3$ $g^{-1}$ for Mo-55. In both cases, ρ was 1.01 g $cm^{-1}$.

### *NTCB cleavage*

Mo-92 and Mo-55 were oxidized at 100 μM by stirring overnight in 20 mM Tris (pH 8.8). Samples were brought to 5 % acetic acid, dialyzed against 5 % acetic acid for 24 h, and lyophilized. Oxidized Mo-92, Mo-55, or reduced cysteine-to-alanine mutants were dissolved at 1 mM in 20 mM Tris (pH 8.8). NTCB was added to 10 mM, followed by GuHCl to saturation. The sample was placed in the dark for 15 min at 37 °C. NaOH (1N) was added until a pH ~ 9.5 was achieved, and the cleavage reaction was allowed to proceed in the dark for 8 h at 37 °C [17]. An aliquot was diluted 1:10 in 70 % $CH_3CN$, 0.1 % TFA for mass spectrometry. The observed

masses for NTCB cleavage of Mo-92 were 5597 and 5 081 Da. Expected masses for the amino- and carboxy-terminal fragments of cleavage before the third cysteine in Mo-92 are 5 593 and 5 063 Da, respectively. A methionine sulfoxide in the carboxy-terminal fragment would lead to an expected mass of 5 079 Da. Cleavage of oxidized Mo-55 yielded an amino-terminal fragment of 5 600 Da.

### *Mass spectrometry*

Laser desorption mass spectrometry was performed on a Voyager Elite BioSpectrometry Research Station (PerSeptive Biosystems), using a matrix of 10 mg $ml^{-1}$ α-cyano-4-hydroxycinnamic acid in 70 % $CH_3CN$, 0.1 % TFA.

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