

Trimeric Transmembrane Domain Interactions in Paramyxovirus Fusion Proteins

ROLES IN PROTEIN FOLDING, STABILITY, AND FUNCTION*

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Background: Mutations in transmembrane domains can affect activity of viral fusion proteins, but little is known about potential interactions between these domains.

Results: Isolated paramyxovirus fusion protein transmembrane domains interact as trimers.

Conclusion: Viral fusion protein transmembrane domains self-associate.

Significance: Transmembrane domain associations may regulate stability of the prefusion conformation.

Paramyxovirus fusion (F) proteins promote membrane fusion between the viral envelope and host cell membranes, a critical early step in viral infection. Although mutational analyses have indicated that transmembrane (TM) domain residues can affect folding or function of viral fusion proteins, direct analysis of TM-TM interactions has proved challenging. To directly assess TM interactions, the oligomeric state of purified chimeric proteins containing the *Staphylococcal* nuclease (SN) protein linked to the TM segments from three paramyxovirus F proteins was analyzed by sedimentation equilibrium analysis in detergent and buffer conditions that allowed density matching. A monomer-trimer equilibrium best fit was found for all three SN-TM constructs tested, and similar fits were obtained with peptides corresponding to just the TM region of two different paramyxovirus F proteins. These findings demonstrate for the first time that class I viral fusion protein TM domains can self-associate as trimeric complexes in the absence of the rest of the protein. Glycine residues have been implicated in TM helix interactions, so the effect of mutations at Hendra F Gly-508 was assessed in the context of the whole F protein. Mutations G508I or G508L resulted in decreased cell surface expression of the fusogenic form, consistent with decreased stability of the prefusion form of the protein. Sedimentation equilibrium analysis of TM domains containing these mutations gave higher relative association constants, suggesting altered TM-TM interactions. Overall, these results suggest that trimeric TM interactions are important driving forces for protein folding, stability and membrane fusion promotion.

Entry of enveloped viruses requires fusion between the viral envelope and a target cell membrane. This critical early event in infection is promoted by specific viral glycoproteins, termed fusion (F)² proteins, which undergo conformational rearrangements that ultimately drive membrane fusion (1). Paramyxoviruses are nonsegmented negative-strand RNA viruses and include important human pathogens such as measles, mumps, respiratory syncytial virus, human metapneumovirus (HMPV), and the zoonotic Hendra and Nipah viruses (2). Attachment and entry of paramyxoviruses are generally promoted by two membrane glycoproteins: the attachment protein, which facilitates binding to cellular receptors, and the F protein, which promotes fusion between the viral envelope and a target cell membrane (3).

Like other class I viral fusion proteins, paramyxovirus F proteins are synthesized as inactive precursors (F₀), which are proteolytically processed to the mature and fusogenic (F₁ + F₂) form (see Fig. 1A). This cleavage event can be promoted by furin in the *trans*-Golgi network, as in the case of parainfluenza virus 5 F (PIV5 F (4)); by exogenous proteases, as seen with HMPV F (5); or by cathepsin L after endocytosis of the F protein, a unique pathway utilized by the Hendra and Nipah F proteins (6). The processed F₁ + F₂ form (see Fig. 1B, part a) exists in a metastable state that can be triggered to undergo the massive conformational changes associated with fusion. Paramyxovirus F proteins, along with other class I viral fusion proteins, share conserved elements that play fundamental roles in the fusion process. Crystal structures of the prefusion form of the PIV5 F protein (7, 8) and of the postfusion forms of the Newcastle disease virus and HPIV3 F proteins (9–11), combined with research to date on a number of paramyxovirus F proteins, suggest a model for fusion that elucidates the role of these conserved regions (2, 3). The fusion peptide, a hydrophobic region at the N terminus of the F₁ subunit, inserts into the target mem-

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² The abbreviations used are: F, fusion protein; HMPV, human metapneumovirus; PIV 5, parainfluenza virus 5; TM, transmembrane; SN, *Staphylococcal* nuclease; HRA, heptad repeat A; HRB, heptad repeat region; C14SB, 3-(*N,N*-dimethylmyristyl-ammonio)propanesulfonate; Bis-Tris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)propane-1,3-diol.

Transmembrane Domain Interactions in Viral Fusion Proteins

nio)propanesulfonate (C14SB) (Sigma/Fluka (40)). Recombinant protein was eluted using the C14SB-containing solution with 1 M NaCl and dialyzed using Slide-A-Lyzer MINI dialysis units (10,000 molecular weight cut-off; Pierce). Protein concentrations were determined by spectrophotometry, using $\epsilon_{280} = 17,420 \text{ M}^{-1} \text{ cm}^{-1}$.

Hendra F TM Synthesis and Purification—A thrombin cleavage site was introduced between the SN coding region and the Hendra TM coding region. Recombinant protein was expressed as described above. Following the first FPLC purification step, protein was eluted in buffer containing C14SB, and dialysis was performed to reduce salt concentration to 200 mM. Thrombin (ZymoGenetics, Seattle, WA) was added at a 1:100 concentration, and the sample was incubated at room temperature for 4 h. The sample was repurified through a 1-ml HiTrap SP FF column as described above.

Analytical Ultracentrifugation—Sedimentation equilibrium measurements were obtained at three different rotor speeds using a Beckman XL-A analytical ultracentrifuge equipped with an An-60 Ti rotor operated at 25 °C. Three protein concentrations corresponding to 280 nm absorbances between 0.25 and 0.8 were utilized for determination of the best fit models for three TM domains (see Fig. 2). For analysis of relative association constants, duplicate samples with an absorbance of 0.25 at 280 nm were utilized. Attainment of sedimentation equilibrium was monitored by comparing radial scans. Equilibrium was considered to be obtained when scans taken 6 h apart were indistinguishable. Further verification of attainment of equilibrium included approach to equilibrium from higher and lower rotor speeds and approach to equilibrium by temperature shift. On attainment of apparent sedimentation equilibrium by these different paths, concentration distributions and equilibrium constants were statistically indistinguishable. In addition, samples cycled between sedimentation equilibrium states at two different rotor speeds gave closely reproducible concentration profiles, indicating the absence of hysteresis. Together, these tests suggest that chemical and transport equilibria were attained. The buffer density was matched to that of C14SB detergent ($\rho = 1.04 \text{ g/ml}$) using D_2O , as described previously (40). Partial specific volumes of each protein were estimated using SEDNTERP (69), and data analysis was performed using KaleidaGraph (Synergy Software, Reading, PA) and Hetero-Analysis (41). For analysis, molecular weight values were fixed, and baselines were experimentally determined from absorbance near the meniscus after overspeeding.

Biotinylation of Cell Surface Proteins—Subconfluent Vero cells in 60-mm dishes were transiently transfected with 3 μg of either wild-type or mutant Hendra F in pCAGGS using Lipofectamine and Lipofectamine Plus (Invitrogen) according to the manufacturer's instructions. Eighteen to twenty-four hours after transfection, cells were starved and metabolically labeled, and cell surface proteins were biotinylated as described previously (42). Anti-peptide polyclonal antibodies (43) were used to immunoprecipitate Hendra F, and protein was analyzed via 15% SDS-PAGE. Surface-expressed Hendra F was visualized using a Typhoon Imaging System (GE Healthcare), and band densitometry was performed using ImageQuant 5.2 software (GE Healthcare).

Luciferase Reporter Gene Assay—Vero cells in 6-well plates were transiently transfected with 0.3 μg of either wild-type or mutant Hendra F, 0.9 μg of wild-type Hendra G, and 0.8 μg of a plasmid encoding luciferase under control of the T7 promoter using Lipofectamine and Lipofectamine Plus (Invitrogen). Eighteen hours after transfection, Vero cells were overlaid for 3 h with BSR cells, which stably express the T7 polymerase (44), and luciferase activity was measured using an Lmax luminometer (Molecular Devices, Sunnyvale, CA). Background (Hendra G alone) values were subtracted, and luciferase activity was expressed as the percentage of wild-type Hendra F.

RESULTS

Construction of Chimeric Proteins and Analysis of TM-TM Interactions—To analyze TM interactions, we utilized chimeric proteins containing the SN protein linked to the TM segment of interest, a system that has been used extensively for analysis of glycoprotein A (37, 45, 46), synaptobrevin and syntaxin (39), and the epidermal growth factor (ErB) receptor (47). SN is a monomeric protein under the conditions utilized for analytical ultracentrifugation (37). The use of SN-TM chimeras gives high yield expression in *Escherichia coli*, allows the use of lower protein concentrations during analysis due to the larger molar extinction coefficient *versus* an isolated TM peptide, and permits centrifugation at lower speeds due to the higher molecular weight of the chimeric protein (37). We confirmed that the SN-glycoprotein A TM construct, kindly provided by Dr. Karen Fleming (Johns Hopkins University), is in monomer-dimer equilibrium, as had been previously shown using this assay (37). Chimeric proteins were expressed, purified, and exchanged into C14SB detergent (40). Samples at three concentrations were brought to sedimentation equilibrium in a Beckman XL-A analytical ultracentrifuge, radial absorbance data were obtained at 20,000, 30,000, and 40,000 rpm, and the data to determine best fit were analyzed using both KaleidaGraph and Hetero-Analysis (41).

Analysis of a chimeric protein containing the wild-type Hendra F TM domain region (residues 484–521; Fig. 1A) at three concentrations and speeds indicated that a monomer-trimer equilibrium was the best fit model (Fig. 2A). The small, symmetrically distributed residuals (Fig. 2A, upper panels) indicated that this model was consistent with the mass distributions present. The molecular weights of the species and the third-power concentration dependence of the higher molecular weight species on the concentration of monomer support this conclusion. Other single-species fits, such as monomer-only (Fig. 2, D–F) or two species models, fit the data significantly less well or gave nonrandom residual distributions. Inclusion of additional species (such as monomer-dimer-trimer) gave amplitudes for the additional terms that were within error equal to zero (results not shown). These results demonstrate for the first time that an F protein TM domain, separate from the rest of the protein, is in a monomer-trimer equilibrium. Analysis using pentaethylene glycol monoethyl ether (C8E5) (40) instead of C14SB yielded similar results, indicating that the trimeric interactions between the F protein TM domains are not dependent on the identity of the detergent. The Hendra F TM domain contains a cysteine residue (Cys-506), but analysis per-

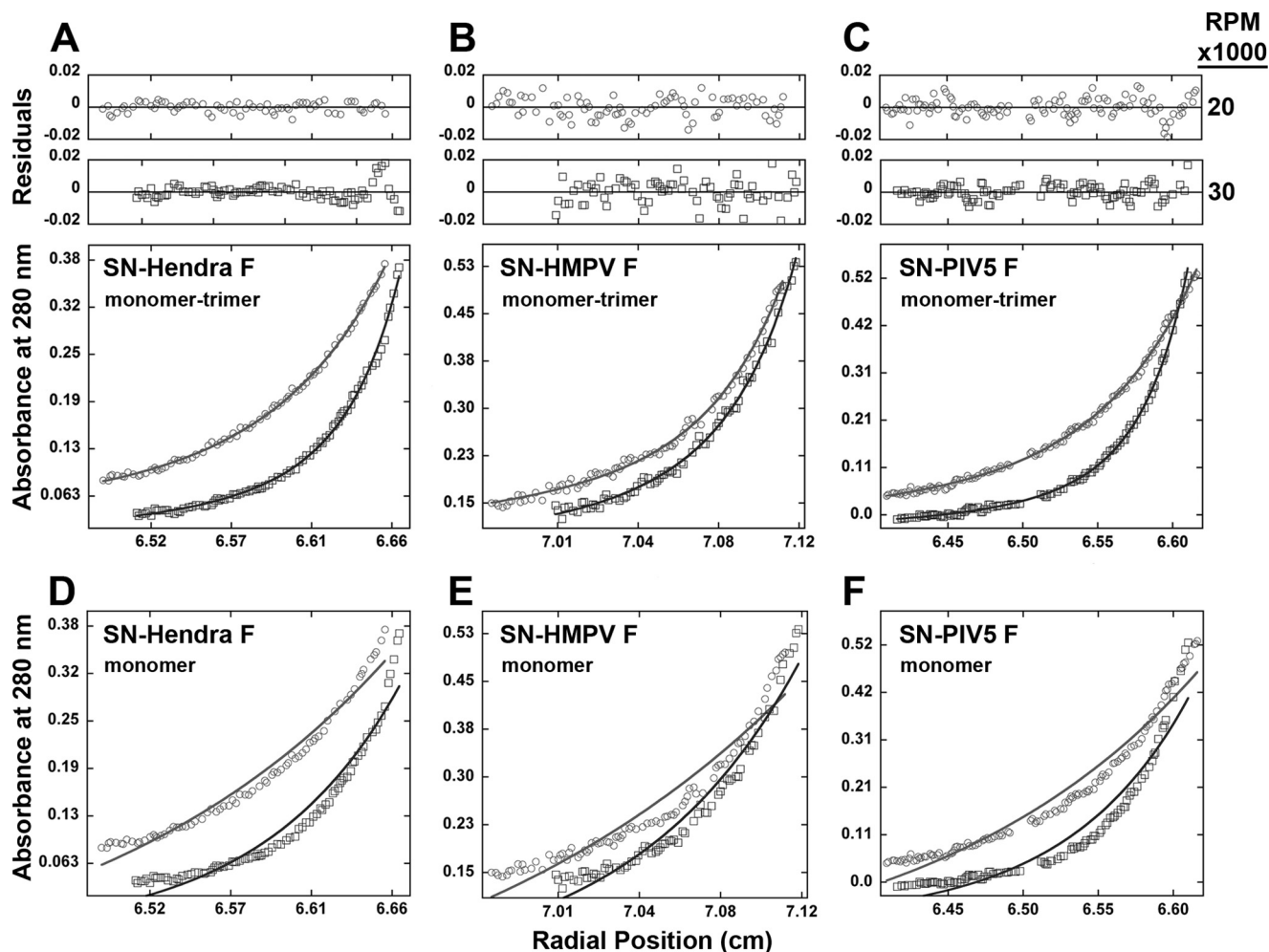


FIGURE 2. **Sedimentation equilibrium analysis of the chimeric SN-F-TM proteins demonstrates a monomer-trimer equilibrium.** Protein samples were prepared in C14SB detergent. A_{280} data were collected at 20,000, 25,000, and 30,000 rpm on a Beckman XL-A analytical ultracentrifuge, with only the 20,000 and 30,000 displayed for clarity. *A–C*, monomer-trimer fits, with data (points) shown along with the predicted theoretical fit for a monomer-trimer. *D–F*, monomer-only fits, with data (points) shown along with the predicted theoretical fit for a monomer-trimer. *A* and *D*, SN-Hendra F TM. *B* and *E*, SN-HMPV F TM. *C* and *F*, SN-PIV5 F TM.

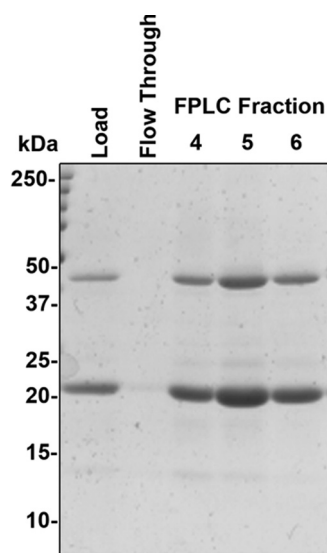


FIGURE 3. **Purified SN-Hendra F protein.** Samples from load, flow-through, and the three FPLC fractions containing the majority of the Hendra SN-TM protein were separated on a 15% SDS-PAGE gel and visualized by Coomassie Blue Staining. Bands corresponding to the monomeric and an oligomeric size are apparent.

protein expression and localization were analyzed by biotinylation of cell surface proteins as described previously (35). Mutation of Gly-508 to alanine resulted in a small decrease in F_1 cell surface expression ($72.3 \pm 20.4\%$ of wild-type F levels), whereas mutation to isoleucine or leucine caused significant reductions (19.9 ± 4.4 and $11.8 \pm 2.2\%$ of wild-type F levels, respectively) in F_1 expression as compared with wild type (Fig. 7A). Interestingly, cell surface expression levels of the uncleaved F_0 form were similar to wild type. The fraction of the mutant proteins present on the cell surface was also reduced as compared with WT F (surface $(F_0 + F_1)/\text{total } (F_0 + F_1) = 1.0 \pm 0.2$ for WT F, 0.8 ± 0.1 for G508A, 0.6 ± 0.2 for G508I, and 0.7 ± 0.3 for G508L). These results suggest that the G508I and G508L mutations affect the stable expression of the cleaved prefusion form on the cell surface, potentially through alterations in stability or endosomal trafficking. Several regions within Hendra F have been previously implicated in maintenance of prefusion metastability (3); however, such a role for the TM domain has yet to be described. As mutations within TM domains can affect membrane fusion promotion, cell-cell fusion levels were examined using a luciferase reporter gene assay (6). Each mutant

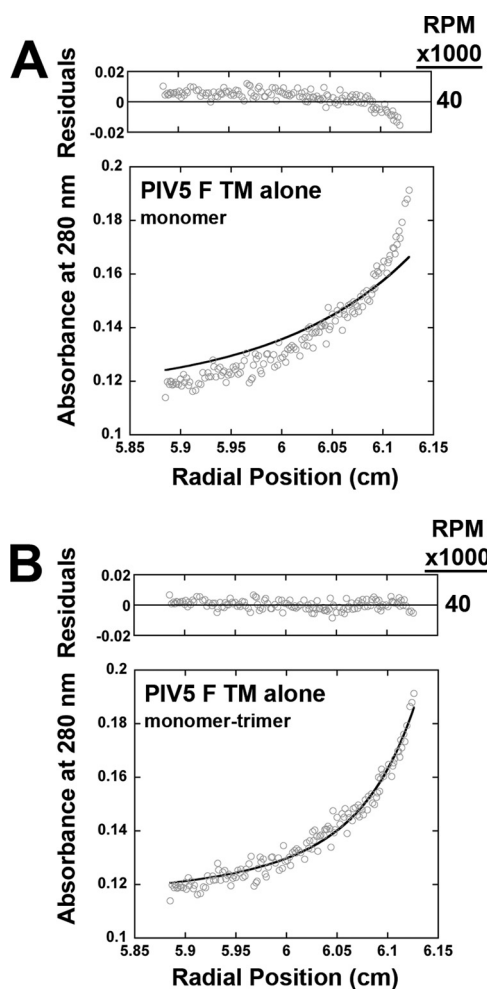


FIGURE 4. Sedimentation equilibrium analysis of a PIV5 F TM domain peptide confirms a monomer-trimer equilibrium. The PIV5 TM peptide was prepared in C145B detergent (protein:lipid molar ratio 1:280), and A_{280} data were collected at 40,000 rpm on a Beckman XL-1 analytical ultracentrifuge. *A* and *B*, the predicted curves (dark lines) for monomer (*A*) and monomer-trimer (*B*) fits are shown and superimposed with actual data points.

promoted cell-cell fusion at levels consistent with F_1 cell surface expression (Fig. 7*B*), indicating that substitutions at Gly-508 do not disrupt F-mediated membrane fusion beyond the effect on cell surface expression.

To assess the effect of these mutations on TM-TM interactions, the G508A, G508I, and G508L mutations were introduced into the SN-TM chimera system, the recombinant proteins were expressed and purified, and sedimentation equilibrium analysis was performed. To maintain a constant protein:lipid ratio, all samples were examined at the same protein concentration. An apparent molar association constant of $8.38 \pm 0.84 \times 10^8 \text{ M}^{-2}$ was calculated for the SN-Hendra F TM at the lowest speed (Table 1), and this value decreased with increasing rotor speed, displaying an ~ 2 -fold reduction at the highest speed, suggesting that the equilibrium is pressure-dependent (50, 51). Pressure effects are expected when the oligomeric form displays alterations in specific volume from what would be calculated as the sum of the specific volumes of each monomer, and thus this finding is consistent with recent work indicating a potential bulge in the PIV5 TM helix region upon oligomerization (22). Similar results were obtained with multi-

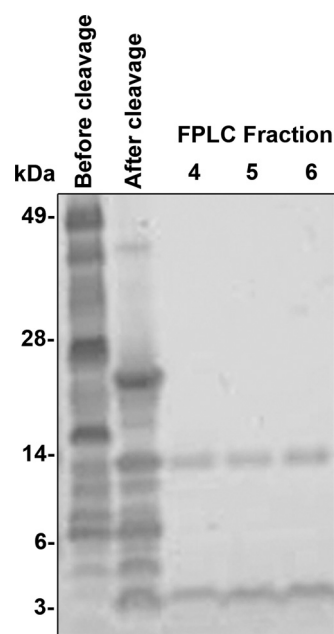


FIGURE 5. Purification of the Hendra F TM domain peptide. Samples from before (pre-digest) and after (post-digest) thrombin cleavage and the three FPLC fractions containing the Hendra TM peptide were separated on a 4–12% NuPAGE Bis-Tris gel and visualized by Simply Blue SafeStain (Invitrogen). Bands correspond to the predicted molecular weight of a monomer (molecular weight 4297) and trimer.

ple preparations and with samples analyzed either immediately following purification or after prolonged storage. When TM-TM interactions were analyzed, the G508A mutant displayed a moderate increase in TM-TM association (Table 1; relative association constant as compared with the WT TM at 20,000 rpm of 3.29 ± 0.51), whereas the G508I or G508L mutations resulted in an even stronger TM-TM association (relative association constants at 20,000 rpm of 14.91 ± 3.56 and 6.46 ± 1.13 , respectively). These results indicate that interactions involving the GXXXA motif are not the driving force behind the association of Hendra F TM domains as TM association increased when the glycine residue was removed. In addition, these results demonstrate that substitutions at Gly-508 can alter the stability or trafficking of the cleaved F_1 form, likely through modulating TM-TM interactions, with modest effects observed with an alanine substitution and more dramatic effects observed upon introduction of larger, more hydrophobic amino acids (Ile and Leu).

Role of Noncharged Polar Residues in the Hendra F TM Domain—Noncharged polar residues can provide a driving force for association of TM domains (52, 53), with serine and threonine residues playing critical roles in some systems (54). The Hendra F TM domain contains four serine or threonine residues at positions Ser-493, Ser-501, Thr-511, and Ser-514, with two additional serine residues at positions 487 and 490 predicted to be at or near the TM-ectodomain border. To analyze the role of the TM Ser and Thr residues in folding and function of the F protein, individual alanine substitutions were created. Interestingly, although alanine replacements at Ser-501, Thr-511, or Ser-514 did not significantly impact cell surface expression or fusion, an S493A mutation, predicted to be at the TM-ectodomain border, gave surface expression at 70% of

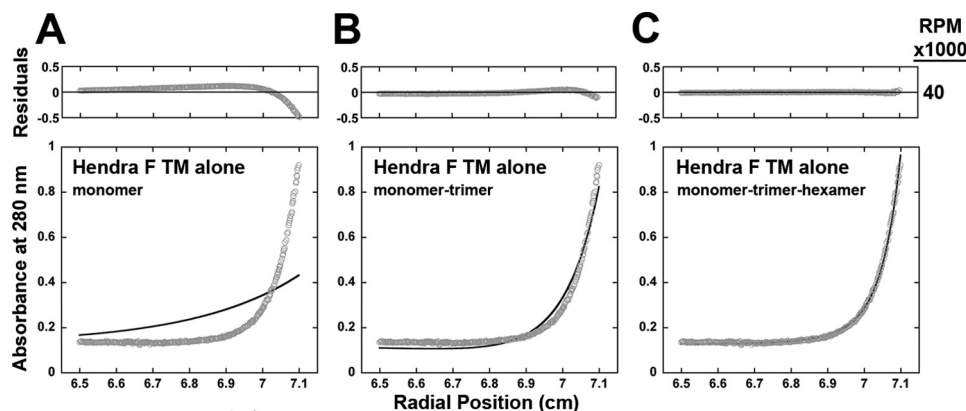


FIGURE 6. **Sedimentation equilibrium analysis of a Hendra F TM domain peptide indicates a best fit monomer-trimer-hexamer equilibrium.** The Hendra TM peptide was prepared in C14SB detergent (protein:lipid molar ratio 1:50), and A_{280} data were collected at 40,000 rpm on a Beckman XL-1 analytical ultracentrifuge using a two-channel centerpiece. A–C, the predicted curves (dark lines) for monomer (A), monomer-trimer (B), and monomer-trimer-hexamer (C) fits are shown and superimposed with actual data points.

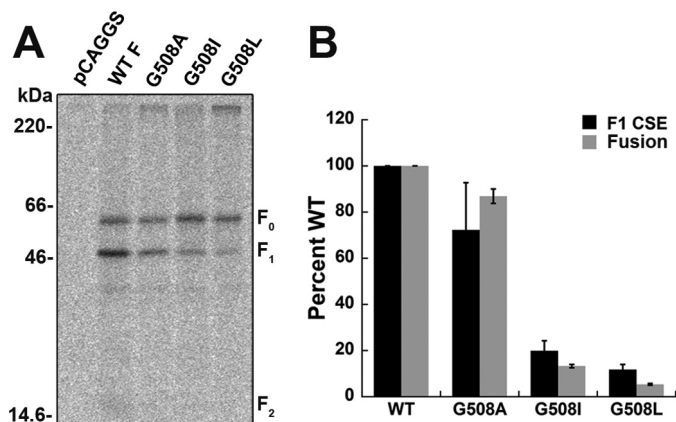


FIGURE 7. **Mutations at Hendra F Gly-508 can modulate expression of the cleaved, fusogenic form.** A, cell surface expression of transiently transfected wild type and Hendra F mutants in Vero cells following a 3-h metabolic label. Surface proteins were biotinylated prior to immunoprecipitation, and the total and surface populations were separated by streptavidin pull-down. Proteins were analyzed via 15% SDS-PAGE and visualized using autoradiography. B, cell surface expression (CSE) and fusion for the wild type and Gly-508 mutants. F_1 band quantitation via densitometry was normalized to wild-type levels plus or minus one standard deviation. Cell surface expression represents the average of three independent experiments. Fusion was analyzed using a reporter gene assay, $n = 9$; \pm S.E.

wild-type levels, but presented a dramatic loss in fusion promotion (Fig. 8, A and B), indicating that this mutation directly affects membrane fusion. A recent study of the PIV5 F protein has also implicated residues in the N-terminal end of the TM domain in fusion promotion (22). TM-TM interactions were not significantly altered when the S493A mutation was introduced into the SN-Hendra F TM construct, as shown by the preservation of a monomer-trimer fit with association constants similar to that of the wild-type protein. Thus, these data suggest that the reductions in fusion observed for the S493A mutation are not directly due to changes in TM-TM association. Although single polar residues may impact TM-TM association, cooperative interaction of several polar residues has been implicated in helix packing (55–58). To test this, double (T511A/S514A), triple (S501A/T511A/S514A), and quadruple (S493A/S501A/T511A/S514A) mutations were created and assessed for their effects on cell surface expression and fusion. Cell surface expression and fusion were moderately reduced for

the T511A/S514A and S501A/T511A/S514A mutants, whereas the S493A/S501A/T511A/S514A mutant displayed greatly reduced cell surface expression, suggesting that at least one noncharged polar residue is needed for protein stability (Fig. 8A). Fusion promoted by S493A/S501A/T511A/S514A was similar to the low levels observed with the S493A single mutant, consistent with defects in fusion driven primarily by substitutions at Ser-493 (Fig. 8B). These data suggest that substitution of polar residues within the Hendra F TM domain can impact overall protein folding and stability, potentially by altering TM-TM interactions. Additionally, these data indicate that a single polar residue near the N terminus of the TM (S493A) is the only one that significantly modulates membrane fusion.

DISCUSSION

Historically, the role of TM domains in viral fusion proteins has been difficult to address. The work presented here demonstrates that isolated TM domains from class I fusion proteins are present in a monomer-trimer equilibrium. Furthermore, our data indicate that TM-TM interactions contribute to the folding, stability, and fusion function of these important viral proteins. As similar interactions were observed using the TM domains from Hendra F, PIV5 F and HMPV F (Fig. 2), it is likely that trimeric TM-TM interactions are characteristic of F proteins throughout the paramyxovirus family. Because all class I viral fusion proteins, including influenza HA and Ebola GP, are trimeric molecules, TM-TM interactions may represent a critical and conserved structural feature of viral fusion proteins. Consistent with our findings, Bissonnette *et al.* (22) found evidence for TM-TM interactions using cysteine scanning of the PIV5 F protein, several groups have reported multiple oligomeric species of the influenza HA TM domain (59, 60), and a recent study confirmed TM oligomerization of the HIV env protein (32). Interestingly, a recent study of the PIV5 F protein, which also employed sedimentation equilibrium analysis, found evidence for a fusion peptide-TM domain interaction, but did not observe TM-TM association in dodecylphosphocholine micelles (48). However, our results with both synthesized PIV5 TM peptides (Fig. 4) and isolated Hendra TM peptides (Fig. 6) confirm trimeric association of the TM regions in the absence of the SN construct. The PIV5 peptide utilized by

TABLE 1

Best fit model and relative association constants

SN-Hendra F TM constructs were purified and analyzed by sedimentation equilibrium analysis.

Sample	20,000 rpm		25,000 rpm		30,000 rpm	
	Best fit model	Relative association constant ^a	Best fit model	Relative association constant ^a	Best fit model	Relative association constant ^a
WT F TM	1:3	1	1:3	0.54 ± 0.04	1:3	0.45 ± 0.02
G508A	1:3	3.29 ± 0.51	1:3	0.82 ± 0.07	1:3	0.71 ± 0.02
G508I	1:3	14.91 ± 3.56	1:3	2.56 ± 0.19	1:3	1.49 ± 0.11
G508L	1:3	6.46 ± 1.13	1:3	2.01 ± 0.14	1:3	1.71 ± 0.16

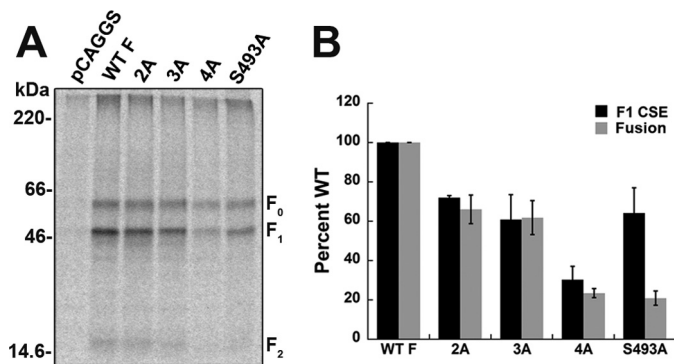
^a Relative association constant as compared with WT F at 20,000 rpm, $8.38 \pm 0.84 \times 10^8 \text{ M}^{-2}$.

FIGURE 8. Mutations at Hendra F Ser-493 inhibit fusion. *A*, cell surface expression of transiently transfected wild type and Hendra F mutants in Vero cells following a 3-h metabolic label. 2A = T511A/S514A; 3A = S501A/T511A/S514A; and 4A = S493A/S501A/T511A/S514A. Proteins were analyzed via 15% SDS-PAGE and visualized using autoradiography. *B*, cell surface expression (CSE) and fusion for the wild type and TM mutants. F₁ band quantitation via densitometry was normalized to wild-type levels plus or minus one standard deviation. Cell surface expression represents the average of three independent experiments. Fusion was analyzed using a reporter gene assay, $n = 5-9$; \pm S.E.

Donald *et al.* (48) included mutation of an internal cysteine residue in the PIV5 TM sequence to alanine and lacked the two C-terminal leucine residues present in our peptide, and these changes may account for the difference in findings.

The factors that drive TM-TM interactions remain poorly understood (30, 31), but GXXXG motifs or similar motifs with small (Gly, Ala, or Ser) residues spaced four apart are hypothesized to provide a framework to facilitate interactions of some TM helices. TM glycine residues are important for the fusion activity of the vesicular stomatitis virus G protein (13) and for the trafficking and function of the HIV env protein (16, 23, 49), potentially due to alterations in TM-TM interactions (32). In our studies, mutation of the Hendra F TM Gly-508 residue resulted in both an increase in the relative association of TM domains and a decrease in cell surface expression of the fusogenic F₁ protein. An alanine substitution, yielding an AXXXA motif, resulted in modest effects, potentially due either to a requirement for backbone flexibility for optimal helix interactions or to steric effects. Although replacement of the G/A in these motifs with bulkier side chains often results in decreased association in dimeric systems, a significant increase in trimer association was noted when such substitutions were made in the Hendra F TM (Table 1). In addition, the G508I or G508L mutations in the context of the whole protein led to greatly decreased cell surface expression of the fusogenic F₁ form (Fig. 7). An alteration in TM association driven by introduction of the bulky Ile or Leu side chain may result in changes to the transmembrane domain region and subsequent alterations to

the ectodomain structure, thereby altering protein stability. Alternatively, these modifications to TM structure may affect trafficking through the endocytic pathway. Integrin TM domains, like viral fusion protein TM domains, must undergo changes in association for proper function, and their association has been shown to be facilitated by complementary small and large side chains on neighboring helices (62), consistent with the idea that bulky residues can drive TM helix interactions.

Polar residues can also promote TM-TM interactions (52–54) and are present at higher than average percentages in paramyxovirus F TM domains. Interestingly, only Hendra F Ser-493 is critical for the fusion process (Fig. 8), but no alterations were observed in TM-TM association when this residue was replaced with alanine. As Ser-493 is located near the N-terminal end of the TM segment, the fusion effects may reflect a critical role for side chain interactions with the environment at the lipid interface. No other significant folding or fusion defects were observed with replacement of one, two, or three TM serine or threonine residues (Fig. 8), although significant folding and fusion defects were observed when all four TM S/T residues (including S493A) were changed to alanine. Thus, cooperative interaction of several polar residues, previously implicated in helix packing (55–58), may regulate F protein TM interactions, but a series of residues spread throughout the TM domain would likely be involved. Recent work has demonstrated that dimerization of TM domains is driven by factors extending beyond recognizable sequence motifs (66). Our results suggest that paramyxovirus TM trimerization is also likely driven by multiple sequence and structural determinants, consistent with the apparent lack of conservation observed between different F protein TMs.

TM-TM interactions could potentially drive both F protein folding and fusion. One possibility is that these interactions stabilize the HRB coiled-coil in the prefusion form (Fig. 1B) (7). Support for this comes from peptide studies indicating that stable HRB interactions do not occur for the isolated domain (63) and from the requirement for trimerization domains in place of the PIV5 F and respiratory syncytial virus F TM domains to obtain prefusion structures (7, 33). Consistent with the hypothesis that TM-TM interactions stabilize the prefusion form, and particularly the HRB coiled-coil, we have recently reported that the addition of the HRB segment to the Hendra F TM domain results in destabilization of the TM trimer (64). A recent study also suggested a requirement for the herpes simplex virus gB fusion protein TM domain in stabilization of a prefusion structure (65). Combined, these data strongly support a critical role for the TM domain in maintaining prefusion

stability and suggest that this function may extend beyond the paramyxoviruses.

TM-TM interactions could also influence triggering of membrane fusion. Interactions with the viral attachment protein are hypothesized to trigger fusion for most paramyxovirus F proteins (2, 3) except for HMPV F (67), and altered association of the measles attachment protein with the cleaved fusogenic $F_1 + F_2$ protein was observed when specific mutations were introduced into the F TM domain (17). Alterations in TM-TM association due to attachment protein interactions or alterations in the lipid environment could regulate the dissociation of the heptad repeat B coiled-coil needed to reach the final fusogenic structure (Fig. 1B). Information on factors that stabilize or destabilize F protein TM interactions may therefore provide critical new insight into the triggering process. TM domain β -branched residues such as valine or isoleucine have been implicated in backbone dynamics and fusogenicity of peptides (68) and the fusion function of the reovirus fusion-associated small transmembrane proteins (61). β -Branched residues are present throughout the paramyxovirus F protein TM domains, and we have recently demonstrated that mutation of these residues can alter membrane fusion without affecting surface expression (64); however, the effect of these mutations on TM interactions remains to be determined. The work presented here presents a new system for the study of TM mutations and interactions in viral fusion proteins, providing a foundation to begin dissecting the role of trimeric TM interactions in the folding, stability, and fusion activity of these important viral proteins.

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