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 folding or function of viral fusion proteins, direct analysis of the oligomeric state of purified chimeric proteins containing the TM interactions has proved challenging. To directly assess folding or function of viral fusion proteins, direct analysis of 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) proteases, 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 F0 subunit, inserts into the target mem-

* This work was supported, in whole or in part, by National Institutes of Health Grants R01AI051517 and U54 AI057157 (through the NIAID) from the Southeastern Regional Center of Excellence for Emerging Infections and Biodefense (to R. E. D.), R01GM070662 (to M. G. F.), and 2P20 RR020171 from the National Center for Research Resources (to R. E. D. and M. G. F.).

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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-dimethylamino)-2-(hydroxymethyl)propane-1,3-diol.
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EXPERIMENTAL PROCEDURES

Cell Lines and Culture—Vero and BSR (provided by Karl-Klaus Conzelmann, Max Pettenkofer Institut) cells were grown in DMEM (Invitrogen) containing 10% FBS and 1% penicillin and streptomycin. Every third passage, BSR cells were selected for expression of T7 polymerase by the addition of 0.5 mg/ml G-418 sulfate (Invitrogen) to the growth medium.

Plasmids, Peptides, and Mutagenesis—Plasmids containing Hendra F and G were generously provided by Dr. Lin-Fa Wang (Australian Animal Health Laboratory), and all Hendra F mutants were generated as described previously (34–36). Staphylococcal nuclease (SN) fused to the glycophorin A (GpA) TM domain in the pET-11a expression vector (37) was generously provided by Dr. Karen Fleming (Johns Hopkins University). The TM domains of wild-type or mutant Hendra F, HMPV F and PIV5 F were identified using bioinformatics (using the TMHMM Server v. 2.0), with the C-terminal charged residues left in place to aid in solubility. The TMs were PCR-amplified from pCAGGS-Hendra F, -HMPV F, or -PIV5 F, respectively, and ligated into pET-11a using the Xmal (5’S) and BamHI (3’) restriction sites. All constructs were sequenced in their entirety prior to use. A peptide corresponding to residues 485–512 of the PIV F protein with the sequence (VLSIIAICLG-SLGIIILCSVVWVKLL) was synthesized by Peptide Protein Research Ltd. The peptide was dissolved in the appropriate buffer for analytical ultracentrifugation, dialyzed, and analyzed at a concentration of 35 μM.

Recombinant Protein Expression and Purification—Constructs expressing chimeric proteins containing the TM of either wild-type or mutant Hendra F, HMPV F or PIV5 F fused to SN in pET-11a were transformed into Rosetta-gami cells (EMD Chemicals, Gibbstown, NJ) and plated on LB plates containing 100 μg/ml ampicillin. Colonies were grown in 25 ml of 2×YT under the selection of 15 μg/ml kanamycin, 12.5 μg/ml tetracycline, 50 μg/ml streptomycin, 34 μg/ml chloramphenicol, and 100 μg/ml ampicillin overnight at 37 °C. Cultures were transferred to 500 ml of 2×YT containing100 μg/ml ampicillin and grown to an OD of 0.6–1.0 at 37 °C (approximately 2 h), induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (Sigma), grown for 4 h, and harvested by centrifugation. Cells were resuspended in a 1:20 culture volume of lysis buffer (50 mM HEPES, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, pH 8.0), and recombinant protein was purified using the detergent Triton X-100 (Sigma) and a single salt (1 mM NH4OAc) extraction as described previously (38), except using 50 mM HEPES instead of 20 mM Tris-HCl. Following supernatant containing the recombinant protein was dialyzed (6000–8000 molecular weight cut-off) overnight at 4 °C against lysis buffer containing 0.1 M NH4OAc and 0.2% v/v Triton X-100 and clarified by centrifugation at 15,000 × g for 15 min. Recombinant protein was then FPLC-purified by cation-exchange chromatography using a 1-ml HiTrap SP FF column (GE Healthcare (39)) and eluted in lysis buffer containing 0.1 M NH4OAc, 1 M NaCl, and 0.2% v/v Triton X-100. A second round of FPLC was performed to exchange the protein into a solution containing 200 mM NaCl, 20 mM Na2HPO4/NaH2PO4 (pH = 7), 29% D2O, and the Zwittergent detergent 3-(N,N-dimethylmyristyl-ammo-

brane, and the adjacent heptad repeat A (HRA) region forms a trimeric coiled-coil (see Fig. 1B, part b). A second heptad repeat region (HRB) proximal to the transmembrane (TM) domain separates and refolds with the HRA coiled-coil, forming an extremely stable six-helix bundle structure that is critical for fusion promotion (see Fig. 1B, part c).

A number of studies have observed that modifications to the TM domain can result in modulation of class I viral fusion protein activity (12–18), including impairment of fusion pore opening or enlargement (13–15, 19–21) or alterations in the efficiency of fusion promotion (18, 22, 23). Recent studies have also demonstrated an important role for the TM domains of class II fusion proteins (24) and reovirus fusion-associated small transmembrane (FAST) proteins (25) in the fusion process. Specific sequences within the TM domain are not obligately required as replacement with alternative TM domains can in some cases produce a functional fusion protein (26–29). However, fusion alterations observed following the introduction of point mutations within some TM domains indicate that specific sequence elements or residues are important. Replacement of the paramyxovirus PIV5 F protein TM domain with polyleucine resulted in a misfolded protein (22), confirming that the TM domain serves as more than a simple membrane anchor. However, the mechanisms by which TM domains contribute to the folding and fusion process remain poorly understood, partially due to the difficulties in obtaining direct structural information about these regions.

TM-TM interactions are critical for a number of important biological processes (30, 31), and TM domains from the HIV env protein have been shown recently to interact (32), although the oligomeric form could not be assessed. Several lines of evidence suggest a potential role for TM-TM interactions in paramyxovirus F protein targeting and function. Results of a cysteine-scanning study of the PIV5 F TM domain with polyethylene resulted in a misfolded protein (22), confirming that the TM domain serves as more than a simple membrane anchor. However, the mechanisms by which TM domains contribute to the folding and fusion process remain poorly understood, partially due to the difficulties in obtaining direct structural information about these regions.

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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 protein was eluted in buffer containing C14SB, and dialysis was 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 (40). Partial specific volumes of each protein were estimated using SEDNTERP (69), and data analysis was performed using KaleidaGraph (Synergy Software, Reading, PA) and HeteroAnalysis (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 5 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).

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 glycoporin 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-glycoporin 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 HeteroAnalysis (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 monooyctyl 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-
formed in the presence of 2 mM β-mercaptoethanol did not lead to alterations in the best fit monomer-trimer model, indicating that disulfide bond formation was not a significant contributing factor to the TM-TM interactions in this system (data not shown). Consistent with this, a previous study found that no difference in oligomeric forms on a nonreducing gel was seen between the WT PIV5 F protein and a mutant lacking the one TM cysteine residue (22). Analysis of the SN-TM protein by SDS-PAGE, both before (Fig. 3) and after centrifugation, showed both a monomer and a fainter oligomer band, and no proteolytic degradation was observed after centrifugation.

To determine whether these results were specific for the TM domain from Hendra F, TM regions from PIV5 F (residues 485–512) and HMPV F (residues 489–516) were analyzed by the same method. Data for both were again best fit by a monomer-trimer model in the majority of speeds and concentrations (Fig. 2, B and C), with a slightly better fit for monomer-trimer-hexamer at the lowest speeds for HMPV. As Hendra, PIV5, and HMPV represent diverse branches of the paramyxovirus family, these results suggest that trimeric TM-TM interactions are a common paradigm for paramyxovirus F proteins.

Role of the Hendra F TM Residue Gly-508—Our results strongly support a trimeric association between isolated TM domains of paramyxovirus F proteins, but the potential impact of TM association on F protein folding and function and the identity of the residues that promote this association were unclear. We therefore performed mutational analyses of residues or motifs previously implicated in promotion of TM-TM interactions (30, 31). Motifs containing one or more glycine residues (GXXX or GXXXY, where Y is a small residue) have been demonstrated to promote interactions between the TM domains of HIV env (16, 32, 49). One glycine residue is present in the TM domain of Hendra F, forming a portion of an AXXXG motif. To test the role of this motif in the whole protein, Gly-508 was mutated to alanine, isoleucine, or leucine, and

![Diagram of a paramyxovirus F protein and model of membrane fusion.](image-url)

**FIGURE 1.** Schematic of a paramyxovirus F protein and model of membrane fusion. **A**, diagram of a mature paramyxovirus F protein, with TM sequences indicated. **B**, model for F protein-promoted membrane fusion. **Part a**, the HRB domains adjacent to the TM form a coiled-coil in the prefusion state, whereas the HRA domains are present as a series of small helices. **Part b**, triggering of fusion leads to insertion of the fusion peptide into the target membrane and formation of an HRA coiled-coil, and separation of the HRB helices and TM domains. **Part c**, fusion pore opening occurs concomitant with formation of a six-helix bundle with the HRA segments on the interior of the bundle, placing the HRB and TM segments on the exterior.
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 F1 cell surface expression (72.3% of wild-type F levels), whereas mutation to isoleucine or leucine caused significant reductions (19.9% and 11.8% of wild-type F levels, respectively) in F1 expression as compared with wild type (Fig. 7A). Interestingly, cell surface expression levels of the uncleaved F0 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 (F0 + F1)/total (F0 + F1) = 1.0 ± 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 2. Sedimentation equilibrium analysis of the chimeric SN-F-TM proteins demonstrates a monomer-trimer equilibrium. Protein samples were prepared in C14SB detergent. A280 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.
promoted cell-cell fusion at levels consistent with F1 cell surface expression (Fig. 7B), 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 $8.38 \pm 0.84$ M$^{-1}$ was calculated for the SN-Hendra F TM at the lowest speed (Table 1), and this value decreased with increasing rotor speed, displaying a two-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 multiple 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 $3.29 \pm 0.51$), whereas the G508I or G508L mutations resulted in an even stronger TM-TM association (relative association constants at 20,000 rpm of $14.91 \pm 3.56$ and $6.46 \pm 1.13$, 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 F1 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
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 substitution 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
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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), activity of the vesicular stomatitis virus G protein (13) and for the 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; ± S.E.

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

| Sample          | Best fit model | Relative association constant* | Best fit model | Relative association constant* | Best fit model | Relative association constant* |
|-----------------|----------------|--------------------------------|----------------|--------------------------------|----------------|--------------------------------|
| 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                    |

* Relative association constant as compared with WT F at 20,000 rpm, 8.38 ± 0.84 × 10^3 M^-1 s^-1.

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 TM.

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.
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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.

Acknowledgments—We are grateful to Katie Routt for technical assistance and Dr. Karen Fleming for the kind gift of the pet11A-MeV-1 protein and fusogenic F_1 protein. We thank Dr. Karen Fleming for the kind gift of the pet11A-MeV-1 protein and fusogenic F_1 protein. We also thank the members of the Dutch laboratory and Dr. Trevor Creamer for critical reviews of the manuscript.

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