Homomultimerization of the reovirus p14 fusion-associated small transmembrane protein during transit through the ER–Golgi complex secretory pathway

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The reovirus fusion-associated small transmembrane (FAST) proteins are the smallest known viral membrane-fusion proteins. How these diminutive fusogens mediate cell–cell fusion and syncytium formation is unclear. Ongoing efforts are aimed at defining the roles of the FAST protein ecto-, endo- and transmembrane domains in the membrane-fusion reaction. We now provide direct evidence for homomultimer formation by the FAST proteins by using an anti-haemagglutinin (HA) mAb to co-precipitate the untagged p14 FAST protein from cells co-transfected with HA-tagged p14. Disrupting the intracellular endoplasmic reticulum–Golgi complex vesicle transport pathway prevented p14 homomultimer formation, while lower pH disrupted p14 multimers. The p14 endodomain or transmembrane domains are not required for multimer formation, which, along with the pH sensitivity and the distribution of histidine residues, suggests the 36 aa p14 ectodomain is a multimerization motif.

The fusion-associated small transmembrane (FAST) proteins are a novel family of viral membrane-fusion proteins encoded by the non-enveloped fusogenic orthoreoviruses and aquareoviruses. Unlike their enveloped virus counterparts (White et al., 2008), the FAST proteins are non-structural proteins and are therefore not involved in mediating virus–cell membrane fusion and virus entry (Dawe & Duncan, 2002; Shmulevitz & Duncan, 2000). Instead, the FAST proteins are expressed and localized to the plasma membrane in reovirus-infected cells, where they function as dedicated cell–cell fusogens to induce syncytium formation, thus promoting virus dissemination and contributing to the pathogenic potential of the fusogenic reoviruses (Brown et al., 2009; Salsman et al., 2005). The structures of the FAST proteins also differ dramatically from all of the enveloped-virus membrane-fusion proteins. Ranging in size from just 98 to 198 aa (Corcoran & Duncan, 2004; Dawe & Duncan, 2002; Racine et al., 2009; Shmulevitz & Duncan, 2000), the FAST proteins are the smallest known viral fusogens. They all assume an asymmetrical, single-pass N-extracellular–C-cytoplasmic topology in the membrane that positions small (~20–40 aa) ectodomains outside the plasma membrane, with the majority of their masses being contained within the transmembrane and endodomains. It is unclear how the FAST proteins could mediate membrane fusion in a similar manner to the enveloped virus fusogens, which use extensive structural rearrangements of their complex, oligomeric ectodomains to promote close membrane apposition and the merging of the two bilayers (Melikyan, 2008).

The FAST protein family consists of four members: p14 of reptilian reovirus, p15 of baboon reovirus and the p10 proteins encoded by the avian and Nelson Bay reoviruses (ARV and NBV, respectively), all of which are members of the genus Orthoreovirus, and the recently discovered p22 protein of Atlantic salmon reovirus, a member of the genus Aquareovirus. The FAST proteins lack significant sequence similarity but share a subset of structural features. These include a single transmembrane domain flanked on the cytosolic side by a cluster of basic residues, a small region of moderate hydrophobicity (termed the hydrophobic patch) that can reside on either side of the transmembrane domain and a fatty acid modification (either an N-terminal myristate or a palmitoylated dicysteine motif), all of which are required for membrane fusion activity (Corcoran et al., 2004; Dawe et al., 2005; Shmulevitz et al., 2003). Defining the roles of these motifs in the fusion reaction requires a more detailed understanding of the structure–function relationships of the FAST proteins.

Previous mutagenesis studies of the 125 aa p14 FAST protein identified several positions in the 36 aa ectodomain where point substitutions eliminated membrane fusion activity. For example, a G2A substitution removing the myristoylation consensus sequence, a V9T substitution creating an N-glycosylation site and substitutions of His-11 with either glutamic acid or arginine (H11E and H11R, respectively) all eliminated syncytium formation (Corcoran et al., 2004). Interestingly, co-expression of these p14 ectodomain mutants with wild-type p14 had variable effects on p14-induced syncytium formation (Fig. 1). Co-expres-
sion of p14 with the fusion-incompetent V9T and H11E mutants, using a 1:1 ratio of plasmid DNA, severely impaired p14-induced cell–cell fusion, as observed by light microscopy of Giemsa-stained monolayers (Fig. 1a). A quantitative syncytial assay based on the mean number of syncytial nuclei per microscopic field (Corcoran & Duncan, 2004) revealed that these p14 ectodomain mutants, and the fusion-incompetent G2A mutant, inhibited p14-induced cell–cell fusion by >80–90 % (Fig. 1b). In contrast, co-expression of p14 with the V9I or H11A constructs, both of which retain substantial levels of cell–cell fusion activity (Corcoran et al., 2004), had no significant inhibitory effect on syncytogenesis (Fig. 1b).

![Image of Vero cells co-transfected with p14 ectodomain mutants.](http://vir.sgmjournals.org)

**Fig. 1.** Fusion-defective p14 ectodomain substitutions exert a dominant-negative effect on the syncytogenic ability of wild-type p14. (a) Vero cells were co-transfected at a 1:1 DNA ratio with plasmid DNA expressing wild-type p14 and either vector DNA or the p14 mutants V9T, V9I, H11E, H11A or H11R. Syncytium formation was visualized by light microscopy of Giemsa-stained cell monolayers 19 h after transfection. (b) Cells were co-transfected with p14 and the indicated p14-ectodomain mutants and the mean number of syncytial nuclei per microscopic field of Giemsa-stained monolayers was determined. Results from one representative experiment performed in triplicate are reported as the mean percentage of fusion (±SEM) relative to cells transfected with wild-type p14 and empty vector.

Therefore, specific substitutions in the p14 ectodomain exert a dominant-negative effect on p14-induced syncytogenesis.

One straightforward explanation for a dominant-negative phenotype is that the protein functions as a multimer, where the presence of one or more mutant monomers in the multimeric complex can disrupt activity. The number of monomers present in a FAST protein remains unclear. Attempts to co-precipitate untagged p10 when it was co-expressed with haemagglutinin (HA)-tagged p10 using anti-HA antibody were unsuccessful; chemical cross-linking also failed to detect p10 multimers (Shmulevitz et al., 2003). However, a band present in non-reducing gels of the NBV p10 protein may represent a p10 dimer (Cheng et al., 2005). To determine whether p14 forms multimers, we employed an anti-HA co-immunoprecipitation approach. QM5 cells were co-transfected, at ratios of either 1:1 or 3:1 of plasmid DNA, with p14 and a C-terminally tagged version of p14 containing two, 9 aa HA epitopes separated by a flexible glycine linker (hereafter referred to as p14HAC). The p14HAC construct developed large multinucleated syncytia at a rate similar to wild-type p14 (Fig. 2a). Co-transfected cells were radiolabelled at 14 h post-transfection and then lysed using either a stringent ionic-detergent-containing (RIPA) buffer [50 mM Tris/HCl, pH 8, 150 mM NaCl, 1 mM EDTA, 1 % (w/v) Igepal, 0.5 % (w/v) sodium deoxycholate, 0.1 % (w/v) SDS] or a less stringent Triton X-100 buffer [50 mM Tris/HCl, pH 8, 150 mM NaCl, 1 mM EDTA, 1 % (w/v) Triton X-100]. Both buffers contained protease inhibitors (200 mM aprotinin, 1 μM leupeptin and 1 μM pepstatin). Radio-immunoprecipitation with polyclonal anti-p14 antiserum followed by SDS-PAGE and autoradiography detected both versions of p14, which were easily distinguishable by the retarded gel mobility of the HA-tagged p14 construct relative to untagged p14 (Fig. 2b, lanes 2 and 8). Most importantly, radioimmunoprecipitation with anti-HA mAb precipitated not only p14HAC but also untagged p14 under both detergent conditions and at both plasmid DNA ratios (Fig. 2b, lanes 5 and 6, and lanes 11 and 12, respectively). Immunoprecipitation carried out with normal rabbit serum or an isotype control for the anti-HA (IgG2b) antibody did not precipitate either form of p14 (Fig. 2b, lanes 1 and 7, and lanes 4 and 10, respectively), confirming the specificity of the immunoprecipitations and that p14 was not present in the immunoprecipitates because of inadequate detergent solubilization of integral membrane proteins. As a further control, cells were co-transfected with empty vector and either p14 or p14HAC, and the presence of p14 in the anti-HA immunoprecipitates was detected by immunoblotting with anti-p14 antiserum. The anti-HA antibody precipitated p14 from cells co-expressing p14 and p14HAC, but not from cells co-expressing p14 and empty vector (Fig. 2c, lanes 2 and 3 versus lanes 8 and 9).

Radioimmunoprecipitation and immunoprecipitation–immunoblotting approaches therefore both provided direct evidence that p14 forms homomultimers.

Plasma membrane-localized integral membrane proteins
secretory pathway (Arvan proteins can occur in different compartments of the plasma membrane. Multimerization of these membrane proteins towards the ER via retrograde transport (Klausner et al., 1992). Immunofluorescent staining of p14-multimers are generally trafficked through the endoplasmic reticulum (ER)–Golgi complex vesicle transport pathway to reach the plasma membrane. Most importantly, detection of p14 homotypic interactions via co-immunoprecipitation under both detergent extraction conditions was eliminated by disrupting the secretory pathway with BFA (Fig. 3b). Normal transit through the ER–Golgi complex pathway is therefore required for p14 homotypic interactions to occur.

To determine which domain(s) of p14 is responsible for mediating homotypic interactions, chimeric p14 constructs were created in which individual domains of p14 were replaced with the complementary domain from the heterologous ARV p10 FAST protein. These three chimeric p14 constructs contained the p10 ecto- (p14ect10), endo- (p14end10) or transmembrane (p14TM10) domains, (Fig. 3c). The p14TM10 construct retained fusion activity, fusing monolayers to completion, albeit with reduced kinetics of syncytigenesis (Clancy & Duncan, 2009). The p14end10 construct retained some minimal fusion activity, generating small syncytia by 24 h after transfection, while the p14ect10 construct showed no fusion (not shown). These three constructs were individually co-transfected with p14HAC and assessed for their ability to form homotypic interactions by co-immunoprecipitation. The anti-HA antibody successfully precipitated both p14end10 (Fig. 3c, top panel) and p14TM10 (Fig. 3c, middle panel). Since the same co-immunoprecipitation approach did not co-precipitate tagged and untagged p10 (Shmulevitz et al., 2003), it is unlikely that the homotypic interactions observed for the p14TM10 or p14end10 constructs were due to interactions between the p10 components of these chimeric FAST proteins. The p14 endodomain and transmembrane domain are therefore dispensable for p14 homotypic interactions.

Three additional lines of evidence suggested that the p14 ectodomain is responsible for homomultimer formation. Firstly, we were unable to detect co-precipitation of the p14ect10 construct with the anti-HA antibody (Fig. 3c, bottom panel). However, the p14ect10 construct was not expressed as well as the other constructs, which could have contributed to the inability to detect this construct by co-immunoprecipitation. Secondly, immunoprecipitations of co-transfected cell lysates at pH 5.3 did not co-precipitate untagged p14 (Fig. 3d), suggesting that protonation of histidine residues (side chain pKₐ=6.1) disrupts homomultimer formation. There are only three histidine residues in p14, and two of these reside in the p14 ectodomain. This fungal metabolite disrupts the Golgi complex, resulting in a redistribution of Golgi complex-resident proteins towards the ER via retrograde transport (Klauser et al., 1992). Immunofluorescent staining of p14-transfected QM5 cells using anti-β-coatamer protein (COP) antibody (a marker of the ER–Golgi complex intermediate compartment) revealed the slightly diffuse perinuclear staining typical for this compartment (Fig. 3a). As previously reported by Donaldson et al. (1990), BFA treatment disrupted ER–Golgi complex trafficking, resulting in a loss of the localized perinuclear staining with β-COP antibody and a more disseminated cytoplasmic staining pattern (Fig. 3a). Strong co-fluorescence of p14–EGFP with the β-COP marker was obvious in both untreated and BFA-treated cells (Fig. 3a), consistent with p14 trafficking through the ER–Golgi complex pathway to the plasma membrane.

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Fig. 2. Co-immunoprecipitation indicates that p14 forms homomultimers. (a) Vero cells were transfected with empty vector or plasmid DNA expressing wild-type p14 or p14HAC. Syncytium formation was visualized by light microscopy of Giemsa-stained monolayers 19 h after transfection. (b) QM5 cells co-transfected with p14 and p14HAC at 1 : 1 or 3 : 1 (indicated by *) DNA ratios were radiolabelled and then lysed, in either RIPA or Triton X-100 buffer 14 h after transfection. Immunoprecipitations were performed using anti-p14 antibody (14), anti-HA antibody (HA) (produced in-house), normal rabbit serum (N) or an isotype control (I). Precipitates were resolved by SDS-PAGE and detected by autoradiography. Locations of the tagged (p14HAC) and untagged (p14) polypeptides are indicated on the right. (c) QM5 cells were co-transfected with a 1 : 1 ratio of empty vector (vec) and p14, empty vector and p14HAC, or p14 and p14HAC. Cells were lysed in RIPA (R) or Triton X-100 (T) buffer 14 h after transfection. Cell lysates were immunoprecipitated with anti-HA antibody, and the lysates (L) or the immunoprecipitates were analysed by immunoblotting with anti-p14 antisera.

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While C-terminal truncations that remove the endodomain histidine retain fusion activity (Corcoran & Duncan, 2004), at least one of the histidine residues in the ectodomain (His-11) is sensitive to substitution; an H11A substitution decreases the fusion kinetics of p14, while H11E and H11R substitutions are fusion defective (Corcoran et al., 2004) (Fig. 1). The other His residue in the ectodomain (His-34) might also contribute to pH-dependent disruption of p14 multimers, but this residue has not been investigated by substitution analysis. Thirdly, if protonation of His-11 contributes to disruption of multimers, we would predict that a positive charge at this location should also disrupt multimers and p14 function. The dominant-negative results were consistent with this prediction. While both H11E and H11R are fusion incompetent (Corcoran et al., 2004), only H11E exerted a strong dominant-negative effect (Fig. 1b). The simplest explanation for these results is that the negative charge in H11E eliminates p14 fusion activity but does not disrupt p14 multimers, hence the dominant-negative effect. Conversely, the H11R substitution is also non-functional (Corcoran et al., 2004) but has no strong dominant-negative effect (Fig. 1), consistent with this substitution disrupting multimer formation. Several lines of evidence therefore implicate the p14 ectodomain in multimer formation during trafficking through the secretory pathway.

The present results provide the first direct evidence of multimer formation by any FAST protein. They also indicate that the p14 FAST protein forms pH-sensitive homomultimers during transit through the ER–Golgi complex pathway, probably mediated by homotypic...
ectodomain interactions. The small size of the p14 ectodomain would probably limit the extent of these interactions, which suggests that the multimeric interactions may not be highly stable, thereby allowing structural rearrangements of the p14 ectodomains during the fusion reaction. Transient or weak homotypic interactions may also explain why multimers of the p10 FAST protein cannot be detected by co-immunoprecipitation (Shmulevitz et al., 2003). Presently, it is unclear how transit through the ER–Golgi complex pathway influences p14-multimer formation, but factors such as post-ER p14 localization to membrane microdomains (Corcoran et al., 2006) or the effects of the different lipid compositions of secretory membrane compartments on p14 interactions could influence this process. Additional NMR structural analysis might further define the nature of the homotypic p14 ectodomain interactions and how these interactions should be integrated into working models of how this unusual family of non-enveloped virus proteins drives cell–cell membrane fusion.

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**References**


