The subfamily Pneumovirinae of the family Paramyxoviridae includes many important pathogens of humans and animals, such as Human respiratory syncytial virus (HRSV), bovine RSV, pneumonia virus of mice, human metapneumovirus and avian pneumovirus (Lamb & Kolakofsky, 2001). HRSV infection is the single most common cause of hospitalization of infants and young children due to bronchiolitis and pneumonia, and is a significant cause of morbidity and mortality among the elderly and transplant recipients (Han et al., 1999; Ison & Hayden, 2002; Thompson et al., 2003; Whimbey & Ghosh, 2000). The fusion (F) proteins of pneumoviruses play critical roles in promoting the entry and assembly of infectious virions and have an overall structural organization similar to other type I membrane viral fusion proteins (reviewed by Colman & Lawrence, 2003). The HRSV F mRNA is translated into a 574 aa precursor protein designated F0, which is cleaved at two sites (Gonzalez-Reyes et al., 2001) by furin in the trans-Golgi (Bolt et al., 2000; Collins & Mottet, 1991), generating two subunits designated F1 (~50 kDa) and F2 (~20 kDa) (Rixon et al., 2002). The F1 and F2 chains are joined together by disulfide-bond formation (Gruber & Levine, 1983; Scheid & Choppin, 1977). The mature form of the F protein present on the surface of the virus and infected cells is believed to consist of a homotrimer of three non-covalently associated units of F1–F2. The F1 subunit contains an N-terminal hydrophobic fusion peptide region followed by two heptad repeat regions (HRA and HRB) separated by an intervening cysteine-rich region and a hydrophobic transmembrane domain located near the C-terminus of the protein followed by a short (26 aa) cytoplasmic domain containing a single cysteine residue (C550), which has been shown to be the site of addition for a palmitoyl group (Arumugham et al., 1989). Previous studies of the F protein of the paramyxovirus Newcastle disease virus (NDV) demonstrated that deletion of the entire cytoplasmic domain altered protein processing and abolished syncytia formation (Sergel & Morrison, 1995). To determine whether the cytoplasmic domain of the F protein or its single cysteine (C550) plays a similar role in fusion, we prepared a construct in which aa 549–574 were deleted (ACT), as well as one in which C550 was changed to a serine (C550S). The effect of these mutations on protein expression, processing and the ability to cause cell–cell fusion was examined.

Plasmid pHRSVF0ptA2 containing the coding region of the HRSV F protein (subgroup A, strain A2; amino acid sequence derived from a known infectious HRSV cDNA and codon optimized) cloned into pcDNA3.1/Hygro(−) (Invitrogen) has been described previously (Branigan et al., 2005; Morton et al., 2003). The codon encoding HRSV F C550 in pHRSVF0ptA2 was changed to serine by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene). Plasmid pACT truncates the HRSV F protein-coding region in pHRSVF0ptA2 immediately after the transmembrane domain, deleting the entire
The cytoplasmic domain (aa 549–574). Plasmid pCMVβ (BD Biosciences Clontech) expresses the β-galactosidase gene under the control of the human cytomegalovirus promoter. 293T cells were transfected with plasmids pHRSVF0ptA2, pC550S, pACT or the negative control plasmids pCMVβ or pcDNA3.1/Hygro (−) vector only, and expression and protein processing were assayed by metabolic labelling, followed by immunoprecipitation with a cocktail of anti-HRSV F monoclonal antibodies (mAbs) as described previously (Branigan et al., 2005). A band migrating at approximately 45 kDa was detected in all immunoprecipitates, including those from cells transfected with the negative control plasmid pCMVβ or pcDNA3.1/Hygro (−) and most likely represents a non-specific, co-immunoprecipitating protein. Labelled bands migrating at the expected sizes for the F1 and F2 subunits (~50 and ~20 kDa, respectively) of the wild-type (WT) HRSV F protein were detected readily as indicated (Fig. 1, lane 2). Neither mutation of C550 to serine (Fig. 1, lane 1, C550S) nor deletion of the entire cytoplasmic tail (Fig. 1, lane 3, ΔCT) had any effect on protein processing relative to WT, although as expected, the F1 subunit encoded by pACT migrated faster than the WT (Fig. 1, lane 3, ΔCT) due to the truncation of the cytoplasmic domain.

To assess cell-surface expression, 293T cells were seeded into 96-well plates and transfected with plasmids pHRSVF0ptA2, pC550S, pACT or negative controls, and the levels of the HRSV F protein on the cell surface were determined by flow cytometry and ELISA. At 20–24 h post-transfection, cells were assayed for binding of the anti-HRSV F protein mAb, palivizumab (Johnson et al., 1997), under permeabilizing or non-permeabilizing conditions. Cells were fixed by the addition of 0·05 % glutaraldehyde (Sigma) in 1× PBS for 15 min at room temperature and washed under either permeabilizing (0·1 % Triton X-100 in PBS) or non-permeabilizing (0·05 % Tween 20 in PBS) conditions. These conditions were verified using an anti-HRSV N protein mAb (clone # M291207; Fitzgerald Industries International) and HRSV-infected cells. HRSV N protein is produced only within the cytoplasm of HRSV-infected cells. The anti-N mAb yielded a strong positive signal in infected cells when wash buffer containing 0·1 % Triton X-100 was used, but not when wash buffer containing 0·05 % Tween 20 was used (data not shown). Cells were blocked for 1 h with Super-Block (Pierce Biotechnology), followed by incubation with 1 μg palivizumab ml⁻¹ for 1 h at room temperature. Samples were then incubated with a horseradish peroxidase-conjugated anti-human IgG (Amersham Biosciences) at a dilution of 1:800 for 1 h at room temperature, followed by detection with TMB substrate (Sigma). The reaction was stopped with the addition of 1 M sulfuric acid and the optical density was read at 450 nm. Values were calculated as percentage relative to WT HRSV F protein after adjusting for background signal from the vector-only control. Neither mutation of C550 to serine nor deletion of the entire cytoplasmic domain had any effect on the levels of cell-surface HRSV F protein expression as assayed by ELISA (Fig. 2a). This finding was confirmed by using flow cytometry (Fig. 2b) using conditions described previously (Branigan et al., 2005).

The ability of the C550S mutant and cytoplasmic domain deletion mutant to promote cell fusion was assayed using methods described previously (Branigan et al., 2005). In contrast to NDV F protein, where deletion of the entire cytoplasmic domain abolished syncytia formation (Sergel & Morrison, 1995), deletion of the entire cytoplasmic domain reduced fusion activity by approximately 36 % relative to WT HRSV F protein (Fig. 3), but still retained significant fusion activity at 37 °C. This finding indicated that the cytoplasmic domain of the HRSV F protein is not required for fusion and that the extracellular and transmembrane domains are sufficient to mediate fusion. The fusion activity of the C550S mutation was slightly increased (128 %) relative to WT (100 %) (Fig. 3). As mutation of cysteine residues in other viral fusion proteins has been reported to cause a temperature-sensitive (ts) phenotype (Long et al., 1990), we also examined fusion activity at 32 and 39·5 °C. These temperatures were selected as HRSV mutants sensitive for these two temperatures have been described previously (Gharpure et al., 1969; Hsu et al., 1995). The overall levels of WT HRSV F-mediated cell fusion were reduced by approximately 50 % at either 32 or 39·5 °C relative to 37 °C (Fig. 3). It is possible that the reduction in fusion observed at 32 and 39·5 °C was due to slower kinetics of overall fusion at the lower temperature and lower levels of cell-surface protein expression at the higher temperature, although further.

**Fig. 1.** Protein processing of HRSV F proteins. 293T cells were transfected with plasmid vector only (−), a plasmid expressing β-galactosidase (β-Gal) or plasmids encoding the WT HRSV F protein, the C550S mutation (C550S) or the cytoplasmic deletion mutation (ΔCT), followed by metabolic labelling with [³⁵S]methionine/cysteine mixture and immunoprecipitation with a cocktail of HRSV F-specific mAbs. The positions of molecular size markers are shown. Positions of the F2 subunits, the F1 subunits of the WT and C550S OS mutants, and the truncated F0 precursor and truncated F1 subunit of the cytoplasmic deletion mutation are indicated.
experimentation is needed to support this hypothesis. Although the absolute levels of cell fusion were reduced at both 32 and 39.5 °C relative to 37 °C for all proteins including WT (Fig. 3), there were no differences observed in their relative fusion activities at either 32 or 39.5 °C relative to 37 °C, suggesting a lack of a ts phenotype for fusion for either the C550S mutation or the cytoplasmic domain deletion mutation (Fig. 3).

The results presented here demonstrated that the cytoplasmic domain of the HRSV F protein is not required for cell fusion. These findings are intriguing, as deletion of the entire cytoplasmic domain of NDV F protein dramatically reduced precursor protein processing and syncytium formation in a previous study (Sergel & Morrison, 1995); however, the assays used to measure fusion in those studies and those presented here are different and cannot be compared directly. This finding is intriguing, as the NDV F protein X-ray structure has been used to generate a structural model of the HRSV F protein (Morton et al., 2003; Smith et al., 2002). Although the NDV and HRSV F proteins appear to be related structurally, these results highlight a functional difference between these proteins and give further support to the difference in classification between the paramyxovirus and pneumovirus genera. C550 is the only cysteine present in the cytoplasmic domain of the HRSV F protein and is the site of addition of a palmitoyl group (Arumugham et al., 1989). Palmitoylation of viral fusion proteins affects trafficking to lipid rafts, which can play a role in virus assembly, as described for human immunodeficiency virus type 1 (Bhattacharya et al., 2004). Neither palmitoylation nor association with lipid rafts is required by NDV F protein for cell fusion (Dolganovic et al., 2003; Sergel & Morrison, 1995). HRSV proteins associate with lipid rafts (Brown et al., 2004) and this association is thought to play a role in viral assembly. Although both the C550S mutation and the deletion of the entire cytoplasmic domain support the hypothesis that palmitoylation is not required for HRSV F protein fusion, we are currently investigating the role of the HRSV F protein cytoplasmic domain and palmitoylation in lipid raft trafficking and virus assembly.

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References


