Membrane orientation and oligomerization of the small hydrophobic protein of human respiratory syncytial virus

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Previous work has demonstrated that the small hydrophobic (SH) protein of human respiratory syncytial virus (RSV) A2 strain is a 64 amino acid integral membrane protein that accumulates intracellularly as an unglycosylated major species (SH₀), a minor species truncated at the amino terminus and two N-glycosylated species one of which contains a further addition of polylactosamine. In this study, the membrane orientation of SH₀ was mapped by trypsinization of intact RSV-infected cells followed by washout, lysis and immunoprecipitation of protected fragments with antisera specific for the protein termini. This showed that the C terminus is extracellular and the SH protein was not detectably palmitylated. Analysis of the SH protein by sedimentation on sucrose gradients showed that it rapidly assembles into a homo-oligomer that sediments with the F protein tetramer. Interestingly, all forms of the SH protein were found in the oligomeric fraction. Chemical cross-linking generated species which appeared to represent dimers, trimers, tetramers and pentamers as well as a minor species of 180K which might correspond to the oligomeric form detected by sucrose gradient sedimentation.

Human respiratory syncytial virus (RSV), a member of the genus Pneumovirus of the family Paramyxoviridae, encodes three transmembrane surface proteins, the fusion (F) protein responsible for viral penetration, the heavily glycosylated (G) protein responsible for viral attachment, and the small hydrophobic (SH) protein of unknown function (Collins & Wertz, 1985; Olmsted & Collins, 1989; Collins, 1991; Collins et al., 1990). Some other paramyxoviruses, such as simian virus 5, also encode SH proteins (Hiebert et al., 1988) but others do not (Collins, 1991). Influenza A and B viruses also encode small integral membrane proteins, the M2 and NB proteins respectively (Williams & Lamb, 1986; Holsinger & Lamb, 1991; Pinto et al., 1992), although it is not known whether any of these small proteins are functional counterparts.

Following short labelling pulses, the SH protein of RSV strain A2 appears in three forms: two unglycosylated species of 7-5K (SH₀) and 4-5K (SH₁) that represent translational initiation at the first and second methionyl codons, respectively, in the translational open reading frame, and a 13K to 15K species (SH₂) which is an N-glycosylated form of SH₀ (Olmsted & Collins, 1989; P.L. Collins & G. Mottet, unpublished). Interestingly, therefore, SH protein accumulates in glycosylated and non-glycosylated forms. A proportion of SH₂ is modified subsequently by the post-translational addition of polylactosaminoglycan to the N-linked carbohydrate to yield a heterodisperse species (SH₃p) of 21K to 40K (Olmsted & Collins, 1989; Anderson et al., 1992; H. Sheshberadaran, R. A. Olmsted, P. L. Collins & R. A. Lamb, unpublished). All RSV strains examined to date, representing both human antigenic subgroups and a bovine strain, direct the synthesis of SH₀, SH₂, and SH₃p, suggesting that each form has some role in the RSV replicative cycle (Collins et al., 1990; Anderson et al., 1992). These three proteins are expressed at the cell surface in the same relative proportions as found intracellularly but differ in their rates of movement, as described below (Olmsted & Collins, 1989; P.L. Collins & G. Mottet, unpublished). SH₀ and SH₂ appear to be virion structural proteins (Collins et al., 1990).

It was suggested that the carboxyl terminus of the SH protein is orientated extracellularly in previous work where (i) there was positive surface immunofluorescence of live RSV-infected cells with an antisera specific to a synthetic peptide, SH-CT, that represents the carboxyl terminus of the SH protein, and (ii) the same antisera was able to form antibody-antigen complexes with the SH protein when reacted with intact RSV-infected cells in surface immunoprecipitation experiments (Olmsted & Collins, 1989). The membrane orientation of the SH protein was investigated by surface proteolysis (Fig. 1).
CV-1 cells were infected with RSV at an m.o.i. of 2 or were mock-infected. At 19.5 h post-infection, the cells were radiolabelled by incubation with 100 μCi/ml [35S]methionine for 1 h followed by a 1 h non-radioactive chase. The cells were washed and scraped into PBS pH 8.1, and aliquots were incubated at 37 °C for 30 min in the presence of TPCK-treated trypsin (Worthington) at the protease concentrations indicated in Fig. 1. Aprotinin (Boehringer) and trypsin inhibitor (Sigma) were added, to a concentration of 100 μg/ml each, and the treated cells were centrifuged (SW50.1 rotor, 35,000 r.p.m., 1 h, 4 °C) through a 3 ml cushion of 10 % w/w sucrose in 1 mM-Tris–HCl pH 8.0, containing 1 mM-EDTA. The pelleted cells were lysed and re-suspended in immunoprecipitation buffer (50 mM-Tris–HCl pH 7.5, 150 mM-NaCl, 1 mM-EDTA, 1% v/v Triton X-100 and 0.1% w/v SDS) containing 100 μg/ml each of aprotinin and trypsin inhibitor and the mixture was clarified by centrifugation. Aliquots of the lysates were analysed by immunoprecipitation with antisera specific for the amino (NT) or carboxy terminus of the SH protein, prepared against peptides SH-NT and SH-CT, respectively (Olmsted & Collins, 1989; Collins et al., 1990) followed by SDS–PAGE (Fig. 1).

The most abundant form of the SH protein is SH0, and the mapping data are limited to this species. Following trypsin treatment, the amount of SH0 protein immunoprecipitated with SH-CT-specific antiserum (Fig. 1) was substantially diminished. This suggested that the trypsin digested the carboxyl-terminal domain recognized by the antipeptide antibodies; the residual immunoprecipitated full-length SH0 protein presumably represented an undigested fraction. The fact that only full-length SH0 protein was detected indicated that all of the digested forms lacked the carboxy terminus and thus could not be immunoprecipitated with this antiserum. There was no evidence of proteolysis of the amino terminus.

In contrast, the trypsin treatment did not diminish the quantity of SH0 protein immunoprecipitated by antiserum to peptide SH-NT, but most of the immunoprecipitated material was reduced in Mr from 7.5K to 5.8K. That the amount of immunoprecipitated protein was undiminished indicated that the amino-terminal domain recognized by the antipeptide antibodies was not digested, whereas the reduction in size confirmed the idea that the carboxy terminus was sensitive to trypsinization of intact cells. In control experiments (not shown) in which lysates were prepared from parallel cultures of radiolabelled RSV-infected cells by solubilization in 1% Triton X-100, the SH protein was completely digested by trypsin. This supports the premise that the 5-8K fragment described above was protected by membrane rather than being a protease-resistant core.

This study demonstrated that the SH protein is anchored in the membrane such that the carboxy-terminal domain is extracellular and the amino-terminal domain is either cytoplasmic or embedded in membrane. A similar membrane orientation was demonstrated for the SH protein of simian virus 5 (Hiebert et al., 1988), whereas the M2 and NB proteins of influenza viruses A and B have the opposite orientation (Williams & Lamb, 1986; Holsinger & Lamb, 1991).

Examination of the amino acid sequence of the SH protein of strain A2 (Collins & Wertz, 1985) indicated that only two potential cleavage sites could be compatible with the observed protected fragment: cleavage beyond lysine43 would remove 21 amino acids with a calculated Mr of 2.55 K, leaving an SH protein of Mr approx. 4.95K, whereas cleavage beyond lysine53 would remove 11 amino acids of Mr, 1.302K, leaving an SH protein of...
membrane-embedded, were relatively well conserved whereas not evenly distributed. Amino acids 23 to 41, which (84% and 91% identical, respectively, between the two strains) are likely to span the membrane, and amino acids 1 to 22, which are likely to be cytoplasmic or cytoplasmic domains which are relatively well conserved, corresponding subgroup B) showed that the proteins were 76% identical overall, but that the amino acid differences were not evenly distributed. Amino acids 23 to 41, which this work suggests are likely to span the membrane, and amino acids 1 to 22, which are likely to be cytoplasmic or membrane-embedded, were relatively well conserved (84% and 91% identical, respectively, between the two strains). In contrast, the carboxy-terminal domain (amino acids 42 to 64) was only 50% conserved. Here, this latter domain was shown to be extracellular. A similar asymmetrical distribution of amino acid differences was observed between the human and bovine RSV strains (Anderson et al., 1992). Thus, the SH protein resembles the G protein in having a relatively divergent ectodomain [that of the G protein is 43% identical between strains A2 and 18537, consisting of two large divergent regions flanking a single, short conserved region suggested to be a functional domain (Johnson et al., 1987)] and in having membrane-spanning and cytoplasmic domains which are relatively well conserved, exhibiting a level of inter-subgroup identity (84%) similar to that of the other proteins (≥87%). As has been suggested elsewhere (Collins, 1991, and references therein), diversity among strains in the amino acid sequences of extracellular domains might reduce the effectiveness of host immunity against heterologous strains and might be one of numerous factors contributing to the ability of RSV to reinfect. The finding that the extracellular domain of SH is divergent also suggests that it does not contain a specific sequence that is critical for RSV replication. Indeed, one possibility is that the ectodomain might not be involved directly in its function and might instead have the non-specific role of securing the protein in the membrane.

To investigate whether the SH protein assembled into an oligomeric form, lysates of RSV-infected cells were exposed to a series of chemical cross-linking agents. The SH protein was recovered by immunoprecipitation with antisera specific to peptide SH-CT and was analysed by gel electrophoresis (Fig. 2). In initial experiments (not shown), the identification of cross-linked species was found to be complicated by the presence of the glycosylated forms. Therefore, the SH protein used for cross-linking analysis was radiolabelled in the presence of 2 μg/ml tunicamycin, first added 2 h prior to labelling. The SH0 species, the predominant species labelled under these conditions, was not detectably cross-linked by a series of dimethyl diimidates and other cross-linking agents under conditions where dimers, trimers and tetramers of the F protein were cross-linked in parallel reactions (Collins & Mottet, 1991). However, cross-linked species corresponding in size to homodimers, homotrimers, homotetramers and homopentamers of the SH0 protein (species a to d in Fig. 2a) were detected with the use of the more stable cross-linker disuccinimidyl suberate (bridge distance 11.4 Å). In addition, a minor species of Mr approx. 180K was detected (Fig. 2b, species e). None of these species were detectably precipitated with antisera specific to the F or G proteins (not shown), consistent with the interpretation that these two other RSV transmembrane proteins were not significantly involved. It was not possible, however, to determine whether the cross-linked species contained SH protein alone because this particular cross-linker is not

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**Fig. 2.** Chemical cross-linking analysis of the SH protein. RSV-infected cells were labelled with [35S]methionine for 4 h followed by a non-radioactive chase of 1 h in the presence of 2 μg/ml tunicamycin, which was added and maintained from 2 h prior to the labelling period. Lysates were prepared and replicate aliquots were incubated with 10 mg/ml of each of the following cross-linkers: suberimidate (lane 2), azelainamidate (3), sebacimidate (4), disuccinimidyl suberate (5) or no added cross-linker (1). SH protein was immunoprecipitated with antiserum against peptide SH-CT and analysed by SDS-PAGE on (a) 17% or (b) 8% gels under non-reducing conditions. Lanes M give the positions of Mr (× 103) markers. The positions are shown for the SH0 protein monomer (a, lanes 1 to 4) and possible di-, tri-, tetra- and pentameric cross-linked species as well as (b, lane 5) a larger multimeric form (see text).
Oligomeric forms of the SH protein were not detected under non-reducing conditions in the absence of chemical cross-linking (Fig. 2a, lane 1), indicating that disulphide bonds are not involved in the native structure (the SH protein of strain A2 contains a single cysteine residue at position 45, near the inner membrane face). This is in contrast to the situation with the M2 and NB proteins of influenza viruses A and B, respectively (Williams & Lamb, 1986; Holsinger & Lamb, 1991), which form disulphide-linked dimers that, at least in the case of M2, associate pairwise by non-covalent interactions to form tetramers.

To investigate oligomerization of the SH protein further, RSV-infected cells were labelled for 5 min with [35S]methionine followed by non-radioactive chase incubations of up to 60 min (Fig. 3). Lysates were prepared and subjected to sedimentation in sucrose gradients at pH 5-8 in the presence of Triton X-100. In initial control experiments, lysis and analysis were performed at pH 6-5, 7.0 or 7-5 (for experimental details see Collins & Mottet, 1991) and yielded similar results (not shown). When the total protein profile across the fractionated gradients was analysed directly without immunoprecipitation (not shown), most intracellular proteins were present as monomeric species in fractions 8, 9 and 10 (fraction 1 represents the bottom of the gradient and fraction 12 the top). The gradient positions of the SH and F proteins were then determined independently by immunoprecipitation with antisera to their respective carboxy termini.

Following the short labelling pulse, most of the SH

cleavable. With this caveat, these data indicated that the SH protein assembles into oligomers which were of the appropriate Mr's to range from dimers to pentamers. The low Mr's of these multimeric forms make it unlikely that they included RSV proteins other than the 64 amino acid SH protein, although the involvement of cellular species of low Mr cannot be ruled out.

Fig. 3. The SH protein forms a homooligomer that assembles rapidly, contains all forms of the SH protein, and cosediments with the F protein tetramer. RSV-infected cells were labelled with 200 μCi/ml [35S]methionine for 5 min and (a) harvested immediately or incubated in non-radioactive chase medium in the presence of 2 mM-methionine for (b) 2 min or (c, d) 60 min. The cells were lysed in 50 mM-MES pH 5.8, 150 mM-NaCl and 1% (v/v) Triton X-100 containing 20 mM-iodoacetamide, clarified and centrifuged on 5 to 25% (w/v) sucrose gradients in 50 mM-MES pH 5.8, 100 mM-NaCl, 0.1% Triton X-100 for 16 h at 4 °C. Each gradient was fractionated into 12 1 ml aliquots, fraction 1 representing the gradient bottom and fraction 12 the top (not all fractions are shown) together with an additional fraction (lane P) containing the resuspended pellet from the gradient bottom. These fractions were analysed by immunoprecipitation with antisera to peptide SH-CT (a to c) or a peptide representing the C terminus of the F protein (d) (Collins & Mottet, 1991). The samples were analysed on 17% (a to c) or 13% (d) polyacrylamide gels.
protein migrated in fractions 3 and 9 of the gradients shown in Fig. 3; the former and latter peaks are thought to represent oligomeric and monomeric species, respectively. Thus, the SH protein appeared to oligomerize rapidly. The apparent absence of other coprecipitating RSV proteins suggests that the SH protein was contained in homo-oligomers, although we cannot discount the possibility that the SH protein was associated with another labelled protein during sedimentation and subsequently dissociated completely during immunoprecipitation, or that the SH protein associated with preformed unlabelled species.

Immunoprecipitation of the F protein from duplicate aliquots of the same gradient fractions (Fig. 3d compared with Fig. 3c) showed that the 280K F protein tetramer (Collins & Mottet, 1991) cosedimented approximately with the SH oligomer. This indicates that the $M_r$ of the native oligomeric form of the SH protein is substantially greater than the 37K pentamer (species d in Fig. 2) detected by chemical cross-linking and would be more consistent with the value of 180K determined for the largest, minor band (species e) in the cross-linked material. The $M_r$ of this minor band corresponds to approximately 19 to 24 molecules of SH$_p$, so the readily cross-linked pentamer could be a subunit of a larger oligomer that might contain four or five such subunits. In the case of the M2 protein of influenza A virus, its sedimentation in sucrose gradients confirmed it might be a tetramer (Holsinger & Lamb, 1991). However, a minor 150K to 180K species was detected by chemical cross-linking and appeared to consist solely of M2 protein (Holsinger & Lamb, 1991). This suggested the possibility that a larger form exists for the native M2 oligomer, analogous to that detected for the RSV SH protein by sucrose gradient sedimentation, which might have escaped detection by sedimentation analysis owing to instability.

The single cysteine residue in the SH protein, C$_{45}$, is located in the cytoplasmic tail near the inner membrane face and might serve as an acceptor site for fatty acids. The M2 protein of influenza A virus has been shown to be palmitoylated (Sugrue et al., 1990; Veit et al., 1991), but the SH protein was not detectably labelled with [$^{3}$H]palmitate under conditions where label was efficiently incorporated into the F and G proteins (not shown).

The intracellular site at which polylactosamine is added to the SH protein was investigated with pulse-chase labelling protocols employing the following inhibitors of exocytosis: carbonylcyanide m-chlorophenylhydrazine (CCCP), an energy poison that blocks protein transport from the endoplasmic reticulum (ER) (Copeland et al., 1988), monensin, which has been shown to inhibit protein transport from the medial compartment of the Golgi complex (Quinn et al., 1983) and brefeldin A (BFA), which blocks transport from the ER. The effects of BFA are somewhat complicated because the drug inhibits outward but not inward vesicular transport (Doms et al., 1989; Lippincott-Schwartz et al., 1990). The retrograde vesicular recycling from the Golgi that continues in the presence of BFA results in the rapid collapse of the cis-, medial and some or all of the trans-Golgi compartments into the ER. In previous studies, a number of Golgi enzymes remained active following relocation to the ER and efficiently processed ER-immobilized proteins. Thus, BFA treatment allows immobilized proteins to interact with enzymes from more distal cisternae, those of the trans-Golgi compartment, than does monensin treatment.

Replicate cultures of RSV-infected cells were labelled for 5 min with [$^{35}$S]methionine followed by a non-radioactive chase for 1 h. Cells were mock-drug-treated (lanes 1 to 4), or treated with 50 µg/ml CCCP (lanes 5 and 6) immediately following the labelling period, or 5 µg/ml BFA (lanes 7 and 8) added 45 min before the labelling period and maintained thereafter, or 25 µM-monensin (lane 9) added 45 min before the labelling period and maintained thereafter. Proteins were immunoprecipitated with antiserum raised against peptide SH-CT and treated with endoglycosidase H (lanes 2, 4, 6 and 8) or mock-treated (lanes 1, 3, 5 and 7). Lane 9 represents a separate experiment in which the sample was neither endoglycosidase H-treated nor mock-treated. The samples were analysed on a 17% gel. $M_r$ (×10$^3$) are indicated in lane M.

**Fig. 4.** The addition of polylactosamine is a late event in exocytosis. RSV-infected (lanes 3 to 9) or mock-infected cells (lanes 1 and 2) were labelled for 5 min with [$^{35}$S]methionine followed by a non-radioactive chase for 1 h. Cells were mock-drug-treated (lanes 1 to 4), or treated with 50 µg/ml CCCP (lanes 5 and 6) immediately following the labelling period, or 5 µg/ml BFA (lanes 7 and 8) added 45 min before the labelling period and maintained thereafter, or 25 µM-monensin (lane 9) added 45 min before the labelling period and maintained thereafter. Proteins were immunoprecipitated with antiserum raised against peptide SH-CT and treated with endoglycosidase H (lanes 2, 4, 6 and 8) or mock-treated (lanes 1, 3, 5 and 7). Lane 9 represents a separate experiment in which the sample was neither endoglycosidase H-treated nor mock-treated. The samples were analysed on a 17% gel. $M_r$ (×10$^3$) are indicated in lane M.
It was interesting that all forms of the SH protein, SH\(_0\), SH\(_t\), SH\(_g\) and SH\(_p\), were represented in oligomers with no apparent differences in sedimentation rate (Fig. 3). Whether the different forms co-assemble or are segregated into separate oligomers is unknown, although the cosedimentation would suggest the former. Also, Anderson et al. (1992) reported that the purification of SH\(_p\) by lectin chromatography specific for polylactosaminoglycan resulted in the copurification of SH\(_0\), which is suggestive of mixed oligomers. On the other hand, the different forms of the SH protein differ in their rate of transport to the cell surface: the SH\(_0\) protein appeared almost immediately after a 5 min chase compared with approximately 10 min for SH\(_g\) and 20 to 30 min for SH\(_p\), and SH\(_t\) has never been detected by surface immunoprecipitation (Olmsted & Collins, 1989; P. L. Collins & G. Mottet, unpublished). Only SH\(_0\) and SH\(_p\) were detected in virions. These data would be more consistent with the different SH species being segregated into distinct oligomers and transported at different rates and to different final destinations.

The function of the SH protein remains unknown. Its status as an integral membrane protein is suggestive of a role either in early events that lead to uncoating or in late events involving virion morphogenesis. One hypothesis (Belshe & Hay, 1989) is that it forms ion channels, as does the M2 protein of influenza A virus (Pinto et al., 1992). But it is not clear what role such an activity would play in the RSV replicative cycle since this virus, in contrast to influenza virus, is thought to enter cells by fusion at the cell surface rather than from acidified endosomes.

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References


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