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

Peter L. Collins* and Geneviève Mottet†

Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Building 7, Room 100, Bethesda, Maryland 20892, U.S.A.

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 un-glycosylated major species (SH$_0$), 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 SH$_0$ 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 co-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, tetratmers 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 un-glycosylated species of 7-5K (SH$_0$) and 4-5K (SH$_t$) that represent translational initiation at the first and second methionyl codons, respectively, in the translational open reading frame, and a 13K to 15K species (SH$_g$) which is an N-glycosylated form of SH$_0$ (Olmsted & Collins, 1989; P. L. Collins & G. Mottet, unpublished). Interestingly, therefore, SH protein accumulates in glycosylated and non-glycosylated forms. A proportion of SH$_g$ is modified subsequently by the post-translational addition of polylactosaminoglycan to the N-linked carbohydrate to yield a heterodisperse species (SH$_p$) 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 SH$_0$, SH$_g$ and SH$_p$, 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). SH$_0$ and SH$_g$ 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 antiserum specific to a synthetic peptide, SH-CT, that represents the translational initiation at the first and second methionyl codons, respectively, in the translational open reading frame, and a 13K to 15K species (SH$_g$) which is an N-glycosylated form of SH$_0$ (Olmsted & Collins, 1989; P. L. Collins & G. Mottet, unpublished). Interestingly, therefore, SH protein accumulates in...
Fig. 1. Mapping the extracellular domain of the SH protein by trypsin treatment of intact RSV-infected cells. RSV-infected or mock-infected cells were labelled with [35S]methionine for 1 h, incubated in non-radioactive medium for 1 h, and treated with trypsin. The cells were pelleted, solubilized and analysed by immunoprecipitation with antibodies specific to the amino or carboxy terminus of the SH protein, followed by SDS-PAGE. Marker lanes contained total proteins from (lane 1) mock-infected or (lane 2), RSV-infected cells without trypsin treatment. Other lanes represent RSV-infected (3 to 8) or uninfected cells (9 and 10), with the concentration of trypsin and the antiserum used for immunoprecipitation as follows: (3 and 9) no trypsin, anti-SH-CT antiserum; (4) 100 µg/ml trypsin, anti-SH-CT; (5) 500 µg/ml trypsin, anti-SH-CT; (6 and 10) no trypsin, anti-SH-NT; (7) 100 µg/ml trypsin, anti-SH-NT; (8) 500 µg/ml trypsin, anti-SH-NT. The major, 5-8K digestion product, reactive only with antisera to SH-NT, is indicated with a filled arrowhead.
Membrane-embedded, were relatively well conserved. This latter domain was shown to be extracellular. Amino acids 23 to 41, which 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 antiserum specific to peptide SH-CT and 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 Mₚ 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...
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

Fig. 3. The SH protein forms a homooligomer that assembles rapidly, contains all forms of the SH protein, and cosegregates 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 antiserum 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. 3 d compared with Fig. 3 c) showed that the 280K F protein tetramer (Collins & Mottet, 1991) cosedimented approximately with the SH oligomer. This indicates that the $M_\text{r}$ 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 $M_\text{r}$ of this minor band corresponds to approximately 19 to 24 molecules of SH protein, 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 palmitylated (Sugrue et al., 1990; Veit et al., 1991), but the SH protein was not detectably labelled with $[^{14}$C$]$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 1 h chase in the presence of excess unlabelled methionine. One culture received CCCP immediately following the pulse and throughout the chase. Other cultures received monensin or BFA 45 min prior to labelling and again maintained throughout the labelling and chase. The generation of SHp from SHg by the addition of polylactosamine was blocked by CCCP, monensin or BFA (Fig. 4), indicating that this is a late event, occurring in or beyond the trans-Golgi compartment.
It was interesting that all forms of the SH protein, SH₀, SH₁, SH₂ and SH₃p, 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 SH₃p by lectin chromatography specific for poly lactosaminoglycan resulted in the copurification of SH₀, 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 SH₀ protein appeared almost immediately after a 5 min chase compared with approximately 10 min for SH₂ and 20 to 30 min for SH₃p, and SH₁ has never been detected by surface immunoprecipitation (Olmsted & Collins, 1989; P. L. Collins & G. Mottet, unpublished). Only SH₀ and SH₃p 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.

We thank Ena Camargo and Myron Hill for technical assistance to P. L. Collins, and Drs Robert M. Chanock and Brian R. Murphy for their support and for reviewing the manuscript.

References


