The small hydrophobic (SH) protein accumulates within lipid-raft structures of the Golgi complex during respiratory syncytial virus infection

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Received 30 October 2003
Accepted 19 January 2004

The cellular distribution of the small hydrophobic (SH) protein in respiratory syncytial virus (RSV)-infected cells was examined. Although the SH protein was distributed throughout the cytoplasm, it appeared to accumulate in the Golgi complex within membrane structures that were enriched in the raft lipid, GM1. The ability of the SH protein to interact with lipid-raft membranes was further confirmed by examining its detergent-solubility properties in Triton X-100 at 4°C. This analysis showed that a large proportion of the SH protein exhibited detergent-solubility characteristics that were consistent with an association with lipid-raft membranes. Analysis of virus-infected cells by immuno-transmission electron microscopy revealed SH protein clusters on the cell surface, but only very low levels of the protein appeared to be associated with mature virus filaments and inclusion bodies. These data suggest that during virus infection, the compartments in the secretory pathway, such as the endoplasmic reticulum (ER) and Golgi complex, are major sites of accumulation of the SH protein. Furthermore, although a significant amount of this protein interacts with lipid-raft membranes within the Golgi complex, its presence within mature virus filaments is minimal.

INTRODUCTION

Respiratory syncytial virus (RSV) is the major cause of lower respiratory tract disease in young children. Although RSV causes severe bronchiolitis in infants, milder respiratory tract infections are generally observed in most adults. However, in certain high-risk groups within the adult population (e.g. the elderly and immunocompromised), disease progression can lead to more severe complications and currently no effective vaccine exists to protect such individuals within these high-risk groups from RSV infection.

The formation of the RSV envelope occurs within lipid-raft platforms on the surface of virus-infected cells (Brown et al., 2002b; McCurdy & Graham, 2003; Jeffree et al., 2003) and the mature virus can be visualized as long filamentous structures on the cell surface (Parry et al., 1979; Roberts et al., 1995; Brown et al., 2002a). The virus encodes three membrane-bound glycoproteins, namely the fusion (F), attachment (G) and small hydrophobic (SH) proteins. The F protein mediates fusion of the virus and cell membranes, and the G protein is involved in virus attachment. The biological properties of the F and G glycoproteins and the role that they play during virus replication are relatively well understood, but the functional significance of the SH protein during virus replication remains unclear.

The amino acid sequence of the SH protein is highly conserved among all RSV A subtypes (Chen et al., 2000) and the SH protein of the RSV A2 strain is expressed as several different forms in virus-infected cells (Olmsted & Collins, 1989; Collins et al., 1990; Anderson et al., 1992). In the human RSV A2 subtype, the full-length translated SH protein contains 65 amino acids and appears in a variety of forms depending upon its glycosylation status. These are a 7.5 kDa non-glycosylated form (SH0), a 13–15 kDa N-linked glycosylated form (SHg) and a polylactosaminoglycan-modified form of the protein (SHp) that ranges in size from 21–30 kDa. Evidence suggests that SH0 is first modified by N-linked glycosylation to SHg, which is subsequently transformed into SHp by the addition of polylactosaminoglycan. In addition, a fourth form of the SH protein can be detected in which initiation of translation occurs at an alternative methionine, giving rise to a 4-6 kDa truncated form of the non-glycosylated protein (SHt).

The role played by the SH protein during RSV replication is at present unclear. Studies which have employed reverse genetics to produce viruses in which the SH gene has been deleted, appear to suggest that it is dispensable for virus growth but may play a role in evading the host’s immune system (Bukreyev et al., 1997). In contrast, several publications have indicated that this protein may play an ancillary...
role in virus-mediated cell fusion (Heminway et al., 1994; Perez et al., 1997; Techaprornkul et al., 2001) and in this regard, the ability of the SH protein to interact with the F protein in virus-infected cells has been demonstrated (Feldman et al., 2001). A greater understanding of its biological properties should aid our understanding of the role played by the SH protein during virus replication. This report examines the cellular distribution of the SH protein in virus-infected cells. The results provide evidence for an association of the SH protein with lipid-raft membrane structures, although only low levels of the protein are associated with the envelope of mature virus filaments. Furthermore, the Golgi complex appears to be a major site of SH protein accumulation within the cell.

METHODS

Cells and viruses. The RSV A2 strain was used throughout this study. Vero C1008 cells were purchased from the European Cell and Culture Collection and maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% foetal calf serum (FCS) and antibiotics.

Antibodies. The SH protein monoclonal antibody (MABSH) was prepared as follows. A branched peptide (SHH2-64) corresponding to the C-terminal 13 amino acids (NKTFELPRARVNT) of the RSV A2 strain (Collins & Wertz, 1985) was synthesized on a tetravalent (Lys)2-Lys-β-Ala-Wang resin (Novabiochem) using standard 9-fluorenylmethoxy carbonyl solid-phase chemistry and an Advanced ChemTech 348 (automated peptide synthesizer). The resulting peptide, SHH2-64, was coupled to a mouse T-cell epitope from sperm whale myoglobin (NKALEFLRDIAAKYKE) using standard procedures. This peptide was used to immunize BALB/c mice and monoclonal antibodies were prepared using standard protocols. The tissue culture medium was harvested from MABSH-expressing hybridoma cells, concentrated and filtered through a 0.2-μm filter prior to use.

The F protein monoclonal antibody (MAB169) was prepared from recombinant F protein expressed in E. coli (H. Rixon, S. Graham and R. Sugrue, unpublished data). MAB19 and anti-M were gifts from Geraldine Taylor (IAH, Compton, UK) and Paul Yeo (MRC Virology Unit, Glasgow, UK), respectively. The Golgi-specific marker, GM130, was provided by Martin Lowe (School of Biological Sciences, University of Manchester, UK), the Calnexin antibody was purchased was provided by Martin Lowe (School of Biological Sciences, University of Manchester, UK), and the Calnexin antibody was purchased from Stressgen and cholera toxin B subunit conjugated to FITC (CTX-B–FITC) was purchased from Sigma.

Radioimmunoprecipitation (RIP). The radio labelling and RIP procedures were performed as previously described (Rixon et al., 2002). Briefly, mock- and RSV-infected Vero cells were incubated at 33°C for 18 h, after which the DMEM plus 2% FCS was replaced with DMEM minus either methionine or glucose and the cells were incubated for a further 18 h in the presence of either 100 μCi (3.7 MBq) [35S]methionine ml⁻¹ or 150 μCi (5.5 MBq) [3H]glucosamine ml⁻¹, respectively. Immunoprecipitated proteins were separated by using 15% SDS-PAGE and the [35S]methionine-labelled proteins were detected by using a Bio-Rad personal FX phosphor imager while the [3H]glucosamine-labelled proteins were detected by fluorography.

Immunofluorescence. Cells were seeded on 13 mm glass coverslips and incubated overnight at 37°C prior to infection with RSV. Mock- or RSV-infected cells were fixed with 3% paraformaldehyde for 30 min at 4°C. The fixative was removed and the cells were washed once with PBS + 1 mM glycine and then four times with PBS. The cells were incubated at 25°C for 1 h with primary antibody after which they were washed and incubated for a further 1 h either with anti-mouse or anti-rabbit IgG (whole molecule) conjugated either to FITC, TRITC or Cy5 (1/100 dilution). The stained cells were mounted on slides using Citifluor and visualized in a Zeiss Axioplan 2 confocal microscope. The images were processed using LSM 510 v2.01 software.

Flotation gradient analysis of detergent-insoluble lipid-raft membranes. Cell sheets were washed twice with PBSA (20 mM sodium phosphate, 150 mM NaCl pH 7.2) and then twice with TM buffer (10 mM Tris/HCl pH 7.4; 1 mM MgCl₂). The cells (1 × 10⁸) were scraped into TM buffer supplemented with an EDTA-free protease-inhibitor mixture (Roche Molecular Biochemicals) and Dounce homogenized (80 strokes). Unbroken cells and nuclei were removed by centrifugation at 1000 g for 5 min. The 1000 g supernatant was centrifuged at 45000 g for 15 min to pellet the cell membranes, which were then washed in TM buffer. The membranes were resuspended at 4°C in PBSA containing 1% Triton X-100 by using a Dounce homogenizer, after which they were incubated for a further 1-5 h at 4°C. The homogenate was added to an equal volume of 80% (w/v) sucrose in 10 mM Tris/HCl, pH 7.4; 150 mM NaCl; 1 mM EDTA (TNE). The solubilized membranes (in 40% sucrose) were placed at the bottom of a centrifuge tube and overlaid successively with 7 ml 35% sucrose and 2 ml 5% sucrose (in TNE). After centrifugation at 210 000 g for 18 h, the gradient was fractionated and the individual fractions were analysed by Western blotting for the presence of specific proteins using appropriate antibodies.

Western blotting. Protein samples were separated using SDS-PAGE and were transferred by Western blotting onto a PVDF membrane. After transfer, membranes were washed with PBSA and blocked for 18 h at 4°C in PBSA containing 1% Marval and 0.05% Tween 20. They were then washed twice in PBSA, prior to incubation with the appropriate primary antibody for 1 h, washed four times in PBSA containing 0.05% Tween 20 and probed either with goat anti-mouse or anti-rabbit IgG (whole molecule) peroxidase conjugate (Sigma). The protein bands were visualized by using the ECL protein detection system (Amersham). Apparent molecular masses were estimated using Rainbow protein markers (Amersham) in the molecular mass range 14 3–220 kDa.

Immuo-transmission electron microscopy (I-TEM). Cell monolayers were pelleted in BEEM capsules and fixed for 5 h at 4°C with 0.5% glutaraldehyde in PBS after which the fixed pellet was extensively washed with PBS and dehydrated by transfer through a gradient of 30, 50, 70, 90 and 100% ethanol. The cell pellet was subsequently infiltrated with Unicryl (TAAB Laboratories) and the resin polymerized by UV irradiation at −15°C. Ultrathin sections were placed on nickel-coated grids and incubated either with MABSH (1/50 dilution), or anti-M (1/100 dilution) for 5 h at 25°C. The grids were washed with PBS and incubated for 2 h either with anti-rabbit or anti-mouse IgG (whole molecule) 5 or 10 nm colloidal gold conjugate (Sigma) or anti-rabbit IgG (whole molecule) 20 nm colloidal gold conjugate (British BioCell) as appropriate. They were then washed in PBS and fixed in osmium tetroxide vapour for 2 h prior to staining with uranyl acetate (saturated in 50:50 ethanol/ water), washing in distilled water and counter-staining with lead citrate.

RESULTS AND DISCUSSION

The specificity of MABSH in RSV-infected Vero C1008 cells

The data presented in this report were obtained with the Vero C1008 cell line and the SH protein was detected in
RSV-infected cells by using the monoclonal antibody MAbSH (see Methods). This antibody was raised against a peptide whose sequence is present within the C terminus of the SH protein and which represents a part of the protein that is orientated on the extracellular side of the cell membrane (Collins & Mottet, 1993).

The specificity of MAbSH for the SH protein was confirmed by Western blotting analysis (Fig. 1A). Probing RSV-infected cell lysates with MAbSH revealed the presence of a major protein band of approximately 10 kDa and a minor band of approximately 5 kDa. The protein profiles and sizes of the products detected are similar to the SH protein species that have been previously described (Olmsted & Collins, 1989; Collins et al., 1990; Anderson et al., 1992). The size of the 10 kDa species is consistent with it being the non-glycosylated form of the SH protein (SH0) and the 5 kDa species is similar to the expected size of the truncated form of the SH protein (SHt). No protein bands corresponding to SHg or SHp were detected by Western blotting, confirming previous findings that these glycosylated forms represent minor amounts of the total expressed SH protein (Olmsted & Collins, 1989; Anderson et al., 1992). As expected, no protein bands were detected in mock-infected cells by Western blotting, thus demonstrating the specificity of MAbSH.

Although no significant SHg or SHp was detected by Western blotting, low levels of these proteins could be detected by immunoprecipitation of the SH protein from lysates prepared from RSV-infected cells. In RSV-infected cells radiolabelled with $^{35}$S methionine (Fig. 1B), only SH0

![Fig. 1. Specificity of MAbSH and MAb169 in RSV-infected cells.](http://vir.sgmjournals.org)
could be detected clearly, whereas in RSV-infected cells radiolabelled with $^3$H]glucosamine (Fig. 1C), low levels of a 15 kDa labelled protein band together with diffusely labelled protein species between 20 and 30 kDa could be detected. Similar protein species have been previously detected (Olmsted & Collins, 1989; Collins et al., 1990; Anderson et al., 1992) and these presumably represent SHg and SHp, respectively. This therefore shows that while SHO is the major form of the SH protein detected in RSV-infected cells, MAbSH is also able to recognize the glycosylated forms of the SH protein. In this report, the SHO form will be referred to as the SH protein unless otherwise stated.

In this study, two monoclonal antibodies were used to detect the RSV F protein, namely MAb19, whose use has been previously described (Taylor et al., 1992; Brown et al., 2002a, b), and MAb169 (see Methods). Analysis of virus-infected cell lysates by Western blotting with MAb169 showed a single protein band whose size is consistent with that of the F1 subunit of the F protein (Fig. 1D). No protein bands were detected in lysates prepared from mock-infected cells, thus demonstrating the specificity of this reagent.

**The SH protein interacts with lipid-raft membrane structures**

Although several publications have shown that the SH protein is an integral membrane protein (Olmsted & Collins, 1989; Collins & Mottet, 1993), there is little information about how the protein is distributed within the virus-infected cell. Mock- and virus-infected cells were labelled with MAbSH and analysed by fluorescence microscopy (Fig. 2A). No significant staining was observed in mock-infected cells whereas in virus-infected cells, MAbSH exhibited a diffuse staining pattern across the cell, the staining appeared to concentrate in the perinuclear region. This observation, together with the known association of the SH protein with cellular membranes (Olmsted & Collins, 1989), suggested that the SH protein may accumulate at specific locations in the secretory pathway. Therefore, infected cells were examined by fluorescence microscopy after double-labelling with MAbSH and cellular markers specific for the endoplasmic reticulum (ER) and Golgi complex. Cells stained with GM130 exhibited a perinuclear pattern of labelling that was characteristic for Golgi staining (Fig. 2B). Although the SH protein partly co-localized with ER markers such as Calnexin (Fig. 2C), the co-localization between the SH protein and the Golgi marker GM130, was much stronger. The strong level of co-localization between GM130 and the SH protein (Fig. 2B, yellow staining pattern) suggested that despite being detected in the cytoplasm, a large proportion of the total expressed SH protein was located in the early compartments of the secretory pathway and in particular the Golgi compartment.

The F protein, like the SH protein, is transported through the secretory pathway of the cell. Therefore, the double-labelling pattern of the F protein and GM130 (Fig. 2D, plate A) was compared with that of the SH protein and GM130 shown in Fig. 2(B). In contrast to the strong co-localization observed between the SH protein and GM130, the F protein showed very little co-localization with GM130 (Fig. 2D, plate A), which is consistent with its rapid transit through the secretory pathway (Collins & Mottet, 1991). In addition, no significant co-localization of the M protein, a major structural protein of the mature virus, with the Golgi was detected, confirming previous observations (Henderson et al., 2002). This, therefore, suggests that co-localization of the SH protein with the Golgi compartment is not just a consequence of its transport through the Golgi complex, but reflects more an accumulation of the SH protein at this site in the cell.

Recent evidence has suggested that RSV assembly occurs within lipid-raft structures on the cell surface (Brown et al., 2002a, b; McCurdy & Graham, 2003; Jeffree et al., 2003) and several reports have demonstrated that specific proteins are sorted into lipid-raft domains in the Golgi network (reviewed by van Meer & Simons, 1988; Brown & London, 1998; Ikonen, 2001). It was therefore interesting to determine if the SH protein was able to interact with lipid rafts, since very little of the protein was detected in structures which are associated with mature virus (see below). In this experiment, the cholera toxin B subunit (CTX-B) was used to detect the presence of the raft lipid, GM1, within the cell. CTX-B binds to GM1 at the cell surface and is then transported via endocytosis to the Golgi apparatus (Lencer et al., 1992, 1995, 1999; Le & Nabi, 2003). Exposure of cells to CTX-B at 4°C allows the detection of cell surface GM1 since at this temperature CTX-B is not internalised. At higher temperatures (e.g. between 25°C and 37°C), the GM1-bound CTX-B is internalized and transported to the Golgi complex. In this way, CTX-B-conjugated to FITC (CTX-B–FITC) can be used to locate GM1-enriched micro-domains at both the cell surface and within the Golgi compartment depending upon the temperature at which the cells are exposed to CTX-B. Virus-infected cells were exposed to CTX-B–FITC under normal culturing conditions (i.e. 33°C), which allowed internalization and uptake of CTX-B–FITC, after which the cells were fixed and labelled with MAbSH and GM130 and visualized using a three-colour analysis that has been previously described (Henderson et al., 2002). In this way, the distribution of

**Fig. 2.** Distribution of the SH protein in virus-infected Vero cells examined by fluorescence microscopy. (A) Mock- and virus-infected cells were labelled with MAbSH. (B and C) RSV-infected cells were labelled with MAbSH (green) and (B) Golgi marker, GM130 (red) or (C) ER marker, Calnexin (red). (D) Distribution of GM130 (red) in relation to the F (plate A) and the M (plate B) proteins (green) in virus-infected cells showing the merged images. In all cases, co-localization of antigens is shown by the yellow staining pattern in the merged images.
GM1, the Golgi compartment and the SH protein was compared in the same cell. This revealed that GM1 (green), GM130 (blue) and the SH protein (red) co-localized strongly in the perinuclear region of the cell (Fig. 3A, indicated by white staining pattern in the merged image), providing evidence that the SH protein is located within regions of the Golgi compartment that contain the raft lipid GM1. The presence of the SH protein within GM1-rich regions of the cell can be seen clearly by comparing the SH protein and GM1 distribution within the cell, revealing a significant level of co-localization between CTX-B–FITC and MAbSH (Fig. 3B, indicated by yellow staining in the merged image). However, no significant co-localization of the SH protein with GM1 was detected at the surface of virus-infected cells following exposure of the cells to CTX-B–FITC at 4 °C (data not shown).

The ability of the SH protein to associate with lipid-raft structures was investigated by examining its detergent solubility. Although proteins associated with lipid-raft membranes can be solubilized with detergents such as Nonidet P 40 (NP40) and octyl β-glucoside (O-β-G), they are resistant to solubilization when treated with other detergents such as Triton X-100 or Brij 58 at 4 °C (Brown &

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**Fig. 3.** SH protein is located in GM1-enriched microdomains within the Golgi complex. Virus-infected cells were incubated at 33 °C with CTX-B–FITC and processed for fluorescence microscopy using procedures previously described (Brown et al., 2002b). The treated cells were then labelled with MAbSH and anti-GM130. (A) Distribution of the SH protein (red), GM1 (green) and the Golgi marker GM130 (blue) using a three-colour analysis routine. In the merged image, co-localization of all three colours is indicated by the white staining pattern. (B) Cellular distribution of the SH protein (red) and GM1 (green) is shown and co-localization is indicated by the yellow staining pattern in the merged image.
London, 1998). In this way, the detergent solubility of the SH protein was compared with those of three other proteins which are known to reside in lipid rafts, namely caveolin-1 (cav-1), flotillin-1 (flot-1) and the RSV F glycoprotein. Infected cell monolayers were extracted with the respective detergent and separated by centrifugation into a detergent-soluble (S) and an insoluble cell pellet (P) fraction. Each fraction was examined for the presence of the F protein (indicated by the F1 subunit), cav-1 and the SH protein by Western blotting (Fig. 4A). In lysates prepared with Triton X-100 and Brij 58, both the F protein and cav-1 were found to be in the insoluble pellet fraction, whereas in lysates prepared with NP40/O-β-G, these proteins were located almost entirely in the soluble fraction. Similarly, the SH protein was soluble in NP40/O-β-G, but although this protein remained largely associated with the pellet fraction following Triton X-100 extraction, a significant proportion of the SH protein was soluble in Triton X-100. This suggests that, in contrast to the F protein, flot-1 and cav-1, a population of the SH protein exists which is distributed in non-raft membranes. However, these results indicate that significant levels of the SH protein appear to show detergent-solubility characteristics which are consistent with it being able to associate with raft membranes.

The detergent-solubility characteristics of the SH protein were further examined by flotation gradient analysis. Total membranes, prepared from virus-infected cells, were extracted with Triton X-100 at 4 °C and placed at the bottom of a step gradient containing 35% and 5% sucrose.
infected cells had been extracted with NP40/O-flotation gradients in which cell membranes from virus-5–35 % sucrose interface (Fig. 4C). In contrast, analysis of cellular proteins, cav-1 and flot-1, were also detected at the density (fractions 3–5). The endogenous raft-associated lipid-raft membranes migrate due to their higher buoyant close to, the 5–35 % sucrose interface proportion of each protein was found in the fractions at, or presumably as a result of protein aggregation, a significant protein was detected in the pellet fraction (fraction 11), presence of the SH and F proteins. Although some of each fractionated and examined by Western blotting for the (see Methods). After centrifugation, the gradient was restricted entirely to the lower fractions (fractions 10 and 11) [Fig. 4B, (c)], which is consistent with its solubilization in this detergent. Although the F and SH proteins migrated into the region of the flotation gradient at which raft membranes were present, a proportion of the SH protein could also be detected in the Triton-X-100-soluble fraction (fractions 9 and 10). This supports the above suggestion that a proportion of the SH protein resides in non-raft membrane systems. However, the fluorescent microscopy data and detergent-solubility characteristics of the SH protein suggest that a significant proportion accumulates within lipid-raft membranes of the Golgi complex.

Low levels of the SH protein are incorporated into the virus envelope

It is currently unclear to what extent the SH protein interacts with mature RSV filaments. The cellular distribution of the SH protein in infected cells was therefore examined using two complementary techniques, fluorescence microscopy and immuno-transmission electron microscopy (I-TEM).

Previously, fluorescent staining of RSV filaments had been observed following labelling of infected cells with antibodies against the F and G glycoproteins (Roberts et al., 1995; Brown et al., 2002a; Jeffree et al., 2003) and the M protein (Henderson et al., 2002). However, in the fluorescence microscopic analysis described above (Fig. 2), no obvious staining of the virus filaments with MAbSH was observed. The absence of detectable SH protein in virus filaments studied by confocal microscopy was confirmed by comparing the surface-labelling pattern of the SH and F proteins (Fig. 5A, plates A and B). Non-permeabilized virus-infected cells were labelled either with MAbSH or MAb19 and the labelling pattern visualized by confocal microscopy. Low-level surface-labelling for the SH protein was observed, which appeared to be punctate in appearance rather than filamentous. This contrasted with the surface-labelling of cells stained with MAb19 which revealed the presence of clearly defined virus filaments.

The labelling pattern of the M protein, a major virus structural protein, was compared with that of the SH protein in permeabilized virus-infected cells using confocal microscopy. In this analysis, a Z-stack gallery of images from the same cells at different focal planes was obtained, thus allowing a comparison of the internal and surface-labelling patterns (Fig. 5B, plates A–C). As expected, anti-M efficiently labelled both the virus filaments and the inclusion bodies (IB). The latter structures contain a large amount of the N and P proteins in addition to the M protein (Garcia et al., 1993; Garcia-Barreno et al., 1996; Ghildyal et al., 2002). In contrast, no significant labelling of the filaments was observed with MAbSH but a low level of SH protein staining could be detected within IBs, observed as a weak co-localization between anti-M and MAbSH (Fig. 5B, visualized as speckled yellow staining within the IB structures).

The fluorescence microscopy data suggested that the SH protein was not present within mature virus filaments. However, a major limitation of light microscopy is its lower resolution compared with that of electron microscopy and thus it seemed possible that fluorescence microscopy may not detect low levels of virus-associated SH protein. Previous studies which employed an indirect biochemical analysis suggested that low levels of the SH0 and SHp forms may be present within purified RSV particles (Collins et al., 1990; Anderson et al., 1992). However, a major problem in interpreting the results with this type of approach is that RSV-infectivity remains largely cell-associated (Roberts et al., 1995) and purification of infectious RSV is a difficult process. It is possible that low levels of contaminating virus proteins may also co-purify with the RSV particles, which is particularly relevant since the failure to detect the SH protein in virus filaments by fluorescence microscopy suggested that if it was present in mature virus filaments, it was only present at low levels. To overcome these two potential problems, immuno-transmission electron microscopy (I-TEM) was employed to analyse RSV-infected cells in situ. In this study, infected cells were processed for I-TEM as previously described (Brown et al., 2002a) and the SH protein was detected using MAbSH. In the I-TEM images, the mature virus filaments were visible as were the virus inclusion bodies (Fig. 6A and B). The thin sections prepared from virus-infected cells were labelled with MAbSH and the presence of bound antigen was detected using 5 nm colloidal gold-labelled second antibody. Although it was possible to visualize the SH protein in virus filaments, only between one and four gold particles per filament were detected, despite extensive examination of the samples. The significance of this low level of gold signal within the virus filaments is unclear. However, it is unlikely to be due to non-specific binding of the gold conjugate since gold label was not observed in sections in which either MAbSH was omitted or MAbSH was replaced with a non-specific mouse IgG (Fig. 6C and D).

The SH protein within IBs appeared to be more readily detected than in virus filaments. This is presumably a reflection of the larger surface area of the IBs within the thin sections compared with that of the virus filaments,
which allows easier visualization of the SH protein. In addition, the I-TEM data revealed regions on the infected-cell surface, in the absence of virus filaments, that exhibited a relatively higher level of SH protein labelling (Fig. 6B). In many instances, this was visualized as small clusters of gold particles, suggesting that the SH protein is concentrated at specific regions of the plasma membrane. This is consistent with the data which show surface expression of the SH protein (Fig. 5A, plate A) and with previous reports in which expression of the SH protein on the surface of virus-infected cells has been observed (Olmsted & Collins, 1989).

I-TEM was used to examine and compare the relative distribution of the SH and M proteins. Although such a comparison may not provide an accurate quantitative measure of SH and M protein levels, it was reasoned that the levels of each protein in the virus-induced structures, i.e. virus filaments and IBs, could be compared qualitatively. The relative distribution of the M and SH proteins within virus filaments was examined in sections labelled with both anti-M and MAbSH, and the presence of the bound antibody detected using 5 and 10 nm colloidal gold-conjugated second antibodies, respectively (Fig. 7A and B). An abundance of labelling with anti-M was identified by the relatively large amount of 5 nm gold probe (highlighted by white arrow) associated with mature virus filaments and newly budded virus. In contrast, very little 10 nm gold probe (highlighted by black arrow) appeared to be
associated either with the mature virus filaments or with newly budding virus.

A similar analysis to that above was performed to examine the relative amounts of each protein in the virus IBs. In this case, the presence of the SH protein was detected using a gold probe of larger size (20 nm) (Fig. 7C). It is clear that while there is an abundance of the 5 nm gold probe (white arrow, representing the M protein), there are many fewer 20 nm gold particles (black arrow, representing the SH protein), indicating that the SH protein is present in IBs but in low amounts. The presence of the SH protein, an integral membrane protein, suggests that host-cell derived membranes may be associated with IBs. It should be noted that the results of the labelling experiment were consistent irrespective of the size of the gold probe used for detection since this analysis has been performed extensively using a variety of gold conjugate sizes (between 5 and 20 nm) to detect each of the two proteins.

Although direct evidence has been provided that a low level of the SH protein is present within mature RSV filaments, our results show that raft membranes within the Golgi complex are a major site of SH protein accumulation.

**Fig. 6.** SH protein is detected in mature virus filaments (VF) and inclusion bodies (IB) by I-TEM, but at low levels. (A and B). Virus-infected cells were prepared for I-TEM (see Methods), labelled with MAbSH, and the presence of bound antibody detected using 5 nm colloidal gold (indicated by black arrows). (B) The presence of SH protein clusters can be seen at the cell surface in the absence of visible virus structures (highlighted by *). (C and D) Representative images from sections, showing both VF and IB, incubated in the presence of secondary antibody conjugated to 5 nm colloidal gold only. Bar, 200 nm.
during virus infection. Although the function of the SH protein has not been defined, it is possible that it may modify some property of the Golgi complex to aid virus protein transport through the secretory pathway. In this respect, it is interesting to note that several small membrane proteins encoded by other viruses are able to alter the protein transport machinery of the host-cell, a list that includes the M2 and BM2 proteins of influenza virus. The M2 protein of influenza A virus modifies the acidity of the Golgi compartment (Sugrue et al., 1990; Ciampor et al., 1992), a process that has been shown, in polarized cells, to decrease the rate of transport of glycoproteins to the apical cell surface (Sakaguchi et al., 1996; Henkel & Weisz, 1998; Henkel et al., 1998, 2000). More recent work has demonstrated that the BM2 protein of influenza B virus is able to elicit similar effects to those observed for the M2 protein (Mould et al., 2003). However, at present, the effect exerted by the SH protein on the physiology of the host cell, and in particular the secretory pathway (e.g. the Golgi complex), remains to be established as does its functional significance within the virus.

ACKNOWLEDGEMENTS

We thank Duncan McGeoch for critical review of the manuscript. We are grateful to Geraldine Taylor at the Institute of Animal Health, Compton, UK, for providing MAb19, Paul Yeo for providing anti-M and Martin Lowe from the University of Manchester for providing anti-GM130.

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