The respiratory syncytial virus small hydrophobic protein is phosphorylated via a mitogen-activated protein kinase p38-dependent tyrosine kinase activity during virus infection

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INTRODUCTION

Respiratory syncytial virus (RSV) encodes three integral membrane proteins, 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, whereas the functional significance of the SH protein during virus replication remains unclear. 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) 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), which ranges in size from 21 to 30 kDa. A fourth form of the SH protein can also 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). Of these different forms, SH0 is by far the most abundant protein detected during virus infection (Olmsted & Collins, 1989; Anderson et al., 1992).

During RSV infection, the bulk of the expressed SH protein remains cell-associated and a large proportion of the protein appears to localize in the early compartments of the secretory pathway, such as the endoplasmic reticulum (ER) and Golgi complex (Olmsted & Collins, 1989; Rixon et al., 2004). Although the SH protein is expressed on the surface of infected cells, only very low levels are detected within virions (Collins et al. 1990; Anderson et al., 1992; Rixon et al., 2004). The function of the SH protein during virus infection is currently unknown, although studies have suggested a function either in evading the host’s immune system (Bukreyev et al., 1997) or in providing an ancillary role in virus-mediated cell fusion (Heminway et al., 1994; Perez et al., 1997; Techaarpornkul et al., 2001). A greater understanding of the biochemical properties of the SH protein is a prerequisite to understanding its function during virus infection.
Several RSV proteins undergo post-translational modification leading to their glycosylation (Lambert & Pons, 1983; Gruber & Levine, 1985), acylation (Arumugham et al., 1989) and phosphorylation (Lambert et al., 1988). In the case of the SH protein, glycosylation is the only modification that has been proved experimentally (Olmsted & Collins, 1989; Collins et al., 1990; Anderson et al., 1992). There is no published experimental evidence that the SH protein is either acylated or phosphorylated. However, mammalian cells elicit biological responses to different stimuli, including virus infection, through the activation of the mitogen-activated protein kinase (MAPK) signalling pathways. These pathways both induce the expression of specific cellular proteins and lead to the activation of specific host proteins, including kinases, by phosphorylation. The best-characterized of these are the extracellular signal-regulated kinase (ERK) and the p38 kinase signalling pathways (reviewed by Roux & Blenis, 2004). Several host-cell kinases are activated during RSV infection, through the activation of the mitogen-activated protein kinase (MAPK) signalling pathways.

Methods and viruses. The RSV A2 strain was used throughout this study. The Vero C1008 cell line was purchased from the European Collection and maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum (FCS) and antibiotics.

Antibodies and inhibitors. The SH protein monoclonal antibody (mAbSH) was prepared as described previously (Rixon et al., 2004); anti-RSV antibody (RCL-3) was purchased from Novacastra and mAb30 was a gift from Geraldine Taylor (IAH, Compton, UK). The Golgi-specific marker GM130 was provided by Martin Lowe (School of Biological Sciences, University of Manchester, UK). The antibodies PY20, anti-phospho-p38 and anti-phospho-ERK1/2 were purchased from New England Biolabs. The kinase inhibitors SB203580, PD98059 and genistein were purchased from Calbiochem (School of Biological Sciences, University of Manchester, UK). The SH protein monoclonal antibody was detected by phosphorimager analysis and compared with the position of phosphorylase b (97.5 kDa) and myosin (220 kDa).

Western blotting. Protein samples were separated by SDS-PAGE and transferred by Western blotting on to a PVDF membrane. After transfer, the membrane was washed with Tris-buffered saline containing 0.05% Tween 20 (TBS/Tween) and blocked for 18 h at 4°C in TBS/Tween containing 1% BSA. It was then washed twice in TBS/Tween prior to incubation with the primary antibody for 60 min. The membrane was washed four times in TBS/Tween and probed using either goat anti-mouse or anti-rabbit IgG (whole molecule) conjugated to peroxidase (Sigma) as appropriate. Protein bands were visualized using the ECL protein detection system (Amersham). Apparent molecular masses were estimated using Rainbow molecular mass markers in the range 14–220 kDa (Amersham).

Phosphoamino acid (PAA) analysis. The SH protein was isolated from 35Porthophosphate-labelled cells by RIP using mAbSH and transferred by Western blotting on to PVDF membranes. The labelled SH protein band was visualized by phosphorimaging and excised from the membrane. Amino acids were hydrolysed from the membrane slice by incubating it in 6 M HCl and heating at 110°C for 90 min. The released amino acids were dried under vacuum and then resuspended in pyridine acetate, pH 3.5. Each sample was spotted on to a cellulose-coated thin layer chromatography (TLC) plate (Merck) and electrophoresed at 1000 V for 75 min in a Multiphor II electrophoresis tank (Amersham Biosciences), with pyridine acetate (pH 3.5) as the solvent. The TLC plate was air dried and the positions of the separated PAs were visualized by incubating the plate for 1 min in 0.2% (w/v) ninhydrin in acetone and air drying for a further 10 min. The positions of the 35P-labelled PAs was detected by phosphorimager analysis and compared with the position of phosphothreonine, phosphoserine and phosphotyrosine standards (Sigma-Aldrich).

Immunofluorescence. RSV-infected cells were fixed with 3% paraformaldehyde for 30 min at 4°C. The fixative was removed and the cells washed once with PBS plus 1 mM glycine and four times with PBS. Cells were incubated at 25°C for 1 h with primary antibody after which they were washed and incubated for a further 1 h with either anti-mouse or anti-rabbit IgG (whole molecule) conjugated to FITC or cy5 (1:100 dilution). Stained cells were mounted on slides using Citifluor and visualized using a Zeiss Axiosplan 2 confocal microscope. The images were processed using LSM 510 v. 2.01 software.

Protein cross-linking. This was performed as described previously (Sugrue & Hay, 1991). Briefly, a stock solution of dithiobis(succinimidyl) propionate (DSP; Pierce) in DMSO (100 mM) was added to PBS, pH 8.0, to give the final concentration required. This solution was then added to the cell monolayers and incubated at 4°C for 1 h. The cross-linking solution was then removed and the reactions quenched by washing the cells extensively with PBSA plus 20 mM lysine. Cell extracts were prepared and analysed either by Western blotting or by RIP.
RESULTS AND DISCUSSION

The SH protein is modified by tyrosine phosphorylation

In this report, the SH protein monoclonal antibody mAbSH (Rixon et al., 2004) was used to detect the SH protein. Confluent Vero C1008 cell monolayers were infected with the RSV A2 strain and between 8 and 20 h p.i. the cells were pulse labelled with either $[^{35}S]$methionine or $[^{33}P]$orthophosphate. The SH protein was then isolated by RIP using mAbSH and analysed by SDS-PAGE (Fig. 1a). In lysates prepared from either $[^{35}S]$methionine- or $[^{33}P]$phosphate-labelled cells, a protein species of approximately 10 kDa was detected, which migrated similarly to SH0. Under these conditions, protein species corresponding in size to either SHg or SHp were not detected, suggesting, as noted previously, that they are minor forms of the total SH protein population expressed in virus-infected cells (Olmsted & Collins, 1989; Anderson et al., 1992). In mock-infected cells, this protein species was not detected, confirming its viral origin. These data provide the first direct evidence that the SH protein is modified by phosphorylation.

Examination of the SH protein amino acid sequence revealed the presence of several sites that had the potential to be phosphorylated (Fig. 1b). An SSK motif was located at aa 11–13; this is a consensus sequence for protein kinase C phosphorylation and therefore could potentially give rise to serine-linked phosphorylation. In addition, two tyrosine residues were located at aa 17 and 47 that could potentially be modified by tyrosine-dependent phosphorylation. However, neither of these tyrosine residues was located within the typical consensus sequence for tyrosine-dependent phosphorylation, although each residue was located within a potential tyrosine-based sorting signal sequence, YXXΦ, where X is any amino acid and Φ is any hydrophobic amino acid (reviewed by Bonifacino & Traub, 2003). Therefore, examination of the SH protein sequence suggested that this protein had the potential to be modified by serine- and/or tyrosine-dependent phosphorylation.

Two standard procedures were employed to identify the type of amino acid that was phosphorylated in the SH protein, namely PAA analysis and immunoreactivity with the phosphotyrosine-specific antibody PY20.

Identification of the phosphorylated amino acid residue present in the SH protein was carried out by a standard PAA analysis (Fig. 2a), performed as described in Methods. Migration of the $[^{33}P]$phosphate-labelled amino acid derived from the SH protein was compared with that of the PAA markers phosphoserine, phosphothreonine and phosphotyrosine (Fig. 2a). This revealed that the only labelled amino acid detected in the SH protein hydrolysedate migrated similarly to phosphotyrosine, and no evidence for the presence of either phosphoserine or phosphothreonine in this protein was obtained.

The result obtained by PAA analysis was confirmed by analysing the immunoreactivity of the SH protein with the antibody PY20 [Fig. 2b(i)]. PY20 is an established immunological reagent that is specific in its reactivity towards phosphotyrosine-modified proteins. In this analysis, lysates were prepared from mock- and virus-infected cells and the SH protein immunoprecipitated with mAbSH. Immunoprecipitates were then separated by SDS-PAGE, transferred by Western blotting on to PVDF membranes and probed with either PY20 or mAbSH. Probing with PY20 revealed the presence of a single protein species of approximately 10 kDa, similar in size to SH0. This protein band was not detected in a similar analysis using mock-infected cells. The identity of this phosphotyrosine-modified protein band was confirmed by reprobing the PY20 blot with mAbSH, which showed the PY20 band to be identical to that of the SH protein [Fig. 2b(ii)].

Further evidence for the role of tyrosine kinase activity in SH protein phosphorylation was obtained using the broad-spectrum tyrosine kinase inhibitor genistein (Fig. 2c). This reagent inhibits phosphorylation by blocking the binding of ATP to the ATP-binding site of tyrosine kinases, which is an essential step during this enzymic process. Virus-infected cells were exposed to varying concentrations of genistein between 8 and 20 h p.i., after which the SH protein was transferred on to a PVDF membrane by Western blotting and probed with PY20 as described above. A
comparison of the non-treated and gentistein-treated samples showed that the PY20 reactivity of the SH protein was significantly reduced in the presence of concentrations of genistein as low as 25 μM, whereas SH protein levels remained unchanged at these concentrations.

**The SH protein forms a phosphorylated pentameric structure that interacts with the RSV attachment protein**

Previous studies have suggested that the SH protein is able to assemble into a homo-pentameric structure (Collins & Mottet, 1993; Kochva et al., 2003). We used protein cross-linking reagents to determine whether the phosphorylated SH protein could also form similar oligomeric structures. Several different cross-linking reagents were used in this analysis, but due to the similarity of the results obtained, only the data obtained with one of these, DSP, are presented. DSP is a reversible cross-linking reagent; the intermolecular covalent bonds introduced by DSP can be removed by treatment with a reducing agent such as β-mercaptoethanol.

Virus-infected cells were mock-treated or treated with DSP and analysed directly by Western blotting using mAbSH (Fig. 3a). Under non-reducing conditions and in the presence of DSP, the appearance of at least four higher molecular mass species, in addition to SH0, of approximately 17, 24, 30 and 38 kDa, was noted. These were similar in size to the SH0 cross-linked species reported in a previous study (Collins & Mottet, 1993). Under reducing conditions, only a single protein species corresponding in size to SH0 was observed. In a further analysis, [35S]methionine- or [33P]phosphate-labelled RSV-infected cell monolayers were mock-treated or treated in situ with 0.5–2.0 mM DSP (Fig. 3b and c). The SH protein was isolated by RIP, separated by 15% SDS-PAGE and the protein profile of the SH protein from non-treated and treated cells was compared. Following DSP treatment, four higher molecular mass species were detected in both [35S]methionine- and [33P]phosphate-labelling experiments; these were similar in size to the SH products detected by Western blotting (Fig. 3a). These data were consistent with the pentameric structure that has been reported for the SH protein (Collins & Mottet, 1993; Kochva et al., 2003).

It was observed that the level of recoverable labelled SH protein was significantly higher than in the RIP from non-treated cells, as judged by comparison of the intensity of the [35S]methionine-labelled SH0 protein band in the non-treated and in the DSP-treated, reduced samples. This allowed better visualization of the different glycosylated
forms of the SH protein. Additionally, this suggested that the process of cross-linking may, in addition to stabilizing the SH protein-containing complexes, protect the SH protein from degradation during the detergent-extraction procedure. A reduction in the levels of recoverable SH protein following detergent extraction has been reported (Olmsted & Collins, 1989), which is presumably a result of its increased sensitivity to proteolytic degradation.

Under non-reducing conditions, a large amount of an additional, much higher molecular mass protein complex was apparent whose size approached 200 kDa. This material, which was not present in the non-DSP-treated samples, accounted for most of the radiolabelled protein recovered by RIP and could not be resolved, even on low-percentage polyacrylamide gels, which can resolve proteins up to 200 kDa [Fig. 3b(ii) and c(ii)]. A similar-sized product was reported in earlier studies (Collins & Mottet, 1993) but remained uncharacterized. After treatment with reducing agent, this high molecular mass protein complex disappeared and correlated with the appearance of an 80 kDa protein species together with a large amount of SH0. The 80 kDa protein was only detected in [35S]methionine-labelled samples suggesting that it was not phosphorylated, and it was not detected by Western blotting using mAbSH, suggesting that it was not the SH protein.

In a similar study, mock- and RSV-infected cells were [3H]glucosamine labelled and treated with DSP prior to analysis by SDS-PAGE (Fig. 4a). This labelling procedure enhances the visualization of the glycosylated SH protein forms, which are expressed at relatively low levels and are difficult to detect by [35S]methionine labelling. Under non-reducing conditions, a labelled smear was observed that extended from 40 kDa up to a molecular mass that exceeded 200 kDa, with the majority of the recovered labelled protein being >200 kDa. Treatment of the samples with reducing agent revealed the presence of a smear from 21 to 40 kDa, which is consistent with the molecular mass of SHp (Anderson et al., 1992), and a strongly labelled protein product of 80 kDa (Fig. 4a, filled arrowhead). This was identical in migration to the 80 kDa protein detected by

**Fig. 3.** The phosphorylated SH protein forms pentamers. (a) RSV-infected cells were not treated (NT) or treated with DSP as described in Methods. Cells were extracted in boiling mix and heated at 100 °C for 10 min in the absence or presence of 5% β-mercaptoethanol. Proteins were separated by 15% SDS-PAGE and analysed by Western blotting using mAbSH. (b, c) (i) [35S]methionine-labelled (b) or [33P]orthophosphate-labelled (c) virus-infected cells were not treated (NT) or treated with DSP at the indicated concentrations. The SH protein was isolated by RIP and examined by either non-reducing or reducing 15% SDS-PAGE. A mock-infected (M) [35S]methionine-labelled control is also shown. The different SH protein species are indicated, as are the cross-linked SH-containing complexes (filled arrowheads), the 80 kDa protein (arrow) and a 50 kDa phosphorylated cellular protein that co-precipitates with mAbSH (*). (ii) The SH protein was isolated by RIP from [35S]methionine- or [33P]orthophosphate-labelled cells that were treated with 1.0 mM DSP and examined using non-reducing 7.5% SDS-PAGE. The high molecular mass cross-linked material observed by non-reducing SDS-PAGE (open arrowhead) is indicated.
methionine labelling (Fig. 3b) and suggested that this protein was a glycoprotein. Given the size of the protein and its glycosylated nature, it appeared possible that this might be the RSV G protein, since previous studies have suggested that the SH protein is able to form a complex with the G and F proteins during virus infection (Feldman et al., 2001). Lysates were prepared from DSP-treated cells and the SH protein isolated by immunoprecipitation, separated by reducing SDS-PAGE and transferred by Western blotting on to PVDF membranes. The membranes were probed with mAb30 [Fig. 4b(i)], an anti-G protein monoclonal antibody that has been described previously (Taylor et al., 1992), followed by mAbSH [Fig. 4b(ii)]. In these samples, low levels of a protein band corresponding in size to the RSV G protein were detected. In a similar analysis using antibodies that recognize other virus structural proteins, no reactivity with the F or other viral proteins was detected (data not shown). These results showed therefore that the different glycosylated forms of the SH protein were phosphorylated and that the phosphorylated SH protein was able to oligomerize into pentamers. Our results also suggested that the phosphorylated form of the SH protein was able to interact with the virus attachment protein during virus infection. However, at present the functional significance of this interaction is not clear.

The SH protein is phosphorylated via an MAPK p38-dependent pathway

Several reports have demonstrated that cells respond to RSV infection by the induction of cytokine expression via the ERK and p38-dependent pathways (Pazdruk et al., 2002; Meusel & Imani, 2003; Kong et al., 2004; Monick et al., 2004). Mock- and virus-infected Vero C1008 cells were analysed by Western blotting using two antibodies, anti-phospho-p38 and anti-phospho-ERK1/2, which specifically recognize the phosphorylated forms of p38 and ERK1/2, respectively. This analysis showed the presence of similar levels of phosphorylated ERK1/2 and p38 in mock- and virus-infected cells (Fig. 5), suggesting that in this cell line these kinases are phosphorylated prior to infection. We were therefore interested to determine whether or not the phosphorylation of the SH protein was dependent upon either or both of these different signalling pathways, since they have been implicated in RSV infection. In this analysis, two standard MAPK inhibitors that specifically inhibit p38 kinase and ERK were used, thus allowing determination of the effect that these different pathways have on SH protein phosphorylation. SB203580 is a specific inhibitor of MAPK p38 kinase (Gallagher et al., 1997) and hence inhibits phosphorylation of p38 protein substrates. ERK1/2 is phosphorylated by MAP kinase kinase 1 (MEK1), and PD98059, which inhibits phosphorylation of MEK1 (Dudley et al., 2000), was used to inhibit pathways independent of p38. In these experiments, the levels of phosphorylated p38 and ERK were determined by Western blotting of mock- and virus-infected cells with antibodies specific for phosphorylated p38 and ERK1/2 (P-p38 and P-ERK1/2, respectively).

Fig. 4. The SH protein interacts with the virus attachment (G) protein. (a) Mock-infected (M) or virus-infected (I) cells were labelled with [35S]methionine and treated with 1 mM DSP. The SH protein was isolated by RIP and examined by either non-reducing or reducing SDS-PAGE. The location of the 200 kDa cross-linked protein species (open arrowhead) observed under non-reducing conditions and the 80 kDa protein (filled arrowhead) and protein species corresponding to SHp observed under reducing conditions are indicated. (b) Mock- or virus-infected cells were treated with 1 mM DSP and the SH protein isolated by immunoprecipitation using mAbSH. The immunoprecipitates were separated by reducing SDS-PAGE, transferred by Western blotting on to a PVDF membrane and probed with mAb30 (i) followed by mAbSH (ii). The position of the G protein is indicated. Asterisks highlight the positions of the IgG heavy and light chains.

Fig. 5. MAPK p38 and ERK1/2 kinases are phosphorylated in Vero C1008 cells. Mock-infected (M) and virus-infected (I) cells were extracted in boiling mix and heated at 100 °C for 5 min. Lysates were separated by SDS-PAGE and transferred by Western blotting on to PVDF membranes, which were then probed with an antibody against phospho-p38 (P-p38) or phospho-ERK1/2 (P-ERK1/2), as described in Methods.
et al., 1995), thus blocks phosphorylation via the ERK1/2 pathway.

Different concentrations of these inhibitors were added to RSV-infected Vero cells at 8 h p.i. and the cells were harvested at 20 h p.i. The SH protein was isolated by immunoprecipitation, transferred by Western blotting on to a PVDF membrane and probed with PY20 and mAbSH. Following the addition of SB203580 to virus-infected cells, the phosphotyrosine signal detected by Western blotting appeared to disappear in a dose-dependent manner (Fig. 6a). In contrast, the addition of PD98059 appeared to have no significant effect on the PY20 signal, which was similar to that detected in non-treated control cells (Fig. 6b). Probing these membranes with mAbSH showed similar levels of the SH protein in both treated and non-treated cells, indicating that addition of these inhibitors had no effect on the SH protein levels. Furthermore, the addition of equivalent levels of DMSO, the solvent used to solubilize the inhibitors, had no effect on SH protein detection levels in the presence of either PY20 or mAbSH. This indicated that the reduction in PY20 immunoreactivity was not due to a reduction in SH protein levels following the addition SB203580, but was due to a reduction in the levels of tyrosine phosphorylation.

In a final analysis, we examined the effect of SB203580 on the cellular distribution of the SH protein using fluorescence microscopy (Fig. 7). In non-treated cells labelled with mAbSH, the SH protein staining pattern was similar to that reported previously (Rixon et al., 2004). Although there was diffuse staining across the cell, there appeared to be a concentration of the SH protein in the perinuclear region, consistent with its localization within the ER/cis-Golgi

Fig. 6. The SH protein is phosphorylated by a MAPK p38-dependent pathway. RSV-infected cells were incubated with increasing concentrations of SB203580 or DMSO (a) or PD98059 (b). The SH protein was isolated by immunoprecipitation and following SDS-PAGE was transferred on to PVDF membranes by Western blotting. Membranes were then probed with PY20 or mAbSH. The presence of the phosphorylated SH protein (P-SH) and the SH protein is indicated. NT, immunoprecipitation assay from non-treated cells.

Fig. 7. Distribution of the SH protein in virus-infected Vero cells after treatment with 40 μM SB203580 between 8 and 20 h p.i. (a, b) Non-treated or SB203580-treated cells were labelled with mAbSH and examined by fluorescence microscopy. Regions of the cell where the SH protein accumulated are indicated by white arrows. Inset, higher magnification of single cells. (c, d) Cells were also stained with anti-RSV antibody. The virus filaments (VF) and inclusion bodies (IB) are indicated.
complex (Fig. 7a). A similar observation was noted for cells treated with PD98059 (data not shown). In contrast, in the presence of 40 μM SB203580, the SH protein staining pattern appeared to undergo a distinct change in its distribution. There appeared to be a marked increase in the SH protein staining, which showed a typical Golgi localization (Fig. 7b, white arrow). This was confirmed by comparing the distribution of the SH protein and the resident Golgi protein GM130 (Fig. 8), the latter being a commonly used Golgi complex marker. The GM130 protein is one of several resident Golgi proteins called Golgins that together form the \textit{cis}-Golgi matrix. These proteins play roles in both the maintenance of the Golgi cisternae and in the transport of cargo from the ER to the Golgi compartment (reviewed by Barr & Short, 2003; Short & Barr, 2003). Anti-GM130 antibody therefore labels the \textit{cis}-Golgi compartment of the cell. We noted that both non-treated and SB203580-treated cells labelled with anti-GM130 antibody showed a similar staining pattern in the perinuclear region of the cell (Fig. 8, asterisks). In the absence of SB203580, although the SH protein exhibited a diffuse staining across the cell, some co-localization of the SH and GM130 proteins was observed. However, in the presence of SB203580, a significant increase in the SH protein staining pattern was observed that matched, almost exactly, the distribution of the GM130 protein (Fig. 8, white arrow). This suggested an increase in the levels of the SH protein that is located in the early compartments of the secretory pathway e.g. the \textit{cis}-Golgi compartment. SB203580 treatment did not appear to have a significant effect on the distribution of other virus structures. Anti-RSV antibody, a reagent that we have used previously to examine RSV assembly (Brown \textit{et al.}, 2002), is able to recognize the N, P, F and M2-1 proteins (Wright \textit{et al.}, 1997) and can be used in confocal microscopy to visualize both virus filaments and inclusion bodies. Cells stained with anti-RSV antibody in the presence or absence of SB203580 showed both virus filaments and inclusion bodies (Fig. 7c and d), although there appeared to be a slight reduction in the density of the former in the presence of SB203580. SB203580 appeared not to have a generalized effect on virus maturation at the concentration used, suggesting that phosphorylation of the SH protein influences its distribution within the secretory pathway.

**Fig. 8.** The SH protein shows an increased localization in the Golgi complex following treatment with SB203580. Virus-infected cells, either non-treated or treated with 40 μM SB203580, were labelled with mAbSH (green) and anti-GM130 antibody (red) and examined using fluorescence microscopy. Asterisks indicate the GM130 staining pattern. In all cases, co-localization of antigens was indicated by yellow staining in the merged images. In each case the images were recorded using the same machine settings (e.g. the same laser intensity). Inset, an enlarged image highlighting the differences in co-localization between non-treated and SB203580-treated cells. Regions of the cell where the SH protein accumulated in the presence of SB203580 are highlighted by white arrows and correlated with an increase in the levels of yellow staining in the merged image.
Phosphorylation of the RSV SH protein

At present, the role played by the p38 kinase in SH protein phosphorylation is unclear since it has been shown to activate a plethora of cellular proteins by serine/threonine phosphorylation, including other cellular kinases (Roux & Blenis, 2004). Since the SH protein is tyrosine phosphorylated, our data suggest that p38 does not act directly on the SH protein, but may regulate the tyrosine kinase activity of one or more host factors. A recent observation has been reported where the tyrosine phosphorylation-dependent cleavage of angiotensin-converting enzyme (ACE) appeared to be dependent upon p38 activation (Santhamma et al., 2004). The studies on ACE cleavage show that this event occurs in the ER and it is interesting to note that the SH protein accumulates in the early compartments of the secretory pathway, such as the ER and cis-Golgi (Rixon et al., 2004). It is therefore conceivable that a similar tyrosine kinase activity is involved in SH protein phosphorylation and that this activity is located in the early compartments of the secretory pathway.

Although SH protein phosphorylation occurs via a MAPK p38-dependent pathway, RSV infection appears to be required for efficient phosphorylation of the protein. We have made several attempts to identify a phosphorylated form of the SH protein using recombinant protein expression in Vero C1008 cells. In these studies, efficient expression of the SH protein was obtained, but we failed to detect SH phosphorylation either by radiolabelling or by the use of phosphorylation-specific antibodies (H. W. McL. Rixon and R. J. Sugrue, unpublished observations). It is therefore possible that host-cell changes induced by RSV infection are required for SH protein phosphorylation.

At present we have not identified the tyrosine residue(s) that are phosphorylated within the SH protein sequence. However, examination of the RSV A2 strain SH protein sequence revealed that both tyrosine residues are located within a tyrosine-based sorting signal consensus sequence. These motifs have been shown to be involved in the interaction between specific cellular proteins and adapter protein (AP) complexes that allow their sorting into specific cellular compartments such as lysosomes (e.g. LAMP-1), the Golgi complex (e.g. TGN38 and furin) and specialized endosomal compartments such as antigen-processing compartments (e.g. HLA-DEM) (reviewed by Bonifacio & Traub, 2003). In addition, the Golgi localization of several virus proteins has been shown to be mediated via similar tyrosine-containing sorting signals, a list that includes the gB and gE proteins of varicella-zoster virus (Alconada et al., 1996; Heineman & Hall, 2001), the gE protein of herpes simplex virus (Alconada et al., 1999) and the B5R protein of vaccinia virus (Ward & Moss, 2000). In all cases, these sorting signals are oriented on the cytoplasmic side of the particular membrane-associated protein in question. Analysis of the SH protein has shown that the carboxyl terminus of the protein is orientated extracellularly, while the amino terminus is located on the cytoplasmic side of the membrane (Olmsted & Collins, 1989; Collins & Mottet, 1993). Although it has not been possible to demonstrate that these tyrosine-containing sequences function as sorting signals during infection, this suggests that the sequence 1YNFL20 would be located at a position in the protein that would favour this as a sorting signal. This sequence is conserved in all known SH protein sequences (Chen et al., 2000), whereas the putative sorting sequence present within the extracellular domain (47YNFL26) is specific only to the RSV A2 strain.

The tyrosine residue within this type of sorting signal is critical for recognition by the AP complex and several reports have suggested that tyrosine phosphorylation may regulate the interaction between the sorting signal and the AP complex (Zhang & Allison, 1997; Shiratori et al., 1997). We have observed that the inhibition of SH protein phosphorylation correlates with a large increase in the levels of the SH protein associated with the Golgi complex. If one, or both, of these tyrosine-containing sequences within the SH protein functions as a sorting signal, tyrosine phosphorylation may be one method by which its transport through the secretory pathway is controlled, although this possibility requires further investigation.

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