Furin cleavage of the respiratory syncytial virus fusion protein is not a requirement for its transport to the surface of virus-infected cells

Richard J. Sugrue,1 Craig Brown,1 Gaie Brown,1 James Aitken2 and Helen W. McL. Rixon1

1 MRC Virology Unit, Institute of Virology, Church Street, G11 5JR, Glasgow, UK
2 Division of Virology, University of Glasgow, Institute of Virology, Church Street, G11 5JR, Glasgow, UK

The intracellular cleavage of respiratory syncytial virus (RSV) fusion (F) protein by furin was examined. In RSV-infected LoVo cells, which express an inactive form of furin, and in RSV-infected Vero cells treated with the furin inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone (dec-RVKR-cmk), the F protein was expressed as a non-cleaved 73 kDa species. In both cases the F protein was initially expressed as an endoglycosidase H (Endo H)-sensitive precursor (F0EHs) which was modified approximately 40 min post-synthesis by the addition of complex carbohydrates to produce the Endo H-resistant form (F0EHr). The size and glycosylation state of F0EHr were identical to a transient intermediate form of non-cleaved F protein which was detected in RSV-infected Vero cells in the absence of inhibitor. Cell surface biotinylation and surface immunofluorescence staining showed that F0EHr was present on the surface of RSV-infected cells. RSV filaments have been shown to be the predominant form of the budding virus that is detected during virus replication. Analysis of the RSV-infected cells using scanning electron microscopy (SEM) showed that, in the presence of dec-RVKR-cmk, virus budding was impaired, producing fewer and much smaller viral filaments than in untreated cells. A comparison of immunofluorescence and SEM data showed that F0EHr was routed to the surface of virus-infected cells but not located in these smaller structures. Our findings suggest that activation of the F protein is required for the efficient formation of RSV filaments.

Introduction

Human respiratory syncytial virus (RSV) fusion (F) protein is initially synthesized as an inactive precursor (F0), which undergoes oligomerization before transport through the secretory pathway. F0 is processed by proteolytic cleavage into the disulphide-linked F1 and F2 subunits (Scheid & Choppin, 1977; Gruber & Levine, 1985) producing the mature and active form of the fusion protein (F2s–sF1). The available evidence suggests that this cleavage occurs during transport in the trans-Golgi by a host cell protease (Collins & Mottet, 1991; Anderson et al., 1992). A polybasic amino acid sequence (Lys-Lys-Arg-Lys-Arg) (Collins et al., 1984; Lopez et al., 1988) is located between those sequences in F0 that eventually form the F1 and F2 domains in the mature protein. This sequence constitutes a furin cleavage site, which triggers the activation of the fusion protein. A recent report has identified a calcium-dependent protease activity that is responsible for activation of the F protein, and furin was suggested as the cellular protease involved (Bolt et al., 2000). The proteolytic cleavage site is located immediately proximal to a stretch of hydrophobic residues which constitute the N-terminal 26 amino acid residues of the F1 domain (Collins et al., 1984; Lopez et al., 1988). The proteolytic cleavage of F0 thus creates the hydrophobic N terminus of the F1 subunit which, by analogy with other viral fusion proteins, is presumed to be inserted into the target membrane during virus-mediated fusion. Although the factor that triggers this fusion event in RSV remains unidentified, the proteolytic activation of the F protein is an essential feature of this process. In this communication, the role played by furin in the post-translational cleavage of the RSV F protein is examined.

Methods

Cells and viruses. The RSV A2 strain was used throughout this study. The recombinant vaccinia virus expressing human furin (vacc:hfur) was provided by Gary Thomas, Vollum Institute. Vero C1008 and LoVo cell lines were purchased from the European Cell and Culture Collection.
Fig. 1. Processing and intracellular transport of the RSV F protein in Vero C1008 cells. Virus-infected cell monolayers were labelled for 5 min with \[^{35}\text{S}\]methionine, washed and incubated in non-radioactive chase medium for between 0 and 80 min. Lysates were prepared, and the F protein isolated by RIP using MAb19. The immunoprecipitates were treated with Endo H (H), PNGase F (F) or mock-treated (fi) prior to analysis by 12–5% SDS–PAGE. The positions of the glycosylated (n) and deglycosylated (l) forms of F0EHs, F0EHr, F1 and F2 are indicated.

Vero C1008 and LoVo cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) and in Nutrient Mixture F12 (HAM) respectively, supplemented with 10% foetal calf serum (FCS) and antibiotics.

■ Antibodies. The F protein monoclonal antibody, MAb19, was provided by Geraldine Taylor. The anti-RSV monoclonal antibody (NCL-RSV3) was purchased from Novocastra Laboratories.

■ Radiolabelling. Cell monolayers were infected with RSV at a multiplicity of 2 and following adsorption at 33 °C for 2 h, were incubated at 33 °C for 18 h. At 20 h post-infection, the medium was removed and the cells were incubated in DMEM minus methionine for 1 h prior to radiolabelling with 100 µCi/ml \[^{35}\text{S}\]methionine. In pulse–chase experiments, the chase was carried out using DMEM supplemented with 1 mM methionine.

■ Immunoprecipitation. Mock-infected or RSV-infected monolayers (60 mm) were extracted at 4 °C for 10 min with 500 µl lysis buffer (1% NP-40, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 2 mM PMSF, 20 mM Tris–HCl, pH 7.5) and clarified by centrifugation. Clarified lysate (100 µl) and 1 µl of MAb19 were added to 600 µl binding buffer (0.5% NP-40, 150 mM NaCl, 1 mM EDTA, 0.25% BSA, 20 mM Tris–HCl, pH 8) and incubated overnight at 4 °C. The immune complexes were isolated by adding protein A–Sepharose for 2 h at 4 °C. The protein A–Sepharose was washed six times with high salt buffer (1% Triton X-100, 650 mM NaCl, 1 mM EDTA, 10 mM sodium phosphate, pH 7.0) and once with low salt buffer (1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 10 mM sodium phosphate, pH 7.0). The protein A–Sepharose-bound immune complexes were resuspended in 40 µl boiling mix (1% SDS, 15% glycerol, 1% β-mercaptoethanol, 60 mM sodium phosphate, pH 6.8) and heated at 100 °C for 2 min. After removing the protein A–Sepharose by centrifugation, the samples were analysed by SDS–PAGE. The labelled protein bands were detected using a Bio-Rad personal Fx phosphorimager and quantified by density volume analysis using Quantity one software (Bio-Rad, ver. 4). Apparent molecular masses were estimated using 13C-methylated proteins (Amersham) in the molecular mass range 14-3–220 kDa: lysozyme (14-3 kDa), soyabean trypsin inhibitor (21-5 kDa), carbonic anhydrase (30 kDa), ovalbumin (46 kDa), serum albumin (66 kDa), phosphorylase b (97-5 kDa) and myosin (220 kDa).

■ Endoglycosidase digestion. Immunoprecipitates were incubated at 100 °C for 10 min in 0.5% SDS, 1% mercaptoethanol. The samples were then made up to a final concentration of either 50 mM sodium phosphate, 1% NP-40, pH 7-5 or 50 mM sodium citrate, pH 5.5 and incubated at 37 °C for 14 h with 1000 u PNGase F (NEB) or 1000 u Endo H (NEB) respectively.

■ Dec-RVKR-cmk. The furin inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone (dec-RVKR-cmk) was purchased from Bachem (UK)
Cleavage of the RSV F protein

**Results**

**Post-translational cleavage of the RSV fusion protein in Vero C1008 cells**

Vero C1008 cell monolayers were infected with the RSV A2 strain and at 20 h post-infection, pulse–chase labelled with \(^{35}\)S)methionine. The labelled F protein was isolated by radioimmunoprecipitation (RIP) using MAb19 (Taylor et al., 1992), and analysed by SDS–PAGE (Fig. 1). This enabled the
Table 1. Effect of dec-RVKR-cmk on RSV infectivity in Vero cells

<table>
<thead>
<tr>
<th>Virus preparation</th>
<th>Virus titre (p.f.u./ml)</th>
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<tbody>
<tr>
<td>RSV-infected, HEp-2</td>
<td>3 × 10⁸</td>
</tr>
<tr>
<td>RSV-infected, Vero</td>
<td>2 × 10⁸</td>
</tr>
<tr>
<td>RSV-infected, Vero + 100 µM</td>
<td>3 × 10¹</td>
</tr>
<tr>
<td>dec-RVKR-cmk</td>
<td></td>
</tr>
<tr>
<td>RSV-infected, LoVo</td>
<td>8 × 10²</td>
</tr>
<tr>
<td>Mock-infected, Vero</td>
<td>None detected</td>
</tr>
<tr>
<td>Mock-infected, LoVo</td>
<td>None detected</td>
</tr>
</tbody>
</table>

post-translational cleavage of the F protein to be monitored during its transport via the secretory pathway. The data obtained clearly show that the immature fusion protein (F₀, 70 kDa) is processed into the F₁ (50 kDa) and F₂ (20 kDa) subunits approximately 30–40 min after the initiation of the chase. In addition, a small proportion of non-cleaved fusion protein was detected following an extended chase of up to 80 min. This is consistent with previous reports (Collins & Mottet, 1991; Anderson et al., 1992). Taken together, these data show that the cleavage of the F protein was concomitant with the onset of Endo H resistance and is consistent with the involvement of a protease activity that is resident in a trans-Golgi compartment. Furthermore, the data suggest that F₀ is modified by the addition of complex sugars immediately prior to its cleavage by this intracellular protease activity.

**RSV fusion protein is processed by furin**

The LoVo cell line is derived from a human colon adenocarcinoma which has a specific genetic defect resulting in the expression of an inactive form of furin (Takahashi et al., 1993). It is recognized that furin is only one of a family of pro-protein convertases, which include PC2, PC1/PC3, PC4, PC5/6, PC7 and PACE4, and which constitute the mammalian subtilisin-like pro-protein convertases (reviewed in Nakayama, 1997). Although LoVo cells synthesize an inactive form of furin, they do express a variety of other pro-protein convertases (Bolt & Pedersen, 1998). Processing of the F protein in RSV-infected Vero and LoVo cells was compared to assess the importance of furin in the activation of the fusion protein.

Vero and LoVo cell monolayers were infected with RSV, pulse–chase labelled with [³⁵S]methionine and the F protein detected by RIP and SDS–PAGE (Fig. 2 A). In this analysis, the amount of labelled F protein that was immunoprecipitated from each cell line was equivalent, but the pattern of the labelled F protein differed. As expected, in RSV-infected Vero cells, both F₀ and F₂–sF₁ were detected after 60 min pulse-label but only the latter was detected following the 60 min chase, due to cleavage of F₀. In addition, a 60 kDa protein was detected in RSV-infected Vero cells during the pulse-label which was not present in either mock-infected cells or in RSV-infected Vero cells following the long chase. In addition to F₁ and F₂, the 60 kDa protein could be efficiently labelled using [³⁵S]methionine and the F protein that was fully resistant to deglycosylation by Endo H treatment (F₀EHr). Analysis by SDS–PAGE showed that the levels of F₀EHr (73 kDa) were maximal at 40 min chase and coincided with the appearance of the F₁ and F₂ domains. The population of F₀EHr appeared as a diffuse protein band whose electrophoretic mobility was approximately 3 kDa greater than that of F₀EHs (70 kDa). However, both F₀EHs and F₀EHr were deglycosylated following PNGase F treatment, to a single 58 kDa product. As expected, the electrophoretic mobility of the F₁ and F₂ subunits remained unchanged following Endo H treatment, but were deglycosylated, to a 45 kDa and 10 kDa species respectively, following PNGase F treatment. This is in agreement with previous reports (Collins & Mottet, 1991; Anderson et al., 1992). Taken together, these data show that the cleavage of the F protein was concomitant with the onset of Endo H resistance and is consistent with the involvement of a protease activity that is resident in a trans-Golgi compartment. Furthermore, the data suggest that F₀ is modified by the addition of complex sugars immediately prior to its cleavage by this intracellular protease activity.
Cleavage of the RSV F protein

In contrast, analysis of the F protein expressed in RSV-infected LoVo cells after a 60 minute pulse-label showed the presence of two forms of the non-cleaved F protein. In addition to F0, a second form of non-cleaved F protein was detected (F0*) that migrated with a slightly higher electrophoretic mobility in SDS–PAGE. However, following the 60 min chase, F0* was the only form of non-cleaved F protein detected. Analysis by SDS–PAGE showed that the F0 and F0* detected in LoVo cells migrated identically to the F0EHs and F0EHr identified in Vero cells (Fig. 1), which suggests that they are identical. In addition, low levels of F1 and gp60 were detected in RSV-infected LoVo cells following the 60 min chase. Densitometric scanning of the immunoprecipitation data in Fig. 2(A) indicated that only approximately 1% of the F protein was cleaved in LoVo cells. Virus preparations obtained from LoVo cells showed a 2500-fold reduction in infectivity compared with those obtained from Vero and HEp-2 cells (Table 1). This may largely reflect the failure of the F protein to undergo efficient proteolytic activation in LoVo cells.

The F0* expressed in RSV-infected LoVo cells was assayed for its sensitivity to deglycosylation by Endo H and PNGase F (Fig. 2B). The electrophoretic mobility of F0* remained unchanged following Endo H treatment (lane 2) but migrated with an increased electrophoretic mobility following treatment with PNGase F (lane 3). This indicates that F0* is modified by the addition of complex carbohydrates via N-linked glycosylation and provides clear evidence that F0* is transported through the trans-Golgi network. It was also noted that, following PNGase F treatment, the non-cleaved F protein expressed in Vero and LoVo cells migrated identically in SDS–PAGE (data not shown). This indicates that the observed difference in electrophoretic mobility between F0* and F0 is due to a variation in the N-linked glycosylation pattern, rather than a difference in the proteolytic processing.

The above data suggest that furin is the most important host cell protease involved in activating the RSV fusion protein. To strengthen this conclusion, the recombinant vaccinia virus vacc:hfur, which expresses the human form of furin, was used to restore furin protease activity in LoVo cells. The use of vacc:hfur to assay the furin dependence of viral
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Fig. 5. Kinetics of F protein processing in Vero cells treated with dec-RVKR-cmk. RSV-infected Vero cells were either (A) treated with 80 µM dec-RVKR-cmk or (B) mock-treated 1 h before pulse-labelling with [35S]methionine for 5 min. The cells were chased in medium containing non-radioactive methionine for up to 120 min with (A) 80 µM dec-RVKR-cmk or (B) without dec-RVKR-cmk. The F protein was isolated by RIP and analysed using 12.5% SDS–PAGE; * indicates the position of unknown host cell proteins that are co-precipitated.

Fig. 6. Cell surface biotinylation of F protein. Proteins on the cell surface were labelled using biotin as described in Methods. (A) Vero cells: 1, mock-infected; 2, RSV-infected; 3, RSV-infected +100 µM dec-RVKR-cmk. (B) LoVo cells: 1, mock-infected; 2, RSV-infected.

Temporal aspects of F0* formation in RSV-infected LoVo cells were examined using pulse–chase labelling (Fig. 3). This experiment showed that in LoVo cells, the F protein is initially expressed as F0 but that this is subsequently converted into F0*, approximately 30–40 min post synthesis (Fig. 3 A). During the chase, the F protein was assayed for its sensitivity to deglycosylation by treatment with Endo H and PNGase F (Fig. 3 B). The data showed that the onset of Endo H resistance occurred at approximately 30–40 min after the initiation of the chase and coincided with the appearance of F0*.

Processing of the F protein is inhibited by dec-RVKR-cmk

Previous studies have shown that the specific furin inhibitor dec-RVKR-cmk is able to inhibit the activation of a variety of different viral glycoproteins (Stadler et al., 1997; Volchkov et al., 1998). The effectiveness of dec-RVKR-cmk in preventing cleavage of F0 was therefore examined in RSV-infected Vero cells (Fig. 4). Analysis by SDS–PAGE showed that addition of dec-RVKR-cmk resulted in the formation of a non-cleaved F protein species whose electrophoretic migration was identical to F0*EHr and which was resistant to Endo H treatment (data not shown). In the 10–40 µM inhibitor concentration range, approximately 10% of the total F protein detected was processed by host cell protease activity, as evidenced by the
appearance of the F1 domain. In addition, gp60 was also detected in this dec-RVKR-cmk concentration range. This residual protease activity was largely ablated at higher dec-RVKR-cmk concentrations (> 40 µM), as inferred from the disappearance of both the F1 domain and gp60. The concomitant disappearance of both the F1 domain and gp60
suggests that the presence of the latter is dependent upon the cleavage of the F protein. This correlated with an approximate 60,000-fold reduction in the level of infectious virus in RSV preparations obtained from Vero cells treated with dec-RVKR-cmk when compared with virus preparations from mock-treated cells (Table 1).

The concentration range over which dec-RVKR-cmk was effective in inhibiting cleavage of F0 was similar to that reported for other furin-processed glycoproteins (Ortmann et al., 1994; Vey et al., 1995; Stadler et al., 1997; Volchkov et al., 1998). However, the available published data indicate subtle variations in the sensitivity to inhibition by dec-RVKR-cmk. The cleavage of some viral fusion proteins is inhibited at concentrations of approximately 25–30 μM, while other proteins are inhibited at concentrations greater than 40 μM, and the RSV F protein falls into this latter group. Differences in sensitivity to dec-RVKR-cmk may reflect variations in the protein structure of particular proteins which may influence the ease with which furin can access the cleavage site.

In a separate experiment, temporal aspects of F protein processing in the presence of dec-RVKR-cmk were investigated. In the presence of 80 μM dec-RVKR-cmk, F0 was initially expressed as F0EHs which was subsequently processed into F0EHr approximately 30–40 min after the initiation of the chase (Fig. 5A). This was similar to the time that F2s–sF1 is detected, following the cleavage of F protein, in mock-treated cells (Fig. 5B). As these two events are accompanied by the onset of resistance to Endo H digestion, both F0EHr and F2s–sF1 appear to be transported through the trans-Golgi at a similar rate in Vero cells.

Non-cleaved F protein is transported to the surface of RSV-infected cells

We were interested to determine if non-cleaved RSV F protein was transported to the cell surface, either in virus-infected LoVo cells or in virus-infected Vero cells treated with dec-RVKR-cmk. Expression of the F protein on the surface of virus-infected cells was assayed using two techniques, namely cell surface biotinylation and indirect surface immunofluorescence.

Cell surface biotinylation of RSV-infected cells was performed using sulfo-NHS-LC-biotin. This N-hydroxysucciminate ester is unable to cross the plasma membrane and specifically couples biotin to cell surface proteins. RSV-infected monolayers were treated with sulfo-NHS-LC-biotin and the surface-labelled F protein transferred by Western blotting onto PVDF membranes, which were subsequently probed using streptavidin–HRP (Fig. 6). In the absence of inhibitor F2s–sF1 was the predominant F protein species detected on the surface of virus-infected Vero cells (Fig. 6A, lane 2). In contrast, in the presence of inhibitor, the vast majority of the F protein population detected on the surface of infected cells corre-
Cleavage of the RSV F protein

Fig. 7. Cell surface expression of the F protein on RSV-infected cells. (A) Confocal microscopy: 1, mock-infected Vero cells; 2, 6, RSV-infected Vero cells; 3, 7, RSV-infected Vero cells + 100 µM dec-RVKR-cmk; 4, mock-infected LoVo cells; 5, RSV-infected LoVo cells. The budding viral filaments (V) are indicated. Magnification: 1–5, ×40 objective; 6–7, ×63 objective (oil immersion). (B) Quantification of surface fluorescence in RSV-infected Vero cells by FACS analysis. M1 and M2 represent the regions of background and positive fluorescence staining respectively. FL1-H is the measurement of fluorescence intensity. 1, mock-infected; 2, RSV-infected cells; 3, RSV-infected cells + 100 µM dec-RVKR-cmk. (C) Scanning electron micrographs of RSV-infected Vero cells. The samples were prepared as described in Methods. 1, mock-infected; 2, RSV-infected cells; 3, RSV-infected cells + 100 µM dec-RVKR-cmk. Arrows indicate budding virus; mv indicates the presence of microvilli. Bar, 2 µm. Magnification ×1400.

Confocal fluorescence microscopy was employed to examine surface expression of the F protein on virus-infected Vero cells treated with dec-RVKR-cmk (Fig. 7A). In this assay, a similar level of immunofluorescence staining was observed on the surface of RSV-infected cells, both in the absence (Fig. 7A, panels 2 and 6) and presence (Fig. 7A, panels 3 and 7) of dec-RVKR-cmk. The surface fluorescence was quantified using

responded in size to F0\textsuperscript{EHr}. Similarly, in RSV-infected LoVo cells, a significant level of biotinylated F0\textsuperscript{*} was detected (Fig. 6B, lane 2) on the cell surface. In this assay, LoVo cells exhibited a 2- to 3-fold reduction in the level of biotinylated F protein when compared with Vero cells. This presumably reflects differences in the surface expression of non-cleaved F protein in the two cell lines.
FACS analysis (Fig. 7B) which confirmed similar levels of cell surface F protein expression. The pattern of immunofluorescence staining on the surface of RSV-infected LoVo cells (Fig. 7A, panel 5) was similar to that on Vero cells treated with inhibitor but the intensity of surface immunofluorescence was lower than in Vero cells. This observation is consistent with the cell surface biotinylation assay described above. However, these two experimental procedures do suggest that the non-cleaved F protein is transported to the cell surface in both cell types. Taken together, the results described above (Figs 6 and 7) clearly show that cleavage of the F protein is not a prerequisite for transport to the cell surface.

Examination of cell surface fluorescence using confocal microscopy revealed a significant difference in the pattern of surface staining (Fig. 7A). RSV filaments have been shown to be the predominant form of the budding virus that is detected during virus replication (Faulkner et al., 1976; Parry et al., 1979; Roberts et al., 1995). These viral filaments mature on the apical surface of polarized epithelial cells and can be visualized by light microscopy (Parry et al., 1979; Roberts et al., 1995). As expected, in the absence of the furin inhibitor, the fluorescence staining pattern was largely confined to these filamentous structures (Fig. 7A, panel 6), showing that the F protein is efficiently incorporated into maturing virions. However, in the presence of dec-RVKR-cmk, we observed punctate staining of the infected cell surface and viral filaments were not detected (Fig. 7A, panel 7). A detailed examination of the surface of RSV-infected cells was performed using SEM (Fig. 7C). In the absence of viral infection, only small clusters of microvilli were detected and the surface of the Vero cells appeared relatively featureless (Fig. 7C, panel 1). A comparison of the surface of mock-infected and RSV-infected cells showed the latter to be densely covered with viral filaments up to 8 µm in length (Fig. 7C, panel 2). It is these structures that give rise to the characteristic fluorescence staining described above (Fig. 7A, panel 6). Addition of dec-RVKR-cmk to RSV-infected cells resulted in a reduction in both the number and size of the detected viral filaments (Fig. 7C, panel 3). Most of the budding virus that could be detected on the cell surface appeared up to 2 µm in length. A comparison of the immunofluorescence and SEM data suggests that the non-cleaved F protein is not associated with these shortened virus filaments. This suggests that cleavage of the F protein is required for its incorporation into viral filaments. In addition, virus budding and hence maturation is impaired in the presence of dec-RVKR-cmk. These data provide evidence that cleavage of the F protein is required for egress of RSV from the surface of infected cells.

Discussion

Endoproteolytic cleavage is a common post-translational modification of many viral glycoproteins. Inhibiting this intracellular cleavage during transport can have a variety of different outcomes, depending upon the virus in question. Inhibiting the furin-dependent cleavage of the avian influenza virus haemagglutinin prevents its transport to the cell surface, whereas transport of human immunodeficiency virus type 1 gp160 is unimpaired, but it is not incorporated into virus particles (Guo et al., 1990; Moulard et al., 1999). In measles virus, the non-cleaved fusion protein is incorporated into virus particles (Bolt & Pedersen, 1998). In the case of RSV, our studies clearly show that proteolytic activation of the F protein is not a requirement for its cell surface expression, unlike other post-translational modifications such as N-linked glycosylation (Pastey & Samal, 1997). However, examination of the cell surface using SEM indicates that dec-RVKR-cmk strongly impairs the formation of RSV filaments. This suggests that proteolytic activation of the F protein is a prerequisite for the efficient formation of these filaments. Previous reports indicate that in many cases inhibiting furin activation of viral spike proteins impairs virus budding (Guo et al., 1990; Bolt & Pedersen, 1998), resulting in a significant reduction in viral infectivity.

Our observations differ slightly from those presented previously (Bolt et al., 2000) which suggested that the cleavage of F0 is a requirement for its surface expression. However, Bolt et al. reported erroneous processing of F0 when cleavage of the fusion protein was inhibited, presumably a consequence of cellular protease activity. We did not observe any such aberrant processing of the non-cleaved fusion protein in our experiments, which may account for differences in the surface detection of this protein.

In this study, we observed the presence of a 60 kDa protein whose detection was dependent upon cleavage of the F protein. The identity of this transient species is at present unclear. However, examination of the F protein amino acid sequence from a variety of different virus isolates reveals an additional potential furin cleavage site (Collins et al., 1984; Baybutt & Pringle, 1987; Lopez et al., 1988; Lerch et al., 1991). In addition to KKRKRR, which is located between the F2 and F1 sequences, a second sequence, RARR, is located within the C-terminal half of the F2 amino acid sequence (aa 106–109) of the RSV A2 strain. This conforms to the general consensus sequence for a furin recognition site (RxK/RR, where x is any amino acid). It is interesting to note that this second consensus sequence is conserved among human A and B serotypes, in addition to isolates of bovine RSV. In addition, the sequence RARR is present in the putative cleavage site of several other viral glycoproteins (Wunsch et al., 1983; Keller et al., 1986; Perez & Hunter, 1987). However, at present it is not clear if this additional sequence in the RSV F protein is cleaved by furin and this is currently under investigation.

Two different forms of the non-cleaved F protein have been identified in this study, distinguished by differences in their electrophoretic migration during SDS–PAGE and susceptibility to deglycosylation by Endo H. These were designated F0EHs and F0EHr, and they are both intermediates in the
processing pathway that leads to the formation of F2s–SF1. F0\textsubscript{EH}, which is located in the endoplasmic reticulum, is subsequently modified by the addition of complex carbohydrates in the trans-Golgi to form F0\textsubscript{EH}. The latter is transiently detected in pulse–chase experiments and its appearance coincides with cleavage of the fusion protein. However, when cleavage of the fusion protein is inhibited, F0\textsubscript{EH} can assemble into an oligomeric structure that is transported to the cell surface. It is not clear to what extent cell surface expression of the immature F protein occurs during natural infection. Although the immature F protein is not present in virions, it has been detected on the surface of virus-infected cells by some workers (Gruber & Levine, 1985), while others have noted its absence (Collins & Mottet, 1991). We were able to detect a small proportion of a non-cleaved F protein species, identical in size to F0\textsubscript{EH}, on the surface of RSV-infected Vero cells in the absence of inhibitor (Fig. 6A, lane 2). A recent report has suggested that the immune response to the immature form of the F protein during natural infection may provide a mechanism by which the virus can evade the host’s immune system (Sakurai \textit{et al}., 1999). It was suggested that this may arise from the liberation of immature F protein as a result of cell lysis. However, our data provide an alternative mechanism by which the immature F protein can interact with the host’s immune system, namely its presentation on the surface of virus-infected cells.

The data presented in this report provide clear evidence that furin is the major pro-protein convertase that is involved in the post-translational cleavage of the F protein during virus maturation. However, our data suggest that an additional pro tease activity, distinct from furin, is able to process the F protein. This unidentified protease activity appears to be less efficient than furin and its functional significance is unclear. An increasing body of information suggests that other members of the pro-protein convertase family, in addition to furin, can process viral glycoproteins. It was previously demonstrated that PC1/3 was able to partially process the Newcastle disease virus fusion protein in the absence of furin, but with a vastly reduced efficiency (Gotoh \textit{et al}., 1992). Recent studies have shown that PC5/6 and PC7, in addition to furin, can process gp160 into gp120/gp41 (Onishi \textit{et al}., 1994; Decroly \textit{et al}., 1996; Hallenberger \textit{et al}., 1997). In many cases, the cleavability of a fusion protein is an important factor both in viral transmission and tissue tropism (reviewed in Nagai, 1993; Klenk & Garten, 1994). In the case of RSV, the infection is usually restricted to the superficial layers of the respiratory tract, resulting in the familiar acute infection. However, under certain conditions, e.g. immunosuppressive therapy, it has been reported that the virus can infect tissues other than those of the respiratory epithelium (Fishaut \textit{et al}., 1980; Johnson \textit{et al}., 1982; Padman \textit{et al}., 1985; Milner \textit{et al}., 1985). It is likely that cleavage of the fusion protein by furin, which is distributed ubiquitously throughout different tissues, is a major factor in the disease progression.

We acknowledge David Blackbourn and Reg Clayton for advice in obtaining the FACS data and Jill Murray for technical assistance in obtaining the confocal microscope images. 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 and Gary Thomas at the Vollum Institute, Portland, Oregon, USA for providing vacc:hfur. C.B. was funded by a Wellcome Trust Vacation Scholarship.

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Received 28 November 2000; Accepted 16 February 2001