Structural Differences between Subtype A and B Strains of Respiratory Syncytial Virus

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SUMMARY

Differences in the properties of homologous intracellular structural components of eight strains of subtype A and eight strains of subtype B of human respiratory syncytial (RS) virus were examined. The size of the fusion (F) protein cleavage products and the phosphoprotein (P) showed systematic differences between virus strains representing the two subtypes. The apparent mol. wt. in SDS–polyacrylamide gels under reducing conditions was 48000 (48K) and 46K to 47K for the cleavage product F1 in subtype A and B strains, respectively. The size of the F2 protein was 18K to 20K. The subtype B strains showed a slightly higher mol. wt. of this protein compared to the subtype A strains. The size of the P protein was 36K in subtype A strains, but only 34K in subtype B strains. Variations also occurred in the size of the glycoprotein (G) and the 22K to 24K structural protein. These variations did not correlate with the virus subtypes, but were strain-specific. The size of non-glycosylated forms of the F protein cleavage products was determined by use of material from tunicamycin-treated cells. A 44K to 45K non-glycosylated form of the F1 protein was detected with subtype A virus strains, but the corresponding protein of subtype B strains was not reproducibly identified, presumably due to instability in the absence of glycosylation or altered antigenicity. Monoclonal antibody immunosorbent-bound viral glycoproteins were partially digested with proteases. The pattern of breakdown products of the F1 protein was distinctly different between subtype A and B strains, but it was similar among strains of the same subtype. No subtype-specific pattern was seen in proteolytic digests of monoclonal antibody-bound G protein.

INTRODUCTION

For the 3 decades since its discovery human respiratory syncytial (RS) virus has been considered to represent a single homogeneous serotype. Although cross-neutralization tests with animal hyperimmune sera demonstrated differences between isolates of the virus (Coates et al., 1963; Wulff et al., 1964; Doggett & Taylor-Robinson, 1964) no corresponding differences were observed with infant sera and they were therefore not considered to be of any relevance. With the advent of monoclonal antibodies (MAbs) the antigenic stability of RS virus could be assessed again. Studies with such reagents revealed that isolates of RS virus exhibited variations in epitope occurrence (Gimenez et al., 1984) but that these variations were more extensive than could be explained by incidental mutational changes (Anderson et al., 1985; Mufson et al., 1985). The isolates could be separated into two categories based on their characteristic reaction pattern with different MAbs. The two groups were named RS virus subtype A and B (Mufson et al., 1985). These subtypes showed different properties in at least four different structural proteins emphasizing that they had become ecologically adjusted after a period of separate evolution. Characterization of the epidemiological occurrence of the two RS virus subtypes
confirms that they may circulate concurrently, but that both temporal and geographical clustering can be distinguished (Hendry et al., 1986; Åkerlind & Norrby, 1986). Previous studies have shown that different strains of RS virus have phosphoproteins (P) of different sizes (Cash et al., 1977). The occurrence of such differences was confirmed in this study and the size variation could be correlated with subtype A or B characteristics of different virus strains. In addition we observed distinct structural differences between other cell-associated components of RS virus subtype A and B strains by use of physicochemical methods. This presentation describes subtype-specific differences in the size of homologous structural proteins of both the fusion (F) protein cleavage products and P protein, the stability of the F glycoprotein of both the fusion (F) protein cleavage products and P protein, the stability of the F glycoprotein.

METHODS

Virus strains. The subtype A strains included the prototype laboratory strains A2 and Long, the strain CH287 which was evaluated as a parenterally administered live vaccine (Belshe et al., 1982), the strains WV9894 and WV12138 characterized in a previous study (Mufson et al., 1985) and three strains (V214, V316, V401) isolated in Bristol, U.K. between 1983 and 1984 and provided by Dr E. J. Stott, Institute for Research on Animal Diseases, Compton, Newbury, U.K. The subtype B strains included two strains isolated during the early 1960s, CH18537 (Coates et al., 1963) obtained from Dr R. M. Chanock, NIAID, National Institutes of Health, Bethesda, Md., U.S.A. and a Swedish strain, 8/60 (Doggett & Taylor-Robinson, 1964), obtained from Dr E. J. Stott (both have been defined as B subtypes; E. Norrby, unpublished), the strains WV1293, WV3212, WV4843 and WV6873 characterized previously (Mufson et al., 1985) and two strains (V422 and V463) isolated in Bristol between 1983 and 1984 and provided by Dr E. J. Stott. The LEC strain of measles virus (see Sheshberadaran et al., 1983) was used as a control in experiments with tunicamycin-treated cells.

Cells. Vero and HeLa cells were grown in Eagle's MEM supplemented with 3% heat-inactivated (56°C for 30 min) foetal calf serum. The cells were maintained in medium with 1% serum.

Antibody-containing samples. Ascites materials prepared by injection of hybridoma cells producing RS virus-specific MAbs described in an earlier publication (Mufson et al., 1985) were used. Antibodies with specificities for the glycoprotein (G), F, nucleocapsid (NP), P and matrix (M) proteins were employed. In addition two MAbs specific for the 22K to 24K envelope-associated protein (Huang et al., 1985) were used as a control in experiments with tunicamycin-treated cells.

Radioimmune precipitation assay (RIPA). Viral structural proteins were labelled for 24 h with [35S]methionine or [3H]glucosamine (50 μCi/ml of medium) prior to development of extensive cytopathic effects in Vero and HeLa cells. Cells were scraped into the supernatant, pelleted, washed twice with cold phosphate-buffered saline and dissociated on ice with a buffer containing 2% Triton X-100, 0.15 M-NaCl, 0.6 M-KCl, 0.5 mM-MgCl2, 1 mM-PMSF and 1% trisoyl aprotinin in 0.01 M-Tris-HCl pH 7.8 (RIPA buffer). The supernatant was collected, clarified in an Eppendorf centrifuge and then stored at −20°C until used. The antigen preparation was precipitated with MAbs in the form of ascites fluid and fractionated by SDS-PAGE under reducing conditions. The details of these procedures were described previously (Örvell & Norrby, 1980; Sheshberadaran et al., 1983).

Protein footprinting. This method in brief was as follows. Isotope-labelled antigen and MAB were allowed to bind on ice for at least 2 h and thereafter the immunocomplexes were precipitated with Staphylococcus aureus Protein A-Sepharose CL-4B beads. The beads were washed three times with RIPA buffer and twice with TBS (0.01 M-Tris-HCl, 0.15 M-NaCl pH 8.0) containing 1-2% Triton X-100 and 0.1% SDS. The excess buffer was removed from the pelleted beads and 30 μl of stock trypsin or V8 protease solution (1 mg/ml in H2O) was added per sample. The samples were held at 37°C with occasional agitation for the time periods stated in the text. At the end of the digestion samples were placed on ice and washed twice with RIPA buffer and once with TBS. Excess buffer was removed and beads were dried at 37°C. Samples were analysed by SDS-PAGE under reducing conditions unless otherwise stated.

The above procedure was employed for anti-F protein MAB footprinting. For G protein footprinting the procedure was modified because the single available anti-G protein MAB reacting with both subtypes was degraded by the proteases too rapidly. Prior to addition of protease, 20 μl of undiluted rabbit anti-mouse immunoglobulins was added per sample and the beads were held at 4°C for 1 h and occasionally agitation. Protease was then added and digestion performed as described above. At the end of the digestion period, 20 μl of fivefold concentrated SDS-PAGE reducing sample buffer was added to each sample which was then held at 100°C for 3 min. Samples were analysed by SDS-PAGE without further treatment.

Isotopes, molecular weight markers and chemicals. [35S]Methionine (sp. act. 1000 to 1200 Ci/mmol), d-[1,6-
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$^3$H(N)glucosamine hydrochloride (sp. act. 31 to 33 Ci/mmol), and reference $^{14}$C-methylated protein mixture were purchased from New England Nuclear. The reference protein mixture contained phosphorylase b (97400 mol.wt., 97-4K), bovine serum albumin (69K), ovalbumin (46K), carbonic anhydrase (30K), lactoglobulin A (18-4K) and insulin (5-8K). S. aureus V8 protease, TPCK-treated trypsin, tunicamycin, PMSF and trasylol aprotinin were purchased from Sigma, Protein A-Sepharose CL-4B beads were bought from Pharmacia, and the rabbit anti-mouse immunoglobulins from Dako, Copenhagen, Denmark.

RESULTS

Sizes of cell-associated structural proteins of RS virus subtypes A and B strains in SDS–PAGE

RIPA with $[^{35}$S]methionine-labelled virus strains incubated with polyclonal human convalescent sera and a MAb specific for the F protein is illustrated in Fig. 1 (a). No variations in apparent mol. wt. in SDS–PAGE of the NP and M proteins of six virus strains representing different subtypes was found, but there was subtype-specific variation in the size of the F proteins. Thus the size of the F1 protein was 48K in all eight subtype A strains, but only 46K to 47K in all eight subtype B strains (see also Fig. 2, Table 1). Further, the characteristics of lower mol. wt. F cleavage polypeptides also differed (Fig. 1 a and tests with anti-F MAbs in Fig. 2 and Fig. 3). In subtype A strains two bands were seen in the 18K to 23K range. As is shown below (Table 1) these were apparently the 18K to 20K bona fide F2 protein and a 23K non-glycosylated fragment of the F1 protein. In subtype B strains only one band (18.5K to 20K) was visible in this

![Fig. 1](https://example.com/fig1.png)

Fig. 1. Comparison of the size of homologous proteins of RS virus subtype A and B strains in RIPA. Viral proteins were labelled in Vero cells with $[^{35}$S]methionine (a and b) and in HeLa cells with $[^1$H]glucosamine (c). (a) The four samples to the left [virus strains CH287 and WV 12138 (subtype A), and CH18537 and WV4843 (subtype B)] were precipitated with a human convalescent serum and the samples on the right (virus strains WV 12138, WV4843) with an anti-F MAb (B46). (b) The two left lanes contain one subtype A strain (CH287) and one subtype B strain (WV3212) precipitated with an anti-P MAb (C771) and the two right lanes contain the corresponding antigen preparations precipitated with an anti-22K–24K MAb (5H5). The 41K band represents co-precipitated NP protein and the low mol. wt. band in the two left lanes represents a P protein breakdown product or co-precipitated 22K–24K protein. (c) Two subtype B strains labelled on three different occasions and precipitated with an anti-G MAb (C793). Samples were analysed on 10% (a, c) or 12.5% (b) gels under reducing conditions. Lane R shows mol. wt. markers in (a) and arrows indicate the position of these markers in (b) and (c).
Fig. 2. Comparison of the size of F cleavage products of a subtype A strain (CH287) and a subtype B strain (WV4843) grown in Vero cells in the absence (a) and presence (b) of 15 μg/ml of tunicamycin. Samples were analysed in a 12.5% gel under reducing conditions. Mol. wt. markers are indicated by arrows.

Table 1. Summary of size characteristics of homologous structural proteins of RS virus subtype A and B strains, including the size of the non-glycosylated form of the F1 protein and of different proteolytic breakdown products of this protein

<table>
<thead>
<tr>
<th>Component</th>
<th>RS virus subtype A</th>
<th>RS virus subtype B</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>90K*</td>
<td>90K*</td>
</tr>
<tr>
<td>F1</td>
<td>48K</td>
<td>46K–47K</td>
</tr>
<tr>
<td>F1, non-glycosylated</td>
<td>44K–45K</td>
<td>(43K–44K)†</td>
</tr>
<tr>
<td>F1, trypsin breakdown</td>
<td>32K, 23K</td>
<td>30K, 21K</td>
</tr>
<tr>
<td>breakdown fragments</td>
<td>18K–20K</td>
<td>18–5K–20K</td>
</tr>
<tr>
<td>NP</td>
<td>41K</td>
<td>41K</td>
</tr>
<tr>
<td>P</td>
<td>36K</td>
<td>34K</td>
</tr>
<tr>
<td>M</td>
<td>26K</td>
<td>26K</td>
</tr>
<tr>
<td>22K–24K</td>
<td>23K–24K*</td>
<td>22K–24K*</td>
</tr>
</tbody>
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* Certain strain-specific variations.
† Detected in only one out of four experiments.
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mol. wt. range but this band contains two polypeptides which migrate closely (see below). One of these, the F2 glycoprotein, in repeated experiments had a marginally higher mol. wt. than the corresponding protein in subtype A strains (not readily observable in Fig. 1a or by comparison of lanes 1 to 3 and 4 to 5 in Fig. 3b, but better seen in Fig. 2). Other consistent differences were detected in the size of the F protein in four strains representing each subtype (exemplified in Fig. 1b); 36K in subtype A virus strains but only 34K in subtype B virus strains (Table 1).

Occasional variations were seen also in the size of other proteins in different strains, but they did not correlate with the subtype A and B classification. The 22K to 24K protein of unknown function had an apparent size of 22K in the subtype B strain WV3212, but in another subtype B strain, WV4843, as well as in two subtype A strains (A2 and CH287), it had a size of about 23K to 24K (exemplified in Fig. 1b). The relative size of the G protein was compared in RIPAs with [3H]glucosamine-labelled HeLa cell material with five strains each representing subtype A and subtype B strains. There was a tendency for the broad G band to migrate slightly more slowly in subtype A strains than in subtype B strains, but one subtype B strain, WV4843, had a G protein similar in size to that of most subtype A strains (exemplified in Fig. 5a). This difference in size of the G proteins of WV4843 and another subtype B strain was seen in three consecutive experiments (Fig. 1c). Therefore the size of the G protein may be strain- but not subtype-specific.

Relative size in SDS-polyacrylamide gels of non-glycosylated F protein cleavage products of RS virus subtypes A and B

The F protein of RS virus contains N-linked oligosaccharides as evidenced by the effect of treatment of infected cells with tunicamycin (Gruber & Levine, 1985). Since the size of the F protein cleavage products differs between subtype A and B strains it was considered of interest to compare the relative size of these proteins in their non-glycosylated form. Cells showing partial cytopathic effects were transferred to [3SS]methionine medium containing 5 or 15 μg/ml of tunicamycin or as control without addition of the drug. Cell harvests were made 24 h later.

For comparison Vero cell cultures infected with the LEC strain of measles virus were maintained on the corresponding medium with and without tunicamycin. The virus-specific polypeptides were identified by immunoprecipitation with MAbs. The measles virus haemagglutinin was reduced in relative size from 79K to 65K or 66K (not illustrated) by treatment with tunicamycin as described previously (Bellini et al., 1983), but in the presence of even the lower concentration of tunicamycin, 5 μg/ml, no non-glycosylated forms of the 40K and 18K measles virus F1 and F2 proteins were detectable. This was interpreted to reflect an accentuated sensitivity to proteolysis of these forms of the F products.

The effect of tunicamycin treatment on the F cleavage products of RS virus differed for the subtype A and B strains. A distinct 44K to 45K non-glycosylated form of the F1 protein was detected in cells infected with subtype A virus strains (Fig. 2) confirming previously published data (Gruber & Levine, 1985). However, in cells infected with subtype B strains one out of four experiments revealed a low concentration of a 43K to 44K protein, but in the three other experiments no non-glycosylated F cleavage products could be discerned as in the case of measles virus F protein in tunicamycin-treated cells. Fig. 2 also shows a weak protein band with a high migration rate (< 10K) in tunicamycin-treated cells infected with subtype A RS virus. This protein may represent the non-glycosylated F2 protein, but it may also represent a degradation product of the F1 protein. In most experiments with subtype A strains this low molecular weight protein was not seen and it was never observed in tunicamycin-treated RS subtype B virus-infected cells.

Footprinting of F and G glycoproteins: proteolytic cleavage of MAb-bound antigen

MAb immunosorbent-bound F protein was partially digested with protease and washed to remove unbound fragments. [35S]Methionine- and [3H]glucosamine-radiolabelled F protein from subtype A and B strains were compared. The anti-F MAbs reacting with epitope I on the F protein (Mufson et al., 1985) generated the digest pattern shown in Fig. 3 in which the major MAb-bound F1-derived fragment was 32K in subtype A and 30K in subtype B strains. The
molecular weight difference in the undigested F1 of the two subtypes therefore was maintained in this fragment. This 30K/32K F1 fragment was non-glycosylated in both subtypes.

The single anti-F MAb which reacts with a second, distinct epitope (B151; Mufson et al., 1985) generated a slightly different F digest pattern. This major F1-derived fragment was a non-glycosylated fragment of mol. wt. 23K in subtype A and 21K in subtype B strains (Fig. 4a). This 23K fragment was seen also in RIPA with undigested F protein of subtype A strains, but its equivalent in subtype B strains (21K fragment) was commonly masked in such RIPAs due to its close migration with subtype B F2 (Fig. 4b). As with the 32K/30K F1 fragment, the molecular weight difference between the undigested F1 of the two subtypes was maintained in the 23K/21K fragment (Table 1). In addition to this fragment 13K fragments were generated. Further, the 30K fragment was seen with MAb B151 in subtype B strains, but the corresponding 32K fragment of subtype A strains was absent. The MAb-bound F digests were examined also by non-reducing SDS–PAGE. The results (Fig. 4b) indicate that the 32K/30K, 23K/21K and F2 proteins are disulphide-bonded in both subtypes (data for a subtype B strain not illustrated).

Comparison of partial proteolytic digests of MAb bound G protein of the two subtypes did not reveal any subtype-specific pattern as seen for the F protein (Fig. 5).

**DISCUSSION**

The results of this study further emphasize the distinctive nature of RS virus subtypes A and B. Previously it was shown by use of monoclonal antibodies that subtypes A and B differed in the antigenic properties of at least four structural proteins (Mufson et al., 1985). The present study demonstrates that distinct differences exist in the apparent size in SDS–PAGE of the homologous cell-associated F1 and P proteins of RS subtype A and B viruses. Furthermore the footprinting analysis by proteolysis of MAb-bound anti-F proteins shows patterns of breakdown
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Fig. 4. Footprinting of MAb151-bound F protein. (a) $[^{35}S]$Methionine-labelled subtype A strain A2 and subtype B strain CH18537. (b) $[^{3}H]$Glucosamine (GLU) and $[^{35}S]$Methionine (MET)-labelled subtype A strain CH287. All labelling was performed in Vero cells. In all panels lanes u show the undigested control material, lanes 1 show footprints after digestion for 1 h and lanes 2 after digestion for 6 h with trypsin. Lane R in (a) shows mol. wt. markers (see text) and arrows indicate the positions of these markers in (b). Samples were analysed on 10 to 20% gradient gels under reducing conditions except the MET samples in (b), which were run without addition of mercaptoethanol.

products characteristic of subtype A and B viruses. In studies of one RS virus strain from a local epidemic, Ward et al. (1983) noted that their anti-F MAb immunoprecipitated a non-glycosylated 23K protein in addition to the glycopolypeptides F1 and F2. Judging from the present data it is likely that the epidemic studied was caused by a subtype A virus strain.

As with other paramyxoviruses, both surface glycoproteins G and F may play a role in immune protection (Walsh et al., 1984a, b, 1985). However, it appears that the involvement of RS virus F protein is relatively more important both for neutralization phenomena in vitro and for events restricting replication in vivo. It remains to be determined whether the difference between the homologous and heterologous neutralization titres of strain-specific polyclonal hyperimmune sera (Coates et al., 1963; Wulff et al., 1964, Doggett & Taylor-Robinson, 1964) which exemplify differences between subtype A and B strains (E. Norrby, unpublished data) reflect antigenic differences between G or F components. Since it is more likely that the F antigen is involved, the finding in this study of distinct differences between the F components of subtypes A and B assume a special significance.

The identification of antigenic fragments after proteolytic digestion may allow dissection of structural–functional relationships of the F component with reference to subtype-unique and subtype-common antigenic sites. Information on the primary structure of the F polypeptide from cloning of RS virus subtype A genes (Collins et al., 1984; Elango et al., 1985) and forthcoming similar data on the F gene of subtype B strains will be of considerable value in this context.

Concerning the cell-associated G protein, no reproducible size differences between subtype A and B strains (five of each were examined) were found, although in the majority of strains the protein of subtype A strains was larger. The footprinting experiments using proteolysis of MAb-bound antigen also did not reveal any uniform pattern characteristic of this protein in subtype A and B strains. The protein part of the G protein is a 33K polypeptide (Satake et al., 1985; Wertz
et al., 1985) and the 90K apparent mol. wt. in SDS–PAGE is due to heavy glycosylation. This glycosylation involves both N- and O-linked oligosaccharides (Gruber & Levine, 1985). Since mutational events could readily change any of the many sites of glycosylation, a variation in molecular weight of the G protein in different strains would be expected to occur. Repeated analysis of individual strains showed that the G protein had stable size characteristics.

The stability of non-glycosylated F proteins of subtype A and B strains was different. By analogy with other systems it would appear that glycosylation contributes to the resistance of the RS virus F proteins to proteolysis or possibly alters the capacity of the protein to react with the MAbs employed in RIPA. In most experiments the use of Vero cells, which show a relatively high endogenous proteolytic activity, may have assisted in revealing the difference in stability of the F proteins of RS virus subtype A and B strains. It can not be deduced whether the differences in glycosylation which cause the high sensitivity of non-glycosylated RS virus subtype B F1 protein to proteolysis is quantitative or qualitative. The present data do not indicate major differences in glycosylation of the F1 and F2 proteins of viruses representing the two subtypes, suggesting that the main difference may be qualitative. In fact this finding may not be a reflection of differences in glycosylation, but may be due to variations in protein tertiary structure.

During the preparation of this manuscript an article representing an extension of previously published data (Gimenez et al., 1984), dealing with antigenic variation between human RS virus isolates appeared (Gimenez et al., 1986). Twenty-one out of 42 strains lacked the capacity to react with one of two anti-P MAbs. The mol. wt. of the P protein of representative isolates of these 21 strains was 3K higher (35K compared to 32K) than in other strains. Judging from the present data the former 21 strains represent RS subtype A viruses. As a corollary it can be concluded that the strain RSN-2 used to generate the MAbs has subtype B characteristics. Two
of the three MAbs used therefore are subtype B-specific. In recent studies in this laboratory a large collection of subtype B-specific MAbs have been generated (C. Örvell et al., unpublished results).

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