Heterogeneity of the Respiratory Syncytial Virus 22K Protein Revealed by Western Blotting with Monoclonal Antibodies

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(Accepted 18 December 1986)

SUMMARY

Respiratory syncytial (RS) virus-infected HeLa, HEp-2, Vero and BS-C-1 cell lysates were electrophoresed on SDS–polyacrylamide gels under reducing conditions and analysed by Western blotting and immunoperoxidase using monoclonal antibodies specific for the 22K protein (relative mol. wt. of 23000 in our gel system). Three novel polypeptides with mol. wt. of 24000, 21000 and 17000 were stained in addition to the 23000 polypeptide which was present in the greatest amount in all three virus strains tested regardless of host cell line. When samples were electrophoresed under non-reducing conditions each of the three higher mol. wt. polypeptides seen in reducing gels migrated as two bands (total of six bands) with altered electrophoretic mobilities. In experiments using the alkylating agent iodoacetamide under conditions where the novel 24000, 21000 and 17000 polypeptides were not visible, the number of mobility variants of the 23000 polypeptide which could be detected in non-reducing conditions was increased from two to four. At least one, and possibly three, of these variants was the result of conformational variation in the 23000 polypeptide caused by the generation or rearrangement of intrachain disulphide bonds after the infected cells were lysed in SDS-PAGE sample buffer. Post-lysis conformational changes were minimized by treatment of the infected cells with iodoacetamide before solubilization or by decreasing the SDS concentration or using milder detergents in the lysis buffer.

Respiratory syncytial (RS) virus is classified as a pneumovirus in the paramyxovirus group. Unlike other paramyxoviruses, RS virus possesses two non-glycosylated proteins associated with the virion membrane, the matrix (M) protein and the 22000 Mr (22K) protein (Wunner & Pringle, 1976; Peeples & Levine, 1979; Huang et al., 1984, 1985). Immunofluorescent staining of unfixed infected cells with monoclonal antibodies (MAbs) indicates that the 22K protein is expressed on the cell surface (Routledge et al., 1985) and may be accessible to the host immune system. In this paper we present evidence suggesting that several species of 22K protein exist in infected cells and that when solubilized in detergent buffers containing SDS they undergo conformational changes which involve disulphide bond formation.

Lysates of HeLa cells infected with the A2 strain of RS virus were electrophoresed on SDS–polyacrylamide gels under reducing conditions. Western blotting followed by immunoperoxidase staining with the anti-22K MAb 1C1 (Fig. 1) revealed the 22K protein as a major band which migrated with a Mr of 23000. In addition three novel minor bands of Mr 24000, 21000 and 17000 were also detected. When the same lysate was electrophoresed under non-reducing conditions a completely different staining pattern consisting of five bands was observed. Two major bands of Mr 19000 and 16000 were detected which we have termed 22Ka and 22Kb respectively, as they were identified with 1C1. An additional three minor bands of 20000, 17000 and 14000 were also seen.
Fig. 1. Altered mobility of the 22K protein in non-reducing and reducing SDS-PAGE. Confluent monolayers of HeLa cells were mock-infected (lanes 2) or infected (lanes 1) with RS virus A2. When c.p.e. was extensive the cells were scraped into the medium and collected by centrifugation at 100 g for 5 min. Cell pellets were solubilized in boiling non-reducing (a) or reducing (b) SDS-PAGE sample buffer for 2 min, electrophoresed on 10% SDS-polyacrylamide gels and electroblotted on to cellulose nitrate paper (Burnette, 1981). Papers were stained with 1/1000 anti-22K MAb 1C1 ascites (Routledge et al., 1985) and anti-mouse peroxidase conjugate (Samson et al., 1986). Mr markers (× 10^-3) (Sigma) were run on reducing gels; the Mr values assigned to virus-specific bands in non-reducing gels were calculated from these markers. DF, dye front.

In addition to the five bands described above, we have also occasionally detected a band of Mr 55000 in Western blots of non-reducing gels (Fig. 2). This band was also formed by partially purified 22K protein preparations obtained by preparative SDS-PAGE in non-reducing Laemmlli sample buffer containing 0.1% (w/v) SDS (not shown). This band was resistant to boiling in non-reducing sample buffer but disappeared on reduction. Whether it represents a multimeric form of the 22K protein or is an artefact is unknown. However, disulphide-linked multimeric forms of proteins have been reported for other paramyxoviruses (Markwell & Fox, 1980).

None of the bands described above were caused by non-specific binding of the peroxidase conjugate, nor were they detected in blots stained with MAbs to the RS virus fusion protein, large glycoprotein or phosphoprotein (not shown).

There existed the possibility that the multiple bands detected resulted from infection with a heterogeneous population of RS virus. Plaque purification of RS virus stocks would not necessarily guarantee homogeneity as multiple virus nucleocapsids may be packaged within a
Fig. 2. The 22K protein of three strains of RS virus grown in four cell lines. Confluent monolayers of BS-C-1 cells were mock-infected (lanes 1) or infected with RS virus strain A2 (lanes 2), the 1984 clinical isolate VK2425 (lanes 3) or strain 8/60 (lanes 4) (supplied by Dr J. Stott, Centre for Research on Animal Disease, Compton, U.K.). Samples were prepared and analysed as for Fig. 1. Papers were stained with anti-22K MAb 5H5 (a) or 1C1 (b) (Routledge et al., 1985). (c to f). Confluent monolayers of HeLa (c), HEp-2 (d), Vero (e), and BS-C-1 cells (f) were mock-infected or infected and analysed as in (a).

single virion envelope (Bachi & Howe, 1973; Berthiaume et al., 1974). The possibility of a heterogeneous infection was therefore investigated by staining Western blots of three different RS virus strains with two MAbs, 1C1 and 5H5, which recognize different epitopes on the 22K protein (Fig. 2). The profile of bands was basically the same for each strain tested although
strains 8/60 showed a more rapidly migrating 22Ka and a more slowly migrating 22Kb than did strains A2 and VK2425. MAb 5H5 reacted with all three strains whereas MAb 1C1 reacted with only strain A2. When the epitope concerned was present in a strain it was carried on all five non-reduced and four reduced (not shown) bands, suggesting that all of the bands had a common origin. When absent, as shown with MAb 1C1 on strains 8/60 and VK2425, it was absent from all bands. This observation held true when an additional seven RS virus strains from the years 1965 to 1984 were similarly tested and suggests that the detection of multiple bands was not due to infection with a mixture of strains.

The effect of changing the host cell line upon the 22K protein band pattern was studied using HeLa, HEp-2, Vero and BS-C-1 cells (Fig. 2, c to f) and only slight differences were observed. The intensity of the minor bands relative to the major bands varied slightly but this variation was not consistent from virus strain to virus strain, possibly reflecting differences in the interaction between a given virus and a particular cell line.

In order to investigate the relationship between the reduced and non-reduced bands an infected cell lysate was analysed by Western blotting of two-dimensional SDS-polyacrylamide gels (Samson, 1986). The first dimension electrophoresis was carried out in non-reducing conditions, and the second either in non-reducing (Fig. 2a) or reducing conditions (Fig. 2b). Non-reducing conditions in both dimensions resulted in a diagonal track of proteins just visible due to non-specific conjugate binding. The two major non-reduced 22K protein bands 22Ka and 22Kb, and the three minor bands, were seen as spots lying on the diagonal. Reducing conditions in the second dimension caused a decrease in the mobility of both the 22Ka and 22Kb spots. The magnitude of this decrease was unequal and resulted in the comigration of the two spots in the second dimension, causing them to lie on the same horizontal line above the diagonal track. This observation indicated that the major reduced 22K band (Mr, 23000) seen in standard single dimension gel blots contained both the 22Ka and 22Kb bands (Fig. 1). The minor non-reduced spots behaved in a similar fashion. The non-reduced bands can therefore be divided into two families of three bands (Fig. 3): the a family (22Ka, a' and a''), which show a small decrease in
mobility on reduction, and the b family (22Kb, b' and b''), which undergo a large decrease in mobility on reduction to comigrate with the a family. No non-reduced equivalent of the reduced minor 17000 Mr band was detected.

The comigration of 22Ka and 22Kb at a higher Mr on reduction suggests that 22Kb is unlikely to be a breakdown product of 22Ka. Instead they appear to be variants of the same molecule differing in a manner which is reduction-sensitive, suggesting an involvement of disulphide bond formation. The cause of the difference between 22Ka and 22Kb probably also accounts for those between a' and b', and a'' and b'').

According to the published gene sequence (Collins & Wertz, 1985) the 22K protein contains four cysteine residues and therefore has the potential for a variety of intrachain S-S bonds. Changes in S-S bond position could cause conformational variation in the 22K protein and account for the detection of 22Ka and 22Kb after denaturation in non-reducing SDS-PAGE. Shifts in S-S bond position in proteins dissolved in SDS or urea have previously been reported (Huggins et al., 1951; Cebra, 1964; Gerber, 1964) and the sulphhydryl–disulphide interchange reaction involved requires the presence of sulphydryl groups. The reaction may be prevented using the alkylating agent iodoacetamide which binds irreversibly to sulphydryl groups, preventing their participation in the interchange (Friedman, 1973).

To investigate the role of sulphhydryl–disulphide interchange in the generation of 22Ka and 22Kb, RS virus-infected cells were treated with iodoacetamide either before or immediately after solubilization in non-reducing sample buffer (Fig. 4). As mobility shifts in the minor non-reduced bands (a', a'', b' and b'') complicated data interpretation, the samples were diluted until these bands were below the detection threshold. Samples not treated with iodoacetamide showed the typical 22K a or b pattern (Fig. 1). However, when the cells were treated with iodoacetamide before lysis (pre-lysis) in non-reducing sample buffer two new bands of lower mobility with Mr 21000 and 20000 were detected. A small quantity of 22Ka at 19000 was also

Fig. 4. Pre- and post-SDS lysis treatment of RS virus-infected cells with iodoacetamide. Fifteen ml suspension of mock-infected (lanes 2) and RS virus A2 strain-infected (lanes 1) HeLa cells were prepared from 4 oz bottle cultures as described in Fig. 1. Cells from 1 ml of each suspension were collected as before by centrifugation and resuspended and homogenized in 0.5 ml volumes (a, e) freshly prepared 0.1 M-iodoacetamide (Sigma) in 0.01 M-Tris pH 8.0, (b, f) 0.01 M-Tris pH 8.0, (c, d, g, h) twice normal strength non-reducing sample buffer. Immediately following solubilization, lysates (c), (d), (g) and (h) were mixed with equal volumes of 0.1 M-iodoacetamide in 0.01 M-Tris pH 8.0 (c, d) or 0.01 M-Tris pH 8.0 (d, h). All lysates were then allowed to stand at room temperature for 30 min, after which 0.5 ml of twice normal strength non-reducing sample buffer was added to lysates (a), (b), (e) and (f). Samples were boiled, electrophoresed on 10% non-reducing (a to d) or reducing (e to h) polyacrylamide gels, electroblotted and stained with a pool of MAbs 1C1 and 5H5.
resolved, but 22Kb was absent. Iodoacetamide treatment immediately after solubilization of cells in non-reducing sample buffer resulted in a band profile intermediate between those of the pre-lysis treated and untreated samples, with an increase in the quantities of the 20000 and 19000 bands and only a trace of the 21000 band. 22Kb was again absent. In a separate experiment a delay in the addition of iodoacetamide to lysates from 0 to 48 h post-lysis resulted in a progressive reduction in the amounts of the low mobility forms of the protein and appearance and accumulation of the 16000 22Kb form (not shown). In all cases reduction of the samples resulted in the generation of the previously described reduced 22K protein profile, demonstrating that the differing mobilities observed in iodoacetamide-treated and untreated samples in non-reducing conditions were due to conformational differences rather than to real changes in Mr.

The results with iodoacetamide suggest that 22Kb is an artefactual form of the 22K protein generated in non-reducing sample buffer as a result of disulphide bond formation. Which, if any, of the three bands seen in the pre-lysis iodoacetamide-treated sample represents the native form of the 22K protein is unknown. Possibly different conformational forms of the 22K protein exist inside the infected cell. However, as solubilization in non-reducing sample buffer immediately prior to iodoacetamide treatment increased the amount of the 20000 and 19000, and decreased the amount of the 21000 species detected, it is probable that the 20000 and 19000 bands in the pre-lysis treated sample were generated during cell disruption before the iodoacetamide blocked all available sulphhydryl groups. Therefore the 21000 band is most likely to represent the natural configuration of the 22K protein.

Of the three bands described above, the 20000 band corresponds to the fully reduced 22K protein (migrating at 23000 on reducing gels). This was determined by iodoacetamide treatment of a reduced cell lysate, which was subsequently dialysed to remove the reducing agent and then subjected to non-reducing SDS–PAGE (not shown).

Conditions for the preservation of the 22K protein in its native state without recourse to cell lysis in alkylating agent were investigated. Infected cells were solubilized in a variety of detergent buffers and allowed to stand for 48 h at room temperature. Ammonium persulphate (the oxidizing agent used in gel polymerization) enhanced the speed of 22Kb formation; therefore lysates were treated with iodoacetamide before analysis by SDS–PAGE to freeze the 22K protein in the configuration that had arisen during solubilization (not shown). Lysates made in non-reducing PAGE sample buffer (2% w/v SDS, 10% w/v glycerol, 0-005% w/v bromophenol blue 0-0625 m-Tris pH 6-8) or RIPA buffer (0-1% w/v SDS, 1-0% w/v sodium deoxycholate, 1-0% v/v Triton X-100, 0-15 m-NaCl, 0-01 m-Tris pH 7-4) contained only 22Kb after 48 h. Lysates prepared in Tris–saline (0-15 m-NaCl, 0-01 m-Tris pH 7-4), in Tris–saline containing 2% w/v sodium cholate or in non-reducing PAGE sample buffer with 0-1% rather than 2% w/v SDS contained mainly slowly migrating forms.

The 22K protein is expressed on the surface of infected cells where it is presumably available to the immune response. It is possible that the conformational changes which occur when this protein is solubilized in detergent buffers containing SDS cause the loss of important antigenic epitopes on the native molecule. These changes are therefore clearly relevant to the study of the immune response to the 22K protein when techniques liable to generate them are used.

Previous reports describing the 22K protein of RS virus in radiolabelled cell lysates or purified virions (Wunner & Pringle, 1976; Peeples & Levine, 1979; Huang et al., 1984, 1985; Routledge et al., 1985) have made no mention of the detection of multiple bands. In Western blots of reduced cell lysates (leaving aside conformational variants) we have detected a total of four bands with Mr of 24000, 23000, 21000 and 17000 (Fig. 1), probably because of the different analytical technique employed. We have assumed that the major 23000 band represents the intact, mature form of the 22K protein. If this is so, the presence of the minor 24000 band is difficult to explain, as no evidence for a precursor for this protein has been presented, the 22K protein extracted from infected cells and virions having the same electrophoretic mobility and peptide map as the protein synthesized in vitro from purified mRNA (Collins et al., 1984; Huang et al., 1984). Neither has any evidence for glycosylation or phosphorylation of the 22K protein been found (Cash et al., 1979; Lambert & Pons, 1983). An alternative possibility for the
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detection of multiple bands is that the 24000 band represents the mature form of the 22K protein and all the lower Mr bands, including the major 23000 band, are breakdown products. If this is the case, proteolysis must have occurred before rather than after cell solubilization as all four bands were detected in cells lysed directly in boiling reducing SDS–PAGE sample buffer (Fig. 1). An examination of the 22K protein in virions by Western blotting and pulse–chase labelling of the 22K protein in infected cells may shed light on the interrelationships of these bands.

We would like to thank Professor Roger Pain (Department of Biochemistry, University of Newcastle upon Tyne) for his helpful advice. This work was supported by MRC grant number 425030/12 to G.L.T. and A.C.R.S.

REFERENCES


(Received 8 October 1986)