Measles virus glycoproteins: studies on the structure and interaction of the haemagglutinin and fusion proteins

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We have investigated the structure and interaction of the measles virus (MV) glycoproteins expressed at the cell membrane. Cross-linking studies with a variety of chemicals stabilized dimeric forms of the haemagglutinin (HA) or fusion (F) proteins, although by sucrose density gradient analysis, oligomers corresponding to tetramers and larger were observed for both proteins. In cells in which both HA and F were expressed at the surface, their close association was shown by cross-linking and co-immunoprecipitation.

Introduction

Viruses attach to their host cells by specific receptors expressed at the cell surface. In a second phase, the virus enters the cell either by endocytosis or, in the case of certain enveloped viruses, may fuse with the cell membrane liberating the viral genome and non-membrane proteins into the cytoplasm. Enveloped viruses that use the latter mechanism may effect their entry into the cell by a single viral glycoprotein or by the combination of several. Measles virus (MV), a paramyxovirus, has two glycoproteins, the haemagglutinin (HA) and fusion (F) protein, that are responsible for virus attachment and entry. Studies with peptide analogues of the F protein have shown that virus entry can be differentiated into a two-step process (Norrby, 1971; Richardson et al., 1980). In the presence of these fusion inhibitors, virus can attach to the host cells, but cannot fuse with the cell membrane.

During MV infection, both the viral glycoproteins are synthesized in the endoplasmic reticulum and are transported to the cell surface where they cause fusion (syncytium formation) with the neighbouring cells. The F protein is synthesized as a precursor, F₀, and during transport to the membrane is cleaved to its functionally active forms, F₁ and F₂ (Scheid & Choppin, 1974, 1977; Sato et al., 1988). The two subunits are covalently bound by a single disulphide bridge. The HA is not cleaved, but after synthesis forms disulphide-bound dimers (Hardwick & Bussel, 1978; Kohama et al., 1985; Ogura et al., 1991). During transport to the membrane, the viral glycoproteins undergo a series of modifications giving rise to an oligomeric structure with which is associated a number of biological properties (Kreis & Lodish, 1986; Copeland et al., 1986). Of the viral glycoproteins which have been studied, the oligomeric forms may be either tri- or tetrameric (Wiley et al., 1977; Varghese et al., 1983; Gaudin et al., 1992). Although there is not a general rule which determines their structure, studies on paramyxoviruses have suggested that both glycoproteins are tetramers (Markwell & Fox, 1980; Sechoy et al., 1987; Ng et al., 1989; Spriggs & Collins, 1990; Collins & Mottet, 1991a, b).

To study the structure and biological properties of the MV HA and F proteins, the latter have been expressed in a number of vectors. When expressed in adenovirus, the F protein induces fusion in the cultures but in contrast, in both vaccinia virus and canary poxvirus systems, both HA and F are required (Alkhatib et al., 1990; Vialard et al., 1990; Wild et al., 1991; Taylor et al., 1992). Studies with other paramyxoviruses showed that both HA and F are required for cell fusion; however, in some systems, the results suggest that when F alone does give fusion, the presence of HA enhances this phenomenon (Sakai & Shibuta, 1989; Morrison et al., 1991; Horvath et al., 1992; Tanabayashi et al., 1992). Furthermore, this enhancement is specific, as substitution of a related fusion glycoprotein, for example that of human para-influenza virus types 2 and 3 does not restore the fusion activity (Hu et al., 1992). This may imply that in the homologous system there has to be a specific interaction between HA and F proteins to produce fusion.

In the present study, we have shown by cross-linking studies that the HA and F expressed at the cell surface can be chemically cross-linked. Furthermore, we have investigated the oligomeric form of the two MV glycoproteins presented at the cell surface.
Methods

Chemicals. Tran$^{35}$S (1004 Ci/mmol) was from ICN Biomedicals. All cross-linking agents were purchased from Pierce Chemical Company. Those used were: DTT, di-thiobis(succinimidylpropionate) (DSP; span of 1.2 nm) and 3,3'-dithiobis(sulpho)csuccinimidylpropionate) (DTSSP; span of 1.2 nm) both of which are reversible with DTT, ethylene glycolbis(succinimidylsuccinate) (EGS; span of 1.6 nm) and disuccinimidylsuberate (DSS; span of 1.14 nm).

Cells and viruses. HeLa cells were grown in RPMI medium containing 5% fetal calf serum. The recombinant vaccinia virus expressing the MV HA (VV-HA) and F (VV-F) protein or both (VV-HA/F) have been described previously (Drillien et al., 1988; Wild et al., 1992). To radiolabel virus-induced proteins, 2 × 10^6 cells were infected with the vaccinia virus recombinants (0.1 p.f.u./cell) and incubated 18 h later in medium lacking methionine and cysteine, but containing 50 μCi/ml Tran$^{35}$S for a further 4 h. The cells were then washed in PBS and lysed either in RIPA buffer (1% Triton, 0.6 M-KCl, 0.15 M-NaCl, 10 mM-Tris–HCl pH 7.4) or 1% octylglucoside in PBS. The cell lysate was clarified at 13000 r.p.m. for 5 min in a microcentrifuge before further analyses.

Immunoprecipitation analysis. Tran$^{35}$S-radiolabelled antigens solubilized either in RIPA or octylglucoside buffers were incubated with 2 μl of monoclonal antibody (MAb) (ascites fluid) overnight at 4 °C. After adsorption onto Sepharose–Protein A (Pharmacia), the complex was washed three times in RIPA buffer, once in TNE buffer (0.15 M-NaCl, 1 mM-EDTA, 0.05 M-Tris–HCl pH 7.2) and then denatured in sampling buffer by boiling for 2 min with or without 1% 2-mercaptoethanol (2-ME). The samples were then analysed by SDS–PAGE (Laemmli, 1970). The anti-F MAbs used in these studies were MAbs 186 and 319 which recognize conformational epitopes (Malvoisin & Wild, 1990), MAbs 7-21-17-8 and 3-5-9 against a linear epitope (De Vries, 1988); the anti-HA MAb 55 recognizes a conformational epitope (Giraudon & Wild, 1985).

Sucrose gradient centrifugation. Cells infected with VV recombinant were lysed in 1% octylglucoside/PBS containing 20 mM-iodoacetamide and the cleared cell lysates (300 μl) were centrifuged on 5 to 21.5% (w/v) sucrose gradients (in 0.1% octylglucoside/PBS) over a 200 μl 60% sucrose cushion at 35000 r.p.m. for 16 h in the SW50.1 rotor at 5 °C. Thyroglobulin (670K), ferritin (440K), catalase (232K), human γ-globulin (158K), lactate dehydrogenase (140K) and albumin (67K) were used as standards to calibrate the gradients. Fractions were collected from the bottom of the tube and diluted twofold in RIPA buffer prior to immunoprecipitation.

Chemical cross-linking. The reagents were dissolved in DMSO. Cross-linking was performed either on cells in suspension in PBS or after lysis with detergent. The reaction was incubated for 45 min at room temperature and was stopped by the addition of an equal volume of 200 mM-glycine.

Results

SDS resistance of MV F oligomers

Some membrane proteins display partial resistance to SDS-induced dissociation. When resistance is observed, there is a high probability that the interaction represents a biologically relevant structure (Doms, 1990). Initially, we investigated whether the F oligomer had a partial resistance to SDS. Previous analyses of the MV F protein have shown that the protein is synthesized as a 60K glycosylated precursor (F0) which is subsequently cleaved (F1 and F2), the two subunits being covalently linked by a disulphide bridge. To investigate whether the F protein formed SDS-stable oligomers, HeLa cells were infected with the VV-F recombinant and after radiolabelling with Tran$^{35}$S, the antigen expressed at the surface was immunoprecipitated by incubating the cells with an anti-F (conformation-dependent) MAb prior to lysis. The immune complexes were subsequently analysed by one- and two-dimensional SDS–PAGE (Fig. 1). In the absence of 2-ME, the major band migrated to the monomer position (60K), but minor bands corresponding to the dimer and tetramer were observed. Incubation with 2-ME and migration in a second dimension reduced the polypeptide size of all three bands to 40K (F1). However the band corresponding to the presumed tetrameric form migrated as a faint band. Minor bands of 60K and 120K after 2-ME treatment probably represent monomeric and dimeric forms of the uncleaved F0, whereas the band with the highest Mr is an unidentified product probably of cellular origin.

Cross-linking of the F and HA proteins

Several studies have shown that oligomeric forms of viral proteins can be stabilized by chemical cross-linking reagents (Markwell & Fox, 1980; Collins & Mottet, 1991a, b). To investigate the oligomeric associations of MV glycoproteins we have examined the latter by expressing them from vaccinia virus recombinants. To
Fig. 2. Chemical cross-linking of the MV F protein (a) and the MV HA protein (b). (a) HeLa cells infected with VV-F and radiolabelled with Tran\(^{35}S\) were solubilized in 1% octyl glucoside. Aliquots of the cleared lysate were incubated with 0 (lanes 1), 4.4 mM- (lanes 2), 1.45 mM- (lanes 3), 0.5 mM- (lanes 4), 0.15 mM- (lanes 5) or 0.05 mM-DTSSP (lanes 6) and, after neutralization with glycine, immunoprecipitated with anti-F MAb 7-21-17-8 and analysed by SDS-PAGE. The proteins were analysed either under non-reducing (5% acrylamide) or reducing conditions (8% acrylamide), as shown. In the lane labelled M, arrowheads indicate Mr markers corresponding to 200K, 97K, 69K and 46K. On the non-reducing gel, the arrows designate the presumed cross-linked F dimer. (b) Cells infected with VV-HA and radiolabelled with \([\alpha^{35}S]\)methionine were solubilized in 1% octyl glucoside. Aliquots of the cleared lysate were incubated with the cross-linking agent and after neutralization with glycine, immunoprecipitated with an anti-HA MAb and analysed by SDS-PAGE. (i) \(^{35}S\)-HA from RK13 (lanes 1 and 2) or Vero cells (lanes 3 and 4) untreated (lanes 1 and 3) or incubated with 7.4 mM-DSS (lanes 2 and 4). Lane M, Mr markers. The proteins were analysed on a 5% acrylamide gel. (ii) \(^{35}S\)-HA from HeLa cells incubated with 0 (lane 1), 0.06 mm- (lane 2), 0.18 mm- (lane 3), 0.54 mm- (lane 4) or 1.6 mm-DSS (lane 5). The lane labelled M contains Mr markers: 200K, 97K, 69K, 48K and 30K. (iii) \(^{35}S\)-HA from HeLa cells incubated with 0 (lane 1), 0.08 mm- (lane 2), 0.24 mm- (lane 3), 0.72 mm- (lane 4) or 2.1 mm-EGS (lane 5). Panels (ii) and (iii) show 8% acrylamide gels under reducing conditions.

investigate whether the oligomeric forms of F could be stabilized by cross-linking, \(^{35}S\)-labelled F antigen solubilized from VV-F-infected HeLa cells was incubated with different concentrations of the reversible cross-linking agent DTSSP and then immunoprecipitated with the anti-F MAb and analysed by SDS-PAGE (Fig. 2a). In the presence of DTSSP a minor band at 110K was observed, but tetramers were not found. The 110K band may represent a cross-linked dimeric form of F which has an enhanced electrophoretic mobility due to a more compact structure caused by cross-linking. In contrast, aggregates that did not enter the gel were observed after cross-linking. Reversal of the cross-linking with 2-ME led to the recovery of the F\(_1\) protein. However, increasing the concentration of DTSSP led to lower recoveries of the F\(_1\) protein. This suggests that, after cross-linking with DTSSP, the antigen became progressively denatured. Similar results were obtained with the irreversible cross-linking agent EGS which has a longer bridge length (not shown).
To analyse the oligomeric forms of the HA protein by SDS–PAGE, we studied the effect of cross-linking agents. $^{35}$S-labelled HA solubilized from VV-HA-infected cells was incubated in 7.4 mM-DSS and after immunoprecipitation examined under non-reducing conditions by SDS–PAGE. Fig. 2(b, i) shows the results of experiments in RK13 and Vero cells, but similar results were obtained in HeLa and CCL64 cells (not shown). After cross-linking the major peak was a dimer but migrated ahead of the non-cross-linked HA dimer. After cross-linking a minor band appeared with an apparent $M_r$ of 240K to 250K. However, it has been shown previously that cross-linked proteins often have modified migration rates, making $M_r$ calculation difficult (Doms, 1990). We obtained similar results with the cross-linking agent DSP (not shown). To distinguish between the naturally and chemically linked dimers, the solubilized $^{35}$S-labelled HA was incubated with different concentrations of DSS and examined on gels after reduction with 2-ME (Fig. 2b, ii). At the lowest concentration of DSS (0.06 mM) a dimer was observed. This decreased in intensity with increasing concentrations of DSS. Similar observations were made when EGS was used as a cross-linking agent (Fig. 2b, iii). It appears in all these experiments that increasing the concentration of the chemical cross-linking agents decreased the immunoprecipitability of the protein.

**Sucrose gradient analyses of the F and HA proteins**

The above observations showed that after SDS treatment the major form of the F protein is a monomer whereas the HA is a dimer which can be reduced to a monomer with 2-ME. To establish whether a higher oligomeric
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Fig. 4. Co-immunoprecipitation of HA and F proteins. VV-HA/F-infected HeLa cells were labelled for 4 h and then washed with PBS and treated with different concentrations of cross-linking reagent DSP: lanes 2, 7 and 12, 8.5 mM; lanes 3, 8 and 13, 2.85 mM; lanes 4, 9, 14 and 17, 0.95 mM; lanes 5, 10 and 15, 0.3 mM. Control cultures (lanes 1, 6, 11 and 16) were treated with the same amount of DMSO. After quenching of DSP, intact cells were incubated overnight at 4 °C with MAb 186, MAb 3-5-9, MAb 55 or MAb 319. The cells were lysed in RIPA buffer and the proteins immunoprecipitated from the cleared lysate were analysed on a 9 % polyacrylamide gel in reducing conditions. The lane labelled M contains markers corresponding to 200K, 97K-2, 69K, 46K and 30K.

form could be isolated, we examined the size of the F and HA complexes on sucrose gradients. The F and HA proteins were prepared from HeLa cells infected with a vaccinia virus recombinant virus expressing F and HA proteins (VV-HA/F). The cells were labelled with Tran35S for 45 min and then chased with medium containing unlabelled methionine and cysteine for a further hour. The antigens were solubilized with 1 % octylglucoside/PBS and analysed on sucrose gradients. After immunoprecipitation of the fractions with an anti-F or anti-HA MAb, the complexes were analysed by SDS-PAGE in the absence or presence of 2-ME. As shown in Fig. 3, the HA and F sedimented from fraction 5 to the bottom of the gradient. By comparison with standard proteins centrifuged in parallel tubes, we were able to evaluate the molecular size of the oligomeric forms. The HA peak corresponded to a tetramer, i.e. two disulphide-bonded dimers. In contrast the F protein sedimented as a more diffuse band starting with a tetramer, but with increasing amounts of faster sedimenting forms. These latter proteins were composed of approximately equal amounts of cleaved (F1 and F2) and uncleaved forms whereas those sedimenting in the tetrameric position were mainly in the cleaved forms.

Coprecipitation of F and HA glycoproteins

Previously we have shown that fusion activity requires both HA and F MV proteins (Wild et al., 1991). We have observed that substitution of the MV F protein with the closely related F protein of canine distemper virus (CDV) does not lead to fusion suggesting that the HA and F proteins may need to be closely associated during fusion activity. To demonstrate the interaction between HA and F proteins, VV-HA/F-infected cells were labelled with Tran35S for 4 h. Intact cells were then treated with various concentrations of DSP followed by immunoprecipitation with an anti-HA or anti-F MAb (Fig. 4). After DSP treatment, HA and F were coprecipitated with anti-F MAb 186 and 319 (lanes 2 to 5, lane 17). Conversely, precipitation of F (F1 and F2) with HA was obtained with anti-HA MAb 55 (lanes 12 to 15). Coprecipitation of HA with F also was obtained with anti-F MAb 3-5-9 but only at the lowest concentration of DSP, indicating a possible denaturation of the epitope with the higher concentrations of DSP (lane 10). To analyse further the interaction of the HA and F proteins, HeLa cells infected with either VV-HA/F, VV-HA or VV-F recombinants were labelled with 35S for 3 h and then chased for a further 1 h in non-radioactive medium containing methionine and cysteine. The intact cells were then incubated directly with DTSSP followed by lysis in 1 % octylglucoside. The lysates were then analysed by sucrose gradient centrifugation and immunoprecipitation (Fig. 5). In the VV-HA/F-infected cells, HA and F antigens coprecipitated with either anti-HA or anti-F MAbs. This was observed in the fractions corresponding to all the higher oligomeric forms.

Discussion

In the present study, we have examined the structure of the MV glycoproteins and their interaction with each other. Incubation of HA and F proteins with a number of cross-linking agents stabilized a minor band in the form of a dimer. After such chemical reactions, the
majority of the F protein migrated as an aggregate. The chemically cross-linked HA dimer migrated slightly faster in SDS–PAGE than the naturally occurring oligomer. This type of modification has been previously described for other proteins (Earl et al., 1990; Collins & Mottet, 1991a). Our analysis was hindered by the loss of antigenicity with increasing concentrations of cross-linking agents. In a previous study of the F protein of Sendai virus, a related paramyxovirus, the glycoprotein that was stabilized in the form of a dimer after cross-linking also gave rise to aggregates (Sechoy et al., 1987). In our studies, we have been unable to stabilize higher oligomeric forms of the HA and F proteins. In addition the presence of aggregates impaired the interpretation of the gradients. Preliminary results indicate that the HA and F proteins that sedimented to the bottom of the gradient are higher multimers of correctly folded oligomers (E. Malvoisin, unpublished). However the existence of SDS-resistant dimers of F and of disulphide-linked dimers of HA argues most strongly in favour of a tetrameric form, rather than a trimeric form, for the mature form of both glycoproteins.

Studies in our laboratory using vaccinia–measles recombinant viruses established that both HA and F are required for fusion (Wild et al., 1991). In further studies we have shown that substitution of the MV F protein with the closely related F protein from CDV did not lead to fusion (E. Malvoisin, R. Buckland & T. F. Wild, unpublished). This suggests that the two MV glycoproteins have a close association. In the present study, we were able to show that after cross-linking the HA and F antigens expressed within the same cell, the antigens co-immunoprecipitated. However, if the cells were lysed prior to cross-linking, co-immunoprecipitation was not
observed (not shown). This shows that the HA and F proteins are loosely bound and that the interactions observed in membranes are probably electrostatic in nature. Only the cleaved form of the F protein was co-immunoprecipitated with the HA protein although a certain amount of uncleaved F was present at the cell surface. Cleavage of the F protein is associated with conformational change and is probably obligatory for its interaction with the HA protein (Hsu et al., 1981). This is in agreement with Citovsky et al. (1986), who have shown by circular dichroism that to be fusogenic, the viral envelope glycoproteins of Sendai virus must possess a certain conformation which exists only when they are present within the same membrane.

Studies on respiratory syncytial virus, another paramyxovirus, have failed to cross-link the two glycoproteins (Collins & Mottet, 1991b). However this may be due to a variety of reasons including the highly glycosylated nature of the glycoproteins and difficulty in radiolabelling. It will now be interesting to define the regions on the HA and F proteins that associate.

References


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