Kinetics of Synthesis of Respiratory Syncytial Virus Glycoproteins

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SUMMARY

The synthesis of the two respiratory syncytial (RS) virus glycoproteins (VP66 and VP84) was examined under standard conditions and after treatment with tunicamycin and monensin. The protein backbone for VP66, the fusion protein (F1,2) is cotranslationally glycosylated to form F0, which is cleaved to form F1,2 by 20 min of chase. Monensin treatment inhibited the cleavage of F0 over an 80 min chase period, indicating that this occurred late in the transit of F0 through the Golgi apparatus or after exit from the Golgi apparatus. Tunicamycin treatment resulted in the synthesis of a 50K to 55K unglycosylated F0 which is cleaved to a 40K protein. VP84, the large glycoprotein, contains a protein backbone of only 26K to 30K which is modified by N-linked and probable O-linked glycosylation. Tunicamycin treatment results in the synthesis of a 70K protein (p70) which incorporates [3H]glucosamine and [3H]fucose but not [3H]mannose. Glycosylated precursors varying in mol. wt. from 29K to 45K (p45) are found in infected cells at regular 2K to 3K intervals, producing a 'ladder' effect. The step from p45 to VP84 is severely delayed by monensin treatment thereby enhancing the 'ladder' effect of the precursors.

INTRODUCTION

Respiratory syncytial (RS) virus is a pneumovirus of the family Paramyxoviridae (Kingsbury et al., 1978). Its importance as a cause of lower respiratory tract infection in young children has been well documented (Jacobs et al., 1971). Equally important, re-infection with RS virus readily occurs in the presence of an apparently adequate humoral response (Chanock et al., 1967). Since a detailed knowledge of the synthesis of RS virus glycoproteins might be helpful in dissecting the humoral immune response, we have investigated this matter in some detail.

Like other paramyxoviruses, RS virus contains two glycoproteins. VP84 (Fernie & Gerin, 1982) is a strong candidate for the cell attachment protein (Walsh et al., 1984) while the disulphide-linked VP43–VP19 (Fernie & Gerin, 1982) complex is the fusion protein (F1,2) as shown by Walsh & Hruska (1983). Since the mol. wt. designations of these proteins vary among different groups, we shall refer to these proteins as G and F1,2 respectively. The synthesis of these two glycoproteins was examined in order to learn more about the maturation processes of these proteins within the cell. Tunicamycin treatment was used to study the N-linked glycosylation (for review, see Waechter & Lennarz, 1976) of the F1,2 and G proteins, while the monovalent ionophore, monensin, which slows or blocks migration of proteins through the Golgi apparatus (Tartakoff, 1983) was used to study the maturation of these proteins. We have found that the synthesis of these two proteins is quite different and that VP84 contains a substantial amount of carbohydrate that is apparently O-linked.

METHODS

Virus and cells. The growth of HeLa Ohio (HO) cells in suspension culture and the propagation of RS virus (Long strain) have been previously described (Fernie et al., 1982).

Infection and labelling. HO cells were infected with RS virus at 1 p.f.u./cell. After a 2 h adsorption period at 37 °C, the cells were pelleted and resuspended at 2 × 10⁶ cells/ml in MEM suspension medium containing 5%...
heated (56 °C/45 min) foetal bovine serum. Tunicamycin (Calbiochem), when used, was added after the adsorption period at 2 μg/ml. If the cells were to be labelled with [3H]leucine or [35S]methionine, they were transferred to suspension medium containing 10% of the normal concentration of amino acids and 5 μg actinomycin D (Sigma) per ml at 16 h post-infection (5 h before labelling). If monensin (Sigma) was used, it was added 2.5 h before labelling at a final concentration of 10 μM.

Infected cells were pulse-labelled in the following manner. All procedures were performed in a 37 °C warm room. The cells were pelleted at 200 g for 3 min using a Sorvall Model GLC-1 tabletop centrifuge and resuspended, at 2 x 10⁷ cells/ml, in a labelling mixture containing equal volumes of spent medium and radioactive label (Dubovi, 1982) giving a final label concentration of 400 to 500 μCi/ml. The solution of label was made isotonic by the addition of an appropriate volume of 2.5 M-NaCl and the pH adjusted to approximate neutrality with 1 M-NaOH. The cells were exposed to label for 6 min, re-pelleted for 3 min, the label was decanted and enough fresh suspension medium added to resuspend the cells at 2 x 10⁶/ml. The total elapsed time from addition of label to resuspension in chase medium was 10 min. The labelled cells were chased for various time periods and then pelleted, washed once in cold Hanks' balanced salt solution containing 25 mM-HEPES pH 7.5, and the protease inhibitor, phenylmethylsulphonyl fluoride (PMSF; 0.1 mg/ml). The washed cells were resuspended at 1 x 10⁷ cells/ml in the same buffer and stored at -80 °C.

**Immunoprecipitation.** Labelled cells (1 x 10⁷ per immunoprecipitation) were lysed at 4 °C by adding NP40 (Sigma) and sodium deoxycholate to final concentrations of 1% and 0.5%, respectively (Morrison & Ward, 1984). Nuclei were removed by centrifugation in an Eppendorf Model 5414 microfuge and the supernatant was immunoprecipitated using appropriate monoclonal antibodies as described by Fernie et al. (1982).

**endonuclease treatment.** Digestion with endo-β-N-acetylglucosaminidase H (Endo H; New England Nuclear) was performed using a modification of the method of Zilberstein et al. (1980).

Immunoprecipitates (one pair at each time point for digestion or control) were eluted from their Staphylococcus aureus carrier by boiling for 10 min in 10 mM-sodium citrate pH 5.5, 1% SDS, and 1 mg ovalbumin per ml. The supernatant was then frozen at -80 °C. Digestion was performed by adding 2 μl Endo H solution (45 μg/ml diluted to 30 μg/ml in the above buffer) to 45 μl of immunoprecipitate and incubated for 3 h at 37 °C followed by an additional 2 μl of enzyme and a 4 h incubation at 37 °C. The reaction was stopped by adding 45 μl of Laemmli's (1970) sample buffer and boiling for 10 min. Control immunoprecipitates were treated similarly except no enzyme was added.

**Polyacrylamide gel electrophoresis.** Immunoprecipitates and appropriate molecular weight markers (Bethesda Research Laboratories) were electrophoresed on 14% polyacrylamide-NN-diallyltartardiamide gels using the discontinuous system of Laemmli (1970) as previously described (Fernie & Gerin, 1982). 14C-labelled protein mol. wt. standards (Bethesda Research Laboratories) were included with each gel. The marker proteins included myosin (H-chain) (200K), phosphorylase b (92.5K), bovine serum albumin (68K), ovalbumin (43K), α-chymotrypsinogen (25-7K), α-lactoglobulin (18.4K) and cytochrome c (12.3K). The gels were prepared for fluorography by immersing in En3Hance (New England Nuclear) according to the manufacturer's instructions. After drying, the gels were exposed to preflashed (Laskey & Mills, 1975) Kodak XAR film at -76 °C.

**Radioisotopes.** D-[6-3H]Glucosamine hydrochloride, 20 to 40 Ci/mmol, and L-[35S]methionine, 40 to 500 mCi/mmol, were both from Amersham. L-[4,5-3H]Leucine, 30 to 50 Ci/mmol, was from ICN. D-[1,6-3H(N)]Glucosamine hydrochloride, 30 to 60 Ci/mmol, D-[3,4-3H(N)]mannose, 40 to 60 Ci/mmol, and L-[6-3H]fucose, 70 to 90 Ci/mmol, were from New England Nuclear. These were used for the pulse-labeling experiments.

**RESULTS**

Since the processing of the two RS virus glycoproteins is different, each protein will be discussed separately.

**F1,2 protein**

This protein was originally designated as the fusion protein on the basis of its biochemical similarity to other paramyxovirus glycoproteins (Fernie & Gerin, 1982). It has recently been shown to be the fusion protein on the basis of specific fusion inhibition using a monoclonal antibody to F1,2 (Walsh & Hruska, 1983). The cellular processing of this protein appeared to be straightforward and similar to that of the Newcastle disease virus (NDV) fusion protein (Morrison & Simpson, 1980). After the 10 min pulse, uncleaved F₀ was found in cells labelled with [3H]leucine (Fig. 1 a) or [35S]methionine (not shown). Cleavage of F₀ to F₁ (VP43) and F₂ (VP19) was detected by 20 min of chase. Labelling experiments with [3H]glucosamine, [3H]mannose or [3H]fucose showed that F₀ was glycosylated during the 10 min pulse; some
Fig. 1. Kinetics of F₁₂ synthesis. RS virus-infected HeLa cells were pulse-labelled for 10 min with [³H]leucine or [³H]mannose and chased for the various times (min) above each lane. Cell lysates were immunoprecipitated using a Class 66A (Fernie et al., 1982) monoclonal antibody (RS/H4 661) or a control monoclonal antibody specific for human serum albumin. Specific [³H]leucine-labelled viral proteins were differentiated from cellular background proteins by comparing specific and control precipitation lanes (not shown). The migration of the mol. wt. markers are marked on the left and are 92.5K, 68K, 43K, 25.7K and 18.4K respectively. Myosin (200K) is not readily detected and cytochrome c (12.3K) runs with the dye front. The specific viral protein designations are indicated on the right of the autoradiographs. O indicates the origin of each gel. (a) Synthesis of F₁₂, [³H]leucine label. (b) [³H]Mannose label. (c) Synthesis of F₁₂ in the presence of 1.0 µM-monensin (2.5 h before labelling), [³H]leucine label. (d) Synthesis of F₁₂ in the presence of tunicamycin (2 µg/ml for 19 h before labelling), [³H]leucine label.
cleavage had already occurred since glycosylated F\textsubscript{1} and F\textsubscript{2} were also found (not shown). The precipitation of F\textsubscript{0} by monoclonal antibody 661 was in contrast to the failure of this antibody to precipitate an iodinated virion protein of similar mol. wt. (Fernie & Gerin, 1982; Fernie et al., 1982). This supports the observation by Ward et al. (1983) that the 66K virion protein is not F\textsubscript{0} or undissociated F\textsubscript{1.2}; however, the possibility remains that iodination blocked the epitope on the uncleaved precursor (F\textsubscript{0}).

**Monensin**

The monovalent ionophore, monensin, was used to examine the passage of F\textsubscript{0} through the Golgi apparatus. Glycosylation appears to occur normally since F\textsubscript{0} was found (Fig. 1c). Cleavage of F\textsubscript{0} to F\textsubscript{1,2} was significantly inhibited since no cleavage was detectable (compare Fig. 1a and 1c). This indicates that cleavage occurred at a stage late in transit through the Golgi apparatus or after F\textsubscript{0} had left it.

**Tunicamycin**

Treatment of infected HeLa cells with tunicamycin caused a greater than 90% reduction of labelled sugar incorporation and resulted in the synthesis of unglycosylated F\textsubscript{0} (F\textsubscript{0u}) which has a mol. wt. of 50K (Fig. 1d). F\textsubscript{0u} was not found by approximately 40 to 60 min into the chase period. During this time a 40K protein began to appear. This protein probably represented unglycosylated F\textsubscript{1} (F\textsubscript{1u}) (Morrison & Simpson, 1980). A protein corresponding to F\textsubscript{2u} was not seen and was thought to migrate with the dye front.

**G protein**

The synthesis of G was completely different from that of F\textsubscript{1,2} and was different in many ways from the synthesis of the glycoproteins from other paramyxoviruses.

After the 10 min pulse, VP84 was found with either \[^{3}H\]leucine or \[^{35}S\]methionine label. The level of incorporation appeared to increase during the first 50 min of chase (Fig. 2a). Contrary to previous reports (Bernstein & Hruska, 1981; Dubovi, 1982; Walsh & Hruska, 1983), VP84 could be labelled with \[^{35}S\]methionine, although not as readily as with \[^{3}H\]leucine (not shown). Also present at the end of the pulse period were two other proteins, p45 (mol. wt. 45K) and p29 (mol. wt. 29K) which disappeared during the chase, indicating that they are probably precursors to VP84. \[^{3}H\]Glucosamine labelling showed that VP84, p45 and p29 were glycosylated, as were several other intermediate-sized proteins which were not readily found with amino acid labels (Fig. 2b, last lane).

**Monensin**

The transitional steps from p45 to G were severely inhibited in the presence of monensin (Fig. 2c) which inhibited the release of G by more than 90% (not shown). The precursor protein, p45, was found in monensin-treated, amino acid-labelled cells, but G was not found during the 80 min chase period. Glycosylated G was seen (Fig. 2d), however, probably due to the greater sensitivity of detection possible with sugar labels. In addition, labelling with \[^{3}H\]glucosamine and \[^{3}H\]mannose (but not \[^{3}H\]leucine, Fig. 2c) very clearly showed a 'ladder' of glycosylated bands ranging in size from 26K to 44K or 46K (Fig. 2d). The relationship of the 'ladder' proteins to G was further studied using Endo H digestion. This enzyme cleaves between the proximal N-acetylglucosamine residues of high-mannose (or immature) oligosaccharide side-chains but does not cleave the complex (or mature) type of oligosaccharide side-chain (Tarentino & Maley, 1974; Zilberstein et al., 1980). All of the 'ladder' protein oligosaccharide side-chains were sensitive to Endo H digestion whereas the oligosaccharide side-chains of G were Endo-H-resistant (Fig. 3). This indicates that the 'ladder' proteins were not breakdown products of G. Although we used PMSF to minimize proteolytic breakdown of the G protein (similar results were obtained with apronin and leupeptin), we cannot exclude the possibility that the 'ladder' proteins were breakdown products of p45.
Fig. 2. Synthesis of G. RS virus-infected HeLa cells were pulse-labelled for 10 min with [\textsuperscript{3}H]leucine or [\textsuperscript{3}H]glucosamine. Cell lysates were precipitated using a Class 84B monoclonal antibody (RS/H4 91-4) after the various periods of chase (min) above each lane. G-specific proteins were determined by comparison with control precipitates. Mol. wt. markers as given in the legend to Fig. 1. Precursor proteins are indicated by the small letter p before the numerical designation. Several unmarked protein bands that appear to chase are non-specific proteins. (a) Synthesis of G, [\textsuperscript{3}H]leucine label. (b) Synthesis of G in the presence of tunicamycin (2 μg/ml for 18 h before labelling), [\textsuperscript{3}H]leucine label. The last lane contains [\textsuperscript{3}H]glucosamine-labelled G and associated ‘ladder’ proteins from cells not treated with tunicamycin. (c) Synthesis of G in the presence of 1.0 μM-monensin (2.5 h before labelling), [\textsuperscript{3}H]leucine label. (d) Synthesis of G in the presence of 1.0 μM-monensin, [\textsuperscript{3}H]glucosamine label. The lane marked S contained [\textsuperscript{14}C]-labelled protein markers; see legend to Fig. 1.
Fig. 3. Endo H digestion of G. [3H]Mannose-labelled VP84 immunoprecipitates which had been chased for the times (min) shown were incubated for a total of 7 h at 37 °C in the presence (+) or absence (−) of Endo H. The reaction was stopped by boiling and the samples were analysed using SDS gel electrophoresis. Designation of the markers is found in the legend to Fig. 1.

**Tunicamycin**

The presence of tunicamycin did not completely inhibit the incorporation of [3H]glucosamine or [3H]fucose but did inhibit the incorporation of [3H]mannose (not shown). The tunicamycin-resistant form of G had a mol. wt. of 70K (p70, Fig. 2b). Additionally, a 34K protein was found with [3H]leucine at the end of the pulse period (Fig. 2b). The disappearance of this protein during the chase indicated that it was probably involved in the formation of p70. The protein backbone of G apparently underwent some form of early modification in the presence of tunicamycin, since there was a 5K difference in the mol. wt. of p34 and the p29 found in untreated cells (Fig. 2a). A very faint band at 28K to 29K could be detected in tunicamycin-treated cells if the autoradiographs were overexposed (not shown).

**DISCUSSION**

We have examined the synthesis and post-synthetic modifications of the two RS virus glycoproteins F₁₂ and G, and found their synthesis pathways to be completely different. The processing of F₁₂ is straightforward and will be discussed first. F₁₂ is synthesized as a 50K protein which is co-translationally glycosylated to the 66K uncleaved F₀ protein. F₀ is then cleaved at a stage late in transit through, or exit from, the Golgi apparatus. It should be noted that the observed cleavage of the unglycosylated protein found in tunicamycin-treated cells may or may not be at the same site, as discussed for NDV (Morrison & Simpson, 1980). In view of the
fact that RS/H4 661 precipitated F₀ and F₁,₂ in the present experiments but failed to precipitate the 66K protein found in disrupted virions (Fernie & Gerin, 1982; Fernie et al., 1982), it seems likely that this virion protein is not undissociated F as we proposed (Fernie & Gerin, 1982), but an as yet unidentified protein as suggested by Ward et al. (1983). This protein may be related to the family of stress proteins since stress-related proteins have been reported in other paramyxovirus-infected cells (Peluso et al., 1978). Alternatively, iodination may have selectively blocked the 661 epitope on the uncleaved F₀ protein.

In contrast to F₁,₂, the synthesis of G is much more complicated. We have found that the precursor to G contains a protein backbone no larger than 30K. This precursor is then modified by N-linked and probable O-linked glycosylation and possibly other modifications in such a way that a number of intermediates are formed. These intermediate precursors form a 'ladder'-like array of bands approximately 2K to 3K apart [or the expected mol. wt. shift for a single N-linked carbohydrate chain for HeLa cells in suspension culture (Hunt & Summers, 1976)] that results in the formation of the relatively stable p45 protein. The identity of these 'ladder' proteins as precursors and not breakdown products is most clearly shown by Endo H treatment. The fact that G is resistant to Endo H treatment while all the 'ladder' proteins are sensitive indicates that the latter contain N-linked carbohydrate chains that are not completely modified (Tarentino & Maley, 1974). If any of the 'ladder' proteins were breakdown products of G, they should also be Endo H-resistant. Another possibility not excluded by the Endo H sensitivity data is that the 'ladder' proteins are breakdown products of p45. Although protease inhibitors were used during sample preparation, breakdown products of p45 would also be sensitive to Endo H. It is still not clear what steps are involved in the transformation of p45 into G. One possible mechanism is further glycosylation and/or other modifications resulting in the formation of G. This would result in a protein containing an estimated 50 to 75% carbohydrate. Another mechanism could be that p45 is dimerized in a specific manner, resulting in the formation of G. Both mechanisms are being investigated. Since the effect of monensin on the transport of protein through the Golgi complex has been described as a radical slowing (Tartakoff, 1983) and the actual site of the blockage appears to vary with cell type and protein studied (Griffiths et al., 1983), we have not yet determined whether the transition of p45 to G occurs before or after the site of monensin action in HeLa cells.

The fact that p45 is readily found in cells and that further modification is required to produce G could explain some results found by Wunner & Pringle (1976) and others (Cash et al., 1977; Pringle et al., 1981) where G was difficult to detect. If the appropriate modifying functions are not present in the particular virus–cell system under investigation then p45 and not G would be found. Wunner & Pringle (1976) reported the presence of two glycoproteins in the size region of p45 and very little G. This could be explained by the presence of F₁ (mol. wt. 43K) and p45 in the infected cell. This is currently under investigation.

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REFERENCES


B. F. FERNIE AND OTHERS


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