Respiratory Syncytial Virus Polypeptides. V. The Kinetics of Glycoprotein Synthesis

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(Accepted 7 February 1985)

SUMMARY

The cell-associated glycoproteins of respiratory syncytial virus included GP1 (90K), VP70 (70K), VGP48 (48K) and GP26 (26K). The time course of virus protein synthesis in HeLa cells revealed that the nucleocapsid protein (VPN41) was the first to appear at 11 h post-infection followed by the appearance of the other viral proteins at 16 h post-infection. Pulse-chase experiments with [3H]leucine or [35S]methionine demonstrated that the precursor for VGP48 and GP26 was an uncleaved protein of 70K (VP70) which required at least 30 min to chase into its final products, while the precursor for GP1 was a glycoprotein of 46K, and also required at least 30 min to chase into GP1. Trypsin treatment of monensin-treated infected cells suggested that VP70 can be cleaved intracellularly into its products VGP48 and GP26.

INTRODUCTION

Respiratory syncytial (RS) virus is a member of the genus Pneumovirus, which is in the family Paramyxoviridae. RS virus, like other paramyxoviruses, has two envelope-associated glycoproteins. One (GP1) has a molecular weight of 90K and the other (VP70), a processed protein, has a molecular weight of 70K (Gruber & Levine, 1983; Peeples & Levine, 1979). VP70, which has been tentatively identified as the fusion protein (Walsh & Hruska, 1983), is present in the virus as two disulphide-linked glycopeptides (VGP48 and GP26) (Fernie & Gerin, 1982; Gruber & Levine, 1983). In addition, an unprocessed 70K glycoprotein is found in RS virus-infected cells (Bernstein & Hruska, 1981; Dubovi, 1982; Gruber & Levine, 1985).

There has been only one study to determine the time course of appearance of viral proteins in RS virus-infected cells (Cash et al., 1979). Because the multiplicity of infection was low, viral proteins were not found before 18 to 24 h after infection, when four non-glycosylated viral proteins, VP41, VP32, VP27 and VP38 (related to VPN41) were detected. In addition, these authors reported that glycoprotein VGP48 was sulphated and VP32 was phosphorylated. Since there have been no studies on the kinetics of RS virus glycoprotein synthesis, we performed and report here the results of pulse–chase studies to verify that the cell-associated unprocessed 70K glycoprotein is the uncleaved precursor of VGP48 and GP26, and to determine the precursor of the other glycoprotein, GP1. In addition, we report studies to determine where the processing of VP70 occurs in infected HeLa cells.

METHODS

Cells and virus. The Long strain of RS virus was grown on HeLa cell monolayers. The procedures for radiolabelling virus, harvesting infected cells and purifying virus were as described previously (Levine, 1977; Peeples & Levine, 1979; Gruber & Levine, 1983).

Intracellular isotopic labelling. HeLa monolayers in 60 mm plastic tissue culture dishes were mock-infected or infected with virus at a m.o.i. > 3. At 2 h post-infection, Eagle's MEM containing 5% foetal bovine serum (FBS) was added. Infected and mock-infected cells were pulse-labelled for 1 h at 5 h intervals, from 5 h to 25 h after infection with 17 μCi/ml [35S]methionine in MEM containing 5% FBS (2 ml/plate). After each 1 h pulse, the

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radiolabelled monolayers were washed three times with cold Hanks' balanced salt solution (HBSS), scraped and pooled, and a portion of each pool was TCA-precipitated for radioactivity counting. Based on these counts, aliquots from each pool were pelleted at 45000 r.p.m. for 2 h, the pellets were brought up to 50 μl with Laemmli's dissociation buffer (see below) containing 0.1 mM-phenylmethylsulphonyl fluoride (PMSF) and 0.5 trypsin inhibition units (TIU)/ml aprotinin. The samples were solubilized, reduced and a sample from each pool containing equal TCA-precipitable counts was analysed by polyacrylamide gel electrophoresis.

Rabbit antisera. Antisera were prepared to the two RS virus glycoproteins in rabbits and two antisera, E311 against VP70, and E028 against GP1, were used for these studies. The preparation and specificities of these antisera have been described (Gruber & Levine, 1985).

Pulse–chase experiments. RS virus-infected HeLa cells were pulse–chased as described elsewhere (Gruber & Levine, 1985). Briefly, for the [35S]methionine pulse–chase experiments, infected HeLa cells were preincubated with HBSS for 45 min at 24 h post-infection. Then the cells were pulse-labelled for 10 min with [35S]methionine (100 μCi/ml) in methionine-free MEM containing 2% FBS. For the chase, the cell monolayers were washed with cold HBSS and then fed with MEM containing 5% FBS and excess methionine.

For the [3H]leucine pulse–chase experiments, the cell monolayers were preincubated with leucine-free, FBS-free MEM for 4 h starting at 20 h post-infection. Then, they were pulse-labelled for 15 min with [3H]leucine (250 μCi/0.4 ml) in leucine-free, FBS-free MEM. The chase involved washing the monolayers with cold HBSS and feeding with MEM containing 5% FBS and excess leucine. After the pulse–chase, the cells were lysed in Laemmli's dissociation buffer containing 0.1 mM-PMSF and 0.5 TIU/ml aprotinin and immunoprecipitated.

Polyacrylamide gel electrophoresis (PAGE). Polypeptides were separated on 9% SDS–polyacrylamide slab gels with 3% stacking gels, utilizing the discontinuous buffer system of Laemmli (1970), as previously described (Levine, 1977; Peeples & Levine, 1979). Samples for electrophoresis were suspended in dissociation buffer (50 mM-Tris–HCl pH 7.0, 2% SDS, 8% sucrose and bromophenol blue) with 5% 2-mercaptoethanol and dissociated by heating for 2 min in boiling water.

Trypsinization of monensin-treated cells. Monensin, at 0.1 mM in MEM containing 5% FBS, was added to RS virus-infected cells at 2 h post-infection. At 20 h post-infection, the medium was replaced with 1 ml/plate of HBSS containing monensin and the plates were incubated for 30 min to reduce the intracellular pools of amino acids. The HBSS was replaced with [35S]methionine (100 μCi/ml) in methionine-free MEM containing 2% FBS and monensin (1 ml/plate), and the monolayers were again incubated until 28 h post-infection, when the radiolabelled medium was removed and the cell monolayers were washed with cold HBSS. (A set of monensin-treated [35S]methionine-labelled mock-infected cells was also included in this experiment.) Then cold HBSS, 1 ml/plate, was added to three mock-infected and three infected plates, the cells were scraped and each group was pooled. Also, at 28 h post-infection, three additional infected monolayers were trypsinized, suspended in MEM containing 10% FBS and pooled. The pools of scraped and trypsinized cells were centrifuged at 1000 r.p.m. for 10 min at 4 °C, and the cell pellets were solubilized in dissociation buffer and sonicated. Equal quantities of protein from each pool were immunoprecipitated.

Immunoprecipitation. Radiolabelled cell lysates solubilized in dissociation buffer were immunoprecipitated as described elsewhere (Gruber & Levine, 1985).

Fluorography. The method used was that of Bonner & Laskey (1974) as previously described (Peeples & Levine, 1979).

Chemicals and radioisotopes. The trypsin was purchased from Difco and the monensin from Sigma. The D-[1-14C]glucosamine (55-5 mCi/mmol), [35S]methionine (840 to 1385 Ci/mmol) and L-[4,5-3H]leucine (131 Ci/mmol) were all purchased from Amersham.

RESULTS

Kinetics of appearance of RS virus polypeptides

The synthesis of viral polypeptides was studied to determine their time of first appearance in HeLa cells. RS virus-infected and mock-infected cells were pulse-labelled for 1 h, at 5 h intervals from 5 h to 25 h post-infection, with 34 μCi/plate [35S]methionine. After the 1 h pulse, the cells were washed, solubilized and samples containing equal TCA-precipitable radioactivity were reduced and analysed by SDS–PAGE. VPN41 was the first viral protein to appear, at 11 h post-infection (Fig. 1, lane 4). Other viral polypeptides, VP70, VGP48, VPP32, VPM27 and VP25 became visible at 16 h post-infection (Fig. 1, lane 6), but not GP1 which does not label well with [35S]methionine. The viral polypeptides were even more intensely labelled at 21 h, suggesting an increase in the rate of viral protein synthesis over that at 16 h.
RS virus glycoprotein synthesis

Fig. 1. Synthesis of polypeptides in RS virus-infected and mock-infected cells analysed by PAGE under reducing conditions. Cells were pulse-labelled for 1 h with [35S]methionine at various times after infection. Lane 1, [35S]methionine-labelled RS virus; lanes 2, 4, 6, 8 and 10, RS virus-infected cells at 6, 11, 16, 21 and 26 h post-infection, respectively; lanes 3, 5, 7, 9 and 11, mock-infected cells at 6, 11, 16, 21 and 26 h post-infection, respectively; lane 12, [14C]glucosamine-labelled RS virus.

Pulse–chase experiments

To determine the precursor–product relationships among the RS virus glycoproteins, pulse–chase experiments were performed. Based upon the time at which the viral proteins were first detected, infected cells were pulse-chased after 16 h post-infection. At approximately 25 h post-infection, cells were pulsed for 10 min with [35S]methionine (100 μCi/plate) and chased for various times up to 3 h. The cells were washed, solubilized and portions were immunoprecipitated with antiserum E311, which reacts against VP70. Then, equal immunoprecipitated counts were analysed by SDS–PAGE. The precursor for the virion glycoproteins VGP48 and GP26 is the unprocessed glycoprotein VP70, seen in the 10 min pulse (Fig. 2, lane 3). By 40 min after the addition of label, i.e. after a 30 min chase, VP70 was chased into VGP48 and GP26 (Fig. 2, lane 6). [Although GP26 is poorly labelled with [35S]methionine and is not readily visible (Fig. 2, lane 6) it does appear after a longer film exposure.] With longer chases, the label in VP70 continued to decrease as the label in VGP48 and GP26 continued to increase until by the 3 h chase little VP70 remained. Two other proteins, which may be VPN41 and VPM27, appear to be non-specifically immunoprecipitated in this experiment (there is no change in the intensity of labelling in these bands). These results suggest that VGP48 and GP26 are cleavage products of the precursor VP70. Also, this processing took place only after 30 min of chase, suggesting a late event.

Since the other RS virus glycoprotein, GP1, labels poorly with [35S]methionine, as mentioned above, [3H]leucine was used for this pulse–chase experiment. At 24 h, infected cell monolayers were pulse-labelled for 15 min with [3H]leucine (250 μCi/plate) and chased for different times up to 3 h. The cells were washed, solubilized, aliquots were immunoprecipitated with antiserum
Fig. 2. Fluorogram of polyacrylamide gel analysis of the E311-immunoprecipitated proteins \(^{35}S\)methionine-labelled in a pulse-chase experiment. RS virus-infected and mock-infected cells were pulse-labelled for 10 min with \(^{35}S\)methionine and then chased for various times up to 3 h. Lane 1, \(^{14}C\)glucosamine-labelled RS virus; lane 2, mock-infected cells (10 min pulse); lane 3, infected cells (10 min pulse); lanes 4 to 11, infected cells chased for 10, 20, 30, 40, 60 and 90 min, 2 h and 3 h, respectively; lane 12, mock-infected cells (3 h chase); lane 13, \(^{35}S\)methionine-labelled RS virus.

E028 which reacts against GP1, and equal counts of these immunoprecipitates were analysed by SDS-PAGE. The precursor, migrating with a mol. wt. of 46K, was visible in the pulse (Fig. 3, lane 3). The label in this precursor first appeared in the product, GP1, after a 30 min chase (Fig. 3, lane 5). The label in GP1 continued to increase as the label at 46K decreased with increasing times of chase. VPN41 and VPM27 again appear to be non-specifically immunoprecipitated, but there was no change in their labelling with time. This suggests that the 46K protein is the precursor to GP1 and that this processing is also a late event.

Location of VP70 processing in monensin-treated infected cells

Monensin causes glycoproteins to accumulate in the Golgi apparatus, although some glycoproteins can migrate to the plasma membrane in the presence of this ionophore (Alonso & Compans, 1981; Chatterjee et al., 1982; Johnson & Schlesinger, 1980). As shown previously (Gruber & Levine, 1985), VP70 can be proteolytically processed into VGP48 and GP26 in the presence of monensin. Since it has also been demonstrated that most of the VP70 in the infected cell is in the plasma membrane where it can be removed by trypsin (Gruber & Levine, 1985), the cellular location of this cleavage event was examined by determining whether VP70 and its products migrated to the plasma membrane in the presence of monensin. Infected cell monolayers were treated with monensin from 2 h post-infection, labelled with \(^{35}S\)methionine from 20-5 to 28 h post-infection and either treated with trypsin or left untreated. The cells were solubilized, equal amounts of protein were added to the immune precipitation reaction mixture
and the VP70-related proteins were immunoprecipitated with antiserum E311. Fig. 4 shows the results of this experiment. Trypsin treatment did not remove much of the VP70 or the related polypeptides VGP48 and GP26 from infected cells. This suggests that in the presence of monensin, VP70 and its products do not migrate to the plasma membrane and that the cleavage of VP70 can occur intracellularly. However, an abnormal cleavage in the presence of monensin cannot be excluded.

DISCUSSION

We have found by pulse-chase studies that the precursor VP70 chases into the products VGP48 and GP26 only after 30 min of chase (40 min total) and processing of VP70 continues to occur with longer chases up to 2.5 h later (3 h chase). Forty min is ample time for a viral glycoprotein to reach the plasma membrane (Herrler & Compans, 1983; Strous & Lodish, 1980) and since cleavage of VP70 continues beyond 40 min, it suggests that VP70 processing might occur in the plasma membrane. However, the processing of VP70 can occur intracellularly as demonstrated with monensin treatment. Monensin prevents the migration of VP70 from the Golgi apparatus to the plasma membrane, as demonstrated by the inability of trypsin to remove it from monensin-treated infected cells (Fig. 4) and by the absence of syncytia in monensin-treated infected cells (C. Gruber, unpublished observations), indicating that the cleavage of VP70 is not restricted to the plasma membrane. The long interval between synthesis and
processing probably eliminates the possibility that the rough endoplasmic reticulum is the site for processing. This suggests that in the presence of monensin, the processing of VP70 occurs either in the smooth endoplasmic reticulum or the Golgi complex.

Similarly, in parallel pulse-chase studies the precursor of GP1 (46K) was chased into GP1 only after 30 min. Previously, it was found that this 46K protein consisted of N-linked oligosaccharides attached to a 33K polypeptide, and that GP1 contained both N- and O-linked oligosaccharides (Gruber & Levine, 1985). This suggests that the late processing seen in the pulse-chase is probably due primarily to the addition of O-linked oligosaccharides to the 46K precursor within the Golgi apparatus.

It has long been known that 90% or more of the mature RS virus produced by the infected cell remains associated with the plasma membrane (Levine & Hamilton, 1969; Peeples & Levine, 1980). The cause for the extremely inefficient release is not known. Based on the observation that purified released virus contains only the cleaved products of VP70, i.e. VGP48 and GP26 (Gruber & Levine, 1985), and that the delay between the synthesis and cleavage of VP70 is long enough for it to migrate to the plasma membrane before cleavage, we suggested that the proteolytic processing of VP70 was involved in the release of budded virions. However, our evidence showing that VP70 cleavage can occur before it reaches the plasma membrane may cast doubt on the validity of this hypothesis.

We thank Marie Wolley for her able technical assistance. This work was supported by Public Health Service Research Grant AI-10996, Minority Biomedical Research Support Program Project RR-08167, and the Thrasher Research Fund. Chris Gruber was the recipient of an NIH Cancer-Virology and Immunology Predoctoral Training Grant, CA-09304-05.
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(Received 29 November 1984)