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Monoclonal antibodies to varicella zoster virus (VZV) glycoproteins were used to study the processing of three glycoproteins with molecular weights of 83K-94K (gp 2), 64K (gp 3), and 55K (gp 5). Immunoprecipitation experiments performed with VZV-infected cells, pulse labeled with \(^{3}H\)glucosamine in the presence of tunicamycin, suggest that O-linked oligosaccharide is present on the glycoprotein of gp 2. Use of the enzyme endo-\(\beta\)-N-acetylglucosaminidase H revealed that the fully processed form of gp 3 had high-mannose type and that of gp 5 had only complex type of N-linked oligosaccharides. Experiments with monensin suggest that the precursor form (116K) of gp 3 is cleaved during the processing from Golgi apparatus to cell surface membrane. The extension of O-linked oligosaccharide chain and the complex type of N-linked oligosaccharide chains also occurs during this processing.

INTRODUCTION

Varicella zoster virus (VZV) specifies several glycoproteins which are expressed on both virion and infected cell surfaces (Grose, 1980; Shemer et al., 1980; Zweerink and Neff, 1981; Shiraki et al., 1982). Monoclonal antibodies of VZV have been produced in several laboratories and antigenic analysis of VZV has been attempted (Grose et al., 1983; Okuno et al., 1983). In our laboratory, three groups of monoclonal antibodies against VZV were isolated and the processing of VZV glycoproteins using these antibodies has been investigated (Okuno et al., 1983). Recently the glycosylation of viral proteins using inhibitors of glycosylation such as tunicamycin and monensin has been studied in detail for several enveloped viruses including influenza, paramyxoviruses, vesicular stomatitis virus, Sindbis virus, retroviruses, Semliki forest virus, and herpes simplex virus (Schwarz et al., 1976; Leavitt et al., 1977; Nakamura and Compans, 1978; Diggelmann, 1979; Witte and Wirth, 1979; Johnson and Schlesinger, 1980; Pizer et al., 1980; Stallcup and Fields, 1981; Johnson and Spear, 1982; Nakamura et al., 1982; Wenske and Courtney, 1983). Since the antiviral drug tunicamycin (TM) inhibits the formation of lipid-linked N-acetylglucosamine compound (Takatsuki et al., 1971, 1975) and another drug monensin inhibits the glycoprotein pathway from Golgi apparatus to cell surface membrane (Tartakoff and Vassalli, 1978; Uchida et al., 1979), they are useful in identifying and studying the polypeptide moieties of glycoprotein. In this paper, the processing of three VZV glycoproteins (gp 2, gp 3, and gp 5) in the infected cells was investigated using TM and monensin.

MATERIALS AND METHODS

Virus and Cell Culture

The Kawaguchi strain of VZV was used throughout these studies. The preparation of cell-free VZV was described previously (Yamanishi et al., 1980). Human embryonic fibroblast (HuEF) cells, which were passaged 10 to 15 times, were propagated in a mixture of Eagle's MEM and medium 199 containing 10% fetal calf serum and 0.025% NaHCO₃ for growth medium and
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3% fetal calf serum for maintenance medium.

Monoclonal Antibodies

Hybridoma cell lines secreting monoclonal antibodies directed against VZV glycoproteins were isolated in our laboratory (Okuno et al., 1983). The antibodies used (in the form of ascitic fluids) were: clone 9 (cl 9), directed against gp 2 (83K–94K proteins); cl 8, directed against gp 3 (64K protein); and cl 12, directed against gp 5 (55K protein).

Drug Treatment and Radiolabeling

Tunicamycin (Sigma Chemical Co., St. Louis, Mo.) was used at 2 μg/ml. Monensin (Calbiochem-Behring Corp., La Jolla, Calif.) was used at 10−6 M. The monolayers of HuEF cells in 100-mm plastic plates were infected with virus at a multiplicity of approximately 0.05 PFU/cell. After 3 days of incubation at 37°, infected cells were trypsinized and transferred to noninfected HuEF cells at a ratio of 1:3. When approximately 80% of the cell sheet showed cytopathic effect (CPE), cells were used for drug treatment and radiolabeling. When TM or monensin were applied to cells, infected cells were incubated for 1 hr with the maintenance medium containing either drug before labeling and remained present throughout the labeling. In the pulse-chase experiment, infected cells were preincubated with methionine-free medium for 30 min and labeled with 35S)methionine (10 μCi/ml, 1030 Ci/mmol, Amersham) for 20 min. Then the cells were washed three times with ice-cold phosphate-buffered saline (PBS) and cultured with the maintenance medium containing twice concentrated methionine for 4 hr. The infected cells were also labeled with D-[1,6-3H(N)]glucosamine hydrochloride (20 μCi/ml, sp act 24.8 Ci/mmol, Amersham) for 18 hr.

Preparation of Radiolabeled Antigen Extract

Labeled cells were washed three times with PBS, harvested by scraping from the plastic plates, and lysed with RIPA buffer (pH 7.4, 0.01 M Tris–HCl, 0.15 M NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM methionine, 1 mM phenylmethylsulfonyl fluoride). Cells were disrupted in a sonicator and lysates were ultracentrifuged at 30,000 rpm for 2 hr in a RPS 40 T-2 rotor (Hitachi) at 4°, and the supernatant was used for immunoprecipitation.

Radioimmunoprecipitation and Polyacrylamide Gel Electrophoresis (PAGE)

Three to four hundred microliters (approximately 10 to 1.5 × 106 cpm) of lysate of 35S-labeled or 3H-labeled antigen was mixed with 2 μl of ascites fluid overnight at 4°. Thirty microliters of protein A–Sepharose CL-4B beads (Pharmacia Fine Chemicals) (8.6 mg of beads in 10 mM phosphate buffer) was then added, and incubation continued for 2 hr at room temperature with occasional agitation. The immunoprecipitates were washed six times with washing buffer (PBS, pH 8.6, containing 0.1% bovine serum albumin, 0.2% NaNa, 0.5% NP-40, and 0.1% SDS) and solubilized with sample buffer (pH 8.2, 0.125 M Tris, 1% SDS, 5% β-mercaptoethanol, 20% glycerol). The samples were loaded onto 10% polyacrylamide gels crosslinked with N-N' methylene-bis-acrylamide at a 37.5:1 and run at constant voltage (50 V) for 18 hr. The gels were fixed with 50% methyl alcohol containing 7% acetic acid, infused with sodium salicylate, dried on filter paper, and placed for periods of 7–15 days in contact with Fuji X-ray film at −70° for fluorography. The reference 14C methylated protein mixture containing myosin (200,000 molecular weight), phosphorylase b (92,500), BSA (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and lysozyme (14,300) was purchased from Amersham company (England).

Glycosidase Treatment of Immunoprecipitates

Prior to enzyme treatment, the immunoprecipitates were washed with washing buffer and incubated in 0.8% SDS for 20
min at 37°C followed by boiling for 30 sec. The protein A-Sepharose-bound antibodies were removed by centrifugation. Supernatants were diluted to 0.2% SDS and made 50 mM with respect to sodium citrate buffer (pH 5.5). Endo-β-N-acetylglucosaminidase H (10 μl of 1 IU/ml, Miles Laboratories, Elkhart, Ind.) was added to samples. The reaction mixtures were incubated with constant rotation at 37°C for 20 hr and the reactions were terminated by the addition of cold acetone and centrifugation. The pellets were resuspended in sample buffer and analyzed by SDS-PAGE.

RESULTS

Effect of TM on VZV Glycoprotein Synthesis

In order to investigate the effect of TM on the synthesis of VZV glycoprotein, infected cell cultures were labeled with [3H]glucosamine for 18 hr, and cell extracts were immunoprecipitated with three kinds of monoclonal antibodies (cl 9, cl 8, and cl 12) which react with glycoproteins gp 2, gp 3, and gp 5, respectively (Okuno et al., 1983). When [3H]glucosamine was employed as a specific carbohydrate label, three bands with molecular weight of approximately 75K, 83K, and 94K which reacted with cl 9, two bands with molecular weight of 116K (faint) and 64K (major) which reacted with cl 8, and two bands with molecular weight of 94K (faint) and 55K (major) which reacted with cl 12, were observed in VZV-infected cultures (Fig. 1, lanes a, c, e). When [3H]glucosamine was used to label VZV-infected cells, only one diffuse band with molecular weight of 82-85K which reacted with cl 9 was observed in TM-treated cell extract (Fig. 1, lane b). However, no polypeptide was detected in cultures reacted with cl 8 and 12 monoclonal antibodies (Fig. 1, lanes d, f).

Next the kinetics of glycoprotein synthesis were followed by labeling cells with [35S]methionine and immunoprecipitation with monoclonal antibodies. Infected cells with or without TM treatment were pulsed with [35S]methionine for 20 min and the radiolabeled cells were immediately harvested or chased for 4 hr. When cl 9 antibody was used for immunoprecipitation test, radioactive bands at 65K (minor) and 75K (major) were observed in immunoprecipitates from cells labeled for 20 min (Fig. 2, lane a). As shown in Fig. 2 lane c, these polypeptides were mainly replaced during chase by 83K and 94K molecules. In contrast, the immunoprecipitates formed with extracts of TM-treated cells gave prominent band of 73K at pulse labeling and additional 85K polypeptide during chase (Fig. 2, lanes b, d). Next, when cell extracts were reacted with antibodies of cl 8, a band at 106K was seen at pulse labeling, and additional 116K and 64K (prominent) polypeptides were detected during chase (Fig. 2, lanes b, d). In TM-treated cell extracts, the 102K polypeptide (faint) at pulse labeling and 56K band (major) during chase were observed.

Fig. 1. Effect of tunicamycin (TM) on the glycosylation of VZV glycoproteins. Fluorogram of a 10% gel of immunoprecipitates. VZV-infected cell cultures in the absence (−) or presence (+) of TM were labeled with [3H]glucosamine. Labeled cell extracts were reacted with monoclonal antibodies, cl 9 (lanes a and b), cl 8 (lanes c and d), and cl 12 (lanes e and f) and the immunoprecipitates were analyzed by SDS-PAGE. (●) polypeptides related with gp 2, (●) polypeptides related with gp 3, (●) polypeptides related with gp 5.
FIG. 2. Fluorogram of SDS-PAGE of immunoprecipitates obtained from lysates of [35S]methionine pulse labeled and chased infected HuEF cells in the absence (−) or presence (+) of TM. Infected cells were labeled for 20 min (lanes a, b, c, f, i, j, k, l) or pulsed and chased for 4 hr (lanes c, d, g, h, k, l) and cell extracts were immunoprecipitated with cl 9 (lanes a–d), cl 8 (lanes e–h), and cl 12 (lanes i–l). (●) polypeptides related with gp 2, (■) polypeptides related with gp 3, (*) polypeptides related with gp 5.

The Effect of Endoglycosidase H on VZV Glycoproteins

The oligosaccharides of VZV glycoproteins were characterized by use of endo H. Endo H cleaves N-linked oligosaccharides of the high-mannose type, but not the complex type (Koide and Muramatsu, 1974). VZV-infected cells were pulse labeled with [35S]methionine for 20 min or pulsed and chased for 4 hr as described above. Cell extracts were immunoprecipitated with three monoclonal antibodies and immunoprecipitates were treated or not treated with endo H.

The 75K, 106K, and 49K polypeptides, which presumed to be precursor proteins of gp 2, gp 3, and gp 5, respectively, were detected in pulse-labeled cultures (Fig. 3, lanes a, e, i) and they were replaced by the 73K, 103K, and 46K polypeptides after endo H treatment (Fig. 3, lanes b, f, j). This evidence suggests that these precursor polypeptides were sensitive to endo H and contain high-mannose N-linked oligosaccharides. In contrast, the 83K and 94K polypeptides (gp 2) and 55K polypeptide (gp 5) which appeared during chase and are presumed to be final products were insensitive to endo H (Fig. 3, lanes d, l). On the other hand, the 64K polypeptide which reacted with antibodies from cl 8 and corresponds to gp 3 was sensitive to this enzyme (Fig. 3, lane h). This shows that among three VZV glycoproteins only gp 3 contains high-mannose type oligosaccharides.

The Effect of Monensin on VZV Glycoprotein Synthesis

Monensin is an ionophore and blocks transport of glycoprotein from the Golgi
FIG. 3. Endo H treatment of VZV-infected cell extracts. Fluorogram of a 10% gel of immunoprecipitates. VZV-infected cells were labeled with [35S]methionine and immunoprecipitated as described in the legend to Fig. 2. Cell extracts were reacted with monoclonal cl 9 (lanes a-d), cl 8 (lanes e-h), and cl 12 (lanes i-l). Immunoprecipitates were incubated at 37°C for 20 hr with endo H. (○) polypeptides related with gp 2, (■) polypeptides related with gp 3, (★) polypeptides related with gp 5.

to the plasma membrane (Tartakoff and Vassalli, 1978; Uchida et al., 1979). Figure 4 illustrates the results of experiment in which VZV-infected cells were labeled with [35S]methionine for 20 min or pulsed and chased in the presence or absence of monensin. Cell extracts were reacted with the three kinds of monoclonal antibodies described above.

The 75K, 106K, and 49K polypeptides which correspond to the precursor proteins of gp 2, gp 3, and gp 5, respectively, were detected in extracts from monensin-treated or nontreated cells (Fig. 4, lanes a, b, e, f, i, j). When cell extracts from cultures treated or nontreated with monensin were reacted with these monoclonal antibodies, the 94K (gp 2) and 55K (gp 5) polypeptides, predominantly labeled in the absence of monensin, were replaced by the 83K and 52K polypeptides, respectively (Fig. 4, lane d, i). Finally the 64K polypeptide (gp 3) was predominantly observed during chase in nontreated cell culture, whereas this polypeptide was not detected in monensin-treated cells but the 106K to 116K bands were observed (Fig. 4, lane h).

DISCUSSION

The purpose of this report was to extend our previous work (Okuno et al., 1983) in which we demonstrated the synthesis and processing of three VZV glycoproteins (gp 2, gp 3, and gp 5) using monoclonal antibodies. This report demonstrates that three species of VZV glycoproteins differ in several important structural and bio-
synthetic properties. These include the carbohydrate structure and the processing steps at the post-translational level (Scheme 1).

Tunicamycin is known to block addition of N-linked oligosaccharides (Takatsuki et al., 1975). Our experiments (Fig. 2, lanes d, h, i) showed that the formation of the product proteins (gp 2, gp 3, and gp 5) were inhibited by this drug, suggesting that these VZV glycoproteins contain N-linked oligosaccharides. Next, the VZV-infected cells were labeled with $[^3]$H-glucosamine in the presence or absence of TM. If glycoprotein contains only N-linked oligosaccharides, no polypeptide should be labeled with $[^3]$H-glucosamine in the presence of TM. On the contrary, glycoproteins containing O-linked oligosaccharides would be labeled with $[^3]$H-glucosamine in the presence of TM. Only the 82-85K polypeptide which reacted with cl 9 monoclonal antibody was labeled with $[^3]$H-glucosamine in the presence of TM (Fig. 1). From these data, it could be supposed that VZV gp 2 contains O-glycoside oligosaccharides. Recently the presence of O-glycosidically linked oligosaccharides has been reported for virus-specific glycoproteins of corona virus (Nieman and Klenk, 1981), vaccinia hemagglutinin (Shida and Dales, 1981), and herpes simplex virus (Olofsson et al., 1981a, b, 1983; Johnson and Spear, 1983).

Next, of particular interest is the appearance of newly detected proteins in the presence of TM. The clone 9 monoclonal antibodies reacted with the 75K polypeptide (major) in the pulse labeling, and with the 83K and 94K polypeptides during chase (Fig. 2, lanes a, c). The 73K polypeptide was detected in the TM-treated and pulse-labeled cell culture, and the additional 85K polypeptide appeared during chase (Fig. 2, lane d). This suggests that the nascent polypeptide chain elongates and O-glycoside oligosaccharides would be added to the polypeptides. Next, cl 8 monoclonal antibodies, which react with gp 3, immunoprecipitated the 102K polypeptide in the pulse labeling and the 56K polypeptide during chase (Fig. 2, lane h). Since precursor polypeptide (116K) will be considered to cleave to 64K product protein and 64K polypeptide appear in the virions and on the membrane of cells (Okuno et al., 1983), the 56K polypeptide found in TM-treated cells could be postulated to be a product protein cleaved as the case of 64K polypeptide in the absence of TM. It is reported that the cleavage of Semliki forest virus polypeptide occurs in the presence of TM (Garoff and Schwarz, 1978). This supports that the glycosylation of gp 3 is not necessary for the cleavage of polypeptide. Finally, in the case of gp 5, clone 12 monoclonal antibodies reacted with the 45K polypeptide (major) in the pulse labeling, and with the 55K (major) and 94K (minor) polypeptides. The 94K band was always observed in the long labeled or pulse-chased cultures (Figs. 1-4). It was previously supposed that gp 5 and gp 2 glycoproteins would share some
antigenic determinant (Okuno et al., 1983). The 39K polypeptide was mainly detected in the pulse labeling and the 45K polypeptide was observed during chase. Because of the data from vesicular stomatitis virus (Rothman and Lodish, 1977), which indicate the glycosylation of polypeptide proceeds and the nascent chains are elongating, we believe that the 39K and 45K polypeptides are not normally present as precursor proteins, but the antigenic determinants are similar to the viral glycoproteins.

The enzyme endo H have been shown to cleave selectively N-linked oligosaccharide of high-mannose type but not of the complex type (Koide and Muramatsu, 1974). The precursor polypeptides of 75K (pgp 2), 106K (pgp 3), and 49K (pgp 5) were all sensitive to endo H as shown in Fig. 3. While product proteins, 83K and 94K (pgp 2) and 55K (pgp 5) were insensitive to endo H, the 64K (pgp 3) was sensitive to this enzyme (Fig. 3, lane h). This indicates that the N-linked oligosaccharide chain is present in gp 3, and gp 5 contains complex type N-linked oligosaccharide, and that O-linked and N-linked complex type of oligosaccharides exist in gp 2.

VZV-infected cells were treated with monensin and labeled for a short time or pulse labeled and chased. When cell extracts from monensin-treated cells were reacted with monoclonal antibodies, no effect was observed on the synthesis of polypeptide at the pulse labeling. However, the product proteins were not detected in monensin-treated cells but fast-migrating polypeptides were observed in gp 2 and gp 5. The monensin blocks the addition of O-linked oligosaccharides to glycoprotein and also it inhibits the conversion of N-linked high-mannose type oligosaccharide to the complex type (Johnson and Spear, 1983). Considering the data described above, it can be confirmed that gp 2 contains N-linked and O-linked oligosaccharides and gp 5 contains complex type N-linked oligosaccharides. Finally, the diffuse band between 106K and 116K was observed in monensin-treated cell extracts and no major polypeptides like 64K were detected in nontreated cells. Since the 116K (pgp 3) would be cleaved to 64K product protein (Okuno et al., 1983), this result implies that the cleavage occurs between Golgi apparatus and membrane.

Recently it was reported that gp 3 would be the cleaved-product form of gp 140 which has a disulfide-linked structural form (Grose et al., 1984). We also confirmed that a 140K polypeptide was detected besides the 116K one, when immunoprecipitate was heated in sample buffer lacking 2-mercaptoethanol (unpublished data). However, the relationship between the 116K and 140K glycoproteins is not clear now.

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