Synthesis and Processing of the Three Major Envelope Glycoproteins of Epstein-Barr Virus

CLARK M. EDSON^{1*} AND DAVID A. THORLEY-LAWSON^{1,2}

Department of Pathology¹ and Department of Medicine,² Division of Geographic Medicine, Tufts University School of Medicine, Boston, Massachusetts 02111

Received 12 November 1982/Accepted 10 February 1983

In pulse-chase experiments, the three major Epstein-Barr virus envelope glycoproteins, gp350/300, gp250/200, and gp85, were shown to be synthesized from separate precursors of 190,000, 160,000, and 83,000 daltons, respectively. These three pulse-labeled species were chased into the mature forms of the glycoproteins between 1 and 3 h after transfer to nonradioactive medium. Digestion of precursor forms with endo- β -*N*-acetylglucosaminidase H (endo H) yielded polypeptides of 160,000, 120,000, and 75,000 daltons. Comparison of these results with those from experiments with tunicamycin, which specifically blocks N-linked glycosylation, indicated that some other post-translational modification(s), probably O-linked glycosylation, contributes about 100,000 and 60,000 daltons of apparent molecular mass to gp350/300 and gp250/200, respectively. Experiments with endo H showed that mature gp350/300 and gp250/200 contain complex-type (endo H-resistant) N-linked glycosyl chains, whereas gp85 contains both high-mannose (endo H-sensitive)- and complex-type oligosaccharides. In contrast to the results obtained with the three envelope glycoproteins, no precursor forms of the two unglycosylated proteins, p160 (the major Epstein-Barr virus capsid antigen) and p140 (an envelope protein), were detected. The partial proteolytic maps of gp350/300 and gp250/200 were quite similar, suggesting that polypeptide sequence homology could account for at least part of the observed serological cross-reactivity of the two proteins. Taken together, these results demonstrate that the polypeptide portions of gp350/300 and gp250/200 are closely related but not derived from a common precursor. Furthermore, the polypeptide portions comprise half or less of the apparent molecular weight of the mature glycoproteins on sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Activation of Epstein-Barr virus (EBV) replication in human and other primate B lymphoblastoid cell lines results in the synthesis of a number of virus-specific proteins (3-5, 11, 18-24, 29). Of these, four, gp350/300, gp250/200, p140, and gp85, have been shown to be the major components of the virus envelope, and one, p160, has been shown to be the major component of the virus capsid (5, 19). To date, there have been few reports which bear on the physiological role(s) of these five proteins, but two recent studies have presented evidence suggesting functions for two of the glycoproteins in EBV infection. In the first, it was shown that liposomes containing purified gp350/300 and gp250/200 can block virus binding to target cells, suggesting that these glycoproteins may interact with the EBV receptor present on these cells (40). In the second, it was found that the majority of EBV-neutralizing antibodies in human sera was directed against gp350/300 and gp250/200 (39). It has been shown that monoclonal

antibodies, rabbit antibodies, and most importantly human antibodies against gp350/300 and gp250/200 will neutralize the virus in vitro (8, 20, 30, 38, 39). Interestingly, all of these monoclonal antibodies, with one exception, immunoprecipitate both of the glycoproteins. With one monoclonal antibody, only gp350/300 is immunoprecipitated, indicating that gp350/300 and gp250/200 do not exist as detergent-soluble or disulfide-linked complexes in extracts. The most likely explanation for these results is that gp350/300 shares most of its antigenic determinants with gp250/200. It is not known whether the shared determinant(s) is carbohydrate or protein in nature. (After a poll of most investigators working on EBV membrane antigens, a general nomenclature was agreed upon and published recently (37): gp350/300 is the largest glycoprotein; gp250/200 is the next largest glycoprotein; p160 is the major capsid antigen; p140 is the unglycosylated EBV envelope protein; and gp85 is the smallest glycoprotein.)

In the present study, we used two approaches to investigate the relationship between gp350/300 and gp250/200. In the first, we examined the post-translational processing of the intracellular precursors to these glycoproteins, as well as that of the third major EBV envelope glycoprotein, gp85. In a previous report from this laboratory, all three of these glycoproteins were shown to contain asparagine-linked oligosaccharide chains (5). The synthesis and processing of glycoproteins containing such glycans have been studied intensively in a variety of systems and have been shown to proceed over a period of about 15 to 60 min from cotranslational glycosylation with a high-mannose-type precursor oligosaccharide ($\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$) in the endoplasmic reticulum to conversion to complex-type glycans in the Golgi apparatus to insertion of the mature forms in the plasma membrane (reviewed in reference 9). We here report that gp350/300, gp250/200, and gp85 are synthesized from separate intracellular precursor proteins and that half or more of the apparent molecular masses of mature gp350/300 and gp250/200 is the result of extensive glycosylation with N- and probably O-linked glycans.

As a second approach to examining the relationship between gp350/300 and gp250/200, we performed partial proteolytic mapping, a procedure which can be used to demonstrate protein sequence homology (1). The results suggest that the antigenic cross-reactivity between these two glycoproteins may derive, at least in part, from shared amino acid sequences.

(Portions of this work were presented at the Fifth Cold Spring Harbor Meeting on Herpesviruses, 26 to 31 August 1980, Cold Spring Harbor, N.Y., and at the International Workshop on Herpesviruses, 27 to 31 July 1981, Bologna, Italy.)

MATERIALS AND METHODS

Cell lines and media. The following EBV-producing cell lines were used in this study: M-ABA, a marmoset cell line (M. Epstein, University of Bristol Medical School, Bristol, United Kingdom) (2); B95-8, a marmoset cell line (Pfizer Inc., New York, N.Y.); and P3HR-1, a human cell line (M. Nonoyama, Showa University Research Institute, Clearwater, Fla.). The maintenance procedure for these cell lines has been described previously (35). Dialyzed fetal calf serum was purchased from GIBCO Laboratories, Grand Island, N.Y. L-Methionine-free RPMI 1640 was from Associated Biologic Systems, Baltimore, Md., and glucose-free Eagle minimum essential medium was obtained from GIBCO.

Chemicals, enzymes, and radioisotopes. Triton X-100, sodium dodecyl sulfate (SDS), and α -methylmannoside were purchased from Sigma Chemical Co., St. Louis, Mo. The tumor promoter 12-*O*-tetradecanoyl phorbol-13-acetate was obtained from Polysciences, Inc., Warrington, Pa. All reagents for SDS-polyacryl-

amide gel electrophoresis (SDS-PAGE), except for SDS, were from Bio-Rad Laboratories, Richmond, Calif. Sepharose-coupled *Lens culinaris* agglutinin (lentil lectin) was acquired from Vector Labs, Burlingame, Calif. All radioisotopes were purchased from New England Nuclear Corp., Boston, Mass., and included L-[^{35}S]methionine (800 Ci/mmol), D-[^3H]mannose (18.4 Ci/mmol), and ^{14}C -methylated proteins used as molecular weight markers for SDS-PAGE. All other chemicals were of reagent grade. *Staphylococcus aureus* V8 protease was from Miles Laboratories, Inc., Elkhart, Ind., and electrophoretically pure endo- β -*N*-acetylglucosaminidase H (endo H) was the kind gift of P. W. Robbins (Massachusetts Institute of Technology, Cambridge).

Pulse-labeling of cells. For pulse-labeling with [^{35}S]methionine, 5×10^7 cells were induced with 12-*O*-tetradecanoyl phorbol-13-acetate for 4 days (41), washed once with methionine-free RPMI 1640 without fetal calf serum, and suspended at 5×10^6 cells per ml in 10 ml of prewarmed (37°C) RPMI 1640 containing 10% the normal level of L-methionine, 10% dialyzed fetal calf serum, and 10 mCi of [^{35}S]methionine. Incubation was at 37°C in a humidified 5% CO_2 incubator for 15 min. The pulse-labeling was stopped by placing the cells in an ice bath at 4°C, a procedure shown to be effective in stopping protein synthesis in B lymphoblastoid cell lines (15). The cells were harvested by centrifugation at $260 \times g$ for 10 min at 4°C, washed once with RPMI 1640 containing the normal level of L-methionine, and either used immediately or stored frozen at -70°C. It was found that three pulse-label procedures could be carried out with the same batch of radioactive medium, provided the medium was filter sterilized between uses.

To chase, 1×10^7 of the washed cells were taken for the zero time chase point and the rest (4×10^7) were suspended at 10^6 cells per ml in RPMI 1640 containing 10% fetal calf serum and the normal level of L-methionine. At various times, 10^7 cells were removed, harvested, and treated as outlined above.

For pulse-labeling with [^3H]mannose, 5×10^7 cells induced with 12-*O*-tetradecanoyl phorbol-13-acetate were suspended at 5×10^6 cells per ml in 10 ml of prewarmed (37°C) glucose-free Eagle minimum essential medium plus 10% dialyzed fetal calf serum, 10% the normal level of glucose (100 $\mu\text{g}/\text{ml}$), 5 mM sodium pyruvate, and 10 mCi of D-[^3H]mannose. Incubation was for 15 min at 37°C in a humidified 5% CO_2 incubator. The labeling was stopped as above, and after the cells were washed and a zero time of chase sample (10^7 cells) was removed, the rest of the cells were suspended at 10^6 cells per ml in RPMI 1640 plus 10% fetal calf serum and the normal level of glucose (1 mg/ml). Portions (10^7 cells) were removed at various times during the chase and processed as outlined above.

Detergent solubilization and lectin affinity chromatography. The preparation of Triton X-100-soluble extracts and chromatography on 2-ml columns of Sepharose-coupled lentil lectin have been described previously (36).

Antisera and immunoprecipitation. The preparation and characterization of the two rabbit antisera used in this work have been described previously: the rabbit antiserum to the B95-8 strain of EBV (35) and the rabbit antiserum to the 350,000- and 220,000-molecu-

lar-weight EBV glycoproteins (39). Immunoprecipitations with these sera were carried out using Formalin-fixed *S. aureus* as described originally by Kessler (12) with minor modifications (35, 36). In each experiment, the samples were precleared with the appropriate normal rabbit serum.

SDS-PAGE. SDS-PAGE was carried out by the method of Laemmli (17) on 1.5-mm-thick slab gels at the percent acrylamide indicated in each figure legend. After electrophoresis, gels were prepared for fluorography by use of En³Hance (New England Nuclear).

Digestion with endo H. After absorption of the antigen-antibody complexes onto the Formalin-fixed *S. aureus* and appropriate washings, the labeled antigen was released by boiling for 5 min in 1% SDS and the bacteria were removed by centrifugation. The supernatant was diluted 1:1 with 0.1 M Tris-hydrochloride (pH 6.8), and half was digested with endo H (final concentration, 3 μ g/ml) for 1 h at 37°C. The reaction was stopped by adding SDS to 1.5% (wt/vol), 2-mercaptoethanol to 3% (vol/vol), and glycerol to 10% (vol/vol) and boiling for 5 min.

Partial proteolysis. Pulse-labeling of 5×10^7 B95-8 cells with [³⁵S]methionine, preparation of detergent-soluble extracts, and chromatography on lentil lectin-Sepharose were carried out as outlined above. The eluate from the lentil lectin-Sepharose column was first precleared with 10 μ l of normal rabbit serum and Formalin-fixed *S. aureus* and then treated with 10 μ l of the rabbit anti-gp350/220 serum, and the antibody-antigen complexes were removed with Formalin-fixed *S. aureus*. The immunoprecipitate was solubilized by boiling for 5 min in 50 μ l of 1% SDS and centrifuged at $10,000 \times g$ for 1 min. The endo H treatment was carried out as outlined above. The endo H-treated sample was subjected to SDS-PAGE (5%), and a 1-cm-wide strip which included the sample lane was sliced into 0.5-cm pieces. These were subjected to partial proteolysis with *S. aureus* V8 protease by the method of Cleveland et al. (1). Briefly, the slices were placed in wells of a 15% gel and covered with 40 μ l of a solution containing 0.0625 M Tris (pH 6.8), 1% SDS, 10% glycerol, bromophenol blue, and 4 mg of the protease per ml. The current was turned off for 1 h when the dye reached the bottom of the stacking gel, and then electrophoresis was resumed.

RESULTS

[³⁵S]methionine and [³H]mannose pulse-chase of EBV-specific glycoproteins in M-ABA cells. From previous studies, it was known that gp350/300, gp250/200, and gp85 contain asparagine-linked oligosaccharide chains (5). Therefore, only precursor forms of these proteins which contain high-mannose-type N-linked oligosaccharides should be labeled when cells are incubated briefly (20 min or less) with [³⁵S]methionine or [³H]mannose (26, 32). Such pulse-labeled precursors would contain neither O-linked (7) nor complex-type N-linked glycans (9), since these are synthesized at a later stage, after glycoproteins have reached the processing enzymes of the Golgi apparatus.

M-ABA cells were pulse-labeled for 15 min

with [³⁵S]methionine and chased in nonradioactive medium. The M-ABA cell line was chosen for this experiment because it synthesizes a typical EBV glycoprotein profile (5), unlike the more widely studied B95-8 and P3HR-1 cell lines, which are clearly variants from their original clinical isolates (5, 6, 25, 28). Glycoproteins with N-linked oligosaccharides were separated from unglycosylated proteins by affinity chromatography of detergent-soluble extracts on lentil lectin-Sepharose, and EBV-specific glycoproteins were immunoprecipitated from the α -methylmannoside eluate with the rabbit anti-EBV serum. Electrophoretic analysis of the immunoprecipitates gave the following results. First, mature gp350/300 and gp250/200 were undetectable in the 15-min pulse (Fig. 1A) but were present after 1 h of chase (Fig. 1B) and reached their maximum levels by 3 h (Fig. 1C). Second, gp85 was barely detectable as a faint band in the 15-min pulse but was prominent after 1 h or more of chase. Third, previously undescribed EBV-specific glycoproteins of 190,000, 160,000 and 120,000 daltons were present in the 15-min pulse but decreased during the chase, suggesting that they might be precursors to other proteins. Less than 15% of the EBV-specific glycoprotein radioactivity was recovered in the flow-through

FIG. 1. Electrophoretic analysis (10% SDS-PAGE) of immunoprecipitates obtained with the rabbit anti-EBV serum from lentil lectin-Sepharose eluates of extracts of M-ABA cells pulse-labeled with [³⁵S]methionine for 15 min and chased for the indicated times. Time of exposure was 1 week.

FIG. 2. Electrophoretic analysis (7.5% SDS-PAGE) of immunoprecipitates obtained with the rabbit anti-EBV serum from extracts of M-ABA cells pulse-labeled with [3 H]mannose for 15 min and chased for the indicated times. Lanes A, C, E, G, and I were with normal rabbit serum, and lanes B, D, F, H, and J were with the rabbit anti-EBV serum. Time of exposure was 1 month.

fraction of the lentil lectin affinity column (data not shown).

As an alternative to lectin affinity chromatography in studying the synthesis of the EBV glycoproteins, M-ABA cells were pulse-labeled with [3 H]mannose and chased in nonradioactive medium. Detergent-soluble extracts were immunoprecipitated with the rabbit anti-EBV serum. Electrophoretic analysis of the immunoprecipitates (Fig. 2) revealed no detectable radioactive proteins in the 15-min pulse (Fig. 2B), but after 30 min of chase, proteins of 190,000 and 160,000 daltons appeared (Fig. 2D), as well as a faint band at 350,000 daltons. Upon continued chase, the levels of mature gp350/300 and gp250/200 increased, whereas those of the 190,000- and 160,000-dalton proteins decreased; by 3 h they were undetectable. The delayed appearance of the two precursor proteins in the cells pulse-labeled with [3 H]mannose as compared with [35 S]methionine is consistent with a relatively slow labeling of the lipid-linked oligosaccharide pool (10). No [3 H]mannose-labeled proteins corresponding to the 85,000- or 120,000-dalton glycoprotein seen in the [35 S]methionine experiment were detected.

Immunoprecipitation of lectin affinity-purified [35 S]methionine pulsed-labeled proteins with the rabbit anti-gp350/220 serum. To distinguish which of the glycoprotein precursors observed

in Fig. 1 and 2 were related to the synthesis of gp350/300 and gp250/200 and which were related to the synthesis of gp85, we used a rabbit antiserum raised against gp350/300 and gp250/200 purified by immunoaffinity chromatography on a monoclonal antibody-Sepharose column (39). This rabbit serum (rabbit anti-gp350/220 serum) immunoprecipitated only gp350/300 and gp250/200 and not gp85 or the unglycosylated proteins p160 and p140 (39).

M-ABA cells were pulse-labeled with [35 S]methionine, and a detergent extract was prepared. The extract was chromatographed on lentil lectin-Sepharose, and the eluate was immunoprecipitated with the rabbit anti-gp350/220 serum. Electrophoretic analysis of the immunoprecipitates showed that the serum specifically precipitated the 190,000-, 160,000-, and 120,000-dalton polypeptides observed in Fig. 1A (data shown in Fig. 4C). Thus, the 120,000-dalton protein was related to gp350/300 and gp250/200 and was not a precursor to gp85.

To analyze the precursor-product relationships between the three pulse-labeled proteins and the two mature forms, advantage was taken of the observation that two EBV-producing cell lines (B95-8 and P3HR-1) synthesize distinctively different levels of mature gp350/300 and gp250/200 (5, 24). This is shown for review in Fig. 3A and B, where it can be seen that the B95-8 cell line produces mostly gp350/300 and the P3HR-1 cell line produces mostly gp250/200.

FIG. 3. Electrophoretic analysis of immunoprecipitates obtained with the rabbit anti-gp350/220 serum from extracts of B95-8 and P3HR-1 cells labeled with [35 S]methionine. (A and B) B95-8 and P3HR-1 cells, respectively, labeled for 18 h (5% SDS-PAGE); (C and E) lentil lectin-Sepharose eluates of B95-8 and P3HR-1 cells, respectively, pulse-labeled for 15 min and immunoprecipitated with the rabbit anti-gp350/220 serum (7.5% SDS-PAGE); (D) and (F) same as (C) and (E), respectively, but digested with endo H for 1 h. Time of exposure was 1 week.

Both the B95-8 and P3HR-1 cell lines were pulse-labeled with [35 S]methionine, detergent extracts were chromatographed on lentil lectin-Sepharose, and the eluates were subjected to immunoprecipitation with the rabbit anti-gp350/220 serum.

Electrophoretic analysis of these immunoprecipitates revealed that each cell line yielded a characteristically different pulse-labeled protein pattern. The B95-8 cell line contained predominantly the 190,000-dalton protein and barely detectable levels of the 160,000- and 120,000-dalton proteins. On the other hand, the P3HR-1 cell line contained predominantly the 160,000-dalton protein with some 190,000-dalton protein and a small amount of the 120,000-dalton protein. Comparison of the relative intensities of pulse-labeled and mature glycoprotein bands suggests that the 190,000-dalton glycoprotein is a precursor to gp350/300 and that the 160,000-dalton glycoprotein is a precursor to gp250/200. This is the first evidence indicating that although gp350/300 and gp250/200 are antigenically related, they are synthesized from separate precursors.

The origin of the 120,000-dalton protein is not known, but since its presence seems to be dependent on a significant level of the 160,000-dalton precursor (compare Fig. 3C and E) and its level varies widely from experiment to experiment (data not shown), it may represent an intracellular degradation product of the 160,000-dalton protein or cross-reacting material produced by premature termination of translation of the 160,000-dalton protein.

Determination of apparent molecular masses of deglycosylated gp350/300 and gp250/200. From the data presented above, it is clear that a significant fraction of the apparent molecular masses of gp350/300 and gp250/200 was the result of extensive post-transcriptional modification. We wished to determine the molecular masses of the polypeptide portion of these glycoproteins to estimate the actual extent of this modification. In contrast to the mature forms, which might contain fully processed, complex-type N-linked oligosaccharides and possibly O-linked glycans as well, precursor forms of the glycoproteins pulse-labeled for 15 min would contain only high-mannose N-linked glycosyl chains. These would be susceptible to endo H, an enzyme which specifically cleaves between the two proximal N-acetylglucosamine residues of high-mannose- but not complex-type N-linked oligosaccharides (34). Thus, after endo H digestion, only a single N-acetylglucosamine residue per N-linked oligosaccharide attachment site would be left on the polypeptide. We have observed that mature gp350/300 and gp250/200 are resistant to digestion with endo H, indicating

that their N-linked glycans are of the complex type (data not shown).

The results of endo H digestion of the SDS-denatured precursors from B95-8 and P3HR-1 cells revealed that for B95-8 cells, the 190,000-dalton precursor was completely sensitive to digestion and a new band appeared at about 170,000 daltons (Fig. 3C and D). In the case of P3HR-1 cells, the 190,000-, 160,000-, and 120,000-dalton proteins were completely sensitive, and new bands appeared at 160,000, 120,000, and 90,000 daltons, respectively (Fig. 3E and F). Neither increasing the time of incubation or amount of enzyme nor adding reducing agents changed the results.

The apparent molecular masses of the endo H-treated pulse-labeled precursors in this experiment appeared to be slightly larger in B95-8-derived than P3HR-1-derived material. This result was confirmed when pulse-labeled precursor proteins from the two cell lines were electrophoretically analyzed on the same gel with or without prior endo H digestion (data not shown). This result may indicate slightly different conformations for these proteins, which in turn may lead to steric protection of one or more glycosyl chains in the B95-8 protein from endo H digestion.

An independent method to determine the molecular masses of the modified polypeptides is to pulse-label cells in the presence of 5 μ g of tunicamycin per ml, which specifically inhibits N-linked glycosylation (16, 33). B95-8 cells were incubated for 15 min with [35 S]methionine in the presence of tunicamycin, and detergent lysates were immunoprecipitated with the rabbit anti-gp350/220 serum. Electrophoretic analysis of the immunoprecipitates showed an EBV-specific protein of 135,000 daltons which comigrates precisely with the product obtained from *in vitro* translation of B95-8 mRNA which codes for gp350/300 (M. Hummel, E. Kieff, and D. A. Thorley-Lawson, manuscript in preparation). It is likely that this polypeptide is a completely deglycosylated form of gp350/300.

Post-translational modification of gp350/300 and gp250/200 from M-ABA cells. Taken together, our results indicate that mature gp350/300 originates from a polypeptide of 135,000 daltons, whereas mature gp250/200 originates from a polypeptide of less than 120,000 daltons. Thus, extensive post-translational modification of these two precursor proteins results in increases in apparent molecular mass of about 220,000 and 100,000 daltons, respectively. These large size differences are shown more clearly in Fig. 4, which shows the mature forms of gp350/300 and gp250/200 from M-ABA cells (Fig. 4A), as well as the corresponding pulse-labeled forms before or after digestion with endo H (Fig. 4C and D,

FIG. 4. Electrophoretic analysis (5% SDS-PAGE) of immunoprecipitates from the M-ABA cell line labeled with [35 S]methionine under different conditions. (A) Immunoprecipitation with the rabbit anti-EBV serum of the ricin lectin-Sepharose eluate of M-ABA cells labeled for 18 h; (B) immunoprecipitation with the rabbit anti-gp350/220 serum of a ricin lectin-Sepharose eluate of M-ABA cells labeled for 18 h in the presence of 5 μ g of tunicamycin per ml; (C) immunoprecipitation of a lentil lectin-Sepharose eluate of M-ABA cells pulse-labeled for 15 min; (D) same as (C) but digested with endo H of 1 h. Time of exposure was 1 week.

respectively). Also included are the forms synthesized during an overnight incubation in the presence of tunicamycin (Fig. 4B). Since tunicamycin blocks asparagine-linked glycosylation (16, 33), the proteins would have contained no such oligosaccharides. Similarly, the endo H-treated pulse-labeled material would contain almost no N-linked carbohydrate. If no other post-translational modifications occurred, polypeptides produced under these two conditions would have approximately the same molecular masses. In fact, the two largest glycoproteins in the tunicamycin-treated material were larger than those in the endo H-digested pulse-labeled material by about 140,000 and 60,000 daltons of apparent molecular mass, respectively. We have shown that this is at least partly the result of extensive O-linked glycosylation (C. M. Edson and D. A. Thorley-Lawson, manuscript in preparation); thus, gp350/300 and gp250/200 contain both N- and O-linked glycosyl chains.

Partial proteolytic mapping of gp350/300 and gp250/200 from the B95-8 cell line. The fact that a monoclonal antibody which precipitates gp350/

300 but not gp250/200 has been described (20) suggests that the coimmunoprecipitation of these two glycoproteins is due to antigenic cross-reactivity rather than to an association in a detergent-stable complex. This is strengthened by the observation that several monoclonal antibodies will precipitate both gp350/300 and gp250/200 in the presence of concentrations of SDS at which the two polypeptides migrate independently on SDS-PAGE (39). However, the question remains as to the origin of this cross-reactivity. Is it carbohydrate specific and related to the extensive glycosylation of these proteins, or is it polypeptide specific and the result of common amino acid sequences? To compare the polypeptides of the two proteins, we carried out partial proteolysis with *S. aureus* V8 protease on the endo H-treated precursors from the B95-8 cell line. We used the endo H-treated precursors to minimize any differences in digestion or peptide migration due to glycosylation. The results

FIG. 5. Comparative partial proteolytic analysis (15% SDS-PAGE) of the endo H-digested precursors of gp350/300 and gp250/200 from the B95-8 cell line. The endo H digest of the rabbit anti-gp350/220 immunoprecipitate of the lentil lectin-Sepharose eluate from B95-8 cells pulse-labeled with [35 S]methionine for 15 min was electrophoresed on 5% SDS-PAGE. Sequential 0.5-cm gel slices were applied to a 15% gel, and digestion with *S. aureus* V8 protease and electrophoresis were carried out. (A) shows the pattern for the gp350/300 precursor. (C) shows the pattern for the gp250/200 precursor. (B) shows the gel slice separating the two precursors and indicates that the material in (C) was not the result of poor slicing of the gel or overlap due to incomplete separation. Time of exposure was 2 months.

FIG. 6. Electrophoretic analysis (7.5% SDS-PAGE) of immunoprecipitates obtained with the rabbit anti-EBV serum from lentil lectin-Sepharose eluates of M-ABA cells pulse-labeled with [35 S]methionine for 15 min and chased for the indicated times. The lentil lectin-Sepharose eluates were precleared once with 5 μ l of the rabbit anti-gp350/220 serum which was sufficient to remove some but not all of the gp350/300- and gp250/200-related proteins. Half of each immunoprecipitate was digested with endo H for 1 h. Lanes 1, 3, 5, 7, and 9 were with the rabbit anti-EBV serum alone, and lanes 2, 4, 6, 8, and 10 were digested with endo H. Time of exposure was 1 week.

show that the peptide profiles for the gp350/300 precursor (Fig. 5A) and the gp250/200 precursor (Fig. 5C) are quite similar. From the number of common peptides seen, it is likely that the antigenic relatedness of the two proteins is, at least in part, the result of shared amino acid sequences.

Synthesis of gp85. The data presented above indicated that none of the three major proteins observed in pulse-labeled material (Fig. 1A) was a precursor to the 85,000-dalton glycoprotein. To focus on the synthesis of this glycoprotein, M-ABA cells were pulse-labeled with [35 S]methionine, and the lentil lectin-Sepharose eluates were immunoprecipitated first with the rabbit anti-gp350/220 serum to preclear most of the gp350/300- and gp250/200-related material and then immunoprecipitated with the rabbit anti-EBV serum. Electrophoretic analysis of the immunoprecipitates (Fig. 6) revealed that synthesis of gp85 followed exactly the same sequence seen in Fig. 1. In the pulse-labeled material, only a small amount of the 85,000-dalton glycoprotein was observed, but upon chasing in nonradioactive medium, a marked increase in the intensity of this band was observed. This result suggests that gp85 is initially

synthesized in a form which is not recognized by the rabbit anti-EBV serum but which is subsequently converted within 1 h to a form which is recognized. This result is not unique to this serum, since a human serum which recognizes gp85 gives the same profile (data not shown). The endo H digestion of the immunoprecipitates revealed that the 85,000-dalton glycoprotein initially yielded a 75,000-dalton polypeptide (Fig. 6B), but that this was replaced by an 81,000-dalton band during the chase (Fig. 6, lanes 3 through 10). This result suggests that mature gp85 contains both endo H-sensitive (high mannose) and endo H-resistant (complex) glycosyl chains and that the shift of the endo H-treated material from 75,000 to 81,000 daltons reflects the processing of some of the high-mannose to complex oligosaccharides during the chase.

Although they were not clearly resolved at 1 h of chase (Fig. 6B), at 0.5 h of chase (Fig. 7D),

FIG. 7. Electrophoretic analysis (7.5% SDS-PAGE) of immunoprecipitates obtained with the rabbit anti-EBV serum from extracts of M-ABA cells pulse-labeled with [35 S]methionine for 15 min and chased for the indicated times. Lanes A, C, E, G, and I were with normal rabbit serum, and lanes B, D, F, H, and J were with the rabbit anti-EBV serum. Time of exposure was 1 week.

TABLE 1. Post-translational modification of three EBV glycoproteins in M-ABA cells

Treatment	Apparent mol wt ($\times 10^3$) ^a					
	gp350/300		gp250/200		gp85	
	Precursor ^b	Mature ^c	Precursor	Mature	Precursor	Mature
Untreated	190	350	160	220	83	85
+ Endo H	160	350	120	220	75	81
+ Tunicamycin	135	300	ND ^d	180	ND	ND

^a Apparent molecular masses determined from electrophoretic mobilities on SDS-PAGE as described in the text.

^b Immunoprecipitated from cells labeled with [³⁵S]methionine for 15 min.

^c Immunoprecipitated from cells incubated with [³⁵S]methionine for 12 to 18 h. Similar results were obtained with cells pulse-labeled for 15 min and chased in nonradioactive medium for 3 h or more.

^d ND, Not determined.

gp85 was present as a clearly resolved dimer of 83,000 and 85,000 daltons. By 1 h of chase, the 83,000-dalton form was barely detectable (Fig. 7F), and it was not seen in the previous experiments since 1 h was the first time of chase. Thus, gp85 seems to be synthesized via an 83,000-dalton precursor. It is likely that the increase from 83,000 to 85,000 daltons is the result of post-translational processing of the glycosyl chains, but its exact nature remains to be determined.

Immunoprecipitation of unglycosylated proteins pulse-labeled with [³⁵S]methionine. In addition to the three EBV glycoproteins analyzed in detail above, we also studied the two major unglycosylated EBV structural proteins: an envelope protein (p140) and the major virus capsid antigen (p160). We wished to determine whether these proteins were synthesized from precursor forms. M-ABA cells were pulse-labeled with [³⁵S]methionine for 15 min and chased in nonradioactive medium. Detergent-soluble extracts were immunoprecipitated with the rabbit anti-EBV serum. Electrophoretic analysis of the immunoprecipitates (Fig. 7) showed that both p140 and p160 were present as prominent protein bands in the 15-min pulse (Fig. 7B) and did not change in size throughout the chase. The intensity of the 160,000-dalton band increased visibly at 0.5 and 1.0 h of chase, concomitant with an increase in the intensity of the 190,000-dalton glycoprotein precursor of gp350/300 (Fig. 7A through H). Thus, the increase in the intensity of the 160,000-dalton band was most likely the consequence of coelectrophoresis of p160 with the 160,000-dalton glycoprotein precursor of gp250/200. By 3 h of chase, little of either glycoprotein precursor remained (Fig. 1C and 7H). These results indicate that no detectable precursor forms are involved in the synthesis of the unglycosylated polypeptides p140 and p160.

DISCUSSION

In previous studies, we identified three EBV glycoproteins, gp350/300, gp250/200, and gp85,

which are present in virions and on the plasma membranes of producer cells (5). We have now extended this work to an examination of the synthesis and processing of these glycoproteins (Table 1). By analyzing pulse-chase experiments with EBV-specific or, gp350/300, gp250/200-specific rabbit antiserum and digestion with endo H, we have identified separate precursor forms for each of these glycoproteins. All three EBV glycoproteins undergo post-translational modification. Over half of the apparent molecular mass of mature gp350/300, for example, is missing in the endo H-treated precursor form of this molecule. Part of this can be attributed to asparagine-linked glycosyl chains, since treatment of the precursor forms with endo H reduces their molecular mass. The results of experiments with tunicamycin, however, indicate that for gp350/300 and gp250/200, N-linked glycosylation accounts for only 50,000 and 40,000 daltons, respectively, half or less of the total post-translational mass increase. In experiments to be described elsewhere, we have determined that these two glycoproteins are extensively O-glycosylated (Edson and Thorley-Lawson, in preparation). In contrast to the EBV glycoproteins, no evidence was found for precursors to the unglycosylated envelope protein p140 or to the capsid protein p160.

The endo H-sensitive precursor to gp85 acquires partial endo H resistance between 1 and 3 h after its synthesis. This is somewhat later than the corresponding transition during the maturation of RNA viruses such as vesicular stomatitis virus (13, 14, 26, 27, 31) and Sindbis virus (26, 27), which acquire endo H resistance 15 to 30 min after synthesis. The intracellular pathway followed by the maturing glycoproteins of the latter two viruses has been shown to proceed by a well-ordered sequence of events from cotranslational glycosylation in the endoplasmic reticulum to addition of terminal sugars such as fucose and sialic acid in the Golgi apparatus to insertion in the plasma membrane, from which the mature virions bud (9, 13, 26, 32). In contrast, EBV, like

other herpesviruses, acquires its envelope by budding through the nuclear membrane, and gp350/300, gp250/200, and possibly gp85 are present in the nuclear fraction of producer cells (5). Thus, future studies of the intracellular maturation pathway followed by EBV glycoproteins may reveal unusual features not seen with the model systems usually employed.

Before the present study, it was not known whether the antigenic cross-reactivity of gp350/300 and gp250/200 derived from shared carbohydrate or polypeptide determinants. Our observation that the partial proteolytic cleavage profiles of the two proteins were quite similar suggests some polypeptide sequence homology which may be sufficient to account for the antigenic cross-reactivity. It is interesting to speculate on the origin of this homology. One possibility is that gp350/300 and gp250/200 originally arose by duplication of an ancestral gene. Alternatively, the two glycoproteins may be translated from mRNAs produced from the same DNA sequence by alternate splicing. This may be resolved when it is determined whether gp350/300 and gp250/200 are synthesized from separate mRNAs which arise from a common region of the EBV genome or whether the mRNAs map to different regions.

In the pulse-chase studies, we were unable to use monoclonal antibodies specific for gp350/300 and gp250/200, either from our (38) or from another (8) laboratory. Although these monoclonal antibodies efficiently immunoprecipitated the mature and tunicamycin-grown forms, they did not immunoprecipitate the pulse-labeled precursors (Edson and Thorley-Lawson, in preparation). It could be that the antibodies recognize conformations of the proteins which are induced during the maturation pathway or that they recognize some specific late post-translational modification which is unrelated to asparagine-linked glycosylation. Work is in progress to determine the nature of the antigenic site(s) recognized by these monoclonal antibodies.

The finding that the 190,000- and 160,000-dalton precursors to gp350/300 and gp250/200 were the same molecular mass in human (P3HR-1) and marmoset (M-ABA and B95-8) cell lines supports our previous work indicating that the mobility difference between the mature forms of these proteins in human and marmoset cells was the result of the differences in the post-translational processing of the oligosaccharides rather than in the polypeptide portion of the molecules. The nature of the oligosaccharide processing difference between cells from the two species, which results in an approximately 10% higher molecular mass for mature gp350/300 and gp250/200 in the marmoset cells (5), remains to be determined.

Finally, the data presented here are particularly relevant to attempts to map the genes of these EBV glycoproteins to the viral genome. Such studies would involve hybrid selection of EBV mRNA, followed by *in vitro* translation and immunoprecipitation. Under these conditions, little or no glycosylation would occur, so it is of particular importance to know the molecular weights of the polypeptide portions of the glycoproteins under examination. Clearly, the mRNAs which encode gp350/300 and gp250/200 will be much smaller than might have been predicted from the sizes of the mature forms of these glycoproteins. In addition, as mentioned above, the extensive glycosylation of the completely processed forms of these glycoproteins could influence their conformations as compared with the unglycosylated forms; it is also possible that some antigenic sites are themselves carbohydrate in nature. Thus, antibodies raised against the mature glycoproteins may not recognize the unglycosylated forms. This can be a problem even with gp85, which is less highly glycosylated than the two large glycoproteins. The currently available human and rabbit anti-EBV sera immunoprecipitated only a small portion of the immature, pulse-labeled form of gp85 and will probably not be useful in mapping studies.

ACKNOWLEDGMENTS

We thank S. C. Hubbard for helpful discussions and a critical analysis of this manuscript. We also thank Cheryl Stafford for her invaluable secretarial assistance and Emily Ferris for providing cells used in these studies. The work was initiated when we were at the Sidney Farber Cancer Institute, Boston, Mass.

This work was supported by Public Health Service grants AI15310 and CA28737 from the National Institutes of Health.

LITERATURE CITED

1. Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* **252**:1102-1106.
2. Crawford, D. H., M. A. Epstein, G. W. Bornkamm, B. G. Achong, S. Finerty, and J. L. Thompson. 1979. Biological and biochemical observations on isolates of EB virus from the malignant epithelial cells of two nasopharyngeal carcinomas. *Int. J. Cancer* **24**:294-302.
3. Dolyniuk, M., R. Pritchett, and E. Kieff. 1976. Proteins of Epstein-Barr virus. I. Analysis of the polypeptides of purified enveloped Epstein-Barr virus. *J. Virol.* **17**:935-949.
4. Dolyniuk, M., E. Wolff, and E. Kieff. 1976. Proteins of Epstein-Barr virus. II. Electrophoretic analysis of the polypeptides of the nucleocapsid and the glucosamine- and polysaccharide-containing components of enveloped virus. *J. Virol.* **18**:289-297.
5. Edson, C. M., and D. A. Thorley-Lawson. 1981. Epstein-Barr virus membrane antigens: characterization, distribution, and strain differences. *J. Virol.* **39**:172-184.
6. Epstein, M. A., and B. G. Achong (ed.). 1979. *The Epstein-Barr virus*. Springer-Verlag, New York.
7. Hanover, J. A., J. Elting, G. R. Mintz, and W. J. Lennarz. 1982. Temporal aspects of the N- and O-glycosylation of

- human chorionic gonadotropin. *J. Biol. Chem.* **257**:10172-10177.
8. Hoffman, G. J., S. G. Lazarowitz, and S. D. Hayward. 1980. Monoclonal antibody against a 250,000 dalton glycoprotein of Epstein-Barr virus identifies a membrane antigen and a neutralizing antigen. *Proc. Natl. Acad. Sci. U.S.A.* **77**:2979-2983.
 9. Hubbard, S. C., and R. J. Ivatt. 1981. Synthesis and processing of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* **50**:555-583.
 10. Hubbard, S. C., and P. W. Robbins. 1980. Synthesis of the N-linked oligosaccharide and its relation to protein synthesis *in vivo*. *J. Biol. Chem.* **255**:11782-11798.
 11. Kallin, B., J. Luka, and G. Klein. 1979. Immunochemical characterization of Epstein-Barr virus-associated early and late antigens in *n*-butyrate-treated P3HR-1 cells. *J. Virol.* **32**:710-716.
 12. Kessler, S. 1975. Rapid isolation of antigens from cells with a staphylococcal protein A antibody absorbent. *J. Immunol.* **115**:1617-1624.
 13. Knipe, D. M., D. Baltimore, and H. F. Lodish. 1977. Separate pathways of maturation of the major structural proteins of vesicular stomatitis virus. *J. Virol.* **21**:1128-1139.
 14. Knipe, D. M., H. F. Lodish, and D. Baltimore. 1977. Localization of two cellular forms of the vesicular stomatitis viral glycoprotein. *J. Virol.* **21**:1121-1127.
 15. Krangel, M. S., H. T. Orr, and J. L. Strominger. 1979. Assembly and maturation of HLA-A and HLA-B antigens *in vivo*. *Cell* **18**:979-991.
 16. Kuo, S.-C., and J. O. Lampen. 1974. Tunicamycin—an inhibitor of yeast glycoprotein synthesis. *Biochem. Biophys. Res. Commun.* **58**:287-295.
 17. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
 18. Mueller-Lantzsch, N., B. Georg, N. Yamamoto, and H. ZurHausen. 1980. Epstein-Barr virus-induced proteins. III. Analysis of polypeptides from P3HR-1-EBV-superinfected NC37 cells by immunoprecipitation. *Virology* **102**:231-233.
 19. Mueller-Lantzsch, N., B. Georg, N. Yamamoto, and H. ZurHausen. 1980. Epstein-Barr virus-induced proteins. II. Analysis of surface polypeptides from EBV-producing and superinfected cells by immunoprecipitation. *Virology* **102**:401-411.
 20. Mueller-Lantzsch, N., B. Georg-Fries, H. Herbst, H. ZurHausen, and D. G. Braun. 1981. Epstein-Barr virus strain- and group-specific antigenic determinants detected by monoclonal antibodies. *Int. J. Cancer* **28**:321-327.
 21. Mueller-Lantzsch, N., N. Yamamoto, and H. ZurHausen. 1979. Analysis of early and late Epstein-Barr virus associated polypeptides by immunoprecipitation. *Virology* **97**:378-387.
 22. North, J. R., A. J. Morgan, and M. A. Epstein. 1980. Observations on the EB virus envelope and virus determined membrane antigen (MA) polypeptides. *Int. J. Cancer* **126**:231-240.
 23. Qualtiere, L. F., and G. R. Pearson. 1979. Epstein-Barr virus-induced membrane antigens: immunochemical characterization of Triton X-100 solubilized viral membrane antigens from EBV-superinfected Raji cells. *Int. J. Cancer* **23**:808-817.
 24. Qualtiere, L. F., and G. R. Pearson. 1980. Radioimmune precipitation study comparing the Epstein-Barr virus membrane antigens expressed on P3HR-1 virus-superinfected Raji cells to those expressed on cells in a B95-8 virus-transformed producer culture activated with tumor-promoting agent (TPA). *Virology* **102**:360-369.
 25. Raab-Traub, N., T. Dambaugh, and E. Kleff. 1980. DNA of Epstein-Barr virus. VIII. B95-8, the previous prototype, is an unusual deletion derivative. *Cell* **22**:257-269.
 26. Robbins, P. W., S. C. Hubbard, S. J. Turco, and D. F. Wirth. 1977. Proposal for a common oligosaccharide intermediate in the synthesis of membrane glycoproteins. *Cell* **12**:893-900.
 27. Schmidt, M. F. G., and M. J. Schlesinger. 1980. Relation of fatty acid attachment to the translation and maturation of vesicular stomatitis and Sindbis virus membrane glycoproteins. *J. Biol. Chem.* **255**:3334-3339.
 28. Skare, J., C. Edson, J. Farley, and J. L. Strominger. 1982. The B95-8 isolate of Epstein-Barr virus arose from an isolate with a standard genome. *J. Virol.* **44**:1088-1091.
 29. Strnad, B. C., R. H. Neubauer, H. Rabin, and R. A. Mazur. 1979. Correlation between Epstein-Barr virus membrane antigen and three large cell surface glycoproteins. *J. Virol.* **32**:885-894.
 30. Strnad, B. C., T. Schuster, R. Klein, R. F. Hopkins III, T. Witmer, R. H. Neubauer, and H. Rabin. 1982. Production and characterization of monoclonal antibodies against the Epstein-Barr virus membrane antigen. *J. Virol.* **41**:258-264.
 31. Strous, G. J. A. M., and H. F. Lodish. 1980. Intracellular transport of secretory and membrane proteins in hepatoma cells infected by vesicular stomatitis virus. *Cell* **22**:709-717.
 32. Tabas, I., S. Schlesinger, and S. Kornfeld. 1978. Processing of high mannose oligosaccharides to form complex type oligosaccharides on the newly synthesized polypeptides of the vesicular stomatitis virus G protein and the IgG heavy chain. *J. Biol. Chem.* **253**:716-722.
 33. Takatsuki, A., and G. Tamura. 1971. Effect of tunicamycin on the synthesis of macromolecules in cultures of chick embryo fibroblasts infected with Newcastle disease virus. *J. Antibiot. (Tokyo)* **24**:785-794.
 34. Tarentino, A. L., and F. Maley. 1974. Purification and properties of an endo- β -N-acetylglucosaminidase from *Streptomyces griseus*. *J. Biol. Chem.* **249**:811-817.
 35. Thorley-Lawson, D. A. 1979. Characterization of cross-reacting antigens on the Epstein-Barr virus envelope and plasma membranes of producer cells. *Cell* **16**:33-42.
 36. Thorley-Lawson, D. A., and C. M. Edson. 1979. Polypeptides of the Epstein-Barr virus membrane antigen complex. *J. Virol.* **32**:458-467.
 37. Thorley-Lawson, D. A., C. M. Edson, and K. Geilinger. 1982. Epstein-Barr virus antigens—a challenge to modern biochemistry. *Adv. Cancer Res.* **36**:295-348.
 38. Thorley-Lawson, D. A., and K. Geilinger. 1980. Monoclonal antibodies against the major glycoprotein (gp350/220) of Epstein-Barr virus neutralize infectivity. *Proc. Natl. Acad. Sci. U.S.A.* **77**:5307-5311.
 39. Thorley-Lawson, D. A., and C. A. Poodry. 1982. Identification and isolation of the main component (gp350-gp220) of Epstein-Barr virus responsible for generating neutralizing antibodies *in vivo*. *J. Virol.* **43**:730-736.
 40. Wells, A., N. Koide, and G. Klein. 1982. Two large virion envelope glycoproteins mediate Epstein-Barr virus binding to receptor-positive cells. *J. Virol.* **41**:286-297.
 41. ZurHausen, H., G. W. Bornkamm, R. Schmidt, and E. Heckler. 1979. Tumor initiators and promoters in the induction of Epstein-Barr virus. *Proc. Natl. Acad. Sci. U.S.A.* **76**:782-785.