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Biochemical Characterization of Epstein-Barr Virus Membrane Antigen Associated Glycoproteins

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The Epstein-Barr Virus membrane antigen has been associated with at least four proteins on the basis of radioimmunoprecipitation experiments. The largest of these proteins has now been purified to electrophoretic homogeneity by a combination of lentil lectin - Sepharose chromatography and electrophoresis on acrylamide gel, and the amino acid composition of this protein has been determined.

Radioimmunoprecipitation experiments performed in several laboratories have recently established correlations between Epstein-Barr virus (EBV) membrane antigen (MA) and several glycoproteins found in infected cells (1,2,3,4,5,6,7,8). In our laboratory immunoprecipitation experiments were performed with human sera and B95-8 cells which were induced for EBV production with 12-O-tetradecanoylphorbol-13-acetate (TPA) (9) and surface radiolabeled by the neuraminidase-galactose oxidase sodium borohydride [NGO-NaB(^{3}H)$_4$] technique (10). This protocol resulted in the specific precipitation of three glycoproteins at 236,000 (236K), 212K, and 141K. A fourth protein at 86K was specifically immunoprecipitated when TPA induced B95-8 cells labeled with a tritiated amino acid mixture were used as the antigen source (2). Purification of these proteins is a necessary step in firmly establishing their identity as MA. In this paper we report the purification of the largest EBV-MA associated glycoprotein.

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MATERIALS AND METHODS

Lentil lectin (LcH) Sepharose chromatography

B95-8 cells were treated for three days either with TPA alone (29% MA positive cells), or with both TPA and 100 µg of phosphonoacetic acid (PAA) per ml (1.8% MA positive cells) to inhibit the synthesis of late viral proteins (12). Each group of cells was divided in half. One half was radiolabeled with a tritiated amino acid mixture (3 µC per ml of RPMI 1640 containing 5% the normal concentration of amino acids) overnight, prior to harvest on the 3rd day and treatment with neuraminidase and galactose oxidase. The other half of each group was labeled by the NGO-NaB(^{3}H)$_4$ technique immediately following harvest. Cytoplasmic lysates were prepared from all four groups of cells. The lysates were made 0.15M in NaCl, mixed with LcH-Sepharose (E-Y Laboratories, Inc.; 1.5 mg of lectin/ml of Sepharose) at a ratio of 25 mg of cytoplasmic protein per ml of Sepharose, and incubated for 2 h at 5°C with constant mixing. The LcH-Sepharose was washed four times with 10 volumes of 0.01M Tris-HCl, 0.15M NaCl, 1mM $MnCl_2$, 1mM $CaCl_2$, 1mM $MgCl_2$, pH7.6 and then three times with 3 volumes of .01 M Tris-HCl, 0.8 M α-methylD-mannoside, 0.01 M sodium deoxycholate, pH 7.6 (αMMD). The cytoplasmic lysates, LcH-Sepharose flow through, and αMMD eluate fractions were electrophoresed on a 7% acrylamide gel in 0.1% SDS (13) and the mobilities of the proteins determined by fluorography (14). The amount of protein in various regions of the gels was quantitated by slicing the gels into 2 mm wide segments and counting in 1 ml of NCS Tissue Solubilizer (Amersham) and 8 ml of Econofluor (New England Nuclear).

Purification of the 236K EBV MA associated glycoprotein

Cytoplasmic lysates were prepared simultaneously from unlabeled and NGO-NaB(^{3}H)$_4$ labeled, TPA induced B95-8 cells. The lysates were combined at a ratio of 9 mg of nonradioactive protein to 1 mg of labeled protein, and 30-32 mg of protein were chromatographed on 1.3 ml of LcH-Sepharose as described above. The efficiency of protein binding and elution was monitored by counting aliquots of the eluates in Aquasol-2. In replicate experiments the percentage of NGO-NaB(^{3}H)$_4$ labeled glycoproteins bound ranged from 28 to 40 percent depending on the degree of MA induction. All of the bound glycoproteins were eluted with αMMD and then dialysed against two, 500 ml volumes of 0.05% SDS. The protein solution was made 1.4% in SDS and 0.4M in β mercaptoethanol by the addition of concentrated solutions, and electrophoresed on an 8% acrylamide, 0.2% bisacrylamide gel slab (12 cm x 14 cm x 0.15 cm) in the presence of 0.1% SDS. After electrophoresis a thin vertical section was cut from the slab for staining and fluorography. The remainder of the slab was stored at -70°C until the autoradiogram was developed. The glycoprotein pattern on the autoradiogram was used as a guide for removing a horizontal slice from the slab covering the full width of the 236K band. The gel slice was homogenized by passage four times through a 20 gauge orifice. Then the glycoprotein was electrophoresed out of the gel into a dialysis bag in 0.05M Tris-HCl, 0.1% SDS, pH8.5, using a field strength of 200 volts for 3 h. Under these conditions approximately 70% of the protein was recovered. The purified glycoprotein was dialysed in several changes of PBS to remove SDS. Aliquots were reelectrophoresed on acrylamide gels to monitor the accuracy of the slicing procedure.

Amino acid analyses of the 236K glycoprotein

The mole percent of each amino acid except cysteine and tryptophan was determined in duplicate samples on a Durrum D 500 amino acid analyzer following 24 h hydrolysis in 6N HCl, 0.1% phenol at 115°C. Since the protein

was purified by electrophoresis in a buffer containing high molarity glycine, the mole % glycine was corrected for the amount of glycine found in duplicate unhydrolysed samples of the protein. The mole percent cysteine was determined in duplicate samples by converting cysteine to cysteic acid in performic acid (9 volumes of formic acid; 1 volume of 30% H_2O_2) for 16 h at 0°C followed by hydrolysis in 6N HCl, 0.1% phenol.

RESULTS AND DISCUSSION

The glycoprotein nature of these antigens suggested that lectin affinity chromatography would be a useful means of purification. Initial experiments with soybean lectin columns indicated that the 236K glycoprotein bound to this lectin but that it could not be efficiently eluted with N-acetyl-D-galactosamine (2). Therefore, chromatography on Lens culinaris lectin (LcH), which has a lower affinity for some glycoproteins, was tried. Cytoplasmic lysates were prepared from TPA or TPA/PAA B95-8 cells that had been radiolabeled with the NGO-NaB $(^3H)_4$ technique or with a tritated amino acid mixture. The radiolabeled lysates were chromatographed on LcH-Sepharose and Figure 1 illustrates the electrophoretic patterns of the eluted proteins. Tracks A, B,

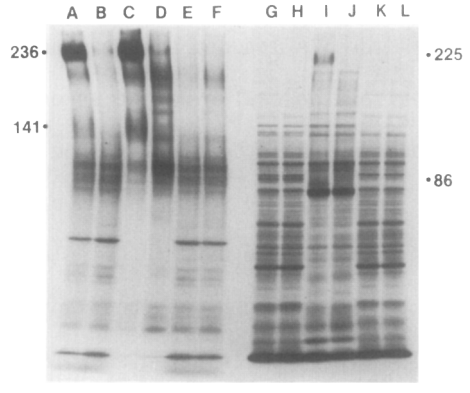

Figure 1 - Autoradiogram of the eluates from the LcH-Sepharose chromatography of TPA and TPA/PAA B95-8 cell cytoplasmic lysates. Tracks A to F, NGO-NaB$(^3H)_4$ surface glycoprotein label. Tracks G to L, ^3H amino acid protein label. Tracks A to C and G to I, TPA B95-8. Tracks D to F and J to L, TPA/PAA B95-8. Tracks A, F, G and L, cytoplasmic lysates. Tracks B, E, H and K, flow through fractions. Tracks C, D, I and J, αMMD eluates. Electrophoresis was carried out on a 7% acrylamide gel slab. Each track contained 30279 dpm of radiolabeled protein. The numbers in the margins indicate the molecular weights in thousands.

and C show that LcH-Sepharose bound the 236 and 141K glycoproteins and that αMMD eluted these glycoproteins efficiently along with a number of other proteins. In contrast, the αMMD eluate obtained following chromatography of the TPA/PAA B95-8 cytoplasmic lysate (track D) contained only a small amount of the 236K glycoprotein. The MA associated 236K glycoprotein found in extracts of TPA/PAA treated cells probably represents the MA synthesized by the subset of cells in the population (i.e., 1.8%) that continue to make MA even in the presence of PAA.

The results of the complementary experiment performed with cells labeled with a tritiated amino acid mixture are shown in tracks G to L. Comparison of the LcH-Sepharose αMMD eluates obtained from TPA and TPA/PAA B95-8 cells (tracks I and J) indicated that two proteins at 225 and 86K were present in TPA B95-8 and absent from TPA/PAA B95-8. On 7% acrylamide gels, the 225K protein detected with an ^{3}H amino acid label has a molecular weight slightly lower than the 236K protein detected with NGO-NaB $(^{3}H)_4$. This result suggests that the NGO-NaB$(^{3}H)_4$ technique predominantly labels the fully glycosylated form of the antigen on the cell surface and that the majority of the antigen inside TPA induced cells is not fully glycosylated. The protein at 86K has the same molecular weight as one of the antigens specifically precipitated from TPA induced cells labeled with an amino acid mixture. This protein is probably a glycoprotein because it binds to LcH-Sepharose and appears as a diffuse band in gel electrophoresis. However, as previously described, the 86K antigen does not radiolabel well with NGO-NaB$(^{3}H)_4$ (2). The 141K glycoprotein detected with NGO-NaB$(^{3}H)_4$ labeling, was not detected when a ^{3}H amino acid label was employed in either the previous immunoprecipitation experiments (2) or the LcH-Sepharose chromatography experiments described above, suggesting that it is present in a smaller amount than the 236 and 86K glycoproteins.

In replicate experiments an average of 5.5% of the ^{3}H amino acid labeled, TPA induced, B95-8 cytoplasmic protein eluted from LcH-Sepharose with

αMMD resulting in an 18 fold purification of the MA associated glycoproteins. When acrylamide gels were sectioned and counted, 3.4% and 0.7% of the ^{3}H amino acid labeled αMMD eluates were found in the 225K and 86K proteins respectively. This means that the 225K and 86K proteins constitute approximately 0.19% and 0.04% of the mass of cytoplasmic protein in TPA induced B95-8 cells. Therefore, the relative number of moles of these two glycoproteins in the cytoplasmic lysate were similar.

Attempts at further purifying the MA associated glycoproteins on DEAE and ECTEOLA celluose columns at pH7 were unsuccessful because although the glycoproteins bound to these columns, they eluted at similar salt concentrations. We therefore turned to the technique of preparative acrylamide gel electrophoresis to prepare pure glycoproteins. We began by purifying the 236K glycoprotein because it could be readily separated from the other components of the LcH-Sepharose αMMD eluate by electrophoresis.

Typical results shown in Fig. 2 indicated that chromatography on LcH-Sepharose followed by electrophoretic separation on SDS acrylamide gels purified the 236K glycoprotein to electrophoretic homogeneity. Amino acid analyses were performed on the 236K antigen and the results are shown in Table 1. An empirical molecular weight of 9,370 was calculated on the basis of the these amino acid analyses assuming the presence of at least one meth-

Figure 2 - Autoradiogram of the purified 236K glycoprotein obtained by sequential LcH-Sepharose chromatography and SDS acrylamide gel electrophoresis. The number in the margin indicates the molecular weight in thousands. IB indicates the ion boundary.

TABLE 1

AMINO ACID COMPOSITION OF THE 236K EBV-MA ASSOCIATED GLYCOPROTEIN

	Mole %
Aspartic acid	9.9
Glutamic acid	11.0
Threonine	9.8
Serine	7.2
Cysteine	1.6
Methionine	1.1
Proline	7.5
Glycine	7.3
Alanine	7.8
Valine	6.3
Isoleucine	3.9
Leucine	9.4
Tyrosine	3.0
Phenylalanine	3.8
Histidine	1.7
Lysine	4.9
Arginine	3.5

ionine residue. It is therefore likely that even the simplest peptide map of this antigen employing a methonine label will have at least 24 spots. A minimum of 0.5 nanomole of protein is required for an N terminal amino acid sequence determination, assuming that the N terminal is not blocked. This corresponds to at least 120 µg of the 236K antigen. The purification procedure described in this paper yields 10 to 15 µg of protein per run. Mice are now being immunized with these preparations to make monoclonal antibody to the 236K antigen. Purification of the larger quantities of protein required for a more complete biochemical analysis of the antigen will be aided by the development of immunoaffinity columns which can be readily scaled up in size. This work is now in progress.

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