Characterization of the Restricted Component of Epstein-Barr Virus Early Antigens as a Cytoplasmic Filamentous Protein

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Four monoclonal antibodies produced against the restricted component of the Epstein-Barr virus (EBV) early antigen (EA-R) precipitated a polypeptide with an approximate molecular weight of 85,000. Three of these antibodies prepared against the native 85,000-molecular-weight protein (85K protein) reacted by immunofluorescence with acetone-fixed smears but not methanol-fixed smears of EBV-producing cells activated with tumor-promoting agent and sodium butyrate. The fourth monoclonal antibody which was produced against the denatured 85K protein reacted with both acetone-fixed cells and methanol-fixed cells. Blocking of direct immunofluorescence by the different monoclonal antibodies established that these monoclonal antibodies were directed against three different epitopes expressed on the 85K protein. The cytoplasmic staining pattern produced by each antibody was granular during the first 24 to 28 h after induction, developed into filamentous structures about 36 h after induction, and then began to aggregate after 48 h. Similar structures were observed in human placental cells transfected by EBV DNA and stained with three of the monoclonal antibodies. These results suggest that the EA-R polypeptide is assembled into filaments during the EBV lytic cycle. The significance of this in regards to replication has yet to be determined. Biochemical characterization of this major EA-R component did not reveal any major differences in this protein isolated from different cell lines.

Several antigen complexes have been identified in Epstein-Barr virus (EBV)-infected cells by immunofluorescence and immunoprecipitation procedures. These include the EBV-induced nuclear antigens, the viral capsid antigens, the EBV-induced early antigens (EA), and the virus-specified membrane-associated antigens (5). EA can be induced in latently infected cell lines by superinfection with P3HR-1 virus or by treatment with chemical inducers (5). The EA complex has been divided into diffuse (EA-D) and restricted (EA-R) components based on staining patterns with different human sera (5). The EA-D staining is located both in the nucleus and the cytoplasm and is resistant to denaturation by methanol. In contrast, the EA-R staining is restricted to the cytoplasm in acetone-fixed cells and is destroyed by methanol fixation. There have been numerous efforts to characterize the EA complex largely because of the specific antibody response patterns to this complex which are of prognostic value. Anti-EA reactivity of sera from patients with infectious mononucleosis and nasopharyngeal carcinoma is directed primarily against the EA-D component, whereas reactivity in sera from patients with African Burkitt's lymphoma is directed mainly against the EA-R component (5-7).

Monoclonal antibodies has been developed which recognize major polypeptides of the EA-D and EA-R components (2, 19). The EA-D component is a protein with multiple molecular weights ranging between 47,000 and 56,000, while the EA-R component is one polypeptide with a molecular weight of about 85,000 (19). The EA-D polypeptides have been mapped to the *Bam*HI M restriction endonuclease fragment of EBV DNA by hybrid selection of early mRNA and by the demonstration that the selected RNA encoded for a polypeptide which was immunoreactive with polyvalent anti-EA positive sera and with the anti anti-EA-D monoclo-

nal antibody (19). More recently, by using amino acid sequencing, the coding region for the EA-D polypeptide was verified (J. Luka and G. R. Pearson, unpublished data). However, the coding region for the EA-R polypeptide still has not been mapped conclusively, although Glaser et al. (4) reported that the gene encoding for this protein mapped to the *Bam*HI H fragment as determined by transfection. Other studies indicated, however, that several regions for EBV DNA might be necessary for the expression of this antigen (23).

In this manuscript, we now report on the further characterization of the EA-R component in superinfected and chemically induced EBV genome-positive cell lines. In addition, through the use of monoclonal antibodies, the EA-R component is shown to be present in cells supporting the virus replication cycle as cytoplasmic filaments which undergo structural changes during the lytic cycle.

MATERIALS AND METHODS

Cell lines. The lymphoblastoid cell lines used in these experiments include the EBV genome-positive Raji, P3HR-1, IB-4, Daudi, and B95-8 cell lines and the EBV genome-negative Ramos, Loukes, and BJAB cell lines. All cell lines were cultivated in RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum. The cells were passaged every 3 or 4 days by reseeding in fresh medium. For antigen induction, the cells were suspended in fresh medium with 3 mM sodium butyrate and 20 ng of tumor-promoting agent (TPA) per ml. In some experiments, Raji cells were also superinfected with P3HR-1 virus for viral antigen production.

Production of monoclonal antibodies to EA-R. K8, K9, and R63 monoclonal antibodies were produced as described earlier (19). BALB/c mice were immunized three times with P3HR-1 cells activated with 20 ng of TPA per ml and 3 mM sodium butyrate for 48 h. Spleen cells were fused with NS-1 cells 4 days after the last immunization. The monoclonal

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antibody designated 85K was produced by immunizing mice with the antibody R63 affinity-purified and denatured form of the EA-R polypeptide. The mice were immunized four times with 25 μ g of purified protein in the presence of 0.1% sodium dodecyl sulfate (SDS). Spleen cells were prepared 4 days after the last immunization, and the cells were fused to NS-1 cells by the method of Vroman et al. (24). Supernatants from wells with visible growth were tested for antibodies by enzyme-linked immunosorbent assay (ELISA) with affinity-purified antigens as the target (10) and by immunofluorescence on virus-producing and nonproducer cell lines (18). Positive clones were also tested by immunoblotting against extracts prepared from producer cell lines as previously described (11).

Labeling of cells with radioisotopes. For labeling viral antigens with [35 S]methionine, viable B-95 or P3HR-1 cells (10^6 cells per ml) were incubated in complete medium supplemented with 20 ng of TPA per ml plus 3 mM sodium butyrate. At different times after induction, the cells were centrifuged at $900 \times g$ for 5 min, washed once with methionine-free medium containing 5% dialyzed fetal calf serum and 2 mM L-glutamine, and then suspended in minimal essential medium containing 3 mM sodium butyrate at a cell concentration of 4×10^6 to 5×10^6 cells per ml. [35 S]methionine was then added to each flask to give a final concentration of 50 μ Ci/ml, and the cultures were incubated at 37°C for 6 h. For labeling with [32 P]phosphate, cells were washed twice in phosphate-free medium and resuspended in this medium supplemented with 5% dialyzed fetal calf serum and 3 mM sodium butyrate at a cell concentration of 10^6 cells per ml. [32 P]phosphate was then added to a final concentration of 200 μ Ci/ml, and the cultures were incubated at 37°C for 6 h. In all cases, after labeling, the cells were pelleted and extracted as described below.

Cell extraction and immune precipitation. Labeled cells were extracted with 0.5% Nonidet P-40 in 20 mM Tris hydrochloride (pH 7.4)–0.15 M NaCl–2 mM EDTA–1.0 mM phenylmethylsulfonyl fluoride and then were sonicated for 15 s. The supernatants were clarified by centrifugation at $38,000 \times g$ for 60 min in an SS34 rotor (Ivan Sorvall, Inc., Norwalk, Conn.), and immune precipitates were formed by mixing portions of the labeled extracts with either antibody-positive or antibody-negative ascitic fluid or human sera as described previously (20–22). After 1 h of incubation at room temperature, immune complexes were removed with protein A-Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.), washed four times with extraction buffer, boiled for 3 min in SDS sample buffer, and analyzed by SDS-polyacrylamide gel electrophoresis as previously described (20–22). Standards were 125 I-labeled myosin (molecular weight, 210,000), phosphorylase B (92,500), bovine serum albumin (67,000), ovalbumin (45,000), and carbonic anhydrase (30,000) (New England Nuclear Corp., Boston, Mass.).

Peptide mapping. Viral proteins were purified by incubating cell extracts with monoclonal antibodies conjugated to Affigel-10 beads for 1 h at room temperature as previously reported (10). The beads were then washed four times with Nonidet P-40 extraction buffer without phenylmethylsulfonyl fluoride and then washed twice with extraction buffer lacking both phenylmethylsulfonyl fluoride and Nonidet P-40. The washed beads were next apportioned into three tubes. Two tubes received 1 or 2 μ g of V8 protease. One tube was designated as the control and received no enzyme. The tubes were incubated at room temperature for 30 min and then immediately diluted with Laemmli sample

buffer (68 mM Tris hydrochloride [pH 6.8], 3% SDS, 5% 2-mercaptoethanol, 0.01% bromphenol blue, 10% glycerol) and boiled for 3 min. The samples were loaded onto a 5 to 15% exponential SDS-polyacrylamide gel, and after electrophoresis, the gel was prepared for fluorography as previously described (20–22).

Two-dimensional gel electrophoresis. The purified protein isolated from the P3HR-1 cell line with affinity columns prepared with different monoclonal antibodies was subjected to two-dimensional gel electrophoresis by using the procedure described by O'Farrell (14) with a pH gradient between 3.5 and 10. The pH in the first dimension was measured on a tube gel run in parallel which was fractionated into 5-mm slices. After homogenization in distilled H₂O, the pH was determined on each slice and plotted. The pI of the 85K protein was calculated from this plot.

Immunofluorescence procedure. Acetone- or methanol-fixed smears of activated cells were stained by immunofluorescence as previously described in detail (8, 19, 24) by using fluorescein-conjugated goat anti-mouse immunoglobulin G (IgG). The stained smears were mounted in 50% buffered glycerol and then examined with a fluorescence microscope (American Optical Corp., Buffalo, N.Y.).

Immunoblotting. After SDS gel electrophoresis, the gels were transferred to a blotting apparatus (Bio-Rad Laboratories, Richmond, Calif.) and electrophoretically transferred into nitrocellulose paper in glycine buffer (pH 8.3) containing 20% methanol at 90 V for 2 h at 10°C. After protein transfer, the nitrocellulose paper was incubated for 30 min at room temperature with 20 mM Tris hydrochloride (pH 7.4)–150 mM NaCl–0.5% Tween 20 (TBS-T). The paper was then incubated for 60 min with the different ascites fluids containing the monoclonal antibodies which were generally diluted 1:400 in TBS-T. The antibody titers in these ascites preparations were generally over 10,000 as determined by immunofluorescence. After the paper was washed twice for 10 min with 50 ml of TBS-T, further incubation was performed with alkaline phosphate-conjugated anti-mouse γ -globulin (dilution 1:400, according to the specifications of the manufacturer [Sigma Chemical Co., St. Louis, Mo.]) for 60 min at room temperature. The paper was then washed for 15 min with TBS-T, and the blots were developed for 2 min in 100 mM Tris hydrochloride (pH 9.4) containing 1 mg of Naphthol AS-BI phosphate (Sigma) per ml and 1 mg of fast violet (Sigma) per ml. The developed blots were washed with water for 5 min and then dried between filter papers.

Labeling of monoclonal antibodies with FITC. One milligram of protein A-Sepharose-purified IgG for each monoclonal antibody was dialyzed against 0.1 M carbonate buffer (pH 9.5) for 4 h. After this initial dialysis, the IgGs were dialyzed further against 10 volumes of borate buffer (0.1 M; pH 8.2) containing 0.1 mg of fluorescein isothiocyanate (FITC) per ml for 24 h. The labeled proteins were then separated from free FITC on a Sephadex G-25 column and then dialyzed against phosphate-buffered saline (pH 7.4).

Blocking of immunofluorescence produced by FITC-labeled monoclonal antibodies by nonlabeled antibodies. Acetone-fixed cells from TPA- and sodium butyrate-activated P3HR1 or Raji cells were incubated with different dilutions (1:10 to 1:640) of unlabeled ascitic fluid containing antibodies to the 85K protein for 1 h. The slides were washed three times with phosphate-buffered saline, and the cells were incubated with FITC-labeled antibodies at a 1:50 dilution (20 μ g of IgG per ml) for 1 h. After a final wash in phosphate-buffered saline, the fluorescence was read on a fluorescence microscope (American Optical Corp., Buffalo, N.Y.). Blocking was

recorded as complete inhibition of the staining of cells by the labeled conjugates.

RESULTS

Characterization of monoclonal antibodies to purified EA(R). The production and characterization of a monoclonal antibody to a major protein of the EA-R complex was previously reported (19). This monoclonal antibody precipitated a major EBV protein with a molecular weight of 85,000. Since that publication, a number of new monoclonal antibodies against this protein have been produced which showed somewhat different precipitation patterns. These patterns are shown in Fig. 1. Three (K8, K9, R63) of the four monoclonal antibodies precipitated a protein with a molecular weight of approximately 130,000 as well as the 85K protein (Fig. 1, lanes D through F). The fourth monoclonal antibody, designated 85K, precipitated only the 85,000-molecular-weight protein (85K protein) (Fig. 1, lane G).

FIG. 1. Immunoprecipitation of polypeptides from TPA- and sodium butyrate-activated P3HR-1 cells by four monoclonal antibodies directed against EA-R. Cells were labeled 32 to 38 h after induction with [35 S]methionine. Lanes A, B, and H, Three human sera containing antibodies to EBV; lane C, human anti-EBV-negative serum; lanes D, E, and F, three monoclonal antibodies (R63, K8, and K9, respectively) prepared against the native EA-R antigen; lane G, a monoclonal antibody (85K) prepared against the denatured form of the major EA-R polypeptide; lane I, an anti-cytomegalovirus antibody-positive monoclonal antibody used as a negative control.

FIG. 2. Immunoblotting with different monoclonal antibodies against the 85K protein of the EA-R complex (lanes 1) or against the 50K to 52K component of the EA-D complex (lanes 2). The monoclonal antibody in lanes A (85K) was prepared against the denatured 85K protein, while the monoclonal antibodies in lanes B (R63), C (K8), and D (K9) were prepared against the native form of this protein. Antibody in lanes E is the monoclonal antibody (R3) directed against the 50K to 52K component of EA-D. The contaminating band (50K) in lane 1 of each precipitate is the heavy chain of IgG which was eluted from the affinity column with the 85K and 50K to 52K proteins and which reacted with the 125 I-labeled anti-mouse IgG reagent in this overexposed gel.

Interestingly, this monoclonal antibody was prepared against the denatured 85K protein and was therefore directed against an epitope expressed on the primary structure of this molecule. The other monoclonal antibodies were prepared against the native 85K protein. Another interesting difference between this monoclonal antibody and those prepared against the native molecule was that only the monoclonal antibody prepared against the denatured protein routinely reacted with the 85K protein in immunoblotting assays and ELISAs (Fig. 2). The four monoclonal antibodies plus a monoclonal antibody prepared against the major EA-D protein with a molecular weight of 50,000 to 52,000 were tested by immunoblotting against the affinity gel-isolated 85K protein and the purified 50K/52K protein. The purified 85K protein in this gel was prepared with antibody R63. However, the same results were obtained by using the 85K protein purified with the other monoclonal antibodies. Only the monoclonal antibody (85K) prepared against the denatured 85K protein reacted strongly with this protein in the immunoblotting experiment (Fig. 2, lane A1). Another monoclonal antibody (R63) prepared against the native EA-R protein sometimes reacted weakly in this assay (Fig. 2, lane B1), while neither of the other two monoclonal antibodies to EA-R (lanes C1 and D1) or the monoclonal antibody to EA-D (lane E1) reacted with the 85K protein. Only the monoclonal antibody to EA-D reacted with the major EA-D protein (Fig. 2, lane E2). A similar pattern of reactivity was noted in the ELISA. Thus, the antibody prepared against the denatured 85K protein appeared to recognize a different epitope from those directed against the native structure.

TABLE 1. Identification of three epitopes on the 85K EA(R) protein by blocking of direct immunofluorescence

Blocking antibody	Reaction with FITC-conjugated antibody ^a			
	K8	K9	R63	85K
K8	+	+	-	-
K9	+	+	-	-
R63	-	-	+	-
85K	-	-	-	+

^a Symbols: +, complete blocking of direct staining of EBV-infected cells by the FITC-conjugated monoclonal antibody (<1% positive cells on the slide); -, no blocking of the direct immunofluorescence reaction (50 to 75% positive cells on the slide).

To examine this question further and to determine whether monoclonal antibodies R63, K8, and K9 were all reacting with the same epitope expressed on the 85K protein, the γ -globulin fractions from mouse ascites fluids containing these monoclonal antibodies were purified and conjugated with FITC. It was then possible to set up experiments to determine whether these different antibodies blocked the direct staining of the 85K protein by each of these conjugates. The blocking patterns noted with the different monoclonal antibodies are shown in Table 1. Direct staining of EBV-infected cells by the K8 conjugate was blocked by the unlabeled monoclonal antibodies K8 and K9 but not with monoclonal antibody R63 or 85K. Similar results were obtained in experiments directed at blocking the staining of infected cells with the K9 conjugate. Only unlabeled antibody R63 blocked the staining pattern produced with the R63 conjugate, and, as expected, monoclonal antibody 85K was the only antibody that blocked the reaction of conjugated antibody 85K with infected cells. Generally, in these experiments, complete blocking was noted up to dilutions of about 160. These results indicated that the four monoclonal antibodies recognized three different epitopes on the 85K protein, two of which were associated with the tertiary structure of this protein while one was expressed on the primary structure.

Another series of experiments was performed to determine whether the 130K polypeptide precipitated in addition to the 85K protein by monoclonal antibodies K8, K9, and R63 was viral or cellular in origin. To determine this, immunoprecipitation experiments were performed with extracts prepared from TPA- and sodium butyrate-induced B95-8, P3HR-1, IB-4, Raji, and Daudi cell lines and from the EBV genome-negative Ramos, BJAB, and Loukes cell lines. Similar observations were noted with all three monoclonal antibodies in regards to this question. Representative results with monoclonal antibody K8 are presented in Fig. 3. Monoclonal antibody 85K was included in this experiment for comparison. The monoclonal antibody prepared against the denatured 85K protein only precipitated this protein from the genome-positive cell lines (Fig. 3, lanes B through F). No proteins were precipitated from the three genome-negative cell lines (Fig. 3, lanes G through I). In contrast, the antibody (K8) prepared against the native EA-R protein precipitated both the 85K and 130K proteins from the genome-positive cell lines (Fig. 3, lanes J through N) and the 130K protein from the EBV genome-negative cell lines (lanes O through R). Thus, this monoclonal antibody also reacted with a cellular protein containing an epitope shared with the major EA-R protein. Table 2 summarizes the differences noted among the different anti-EA-R monoclonal antibodies in the different immunoassays.

Detection of EBV-induced filaments in infected cells with the anti-EA-R monoclonal antibodies. The original published studies with the anti-EA-R monoclonal antibody showed that this antigen was located mainly in the cytoplasm of EBV-producing cell lines (19). However, further immunofluorescence studies showed that the staining pattern changed when cells tested at different times after the activation of the virus replication cycle were examined by this approach. The most striking pattern involved the appearance of cytoplasmic filaments in the activated cells. This was initially noted in the human placental cell strain FF260 transfected with 1 μ g of P3HR-1 DNA by the calcium method (12). After 3 days, the cells were fixed in acetone and reacted with monoclonal antibodies to EA-R (K8, K9, R63) and EA-D (R3). Representative photographs of such cells are shown in Fig. 4. The 85K EA-R protein detected with each antibody was observed as cytoplasmic filamentous (Fig. 4A and C) or granular structures (B) in such cells. In contrast, the monoclonal antibody directed against the major EA-D component produced a diffuse nuclear staining pattern (Fig. 4D) as previously reported (18). Mock-transfected cells gave no fluorescence reaction with any of the antibodies (data not shown), as expected.

This phenomenon was then studied in activated P3HR-1 cells stained at different times after treatment with TPA and

FIG. 3. Immunoprecipitation of proteins from TPA- and sodium butyrate-activated EBV genome-positive and EBV genome-negative cell lines by the monoclonal antibody prepared against the denatured 85K protein (lanes B through I) and a monoclonal antibody (K8) against the native 85K protein (lanes J through R). Lanes B and J, B-95-8 cell line; lanes C and K, P3HR-1; lanes D and L, IB-4; lanes E and M, Raji; lanes F and N, Daudi; lanes G and O, Ramos; lanes H and P, BJAB; lanes I and R, Loukes. The molecular weight standards listed in Materials and Methods are shown in lane A. The upper band in this lane is composed of aggregated ¹²⁵I-labeled markers, while the lowest band is composed of degraded labeled material running on the front of the gel.

FIG. 4. Immunofluorescence patterns on human placental cell strain FF260 in the 27th passage 3 days after transfection with 1 μ g of P3HR-1 virus DNA. Cells were stained with three different monoclonal antibodies (K8, K9, R63) directed against the 85K EA-R protein (A through C) and with a monoclonal antibody against the 50K to 52K EA-D protein (D). Note granular and filamentous staining in panels A through C versus nuclear staining in panel D. Magnification, $\times 400$.

sodium butyrate. Photographs of the different staining patterns produced with monoclonal antibody 85K are presented in Fig. 5. Generally, by 24 to 28 h postactivation, this protein gave a granular type of fluorescence pattern in the cytoplasm of these cells (Fig. 5A and B). By 32 to 36 h postactivation, filamentous structures started to appear in the cytoplasm (Fig. 5C and D), and the structures became pronounced by 40 h postactivation (E). The filaments then appeared to aggregate primarily around the nuclear membrane (Fig. 5F

and G). This pattern was noted with all of the different monoclonal antibodies to the major EA-R protein and in every virus-producing cell line tested by this approach. Other antibodies, including some directed against cellular proteins such as actin, myosin, and tubulin, did not react with the filaments in the activated cells in the immunofluorescence assays. Furthermore, the addition of colchicine to the media had no effect on filament formation. The significance of these morphological changes to the virus lytic cycle is currently under investigation.

Biochemical characterization of the major EA-R protein. Experiments were designed to compare the major EA-R protein isolated from different cell lines and to determine whether this protein was modified during the virus lytic cycle. To determine whether this protein was phosphorylated, as was previously reported for the 50K to 52K EA-D protein (2, 17), induced cells were labeled with [32 P]phosphate between 32 and 38 h after induction and immunoprecipitated by the various monoclonal antibodies. A parallel set of cells was labeled with [35 S]methionine at the same time for comparison. Monoclonal antibody 85K precipitated the [35 S]methionine-labeled protein (Fig. 6, lane B), but no 32 P-labeled protein was precipitated (lane F). In contrast, monoclonal antibody R3 directed against the major EA-D component precipitated both 35 S- and 32 P-labeled proteins at a molecular weight of around 50,000 (Fig. 6, lanes C and G). As a control, monoclonal antibody L2 against one of the late antigens was also included (Fig. 6, lanes D and H).

TABLE 2. Immunoreaction of the EA(R) monoclonal antibodies in different immunoassays

Monoclonal antibody ^a	Mol wt of immunoprecipitated polypeptide(s)	Reaction in:		
		Western blotting	ELISA	Immunofluorescence ^b
K8	85,000, 130,000	—	—	+
K9	85,000, 130,00	—	—	+
R63	85,000	+ ^c	+ ^c	+
85K	85,000	+	+	+

^a Monoclonal antibodies K8, K9, and R63 were prepared against the native 85K component of EA-R; monoclonal antibody 85K was prepared against the denatured 85K component.

^b Monoclonal antibodies K8, K9, and R63 reacted positively against acetone-fixed activated EBV genome-positive cells; monoclonal antibody 85K was positive against both acetone- and methanol-fixed cells.

^c Weak positive reaction.

FIG. 5. Immunofluorescence staining of TPA- and sodium butyrate-activated P3HR-1 cells at different times postactivation with monoclonal antibody 85K against the 85K EA-R protein. Cells were stained at 24 h (A), 28 h (B), 36 h (C and D), 40 h (E), and 48 h (F and G) postinfection. Magnification, $\times 250$.

Previous studies had established that the protein precipitated by this antibody was not phosphorylated (8). This was confirmed in this experiment. In addition, serum from a patient with a nasopharyngeal carcinoma precipitated the 85K antigen from methionine-labeled cells (Fig. 6, lane A) and also precipitated some phosphoproteins in the region of the 85K protein (lane E). However, since the monoclonal antibody did not precipitate these phosphoproteins, they are presumably not related to EA-R. Similar experiments were set up to determine whether the 85K protein was glycosylated, using approaches that have been used successfully in this laboratory for detecting glycoproteins (8, 19, 21, 22). These approaches included efforts to label the 85K proteins with tridiated fucose, mannose and, glucosamine. All experiments produced negative results, indicating that the 85K protein was not glycosylated (data not shown).

Comparative peptide maps of the 85K proteins isolated

from the P3HR-1 cell line with the different monoclonal antibodies did not reveal any difference in the proteins recognized by these antibodies. These data provided further evidence, therefore, that the different monoclonal antibodies were reacting with different epitopes on the same protein. The next question asked was whether the 85K proteins isolated from two different cell lines were identical. To answer this, the 85K proteins isolated from two different cell lines (P3HR-1 and B-95-8) were examined by peptide mapping with *Staphylococcus aureus* protease V8 to determine whether there were detectable differences in this protein. No differences were noted by using this approach (Fig. 7). Treatment with this enzyme produced the same degradation products, indicating that there were no major differences in the amino acid composition of this protein from the two different cell lines. This conclusion was supported by actual determination of the amino acid composition of this protein

purified from the P3HR-1 and B-95-8 cell lines. The amino acid composition of this protein was similar from the two cell lines but significantly differed from the composition of the major EA-D protein (Table 3). Significant differences between these two proteins were noted in the content of proline, glycine, isoleucine, and phenylalanine.

Methionine-labeled proteins immunoprecipitated by two different anti-EA-R monoclonal antibodies (K8, 85K) were also analyzed by two-dimensional gel electrophoresis (Fig. 8). The 85K protein precipitated by both monoclonal antibodies had a pI of approximately 6.8, which was slightly higher than that of actin. In contrast, the 130K cellular protein precipitated by K8 (Fig. 8B) had a very high pI.

DISCUSSION

There have been numerous reports over the past several years on the identification and characterization of proteins composing the EA complex (14). In the majority of these studies, human antisera containing antibodies to a variety of EBV antigens were used, and EBV-specified EA proteins were identified by the fact that they were precipitated from

FIG. 6. Immunoprecipitation of [^{35}S]methionine-labeled (lanes A through D) and ^{32}P -labeled (lanes E through H) proteins from P3HR-1 cells induced with TPA and sodium butyrate. Lanes A and E, human anti-EBV positive serum; lanes B and F, monoclonal antibody 85K; lanes C and G, monoclonal antibody R3 directed against the 50K to 52K component of EA-D; lanes D and H, monoclonal antibody L2 directed against the 125K viral capsid antigen (8).

FIG. 7. SDS gel electrophoresis of [^{35}S]methionine-labeled, affinity-purified 85K protein from P3HR-1 (lane A) and B95-8 (lane D) cell lines after treatment with 1.0 μg (lanes B and E) and 2.0 μg (lanes C and F) of *S. aureus* protease V8.

cells cultivated in the presence of inhibitors of viral DNA synthesis. This resulted in conflicting data on the nature of the proteins that composed this antigen complex. More recently, monoclonal antibodies to different EBV antigens were developed and used in experiments directed at dissecting these antigens into various components. Monoclonal antibodies have now been produced against the major components for the EA-D and EA-R antigens. The gene encoding for the major EA-D component has been mapped to the *Bam*HI M fragment of EBV DNA (19). The gene encoding for the EA-R component has still not been conclusively identified, although evidence implicating the *Bam*HI H fragment has been reported (4).

In the experiments reported in this paper, a number of monoclonal antibodies were used in an attempt to further characterize the EA-R complex. Three of these antibodies (K8, K9, R63) were produced against the native 85K protein, while one (85K) was prepared against the denatured form of the protein. This latter antibody was prepared because the antibodies produced against the native molecule were directed against epitopes associated with the tertiary structure of the major EA-R protein. Because of this, these antibodies

TABLE 3. Comparison of amino acid compositions of 85K and 50K to 52K proteins from P3HR-1 cells

Amino acid residue	Composition (mol%) of ^a :	
	85K protein	50K to 52K protein
Cys	ND ^b	ND
Asx	8.1	6.2
Thr	4.3	5.7
Ser	7.4	8.5
Glx	11.5	11.0
Pro	4.6	8.4
Gly	14.0	9.0
Ala	8.0	9.8
Val	5.8	6.3
Met	1.5	1.2
Ile	3.6	1.8
Leu	7.3	9.3
Tyr	3.8	2.8
Phe	4.8	2.4
Trp	ND	0.8
Lys	4.4	5.4
His	2.0	2.4
Arg	6.3	4.6

^a The mean standard deviation for all values was 0.6, with a maximum of 1.4 and a minimum of 0.2 in values for individual residues; calculations are based on HCl hydrolysis for 24 h.

^b ND, Not determined.

were not used successfully in ELISAs and immunoblotting experiments in which the antigen was subjected to denaturing agents. In addition, they were also not used successfully in mapping experiments with proteins encoded by hybrid-selected species of mRNA. Presumably this was related to the fact that such proteins did not take on the configuration of the native protein. The monoclonal antibody designated 85K produced against the denatured 85K protein overcame some of these problems. It was used successfully to detect this antigen with the ELISA and with the immunoblotting procedures and therefore was more applicable to the characterization studies. It might now be possible to identify conclusively the gene encoding for this protein by the mRNA method with this monoclonal antibody, although such studies have still not been performed.

One other interesting difference was noted between the antibodies produced against the native EA-R protein versus the antibody produced against the denatured protein. All three of the antibodies to the native 85K protein also precipitated a cellular protein with a molecular weight of approximately 130,000. This cellular protein was not precipitated by the antibody directed against an epitope expressed on the primary structure of this protein and did not react with antibodies to different cellular proteins. Whether this protein is complexed to the 85K protein is not known, although the data suggest that this is not the case. Similar reactivity between viral and cellular proteins was previously noted with a monoclonal antibody produced against an antigenic determinant expressed on EBV-induced nuclear antigens (11). The significance of this is still unknown. However, such an antibody directed against an epitope shared by viral and cellular proteins could potentially be involved in the mediation of autoimmune phenomena. Such circumstances could also account, for example, for the presence of antibodies against intermediate filaments in the sera of some patients with infectious mononucleosis (16).

Blocking experiments with FITC-conjugated antibodies showed that these different monoclonal antibodies recog-

nized three different epitopes expressed on the 85K protein. One of these epitopes was recognized by monoclonal antibodies K8 and K9, while different epitopes were identified with monoclonal antibodies R63 and 85K. Two of the epitopes, therefore, were expressed on the native 85K molecule, while the third epitope was associated with the primary structure of this protein. The identification of other epitopes on this protein will require the production of other monoclonal antibodies.

One of the more striking observations made during the course of this study concerned the distribution of this protein in virus-producing cells. The 85K protein was previously shown to reside in the cytoplasm of producer cells by immunofluorescence (19). However, when this technique was used to follow the expression of this antigen at different times after the activation of the virus cycle or in human epithelial cells transfected with viral DNA, a number of different staining patterns were noted. The fluorescence pattern progressed from granular to cytoplasmic filaments and finally to aggregates surrounding the cell nuclei. The location of the aggregates suggested that this protein might involve the Golgi apparatus, although this still has to be determined. Such filaments and aggregates did not react with antibodies prepared against other proteins such as actin,

FIG. 8. Two-dimensional gel electrophoresis of methionine-labeled 85K protein precipitated by monoclonal antibody 85K to EA-R (A) and of the 85K and 130K components precipitated by monoclonal antibody K8 (B). The basic end represents pH 10, and the acidic end represents pH 3.5.

myosin, and tubulin and therefore appeared to be virus specific. Filamentous structures have previously been observed in EBV-infected cells by electron microscopy (13). In addition, filament formation has been noted in cells infected with measles and herpes simplex viruses (1, 3, 25). However, in the last case, these structures were also found in the nucleus. The significance of these structures in the replication of these viruses is still unknown, but their appearance during the early stages of the replication cycle suggests that they could be involved in transport. Further studies will be directed at defining the importance of such structures in the virus lytic cycle.

The monoclonal antibodies were also used in experiments directed at purifying and characterizing the EA-R component from different cell lines. No differences were noted after peptide mapping with protease V8 or by determination of amino acid compositions. To date, it has not been possible to sequence this protein because the N terminus is blocked. It will therefore be necessary to use peptides of this protein for sequencing data. In addition, this protein was not phosphorylated, as was previously noted for the major EA-D protein (2, 17), or glycosylated. Other possible modifications, such as ADP ribosylation or acetylation, are currently being explored. In other experiments not reported here, this protein also did not bind to double-stranded DNA-cellulose, another feature that distinguishes the EA-R protein from the major EA-D component (17).

Finally, by using an experimental approach similar to that reported by Lin et al. (9) for quantitating EA-D in infected cells, we have determined in preliminary experiments that approximately 1 to 2% of the total protein was associated with EA-R (unpublished result). This is similar to the concentration of EA-D in infected cells, as was reported by Lin et al. (9).

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