p105, an Epstein-Barr Virus-Induced, Phosphonoacetic Acid-Insensitive Glycoprotein Target of the Anti-Epstein-Barr Virus Immune Response

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To describe structures and biological functions of targets for antibody-mediated immune responses to Epstein-Barr virus (EBV)-infected lymphoid cells, we have characterized a membrane-associated protein of 105,000 daltons, p105, which was prominently recognized in immunoprecipitates with some EBV antigen-reactive patients' sera. A rabbit antiserum to immunopurified p105 was developed. [³⁵S]methionine-labeled p105 was specific to EBV-superinfected Raji cells, and its synthesis was not blocked with phosphonoacetic acid, indicating that it is an "early" viral antigen. Phosphonoacetic acid treatment of EBV-superinfected Raji cells was associated with the accumulation, mainly in the cytosol, of an 88,000-dalton protein, p88, which was also recognized with anti-p105 serum, but was not detected in superinfected cells which had not been treated with phosphonoacetic acid. Although anti-p105 serum immunoprecipitated a membrane fraction protein, it did not neutralize P3HR-1 virus and was not considered to be an exposed virion component. We conclude that p105 is an early, EBV-induced, membrane fraction antigen to which EBV-infected patients generate a substantial antibody response.

We previously demonstrated the immunoprecipitation of selected EBV superinfection-induced, microsomal membrane-associated molecules with antibodies in hairy cell leukemia patients' sera which uniformly had high titers to EBV antigens (4, 26). Such antibodies to EBV-induced neoantigens could mediate immune attack against EBV by antibody-dependent cellular cytotoxicity (10, 17, 20, 30), by complement-mediated lysis, by virus neutralization (18), or, possibly, by modulation of membrane determinants, thereby affecting viral or host cell function. In fact, high antibody-dependent cellular cytotoxicity titers to EBV-induced membrane antigens correlate positively with survival of patients with nasopharyngeal carcinoma (19) and with response to chemotherapy in patients with BL (21). To characterize the functions of these anti-EBV antibodies and of their membrane targets, we have identified some EBV-induced neoantigens which appear on Raji lymphoblastoid cells after superinfection with P3HR-1-EBV, and we have characterized one 105,000-dalton antigen, which is prominently recognized with BL and hairy cell leukemia

patients' sera, through the use of a rabbit antiserum.

MATERIALS AND METHODS

Abbreviations. The following abbreviations have (not superinfected) Raji lymphoblastoid cells; EA, early antigen; EA-O, EA (diffuse distribution); EA-R, EA (restricted distribution); EBV, Epstein-Barr virus; MA, membrane antigen; PAA, phosphonoacetic acid; p105, protein of 105 kilodaltons (other proteins are identified by this nomenclature, indicating apparent molecular weight in kilodaltons); S-Raji, P3HR-1-EBV-superinfected Raji lymphoblastoid cells; VCA, viral capsid antigen.

Cell lines and antisera. Raji (22) and P3HR-1 (9) cell lines were used. Sera from patients with African BL were the gift of Werner Henle. The anti-EBV immunofluorescence titers of these sera, as reported by Werner Henle, are tabulated in Fig. 2. For the virus neutralization assay, sera from four healthy individuals were used. Sera A and B had titers to EA of less than 1:10, to VCA of 1:160 and 1:160, and to MA of 1:320 and 1:160, respectively. Sera C and D lacked detectable antibody titers (less than 1:10) to EA, VCA, MA and EBV nuclear antigen. Immunofluorescence assays were performed by established methods (5, 7, 8, 14, 24, 29).

Preparation of P3HR-1 virus and superinfection of Raji cells. P3HR-1-EBV was obtained from 10-day-old, 33°C cultures (9). Raji cells (2.5×10^6 cells per ml) were superinfected by suspension in virus 10-fold

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FIG. 1. Correlation of electrophoresis patterns of proteins to serum titers for EA-R, EA-D, and VCA. Immunoprecipitation with various antisera to EBV antigens was compared with titers of these sera as expressed on logarithmic scale [$\log_2$ (serum concentration/10)]. Serum 9 was from an EBV-seronegative person. The titer to EA-R was undetermined (u) in instances in which high titers to EA-D were present. Titers which were less than 1:10 are indicated (-). Precipitation of p19 correlated with high titers to EA-R.

concentrated from P3HR-1 culture supernatants in RPMI 1640 medium with 4% fetal calf serum (6). Cells were incubated at 37°C in a 5% CO₂-in-air atmosphere for various periods of time before analysis. At 48 h 50 to 60% of the cells expressed EA. In some cultures 200 µg of phosphonoacetic acid (Richmond Chemicals; catalog no. 92) per ml was used (16, 28).

Virus neutralization. The ability of antisera to inhibit induction of EA by P3HR-1-EBV superinfection of Raji cells was tested by established techniques (18).

Radiolabeling, immunoprecipitation, and electrophoresis of membrane proteins. S-Raji and C-Raji were labeled with [³⁵S]methionine (20 µCi/ml) for 4 h or with L-[5,6-³H]fucose (40 to 60 Ci/mmol) and D-[1,6-³H(N)]glucosamine (30 to 60 Ci/mmol) (New England Nuclear) at 20 µCi/ml each for 24 h. Microsomal membranes were isolated and subjected to electrophoresis as described previously (3, 13, 25, 31). Alterna-

tively, immunoprecipitates were prepared for electrophoresis with antisera and Formalin-fixed *Staphylococcus aureus* by reported techniques (12, 25).

RESULTS

Proteins recognized with BL patients sera. We sought to analyze membrane proteins immunoprecipitated with BL patients' sera which had defined serological characteristics to test the association between precipitation of individual molecules and high-antibody titers to some EBV-induced antigens (Fig. 1). High titers to EA-R were associated with the immunoprecipitation of p19. High titers to VCA occurred with a loose correlation to precipitation of p105. However, one must note that antibodies to MA are also usually present in sera with high titers to VCA (14). Therefore, on the basis of this experiment, precipitation of p105 could be caused by antibodies to either VCA or MA. In addition, molecules at 44,000, 35,000, and 28,000 daltons were weakly precipitated with some of the antisera. The relationship of these proteins to EBV serological specificities, although they are clearly recognized on a variable basis in immunoprecipitates with the different sera, remains undetermined.

Preparation of anti-p105 serum. A high-titered anti-p105 serum was prepared by immunization of rabbits with antigen which was released from immunoprecipitates formed by a weak rabbit anti-S-Raji serum which had been absorbed with C-Raji membranes until it was fluorescence specific for S-Raji cells and precipitated principally p105 from those cells. The serum from a rabbit which had been immunized with membrane extracts from S-Raji in which there had been a relatively low (20%) induction of EA recognized only four proteins in immunoprecipitates of membranes from S-Raji, including p105. After absorption with microsomal membranes of C-Raji, this antiserum immunoprecipitated only the p105 from S-Raji and no proteins from C-Raji (Fig. 2). This absorbed antiserum was used to immunoprecipitate additional p105 from S-Raji or from 12-O-tetradecanoylphorbol 13-acetate-treated P3HR-1 cells. The antigen and antibody complex from Formalin-fixed *S. aureus* were eluted by treatment with 3.5 M MgCl₂ followed by dialysis of the eluate against phosphate-buffered saline. This dialyzed material was used to immunize two rabbits. After the second booster immunization, the rabbits were exsanguinated. The serum used in the experiments of this paper was generated against p105 precipitated from S-Raji and had a membrane immunofluorescence titer against S-Raji of 1:360. The serum from the rabbit immunized with P3HR-1-derived p105 was essentially similar. The appar-

FIG. 2. Preparation of anti-p105 serum. A rabbit antiserum to P3HR-1-EBV-superinfected Raji (α -Raji-S) was absorbed with uninfected Raji cells (abs α -Raji-S). This absorbed serum was specific in immunoprecipitation studies for p105 of S-Raji cells and was used for the immunopurification of p105 for immunization of a second rabbit.

ent molecular weight of the protein precipitated from S-Raji membranes with anti-p105 serum was determined to be 105,000 daltons after sodium dodecyl sulfate-gel electrophoresis in the phosphate-buffered, Weber-Osborn system (31).

Cytoplasmic and microsomal-membrane localization of anti-p105 immunoprecipitated proteins. To assess the intracellular sites of expression of p105, immunoprecipitates were prepared from detergent-solubilized proteins from the microsomal membranes and from the cytosol (the supernatant of the $100,000 \times g$ centrifugation from which the membranes were pelleted). Proteins from C-Raji, S-Raji, and 200 μ g of PAA-treated S-Raji per ml were compared (Fig. 3). The cytosol of S-Raji had negligible synthesis of p105 in contrast to their membranes, which showed abundant synthesis of p105. We can conclude that synthesized (or fully processed) p105 is microsomal membrane bound and is not appreciably expressed in the cytosol.

Synthesis of p105 not inhibited with PAA. PAA inhibits both replication of the EBV genome and expression of "late" viral antigens and those EBV biological functions which are expressed only after genome duplication (16, 28). EBV-induced proteins which are still synthesized in the presence of PAA are termed "early" viral antigens. In these experiments, PAA completely inhibited VCA synthesis (as judged by immuno-

fluorescence assays), but did not block the synthesis of p105 and, in fact, enhanced by perhaps twofold the intensity of synthesis of p105 (lane 3, Fig. 3). In addition, a second protein of 88,000 daltons was prominently synthesized in PAA-treated cells and was found in the microsomal membrane fraction. More intense levels of synthesis of p88 were found in the cytosol of PAA-treated S-Raji than in the membranes. Minimal synthesis of p88 was seen in cytosol of S-Raji. We would conclude that p88 is principally a cytoplasmic protein possibly sharing antigenic determinants with membranal p105 and is accumulated by PAA treatment of S-Raji. The reasons for PAA enhancement of p88 are unknown, and its structural relationship to p105, e.g., as precursor or product, is not established.

Glycosylation of p105. To assess glycosylation and, by implication, external exposure of p105 on the cell membrane, cells were labeled with [3 H]fucose and [3 H]glucosamine, and their proteins were immunoprecipitated with anti-p105 serum. p105 was found with both rabbit anti-

FIG. 3. Subcellular localization and phosphonoacetate sensitivity of molecules recognized with anti-p105 serum. Microsomal membrane and cytosol molecules precipitated with anti-p105 serum from Raji cells, S-Raji, and PAA-treated S-Raji were compared. p105 appears solely in the microsomal membranes, and its level of synthesis is enhanced by PAA treatment. p88 which appears solely in PAA-treated cells is more prominent in the cytosol, but is also membrane bound. Other molecules are discussed in the text.

FIG. 4. Glycosylation of p105 and EBV-related proteins. Microsomal membrane proteins from [35 S]methionine-labeled C-Raji, S-Raji, and PAA-treated S-Raji and from [3 H]glucosamine- and [3 H]fucose-labeled cells were precipitated with two Burkitt's lymphoma patients' sera (BL-1 and BL-2), with a human serum without detected EBV antibodies, and with anti-p105 serum. p79, p105, p170, and p190 are glycosylated, but p19, p88, p108, and p110 do not appear to be glycosylated.

p105 serum and a BL serum to be metabolically radiolabeled with those tritiated sugars (Fig. 4). By way of control, one can observe that p170, recognized with BL-1 serum, was heavily glycosylated and p19, also recognized with BL-1 serum, was not glycosylated. We can conclude, then, that p105 is a glycosylated and, presumably, external membrane protein. It is noted in these gels that p170 is clearly recognized with BL sera when sugar radiolabeled, but not when [35 S]methionine labeled. Additional glycoproteins at 79,000 and 190,000 daltons can be identified in such immunoprecipitates. Since small amounts of these glycoproteins are also identified with the rabbit anti-p105 serum, we cannot securely draw conclusions from positive functional experiments with this antiserum, although

the preceding chemical observations remain valid.

Lack of virus-neutralizing capacity of anti-p105 serum. To define a functional role for p105 and to establish whether it might be expressed on the EBV virion, a virus-neutralizing assay was performed with anti-p105 serum and with two anti-EBV positive and two negative human sera (Fig. 5). Anti-p105 serum could not neutralize the capacity of P3HR-1-EBV to induce EA at 48 h in Raji cells, whereas two sera with antibodies to VCA and MA were found to neutralize this EA-inducing ability of P3HR-1 virus.

DISCUSSION

Toward the goal of defining the roles of EBV-induced membrane antigens in the immune response to EBV and in viral and host cell cycles, we have first sought to identify virally induced, membrane-associated antigens. A simple procedure for purification of microsomal membranes, yielding 10-fold enrichment for HLA-A,B antigens, permitted separation of this fraction from cytosol and nuclear compartments of the cell. We have identified a series of microsomal membrane-associated proteins (similar to those identified by others; 1, 2, 11, 15, 23, 27) and have found some of them to be immunoprecipitated with patients' sera with known, variable titers to

FIG. 5. Lack of P3HR-1 virus neutralization with anti-p105 antibody. Neutralization indices were determined as a function of serum dilution for the following sera: (○) p-105 rabbit no. 12 serum, (●) preimmune rabbit no. 12 serum, (Δ) EBV antibody-positive human serum A, (▲) EBV antibody-positive human serum B, (◇) EBV antibody-negative human serum C, (□) EBV Ab antibody-negative human serum D. Anti-p105 serum did not neutralize P3HR-1 EBV.

EBV antigens. For example, immunoprecipitation of p19 correlates well with titers to EA-R. Since p19 is not radiolabeled biosynthetically with sugars, including fucose, it might be internal to the cell surface, and, if it were to be proved to be part of the EA-R complex, it probably is associated with cytoplasmic membranes.

The presence of another prominently immunoprecipitated protein, p105, was correlated loosely with VCA titers. However, since antibodies to VCA and MA tend to occur in similar titers in many BL sera (14), this experiment, per se, does not indicate whether p105 is a VCA or MA component. However, PAA, which blocks expression of late viral antigens such as VCA, did not affect synthesis of p105. We would interpret p105 to be an early microsomal membrane-associated, EBV-induced antigen. Since it is glycosylated with fucose, under conditions at which other prominent proteins, viz., p19, are not labeled, we conclude that p105 is a glycoprotein and, therefore, could be an externally exposed membrane protein. This point remains to be solidified by alternative experimental methods.

Although we have demonstrated p105 to be a glycosylated, microsomal, membrane-associated, EBV-induced, early antigen which is prominently recognized with antibodies in BL patients' sera (at least relative to several other EBV-induced proteins which are even more abundantly synthesized), our principal goal remains the definition of the function of p105. If it is external, this function could be as a target for the immune response to EBV-infected cells or as a handle for antibody-induced modulation of cellular or viral processes. Since our rabbit antiserum to p105 contains low reactivities to other external membrane glycoproteins, positive functional experiments with this serum cannot be conclusive with respect to p105 itself. However, toward our goal of defining the function of p105, this rabbit anti-p105 serum can be useful in the development of a monoclonal antibody to p105, for example, in immunopurification of p105 to immunize mice or as an immunoabsorbant for detergent-solubilized antigen in screening for hybridoma clones which are likely to recognize p105.

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