Depletion of Glycoprotein gp85 from Virosomes Made with Epstein-Barr Virus Proteins Abolishes Their Ability To Fuse with Virus Receptor-Bearing Cells

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Entry of an enveloped virus such as Epstein-Barr virus (EBV) into host cells involves fusion of the virion envelope with host cell membranes either at the surface of the cell or within endocytic vesicles. Previous work has indirectly implicated the EBV glycoprotein gp85 in this fusion process. A neutralizing monoclonal antibody to gp85, F-2-1, failed to inhibit binding of EBV to its receptor but interfered with virus fusion as measured with the self-quenching fluorophore octadecyl rhodamine B chloride (R_{18}) (N. Miller and L. M. Hutt-Fletcher, *J. Virol.* 62:2366-2372, 1988). To test further the hypothesis that gp85 functions as a fusion protein, EBV virion proteins including or depleted of gp85 were incorporated into lipid vesicles to form virosomes. Virosomes were labeled with R_{18} , and those that were made with undepleted protein were shown to behave in a manner similar to that of R_{18} -labeled virus. They bound to receptor-positive but not to receptor-negative cells and fused with Raji cells but not with receptor-positive, fusion-incompetent Molt 4 cells; monoclonal antibodies that inhibited binding or fusion of virus inhibited binding and fusion of virosomes, and virus competed with virosomes for attachment to cells. In contrast, virosomes made from virus proteins depleted of gp85 by immunoaffinity chromatography remained capable of binding to receptor-positive cells but failed to fuse. These results are compatible with the hypothesis that gp85 is actively involved in the fusion of EBV with lymphoblastoid cell lines and suggest that the ability of antibody F-2-1 to neutralize infectivity of EBV represents a direct effect on the function of gp85 as a fusion protein.

Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus that causes infectious mononucleosis (17) and is associated with B-cell neoplasia (5), nasopharyngeal carcinoma (8), and oral hairy leukoplakia (13). The virus infects both B lymphocytes and epithelial cells (41). Infection of lymphocytes is initiated by interaction between the major virus envelope protein gp350/220 (34, 43) and a cell surface glycoprotein, known as CR2 (or CD21), which also serves as the receptor for the C3d/C3dg complement fragments (9, 35). Infection of epithelial cells is initiated by attachment to a similar or related molecule (41). Because of the difficulty of establishing epithelial cell cultures in vitro, the only information concerning subsequent steps in virus penetration is derived from studies of normal lymphocytes and lymphoblastoid cell lines.

Entry of enveloped viruses, such as EBV, into host cells involves fusion of the virion envelope with host cell membranes either at the surface of the cell or within endocytic vesicles (44). Electron microscopy studies indicate that fusion of the EBV envelope with lymphoid cell membranes is possible at either site. The virus has been reported to fuse at the surface of Raji lymphoblastoid cells (33, 40) but to enter normal peripheral B cells by endocytosis (33). The virion envelope protein gp85, which is much less abundant than the attachment protein gp350/220, has been implicated as playing a role in the fusion process under both circumstances (31). A monoclonal antibody which neutralizes virus infectivity and recognizes gp85, F-2-1, has been shown to have no effect on virus binding but to interfere with virus fusion, as measured with the self-quenching fluorophore octadecyl rhodamine B chloride (R_{18}). However, although this observation is consistent with the hypothesis that gp85

plays an active role in virus-cell fusion, the experiments provide only indirect evidence for its importance in the process. It remains possible, for example, that binding of antibody constrains the mobility or function of proteins adjacent to gp85.

The unique biology of EBV impedes selection and amplification of mutants for analysis of protein function. Therefore, in an attempt to determine more directly whether gp85 is necessary for fusion of EBV with cell membranes, we set out to prepare artificial deletion mutants of EBV. Our goal was to make virosomes, liposomes incorporating virion proteins, that could bind to and fuse with receptor-positive lymphoblastoid cell lines and then to examine the behavior of virosomes made from virion proteins depleted specifically of gp85. Our results, reported here, indicate that gp85 is actively involved in fusion of EBV with lymphoblastoid cell membranes and suggest that the ability of antibody F-2-1 to neutralize infectivity of EBV represents a direct effect on the function of gp85 as a fusion protein.

MATERIALS AND METHODS

Lymphoblastoid cell lines. Cell lines were grown at 37°C and diluted at least biweekly in RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal calf serum, 100 IU of penicillin per ml, and 100 µg of streptomycin per ml. These cell lines were Raji (38), an EBV genome-positive, nonproducing human B-cell line that expresses CR2; Molt 4 (32), an EBV genome-negative human T-cell line that expresses CR2 but cannot internalize the virus (30); P3HR1-C113 (18), a superinducible EBV-producing human B-cell line; and P3HR1-C15 (18), an EBV genome-positive human B-cell line derived from the same parent line as P3HR1-C113, which currently, in our laboratory, does not produce virus and does not express

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CR2. These last two cell lines were gifts from George Miller, Yale University, New Haven, Conn.

Antibodies. Three antiviral hybridoma antibodies were used: two neutralizing antibodies (F-2-1, an immunoglobulin G2a antibody that recognizes gp85 [42] and 72A1, an immunoglobulin G1 antibody that recognizes gp350/220 [20]) and one nonneutralizing antibody (E1D1, an immunoglobulin G1 antibody that recognizes gp85 [31]). All three antibodies were purified from culture supernatants by chromatography on protein A-agarose. In addition to the monoclonal antibodies, purified mouse immunoglobulin G (Organon Teknika, West Chester, Pa.) was used as a control in some experiments.

Virus production and radiolabeling. Virus-producing P3HR1-Cl13 cells were induced with 30 ng of 12-*o*-tetradecanoylphorbol-13-acetate per ml, and after 7 days virus was collected from the spent culture medium. The medium was centrifuged at $4,000 \times g$ for 10 min to remove cells, 100 μ g of bacitracin per ml was added to the clarified supernatant, and the virus was pelleted by centrifugation at $20,000 \times g$ for 90 min. Pellets were suspended in 1/250 of the original volume of medium containing 100 μ g of bacitracin per ml, reclarified by centrifugation three or four times at $400 \times g$, and filtered through a 1.2- μ m-pore-size filter (Acrodisc; Gelman Sciences, Inc., Ann Arbor, Mich.) and stored at -70°C .

Virus proteins were extrinsically labeled with ^{125}I (Amersham Corp., Arlington Heights, Ill.). Virus was pelleted from concentrated culture supernatant by centrifugation at $100,000 \times g$ for 1 h, suspended in 400 μ l of phosphate-buffered saline, pH 7.4, and labeled with 0.5 mCi of ^{125}I by using Iodogen (tetrachlorodiphenylglycouril; Pierce Chemical Co., Rockford, Ill.) (10). Free iodide was removed by gel filtration on Bio-Gel P-6DG (Bio-Rad Laboratories, Richmond, Calif.). Labeled virus was then repelleted before solubilization.

Preparation of virosomes. Virosomes were made by a modification of standard procedures (15, 22). Briefly, pelleted virions were extracted with lysing buffer containing 1% Triton X-114, 10 mM Tris, pH 7.4, 150 mM NaCl, 3 mM NaN_3 , 1 mM phenylmethylsulfonylfluoride (PMSF), and 100 U of aprotinin per ml, sonicated for 1 min, and then centrifuged at $100,000 \times g$ for 1 h. The supernatant was assayed for protein concentration by using BCA protein assay reagents (Pierce) and bovine serum albumin as a standard. Extracted viral protein at a 1:5 (wt/wt) protein:lipid ratio was added to a dried mixture of L- α -lecithin (egg) and cholesterol (Avanti Inc., Pelham, Ala.) (molar ratio, 1.7:1). The detergent:lipid molar ratio was adjusted to a minimum of 6:1, and virosomes were formed by extensive dialysis against buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 3 mM NaN_3 , and 1 mM PMSF) containing Amberlite XAD-2 (Sigma Chemical Co., St. Louis, Mo.) to remove detergent to below its critical micellar concentration (21, 27); the final dialysis was done against buffer without PMSF or NaN_3 . The dialysate was frozen and thawed three times and sieved over Sepharose 2B-300 (Sigma). Virosomes were collected in the void volume, pelleted by centrifugation at $16,000 \times g$ for 30 min, suspended in buffer, and stored at -70°C .

Immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Immunoprecipitation was carried out as previously described (2). Briefly, virosomes made from radiolabeled protein were solubilized with buffer containing 10 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-114, 1 mM PMSF, and 100 U of aprotinin per ml and mixed with antibody and protein A-agarose beads (Gen-

zyme Corp., Boston, Mass.). The precipitates were washed, dissociated by boiling in sample buffer containing 2-mercaptoethanol, and analyzed by SDS-PAGE (26) in 9% acrylamide cross-linked with 0.28% *N,N'*-diallyltartardiamide. Molecular weight markers (Sigma) were electrophoresed in parallel tracts. Gels were stained, destained, dried on filter paper, and placed in contact with XAR film (Eastman Kodak Co., Rochester, N.Y.) at -70°C for fluorography (3).

Labeling of virosomes with R_{18} . A 13 mM stock solution of R_{18} (Molecular Probes, Inc., Junction City, Oreg.) was prepared in chloroform-methanol (1:1) and stored at -20°C . The probe was incorporated into virosomes by a modification of the method of Hoekstra and colleagues (19). Virosomes were pelleted by centrifugation at $16,000 \times g$ for 30 min and suspended in 1 ml of phosphate-buffered saline. The stock probe (5 μ l) was dried under nitrogen, solubilized in 10 μ l of ethanol, and added to the virosomes with vortexing. The probe and virosomes were incubated at room temperature in the dark for 1 h, after which the virosomes and the unincorporated probe were separated by chromatography on Sepharose 2B-300 (Sigma). Labeled virosomes were used immediately after preparation.

Fluorescence measurement. R_{18} -labeled virosomes (20 to 100 μ l) were added to pellets of 2×10^6 cells and incubated for 35 min on ice in the dark. Cells were washed five times with ice-cold phosphate-buffered saline, suspended in 1 ml, and then transferred (time zero) to a cuvette of an Aminco Bowman spectrophotofluorometer equipped with a chart recorder for continuous monitoring of fluorescence (excitation and emission wavelengths were 560 and 585 nm, respectively). The cuvette was equipped with a magnetic stirrer and was kept in a warming chamber at 37°C . At the end of the assay, Triton X-100 was added to allow the measurement of fluorescence that would be obtained upon infinite dilution of the fluorophore. Fluorescence was expressed in arbitrary units (a.u.). For many experiments, the fluorescence scale was calibrated such that the residual fluorescence of virosomes at time zero was taken as the zero level and the value obtained after the addition of Triton X-100, corrected for sample dilution, was taken as 100% (infinite dilution).

Virosome-binding assay. The ability of virosomes made with radiolabeled protein to bind specifically to CR2-bearing cells was determined by the use of receptor-positive and -negative cells that had been briefly fixed with ice-cold 0.1% paraformaldehyde (Fisher Scientific Company, Fairlawn, N.J.). The virosomes were incubated with fixed cells for 1 h at 4°C , the cells were washed five times, and the acid-precipitable radioactivity remaining attached to cells was counted. The ability of antibody to interfere with the binding was determined by preincubating the virosomes with antibody for 1 h at room temperature. The ability of virus to compete for binding of virosomes to cells was determined by preincubating the receptor-positive cells with virus for 1 h at room temperature.

Depletion of gp85 from virus extracts. Immunoaffinity columns were prepared by coupling antibody E1D1 to Affigel-10 beads (Bio-Rad) according to the instructions of the manufacturer, except that phosphate-buffered saline containing 0.01% NaN_3 was used as the coupling buffer. Control columns were prepared by processing Affigel-10 beads in buffer without antibody. After coupling, the beads were packed in small columns, equilibrated in lysing buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-114, 1 mM PMSF, and 100 U of aprotinin per ml), incubated for 2 h in 2% bovine hemoglobin (Sigma) to minimize nonspecific binding, and washed extensively with lysing buffer. Extracts

FIG. 1. SDS-PAGE in 9% acrylamide of proteins immunoprecipitated by F-2-1 (lane 3), 72A1 (lane 2), or protein A-agarose alone (lane 1) from virosomes made with proteins extracted from virus that had been labeled extrinsically with ^{125}I . Numbers on the right indicate kilodaltons.

of virion protein were passed over the columns five times at 4°C , and the unbound material was concentrated to its original volume by dialysis against polyethylene glycol 20,000 (Sigma) and used in the preparation of virosomes.

RESULTS

Incorporation of radiolabeled proteins into virosomes. Virosomes were first made with protein that had been extracted from virus labeled with ^{125}I . Approximately 15% of the acid-precipitable radioactivity added to the lipid detergent mixture before dialysis remained associated with formed virosomes after they had been sieved and pelleted. Analysis by SDS-PAGE of proteins immunoprecipitated from such liposomes solubilized after preparation indicated that radiolabeled proteins associated with the virosomes included gp350/220 and gp85 (Fig. 1).

Binding of radiolabeled virosomes to cells. Although SDS-PAGE analyses suggested that virion-associated envelope proteins had been incorporated into virosomes, they provided no information as to the orientation of the molecules. The ability of radiolabeled virosomes to bind specifically to the EBV C3d receptor, CR2, was therefore determined to see whether at least the attachment protein gp350/220 was incorporated into virosomes in a functional configuration. Considerably more radioactivity bound to receptor-positive Raji cells than to receptor-negative P3HR1-C15 cells (Table 1). In addition, the amount of radioactivity associated with Raji cells and not with P3HR1-C15 cells was significantly reduced by preincubation of the virosomes with the monoclonal antibody 72A1. This antibody is specific for the attachment protein gp350/220 and is known to neutralize virus because of its ability to block binding to CR2. Equivalent amounts of normal mouse immunoglobulin had relatively little effect. The amount of radioactivity that bound to receptor-positive Molt 4 cell lines was less than that bound

TABLE 1. Effect of antibody on the ability of virosomes made from ^{125}I -labeled virus to bind to receptor-positive and receptor-negative cells

Antibody (amt [μg])	Total acid-precipitable radioactivity (cpm) bound to cells					
	Expt 1		Expt 2		Expt 3	
	Raji ^a	P3HR1-C15 ^b	Raji	P3HR1-C15	Raji	Molt 4 ^c
None	16,873	7,897	12,245	5,595	14,469	10,417
	18,702	7,491	12,347	5,759	13,525	9,313
	14,668	7,073	12,764	5,333		
Normal mouse immunoglob- ulin G (5)	13,441	5,877	11,517	4,339	12,733	9,435
	13,651	5,817	10,491	6,625	13,299	9,001
	13,285	5,899	11,723	4,759		
72A1 (5)	2,939	4,892	2,953	5,006	4,561	2,461
	3,431	4,227	2,125	4,247	3,305	3,405
	2,841	5,384	2,890	5,099		
E1D1 (5)	— ^c	—	—	—	11,003	8,777
					13,007	9,973
F-2-1 (5)	—	—	—	—	11,049	10,783
					12,864	9,751

^a Receptor-positive cells.

^b Receptor-negative cells.

^c —, Not done.

to Raji, which was in keeping with the fact that these cells have a lower density of CR2 receptors. However, once again, the antibody 72A1, and not normal mouse antibody, reduced the association of radioactivity with the cells. No such effect was seen when either of two antibodies of gp85, the neutralizing antibody F-2-1 or the nonneutralizing antibody E1D1, were used. Further confirmation of the specificity of binding of radioactivity to Raji cells was obtained in experiments in which virosomes were added to Raji cells that had been preincubated with increasing amounts of unlabeled virus; virus was able to compete with virosomes for binding to cells (Table 2).

TABLE 2. Effect of preincubation of cells with virus on the ability of virosomes made from ^{125}I -labeled virus to bind to Raji cells

Amt of virus added (μl) ^a	Total acid-precipitable radioactivity (cpm) bound to Raji cells	
	Expt 1	Expt 2
None	16,873	12,245
	18,702	12,347
	14,668	12,764
10	8,248	8,303
	8,397	9,074
	9,197	9,061
20	8,661	8,355
	7,797	7,479
	8,019	7,045
60	3,983	3,083
	4,190	3,266
	3,749	3,543

^a Volumes were adjusted with RPMI 1640 to a final volume of 60 μl .

FIG. 2. Comparison of relief of self-quenching of R_{18} -labeled virosomes bound to Raji cells (●—●), fixed Raji cells (●---●), Molt 4 cells (■—■), or P3HR1-C15 cells (□—□). The increase in fluorescence is expressed as a percentage of the maximum fluorescence obtained with each cell after addition of Triton X-100 (infinite dilution). The average maximum fluorescence values for the cell lines were as follows: Raji, 62 ± 1 a.u.; fixed Raji, 53 ± 2 a.u.; Molt 4, 49 ± 1 a.u.; and P3HR1-C15, 14 ± 2 a.u. Vertical lines indicate the standard deviations of the mean of seven experiments measuring interaction of virosomes with Raji cells, three experiments with fixed Raji cells, six experiments with Molt 4 cells, and two experiments with P3HR1-C15 cells.

Behavior of virosomes labeled with R_{18} . These experiments with virosomes made from radiolabeled protein indicated that we could achieve specific protein binding to receptor-positive cells and suggested that at least some molecules of the attachment protein gp350/220 were in an orientation that allowed specific binding to CR2. However, although most unincorporated soluble protein had presumably been removed in the sieving and pelleting steps involved in preparation of virosomes, we could not be certain that the labeled proteins that were binding were actually incorporated into vesicles. To confirm that this was in fact the case, and, in addition, to examine the ability of virosomes to fuse with cells to which they were bound, we labeled virosomes with self-quenching concentrations of the fluorescent, amphiphilic probe R_{18} . R_{18} -labeled virosomes were then incubated for 1 h on ice with Raji cells, Molt 4 cells, or P3HR1-C15 cells, and the cells were washed and transferred to the warmed cuvette of a spectrophotofluorometer for measurement of any changes in fluorescence emission over time. Over approximately 34 min, there was a steady increase in fluorescence dequenching in samples of virosomes added to the Raji cells (Fig. 2), after which time little further change was detected. This increase was abolished when the Raji cells were prefixed in paraformaldehyde. No increase was seen with the fusion-incompetent Molt 4 cells or with the receptor-negative P3HR1-C15 cells. The average maximum fluorescence values obtained for each cell line after addition of Triton X-100 at the end of the experiment provided a relative estimate of the number of virosomes that were bound initially. Virosomes apparently bound to all the receptor-positive cells, although only the fusion-competent unfixed Raji cells supported dequenching of fluorescence.

Effect of antibodies on relief of self-quenching of R_{18} -labeled virosomes. We next determined whether fluorescence de-

quenching of virosomes was affected by preincubation with antiviral antibodies in a manner that paralleled the effects of antibody on R_{18} -labeled virus (31). Antibody 72A1, which inhibits virus binding to cells, reduced the amount of R_{18} label associated with Raji cells to below the level of detectability in the spectrophotofluorometer (Fig. 3). Antibody F-2-1, which binds to gp85, had no more effect on virosome binding than did normal mouse immunoglobulin, but completely inhibited fluorescence dequenching; this is similar to its effect on the dequenching of R_{18} -labeled virus.

Behavior of virosomes made from proteins depleted of gp85. Since it appeared that R_{18} -labeled virosomes bound to and fused with Raji cells in a manner analogous to that of virus, virosomes were made with extracts of virus protein that had been depleted of gp85 by repeated passage over Affigel coupled to the nonneutralizing antibody E1D1. This antibody was used in preference to the neutralizing antibody F-2-1, in case any of it leached from the affinity column during the procedure. Virus protein extracts were divided into two parts. One sample was depleted of gp85 by passage over the E1D1 column before being used to make virosomes, and the other was passed over a control column and used to make mock-depleted virosomes. Both sets of virosomes were labeled and compared with virosomes made from unmanipulated extracts for the ability to bind to and fuse with Raji cells. Virosomes made from all three differently treated protein extracts bound to cells; all fused, except those made from proteins chromatographed on Affigel coupled to E1D1 (Fig. 4). The failure of E1D1 antibody to block binding or fusion of virosomes made from unmanipulated extracts confirmed that the effects of the E1D1-Affigel could not be attributed merely to leaching of antibody from the matrix.

FIG. 3. Effect of preincubation with 5 μ g of antibody on the relief of self-quenching of R_{18} -labeled virosomes added to Raji cells. $\bullet$ — $\bullet$, No preincubation (62 ± 1 a.u.); $\circ$ — $\circ$, preincubation with normal mouse antibody (55 ± 1 a.u.); $\square$ — $\square$, preincubation with 72A1 (0 a.u.); $\blacksquare$ — $\blacksquare$, preincubation with F-2-1 (55 ± 1 a.u.). a.u. values indicate average maximum fluorescence values obtained after addition of Triton X-100. Vertical lines indicate the standard deviations of the mean of seven experiments with virosomes bound without preincubation, three experiments with virosomes preincubated with normal mouse immunoglobulin, five experiments with virosomes preincubated with 72A1, and five experiments with virosomes preincubated with F-2-1.

DISCUSSION

Several investigators have incorporated virion proteins of enveloped viruses into lipid vesicles and used the resulting virosomes as models to study early interactions of viruses and cells. The majority of studies have been done with enveloped RNA viruses which encode only one or two envelope proteins (15, 22, 23, 27, 28, 39), but there is at least one report of successful preparation of virosomes incorporating envelope glycoproteins of the larger and more complex herpes simplex virus (HSV) (24). Our work, reported here, indicates that attachment and fusion of EBV can also be usefully studied with this technique.

The percentage of solubilized EBV protein incorporated into virosomes was considerably less than that reported for HSV. The reasons for this are unclear, but perhaps in part reflect the fact that the major envelope protein of EBV, gp350/220, is extensively glycosylated and is not a very hydrophobic molecule; during phase separation of a solution with Triton X-114, in contrast to gp85, it partitioned preferentially into the aqueous phase (unpublished data). Several lines of evidence indicate, however, that of those molecules of the attachment protein that were incorporated into virosomes, at least some were in an active state. Virosomes bound preferentially to receptor-positive cells, the monoclonal antibody 72A1 that blocks virus attachment specifically inhibited virosome binding, and virosome binding could be competitively inhibited by virus.

The similarity in behavior of virosomes and virus that had been labeled with the fluorophore R_{18} further encouraged the belief that the functions of viral envelope proteins had been

preserved throughout the extraction and incorporation procedures. Previous studies had shown that virus labeled with R_{18} binds specifically to and fuses with Raji cells, that virus binds to but fails to fuse with Molt 4 cells, and that fusion with Raji cells can be abolished by fixation of the cells in paraformaldehyde (31). Each of these properties was reproduced by R_{18} -labeled virosomes. The failure of virosomes bound to Molt 4 cells or to fixed Raji cells to demonstrate any relief of self-quenching of R_{18} was of particular importance, as it confirmed that dequenching was likely due to membrane fusion and not to lipid transfer, lipid-lipid exchange, or diffusion of label from one membrane to another, and it also eliminated the possibility that virus proteins and fluorophore were incorporated into detergent vesicles that could solubilize cell membranes rather than into liposomes which allowed fusion. This latter possibility had been of concern because of the choice of detergent for the extraction of virus proteins. Detergents of the Triton X series are very effective in extracting viral envelope proteins and are apparently less likely to inactivate viral fusogenic activity than is another commonly used nonionic detergent, octyl glucoside (4, 27). However, they typically have a very low critical micellar concentration and are difficult to remove by dialysis (16); the use of the copolymer Amberlite XAD-2 (21, 27) appears to have successfully overcome this potential disadvantage.

The kinetics of dequenching of labeled virus and virosomes attached to Raji cells were comparable. Dequenching of R_{18} -labeled virus was previously shown to reach a maximum after about 30 min (31). The rate of virosome

FIG. 4. Effect of depletion of gp85 on relief of self-quenching of R_{18} -labeled virosomes added to Raji cells. ●—●, Virosomes made from untreated extracts (62 ± 1 a.u.); ○—○, virosomes made from untreated extracts and preincubated with E1D1 (58 ± 2 a.u.); ●—●, virosomes made from extracts chromatographed on Affigel (56 ± 2 a.u.); ●—●, virosomes made from extracts chromatographed on Affigel coupled to E1D1 (58 ± 2 a.u.). a.u. values indicate average maximum fluorescence values obtained after addition of Triton X-100. Vertical lines indicate the standard deviations of the mean of seven experiments with virosomes made from untreated extracts, four experiments with virosomes preincubated with E1D1, four experiments with virosomes made from extracts chromatographed on Affigel, and five experiments with virosomes made from extracts chromatographed on Affigel coupled to E1D1.

dequenching was only slightly slower. The extent of fluorescence dequenching of virosomes, about 40% of the maximum possible, was less than that of virus, which reached values as high as 75% of the maximum. Nevertheless, this value compares very favorably with that obtained with virosomes made from Semliki Forest virus glycoproteins, which fused with an efficiency of 25% of that observed with the intact virus (28). Our results might reflect a greater preservation of the integrity of incorporated proteins, a higher percentage of molecules inserted in a functional orientation in the membrane, or perhaps a higher ratio of fusion to attachment protein. Although the stoichiometries of individual glycoproteins both in the EBV envelope and in virosomes are unknown, the abundance of the glycoprotein gp85 in the virion is thought to be very low compared with that of gp350/220 (14, 36). In contrast, as a more hydrophobic protein, gp85 might be overrepresented in virosomes.

Of particular relevance to our goal of better defining the role of glycoprotein gp85 in the fusion of EBV with B cells was the finding that the antibody to gp85, F-2-1, which inhibits virus fusion, was also capable of inhibiting the fusion of labeled virosomes. This indicated that whether or not gp85 played a direct or an indirect role in the fusion of virus with cells, the same function was preserved in the virosome. Specific loss of fusion activity after chromatography of proteins on affinity columns made with antibody to gp85 then provided strong evidence in support of the hypothesis that the molecule plays a direct role in fusion.

Glycoprotein gp85 has been mapped to the BXLF2 open reading frame of the EBV genome (14, 36), and homologous sequences have been identified in three other human herpes viruses (6, 7, 25, 29, 37). These encode glycoprotein gH of HSV, gpIII of varicella-zoster virus, and p86 of cytomega-

lovirus. Although none of these proteins ranks as the most abundant in the virion envelope, each carries a neutralization epitope. Antibodies to gH (12), gpIII (25), and p86 (6, 37) have been reported to inhibit spread of virus in tissue culture, a finding consistent with a role for the molecules in membrane fusion. Two sequences have been noted to have counterparts in the homologous proteins of all four viruses, but neither provides much obvious insight into the possible common functions of the molecules. One is a sequence of 4 amino acids, NGTV, which is identical in all viruses, includes a potential N-linked glycosylation site, and is found approximately 15 amino acids N terminal to the putative transmembrane region (6). The other is a 6-amino-acid stretch, SLRFDL, which is identical in EBV and HSV but varies slightly in cytomegalovirus (SGRRDH) and varicella-zoster virus (SLRTDL) and is found at residues 482 to 488 in EBV and in an approximately similar position in the homologs in each of the other three viruses (6, 29, 37). The sequence has an amphiphilic character in EBV, HSV, and varicella-zoster virus, which means that it might serve as a membrane-interactive region, but the amphiphilicity is disrupted by charged residues in the cytomegalovirus sequence. Each contains a potential trypsin cleavage site such as is found in fusion proteins of several RNA viruses (11), but were it to be used (and there is no evidence to date to suggest that it is), the new N terminus that it would create does not have the hydrophobicity expected of a fusion sequence. It is perhaps important to note that the fusion proteins of viruses in which fusogenic sequences have been identified are not merely necessary but are sufficient for fusion. This may not be the case for proteins involved in fusion of the more complex herpesviruses. Three glycoproteins (gH, gB, and gD) are required for penetration of HSV

into cells, and it has been suggested that penetration of this virus involves a cascade of interactions which culminate in fusion of the virion with the cell membrane (45). Analysis of the EBV DNA sequence indicates that the virus may encode glycoproteins in addition to those currently recognized (1), and at least one of these, the product of the BILF2 open reading frame, is present in the virion envelope (M. Mackett, M. Conway, J. Arrand, R. Haddad, and L. Hutt-Fletcher, manuscript in preparation). Virosomes made from EBV proteins, as described here, should provide a valuable tool to evaluate the contribution that such additional molecules might make to penetration of the EBV into its host cells.

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