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Human (HLA-A and HLA-B) and murine (H-2K and H-2D) histocompatibility antigens are cell surface receptors for Semliki Forest virus

(virus receptors/membrane proteins/nonionic detergent/HLA antigens/H-2 antigens)

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ABSTRACT **The proteins coded for by the HLA-A and HLA-B loci in man and the H-2K and H-2D loci in mice were identified as cell surface receptors for Semliki Forest virus. This conclusion is based on the following observations: (*i*) Water-soluble octamers of viral coat proteins inhibit the complement-dependent cytotoxicity of antibodies directed against H-2K and H-2D antigens in mouse cells. (*ii*) Isolated detergent-soluble HLA-A and HLA-B antigens reconstituted in lipid vesicles inhibit the binding of viral proteins to human cells (as do the water-soluble antigens to a lesser extent). (*iii*) Reconstituted HLA-A and HLA-B vesicles interact in solution with Semliki Forest virus (or with vesicles containing viral spike proteins), as demonstrated by coprecipitation with antisera. (*iv*) Complexes between viral spike proteins and HLA-A and HLA-B antigens or H-2K and H-2D antigens can be isolated from the cell surface by utilizing affinity chromatography or immunoprecipitation.**

The initial event in the entry of viruses into cells is the attachment of the virus to the host cell surface. Animal viruses attach via an interaction of viral surface proteins with specific receptors on the host cell membrane (1, 2). Surface proteins, glycolipids, and phospholipids have been implicated (3–6), but no host cell receptor for animal virus has yet been characterized.

In this paper we describe the identification and the isolation of receptors for the Semliki Forest virus (SFV) on mouse and human cells. SFV is a simple membrane virus capable of infecting insect cells as well as most vertebrate cells in culture (7). The virus attaches to the cell surface by glycoprotein spikes that extend 7–8 nm from the viral membrane (8). These spikes are a complex of three glycopolypeptides, E1 (apparent molecular weight 50,000), E2 (50,000), and E3 (10,000) (9). They are anchored to the viral membrane by hydrophobic regions in E1 and E2 (10). The spikes have been isolated and purified from intact virus particles in the presence of nonionic detergents (11). Removal of the detergent from the purified spikes by sucrose density gradient centrifugation results in the formation of water-soluble, lipid-free octamers (29S complexes) held together by their hydrophobic regions (12). Using this octavalent form of the viral spikes as our major tool, we have shown by biochemical and immunological methods that SFV binds to the classical histocompatibility antigens on human and mouse cells. These cell surface antigens, coded for by the *H-2K* and *H-2D* gene regions in mice, and by the *HLA-A* and *HLA-B* regions in humans, have a two-chain structure consisting of a glycopolypeptide with an apparent molecular weight of 44,000 and β_2-microglobulin, which has a molecular weight of 12,000 (13, 14). The larger chain is thought to carry the alloantigenic determinants detected in tissue typing. Amino acid sequence studies show a high degree of homology between these antigens in mouse, guinea pig, and man (15, 16). This homology as well as the presence of HLA-A, HLA-B, H-2K, and H-2D antigens on the surface of all nucleated cells is in agreement with the wide host range of SFV.

MATERIALS AND METHODS

The tissue culture cell lines used were (*i*) of human origin, HeLa (HLA-A and HLA-B positive but type unknown), JY (HLA-A2, 2, B7, 7), CCRF-CEM (HLA-A1, W31; B8, W40), and Daudi (HLA-A and HLA-B negative); and (*ii*) of rodent origin, P815 (H-2^d), SL2 (H-2^d), Eb (H-2^d), and BHK21. The detergent-solubilized and papain-solubilized HLA antigens were isolated from JY cells (15, 17). The [^{35}S]methionine-labeled and the unlabeled SFV, the 29S complexes, and the reconstituted virus membranes were prepared as previously described (12, 18, 19). Antisera against SFV spike proteins and human β_2-microglobulin were raised in rabbits. The antibodies to β_2-microglobulin were purified and coupled to Sepharose 4B (Pharmacia) (17). For isolation of spike protein–receptor complexes from cell membranes, 10×10^6 cells (P815 or CEM) were first surface labeled (20) with diazotized [^{35}S]sulfanilic acid (New England Nuclear). They were then suspended in 0.1 ml of RPMI 1640 medium containing 20 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes) at pH 6.3, 0.5% bovine serum albumin, and 16 μg of 29S complexes. After incubation for 2 hr at 6°, proteolysis inhibitor (Trasylol, Calbiochem) and 1% (wt/vol) Triton X-100 (Rohm and Haas) were added. Unsolubilized material was removed by centrifugation, and crosslinked *Staphylococcus aureus* strain Cowan cells containing protein A (21), previously incubated with antiserum to viral spike protein, were added to the supernatant. After incubation the bacteria were extensively washed and the bound protein was eluted with sodium dodecyl sulfate ($NaDodSO_4$).

To isolate the receptors by using affinity chromatography, 29S complexes (0.75 mg) were coupled to 1.8 ml of Sepharose 2B (Pharmacia) activated by cyanogen bromide (22). The affinity absorbent was equilibrated with 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) at pH 7.0 containing 0.1 M sodium chloride and 0.1% Triton X-100. The lymph node lymphocytes from a BALB/c (H-2^d) mouse were

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Abbreviations: SFV, Semliki Forest virus; IIF, indirect immunofluorescence microscopy; $NaDodSO_4$, sodium dodecyl sulfate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

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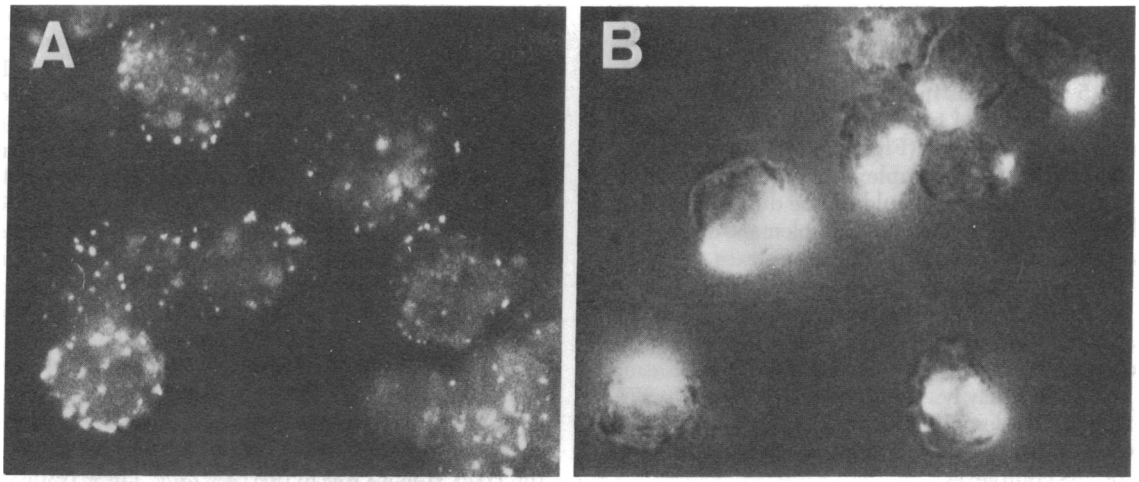

FIG. 1. Detection of cell-bound 29S complexes by IIF in noncapping (*A*) and in capping (*B*) conditions. P815 cells (10^6) were incubated for 1 hr on ice and 15 min at 37° in 25 μl of RPMI 1640 medium containing 0.01 M Hepes/HCl at pH 6.5 and 3.5 μg of 29S complexes. After washing, the cells were suspended in 50 μl of rabbit antiserum to SFV spike protein (1:50) and kept for 30 min on ice. In *A* the cells were washed and suspended in 1:75 diluted ice-cold fluorescein isothiocyanate (FITC)-conjugated pig antiserum to rabbit immunoglobulin (Miles Laboratories). After 30 min on ice the cells were fixed in 1.8% paraformaldehyde in phosphate-buffered saline, washed, and mounted for fluorescence microscopy. In the capping experiments (*B*) the same procedure was followed except that the incubation with FITC-conjugated antibodies was at 37° for 2.5 hr.

surface labeled with ^{125}I by the lactoperoxidase method (23) and extracted with Hepes/saline solutions containing 1% Triton X-100 and 10 units of Trasylol per ml. Cell nuclei and aggregates were removed by centrifugation (100,000 × *g*, 1 hr), and the supernatant was either used directly or subjected to prior fractionation on columns of *Lens culinaris* lectin bound to Sepharose 4B (24) to purify the glycosylated components.

To study the association of HLA-A and HLA-B antigens and SFV, 5 μg (protein) of [^{35}S]methionine-labeled virus was incubated with 2.5 μg (protein) of reconstituted HLA vesicles [prepared by octyl glucoside dialysis (10) and containing ^{32}P-labeled phospholipids] for 1 hr at 37° in 0.13 ml of 0.01 M Hepes/HCl at pH 6.5 containing 0.14 M NaCl and 0.1% bovine serum albumin. Antisera (25 μl) were added and, after 30 min at 37°, 150 μl of suspended *Staphylococcus aureus* bearing protein A. After one hour at 23° the preciptate and the supernatant radioactivities were determined. Reconstituted HLA vesicles were also incubated with ^{35}S-labeled reconstituted virus membranes (19) for 1 hr at 23° in 0.2 ml of 0.03 Tris·HCl at pH 7.4 containing 0.1 M NaCl, 0.1% bovine serum albumin, and 0.05 ml of Sepharose 4B beads coupled with anti-β_2-microglobulin. After incubation radioactivities were determined as above.

The full accounts of the quantitative binding, the immunoprecipitation (E. Fries), the immunofluorescence (B. Morein), and the complement-dependent cytotoxicity (B. Morein and D. Barz) will be published elsewhere.

RESULTS

Binding of 29S Complexes to Cells. The binding of the SFV octamers (the 29S complexes) to cells was demonstrated qualitatively by indirect immunofluorescence microscopy (IIF) and quantitatively by using [^{35}S]methionine-labeled viral proteins. With the IIF technique four human cell lines (HeLa, CEM, JY, and Daudi), three mouse cell lines (P815, SL2, and Eb), mouse lymph node lymphocytes, and one hamster cell line (BHK21) were tested. All cell types, except Daudi, showed distinct surface fluorescence indicative of 29S complex binding. Under noncapping conditions the distribution of fluorescence was "patchy" (Fig. 1*A*) and under capping conditions the receptors on more than 90% of the cells (P815, Eb, BHK21, or JY) were capped (Fig. 1*B*), indicating that the membrane-bound 29S complexes were mobile. Binding isotherms for the 29S complexes obtained using the quantitative binding assay (Fig. 2) showed that the binding was saturable and that the binding capacity varied for different cells. The binding data could be fitted to straight lines in Scatchard plots, allowing estimation of the average number of binding sites ($\bar{n}$) and binding constants (K_a; see Fig. 2). Isotherms obtained with these cells for the binding of complete virus particles gave the same correlation. A lower number of binding sites but higher affinity was, however, observed, which suggests that the virus attaches through a larger number of spike–receptor interactions than the 29S complexes. Moreover, the 29S complexes were found to compete with virus for available receptor sites.

Effect of 29S Complexes on Complement-Dependent Cytotoxicity. The first indication that the 29S complexes were

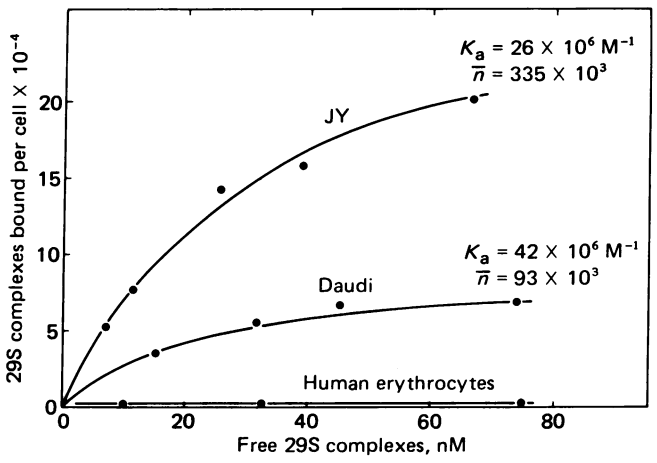

FIG. 2. Isotherms for 29S complex binding to cells. The cells were washed and suspended in RPMI 1640 medium without $NaHCO_3$ containing 0.02 M Mes at pH 6.3 and 0.5% bovine serum albumin. Aliquots containing 5 × 10^6 cells were mixed with appropriate amounts of [^{35}S]methionine-labeled 29S complexes and medium to give a final volume of 0.3 ml. The cells were incubated for 2.5 hr at 6° and pelleted. The concentration of free 29S complexes was determined from the radioactivity of the supernatant and the number of bound 29S complexes from the radioactivity of the cells after washing. The binding constants (K_a) and the average number of binding sites per cell ($\bar{n}$) were obtained from Scatchard plots.

binding to the major histocompatibility antigens was observed in complement-dependent cytotoxicity tests using P815 cells as targets. The alloantiserum CBA anti-BALB/c (H-2^k anti-H-2^d) lyses P815 (H-2^d) cells in the presence of rabbit complement. Prior incubation of target cells with 29S complexes markedly inhibited the cell lysis (Fig. 3). Because one can calculate that, at saturation, 29S complexes cover less than 2% of the total cell surface, the effect could not be due to complete coating of the plasma membrane (12). Complement-dependent cytotoxicity also indicated that the 29S complexes were not inserted as integral membrane components of the plasma membrane during incubation, but remained external to the membrane. Cells infected with SFV, which do have spike proteins incorporated as integral components of the plasma membrane, were effectively lysed with rabbit antiserum to SFV spike protein and complement (Fig. 3). Cytolysis was not observed, however, when the spike proteins were bound to cells in the form of 29S complexes.

Interaction of Viral Proteins and Isolated HLA-Antigens. For inhibition and binding studies, the HLA antigens from JY cells were purified in two forms: as detergent-solubilized complete molecules and as water-soluble papain cleavage products, which lack both the hydrophobic peptide that inserts the molecule in the lipid bilayer and the hydrophilic COOH-terminal region of the heavy chains (26). To prevent lysis of the cells by the detergent needed to maintain the complete HLA molecules in soluble form, the antigens were reconstituted with phospholipids by using the octyl glucoside dialysis method (10). HLA/egg lecithin vesicles were thereby obtained; they contained about 0.5 mg of protein per mg of egg lecithin (27). After negative staining, the HLA proteins could be visualized in the electron microscope as 5- to 6-nm-long knobs studded over the vesicle surface. JY cells were incubated with 29S complexes in the presence of the different HLA preparations, and the relative amount of virus protein bound was estimated by IIF using a cytofluorograph. As shown in Fig. 4, the reconstituted vesicles (which contained a mixture of HLA-A2 and HLA-B7) inhibited 29S complex binding completely. The papain fragments inhibited to a lesser extent. Lipid vesicles formed in the absence of HLA protein had no inhibitory effect. These results indicated that isolated HLA antigens do compete with cell surface receptors for virus protein. Moreover, the lipid vesicles containing multiple HLA antigens were more efficient than the univalent papain-cleaved fragments.

Two approaches were used to demonstrate *in vitro* binding between the SFV spike proteins and the vesicles containing reconstituted HLA protein. [^{35}S]Methionine-labeled virus was first incubated with HLA vesicles containing ^{32}P-labeled phospholipid. Antiserum to SFV spike protein was added and the immune complexes were isolated by secondary precipitation with crosslinked *Staphylococcus aureus* with protein A on its surface. Thirty-one percent of the HLA vesicles and 96% of the virus were precipitated, the values being 14 and 25%, respectively, with control serum. The latter result indicates a rather high level of nonspecific binding to the precipitating agent. Similar results were obtained in the reverse experiment, where reconstituted vesicles containing ^{35}S-labeled viral spike protein were incubated with the ^{32}P-labeled HLA-A and HLA-B vesicles and subsequently precipitated with anti-β_2-microglobulin coupled to Sepharose 4B. Fifty-seven percent of the virus vesicles were coprecipitated with 94% of the HLA-A and HLA-B vesicles. The nonspecific precipitation obtained by omitting the HLA vesicles was in this case 22%. These results show that 20–30% of the virus (or reconstituted viral membrane) had associated with the HLA vesicles in solution. In this system we have so far been unable to detect interaction between the papain fragments of the HLA-A and HLA-B antigens and the virus spike proteins *in vitro*. The reason may be a relatively low affinity between monomeric HLA antigen and spike proteins.

Isolation of SFV Receptors. Two approaches were used to isolate receptor material from cells. First, the surface proteins of CCRF-CEM and P815 cells were specifically labeled with diazo [^{35}S]sulfanilic acid, a reagent that does not penetrate the cell membrane (20). The labeled cells were saturated with

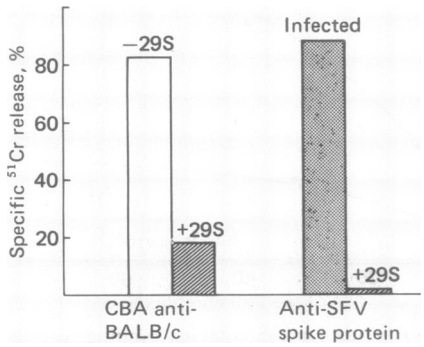

FIG. 3. Inhibition of complement-dependent cytotoxicity by viral spike proteins. P815 target cells were used in three forms: (*a*) untreated (−29S) (*b*) 29S complex-treated (+29S), and (*c*) SFV-infected. For *b*, 10^6 cells were incubated with 10 μg of 29S complexes for 20 min at 37° before ^{51}Cr labeling. For *c*, 10^6 cells in a volume of 0.2 ml were incubated with 100 plaque-forming units of SFV per cell in the presence of ^{51}Cr for 1 hr. After that, 2 ml of complete medium was added and the cells were further incubated for 4 hr. Cytotoxicity was measured by a modified ^{51}Cr release assay (25).

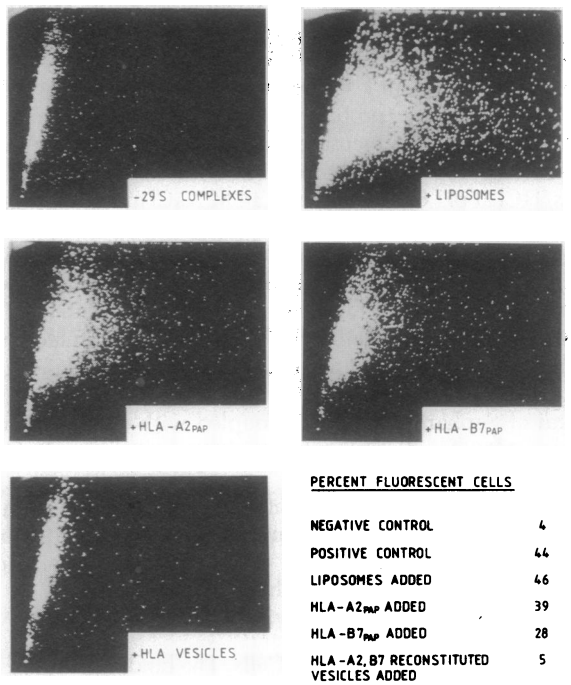

FIG. 4. Inhibition of virus protein binding to JY cells determined by indirect immunofluorescence cytofluorometry. 29S complexes (3.5 μg) were incubated for 1 hr at 37° with 60 μg of egg lecithin liposomes, 48 μg of HLA-$A2_{PAP}$ (cleaved by papain), 20 μg of HLA-$B7_{PAP}$ or 18 μg of reconstituted HLA vesicles in 60 μl of 0.01 M Hepes/HCl at pH 6.5 and 0.14 M NaCl. Cells (10^6) were added and incubation and labeling were done as described for Fig. 1*A*. The percentage of fluorescent cells was measured in a cytofluorograph (Biophysic Systems) adjusted to give the negative controls (29S complexes omitted) a value less than 5% positive cells. Positive control (not illustrated) had no addition other than the 29S complex and was identical to the sample to which liposomes were added. Abscissas, relative fluorescence; ordinates, relative cell size. Each point represents one cell.

unlabeled 29S complexes and solubilized by addition of Triton X-100, whereafter the virus–receptor complexes were precipitated with protein A-bearing *Staphylococcus aureus* that had been preincubated with antiserum to SFV spike protein. The precipitated proteins were eluted with $NaDodSO_4$ and separated by polyacrylamide slab gel electrophoresis. The results are shown in Fig. 5. The autoradiographs reveal two polypeptides of apparent molecular weights of 45,000 and 12,000 that comigrated with the heavy and light chains derived from purified HLA preparations. Other weaker bands of molecular weights 65,000, 26,000, and 15,000 were also observed. If the 29S complexes were omitted, no labeled proteins were precipitated by the procedure.

The second approach relied on affinity chromatography using 29S complexes covalently attached to Sepharose 4B. Lymph node lymphocytes of normal BALB/c ($H\text{-}2^d$) mice were surface labeled with ^{125}I and solubilized with 1% Triton X-100. Either the entire cell supernatant or a glycoprotein fraction obtained by *Lens culinaris* lectin affinity chromatography was passed through columns of spike protein-Sepharose. Fig. 6 shows electrophoretic profiles of the radioactive polypeptides retained by these columns either from whole extracts (Fig. 6*A*) or from the glycoprotein fraction (Fig. 6*B*); both show similar peaks of radioactivity associated with polypeptides of apparent molecular weight 45,000–50,000. A component of apparent molecular weight larger than 100,000 is also seen. The 45,000- to 50,000-dalton peak shows a distinct shoulder indicating two polypeptides with slightly different mobilities. Very similar double peaks are obtained with a mixture of *H-2K* and *H-2D* gene products (29; P. Robinson, unpublished data). The 10% polyacrylamide gels shown did not resolve the small polypeptides in the size range of the β_2-microglobulin, but in 15% gels a distinct peak corresponding to 12,000 daltons was seen.

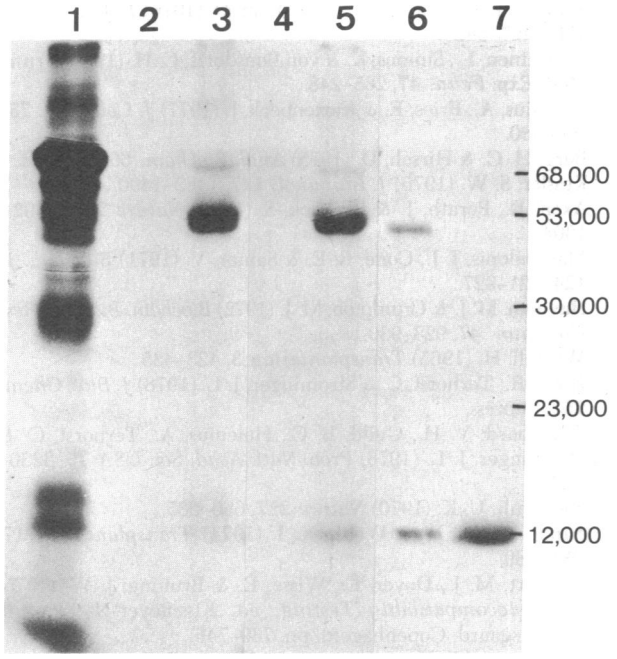

FIG. 5. $NaDodSO_4$ slab gel electrophoresis of ^{35}S-labeled cell surface proteins coprecipitated with 29S complexes by using antibodies to spike protein. (Lane 1) Complete cell extract from CEM cells. (Lanes 2 and 4) Control immunoprecipitates from CEM and P815 cells, respectively, with 29S complexes omitted. (Lanes 3 and 5) Immunoprecipitates from CEM and P815 with 29S complexes present. (Lane 6) HLA antigen, detergent form. (Lane 7) β_2-Microglobulin. Electrophoresis was performed on 12% polyacrylamide gels according to Laemmli (28). Patterns 1–5 were visualized by fluorography and those of 6 and 7 by staining with Coomassie brilliant blue.

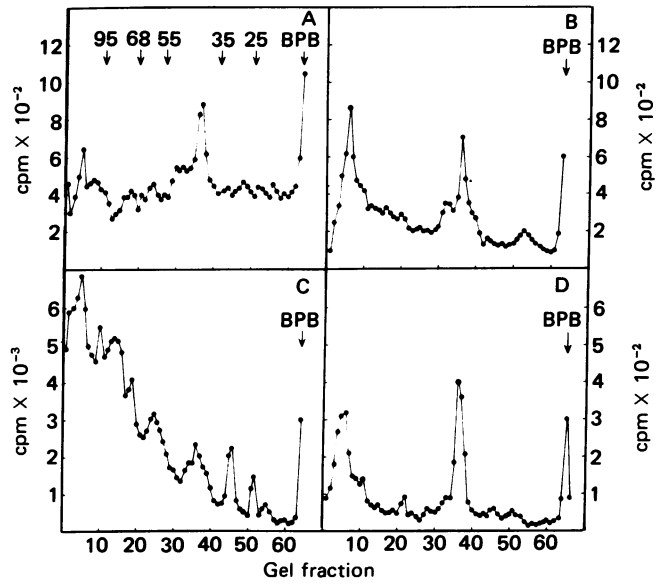

FIG. 6. Affinity chromatography of SFV receptors on 29S complex Sepharose 2B columns. $NaDodSO_4$ slab gel electrophoresis profiles of ^{125}I-labeled surface components of mouse lymphocytes. (*A*) Material from a Triton X-100 cell extract retained by 29S complex-Sepharose. (*B*) As *A*, but material prefractionated on columns of *Lens culinaris* lectin-Sepharose to exclude nonglycosylated components. (*C*) Total glycoprotein fraction before application to 29S complex-Sepharose column. (*D*) Immunoprecipitate formed by antiserum to H-2D and material eluted from 29S complex-Sepharose. The molecular weights ($\times 10^{-3}$) and migrations of marker proteins are indicated in *A*. BPB, bromphenol blue.

To show that the 45,000- to 50,000-dalton polypeptides retained by the affinity column were serologically active H-2K and H-2D antigens, the absorbent with the material bound was incubated with alloantiserum directed against the H-2D antigens. Under these conditions about 60% of the bound radioactivity was released from the affinity absorbent. Immune complexes were then precipitated from the supernatant by using a rabbit antiserum against mouse immunoglobulins. Fig. 6*D* shows an electrophoretic profile of the resulting immunoprecipitate. In addition to the H-2D antigens some of the high molecular weight material was precipitated.

Both these affinity chromatography and receptor precipitation studies show that, among ^{125}I- and diazo[^{35}S]sulfanilic acid-labeled cell surface proteins, the H-2D and H-2K and HLA-A and HLA-B histocompatibility antigens have the highest affinity to the viral spike proteins. Other minor components are also isolated, but whether these represent parts of the virus receptor complex that were labeled to a lesser extent or contaminants remains to be evaluated.

DISCUSSION

The spike octamers (29S complexes) of SFV used in this study have proved very useful in identifying and isolating cell surface receptors for this virus. Although composed of integral membrane proteins, they do not require detergent for solubility and they withstand the detergents used for receptor extraction and purification. Their affinity for the cell surface is sufficient to resist the washes necessary in immunofluorescence staining, cytotoxicity assays, and immunoprecipitation.

Using the 29S complexes as our major tool, we have obtained evidence that strongly points to the histocompatibility antigens on human and mouse cells as receptors for SFV: (*i*) the 29S complexes inhibit the complement-dependent cytotoxicity of antibodies directed against H-2K and H-2D antigens on mouse cells. (*ii*) Isolated HLA-A and HLA-B antigens inhibit the

binding of the viral proteins to cells, especially when presented in a membrane-bound form. (*iii*) Reconstituted HLA-A and HLA-B vesicles interact in solution with SFV and reconstituted SFV membranes. (*iv*) Complexes between viral spike proteins and HLA-A and HLA-B or H-2K and H-2D antigens can be isolated from the cell surface after detergent solubilization. (*v*) When coupled to an insoluble matrix, the 29S complexes selectively bind the H-2K and H-2D antigens from a solubilized mixture of cellular proteins. Our data suggest that the HLA-A, HLA-B, H-2K, and H-2D antigens are in fact the main receptors. This conclusion is supported by the observation that all detectable binding sites for the 29S complexes on JY cells can be capped by using anti-β_2-microglobulin antibodies (B. Morein, unpublished data). The number of binding sites for 29S complexes on the cell line JY (3.35×10^5, see Fig. 2) is consistent with the estimated number of HLA antigens. This estimate is derived by assuming a site density of 2×10^4 HLA antigens on a peripheral blood lymphocyte (29, 30) and a 10- to 30-fold increase of these antigens on a lymphoblastoid cell (31).

It remains to be seen whether the determinants recognized by the viral spike proteins involve the β_2-microglobulin, the heavy chain, or only the carbohydrate moiety. Our data suggest that the viral proteins make no distinction between H-2*D* and H-2*K* gene products, or apparently between the HLA-A2 and HLA-B7 antigens. Daudi cells, which have been reported to carry only the HLA heavy chains (32, 33), do bind appreciable amounts of 29S complexes, suggesting either that the heavy chains alone can serve as receptors or that other components are involved in the receptor sites on Daudi cells. However, these may not be fully functional in virus penetration, because it has been shown that, among numerous human lymphoblastoid cell lines tested, the Daudi cells were the only ones that failed to support SFV infection (34).

Histocompatibility antigens are probably present on all normal nucleated mammalian cells, and their structures and amino acid sequences, as far as known, are highly homologous (15, 16). It has been suggested that the genes coding for these proteins, in fact, form a single evolutionary line from primitive ancestral genes in lower vertebrates to the H-2 complex in mouse and HLA complex in man. This highly conserved structure of the major histocompatibility antigens may provide an explanation for the wide host cell specificity of SFV. The viral spike proteins may recognize determinants that are common to many different histocompatibility antigens. It is desirable to extend our studies using the 29S complexes to other species in which histocompatibility proteins are less well characterized.

The sequence of events in the early interaction of SFV with host cells is not known. Initially, the virus may attach to only a few of the major histocompatibility antigens. Because the antigens are mobile in the plane of the membrane (35), more receptors may subsequently be recruited into the binding site. Sindbis virus, which is closely related to SFV, has been shown to bind evenly over the entire surface of fixed cells, but on unfixed cells they tend to form patches, indicating that the membrane-bound viruses are mobile as well (36). Whether the actual penetration of SFV occurs in specialized areas of the cell membrane and whether it occurs by membrane fusion or by endocytosis are not clear. It is our hope that the identification of the receptors and the reconstitution of these into lipid vesicles will prove helpful in the elucidation of the mechanism of SFV penetration into cells.

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