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Identification of cell surface receptors for the 86-kilodalton glycoprotein of human cytomegalovirus

(anti-idiotype antibody/virus receptor/monoclonal antibody/human cytomegalovirus)

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ABSTRACT **Cell surface receptors for the 86-kDa glycoprotein (gp86) of human cytomegalovirus (HCMV) were identified by using two monoclonal anti-idiotype antibodies that bear the internal image of gp86. These antibodies bound to cells permissive for HCMV infection by both ELISA and immunofluorescence assay and inhibited HCMV plaque formation in human embryonic lung (HEL) cells. Immunoblot analysis showed specific binding of both internal image anti-idiotype antibodies as well as gp86 to an HEL cell membrane protein with an approximate molecular mass of 92.5 kDa. In addition, immunoprecipitation of radiolabeled membrane and cell surface proteins from human foreskin tissue, human foreskin fibroblasts, or HEL cells showed specific binding of anti-idiotype antibody predominantly to the 92.5-kDa protein.**

Anti-idiotype antibodies have been used to identify cell surface receptors for both hormones (1) and viruses, specifically reovirus (2), polyoma (3), and measles virus (4). For the identification of viral receptors, anti-idiotype antibodies can be made against neutralizing antibodies that bind to viral proteins. Anti-idiotype antibodies that bind to cell membrane proteins and inhibit either virus binding and/or viral infection of cells usually bear the internal image of the original ligand and therefore are useful for the identification and isolation of the corresponding cell surface receptor.

The mechanism of entry of human cytomegalovirus (HCMV) into susceptible cells is unknown. However, it is likely that glycoproteins present in the viral envelope mediate virus entry. At least three glycoprotein complexes are known to exist in the HCMV envelope (5, 6): a 130/55-kDa complex (also called gcI), which is homologous to the gB glycoprotein of herpes simplex virus; a 47/52-kDa complex (also called gcII); and a 145/86-kDa complex (also called gp86 and gcIII), which is homologous to the gH protein of herpes simplex virus.

We have described (7) murine monoclonal anti-idiotype antibodies that bear the internal image of an epitope on gp86 and will induce HCMV-neutralizing antibody in mice. Because these anti-idiotype antibodies have characteristics of gp86, we used them to identify a receptor for this viral glycoprotein on cells susceptible to HCMV infection. In this report we identify a 92.5-kDa protein on human fibroblast cells that binds specifically both to anti-idiotype antibodies and gp86 and that can be immunoprecipitated with the anti-idiotype antibody. We suggest that this cell protein may represent a component of a pathway for entry of HCMV into susceptible cells.

MATERIALS AND METHODS

Cell and Virus Propagation. Human embryonic lung (HEL) cells were grown in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum. Human foreskin (HF) tissue was obtained from routine circumcisions performed at Stanford University Hospital. HF fibroblasts were grown in primary culture from foreskins obtained from routine circumcisions as described (14). These cells were grown in MEM supplemented with 10% fetal calf serum and glutamine. HCMV (strain AD169) was passaged in HEL cells, and titer was determined by plaque formation in HEL cells (8).

Anti-Idiotype Antibodies. The preparation of syngeneic monoclonal anti-idiotype antibodies against a monoclonal neutralizing antibody that binds to gp86 of HCMV has been reported (7). The two anti-idiotype antibodies used in this study (4-3-5 and 6-5-1) bear the internal image of an epitope on gp86 and induce titers of neutralizing antibody from 1:16 to 1:80 in sera of hyperimmunized mice. These monoclonal antibodies and a control BALB/c IgM κ light chain (Southern Biotechnology Associates, Birmingham, AL) were purified by affinity chromatography as described (7).

ELISA for Binding of Anti-Idiotype Antibodies to HEL Cells. HEL cells were grown to confluence in 96-well Immulon II plates (Dynatch Laboratories, Alexandria, VA). The cells were washed three times with phosphate-buffered saline (PBS) and then fixed with 0.25% glutaraldehyde. The plates were then blocked with 5% fetal calf serum for 2 hr at 37°C. Fixed cells were then incubated for 1 hr at 37°C with various concentrations of antibody (4-3-5, 6-5-1, control BALB/c IgM) or PBS. Cells were washed three times and then incubated with horseradish peroxidase-labeled goat anti-mouse μ chain-specific antibody (Southern Biotechnology Associates, Birmingham, AL) for 1 hr at 37°C. The cells were finally washed five times, and the assay was developed with 2,2,-azino-di(3-ethylbenzthiazoline) sulfonic acid (ABTS) (Zymed Laboratories). Absorbance was read at 405 nm with a Titertek Multiskan automatic plate reader (Flow Laboratories).

Immunofluorescence Assay. Both glutaraldehyde-fixed and unfixed HEL cell monolayers, grown in four-chamber Labtek tissue culture slides, were incubated with 60 μg of anti-idiotype antibody, control IgM antibody, or PBS per well for 1 hr at 37°C. Cells were washed gently three times with PBS and then incubated with fluorescein isothiocyanate-conjugated goat anti-mouse μ and γ chain-specific antibody (Caltag Laboratories, South San Francisco, CA) for 30 min at 37°C. Cells were washed three times and examined for fluorescence with a Zeiss axioplan research microscope.

Plaque-Inhibition Assay. HEL cells were grown to confluence in Costar 24-well tissue culture plates. Cells were

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Abbreviations: ABTS, 2,2-azino-di(3-ethylbenzthiazoline) sulfonic acid; HCMV, human cytomegalovirus; HEL, human embryonic lung; HF, human foreskin; gp86, 86-kDa glycoprotein.

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preincubated with dilutions of either 4-3-5 antibody or control IgM in PBS for 1 hr at 37°C, after which the cells were inoculated with HCMV for assay of plaques. Inhibition was expressed as the percent of plaque formation in cultures preincubated with either anti-idiotype or control antibody compared to those preincubated with PBS alone.

Membrane Preparations. HEL cell membrane proteins were prepared from hypotonically lysed cells by differential centrifugation (9). Solubilizing buffer was 20 mM Tris·HCL, pH 8.0/0.75% Triton A-100/2 mM phenylmethylsulfonyl fluoride/100 units of aprotinin per ml.

Preparation of Radiolabeled Membrane Proteins. HEL cells (5×10^7 cells) were metabolically labeled with [^{35}S]methionine (0.5 mCi), (New England Nuclear; 1 Ci = 37 GBq) by incubation for 48 hr at 37°C in methionine-free MEM containing 10% dialyzed fetal calf serum. Solubilized proteins were prepared as described above for unlabeled cells. For labeling of HF tissue suspension, tissue was first minced and then cells were further separated by physically homogenizing the tissue until the suspension consisted of approximately 90% single cells as visualized by phase microscopy. Cell suspensions were washed three times and resuspended in PBS. Either pelleted cells from fresh tissue or monolayers of HF fibroblasts with approximately 5×10^6 cells were then surface-labeled with Na^{124}I (Amersham) by the Enzymobead lactoperoxidase reaction (Bio-Rad) according to the manufacturer's instructions. Labeled membrane proteins were solubilized by incubation for 30 min on ice with lysis buffer [0.01 M Tris buffer (pH 8.3) containing 1% deoxycholate, 1 mg of chicken ovalbumin per ml, 0.2 mM phenylmethylsulfonyl fluoride, and 100 units of aprotinin per ml]. Solubilized proteins were separated from pelleted beads and cell debris by centrifugation.

Viral Antigen Purification. Affinity chromatography with monoclonal antibodies that bound gp86 (IG6) or gp130/55 (15D8) was used to prepare purified viral glycoproteins (10).

SDS/PAGE. Nondenaturing 10% polyacrylamide gels were prepared according to the method of Cohen *et al.* (11). Standard 7.5% denaturing polyacrylamide gels were prepared as described by Laemmli (12).

Immunoblot Analysis. After separation by SDS/PAGE, solubilized HEL cell membrane proteins were transferred to nitrocellulose membranes under both nondenaturing and denaturing conditions, and the nitrocellulose membranes were further blocked with "Blotto," as previously described (10, 11). The proteins were then incubated for 2 hr at 37°C with 4-3-5, 6-5-1, or IgM control antibody (100 μg in PBS). Binding of IgM monoclonal antibodies was detected by subsequent incubation with horseradish peroxidase-labeled goat anti-mouse μ chain-specific antibody. For some experiments, affinity-purified gp86 or gp130/55 along with their control preparations from uninfected cells were used. Binding of viral glycoproteins was detected by incubation of the nitrocellulose membranes with guinea pig antisera raised against gp86 or gp130/55 (10) for 1 hr at 37°C, followed by biotinylated goat anti-guinea pig IgG antibody (Vector Laboratories). Assays were developed with Vecta Stain ABC reagents (avidin and biotinylated horseradish peroxidase) (Vector Laboratories) followed by 4-chloro-1-naphthol (Sigma).

Immunoprecipitation. ^{125}I-labeled solubilized HF cell membrane proteins (500,000 cpm) were incubated with 4-3-5 or control IgM antibody (500 μg) for 2 hr at room temperature. Rabbit anti-mouse μ chain antibody (Zymed Laboratories) (50 μg) was then added, and the mixture was further incubated for 1 hr at room temperature. Protein A-Sepharose (Zymed Laboratories) was finally added, and the mixture was incubated on ice for 30 min.

[^{35}S]Methionine-labeled HEL cell membrane proteins (5×10^7 cells) were incubated with 4-3-5, 6-5-1, or control BALB/c IgM at room temperature for 2 hr and then precipitated with Sepharose-coupled rat anti-mouse IgM (Zymed Laboratories) on ice for 30 min. The precipitate was collected by centrifugation and washed five times in buffered detergent solution (pH 8.3) with protease inhibitors as described (13). Immunoprecipitated proteins were separated by SDS/PAGE under denaturing conditions and visualized by autoradiography.

RESULTS

Binding of Anti-Idiotype Antibody to HEL Cells. To determine whether the cell membrane proteins that interact with gp86 of HCMV could be identified by using an internal image anti-idiotype antibody, we first looked for evidence of antibody binding to HEL cells by both ELISA and immunofluorescence assays. For the ELISA, confluent HEL cell monolayers were incubated with either of two internal image IgM anti-idiotype antibodies (4-3-5 or 6-5-1) as well as a BALB/c IgM control antibody. Both anti-idiotype antibodies (referred to as "Ab_2" in Fig. 1) bound better than the control antibody to HEL cells and in a dose-dependent manner.

The immunofluorescent staining of HEL cells with anti-idiotype antibody 4-3-5 is shown in Fig. 2*A*. A diffuse pattern of cytoplasmic fluorescence with evident sparing of the nucleus was seen with 4-3-5 antibody on the fixed cell monolayer. Some background binding of control IgM antibody was again evident (Fig. 2*B*) when compared with negative control cells (Fig. 2*C*). However, it was neither as pronounced nor as extensive as that of the anti-idiotype antibodies, and it was localized to small discrete areas on the cell membranes. A pattern similar to that described for 4-3-5 was also seen with the anti-idiotype 6-5-1 antibody (data not shown).

Inhibition of HCMV Plaque Formation by Anti-Idiotype Antibody. Evidence was next sought that the anti-idiotype antibody was binding to cell surface proteins that were important for HCMV infectivity. HEL cells were preincubated with 4-3-5 or control IgM and infected with HCMV; 5 days later plaques were counted. In addition to having fewer countable plaques when compared with cells treated with control IgM (Table 1), the cultures also had noticeably smaller plaques (data not shown). Preincubation of the cells with 6-5-1 antibody also inhibited HCMV plaque formation in a dose-dependent manner (data not shown).

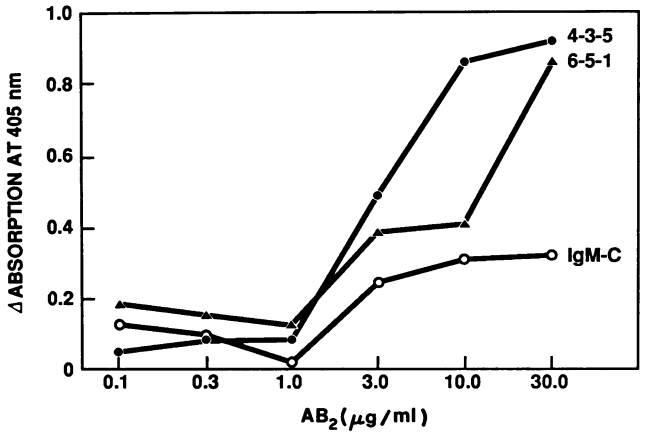

FIG. 1. Specific binding of anti-idiotype antibody by ELISA. HEL cells were incubated with various amounts of anti-idiotype antibody (4-3-5 or 6-5-1), referred to as AB_2, or control antibody (IgM-C) for 1 hr at 37°C. Binding of these antibodies was detected with horseradish peroxidase-labeled goat anti-mouse antibody and ABTS substrate. Values at each concentration represent the average of triplicate samples.

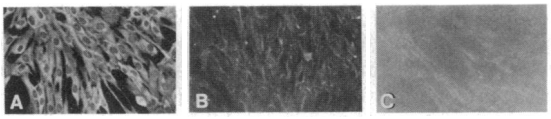

FIG. 2. Detection of anti-idiotype antibody binding to cells by immunofluorescence. Glutaraldehyde-fixed HEL cells were incubated at 37°C for 1 hr with 60 μg of anti-idiotype antibody 4-3-5 (*A*), 60 μg of BALB/c IgM control antibody (*B*), or PBS (*C*). Binding of antibody was detected with fluorescein isothiocyanate-conjugated goat anti-mouse antibody as described. (×120.)

Identification of Specific Cell Membrane Proteins Bound by Both gp86 and Anti-Idiotype Antibody. We next attempted to identify, by immunoblot analysis, the cell membrane protein(s) that bind to the anti-idiotype antibody. Fresh solubilized HEL cell membrane proteins were separated by SDS/PAGE and transferred to nitrocellulose membranes under nondenaturing conditions. Two major bands of 92.5 and 180 kDa were bound by both anti-idiotype 4-3-5 and 6-5-1 antibodies (Fig. 3, lanes 1 and 2). In some immunoblot experiments, the 92.5-kDa protein appeared as two closely migrating species. In addition, 4-3-5 antibody bound strongly to a 45/49-kDa doublet; 6-5-1 also bound to a 35-kDa species. Binding to these same bands was not observed with control IgM (Fig. 3, lane 3). When denaturing conditions were used for SDS/PAGE, there were multiple bands and high background staining, indicating that this technique was not satisfactory for demonstration of receptor with anti-idiotype antibody.

To determine whether gp86 interacted with the 92.5-kDa cell membrane protein, immunoaffinity-isolated gp86 was incubated with nitrocellulose strips to which membrane extracts had been transferred. The gp86 also bound predominantly to a 92.5-kDa doublet cell membrane protein; however, some binding to a 45-kDa protein was also detected (Fig. 3, lane 4). No bands were detected when uninfected cell preparations were eluted from an immunoaffinity column in the same way as lysates from virus-infected cells (Fig. 3, lane 5). Similar bands of approximately 92.5 kDa and 49 kDa were also consistently observed in immunoblots of cell membrane proteins from HF fibroblasts and VERO cells by using 4-3-5 antibody (data not shown).

Preincubation of nitrocellulose-bound HEL cell membrane proteins with 4-3-5 antibody inhibited the subsequent binding

Table 1. Inhibition of HCMV plaque formation by anti-idiotype antibody

Antibody		% plaque formation
Name	μg	
None	—	100
4-3-5	0.04	98
	0.12	99
	0.40	70.5
	1.2	66
	4.0	48
	12	37.5
IgM-C	0.04	98
	0.12	104
	0.4	94
	1.2	90
	4.0	87.5
	12	88

HEL cells were preincubated with anti-idiotype antibody or control BALB/c IgM at the concentrations indicated; medium containing unbound antibody was removed, and a HCMV plaque assay was done. Each value is the average of quadruplicate samples and is expressed as the percent plaque formation compared to a control that was preincubated with PBS alone.

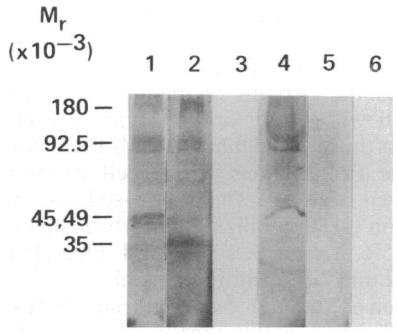

FIG. 3. Immunoblot analysis of p86 and anti-idiotype antibody binding to nondenatured HEL cell membrane proteins. Extracted membrane proteins from HEL cells were separated by SDS/PAGE and transferred to nitrocellulose membranes under nondenaturing conditions. Four hundred microliters of gp86, isolated from 4.8×10^8 CMV-infected cells, was used for a single lane of membrane protein. Control preparations were from an equivalent number of uninfected cells. Nitrocellulose membranes were then blocked and further hybridized to 4-3-5 (lane 1), 6-5-1 (lane 2), control BALB/c IgM (lane 3), affinity-purified gp86 (lane 4), or control uninfected cell proteins prepared in the same manner as gp86 (lane 5); membrane protein in lane 6 was preincubated with 4-3-5 and then hybridized to gp86. Binding of 4-3-5, 6-5-1, and control antibody (lanes 1, 2, and 3) was detected with horseradish peroxidase-labeled goat anti-mouse antibody and developed with 4-chloro-1-naphthol. Binding of gp86 or its control (lanes 4, 5, and 6) was detected by further incubating the membranes with guinea pig antiserum raised against gp86, followed by incubation with horseradish peroxidase-labeled goat anti-guinea pig antibody and development with 4-chloro-1-naphthol.

of gp86 (Fig. 3, lane 6). Affinity-purified gp130/55 and its control from uninfected cells did not bind specifically to the HEL cell membrane preparations (data not shown).

Immunoprecipitation of the gp86 Receptor. Immunoprecipitation of ^{35}S-labeled HEL cell membrane proteins by 4-3-5 (Fig. 4*A*, lane 1) and 6-5-1 (Fig. 4*A*, lane 2) antibodies identified the 92.5-kDa protein as a specific receptor for the anti-idiotype IgM but not control IgM antibodies (Fig. 4*A*, lane 3). To determine whether a similar protein could be immunoprecipitated by these antibodies from another HCMV-susceptible cell type, fresh human foreskin tissue was labeled with ^{125}I. The predominant protein immunoprecipitated specifically from this tissue by antibody 4-3-5 (Fig. 4*B*, lane 1) had an approximate molecular size of 92.5 kDa. Many of the low molecular weight bands as well as the slowly migrating bands of approximately 180 kDa were also reactive with control IgM after immunoprecipitation (Fig. 4*B*, lane 2). Because of a concern that intracellular proteins might also

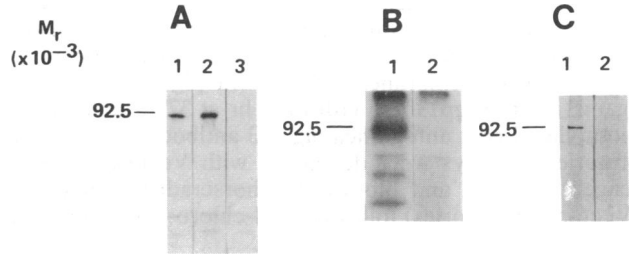

FIG. 4. SDS/PAGE analysis of the immunoprecipitates of radiolabeled fibroblast membranes by anti-idiotype antibodies. (*A*) [^{35}S]Methionine-labeled cell membrane proteins from HEL cells were precipitated with 4-3-5 (lane 1), 6-5-1 (lane 2), or control BALB/c IgM (lane 3). (*B*) ^{125}I-labeled cell membrane proteins extracted from HF tissue were precipitated with 4-3-5 (lane 1) or control BALB/c IgM (lane 2). (*C*) ^{125}I-labeled cell membrane proteins extracted from cultured HF cells were precipitated with 4-3-5 (lane 1) or control BALB/c IgM (lane 2). The dried gels were exposed to Kodak X-Omat AR film at −80° C.

have been labeled in the minced tissue preparation, HF cells grown in cell culture were surface-labeled with ^{125}I, and the solubilized cell membranes were immunoprecipitated by 4-3-5 (Fig. 4C, lane 1) or control IgM (Fig. 4C, lane 2). Again, a 92.5-kDa protein proved to be the predominant cell membrane protein to which anti-idiotype antibody bound.

DISCUSSION

These studies present the first evidence that an envelope glycoprotein from HCMV (gp86) binds to a cell surface receptor. First, anti-idiotype antibodies, known to bear the internal image of gp86, bind to HEL cells that are permissive for HCMV infection. Second, preincubation of HEL cells with the anti-idiotype antibodies results in a decrease in both number and size of HCMV plaques. Third, a cell membrane protein from HEL cells with an approximate molecular size of 92.5 kDa binds to both gp86 and anti-idiotype antibody by immunoblot analysis. A protein of similar molecular size can be immunoprecipitated from HEL cells, HF tissue, and cultured HF fibroblasts by anti-idiotype antibody.

The cell membrane receptors bind specifically to both gp86 "internal image" anti-idiotype antibodies and gp86 since neither control uninfected cell proteins nor gp130/55 bind the predominant band with molecular weight of 92.5 kDa. However, receptor for gp130/55 may not have been identifiable for a number of reasons: (*i*) gp130/55 does not bind directly to the cell membrane, (*ii*) there may not have been sufficient gp130/55 in the affinity-purified preparation to detect binding by the methods used in our study, (*iii*) gp130/55 may bind to a different cell membrane receptor present but be undetectable by our assay, or (*iv*) gp130/55 may bind specifically to cells other than those used in our study.

The cell receptor was clearly demonstrable as a single dominant band after immunoprecipitation with the anti-idiotype antibodies; however, in immunoblots multiple bands were seen. There may be at least two reasons for this: (*i*) the membrane protein may undergo limited proteolysis under the mildly denaturing conditions that exist even in "nondenaturing" gels, or (*ii*) the receptor may be a multimeric unit. Further studies are necessary to define the physical state of the receptor.

The mechanism by which HCMV binds to and penetrates the membrane of susceptible cells is not completely understood. Both gB and gH have been implicated in the penetration and cell-to-cell spread of herpesviruses (15–19).‡ Current evidence suggests that the herpes simplex gH and its homologues in Epstein–Barr and varicella-zoster viruses are not directly involved in attachment, since blocking of this glycoprotein by antibody inhibits infectivity but not adsorption of virus. The ability of our anti-idiotype antibody to block plaque formation is consistent with the examples in other systems and compatible with the idea that gp86 may have a role in HCMV penetration and cell-to-cell spread. Although the control IgM molecules used in this study did not inhibit CMV plaque formation, we must emphasize that they were not specific for membrane glycoproteins. We cannot exclude the possibility that IgMs directed to cell surface determinants could block virus entry nonspecifically.

We have not yet determined whether only cells capable of replicating CMV have the 92.5-kDa receptor described in our study. Virtually all "nonsusceptible" cells so far studied will undergo an abortive cycle of HCMV. Therefore, it is possible that all mammalian cells may possess specific receptors for HCMV viral glycoproteins with the process of intracellular replication being limited by other mechanisms.

‡Fuller, A. O., Santos, R. E. & Spear, P. G. (1988) Proceedings of the 13th International Herpesvirus Workshop, August 7–13, 1988, Irvine, CA, A168.

Class I HLA molecules have been postulated to be cell surface receptors for HCMV to which β_2-microglobulin is bound (20, 21). However, even when using denaturing conditions, we did not find evidence for either gp86 or anti-idiotype IgM binding to a protein that was the 12-kDa size of β_2-microglobulin. Since the largest HCMV protein identified that binds to β_2-microglobulin was reported to be 65 kDa (21), it is likely that a viral protein other than gp86 is involved in this interaction.

The characterization of a cellular receptor for HCMV may lead to generation of new approaches in the therapy of this important human pathogen. The rational design of new human antiviral regimens has been made possible for picornavirus (22–25) and human immunodeficiency virus (25) as a result of the identification of specific cellular receptors for viral proteins. At present, there is only one drug, ganciclovir, that is effective against HCMV disease (26), and it has been reported currently that drug resistant mutants can arise following long-term treatment (27). Because of the impact of HCMV infection on humans, it seems prudent to consider the development of new strategies, such as receptor-mediated inhibition of virus replication for control of this virus infection.

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