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Expression of a Human Cytomegalovirus Receptor Correlates with Infectibility of Cells†

DAWN M. NOWLIN, NEIL R. COOPER, AND TERESA COMPTON*

Department of Immunology, Research Institute of Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, California 92037

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Previous studies have demonstrated that human cytomegalovirus (HCMV) specifically binds to a fibroblast membrane glycoprotein(s) with a molecular mass from 30 to 34 kDa. In this study, the distribution of the putative receptor proteins was analyzed in a variety of cell types, including cell types representative of those that are infected in vivo. Using a sensitive microbinding assay (to score virus attachment) and an indirect detection method (to score HCMV-binding proteins), we found that the 34- and 32-kDa HCMV binding proteins are ubiquitous molecules, broadly distributed among diverse cell types. In addition, the level of virus attachment was found to correlate with the abundance of the 34- and 32-kDa cellular proteins, while the ability of the virus to penetrate cells and initiate infection did not. The results support the hypothesis that the 34- and 32-kDa cellular proteins represent the HCMV (attachment) receptor. The data also support the notion that additional cellular components are required for virus entry and fusion.

Viruses must transfer their genomes across host cell plasma membranes in order to initiate their replicative strategies. Numerous families of viruses are now known to overcome this barrier by binding to a specific protein in the plasma membrane and gaining entry either by receptor-mediated endocytosis or by direct fusion between the viral envelope and the plasma membrane (reviewed in references 27 and 46). The receptor is often a key and in some cases the sole determinant of viral tropism. Because of their pivotal roles in the initiation and spread of infection, viral receptors have gained attention as targets for antiviral strategies. The identification of the cellular receptor (CD4) for human immunodeficiency virus (23), for example, has provided approaches to therapy (7). The finding that infection with human immunodeficiency virus is blocked both in vitro and in animal model systems by a soluble, truncated form of the CD4 molecule has provided a rationale for phase I clinical trials in humans (6, 15, 43).

Among the herpesviruses, only the receptor for Epstein-Barr virus (EBV) has been definitively identified. EBV binding to B cells is mediated by attachment of the virus to the C3dg complement receptor of B lymphocytes, designated CR2 (CD21) (14, 16, 20, 32, 44). Expression of recombinant CR2 on the surface of nonpermissive cells allows low-level EBV infection (2), and soluble recombinant CR2 competitively blocks EBV-induced cell transformation (8, 31). Cell surface heparin sulfate, a glycosaminoglycan, can serve as the initial attachment moiety for both serotypes of herpes simplex virus (47). The porcine herpesvirus may also use a heparinlike molecule for attachment (29). It has been proposed that the interaction of herpes simplex virus with heparin sulfate functions to concentrate the virus on the cell surface to facilitate binding to another as yet unidentified proteinaceous receptor(s) (47). Indeed, a recent report has described the detection of a limited set of cell surface receptors specific for gD which appear to function in virus entry (21).

Human cytomegalovirus (HCMV) is an important human pathogen responsible for significant morbidity and mortality in immunosuppressed patients such as recipients of organ transplants and those suffering from AIDS (3, 5, 26, 33, 34). Previous studies examining the early events in HCMV infection have demonstrated that HCMV exhibits dose-dependent, high-affinity, saturable binding to a membrane glycoprotein(s) in permissive fibroblasts (40). Recent reports from two independent laboratories (1, 41) have shown that HCMV specifically binds fibroblast membrane proteins with apparent molecular masses of 30 to 34 kDa. Taylor and Cooper (41) as well as Adlish et al. (1) observed that the putative receptor proteins were also found in lymphoblastoid or lymphocytic cells. Another recently published study described the detection of a 92.5-kDa fibroblast membrane protein which reacts with gH (gp86) of HCMV (22). This protein was also detected by using an anti-idiotypic monoclonal antibody which was obtained by immunization with an anti-gp86 neutralizing antibody.

Although efficient in vitro productive infection of HCMV occurs only in cultured fibroblasts, HCMV infects epithelial cells, endothelial cells, lymphocytes, and monocytes/macrophages in vivo. In this study, HCMV binding to cell lines representative of these cell types was examined and found to correlate with the presence of 34- and 32-kDa membrane proteins. Viral penetration in these cell lines, however, was found to depend on additional host cell factors.

MATERIALS AND METHODS

Cells. Human foreskin fibroblast (HFF) cells were established and grown at 37°C in 75- or 150-cm^2 plastic flasks (Corning Glass Works, Corning, N.Y.) with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 0.03% L-glutamine (DMEM). All cell lines analyzed are listed in Table 1. Vero, CHO-K1, and FOX-NY cells were cultured in DMEM. Raji, Molt-4, HEp-2, T2, HSB-2, Mono Mac 6, and HPK-1A cells were propagated in RPMI 1640 medium supplemented with 10% fetal bovine

* Corresponding author.
† Publication 6600-IMM from Research Institute of Scripps Clinic, La Jolla, CA 92037.

TABLE 1. Summary of cell lines analyzed

Designation	Description	Type	Source or reference[a]
HFF	Human foreskin fibroblast	Fibroblast	40
HUVEC	Human umbilical vein endothelial	Endothelial	Isolate[b]
HEp-2	Human laryngeal epidermoid carcinoma	Epithelial	ATCC
HPK-1A	Human *ras*-transformed keratinocytes	Epithelial	Isolate[b]
Mono Mac	Human monoblastic leukemia	Monocytoid	48
U937	Human histiocytic lymphoma	Monocytoid	ATCC
Molt-4	Human lymphoblastic leukemia	T lymphoid	ATCC
Raji	Human EBV-positive Burkitt's lymphoma	B lymphoid	ATCC
T2	Human lymphoblastoid, somatic hybrid	B/T lymphoid	12
Vero	African green monkey kidney	Simian	ATCC
CHO-K1 (CHO)	Chinese hamster ovary	Rodent	ATCC
FOX-NY (FOX)	Mouse myeloma	Rodent	ATCC
Sf9	*Spodoptera frugiperda*	Insect	ATCC

[a] ATCC, American Type Culture Collection.
[b] See Materials and Methods.

serum and 0.03% L-glutamine. HPK-1A cells were a generous gift of Johng Rhim (National Cancer Institute, Bethesda, Md.). Human umbilical vein endothelial cells (HUVEC) were a generous gift of David Loskutoff (Research Institute of Scripps Clinic, La Jolla, Calif.).

Virus and virus purification. HCMV AD169 was propagated in HFF cells, and the titer of infectious virus was determined by plaque assays (45). HCMV virions were purified from clarified culture supernatants essentially as described previously (38, 40, 41). Radiolabeled HCMV virions were obtained by adding [^{35}S]methionine to methionine-free DMEM containing 1% (instead of 10%) fetal bovine serum or [^{3}H]thymidine to infected fibroblast cultures exhibiting a cytopathic effect of 50%. Both isotopes were used at concentrations of 50 μCi/ml. Culture supernatants were collected 3 to 5 days later, and virions were purified as described previously (38, 40, 41). Typically, an incorporation level of 10^{-4} cpm/particle was achieved for both isotopes, as measured by using a Beckman LS8000 scintillation counter. The number of virus particles was estimated from the viral DNA content as previously described (40).

Virus attachment assays. Virus attachment was routinely quantified by using radiolabeled virions by a method adapted for herpes simplex virus (17). HFF cells growing as confluent monolayers in 96-well microtiter plates were washed and incubated for 30 min in PBS-GC (10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 140 mM NaCl, 3 mM KCl, 0.5 mM $MgCl_2 \cdot 6H_2O$, 1 mM $CaCl_2$, 0.1% glucose, and 1% inactivated fetal calf serum [pH 6.5]) plus ovalbumin (5 mg/ml) at 4°C. [^{35}S]methionine-labeled HCMV (^{35}S-HCMV) was added at a constant ratio of particle to cell number (generally 250 particles per cell; see figure legends) in PBS-GC and incubated at 4°C for 90 min with frequent, gentle agitation. The virus inoculum was removed, and cells were washed two times with PBS-GC and then lysed in 1% Triton X-100–1% sodium dodecyl sulfate (SDS) in PBS (10 mM $NaPO_4$, 140 mM NaCl [pH 7.4]). The percentage of recovered virus bound to cells was determined by scintillation counting and was calculated from the average of triplicate samples. HCMV binding to cell types other than HFF was done as described above for adherent cell lines, while cells grown in suspension were counted and the reaction was done in microcentrifuge tubes.

Membrane preparation. Cells were initially separated into cytoplasmic and nuclear fractions by a method previously described (10, 11). Briefly, log-phase suspension cells or confluent adherent cell lines which were in 100-mm tissue culture dishes (Corning) and which were harvested by a rubber policeman were washed two times in PBS and suspended in hypotonic lysis buffer (10 mM Tris-HCl, 10 mM KCl, 1.5 mM $MgCl_2$, 1 μM pepstatin, 1 μM leupeptin, 2 mM phenylmethylsulfonyl fluoride [PMSF], pH 7.5) for 10 min at 4°C. Cells were then disrupted with 20 strokes in a Dounce homogenizer (Wheaton), and intact nuclei were removed by centrifugation at 1,000 × *g*. Membrane fragments were pelleted at 100,000 × *g* for 60 min and solubilized in 0.2% SDS–2 mM PMSF–0.25 M Tris-SO_4 (pH 6.8). The solubilized membrane proteins were quantified by a BioRad protein assay, and 25 to 50 μg of protein was diluted with an equal volume of 20% glycerol-bromophenol blue for electrophoresis on a 10% seminative gel system (9).

Detection of cellular membrane proteins which bind HCMV. Electrophoresed proteins were transferred overnight to nitrocellulose membranes (Schleicher & Schuell) by following the method of Towbin et al. (42) and were stained with Ponceau S (30), and nonspecific binding was blocked with 2% nonfat powdered milk in PBS (pH 6.5). Two methods were used to detect electroblotted proteins which bind HCMV. In one procedure, ^{35}S-HCMV was diluted in NETG buffer (50 mM Tris-HCl, pH 6.5; 0.15 M NaCl; 5 mM EDTA; 0.25% gelatin; 0.1% Tween 20) and incubated with the nitrocellulose membranes, which were washed with NETG and subjected to autoradiography. Alternatively, an indirect detection method was utilized. In this procedure, nitrocellulose replicas were initially incubated with unlabeled virus, then with a monoclonal antibody specific for gB (gp55-116) (the 27-156 monoclonal antibody used in these studies was a generous gift of William J. Britt, University of Alabama, Birmingham, Ala.), and subsequently with ^{125}I-protein A. All incubations and washings were performed in NETG as described above.

Penetration of HCMV into cells. To analyze entry or penetration of HCMV into the various cells, cellular uptake of [^{3}H]thymidine-labeled HCMV (^{3}H-HCMV) was determined. Approximately 4 × 10^5 adherent and suspension cells were washed two times with PBS-GC. Nonspecific binding was blocked by a 30-min incubation in a solution containing PBS-GC and 5 mg of ovalbumin per ml. Cultures were incubated for 3 h at 37°C with ^{3}H-HCMV at a ratio of 5,000 particles per cell in PBS-GC. The virus inoculum was removed, and the cells were washed twice with PBS-GC and once with PBS. HCMV remaining on the cell surface was released by a 5-min incubation at room temperature with a

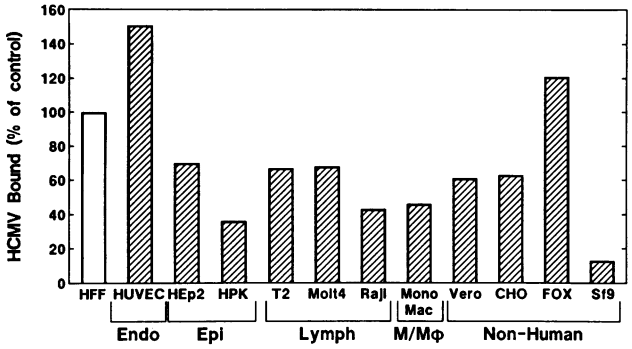

FIG. 1. Attachment of HCMV to cell types of diverse origins. ^{35}S-HCMV (500 particles per cell) was added to the indicated cell lines at 4°C for 90 min. After removal of the unbound virus, the cells were washed extensively and lysed with 1% Triton X-100–1% SDS in PBS. All assays were performed in triplicate, and the percent virus bound was determined by scintillation counting. The data were normalized to HFF cells.

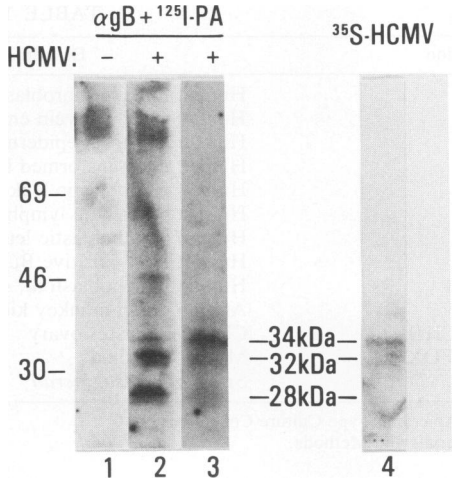

FIG. 2. HCMV binds to 34-kDa and 32-kDa HFF membrane proteins. Membranes were prepared from HFF cells and solubilized in 0.2% SDS–0.25 M Tris-SO_4, pH 6.8. Proteins (25 μg) were resolved on a 10% seminative polyacrylamide gel and then transferred to nitrocellulose. The blotted membrane proteins were incubated in buffer (lane 1), unlabeled virus (lanes 2 and 3), or ^{35}S-HCMV (lane 4). The protein sample in lane 3 contained protease inhibitors (1 μM pepstatin, 1 μM leupeptin, and 2 mM PMSF). Lanes 1 to 3 were incubated with a monoclonal antibody to gB (27-156) and then with ^{125}I-protein A.

trypsin-versine mixture (Whittaker Bioproducts), and the reaction was stopped with an equal volume of fetal bovine serum. The cells were washed again and then lysed with 1% SDS–1% Triton X-100 in PBS and assessed for radioactivity. The amount of radioactivity in the cell lysate represents the amount of internalized HCMV.

Analysis of HCMV immediate-early gene expression. Synthesis of the major immediate-early gene product, the 72-kDa protein (33, 34), was assessed by Western immunoblot analysis. Cells were exposed to HCMV at a multiplicity of infection of ca. 20 to 30 in serum-free media (DMEM or RPMI, depending on the cell type) for 90 min at 37°C, the inoculum was removed, maintenance medium was added (Dulbecco's modified Eagle's medium supplemented with 2% fetal bovine serum and 0.03% L-glutamine), and incubation was continued for 3 to 5 days for all cell types excluding HFF. HFF cells were infected with HCMV at a multiplicity of infection of 1 and harvested 4 to 16 h later. The cells were washed two times with PBS, lysed with 1× SDS-polyacrylamide gel electrophoresis sample buffer, and analyzed by Western blotting as described above. Transferred proteins were incubated with a rabbit anti-peptide antibody (8528) (generously provided by Jay A. Nelson, Research Institute of Scripps Clinic) specific for the 72-kDa immediate early (IE) protein (36) in NETG (pH 7.5), and ^{125}I-protein A was used for detection as described above.

RESULTS

Attachment of HCMV to cell lines of diverse origins. The ability of HCMV to bind to cell lines of various, including nonhuman, origins was examined. A complete list of the cell lines analyzed in these studies is presented in Table 1. ^{35}S-HCMV was added to cells at a constant ratio of particle to cell number for 90 min at 4°C. After removal of the unbound virus, the cells were washed extensively and lysed with detergent. The percent virus bound was determined, and the data were normalized to the values obtained for HFF cells (Fig. 1). Binding of HCMV was detected to all cell lines tested, with the exception of the Sf9 insect cells, for which binding was not significant above the background. The T2 cell line, a chimeric B/T cell line which does not express major histocompatibility complex (MHC) class I antigens (13), also bound HCMV. While the level of HCMV binding within each class of cells was variable, major distinctions were not evident between cell types of diverse origins.

Binding of HCMV to membrane proteins of HFF cells. Previous studies examining the binding of HCMV to electroblotted membrane proteins employed radiolabeled HCMV virions as a probe (1, 41). To potentially enhance the sensitivity of such experiments, an indirect detection method was developed. In this procedure, blotted membrane proteins are initially incubated with purified unlabeled virions and then incubated with a monoclonal antibody specific for the gB homolog of HCMV (gp55-116) (12) and ^{125}I-protein A. As can be seen in Fig. 2 (lane 2), two proteins of 28,000 and 32,000 molecular weight are predominantly detected by this method. The high-molecular-weight proteins which are also detected are seen in the absence of the virus (lane 1) and most likely represent a nonspecific reaction with the antibody or of protein A with membrane constituents. When a cocktail of protease inhibitors was included throughout the preparation process, the proportion of the 28-kDa protein was reduced and a larger, 34-kDa protein was evident (lane 3). Therefore the 28-kDa protein may represent a proteolytic fragment of a larger 34-kDa species, yet this fragment retains the ability to bind the virus. Identical results are obtained when ^{35}S-HCMV is used as the probe (lane 4), confirming earlier results (40).

Distribution of the 34- and 32-kDa HCMV binding proteins in cell lines of diverse origins. To examine the distribution of the 34- and 32-kDa proteins in the various cell lines, membrane fractions were prepared from each of the indicated cell types and 50 μg of solubilized membrane protein was resolved on seminative gels (Fig. 3). The blot shown in Fig. 3A was incubated in the presence of HCMV virions, while the blot in Fig. 3B was incubated with buffer. Both panels were incubated with anti-gB monoclonal antibody and ^{125}I-protein A. The results show that the 34- and 32-kDa HCMV binding proteins were present in the different cell lines. Negligible

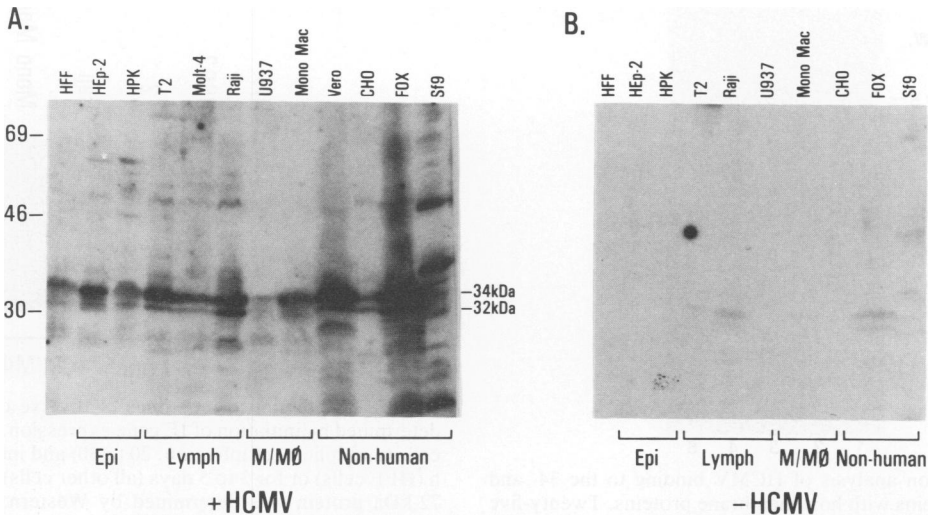

FIG. 3. Detection of the 34- and 32-kDa HCMV binding proteins in cell lines of diverse origins. Membrane fractions were prepared from each of the indicated cell lines as described in Materials and Methods, and 50 μg of solubilized membrane proteins (in the presence of 1 μM pepstatin, 1 μM leupeptin, and 2 mM PMSF) were resolved on 10% seminative gels. After transfer to nitrocellulose, the blotted proteins were incubated in the presence (A) or absence (B) of HCMV virions and then incubated with anti-gB antibody and ^{125}I-protein A.

background was obtained in the absence of virus in most cell lines tested (Fig. 3B). Some nonspecific binding to the 34- and 32-kDa proteins was observed in the Raji and Fox cell lines, which exhibit strong virus binding signals; however, in other cell lines that also exhibit high virus binding signals, no reaction to the 34- and 32-kDa proteins was observed. This is most evident in the T2 cell line (compare Fig. 3A and B) and in Vero cells (data not shown). Other cell lines which have readily detectable 34- and 32-kDa proteins include the endothelial cell line (HUVEC) and a T lymphoid line (HSB2, data not shown). The only cell line we tested which appears not to contain the 34- and 32-kDa proteins was the Sf9 cells, although other high-molecular-weight proteins are recognized. The absence of the 34- and 32-kDa proteins in Sf9 cells is more clearly evident in Fig. 5, lane 4. These data demonstrate that the 34- and 32-kDa HCMV binding proteins are ubiquitous molecules, broadly distributed even in cells of nonhuman origin.

To demonstrate the specificity of binding of HCMV to the 34- and 32-kDa receptor molecules, cold competition analyses were performed. Blotted proteins were incubated with ^{35}S-HCMV (Fig. 4A) or with ^{35}S-HCMV plus a 50-fold excess of unlabeled HCMV (Fig. 4B). Complete blocking was observed, indicating that recognition of the 34- and 32-kDa proteins is specific for HCMV. Specificity of binding of HCMV to the 34- and 32-kDa receptor molecules was also demonstrated in experiments in which ^{35}S-HCMV was incubated with membrane proteins from 34- and 32-kDa protein-positive cells (HEp-2) or -negative cells (Sf9). When ^{35}S-HCMV was incubated with HEp-2 membranes and then used to probe blotted HEp-2 membrane proteins, complete competition for 34- and 32-kDa protein binding is observed (Fig. 5, lane 2 versus lane 3). Similarly, when ^{35}S-HCMV was incubated with Sf9 membranes and used to probe blotted Sf9 proteins, competition for binding to higher-molecular-weight proteins detected in these cells was observed (Fig. 5, lane 4 versus lane 5); however, Sf9-treated virus retained the ability to bind to the 34- and 32-kDa proteins in HEp2 cells (Fig. 5, lane 1). The signal for binding to the 34- and 32-kDa proteins was reduced in Sf9-treated virus, as expected since the virus does react with other Sf9 proteins. It is unlikely that the higher-molecular-weight Sf9 proteins which bind HCMV are related to the 34- and 32-kDa receptors, since the large differences in molecular weight are unaccountable by glycosylation variations between insect and vertebrate cells. Taken together, the data indicate that recognition of the 34- and 32-kDa proteins is specific, which can be demonstrated by competition with excess unlabeled virus or by membrane preparations from 34- and 32-kDa protein-positive cells.

Ability of HCMV to penetrate cell lines of diverse origins. The pathway of HCMV internalization is not known. Specifically, it is not known whether the HCMV envelope fuses

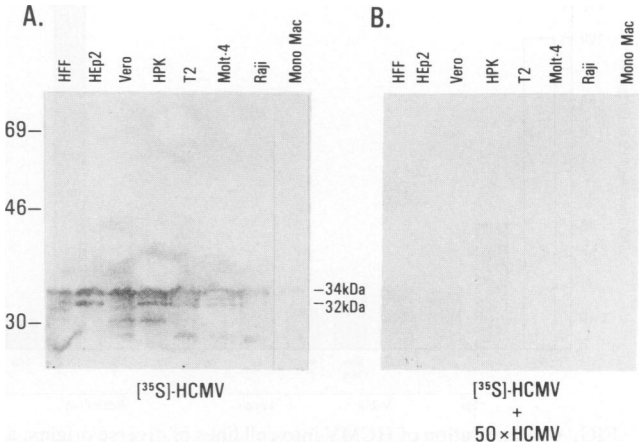

FIG. 4. Specificity of HCMV binding to the 34- and 32-kDa receptor proteins. Cold competition analyses were performed to demonstrate the specificity of binding of HCMV to the 34- and 32-kDa receptor proteins. Membrane proteins from the indicated cell lines were electrophoresed and transferred to nitrocellulose as described in the legend to Fig. 2. The blotted proteins were incubated with ^{35}S-HCMV (A) or with ^{35}S-HCMV plus a 50-fold excess of unlabeled HCMV (B).

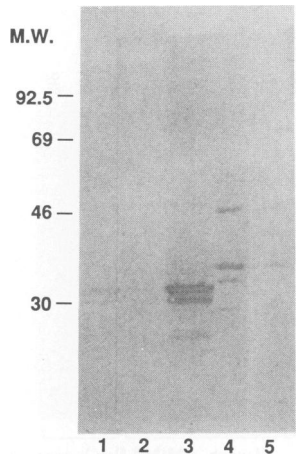

FIG. 5. Competition analysis of HCMV binding to the 34- and 32-kDa receptor proteins with host membrane proteins. Twenty-five micrograms of HEp-2 membrane proteins (lanes 1 to 3) and Sf9 (lanes 4 and 5) were incubated with untreated ^{35}S-HCMV (lanes 3 and 4), ^{35}S-HCMV plus HEp-2 membrane proteins (lane 2), or ^{35}S-HCMV plus Sf9 membrane proteins (lanes 1 and 5). Molecular weight (M. W.) is shown to the left (in thousands).

directly with the plasma membrane (pH-independent fusion) or is internalized and fuses with the endosomal membrane (pH-dependent fusion). An assay was developed that would assess the cellular uptake of ^{3}H-HCMV regardless of the mechanism of penetration. In these experiments, cells were incubated with ^{3}H-HCMV for 3 h at 37°C. After removal of the virus inoculum, the cells were briefly treated with trypsin to remove attached but not internalized virus. The cells were then lysed, and the radioactivity associated with the cells was determined by scintillation counting. As shown in Fig. 6, HCMV was able to penetrate many of the cell lines tested. The variation of the datum points among the various cell

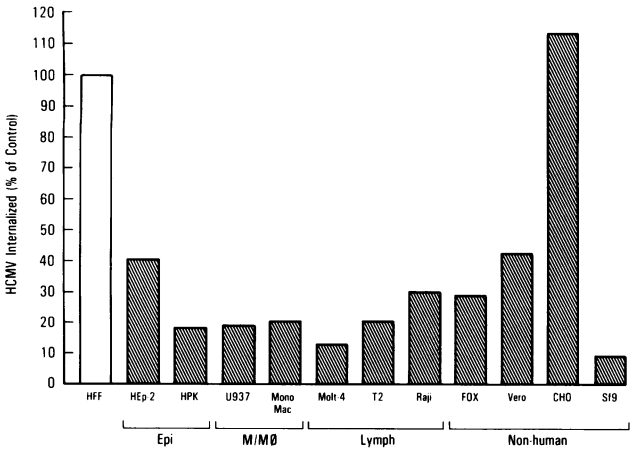

FIG. 6. Penetration of HCMV into cell lines of diverse origins, as determined by cellular uptake of ^{3}H-HCMV. Penetration of HCMV into cells was evaluated by analyzing cellular uptake of ^{3}H-HCMV. Cells were incubated with ^{3}H-HCMV (5,000 particles per cell) for 3 h at 37°C. After removal of the virus inoculum, the cells were briefly treated with trypsin to remove attached but not internalized virus. After lysis of the cells, the percent virus internalized was determined by scintillation counting, and the data were normalized to HFF cells.

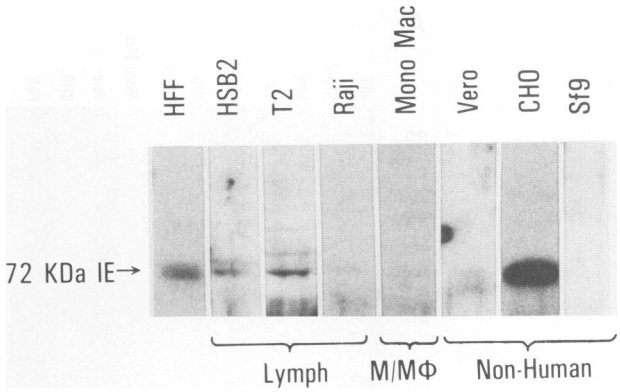

FIG. 7. Infection of cell lines of diverse origins by HCMV, as determined by initiation of IE gene expression. HCMV was added to cells (multiplicity of infection, 20 to 30) and incubated at 37°C for 12 h (HFF cells) or for 3 to 5 days (all other cells). The synthesis of the 72-kDa protein was determined by Western blot analysis, as described in Materials and Methods.

lines was ±10%; therefore, it appears that Molt-4 (12% of control) and Sf9 (10% of control) cells do not support HCMV penetration. Other than CHO cells and HUVEC (80% of control, data not shown), the virus showed a reduced capacity to penetrate most of the cell types, compared with permissive fibroblasts.

Infection of cell lines of diverse origins. The ability to initiate infection, as determined by Western immunoblot analysis of the major immediate early gene product, a 72-kDa protein, was also examined in the various cell lines. IE gene expression indicates that the viral DNA has uncoated and reached the nucleus; however, absence of IE gene expression may merely reflect lack of the appropriate transcription environment for the complex major IE promoter (36, 37, 39). In our studies, IE protein synthesis was readily detectable in T lymphoid cell lines (HSB2 and T2) as well as CHO cells (Fig. 7), while low-level synthesis was discernable in Raji, Mono Mac, and Vero cell lines (more apparent after additional exposure time [data not shown and Fig. 7]) and no expression was evident even with very long autoradiography times in the epithelial cell lines (HEp-2, HPK-1A), Molt-4, and U937, as well as the Fox and Sf9 cells.

DISCUSSION

The 34- and 32-kDa proteins may represent an initial virus attachment receptor. We have shown that HCMV binds predominantly to two membrane proteins of 34 and 32 kDa in fibroblasts. Proteins of the same molecular size are also detected in cell lines of epithelial, endothelial, lymphoid, and monocytoid, as well as nonhuman origin. A summary of the experiments is presented in Table 2. The striking feature of the data is that the ability of the virus to attach to cells correlated with the abundance of the 34- and 32-kDa proteins, while the ability of the virus to penetrate cells and initiate infection (IE gene expression) did not. Although the binding assays were conducted with protein-labeled virions and the internalization assays were conducted with DNA-labeled HCMV, we have found similar levels of binding with DNA-labeled virus (data not shown). These findings indicate that appreciable numbers of particles devoid of DNA do not contribute to the large differences observed in binding versus

TABLE 2. Summary of early events in HCMV infection of cell types of various origins

Cell line	Cell type	Attachment[a]	34- and 32-kDa proteins[b]	Penetration[c]	IE synthesis[d]
HFF	Fibroblast	+++	+++	+++	+++
HEp-2	Epithelial	+++	++++	++	+
HPK	Epithelial	++	++	+	−
U937	Monocytoid	+++	++	+	−
Mono Mac	Monocytoid	++	+++	+	+
Molt-4	Lymphoid	+++	+++	−	−
Raji	Lymphoid	++	+++	+	+
T2	Lymphoid	+++	++++	+	++
Vero	Simian	+++	++++	++	++
CHO	Rodent	+++	+++	++++	++++
Fox	Rodent	++++	++++	+	+
Sf9	Insect	−	−	−	−

[a] Percent HCMV bound to HFF (Fig. 2) was assigned a rating of +++, and the data from other cell lines were ordered and assigned ratings between − and ++++.

[b] An autoradiogram corresponding to Fig. 4 was scanned by densitometry, the area under the curve was calculated, and values were normalized to that of HFF (100%), which was assigned value of +++.

[c] Data from Fig. 6 were ordered, and the percent HCMV internalized in HFF cells was assigned a value of +++; other data from the various cell lines were then ordered and rated from − to ++++.

[d] An autoradiogram corresponding to Fig. 7 was scanned by densitometry, the area under the curve was calculated, and values were normalized to HFF, which was assigned value of +++.

internalization and give further support to the notion that attachment and penetration are uncoupled events. Other examples of this phenomenon include HSV mutants which can attach but not penetrate (24, 25, 35) and the finding that EBV can bind but not penetrate Molt-4 cells (28). In this study, HCMV bound well to Molt-4 and T2 cell lines but penetrated these cell lines poorly. The opposite scenario was also observed in that HCMV binding to CHO and HFF cells was comparable, but the virus penetrated and initiated infection to a greater extent in CHO cells than in HFF cells. These data suggest that the 34- and 32-kDa proteins represent the cellular receptor for HCMV but that other cellular components are required for fusion and penetration. The 92.5-kDa gH-binding protein described by Keay et al. (22) may well represent such a component, since we have demonstrated that neutralizing monoclonal antibodies specific for gH (gp86) permit virus attachment but prevent penetration (11a). Although higher-molecular-weight proteins were variably recognized by HCMV in these studies, the 92.5-kDa protein was rarely detected, even under experimental conditions identical to theirs. The discrepancy in the results likely reflects the difference in the probes, since the 92.5-kDa protein was detected with purified HCMV gH glycoprotein (gp86) or with an anti-idiotypic antibody produced against neutralizing anti-gH monoclonal antibody while we used intact HCMV virions. We are currently applying approaches similar to those presented here toward the complete dissection of the sequence of events in the initial interaction of HCMV with host cells.

The development of a sensitive microassay for virus binding together with the use of an indirect detection procedure for HCMV binding proteins, compared with the use of radiolabeled virus as a probe, has greatly facilitated these studies. Virus binding to as little as 25 μg of extracted membrane protein was readily detected (Fig. 2 and 5), compared with 150 (41) or 100 (1) μg in earlier studies. In addition we have resolved the HCMV-binding proteins into a 34- and 32-kDa doublet; these correspond to the 30-kDa and variably observed 28-kDa membrane proteins described previously (41). The relationship of the 34-kDa protein to the 32-kDa protein is not known at this time. Although it is possible that the 34- and 32-kDa proteins represent a dual component receptor complex, the ratio of the two bands and the amount of the 32-kDa protein varied from cell line to cell line. Similar results were reported by Adlish et al. who found that a 34-kDa HCMV-binding protein predominated in fibroblasts, while both 34- and 23-kDa proteins were detected in B and T lymphocytes (1). Although in our studies great care was taken to prevent proteolysis, it is possible that the 32-kDa protein represents a proteolytic fragment of the larger molecular species.

Other investigators have suggested that a MHC class I antigen may be the receptor for HCMV (18). They proposed that HCMV coated with β2 microglobulin binds to MHC class I molecules after displacing β2 microglobulin from the cell MHC molecules (18, 19). The fact that HCMV binds β-2 microglobulin is attributable to the fact that HCMV encodes an MHC class I homolog (4); however, it has not been demonstrated that the MHC class I homolog is expressed in HCMV-infected cells. It has been demonstrated that intact HCMV virions do not bind the purified electroblotted MHC class I protein (40). In this study, a B lymphoblastoid/T lymphoblastoid chimeric cell line (T2) which does not express the MHC class I antigen was employed (13). Nevertheless, the virus was able to attach, penetrate, and initiate viral gene expression in T2 cells. Therefore, while the presence of an MHC class I homolog in HCMV and the ability of the virus to bind β2 microglobulin could well play a role in the pathogenesis of HCMV infection, it is clear that the presence of an MHC class I molecule on the surface of a host cell is not required for early events in infection and does not represent the cellular receptor for HCMV.

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REFERENCES

1. **Adlish, J. D., R. S. Lahijani, and S. C. St. Jeor.** 1990. Identification of a putative cell receptor for human cytomegalovirus. Virology **176**:337–345.
2. **Ahearn, J. M., S. D. Hayward, J. C. Hickey, and D. T. Fearon.** 1988. Epstein-Barr virus (EBV) infection of murine L cells expressing recombinant human EBV/C3d receptor. Proc. Natl. Acad. Sci. USA **85**:9307–9311.
3. **Alford, C. A., and W. J. Britt.** 1985. Cytomegalovirus, p. 629–659. *In* B. Fields (ed.), Virology. Raven Press, New York.
4. **Beck, S., and B. G. Barrell.** 1988. Human cytomegalovirus encodes a glycoprotein homologous to MHC class-I antigens. Nature (London) **331**:269–272.
5. **Betts, R. F., R. B. Freeman, R. G. Douglas, and T. E. Talley.** 1977. Clinical manifestations of renal allograft derived primary cytomegalovirus infection. Am. J. Dis. Child. **131**:759–763.
6. **Byrn, R. A., I. Sekigawa, S. M. Chamow, J. S. Johnson, T. J. Gregory, D. J. Capon, and J. E. Groopman.** 1989. Characterization of an in vitro inhibition of human immunodeficiency virus by purified recombinant CD4. J. Virol. **63**:4370–4375.
7. **Capon, D. R., S. M. Chamow, J. Mordenti, S. A. Marsters, T. Gregory, H. Mitsuya, R. A. Byrn, C. Lucas, F. M. Wurm, J. E. Groopman, S. Broder, and D. H. Smith.** 1989. Designing CD4 immunoadhesins for AIDS therapy. Nature (London) **337**:525–530.
8. **Carel, J.-C., B. Frazier, T. J. Ley, and V. M. Holers.** 1989. Analysis of epitope expression and the functional repertoire of recombinant complement receptors (CR2/CD21) in mouse and human cells. J. Immunol. **143**:923–930.
9. **Cohen, G. H., V. J. Isola, J. Kuhns, P. W. Berman, and R. J. Eisenberg.** 1986. Localization of discontinuous epitopes of herpes simplex virus glycoprotein D: use of a nondenaturing ("native" gel) system of polyacrylamide gel electrophoresis coupled with Western blotting. J. Virol. **60**:157–166.
10. **Compton, T., and R. J. Courtney.** 1984. Virus-specific glycoproteins associated with the nuclear fraction of herpes simplex virus type 1-infected cells. J. Virol. **49**:594–597.
11. **Compton, T., and R. J. Courtney.** 1984. Evidence for post-translational glycosylation of a nonglycosylated precursor protein of herpes simplex virus type 1. J. Virol. **52**:630–637.

11a. **Compton, T., and R. Nepomuceno.** Unpublished data.

12. **Cranage, M. P., R. Kouzarides, A. T. Bankier, S. Satchwell, K. Weston, P. Tomlenson, B. Barrell, H. Hart, S. E. Bell, A. C. Minson, and G. L. Smith.** 1986. Identification of the human cytomegalovirus glycoprotein B gene and induction of neutralizing antibodies via its expression in recombinant vaccinia virus. EMBO J. **11**:3057–3063.
13. **DeMars, R., R. Rudersdorf, C. Chang, J. Petersen, J. Strandtmann, N. Korn, B. Sidwell, and H. T. Orr.** 1985. Mutations that impair a posttranscriptional step in expression of HLA-A and -B antigens. Proc. Natl. Acad. Sci. USA **82**:8183–8187.
14. **Fingeroth, J. D., J. J. Weis, T. F. Tedder, J. L. Strominger, P. A. Bird, and D. T. Fearon.** 1984. Epstein-Barr virus receptor of human B lymphocytes is the C3d receptor CR2. Proc. Natl. Acad. Sci. USA **81**:4510–4516.
15. **Fisher, R. A., J. M. Bertonis, W. Meier, V. A. Johnson, D. S. Costopoulos, T. Lui, R. Tizard, B. D. Walker, M. S. Hirsh, R. T. Schooley, and R. A. Flavell.** 1988. HIV infection is blocked *in vitro* by recombinant soluble CD4. Nature (London) **331**:76–78.
16. **Frade, R., M. Barel, B. Ehlin-Henriksson, and G. Klein.** 1985. gp140, the C3d receptor of human B lymphocytes, is also the Epstein-Barr virus receptor. Proc. Natl. Acad. Sci. USA **82**: 1490–1493.
17. **Fuller, A. O., and P. G. Spear.** 1985. Specificities of monoclonal and polyclonal antibodies that inhibit adsorption of herpes simplex virus to cells and lack inhibition by potent neutralizing antibodies. J. Virol. **55**:475–482.
18. **Grundy, J. E., J. A. McKeating, and P. D. Griffiths.** 1987. Cytomegalovirus strain AD169 binds β2-microglobulin *in vitro* after release from cells. J. Gen. Virol. **68**:777–784.
19. **Grundy, J. E., J. A. McKeating, P. J. Ward, A. R. Sanderson, and P. D. Griffiths.** 1987. β2-microglobulin enhances infectivity of cytomegalovirus and when bound to virus enables class I HLA molecules to be used as a virus receptor. J. Gen. Virol. **68**:793–803.
20. **Iida, K., L. Nadler, and V. Nussenzweig.** 1983. Identification of the membrane receptor for the complement fragment C3d by means of a monoclonal antibody. J. Exp. Med. **158**:1021–1033.
21. **Johnson, D. C., R. L. Burke, and T. Gregory.** 1990. Soluble forms of herpes simplex virus glycoprotein D bind to a limited number of cell surface receptors and inhibit virus entry into cells. J. Virol. **64**:2569–2576.
22. **Keay, S., T. C. Merigan, and L. Rasmussen.** 1989. Identification of cell surface receptors for the 86-kilodalton glycoprotein of human cytomegalovirus. Proc. Natl. Acad. Sci. USA **86**:10100–10103.
23. **Klatzmann, D., E. Champagne, S. Chamaret, J. Gruest, D. Guetand, T. Hercend, J. C. Gluckman, and L. Montagnier.** 1984. T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. Nature (London) **312**:767–768.
24. **Ligas, M. W., and D. C. Johnson.** 1988. A herpes simplex virus mutant in which glycoprotein D sequences are replaced by β-galactosidase sequences binds to but is unable to penetrate into cells. J. Virol. **62**:1486–1494.
25. **Little, S. P., J. T. Jofre, R. J. Courtney, and P. A. Schaffer.** 1981. A virion-associated glycoprotein essential for infectivity of herpes simplex virus type 1. Virology **115**:149–158.
26. **Macher, A. M., C. M. Reichert, S. E. Strauss, D. L. Long, J. Parrillo, H. C. Lane, and A. J. Fauci.** 1983. Death in the AIDS patient: role of cytomegalovirus. N. Engl. J. Med. **309**:1454.
27. **Marsh, M., and A. Helenius.** 1989. Virus entry into animal cells. Adv. Virus Res. **36**:107–151.
28. **Menezes, J., J. M. Seigneurin, P. Patel, A. Bourkas, and G. Lenoir.** 1977. Presence of Epstein-Barr virus receptors, but absence of virus penetration, in cells of an Epstein-Barr virus genome-negative human lymphoblastoid T line (Molt-4). J. Virol. **22**:816–821.
29. **Mettenleiter, T. C., L. Zsak, F. Zukerman, N. Sugg, H. Kern, and T. Ben-Porat.** 1990. Interaction of glycoprotein gIII with a cellular heparinlike substance mediates adsorption of pseudorabies virus. J. Virol. **64**:278–286.
30. **Muilerman, H. G., H. G. J. Ter Hart, and W. Van Dijk.** 1982. Specific detection of inactive enzyme protein after polyacrylamide gel electrophoresis by a new enzyme-immunoassay method using unspecific antiserum and partially purified active enzyme: application to rat liver phosphodiesterase I. Anal. Biochem. **120**:46–51.
31. **Nemerow, G. R., J. J. Mullen III, P. W. Dickson, and N. R. Cooper.** 1990. Soluble recombinant CR2 (CD21) inhibits Epstein-Barr virus infection. J. Virol. **64**:1348–1352.
32. **Nemerow, G. R., R. Wolfert, M. E. McNaughton, and N. R. Cooper.** 1985. Identification and characterization of the Epstein-Barr virus receptor on human B lymphocytes and its relationship to the C3d complement receptor (CR2). J. Virol. **55**:347–351.
33. **Quinnan, G. V., H. Masur, A. H. Rook, G. Armstrong, W. R. Frederick, J. Epstein, J. F. Manischewitz, A. M. Macher, L. Jackson, J. Ames, H. A. Smith, M. Parker, G. R. Pearson, J. Parrillo, C. Mitchell, and S. E. Strauss.** 1984. Herpesvirus infections in the acquired immune deficiency syndrome. JAMA **252**:72–77.
34. **Rand, K. H., R. B. Pollard, and T. C. Merigan.** 1978. Increased pulmonary superinfections in cardiac-transplant patients undergoing primary cytomegalovirus infection. N. Engl. J. Med. **298**:951–953.
35. **Sarmiento, M., M. Haffey, and P. G. Spear.** 1979. Membrane proteins specified by herpes simplex viruses. III. Role of glycoprotein VP7 (B2) in virion infectivity. J. Virol. **29**:1149–1158.
36. **Stenberg, R. M., A. S. Depto, J. Fortney, and J. A. Nelson.** 1989. Regulated expression of early and late RNAs and proteins from the human cytomegalovirus immediate-early gene region. J. Virol. **63**:2699–2708.
37. **Stenberg, R. M., D. R. Thomsen, and M. F. Stinski.** 1984. Structural analysis of the major immediate early gene of human cytomegalovirus. J. Virol. **49**:190–199.

38. **Stinski, M. F.** 1976. Human cytomegalovirus glycoproteins associated with virions and dense bodies. J. Virol. **19**:594–609.
39. **Stinski, M. F., D. R. Thomsen, R. M. Stenberg, and L. C. Goldstein.** 1983. Organization and expression of the immediate early genes of human cytomegalovirus. J. Virol. **46**:1–14.
40. **Taylor, H. P., and N. R. Cooper.** 1989. Human cytomegalovirus binding to fibroblasts is receptor mediated. J. Virol. **63**:3991–3998.
41. **Taylor, H. P., and N. R. Cooper.** 1990. The human cytomegalovirus receptor on fibroblasts is a 30-kilodalton membrane protein. J. Virol. **64**:2484–2490.
42. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
43. **Watanabe, M., K. A. Reimann, R. A. DeLong, T. Lui, R. A. Fisher, and N. L. Levin.** 1989. Effect of recombinant soluble CD4 in rhesus monkeys infected with simian immunodeficiency virus of macaques. Nature (London) **337**:267–270.
44. **Weiss, J. J., T. F. Tedder, and D. T. Fearon.** 1984. Identification of a 145,000 Mr membrane protein as the C3d receptor (CR2) of human B lymphocytes. Proc. Natl. Acad. Sci. USA **81**:881–885.
45. **Wentworth, B. B., and L. French.** 1970. Plaque assay of cytomegalovirus strains of human origin (36031). Proc. Soc. Exp. Biol. Med. **135**:253–259.
46. **White, J. M., and D. R. Littman.** 1989. Viral receptors of the immunoglobulin superfamily. Cell **56**:725–728.
47. **WuDunn, D., and P. G. Spear.** 1989. Initial interaction of herpes simplex virus with cells is binding to heparin sulfate. J. Virol. **63**:52–58.
48. **Ziegler-Heitbrock, H. W. L., E. Theil, A. Futterer, V. Herzog, A. Wirtz, and G. Riethmuller.** 1988. Establishment of a human cell line (Mono Mac 6) with characteristics of mature monocytes. Int. J. Cancer **41**:456–461.