Cellular integrins function as entry receptors for human cytomegalovirus via a highly conserved disintegrin-like domain

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Human cytomegalovirus (HCMV) is capable of manifesting disease in nearly every organ system in immunocompromised patients. This broad pathogenic tropism correlates with the ability of the virus to infect all tested vertebrate cell types in vitro, a characteristic that has made receptor identification extremely difficult. During virus entry, HCMV induces cellular morphological changes and signaling cascades consistent with engagement of cellular integrins; however, HCMV structural proteins do not possess the widely used RGD integrin-binding motif. We identified an integrin-binding disintegrin-like domain within HCMV envelope glycoprotein B, a protein required for virus entry and fusion throughout the Herpesviridae. Accepted receptor criteria are met through the use of function-blocking integrin Abs, β1 integrin knockout mouse fibroblasts, and glycoprotein B disintegrin-like peptides, all of which support a critical role for α2β1, αβ1, and αVβ3 integrins as HCMV entry receptors and signaling mediators acting during the penetration stage of the entry pathway. Strikingly, the glycoprotein B disintegrin-like domain is conserved in many human and animal herpesviruses, suggesting that integrins may support entry across this medically important virus family.

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terms.

Human cytomegalovirus (HCMV) is a member of the medically significant Herpesviridae family of viruses. Herpesviruses establish a life-long relationship with their hosts and can manifest disease in an opportunistic manner. HCMV is the most common viral cause of congenital birth defects and is responsible for significant morbidity and mortality in immunocompromised patients, including AIDS patients and organ transplant recipients (1, 2). A notable feature of HCMV pathogenesis is its exceptionally broad tissue tropism. HCMV is capable of manifesting disease in most organ systems and tissue types, which directly correlates with its ability to infect fibroblasts, endothelial cells, epithelial cells, monocytes/macrophages, smooth muscle cells, stromal cells, neuronal cells, neutrophils, and hepatocytes (3, 4). In vitro entry into target cells is equally promiscuous since HCMV is able to bind, penetrate, and initiate replication in all tested vertebrate cell types (5). HCMV host cell entry begins with a required tethering step to cell surface heparan sulfate proteoglycans (HSPGs) (6). After HSPG binding, the virus transitions to a more stable docking step by engaging unidentified protein receptors (7), all of which lead to fusion at the cell surface (8). Recently, epidermal growth factor receptor (EGFR) was identified as a potential cellular attachment and signaling coreceptor for HCMV, the expression of which correlated with the ability of the virus to initiate gene expression (9). However, EGFR is not expressed on several HCMV permissive cells, such as hematopoietic cell types. Therefore, postattachment entry mediating coreceptors must exist.

Many of the physiological consequences associated with HCMV infection are consistent with activation of cellular integrins. Host cells respond to HCMV infection by activating numerous signal transduction pathways including initiating Ca2+ influx at the cell membrane, as well as activating phospholipases C and A2, mitogen-activated protein kinase, p38, NF-κB, and SP-1 (10, 11). HCMV also induces a distinct cytopathology, with cells rounding 30–60 min after viral challenge corresponding to the entry event (12).

In recent years, cellular integrins have emerged as attachment or “postattachment” (internalization) receptors for a large number of viruses, including the herpesviruses Kaposi’s sarcoma-associated herpesvirus (KSHV) and Epstein–Barr virus (13, 14). Integrins are expressed on the cell surface as a noncovalently linked heterodimer consisting of α and β subunits, which conveys specificity in cell–cell adhesion, cell–extracellular matrix (ECM) adhesion, immune cell recruitment, extravasation, and signaling (15, 16). There are several known integrin recognition motifs. The most common of these contains the amino acid sequence Arg-Gly-Asp (RGD). However, there are a number of RGD-independent integrin recognition motifs. These include motifs found in certain ECM proteins and the disintegrin domain found in the family of proteins known as ADAMs (a disintegrin and a metalloprotease) (17). ADAM members are multidomain proteins that can modulate a variety of cell–cell and cell–ECM interactions with specific (primarily) β1 integrin heterodimers (18). From the 30 known members of the ADAM family, a disintegrin loop domain consensus and minimum integrin recognition motif has been identified (RX-x-DLXXF/L) (19).

Throughout Herpesviridae, viral glycoprotein B (gB) is essential for virus entry and is a critical member of the conserved basic fusion machinery (20). In addition, HCMV gB has documented cell adhesion and signaling capabilities (7, 21). Analysis of HCMV envelope glycoprotein sequences revealed a domain in gB that is very similar to the ADAM disintegrin loop. Strikingly, the gB disintegrin-like domain is highly conserved in HCMV clinical isolates and most β and γ herpesviruses. Peptides encompassing the gB disintegrin-like domain block both HCMV and mouse CMV (MCMV) entry. Experiments using function blocking Abs and integrin knockout cells indicate that α2β1, αβ1, and αVβ3 are critical determinants of HCMV entry and infection. Together, our findings reveal that both HCMV and MCMV engage cellular integrins via a highly conserved disintegrin-like domain. The conservation of the disintegrin-like sequence in the gB homologs of many herpesviruses suggests that cellular integrins function as receptors across this family of viruses.

Methods

Cell Lines and Viruses. β1 integrin knockout fibroblasts (GD25) and β1 integrin-restored GD25 cells (GD25β1) were a generous gift from D. Mosher (University of Wisconsin, Madison). Normal human dermal fibroblasts (NHDFs), mouse NIH 3T3 cells, GD25, and GD25β1 were cultured in DMEM supplemented with 10% FBS; 1,000 IU/ml penicillin; 1,000 μg/ml streptomycin; 50 μg/ml amphotericin B; and 50 μg/ml gentamicin. HCMV strain AD169 was grown in Hep-2 cells and titrated in plaque assay. The MCMV strain F was grown in JRA cells and titrated similarly.

Abbreviations: NHDF, normal human dermal fibroblast; CMV, cytomegalovirus; HCMV, human CMV; MCMV, mouse CMV; KSHV, Kaposi’s sarcoma-associated herpesvirus; ADAM, a disintegrin and a metalloprotease; pfu, plaque-forming units; HSV-1, herpes simplex virus 1; FAK, focal adhesion kinase; EGFR, epidermal growth factor receptor; ECM, extracellular matrix; gB, glycoprotein B; moi, multiplicity of infection; RGD, Arg-Gly-Asp.

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FCS (GIBCO) in a 5% CO₂ atmosphere at 37°C. GD25β1 media also contained 10 µg/ml puromycin (Sigma). Herpes simplex virus 1 (HSV-1) strain HSV-1(KOS)gB86 marked with the Escherichia coli LacZ gene was a gift from R. Montgomery (University of Wisconsin, Madison) (22). MCMV (Smith strain; ATCC VR-194) was prepared and titered on NIH 3T3 cells as described (23). MCMV-GFP (strain RVG102) marked with enhanced GFP under the control of the native immediate early MCMV-GFP was prepared and titered on NIH 3T3 cells as described (23). HCMV AD169 was grown and titered on NHDFs as a generous gift from A. Campbell (Eastern Virginia Medical College, Norfolk) (23). HCMV AD169 was grown and titered on NHDFs as described (5).

Abs and Peptides. β1 integrin Ab DE9 (IgG) (24), was a generous gift from J. Bergelson (Children’s Hospital of Philadelphia). All other integrin Abs (α1 (FB12), α2 (P1E6), α3 (P1B5), α4 (P1H4), α5 (P1D6), αV (M9), αβ1 (GoH1), αβ2 (25E11), and αβ3 (LMI609)) were purchased from Chemicon. Anti-phospho-FAK (Y397) (MAb144) and total FAK (4-4A) were purchased from Chemicon. Phosphospecific polyclonal anti-integrin β1 [pTfpT788/789] (44-872) and phosphospecific polyclonal anti-integrin β3 [pY785] (44-878) were purchased from BioSource International (Camarillo, CA). mAb 1203, which recognizes the immediate early gene products of HCMV, was purchased from the Rumbaugh–Goodwin Institute for Cancer Research (Plantation, FL). mAb 27–78 recognizes gB and was a gift from W. Britt (University of Alabama, Birmingham) (25). A mAb raised against the tegument protein, pp65, was purchased from Advanced Biotechnologies (Columbia, MD). Rabbit polyclonal anti-MCMV e1 (23), which recognizes MCMV early protein (e1), was a generous gift from A. Campbell. Fluorescein and horseradish peroxidase-conjugated secondary Abs were purchased from Pierce. HCMV gB disintegrin-like peptide (RVCSMAGQTDLIRFERNIIC), HCMV gB disintegrin-like null peptide (AVCSMAQTDAIRERIICC), and gB N terminus (SSSTSHATSTSSHGTSRTTSA) were synthesized and purified by reverse-phase HPLC, confirmed by MS, and purchased from the University of Wisconsin Biotechnology Center Peptide Synthesis Facility. RGDS and RGES peptides were purchased from Sigma.

Virus Entry Assays. Integrin-neutralizing Abs or peptides were incubated with cells plated on glass coverslips for 30 min at 37°C. Cells were washed with PBS and incubated with virus at an approximate multiplicity of infection (moi) of 0.5 plaque-forming units (pfu) per cell for 60 min at 37°C. Nonpenetrated virus was inactivated with low-pH citrate buffer, and 24 h later, immunofluorescence was performed (26). Cells were fixed in 3% paraformaldehyde, followed by reverse-phase HPLC, confirmed by MS, and purchased from the University of Wisconsin Biotechnology Center Peptide Synthesis Facility. RGDS and RGES peptides were purchased from Sigma.

Virus-Binding Assay. NHDFs were treated with integrin-neutralizing Abs, peptides, or heparin for 60 min at 4°C. Cells were infected with HCMV (moi, 5 pfu per cell) for 60 min at 4°C. Unbound virus was removed, and cells were washed and fixed with 3% paraformaldehyde. Bound HCMV was detected with mAb 27–78, horseradish peroxidase-conjugated goat anti-mouse secondary Ab, and ImmunoPure tetramethylbenzidine substrate kit (Pierce) with absorbance measured at 450 nm.

Integrin and Focal Adhesion Kinase (FAK) Activation Assay. NHDFs were serum-starved for 24 h. Cells were stimulated with 2 µM lysophosphatidic acid (Sigma) for 10 min at 37°C or with HCMV (moi, 5 pfu per cell) for the times indicated. Each treatment was lysed in an ice-cold modified radioimmunoprecipitation assay buffer and centrifuged at 14,000 × g for 10 min. Total protein levels were quantitated with a Bradford assay, and equivalent amounts of protein were loaded into each lane; this procedure was followed by Western blot analysis as described (7).

Cytoskeletal Rearrangements Assay. Cells were serum-starved for 24 h, followed by stimulation with the indicated viruses at a moi of 1, with 1.5 µg/ml soluble gB (26) or 300 µl of KSHV for 10 min at 37°C. Cells were fixed in 3% paraformaldehyde, followed by immunofluorescence analysis as described (6), with phalloidin-tetramethylrhodamine B isothiocyanate (Sigma).

Results

The gB Disintegrin-Like Domain Is Highly Conserved Throughout Herpesviridae. Integrin expression patterns on HCMV susceptible cells (13, 27, 28). HCMV-induced cellular morphological changes (12), and overlapping signaling capabilities (9–11) suggest that integrins may be involved in HCMV entry. Because all viruses known to use integrins as entry receptors have been shown to do so by ECM protein mimicry, we inspected all HCMV structural glycoproteins for the integrin-binding sequences LDV, DGE, RGD, NGR, RRETAWA, REDV, SDGR, YIGSR, YIGSE, RGES, RSGIY, RSGD, DRDE, and SRYD by using DNASTAR software (DNASTAR, Madison, WI). We found that all HCMV glycoproteins lack ECM-derived integrin-binding sequences, but the gB protein does contain a sequence very similar to the RX6-8DLXXF found in the ADAM family of proteases, which we have termed disintegrin-like (18, 19). The gB protein contains a sequence that all HCMV glycoproteins lack ECM-derived integrin-binding sequences, but the gB protein does contain a sequence that all HCMV glycoproteins lack ECM-derived integrin-binding sequences, but the gB protein does contain a sequence very similar to the RX6-8DLXXF found in the ADAM family of proteases, which we have termed disintegrin-like (18, 19).
CMV Uses Integrins in a Disintegrin-Like Domain-Dependent Manner. To test the role of the gB disintegrin-like domain in CMV entry, peptides corresponding to the 20 amino acids encompassing this domain, or the same peptide with alanine substitutions in the core consensus residues, along with a distinct N-terminal gB peptide lacking disintegrin sequences, were analyzed for their effects on HCMV entry. We also tested HCMV infectivity of fibroblasts after treatment with RGD and RGE peptides to rule out the possibility of RGD structural mimetics in these glycoproteins. HCMV was able to infect RGD5, RGE5, gB disintegrin-like null, and gB N-terminal peptide-treated cells, however, a dose-dependent decrease in infection foci was observed when fibroblasts were treated with gB disintegrin-like peptide (Fig. 2A). Given the high degree of conservation of the gB disintegrin-like domain throughout the β herpesvirus subfamily (Fig. 1), we tested the effect of the human gB disintegrin-like peptide on MCMV infectivity. Treatment of mouse fibroblasts with the gB disintegrin-like peptide resulted in an even more pronounced reduction in MCMV infectivity (Fig. 2B). By contrast, the gB disintegrin-like peptide had no effect on the ability of a virus that lacks the gB disintegrin-like domain, HSV-1, to infect cells.

Integrin Blocking Abs Inhibit HCMV Infection. Given the inhibitory effects of the gB disintegrin-like peptides, we investigated the role of specific cellular integrins in HCMV entry. We tested a variety of Abs designed to bind the natural ligand-binding pocket of β1 integrin and β3 integrin subunits, the two most broadly distributed integrins. The β1 integrin-neutralizing Ab DE9 has been used in a number of studies to partially inhibit the entry of numerous β1 integrin-dependent viruses (e.g., ref. 24). Treatment of fibroblasts with DE9 Ab or β3 integrin-neutralizing Ab inhibited HCMV infection in a dose-dependent manner (Fig. 3A and B), whereas treatment with control ascites, isotype control, or β1 integrin nonneutralizing Abs exhibited no inhibitory activity. These data are consistent with inhibition levels seen by other β1 integrins using viruses and implicate a role for both β1 and β3 integrin subunits in HCMV entry and infection.

A panel of α integrin subunit neutralizing Abs was used to identify specific integrin heterodimers involved in viral infection. Treatment of human fibroblasts with mAbs to α2 and α6 integrin subunits both inhibited HCMV infection similarly, whereas mAbs to the αV integrin subunit had moderate inhibitory activity (Fig. 3C).

To verify a role for β integrins in CMV infection, we performed virus entry assays in β1 integrin-null fibroblasts (GD25) or in GD25 cells with restored β1 integrin expression (GD25β1). HCMV infectivity was reduced by >60% in GD25 cells, compared with the same cell line with restored β1 integrin expression GD25β1. Moreover, MCMV infectivity was reduced by >90% in β1 integrin-null cells (Fig. 3D).

As expected, GD25 fibroblast treatment with β1 integrin-neutralizing Abs had no effect. In addition, GD25 cells treated with β3-neutralizing Abs had no inhibitory effect on MCMV infectivity. However, in cells expressing β1 integrins, such as GD25β1 or NIH 3T3, β1 integrin-neutralizing Abs inhibited MCMV infectivity by >80% (Fig. 3E), a level consistent with inhibitory levels observed with the disintegrin peptide (Fig. 2B). Similar experiments were performed with HCMV. Although nonpermissive for a complete viral lifecycle, HCMV enters mouse fibroblasts with equal efficiency to that of human-derived fibroblasts. Paralleling the Ab-blocking experiments above (Fig. 3A–D), GD25 fibroblasts exhibited a 60% inhibition of HCMV infectivity and >75% inhibition when treated with β1- and β3-neutralizing Abs (Fig. 3F). Collectively, the data show that MCMV primarily uses a β1 integrin-specific entry pathway, whereas HCMV is capable of interacting with both β1 and β3 integrins; particularly α2β1, α6β1, and αVβ3.

CMV Activates Integrin-Specific Signal Transduction Pathways. Integrins bind ECM proteins and adjacent cells to provide cellular attachment functions; however, integrin-binding events are accompanied by a wide range of signal transduction events. Likewise, engagement of integrins and entry of both KSHV and adenovirus have been shown to trigger integrin-specific signaling pathways including the activation of FAK along with inducing...
distinct cellular morphological changes (32, 33). To directly test for integrin activation and downstream integrin-signaling events in response to HCMV, fibroblasts were treated with virus and blotted for specific phosphorylated residues on the cytoplasmic domains of $\alpha_1$, $\beta_3$, and $\beta_3$ integrins or on the soluble cytoplasmic tyrosine kinase that is specifically activated by integrins during integrin binding, FAK. A marked increase in phosphorylation of $\alpha_1$ pT (788) was observed within 15 min after exposure to HCMV, which diminished after 30 min of HCMV infection (Fig. 5A). Additionally, $\beta_3$ pY (785) levels also were increased in response to HCMV; however, the effect was less dramatic (data not shown). Likewise, NHDFs infected with HCMV responded by activating FAK with kinetics that closely paralleled integrin activation (Fig. 5B).

Gross morphological changes consistent with cytoskeletal remodeling have been observed in response to HCMV for some time (12). To specifically examine CMV-induced cytoskeletal rearrangements and cellular morphological changes, fibroblasts were infected with KSHV, a virus known to enter cells in an integrin-dependent manner and induce cytoskeletal rearrangements, or a virus that does not (vesicular stromatitus virus) (33). Cells also were exposed to HCMV or a soluble form of glycoprotein B. As shown in Fig. 5C, cells treated with either HCMV or gB exhibited cytoskeletal rearrangements, stress fiber, and filopodia formation similar to KSHV (Fig. 5C). Furthermore, these CMV-induced changes were found to be $\beta_1$ integrin-specific, because they were only activated in $\beta_1$ integrin-replaced GD25 cells (Fig. 5D and E).

Combined, these data provide further evidence that CMV engages $\beta_1$ integrins via gB. This interaction triggers the activation of integrin cytoplasmic signaling domains, FAK, and cytoskeletal rearrangements and identifies an integrin-specific signaling pathway that was previously unidentified in HCMV-infected cells.

**Discussion**

The identification of HCMV entry receptors has been elusive because of a variety of biological and technical reasons. Herein, we provide distinct lines of evidence supporting the identification of cellular integrins as HCMV coreceptors. All data support a critical role for integrins as HCMV postattachment coreceptors and fully meet the accepted criteria for the identification of a pathogen receptor. mAbs to specific integrin subunits block both the direct virus-entry event, as measured by viral payload delivery, as well HCMV gene expression in a dose-dependent manner. Cells lacking $\beta_1$ integrins were deficient in both entry and cell–cell spread (data not shown) of virus, and the restoration of $\beta_1$ integrin expression in the same cell line restored both phenotypes. A known integrin-binding motif was discovered within a specific 20-aa stretch on an envelope glycoprotein classic throughout *Herpesviridae*. This motif was conserved in $\beta$ and $\gamma$ herpesviruses, and synthetic peptides of this motif...
efficiently blocked entry and infection of two members within this subfamily.

Abs to α2, α6, αV, β1, and β3 inhibited HCMV virion content delivery and infectivity but did not inhibit cell binding. ECM and viral proteins typically engage multiple integrin heterodimers to seemingly perform redundant functions. In fact, the vast majority of viruses that use integrins as entry coreceptors engage multiple integrin heterodimers. Although ensuring receptor expression on a diverse range of cell types appears advantageous for many pathogens, this characteristic presents technical issues in receptor identification. Both the levels of entry inhibition and the concentration of integrin Abs used are consistent with reports of integrin-engaging viruses. However, more convincingly, we performed entry assays in the presence of multiple integrin Abs on cell types that were deficient in β1 integrin expression and saw substantial additive inhibition of entry. Inhibition of virus entry due to Ab blocking was not influenced by relative abundance of each integrin heterodimer. HCMV entry was inhibited when Abs blocked both highly expressed integrin heterodimers (αV/β3), as well as those with lower levels of expression (αβ1), but not the abundant α5 subunit (or the nonneutralizing β1) or scarce integrins such as α4. These observations eliminate the possibility that blocking of abundant integrins inhibited viral entry through steric hindrance or that blocking scarce heterodimers inhibited viral entry due to complete Ab saturation.

HCMV undergoes fusion at neutral pH, likely at the plasma membrane of fibroblasts (8), although how conserved this entry mechanism is among virus strains remains unknown. HCMV is the first enveloped virus to use integrins in a pH-neutral entry pathway. However, several biologically crucial processes fitting the same criteria regularly occur within the human host. β1 integrins are used in myoblast–myoblast, osteoclast–osteoclast, macrophage–macrophage, and vertebrate sperm–egg attachment and fusion events by means of an unidentified mechanism (34, 35). We provide evidence that integrins function as HCMV coreceptors involved in virus entry, likely during the fusion step, as well as during cell–cell spread. This interaction seems to require the disintegrin-like domain found in the N-terminal region of gB. Interestingly, the vertebrate sperm glycoprotein ADAM 2 contains an N-terminal disintegrin-like domain that binds egg cell surface αβ1 integrin to mediate sperm–egg binding and fusion events. The two motifs are not identical; the disintegrin-like domain in herpesviruses is longer by two to three residues and does not contain an internal cysteine; however, it still remains a possibility that HCMV gB mimics ADAM 2 in its method of binding cellular integrins to promote fusion. The conservation of the disintegrin-like domain among herpesviruses suggests that elucidation of the precise mechanism of HCMV fusion may provide insight toward a conserved fusion mechanism within Herpesviridae, sperm–egg interactions, and other integrin-mediated pH-independent fusion events.

Fig. 4. Effect of integrin-blocking treatments on virus binding and entry. (A) HCMV gB disintegrin-like or null peptides, integrin-neutralizing Abs, or soluble heparin were added to NHDFs at 4°C, followed by infection with HCMV. Attachment was measured by gB ELISA. (B) NHDFs were treated with indicated integrin Abs, infected with HCMV, and assayed for pp65 localization. The number of pp65-positive cells per 1,000 cells is shown.

Fig. 5. Effect of HCMV on the activation of integrin signaling. (A and B) NHDFs were serum-starved and challenged with LPA or HCMV as indicated. (C and D) Samples were blotted with phosphospecific polyclonal anti-integrin β1 [pTpT 788/789] or P-FAK to demonstrate differential activated levels. NHDF (C), GD25 (D Left), or GD25β1 (D Right) cells were stimulated as indicated for 10 min. Cells were fixed and stained with phalloidin to visualize the actin cytoskeleton. Arrows indicate cell rounding, filopodia, and stress fiber formation.
peptides of the unique gB disintegrin-like domain inhibit both HCMV and MCMV infectivity, implicating this sequence in the CMV–integrin interaction. We found that the disintegrin-like domain consensus sequence is completely conserved among β herpesviruses, including human herpesvirus 6, human herpesvirus 7, and other animal herpesviruses. The γ herpesviruses Epstein–Barr virus and KSHV both have been shown to use integrins as entry receptors reportedly via an RGD sequence (13, 14); however, upon further examination, both viruses also contain the conserved gB disintegrin-like domain. Although it is thought that KSHV primarily uses αβ1 in its entry, Ab-blocking experiments also implicate α2β1 (13). Both proposed heterodimers typically engage integrins in an RGD-independent manner (42), provoking questions of the importance of the disintegrin-like domain in the entry of these viruses as well. That MCMV seems to require β1 integrin, but not β3 integrin, and HCMV can use both integrins suggests that these viruses may use different β heterodimers. Although both HCMV and MCMV contain identical disintegrin-like consensus sequences, it is the interstitial amino acid residues that vary and have been shown to convey specificity to a particular integrin heterodimer. All ADAM family members contain the disintegrin loop consensus sequence; however, the sequences flanking each consensus residue vary and allow the ADAM family of proteins to bind several different integrin heterodimers, thus, whereas all β herpesviruses and many γ herpesviruses contain the disintegrin-like consensus, not all are expected to use the exact same integrin heterodimers.

Our sequence analysis determined that, although α herpesviruses lack the gB disintegrin-like domain, HSV-1 contains an RGD sequence in gH, a gene essential for virus fusion. Although it has previously been shown that the RGD in HSV-1 gH does not directly mediate entry (43), this sequence may be important for initiating integrin-specific signaling events important for virus infectivity. The presence of a conserved disintegrin-like domain and/or RGD sequence among almost all herpesviruses implicates cellular integrins as coreceptors throughout the medically important Herpesviridae.