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 $\alpha 2\beta 1$, $\alpha 6\beta 1$, and $\alpha V\beta 3$ 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.

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 hematopoetic 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 Ca^{2+} influx at the cell membrane, as well as activating phospolipases 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 an α and β subunit, 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 ($RX_{5\text{-}7}DLXXF/L$) (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 $\alpha 2\beta 1$, $\alpha 6\beta 1$, and $\alpha V\beta 3$ 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%

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% CO_2 atmosphere at 37°C. GD25β1 media also contained 10 μg/ml puromyocin (Sigma). Herpes implex virus 1 (HSV-1) strain HSV-1(KOS)gL86 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 1/3 promoter was 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), α6 (GoH_3), β3 (25E11), and $\alpha V\beta$3 (LM609)] were purchased from Chemicon. Anti-phospho-FAK (Y397) (MAB1144) and total FAK (4-4A) were purchased from Chemicon. Phosphospecific polyclonal anti-integrin β1 [pTpT 788/789] (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 (RVCSMAQGTDLIRFERNIIC), HCMV gB disintegrin-like null peptide (AVCSMAQGTAAIRAERNIIC), and gB N terminus (SSSTSHATSSTHNGSHTSRTTSA) 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). Experiments were performed in triplicate with a minimum of 1,000 cells scored per coverslip. For the HSV entry assay, peptide was incubated with NHDF cells for 30 min and challenged with HSV-1(KOS)gL86, and any nonpenetrated virus was inactivated with low-pH citrate buffer. Cells were incubated 26 h at 37°C before lysis. β-Galactosidase activity was measured by addition of *o*-nitrophenyl β-D-galactoside, and the absorbance was monitored at 420 nm.

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 $\times$ *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).

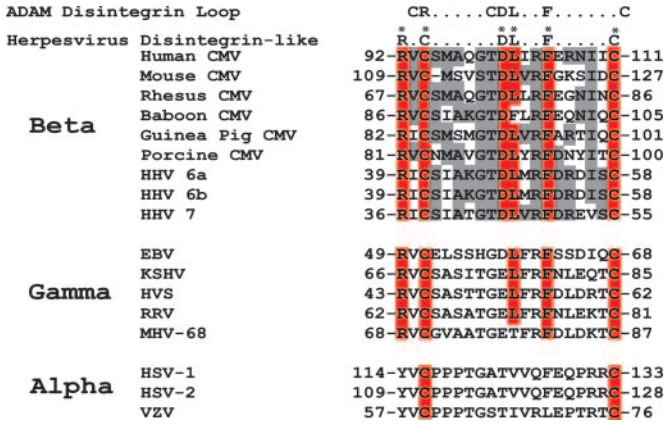

Fig. 1. Conservation of the gB disintegrin-like domain. Alignment of the 20 amino acids encompassing the gB disintegrin-like domain of representative herpesviruses. The conserved herpesvirus gB disintegrin-like consensus sequence is highlighted in red, whereas β herpesvirus conservation is highlighted in gray. The canonical ADAM family disintegrin loop is shown above.

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 immunoflourescence 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 $RX_{6\text{-}8}$DLXXF found in the ADAM family of proteins, which we have termed disintegrin-like (18, 19). The gB sequences of 44 HCMV clinical isolates and two laboratory strains were analyzed for the presence of the disintegrin-like domain. The 20 amino acids encompassing the gB disintegrin-like domain shared a 98% identity with >99% conservation of the disintegrin-like consensus (data not shown). Among other β herpesviruses, the 20 amino acids encompassing the gB disintegrin-like domain share an 86.5% identity with perfect conservation of the disintegrin-like consensus except for a conservative L $\rightarrow$ F substitution in baboon CMV gB (Fig. 1). Furthermore, the gB disintegrin-like domain is present in many γ herpesviruses, but absent in α herpesviruses such as HSV (Fig. 1). The disintegrin-like domain is found in a region of gB implicated in receptor binding and virus–cell fusion (29) and represents disintegrin-like domain mimicry by a virus.

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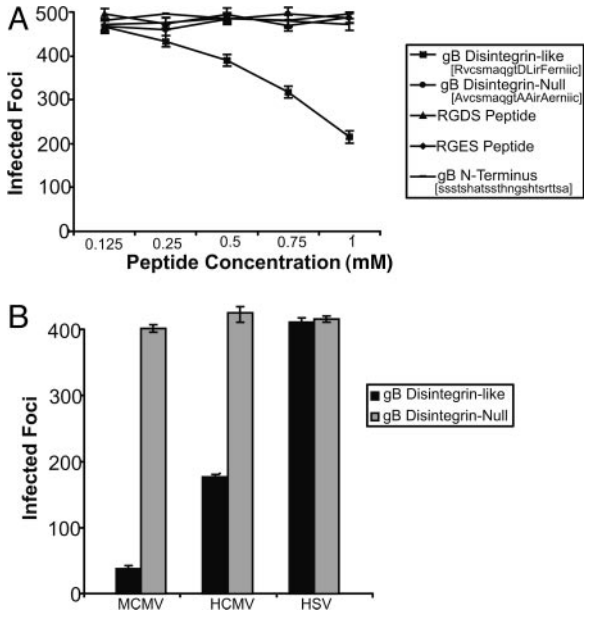

Fig. 2. HCMV gB disintegrin-like peptide inhibits CMV infection. (*A*) NHDFs were treated with the indicated peptides before HCMV challenge (moi, 0.5 pfu per cell). (*B*) The 3T3 or NHDFs were treated with the indicated peptides before virus challenge. Shown is the number of infected foci per 1,000 cells counted.

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 mimicry in these glycoproteins (30). HCMV was able to infect RGDS, RGES, 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. 2*A*). 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. 2*B*). 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. 3 *A* 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. 3*C*).

To verify a role for β1 integrins in CMV infection, we performed virus entry assays in β1 integrin-null fibroblasts (GD25) (31) 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. 3*D*).

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. 3*E*), a level consistent with inhibitory levels observed with the disintegrin peptide (Fig. 2*B*). 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. 3 *A–D*), GD25 fibroblasts exhibited a 60% inhibition of HCMV infectivity and >75% inhibition when treated with β1- and β3-neutralizing Abs (Fig. 3*F*). 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.

HCMV Uses Integrins at a Postattachment Stage During the Entry Pathway. During virus infection, integrins are used as primary viral attachment receptors or as postattachment (fusion-activating) or internalization receptors. To determine at which step in the HCMV entry pathway integrins are functioning, we performed cell-binding experiments (attachment) and an assay to measure viral payload delivery into the cytoplasm (internalization). For the binding assays, virus was bound at 4°C to allow stable virus binding while restricting fusion and internalization. Under conditions that maximally blocked HCMV infection (1 mM gB disintegrin-like peptide, 1:50 DE9, α2, and α6 integrin Abs, 20 μg/ml), there was no effect on HCMV attachment; however, virus attachment was blocked by soluble heparin as described (Fig. 4*A*) (6). These data suggest that integrins are not involved in cellular attachment.

We next performed an assay that directly measures delivery of an internal virion component. The tegument phosphoprotein of 65 kDa, pp65 (UL83), is delivered to the cytoplasm after virus–cell fusion and is targeted to the nucleus by a nuclear localization signal. Thus, the assessment of pp65 uptake is a direct measure of fusion and uncoating but precedes virus gene expression. Treatments that blocked HCMV infectivity, as measured by immediate early gene expression, also blocked uptake of this virion component (Fig. 4*B*). Similarly, cells treated with the gB disintegrin-like peptide but not the gB disintegrin-like null peptide exhibited little uptake of the pp65 tegument protein (data not shown). These data define integrins as HCMV entry receptors mediating HCMV internalization, likely at the level of membrane fusion.

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

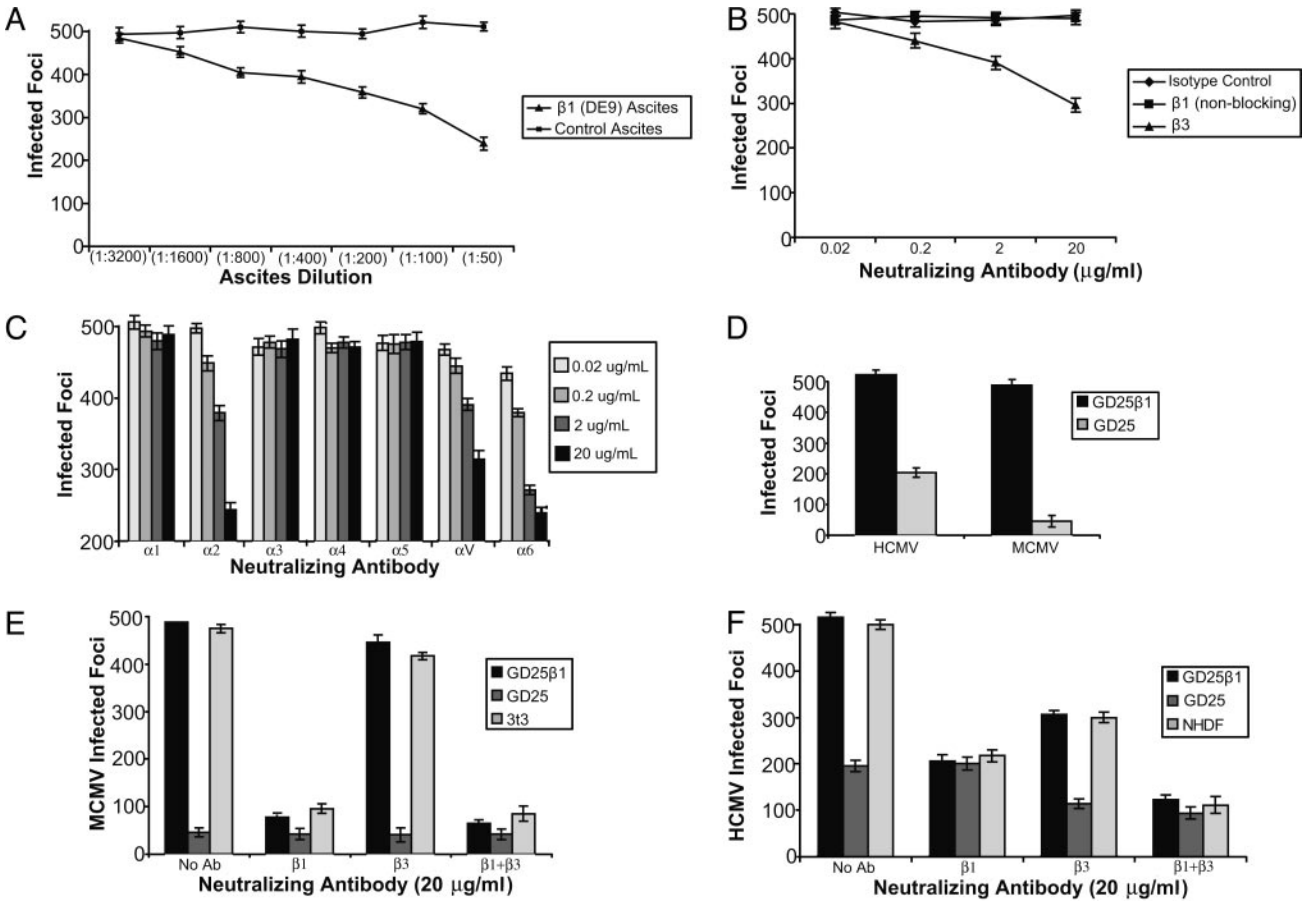

Fig. 3. Integrin-neutralizing Abs inhibit HCMV infectivity. (*A–C*) Integrin-neutralizing Abs were added to NHDFs before HCMV infection (moi, 0.5 pfu per cell). Infectivity was determined by immediate early gene expression with infected foci per 1,000 cells shown. (*D*) β1 integrin knockout fibroblasts (GD25) or GD25 cells with β1 integrin expression restored (GD25β1) were treated with virus, and infected foci were scored. (*E* and *F*) GD25, GD25β1, NHDF, or 3T3 cells were treated with β1, β3, or β1 plus β3 Abs followed by HCMV or MCMV infection (moi, 0.5 pfu per cell). Infectivity was determined by e1 (MCMV) or immediate early (HCMV) gene expression, with infected foci per 1,000 cells counted shown.

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 β1 and β3 integrin or on the soluble cytoplasmic tyrosine kinase that is specifically activated by integrins during integrin binding, FAK. A marked increase in phosphorylation of β1 pTpT (788/789) was observed within 15 min after exposure to HCMV, which diminished after 30 min of HCMV infection (Fig. 5*A*). Additionally, β3 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. 5*B*).

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. 5*C*, cells treated with either HCMV or gB exhibited cytoskeletal rearrangements, stress fiber, and filopodia formation similar to KSHV (Fig. 5*C*). Furthermore, these CMV-induced changes were found to be β1 integrin-specific, becaue they were only activated in β1 integrin-replaced GD25 cells (Fig. 5 *D* and *E*).

Combined, these data provide further evidence that CMV engages β1 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 β1 integrins were deficient in both entry and cell–cell spread (data not shown) of virus, and the restoration of β1 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 essential throughout *Herpesviridae.* This motif was conserved in β and γ herpesviruses, and synthetic peptides of this motif

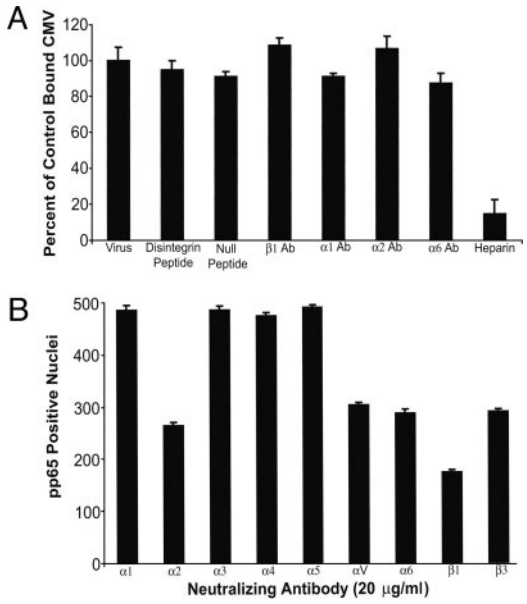

Fig. 4. Effect of integrin-blocking treatments on virus binding and entry. (*A*) HCMV gB disintegrin-like or null peptides, integrin-neutralizing Abs, or soluble heparin were added to NHDFs at 4°C, followed by infection with HCMV. Attachment was measure by gB ELISA. (*B*) NHDFs were treated with indicated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

efficiently blocked entry and infection of two members within this subfamily.

Abs to $\alpha 2$, $\alpha 6$, αV, $\beta 1$, and $\beta 3$ 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 $\beta 1$ 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 ($\alpha V\beta 3$), as well as those with lower levels of expression ($\alpha 6\beta 1$), but not the abundant $\alpha 5$ subunit (or the nonneutralizing $\beta 1$) or scarce integrins such as $\alpha 4$. 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. $\beta 1$ 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 $\alpha 6\beta 1$ 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.

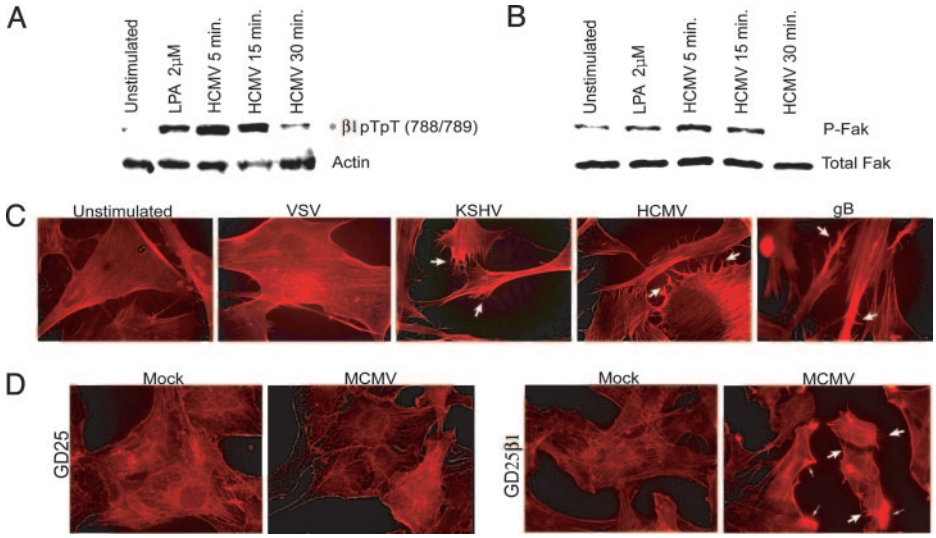

Fig. 5. Effect of HCMV on the activation of integrin signaling. (*A* and *B*) NHDFs were serum-starved and challenged with LPA or HCMV as indicated. (*C* and *D*) Samples were blotted with phosphospecific polyclonal anti-integrin $\beta 1$ [pTpT 788/789] or P-FAK to demonstrate differential activated levels. NHDF (*C*), GD25 (*D Left*), or GD25$\beta 1$ (*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.

A recent report (9) indicates that EGFR functions as a HCMV attachment coreceptor in certain cell types. The data presented herein are completely consistent with the identification of other coreceptors. Our model places cellular integrins in a central ligating role, whereby HCMV can engage multiple receptors and form a multicomponent receptor complex and functional signaling platform. HCMV entry is accompanied by innate immune activation by Toll-like receptors (TLRs) (36). A connection between $\beta1$ and $\beta2$ integrins and an enhancement of TLR signaling has been described (37, 38). Furthermore, both $\beta1$ and $\beta3$ integrins have been shown to associate with EGFR, activate EGFR in a ligand-independent manner (i.e., activate EGFR through integrin binding) (39, 40), and synergistically enhance EGFR signaling (27, 41). The coordination and signaling properties of each of these receptors in both entry and immune detection requires further investigation.

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 $\alpha3\beta1$ in its entry, Ab-blocking experiments also implicate $\alpha2\beta1$ (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 $\beta1$ integrin, but not $\beta3$ integrin, and HCMV can use both integrins suggests that these viruses may use different $\beta1$ 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*.

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