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 enters the cell. This process is highly conserved across this medically important virus family.

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 phospholipases C and A2, mitogen-activated protein kinase, p38, NF-$\kappa$B, 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 $\alpha$ and $\beta$ 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).

Analysis of 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 family of viruses.

**Methods**

**Cell Lines and Viruses.** $\beta$1 integrin knockout fibroblasts (GD25) and $\beta$1 integrin-restored GD25 cells (GD25$\beta$1) were a generous gift from D. Mosher (University of Wisconsin, Madison). Normal human dermal fibroblasts (NHDFs), mouse NIH 3T3 cells, GD25, and GD25$\beta$1 were cultured in DMEM supplemented with 10% FCS. Mouse embryonic fibroblasts expressing the $\alpha$2 and $\beta$1 integrin were cultured in DMEM supplemented with 10% FCS and 5% charcoal-stripped fetal bovine serum (FCS). Mouse P19 cells were cultured in DMEM supplemented with 10% FCS and 10 ng/ml basic fibroblast growth factor (bFGF).

**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; HIV-1, human immunodeficiency 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)gBΔ86 marked with the *Escherichia coli* LacZ gene was a gift from R. Montgomery (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 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 W. Britt (University of Alabama, Birmingham) (25).

**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 (α5 (FB12), α2 (P1E6), α3 (P1B5), α4 (P1H4), α5 (P1D6), αV (M9), α6 (GoH3), β3 (2E5) and αVβ3 (LM609)) were purchased from Chemicon. Anti-phospho-FAK (Y397) (MAb1444) and total FAK (4-4A) were purchased from Chemicon. Phosphospecific polyclonal antibody against integrin β1 [pTyr788/789] (44-872) and phosphospecific polyclonal antibody against 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. Brit (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 (RVCS-MAOGTDLIRFERNI), HCMV gB disintegrin-like null peptide (AVCSMAQTAAIRANI), and gB N terminus (SSSHTSHATSSTHSRTTSA) 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)gBΔ86, and any nonpenetrated virus was inactivated with low-pH citrate buffer. Cells were incubated 24 h at 37°C before lysis. β1-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 incubated 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 radiolmunopro 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. Integran 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, and indeed found all HCMV glycoproteins lack ECM-derived integrin-binding sequences, but the gB protein does contain a sequence very similar to the RX₅₋₈DLXXF found in the ADAM family of proteins. We found that all HCMV glycoproteins lacked the disintegrin-like domain sequence, and instead had a conserved consensus sequence identity with 99% conservation of the disintegrin-like domain (Fig. 1). Furthermore, the gB protein is not conserved in any γ herpesviruses, but absent in α herpesviruses such as HSV (Fig. 1).

The 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 to be present in a region of gB implicated in receptor binding and virus–cell fusion (29) and represents disintegrin-like domain mimicry by a virus.
HSV-1, to infect cells. In contrast, the gB disintegrin-like peptide had no effect on the inhibitory activity. These data are consistent with inhibition levels of fibroblasts with DE9 Ab or control, or/।

The gB disintegrin-like domain throughout the family (Fig. 1), we tested the effect of the human gB disintegrin-like peptide (Fig. 2). 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. 3C).

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. 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. 3 A–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 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 RDGS, 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. 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).

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. 4A) (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 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. 4B).

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 signaling 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 \( \beta_1 \) and \( \beta_3 \) integrin or on the soluble cytoplasmic tyrosine kinase that is specifically activated by integrins during integrin binding, FAK. A marked increase in phosphorylation of \( \beta_1 \) pTyr (788/789) 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 stomatitis 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 essential 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.
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 ligation role, whereby HCMV can engage multiple receptors and form a multimolecular receptor complex and functional signaling platform. HCMV entry is accompanied by innate immune activation by Toll-like receptors (TLRs) (36). A connection between β1 and β2 integrins and an enhancement of TLR signaling has been described (37, 38). Furthermore, both β1 and β3 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 require 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). We are grateful to Dr. Gary Case of the Peptide Synthesis Facility at the University of Wisconsin Biotechnology Center for synthesis and purification of the peptides used in this study and to the members of the Compton laboratory for critical review of the manuscript. This work was supported by U.S. Public Health Service Grant RO1 AI34998 (to T.C.).