

$\alpha_5\beta_1$ -Integrin controls ebolavirus entry by regulating endosomal cathepsins

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Integrins are involved in the binding and internalization of both enveloped and nonenveloped viruses. By using 3 distinct cell systems—CHO cells lacking expression of $\alpha_5\beta_1$ -integrin, HeLa cells treated with siRNA to α_5 -integrin, and mouse β_1 -integrin knockout fibroblasts, we show that $\alpha_5\beta_1$ -integrin is required for efficient infection by pseudovirions bearing the ebolavirus glycoprotein (GP). These integrins are necessary for viral entry but not for binding or internalization. Given the need for endosomal cathepsins B and L (CatB and CatL) to prime GPs for fusion, we investigated the status of CatB and CatL in integrin-positive and integrin-negative cell lines. $\alpha_5\beta_1$ -Integrin-deficient cells lacked the double-chain (DC) forms of CatB and CatL, and this correlated with decreased CatL activity in integrin-negative CHO cells. These data indicate that $\alpha_5\beta_1$ -integrin-negative cells may be refractory to infection by GP pseudovirions because they lack the necessary priming machinery (the double-chain forms of CatB and CatL). In support of this model, we show that GP pseudovirions that have been preprimed *in vitro* to generate the 19-kDa form of GP overcome the requirement for $\alpha_5\beta_1$ -integrin for infection. These results provide further support for the requirement for endosomal cathepsins for ebolavirus infection, identify the DC forms of these cathepsins as previously unrecognized factors that contribute to cell tropism of this virus, and reveal a previously undescribed role for integrins during viral entry as regulators of endosomal cathepsins, which are required to prime the entry proteins of ebolavirus and other pathogenic viruses.

Ebola | filovirus | virus entry | proteolytic priming

Integrins are used by a variety of enveloped and nonenveloped viruses and bacteria to establish infection in host cells. Integrins are heterodimers composed of an α - and a β -subunit. They play an important role in a number of cellular functions, including cell adhesion, migration, proliferation, differentiation, and apoptosis. Integrins are attractive targets for pathogens because they are expressed on a wide variety of different cell types, where they initiate a cascade of signaling events that can facilitate endocytosis and intracellular trafficking of the pathogen (1, 2).

Integrins often serve as primary receptors, allowing viruses to bind to and infect host cells. This is the case with several picornaviruses and hantaviruses (2). For certain adenoviruses and reoviruses, the virus binds to a separate primary cell surface receptor, but interaction with an integrin coreceptor is necessary for internalization. Several herpesviruses have also been shown to bind to host cells via association with other receptors, but require interactions with integrins to initiate cell signaling cascades that promote virus endocytosis and trafficking. Finally, in addition to using integrins for virus internalization, rotavirus encodes an enterotoxin that binds to integrins on intestinal epithelial cells and induces a diarrheal response in mice (3).

β_1 -Integrins have been proposed to facilitate entry of the highly virulent filovirus, ebolavirus (EboV). Kawaoka and colleagues (4) found that β_1 -integrin expression was down-regulated in cells transfected with the EboV glycoprotein (GP). They hypothesized that GP interacts with β_1 -integrins, leading to

its down-regulation, as is the case for the HIV glycoprotein gp120 and its receptor CD4. In support of this hypothesis, they found that treatment of target cells with Abs to β_1 -integrin or with soluble $\alpha_5\beta_1$ -integrin complexes reduced GP-pseudotyped virus infection $\approx 50\%$. Several other cell surface proteins have also been shown to influence GP-mediated entry, including C-type lectins and members of the Tyro3 family (5). So far no single cell surface protein has been found to be both necessary and sufficient for EboV entry. Thus, it is possible that viral entry involves a combination of receptor molecules and could vary by cell type.

Here, we confirmed a role for $\alpha_5\beta_1$ -integrin in EboV infection. Surprisingly, we found that rather than being needed for virus binding or internalization, $\alpha_5\beta_1$ -integrin is required for steps leading to fusion. We further determined that this requirement is at the level of endosomal cathepsins, which have been previously implicated in EboV entry (6, 7).

Results

Expression of $\alpha_5\beta_1$ -Integrin Enhances EboV GP-Mediated Infection. To examine the role of $\alpha_5\beta_1$ -integrin in EboV infection, we took advantage of a series of CHO cells that differ in their $\alpha_5\beta_1$ -integrin expression. CHO K1 cells express endogenous hamster $\alpha_5\beta_1$ -integrin. CHO B2 cells are a clone of CHO K1 cells that was selected for very low cell surface expression of $\alpha_5\beta_1$. These cells still contain the β_1 -integrin chain and increased amounts of a pre- β_1 -integrin moiety, but lack detectable levels of α_5 -integrin and therefore do not express $\alpha_5\beta_1$ -integrin on their surface (8). CHO B2- α_5 cells are a clone of CHO B2 cells engineered to stably express human α_5 -integrin, which can complex with hamster β_1 -integrin to promote surface expression of $\alpha_5\beta_1$ (9). We confirmed expression of α_5 - and β_1 -integrins in these cells by surface biotinylation of the cells and immunoblotting either the whole cell lysate or avidin-precipitated lysate for α_5 - and β_1 -integrins. As expected, only the CHO K1 and CHO B2- α_5 cells express α_5 -integrin in whole cell lysates and on the surface (Fig. 1A, lanes 1, 3, 4, and 6). All 3 cell lines are positive for β_1 -integrin in the whole cell lysates (Fig. 1A, lanes 1–3), but the CHO B2 cells have greatly reduced β_1 -integrin expression on their surface (Fig. 1A, lane 5).

Using VSV pseudotypes encoding GFP that express full-length or mucin domain-deleted EboV GP or VSV G (VSV-GP, VSV-GP Δ , and VSV-G, respectively), we infected this panel of CHO cells and determined the percentage of infected cells by flow cytometry. Although we observed consistently higher in-

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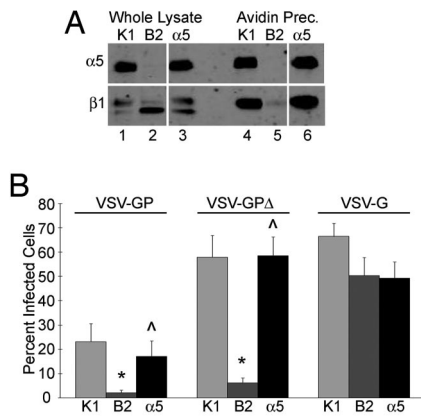


Fig. 1. Expression of $\alpha_5\beta_1$ -integrin enhances EboV GP-mediated infection of CHO cells. (A) Total lysates and surface proteins of CHO K1 (K1), CHO B2 (B2), and CHO B2- α_5 (α_5) cells were immunoblotted for α_5 (Upper) and β_1 (Lower) integrin. Panels shown are from the same blot and same exposure; gaps indicate where lanes were removed. (B) The 3 CHO cell lines were infected with VSV-GP, VSV-GP Δ , or VSV-G, and the percentage of infected cells expressing GFP was measured by flow cytometry. Data shown are the averages from 10 experiments. Error bars indicate SEM. *, $P \leq 0.02$ relative to CHO K1 cells. [^], $P \leq 0.05$ relative to CHO B2 cells.

fection with VSV-GP Δ virus as compared with VSV-GP, in both cases there was an $\approx 90\%$ decrease in infection of the CHO B2 cells compared with CHO K1 cells (Fig. 1B). Reexpression of surface $\alpha_5\beta_1$ -integrin rescued infection by both VSV-GP and VSV-GP Δ . Infection by VSV-G was not significantly different across the 3 cell lines. These results support a previous report that indicated an important role for $\alpha_5\beta_1$ -integrin in EboV GP-mediated infection (4).

To further confirm that $\alpha_5\beta_1$ expression is important for EboV GP-mediated infection, we used siRNA duplexes to knock down expression of α_5 - and β_1 -integrin in HeLa cells. Expression of α_5 -integrin was reduced by 80% in whole cell lysates and 50% on the cell surface in α_5 siRNA-treated cells (Fig. 2A Upper, lanes 2 and 5). This correlated well with a 50% decrease in infection by VSV-GP Δ (Fig. 2B). β_1 -Integrin expression was not reduced, and in fact was increased, on the surface of the α_5 siRNA-treated cells (Fig. 2A Middle, lane 5), suggesting that β_1 is still present on the cell surface in complex with other α subunits. The residual infection in the α_5 siRNA-treated HeLa cells could be due to these other $\alpha\beta_1$ complexes, and/or to the remaining 50% of $\alpha_5\beta_1$ still present on the surface of these cells.

In β_1 -targeted siRNA-treated cells, expression of β_1 -integrin was reduced by 80% in whole cell lysates and by 60% on the cell surface (Fig. 2A Middle, lanes 3 and 6); however, expression of α_5 -integrin was reduced by only 10% on the cell surface (Fig. 2A Top, lane 6). Because α_5 -integrin is not known to form a complex with any other subunit besides β_1 , this result suggests that significant levels ($\approx 90\%$) of $\alpha_5\beta_1$ -integrin are still present on the surface of β_1 siRNA-treated cells, likely explaining why VSV-GP infection of the β_1 siRNA-treated cells was not reduced (Fig. 2B Left, black bar). Infection by VSV-G was not significantly inhibited by either siRNA treatment.

Integrin Expression Is Not Required for EboV GP-Mediated Binding or Internalization. Integrins have been shown to be involved in infection by a wide range of viruses, most often at the stages of virus binding or internalization. The CHO cell lines described above were used to determine if $\alpha_5\beta_1$ -integrin is involved in EboV GP-mediated binding or internalization using a biochemical assay (see Methods). As shown in Fig. 3A, neither virus binding (lanes 1–3) nor internalization (lanes 7–9) was depen-

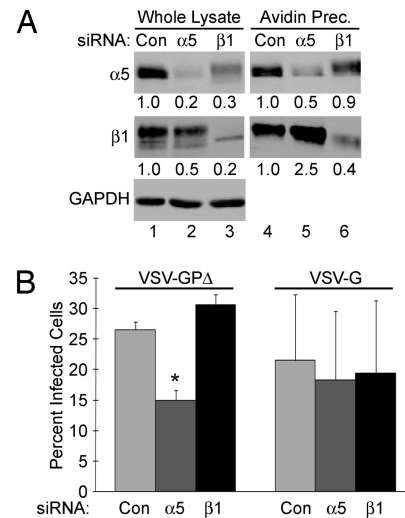
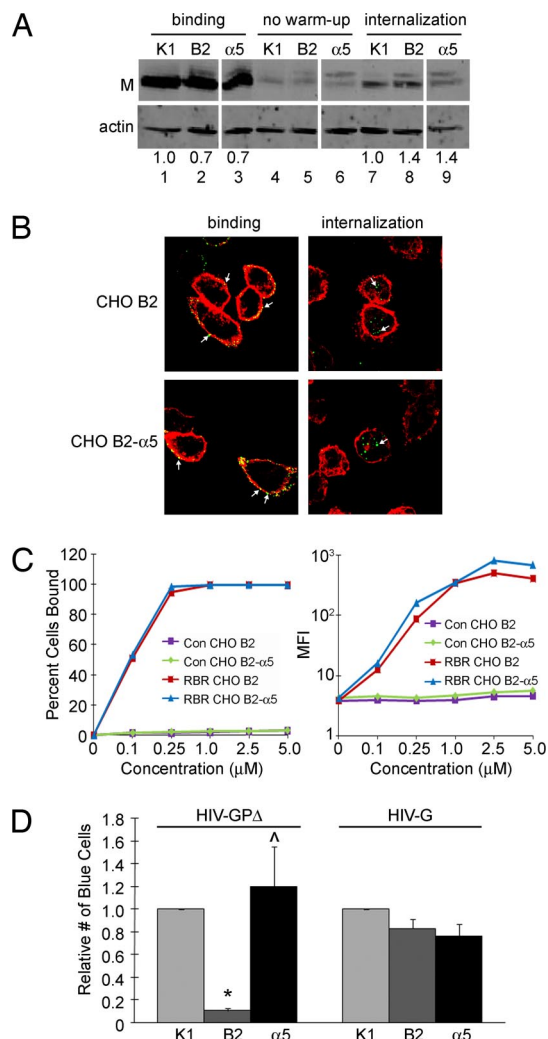


Fig. 2. Knockdown of α_5 -integrin in HeLa cells reduces EboV GP-mediated infection. HeLa cells transfected with a nontargeting control siRNA oligonucleotide (Con) or siRNA oligonucleotides targeting α_5 - or β_1 -integrin were biotinylated and lysed for avidin precipitation and immunoblotting (A) or infected with VSV-GP Δ or VSV-G (B). (A) Representative immunoblots, presented as in Fig. 1, with densitometry values presented below showing the amount of protein, normalized for GAPDH loading, relative to the control siRNA-treated cells. (B) Data shown are the averages from 5 experiments. Error bars indicate SEM. *, $P \leq 0.0008$ relative to control siRNA-treated cells.

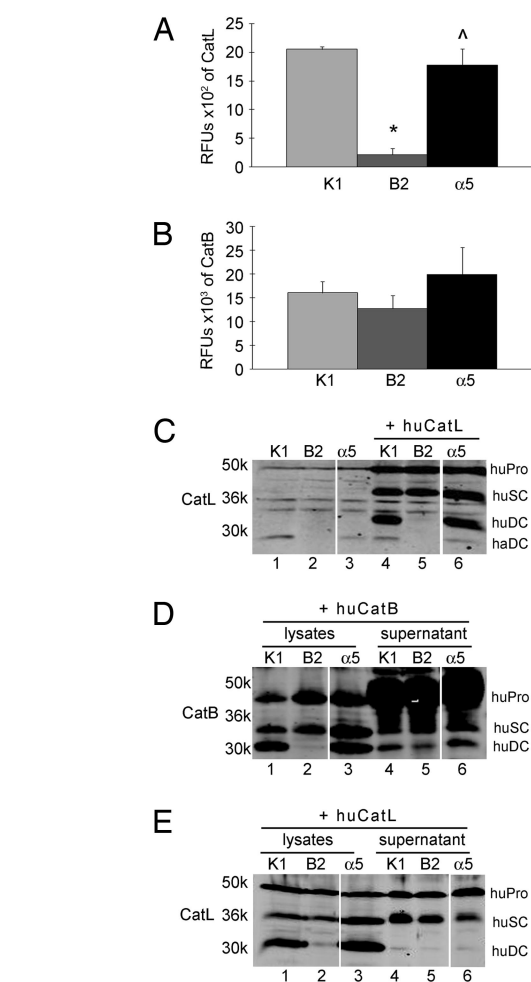
dent on $\alpha_5\beta_1$ -integrin expression. To confirm these results, binding and internalization of VSV-GP Δ in CHO B2 and CHO B2- α_5 cells was visualized by immunofluorescence. In agreement with the above results, we found that both cell lines bound and internalized equivalent amounts of virus (Fig. 3B and Fig. S1A and B). As a further test, we compared binding of increasing amounts of a recombinant EboV receptor binding region (RBR) to CHO B2 and CHO B2- α_5 cells. As shown previously (10, 11), this recombinant RBR protein binds specifically to cells that are susceptible to EboV GP-mediated infection and not to cells that are refractory to EboV infection (Fig. S1C). Consistent with the above data, there was no significant difference in RBR binding between the CHO B2 and CHO B2- α_5 cells (Fig. 3C and Fig. S1C).

$\alpha_5\beta_1$ -Integrin Expression Is Required at or Before EboV GP-Mediated Fusion. After binding and internalization, the virus must undergo fusion with a cellular membrane. To determine if surface expression of $\alpha_5\beta_1$ -integrin is required for events leading up to EboV GP-mediated fusion, the 3 CHO cell lines were infected with HIV pseudotyped viruses containing β -lactamase (BlaM) and bearing EboV GP Δ (HIV-GP Δ). Upon fusion, the BlaM is released into the cytoplasm where it can cleave a fluorogenic substrate, resulting in a shift in fluorescence that can be measured by flow cytometry (12). This differs from the VSV pseudotype system used above in that neither transcription of viral mRNA nor translation of viral proteins is required to generate a positive signal. Our data with the HIV BlaM-containing pseudotypes closely mirrored what was found with the VSV GFP-encoding pseudotypes; EboV GP-mediated fusion was greatly reduced in the CHO B2 cells and was rescued by expression of $\alpha_5\beta_1$ -integrin (Fig. 3D). There were no significant differences in fusion across the 3 cell lines when HIV BlaM pseudotypes bearing VSV G (HIV-G) were used. Taken together, these data indicate that $\alpha_5\beta_1$ -integrin is not required for virus binding or internalization, but is required at or before virus fusion.



Expression of $\alpha_5\beta_1$ -Integrin Correlates with CatL Activity and the Presence of Double Chain (DC) Forms of CatB and CatL. The endosomal cysteine proteases CatB and CatL are required for efficient EboV GP-mediated infection (6, 7). We previously showed that CatB and CatL cleave GP into a 19-kDa primed form, which is then triggered for fusion by an as yet unidentified mechanism. To examine if the block in viral fusion in the $\alpha_5\beta_1$ -integrin-

negative CHO cells was at the stage of GP cleavage by cathepsins, we first looked at CatB and CatL activity in these cells. Using small fluorogenic peptide substrates for CatB and CatL, enzyme activity was measured in whole cell lysates of the 3 CHO cell lines. Surprisingly, CatL activity was reduced by >90% in the CHO B2 cells as compared with the parental CHO K1 cells (Fig. 4A). This activity was significantly rescued by stable transfection of α_5 integrin. In contrast to CatL activity, CatB activity against a small peptide substrate was not significantly different across the panel of CHO cells (Fig. 4B).



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Next, we asked if the decrease in CatL activity seen in the CHO B2 cells correlated with a loss of CatL protein. We found that the CHO B2 cells were missing an ≈ 24 -kDa protein that was detected by the CatL Ab (Fig. 4C, lane 2). The molecular mass of this missing band is consistent with it being the heavy chain of the mature DC form of hamster CatL (haDC), which is generated by sequential processing of the pro- and single chain (SC) forms. The presence or absence of this putative DC form

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of CatL mirrored the CatL activity data (Fig. 4A), and coincided with the pattern seen for EboV GP-mediated infection (Fig. 1B) and fusion (Fig. 3D) in these cells. To determine if overexpression of CatL could rescue infection and expression of the putative DC band in the CHO B2 cells, the CHO cell lines were transiently transfected with a plasmid encoding human CatL and either infected with VSV-GP Δ or lysed for immunoblotting. VSV-GP Δ infection was not rescued in the CHO B2 cells when CatL was ectopically expressed (Fig. S2). Strikingly, although these cells expressed high levels of the precursor (huPro) and SC (huSC) forms of CatL, they specifically lacked the presumptive DC form of the human enzyme (huDC), which runs at a slightly retarded electrophoretic mobility relative to the analogous hamster CatL species (Fig. 4C, lane 5).

We next examined CatB in the $\alpha_5\beta_1$ -integrin-negative cells. As was the case for CatL, the DC form of ectopically expressed CatB was also absent in the CHO B2 cells (Fig. 4D, lane 2), and ectopic expression of human CatB did not rescue infection (Fig. S2). However, in contrast to CatL, the absence of the DC form of CatB did not correlate with a statistically significant decrease in CatB activity in these cells, as measured with a small peptide substrate (Fig. 4B). This is in agreement with a study by Dermody and colleagues (13), who showed that CatB activity toward this peptide substrate correlated with expression of the SC, and not the DC, form of CatB.

To determine if the absence of the DC forms of these enzymes was due to increased secretion, supernatants from equivalent numbers of transfected cells were collected, concentrated, and analyzed by Western blot. The levels of secreted CatB and CatL were not significantly different between the CHO B2 cells and the $\alpha_5\beta_1$ -integrin-positive cells (Fig. 4D and E, lanes 4–6). Moreover, the DC form of CatL was not trapped in high molecular mass or insoluble complexes after cell lysis, since DC CatL was not present when transfected cells were lysed in low pH or boiling sample buffer (Fig. S3A and B). Similarly, pretreating the transfected cells for 24 h with the cysteine protease inhibitor E64d (10–200 μ M) did not result in detection of DC CatL (Fig. S3C), ruling out the possibility that the DC form was being digested by a cysteine protease in the CHO B2 cells. The lack of the DC forms is also unlikely to be due to a defect in processing, because the pro- or SC forms of CatB and CatL did not significantly accumulate in the CHO B2 cells. Finally, bulk CatL protein is not grossly mislocalized in CHO B2 cells, because it colocalized predominantly with the late endosome and lysosome markers LBPA and LAMP1, as in the $\alpha_5\beta_1$ -positive cells (Figs. S4 and S5).

Expression of $\alpha_5\beta_1$ -Integrin Enhances Infection and Expression of the DC Form of CatL in GD25 Cells. As the correlation between $\alpha_5\beta_1$ -integrin expression, EboV GP-mediated fusion, and the presence of the DC form of CatL was highly unexpected, we used an additional cell line to confirm these findings. As shown in Fig. 5A, GD25 cells, which are fibroblastic cells derived from β_1 -deficient mouse embryonic stem cells, do not express α_5 - or β_1 -integrins on their surface. β_1 GD25 cells, which have been stably transfected with the mouse β_1A subunit (14), express both α_5 - and β_1 -integrins on their surface. $\alpha_5\beta_1$ -Integrin expression again led to a significant increase in VSV-GP Δ , but not VSV-G, infection, in this cell system (Fig. 5B). Although we were unable to visualize endogenous CatL (Fig. 5C, lanes 1 and 2) or CatB in these cells, the DC form of ectopically expressed human CatL was only detectable in the cells that express $\alpha_5\beta_1$ (Fig. 5C, lanes 3 and 4). This supports what was seen in the CHO cells; surface expression of $\alpha_5\beta_1$ -integrin correlates with the presence of the DC form of CatL and leads to an enhancement of EboV GP-mediated infection. The basal level of infection of GD25 cells by VSV-GP Δ that is independent of β_1 expression may be due to other integrins, such as $\alpha_V\beta_3$ (not present in CHO B2

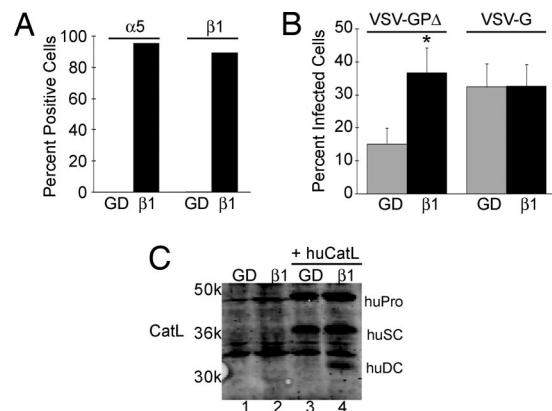


Fig. 5. Expression of $\alpha_5\beta_1$ -integrin enhances EboV GP-mediated infection of GD25 cells. (A) GD25 (GD) and β_1 GD25 (β_1) cells were stained with Abs specific for α_5 - and β_1 -integrins, and the percentage of positive cells was measured by flow cytometry. (B) GD25 and β_1 GD25 cells were infected with VSV-GP Δ or VSV-G, and the percentage of infected cells was analyzed by flow cytometry. Data shown are the averages from 13 experiments. Error bars indicate SEM. *, $P \leq 0.03$ relative to GD25 cells. (C) GD25 and β_1 GD25 cells were either untransfected or transfected with a plasmid encoding human CatL, and lysates were analyzed by immunoblotting for CatL.

cells), that contribute to cathepsin regulation. Alternatively, GD25 cells may possess other proteases that can process GP at a low efficiency.

Prepriming of VSV-GP with Thermolysin Rescues Infection in the $\alpha_5\beta_1$ -Negative CHO Cells. Previously, we found that in vitro treatment of VSV-GP with either a combination of CatB and CatL at low pH or thermolysin at neutral pH cleaves GP into a 20-kDa and subsequently a 19-kDa form. Virus bearing this cleaved form of GP (VSV-GP $_{19k}$) has significantly overcome the block to infection imposed by siRNAs targeting CatB or CatB + CatL (6). To determine if the block in infection of the $\alpha_5\beta_1$ -negative CHO B2 cells was due to the reduction in CatL activity present in these cells, the panel of CHO cells was infected with VSV-GP Δ that had been pretreated with thermolysin to generate VSV-GP $_{19k}$ (Fig. 6A). While infection with mock-treated VSV-GP Δ was again significantly decreased in CHO B2 cells relative to CHO K1 and CHO B2- α_5 cells (Fig. 6B Left), infection of CHO B2 cells by VSV-GP $_{19k}$ was significantly increased as compared with

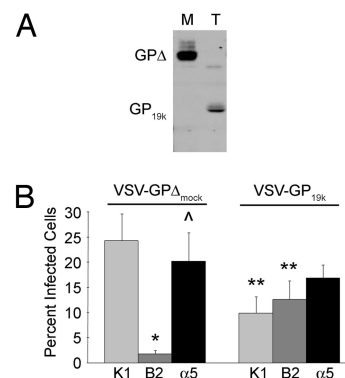


Fig. 6. Pretreatment of VSV-GP Δ to form VSV-GP $_{19k}$ rescues infection in the $\alpha_5\beta_1$ -integrin-negative CHO cells. VSV-GP Δ was either mock-treated (M; VSV-GP Δ_{mock}) or pretreated with thermolysin T to generate VSV-GP $_{19k}$ and analyzed by immunoblotting for GP1 (A) or used to infect the panel of CHO cells (B). Results shown in B are the averages from 9 experiments. Error bars indicate SEM. *, $P \leq 0.007$ relative to CHO K1 cells. \wedge , $P \leq 0.01$ relative to CHO B2 cells. **, $P \leq 0.04$ relative to mock-treated VSV-GP Δ in the same cells.

mock-treated virus and occurred at similar levels to those seen in each of the other cell lines (Fig. 6B Right). These results indicate that the primary block in infection in the $\alpha_5\beta_1$ -negative CHO B2 cells is cleavage of GP to its primed 19-kDa form.

Discussion

In this study, we show that expression of $\alpha_5\beta_1$ -integrin correlates with EboV GP-pseudotyped virus infection postbinding and internalization, but before fusion. Expression of $\alpha_5\beta_1$ -integrin also correlates with expression of the DC forms of CatB and CatL. Cleavage of VSV-GPA to VSV-GP_{19k} rescues infection in $\alpha_5\beta_1$ -integrin-negative CHO B2 cells, indicating that the primary defect in infection of these cells is cathepsin processing of GP, although we have not formally ruled out an additional defect in virus trafficking. These studies provide further support for the requirement for active cathepsins for EboV fusion with host cells and identify the DC forms of CatB and CatL as previously unrecognized factors that contribute to cell tropism of this virus. These findings also identify a previously unrecognized role for integrins in virus entry: regulation of cathepsin expression and activity. This role for integrins in cathepsin regulation may not be restricted to $\alpha_5\beta_1$ -integrin, because a partial rescue in EboV GP-mediated infection and CatB and CatL DC expression was observed in CHO B2 cells engineered to express $\alpha_4\beta_1$ -integrin (Fig. S6).

In addition to EboV, several other viruses have been shown to require endosomal cathepsin activity for cleavage of their fusion proteins. The F proteins of Hendra and Nipah viruses are cleaved by cathepsins before viral release, and this cleavage is required to prime the fusion protein (15). CatL cleavage of the spike proteins of MHV-2 and SARS after endocytosis has been shown to promote fusion (16, 17). CatB and CatL are also required for productive infection by nonenveloped mammalian reoviruses (18). For reoviruses, it has been proposed that CatL or CatB cleavage promotes disassembly of virions to infectious subviral particles (ISVPs) within the endosome in cell culture and non-intestinal tissues, as is seen extracellularly with intestinal proteases (such as chymotrypsin) in natural enteric reovirus infections. Interestingly, reovirus also requires β_1 -integrins for efficient infection. Using the β_1 -integrin positive and negative GD25 cells described above, Dermody and colleagues (19) found that expression of β_1 -integrins significantly enhanced reovirus infection. Furthermore, they showed that pretreating reovirus with chymotrypsin to generate ISVPs relieved the need for β_1 -integrin, similar to what we have shown here for thermolysin-generated VSV-GP_{19k}. In contrast to what we found for EboV pseudotypes, internalization of reovirus was also reduced in the β_1 knockout cells. However, since the block in internalization of reovirus in GD25 cells was not complete and could not account for the full decrease in infection of these cells (19), it is possible that loss of expression of the DC form of CatL may have also contributed to the reduction in reovirus infection.

The NPXY motifs within the β_1 -integrin cytoplasmic tail have been implicated in the process of reovirus trafficking (20). GD25 cells stably expressing β_1 -integrins containing mutations in these motifs (β_1 GD25-NPXF) were able to internalize reovirus, but the virus was trafficked to organelles resembling secondary lysosomes and did not generate a productive infection. A similar phenomenon has recently been demonstrated for EboV entry in the presence of inhibitors of the PI3K-Akt signaling pathway (21). PI3K is activated in response to integrin ligation in a wide range of cellular processes, and during the entry of several viruses (2, 22). PI3K may also be involved in crosstalk between integrins and Tyro3 family members, which have been implicated in ebolavirus entry (5). Davey and colleagues showed that PI3K was activated during EboV entry and that, while EboV was still able to bind to cells and be internalized in the presence of PI3K inhibitors, the virus accumulated in cytosolic compartments and

did not undergo fusion with the endosomal membrane. The observed defects in infection by reovirus in the NPXF-expressing cells and by EboV in PI3K inhibitor-treated cells may have been due to defects in expression of the DC form of CatL in addition to the proposed defects in trafficking. Under these circumstances, uncleaved virus particles may be unable to escape the endosome and therefore may accumulate further down the endosomal pathway than in normally functioning cells.

One of the surprising outcomes of this study is that the DC form of CatL appears to be regulated by $\alpha_5\beta_1$ -integrin and, at least in the CHO cells, the DC form of CatB is similarly regulated. The absence of the DC forms of these enzymes in the $\alpha_5\beta_1$ -integrin-negative cells does not appear to be due to a defect in processing, because there is no significant accumulation of the precursor forms of the cathepsins. The defect is also not due to a mutation in the CatB or CatL genes, because the DC forms of CatB and CatL are also missing when human cathepsins are ectopically expressed in these cells. Finally, secretion of these enzymes is not enhanced in the absence of $\alpha_5\beta_1$ -integrin. Instead, we suggest that surface expression of $\alpha_5\beta_1$ -integrin helps to stabilize the DC forms of CatB and CatL. For example, it is possible that the $\alpha_5\beta_1$ -integrin-negative cells have increased activity of an E64d-insensitive protease that degrades the DC forms of CatB and CatL. Alternatively, $\alpha_5\beta_1$ -integrin expression may be required for expression of a chaperone protein that can form a complex with the DC forms of the cathepsins and stabilize them. Finally, CatB and CatL may form a complex with $\alpha_5\beta_1$ -integrin itself, as has been shown for secreted cathepsin X (CatX) and $\alpha_V\beta_3$ -integrin (23). While neither CatB nor CatL contains an RGD motif, as is found in CatX, it is possible that they can interact with $\alpha_5\beta_1$ -integrin through an RGD-independent mechanism, or via interactions with another molecule, and that this interaction either stabilizes or regulates trafficking of the DC form of the enzymes. Although the bulk of the CatL protein was found in late endosomes and lysosomes irrespective of $\alpha_5\beta_1$ -integrin surface expression, it is possible that the DC forms of the enzymes may be trafficked to a different location in the $\alpha_5\beta_1$ -integrin-negative cells. Such mislocalization could cause the enzymes to be rapidly degraded by bringing them into contact with a cellular protease that they do not normally encounter or by preventing them from interacting with cellular factors that stabilize them.

It has recently been shown that miRNA targeting of the non-receptor tyrosine kinase Abl also results in specific loss of the DC form of CatL (24). Because the activity and localization of Abl has been shown to be regulated by adhesion through α_5 integrins (25), it is possible that Abl may contribute to the regulation of CatL by $\alpha_5\beta_1$ -integrins. Abl can also activate the PI3K-Akt pathway (26), which is required for EboV entry (21). It is therefore possible that Tyro3 family members, PI3K, Akt, and Abl are all involved in the link between $\alpha_5\beta_1$ -integrin and the DC forms of cathepsins.

Based on our data and those of Dermody and colleagues, it appears that at least 2 different viruses have evolved similar entry mechanisms in which they take advantage of widely expressed integrins on the cell surface to promote virus penetration within an endocytic organelle containing active cathepsins. While integrins are well known to regulate binding and signaling activities at the cell surface that lead to viral entry, to our knowledge this is the first clear evidence that integrin expression can also regulate virus entry at subsequent steps within intracellular organelles, in this case by controlling endosomal cathepsins. These results raise the possibility that integrin expression may influence other organelle functions that could be important for infection by a variety of viruses.

Materials and Methods

Cells. CHO K1, CHO B2, and CHO B2- α_5 cells (A. Rick Horwitz, University of Virginia, Charlottesville, VA) were maintained as described in ref. 27. HeLa cells were obtained from American Type Culture Collection and maintained in the recommended growth media. GD25 and β 1GD25 cells (Deane Mosher, University of Wisconsin, Madison, WI) were maintained as described in ref. 14.

Measuring Integrin Expression. In Fig. 1, cells were biotinylated, lysed, and avidin precipitated as described in ref. 27. Eluted proteins were analyzed under non-reducing conditions by immunoblotting with Abs specific for human α_5 (AB1928; Chemicon) or β_1 (Doug DeSimone, University of Virginia) integrins. In Fig. 5, cells were fixed and stained with Abs specific for mouse α_5 (BD Biosciences) or β_1 (19656; Santa Cruz Biotechnology) integrins.

Viruses and Infections. VSV-GP, VSV-GP Δ , and VSV-G were made essentially as described in (6, 28) and infections were performed at approximate multiplicities of infection (m.o.i.) of 1–2 as described in ref. 6. HIV-GP Δ and HIV-G were produced and infections were performed essentially as described in ref. 29. The BlaM-Vpr and HIV Δ Env plasmids were provided by Christopher Broder (Uniformed Services University, Bethesda, MD). Fusion was measured as the shift in fluorescence from green to blue using a CyAn ADP LX 9 Color flow cytometer (DakoCytomation).

siRNA. HeLa cells were transfected with control non-targeting siRNA oligonucleotides or SMARTpool oligonucleotides consisting of 4 different siRNA oligonucleotides targeting α_5 or β_1 integrin (Dharmacon) using oligofectamine (Invitrogen). Forty-eight hours posttransfection cells were infected with VSV-GP Δ or biotinylated and lysed for immunoblotting. Proteins were visualized using the Odyssey infrared imaging system and densitometry was performed using the Odyssey software (LiCor).

Binding and Internalization. VSV-GP Δ was bound to cells at an approximate m.o.i. of 2 at 4 °C. After 2 h unbound virus was washed off and cells were either lysed to measure bound virus, or warmed to 37 °C for 2 h to allow internalization and then treated with 1 mg/mL proteinase K before lysing to remove uninternalized virus. Parallel wells were treated with proteinase K without the warm-up step to confirm that proteinase K effectively removed virus that was bound but not internalized. Lysates were analyzed by immunoblotting using

an Ab specific for the matrix protein (M) of VSV (Michael Whitt, University of Tennessee, Memphis, TN). For immunofluorescence, virus was bound and internalized as above at an approximate m.o.i. of 15. Cells were fixed in 2% PFA, permeabilized with 0.05% Saponin, and stained with a monoclonal Ab to GP (Lisa Hensley, U.S. Army Medical Research Institute of Infectious Diseases, Frederick, MD) followed by anti-mouse AlexaFluor 488. Texas-Red conjugated phalloidin was used to visualize actin. Images were collected with a Nikon C1 laser scanning confocal unit attached to a Nikon Eclipse TE2000-E microscope. Binding of the recombinant EboV receptor binding region was performed as described in (10, 11).

Cathepsin Activity Assays and Immunoblotting. Innozyme CatB and CatL activity assay kits (Calbiochem) were used to measure CatB and CatL enzyme activities against small fluorogenic substrate peptides. To examine protein levels of CatB and CatL, cells were either left untransfected or transfected with plasmids encoding human CatL or CatB (Origene) using FuGENE 6 (Roche). Forty-eight hours posttransfection the cells were lysed and equivalent amounts of protein, measured using the bicinchoninic acid assay (Pierce), were analyzed by immunoblotting with Abs specific for CatL or CatB (Athens Research and Technology). Alternatively, to visualize secreted CatB and CatL, transfection media was replaced with serum-free DMEM 24 h posttransfection. After an additional 24-h incubation, supernatants from equivalent cell numbers were collected and the cells were lysed for immunoblotting as above. Collected supernatants were centrifuged to remove cellular debris and proteins in the supernatant were precipitated using CHCl₃/MeOH containing salmon sperm DNA before analyzing by immunoblotting.

In vitro proteolysis of GP. VSV-GP was mock-treated or treated with 0.5 mg/mL thermolysin (Sigma) for 30 min at 37 °C as described in ref. 6. The reactions were neutralized by the addition of 10 mM EDTA. Virus samples were analyzed by immunoblotting with a polyclonal GP1 Ab raised against sGP-Fc (Paul Bates, University of Pennsylvania, Philadelphia, PA) or used for infection.

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