Andes Virus Recognition of Human and Syrian Hamster β₃ Integrins
Is Determined by an L33P Substitution in the PSI Domain

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Andes virus (ANDV) causes a fatal hantavirus pulmonary syndrome (HPS) in humans and Syrian hamsters. Human αβ₃ integrins are receptors for several pathogenic hantaviruses, and the function of αβ₃ integrins on endothelial cells suggests a role for αβ₃ in hantavirus directed vascular permeability. We determined here that ANDV infection of human endothelial cells or Syrian hamster-derived BHK-21 cells was selectively inhibited by the high-affinity αβ₃ integrin ligand vitronectin and by antibodies to αβ₃ integrins. Further, antibodies to the β₃ integrin PSI domain, as well as PSI domain polypeptides derived from human and Syrian hamster β₃ subunits, but not murine or bovine β₃, inhibited ANDV infection of both BHK-21 and human endothelial cells. These findings suggest that ANDV interacts with β₃ subunits through PSI domain residues conserved in both Syrian hamster and human β₃ integrins. Sequencing the Syrian hamster β₃ integrin PSI domain revealed eight differences between Syrian hamster and human β₃ integrins. Analysis of residues within the PSI domains of human, Syrian hamster, murine, and bovine β₃ integrins identified unique proline substitutions at residues 32 and 33 of murine and bovine PSI domains that could determine ANDV recognition. Mutagenizing the human β₃ PSI domain to contain the L33P substitution present in bovine β₃ integrin abolished the ability of the PSI domain to inhibit ANDV infectivity. Conversely, mutagenizing either the bovine PSI domain, P33L, or the murine PSI domain, S32P, to the residue present human β₃ integrin permitted PSI mutants to inhibit ANDV infection. Similarly, CHO cells transfected with the full-length bovine β₃ integrin containing the P33L mutation permitted infection by ANDV. These findings indicate that human and Syrian hamster αβ₃ integrins are key receptors for ANDV and that specific residues within the β₃ integrin PSI domain are required for ANDV infection. Since L33P is a naturally occurring human β₃ polymorphism, these findings further suggest the importance of specific β₃ integrin residues in hantavirus infection. These findings rationalize determining the role of β₃ integrins in hantavirus pathogenesis in the Syrian hamster model.

Hantaviruses persistently infect specific small mammal hosts and are spread to humans by the inhalation of aerosolized excreted virus (41, 42). Hantaviruses predominantly infect endothelial cells and cause one of two vascular leak-based diseases: hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) (41). Hantavirus diseases are characterized by increased vascular permeability and acute thrombocytopenia in the absence of endothelial cell lysis (36, 41, 42, 54). In general, hantaviruses are not spread from person to person; however, the Andes hantavirus (ANDV) is an exception, since there are several reports of person-to-person transmission of ANDV infection (11, 37, 47, 52). ANDV is also unique in its ability to cause an HPS-like disease in Syrian hamsters and serves as the best-characterized hantavirus disease model with a long onset, symptoms, and pathogenesis nearly identical to that of HPS patients (20, 21, 50).

Hantavirus infection of the endothelium alters endothelial cell barrier functions through direct and immunological responses (8, 14). Although the means by which hantaviruses cause pulmonary edema or hemorrhagic disease has been widely conjectured, the mechanisms by which hantaviruses elicit pathogenic human responses have yet to be defined. Hantaviruses coat the surface of infected VeroE6 cells days after infection (17), and this further suggests that dynamic hantavirus interactions with immune and endothelial cells are likely to contribute to viral pathogenesis. Hantavirus pathogenesis has been suggested to involve CD8+ T cells, tumor necrosis factor alpha or other cytokines, viremia, and the dysregulation of β₃ integrins (7, 8, 13–16, 25–28, 32, 34, 38, 44–46). However, these responses have not been demonstrated to contribute to hantavirus pathogenesis, and in some cases there are conflicting data on their involvement (18, 25–28, 34, 35, 44, 45, 48). Immune complex deposition clearly contributes to HFRS pathogenesis, and in some cases there are conflicting data on their involvement (18, 25–28, 34, 35, 44, 45, 48). Immune complex deposition clearly contributes to HFRS patient disease and renal sequelae (4, 7), but it is unclear what triggers vascular permeability in HPS and HFRS diseases or why hemorrhage occurs in HFRS patients but not in HPS patients (8, 36, 54). Acute thrombocytopenia is common to both diseases, and platelet dysfunction resulting from defective platelet aggregation is reported in HFRS patients (7, 8).

Pathogenic hantaviruses have in common their ability to interact with αβ₃ integrins and αβ₃ integrins present on platelets and endothelial cells (13, 16), and β₃ integrins have primary roles in regulating vascular integrity (1, 2, 6, 19, 22, 39, 40). Consistent with the presence of cell surface displayed virus (17), pathogenic hantaviruses uniquely block αβ₃ directed endothelial cell migration and enhance endothelial cell permeability for 3 to 5 days postinfection (14, 15). Pathogenic hantaviruses dysregulate β₃ integrin functions by binding domains present at...
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the presence of gentamicin (50 μg/ml), amphotericin B (50 μg/ml), and 2% FCS (Clonetics). Andes virus (CCH-7913) (12) was kindly provided by B. Hjelle (University of New Mexico). ANDV and NY-1V were grown as previously described (14) in a biosafety level 3 facility. Briefly, viruses were adsorbed onto VeroE6 monolayers for 1 h, washed, and grown in DMEM containing 2% FCS.

Ligands and antibodies. Fibronectin, collagen, laminin, and chondroitin sulfate were purchased from Sigma and vitronectin was obtained from Chemicon. Polyclonal rabbit antisera to α5 (antibody 1934), α2 (antibody 1936), β3 (antibody 1932), αv (antibody 1950), and blocking monoclonal antibodies (MAbs) to β3 (MB 1962) and αv (MAbs 1976, LM609) were purchased from Chemicon. Goat anti-rabbit horseradish peroxidase conjugate and fluorescein-labeled Goat anti-mouse immunoglobulin G (H+L) were from Kirkegaard & Perry Laboratories, Inc. The generation of rabbit antiserum to the hantavirus nucleocapsid protein was previously described (16).

Plasmids and proteins. cDNA coding regions for human β3, αv, murine β3, and bovine β3 integrin subunits were previously cloned in pcDNA3.1(−)zeo (38). Bovine β3, P33L, human β3 L33P, and murine S32P mutants were generated by oligonucleotide-directed mutagenesis (Stratagene) and sequenced. Clones containing residues 1 to 53 of human β3, bovine β3, bovine β3, P33L, and murine β3 were subcloned from full-length human, bovine, and murine β3 plasmin into pET6His (9) at the BamHIEcoRI site as previously described (38). IPTG (isopropyl-β-D-thiogalactopyranoside) induction (1 mM for 3 h) of pET6His plasmid in BL21(DE3) cells was used to express β3 polypeptides containing residues 1 to 53 and processed at 30°C as previously described (38). Briefly, bacterial pellets were resuspended in 0.1 M NaH2PO4, 10 mM Tris-HCl and 1 M urea, sonicated, and purified on Ni2+-NTA resin (Qiagen) (38). Proteins were eluted with 50 mM EDTA and dialyzed overnight in phosphate-buffered saline (PBS; 3.5-kDa cut-off), and protein concentrations were quantitated by bichinchoninic acid assay (Pierce).

Cloning and sequencing of Syrian hamster β3 integrin subunit. Total RNA was extracted from Syrian hamster cell line BHK-21 and Syrian hamster liver tissue (RNaseiq; Qiagen). RNA was reverse transcribed into cDNA (Transcrip- tor First Strand cDNA Synthesis; Roche) (25°C for 10 min, 42°C for 60 min, and 95°C for 5 min) by using primers containing consensus β3 integrin subunit sequences described from human, murine, rat, and rabbit β3 integrins (Genbank accession numbers NM_00012, NM_016780, NM_153720, and NM_001082066, respectively): antisense primers 5′-ATCACAKACTGTAGCCTGCATGATGG (ending at bp 777 of the human β3 integrin sequence) and 5′-AGCACRT GTTTTGAGCCAAACATGGG-3′ (ending at bp 659 of the human β3 integrin sequence) (R = A or G; K = T or G). cDNA was subjected to nested PCR by using the forward primer 5′-CTGCGCCCTGGGGGCCTGCGCCCTGGT-3′ (ending at bp 480 of the human β3 integrin sequence) (cycle 95°C for 1 min, 60°C for 1 min, and 72°C for 30 s). Amplified cDNA fragments were ligated into pCRII-TOPO (Invitrogen), transformed into XL1-Blue cells, and sequenced. The region corresponding to the N-terminal 53 residues of human β3 integrin was subcloned into pET6His vector for overexpression in the BhamH/EcoRI site, transformed into BL21(DE3) cells, and subsequently expressed as described above.

Ligand and antibody pretreatment of cells. HUVECs, VeroE6 cells, and BHK-21 cells were pretreated with antibodies (0.1 to 1 μg/ml) or potentially competitive ligands (5 to 20 μg/ml) for 1 h at 4°C. Antibodies and ligands were preadsorbed to cells in 50 μl of DMEM with 2% FCS in duplicate wells of a 96-well plate on ice. Sera or ligands were removed, and the cells were washed with PBS. Approximately 1,000 focus-forming units (FFU) of hantavirus were adsorbed to monolayers for 1 h at 37°C. Unbound virus was removed, monolayers were washed three times with PBS, and infected monolayers were incubated 24 h prior to methanol fixation (100% methanol, 30 min at −20°C).

Polypeptide Inhibition of ANDV and NY-1V Infection. Increasing amounts (5 to 20 μg/ml) of human, murine (negative control), S32P murine mutant, bovine, P33L bovine mutant, Syrian hamster, or N39D Syrian hamster mutant β3 polypeptides (residues 1 to 53) were incubated with approximately 1,000 FFU of ANDV or NY-1V (2 h at 4°C) and then adsorbed to HUVECs, VeroE6 cells, or BHK-21 cells in duplicate wells (96-well plate, 100 μl per well) for 1 h at 37°C. Monolayers were washed with PBS, and the cells were incubated for 24 h prior to methanol fixation. Infected cells were detected by immunoperoxidase staining using anti-nucleocapsid antibody and quantitated as previously described (16).

Transfection and infection. CHO cells were transfected (Lipofectamine 2000; Invitrogen) as recommended with equal amounts of human αv, and either murine β3, or bovine β3 expression plasmids (pcDNA3.1) (38). At 2 days posttransfection, transfected CHO cells were washed with ice-cold PBS, and the cells were dissociated with enzyme-free PBS-based cell dissociation buffer (Invitrogen). Cells were resuspended in 100 μl of PBS and 2% FCS and incubated with

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Cells and virus. VeroE6 cells, CHO cells, and BHK-21 cells (ATCC CRL 1586, CCL 61, and CCL-10) were grown in Dulbecco modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS; 56°C inactivated), penicillin (100 μg/ml), streptomycin sulfate (100 μg/ml), and amphotericin B (50 μg/ml) (Gibco). Human umbilical vein endothelial cells (HUVECs; Clonetics) were grown in supplemented endothelial cell basal medium-2 (EBM-2; Clonetics) in

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anti-αvβ3 (MAb 1976) (2 μg/10^6 cells) for 30 min at 4°C. Cells were pelleted (1,000 rpm for 2 min), washed twice with ice-cold PBS, and incubated with anti-mouse fluorescein isothiocyanate for 30 min at 4°C. Cells were repelleted, washed twice with ice-cold PBS plus 2% FCS, resuspended in 500 μl of PBS, and subjected to flow cytometry (FACSCalibur cell sorter; BD Biosciences). The geometric mean titer of β3 integrin fluorescence on CHO cells was used as a measure of integrin expression levels (15), and only cells expressing comparable levels of human or bovine β3 integrins were used in ANDV infection experiments. Transfected and mock-transfected CHO cells were infected with ANDV 48 h posttransfection as described above. Cells were methanol fixed after 24 h and immunoperoxidase stained for hantavirus nucleocapsid protein, and the number of infected cells was quantitated by microscopy as previously described (16).

RESULTS

Syrian hamsters infected with ANDV develop a lethal disease with onset, symptoms, and respiratory distress similar to that of HPS patients (20, 21, 50). To date, all pathogenic hantaviruses have been demonstrated to use αvβ3 integrins for cellular entry and pathogenic hantaviruses inhibit β3 functions days after infection (13–16, 38). However, ANDV interactions with human or Syrian hamster β3 integrins have not been defined, and the role of β3 integrins in regulating endothelial barrier functions provides a rationale for integrin regulation to contribute to viral pathogenesis. We investigated here whether ANDV interacts with human or Syrian hamster β3 integrins and defined residues required for ANDV infection. Initially, we evaluated the ability of ANDV to infect BHK-21 cells that are derived from Syrian hamsters. Figure 1 indicates that BHK-21 cells are highly infected by ANDV, although at a slightly reduced level from the identical infection of VeroE6 cells. However, there was a nearly 3-log reduction in NY-1V infection of BHK-21 cells compared to VeroE6 cells (Fig. 1). This result suggests a fundamental difference in ANDV infection of Syrian hamster cells from that of NY-1V, and this could result from differences in adherence to β3 integrins.

In order to determine whether ANDV uses β3 integrins, we evaluated whether integrin ligands or antibodies inhibited ANDV infection of VeroE6 and BHK-21 cells. Pretreating cells with increasing amounts of collagen, chondroitin sulfate, laminin, or fibronectin had no effect on ANDV infection. In contrast, pretreating VeroE6 cells (Fig. 2A) and BHK-21 cells (Fig. 2B) with vitronectin, a high-affinity αvβ3 ligand, inhibited ANDV infection by 80 and 70%, respectively. Since antibodies that recognize Syrian hamster β3 integrins are not available, we investigated the ability of antibodies to human β3 integrins to inhibit ANDV infection. Figure 3 shows that pretreating cells with antibodies to human β3, or α3β1, dose dependently inhibited ANDV infection of HUVECs and VeroE6 cells (85 and 70%, respectively; Fig. 3). Antibodies to the α3 integrin subunit also reduced ANDV infection of HUVECs and VeroE6 cells (40 to 60%) (Fig. 3), whereas antibodies to α5, α5β1, α2, β2, and β3 had no inhibitory effect on ANDV infection. These results indicate that ligands or antibodies specific to αvβ3 integrins mediate ANDV entry into human, simian, and Syrian hamster cells.

Binding of the NY-1V to β3 integrins was previously shown to be RGD independent, mediated by the N-terminal PSI domain present on human but not murine β3 integrin subunits, and dependent on an aspartic acid at position 39 (16, 38). In order to determine whether ANDV interacts with human β3 integrin PSI domains, we determined whether the expressed β3 integrin PSI domain (residues 1 to 53) or antibody to the PSI domain (residues 17 to 48) inhibited ANDV infection. Pretreating VeroE6 cells (Fig. 4A) and HUVECs (Fig. 4B) with antibodies against the human β3 integrin PSI domain resulted...
in a 70% decrease in ANDV infection compared to control antibodies. Similarly, pretreating ANDV with increasing amounts of the expressed human \( \beta_3 \) PSI domain reduced ANDV infection of HUVECs by 75% (Fig. 5A) and ANDV infection of BHK-21 cells by 85% (Fig. 5B). In contrast, the murine \( \beta_3 \) integrin PSI domain had no apparent effect on ANDV infection of either cell type.

In order to determine whether Syrian hamster \( \beta_3 \) integrins also blocked ANDV infection, we cloned and expressed the \( \beta_3 \) integrin PSI domain from BHK-21 cells. Five independent clones of the Syrian hamster \( \beta_3 \) integrin were sequenced and found to be identical to the cDNA sequenced from Syrian hamster tissue (submitted to GenBank). Figure 6 compares Syrian hamster \( \beta_3 \) PSI domain coding sequences with human, murine, and bovine \( \beta_3 \) PSI domains. The Syrian hamster \( \beta_3 \) integrin PSI domain differs from the human sequence by eight residues. In contrast, murine and bovine PSI domains, which do not confer hantavirus infectivity, differ by 9 and 4 residues, respectively, from human PSI domains, whereas murine sequences differ by five residues from the Syrian hamster PSI domain (positions 30, 32, 42, 43, and 50).

We further evaluated whether the expressed Syrian hamster \( \beta_3 \) integrin PSI domain inhibited ANDV infection. Figure 7A indicates that pretreating ANDV with the Syrian hamster \( \beta_3 \) integrin PSI domain (1 to 53) reduced ANDV infection of BHK-21 cells by 80%, similar to the human \( \beta_3 \) integrin PSI domain (1 to 53). In contrast, the murine \( \beta_3 \) PSI domain had no effect on ANDV infection of BHK-21 cells at any concentration. This indicates that both the Syrian hamster and human \( \beta_3 \) integrin interact with ANDV. Since we previously demonstrated that the presence of an N or D residue at position 39 of \( \beta_3 \) differentiated NY-1V interaction with \( \beta_3 \) (38), we evaluated the effect of mutating residue 39 of the Syrian hamster \( \beta_3 \) PSI domain from N to D. Figure 7A indicates that there was no difference in PSI domain inhibition of ANDV by the N39D mutant. This suggested a fundamental difference in the interaction of \( \beta_3 \) with ANDV and NY-1V.

In order to determine whether the Syrian hamster \( \beta_3 \) or the N39D Syrian hamster \( \beta_3 \) mutant inhibited NY-1V infectivity, we comparatively evaluated the ability of the NY-1V and ANDV to infect HUVECs in the presence or absence of \( \beta_3 \) PSI domains. Figure 7B demonstrates that the human and Syrian hamster \( \beta_3 \) PSI domains inhibited ANDV infection of human endothelial cells irrespective of the N39D mutation. In contrast, the Syrian hamster wild-type \( \beta_3 \) PSI domain had no effect on NY-1V infection of endothelial cells, whereas the Syrian hamster N39D mutant blocked NY-1V infectivity sim-
changes in adjacent residues (32 and 33, Fig. 6 [boxed]) that

similar to the human $\beta_3$ PSI domain. This supports previous find-

ings on the specificity of NY-1V interactions with $\beta_3$ (38) and in-

dicates that ANDV interactions with $\beta_3$ integrins require
discrete PSI domain residues from that of NY-1V.

Specific PSI domain inhibition of ANDV infection further

suggested that PSI domain sequences determine ANDV at-
tachment and infectivity. We previously determined that sub-

stituting residues 1 to 43 of murine $\beta_3$ with human $\beta_3$ se-

quences conferred hantavirus infection (38). When this find-

ing is considered, only two residues in the murine $\beta_3$ (T30 and S32)

and one in the bovine $\beta_3$ (P33) are completely discrete from hu-
nan or Syrian hamster PSI domain sequences (Fig. 6). Mu-

rine and bovine $\beta_3$ PSI domains contain unique proline changes in

adjacent residues (32 and 33, Fig. 6 [boxed]) that differ from the

Syrian hamster and human $\beta_3$ PSI domains and suggest that they are
determinants of ANDV binding to $\beta_3$ integrins.

In order to define the role of proline 33 in ANDV recogni-
tion, we mutated the bovine $\beta_3$ integrin proline 33 to the

leucine (P33L) present in human homologues and then deter-

mined whether the bovine P33L PSI domain mutant was capa-

cible of inhibiting ANDV infection. We similarly mutated the

murine $\beta_3$ PSI domain to contain a proline residue at position

32 (S32P) in order to mimic the human $\beta_3$ PSI domain. Figure

8 indicates that the murine $\beta_3$ PSI domain fails to inhibit

ANDV infectivity, whereas both human and Syrian hamster $\beta_3$

PSI domains block ANDV infection. However, the murine

S32P mutant $\beta_3$ PSI domain also inhibited ANDV infection by

>50%. Similar to murine $\beta_3$, the wild-type bovine $\beta_3$ PSI do-

main failed to inhibit ANDV infection of BHK-21 cells and

VeroE6 cells (Fig. 9). However, the P33L mutant bovine $\beta_3$

PSI domain inhibited ANDV infection of VeroE6 and BHK-21

Cells by 60 and 55%, respectively. These findings suggest that

the absence of proline 32 or the addition of a second proline at

position 33 in the $\beta_3$ integrin PSI domain dramatically reduces

ANDV recognition of PSI domain polypeptides.

To further evaluate the effect of P33 on ANDV infection, we

reciprocally mutated the full-length bovine $\beta_3$ subunit to con-

tain P33L and the full-length human $\beta_3$ to contain L33P. Sub-

sequently, we transfected CHO cells with the $\beta_3$ mutants and

determined the susceptibility of CHO cells expressing mutant

$\beta_3$ subunits to ANDV infection. Equal expression of the $\alpha_\beta_3$

integrins on the surface of CHO cells was confirmed by flow

cytometry, as measured by comparable geometric mean titers

of $\alpha_\beta_3$ cell surface fluorescence. CHO cells expressing either

bovine $\beta_3$ or the human $\beta_3$ L33P mutant failed to confer

ANDV infectivity (Fig. 10). In contrast, CHO cells expressing

either human $\alpha_\beta_3$ or a bovine $\beta_3$ P33L mutant enhanced

ANDV infection by >5-fold (Fig. 10). These results establish

that a proline residue at position 33 inhibits ANDV recogni-
tion of $\beta_3$ integrin PSI domains and further demonstrate the $\beta_3$

integrin sequence specificity of ANDV recognition.

**DISCUSSION**

$\beta_3$ integrins are prominent cell surface receptors on endo-
thelial cells and regulate vascular integrity, permeability, and

hemostasis (2, 6, 19, 23, 39, 40). Interestingly, all pathogenic

hantaviruses analyzed thus far use $\beta_3$ integrin receptors on

human endothelial cells and cause vascular permeability-based
diseases (HTNV, SNV, Puumala virus [PUUV], Seoul virus

[SEROV], and NY-1V), whereas $\alpha_\beta_3$ integrins are used by

nonpathogenic hantaviruses (13, 16, 38). ANDV infection of

Syrian hamsters is a model of HPS pathogenesis that closely

mimics human disease (20, 21, 50); however, the use of $\beta_3$

integrins by ANDV has not been established. We performed

here a series of experiments to determine how ANDV interacts

with $\beta_3$ integrins. Our results indicate that ANDV infection of

either human endothelial cells or Syrian hamster BHK-21 cells

was inhibited by the high-affinity $\alpha_\beta_3$ integrin ligand vitrone-
tin and antibodies against $\beta_3$ subunits. These findings demon-

strate that ANDV infectivity is dependent on the presence of

$\beta_3$ integrins.

$\beta_3$ integrins reportedly form at least two conformations: an

active extended conformation which binds vitronectin and an

inactive bent conformation which forms the basal integrin state

(30, 53, 55). Pathogenic hantaviruses have been shown to bind

PSI domains present at the apex of bent inactive $\beta_3$ integrin



![FIG. 5. Human $\beta_3$ integrin PSI domain polypeptides inhibit ANDV infection. Increasing amounts (5 to 20 $\mu$g) of expressed, and purified polypeptides containing residues 1 to 53 of human or murine $\beta_3$ integrins were incubated for 2 h at 4°C with −1,000 FFU of ANDV. Virus was subsequently adsorbed to HUVECs (A) or BHK-21 cells (B) in 96-well plates for 1 h at 37°C. Monolayers were washed with PBS, and at 0 h postinfection the number of ANDV-infected cells was quantitated after methanol fixation and immunoperoxidase staining of the hantavirus nucleocapsid protein as previously described (38). The results were reproduced in at least two separate experiments and are presented as a percentage of the mock-treated controls.](#)

![FIG. 6. Alignment of human, Syrian hamster, murine, and bovine $\beta_3$ integrin PSI domains. Amino acid sequences of residues 1 to 53 from human, Syrian hamster, murine, and bovine $\beta_3$ integrin (GenBank accession no. XM_616376) subunits are comparatively presented. Residue differences observed in $\beta_3$ integrin homologues that differ from human $\beta_3$ sequences are indicated, and dashes indicate identical residues. Residues 17 to 48 are delineated by a line, and proline residue differences at positions 32 and 33 of the $\beta_3$ integrin homologues are boxed.](#)
subunits, and the binding is species specific (38). The results presented here demonstrate that antibodies against the integrin PSI domain, as well as PSI domain polypeptides derived from either human or Syrian hamster origin, inhibit ANDV infectivity. In contrast, ANDV failed to recognize PSI domain polypeptides of murine or bovine origin. These findings indicate that ANDV selectively recognizes human and Syrian hamster \( \beta_3 \) integrin PSI domains which direct ANDV infectivity. These findings are consistent with ANDV pathogenesis in both humans and Syrian hamsters.

The species-specific use of \( \beta_3 \) integrin PSI domains permitted us to further analyze critical residues within the PSI domain required for ANDV recognition. A comparison of species specific \( \beta_3 \) subunit PSI domains shows that murine and bovine \( \beta_3 \) sequences contain proline substitutions at positions 32 and 33 (P32S and L33P, respectively) resulting in the absence of proline (murine) or the presence of two adjacent prolines (bovine) in this domain. Mutagenesis demonstrated that ANDV recognition of human and Syrian hamster \( \beta_3 \) integrins is abolished by substituting leucine for proline at position 33 of the human \( \beta_3 \) subunit (L33P). Similarly, substituting proline for serine (P32S) in the murine \( \beta_3 \) PSI domain permitted the

FIG. 7. Human \( \beta_3 \) and Syrian hamster \( \beta_3 \) integrin PSI domain inhibit ANDV infection. (A) Increasing amounts (5 to 20 \( \mu \)g) of expressed human (Hu), Syrian hamster (Syr. Ham.), Syrian hamster N39D mutant (D39), or murine (Mu) \( \beta_3 \) integrin PSI domain polypeptides were incubated with \( \sim 1,000 \) FFU of ANDV for 2 h at 4°C. Virus was subsequently adsorbed to BHK-21 cells in a 96-well plate for 1 h at 37°C. At 24 h postinfection, monolayers were immunoperoxidase stained for nucleocapsid protein as previously described (38). (B) Increasing amounts (5 to 20 \( \mu \)g) of human (Hu), Syrian hamster (Syr. Ham.) N39, Syrian hamster D39, and murine (Mu) \( \beta_3 \) integrin PSI domain were incubated with \( \sim 1,000 \) FFU of ANDV or NY-1V for 2 h at 4°C. Virus was adsorbed to HUVECs in a 96-well plate for 1 h at 37°C. At 24 h postinfection ANDV- or NY-1V-infected cells were quantitated by immunoperoxidase staining of the nucleocapsid protein as previously described (38). The results were reproduced in at least two separate experiments and are presented as a percentage of the mock-treated controls.

FIG. 8. Murine \( \beta_3 \) integrin PSI domain mutants inhibit ANDV infection. Increasing amounts (5 to 20 \( \mu \)g) of human, murine, murine S32P, and Syrian hamster \( \beta_3 \) integrin PSI domains were incubated with \( \sim 1,000 \) FFU of ANDV for 2 h at 4°C. Mixtures were adsorbed to VeroE6 cells in a 96-well plate for 1 h at 37°C. After a washing step, the cells were incubated for 24 h, and the number of ANDV-infected cells was quantitated by immunoperoxidase staining of the nucleocapsid protein as previously described (38). The results were reproduced in at least two separate experiments and are presented as a percentage of the mock-treated controls.
FIG. 9. P33L Mutants of the bovine β3 integrin inhibit ANDV infection. Increasing amounts (5 to 20 µg) of human, murine, bovine (Bo) wild-type, or bovine P33L β3, integrin PSI domain were incubated with ~1,000 FFU of ANDV for 2 h at 4°C. Mixtures were adsorbed onto BHK-21 or VeroE6 cells in a 96-well plate for 1 h at 37°C. After a washing step, the cells were incubated for 24 h, and the number of ANDV-infected cells was quantitated by immunoperoxidase staining of the nucleocapsid protein as previously described (38). The results were reproduced in at least two separate experiments and are presented as a percentage of the mock-treated controls.

FIG. 10. Bovine β3 integrin P33L confers cell susceptibility to ANDV infection. CHO cells were cotransfected with recombinant human αv integrin subunits along with human wild-type β3, bovine wild-type β3, mutant bovine P33L β3, or human L33P β3 integrin subunits. Cell surface expression of αvβ3 integrins was determined by flow cytometry, and cells were subsequently infected with ~1,000 FFU of ANDV for 1 h at 37°C. After viral adsorption, inocula were removed, and the cells were washed and incubated 24 h at 37°C before fixation. Infected CHO cells were quantitated by immunoperoxidase staining of the nucleocapsid protein as previously described (38). The results were reproduced in at least two separate experiments and are presented as fold of mock-transfected controls.

PSI domain to function as an inhibitor of ANDV infection. Conversely, when proline 33 of the bovine β3 subunit was replaced by leucine (P33L) the mutant bovine β3 subunit also conferred cell susceptibility to ANDV infection. These findings indicate that ANDV specificity for β3 integrins is directed by residues 32 and 33 or altered by proline-induced changes at these positions. Interestingly, required β3 residues for ANDV binding differ from recognition sequences of NY-1V. In contrast to ANDV, NY-1V binding to β3 is dependent on D39, blocked by introducing N39 within human β3 (38) or the N39 containing wild-type Syrian hamster β3 (Fig. 7), and unaltered by residue 32 or 33 substitutions (38). This is a fundamental difference in β3 recognition between ANDV and NY-1V; however, residues required for binding of either virus reside within the same β3 domain. These findings suggest that additional pathogenic hantaviruses may also have unique β3 residue binding requirements that specify their integrin interactions.

It is unclear whether unique ANDV interactions with β3 integrins are responsible for the discrete ability of ANDV to cause disease in Syrian hamsters or permit person to person transmission. NY-1V is clearly restricted in BHK-21 Syrian hamster cells (Fig. 1), and the data presented here suggest that at one level this difference may be due to species-specific β3 integrin residues (Fig. 7B). It is unclear whether similar differences in β3 residue requirements will be observed in SNV and HTNV, which are also nonpathogenic in Syrian hamsters and yet pathogenic in humans. However, there are many reasons for why a virus may be restricted from causing disease in another species. In fact, analysis of an SNV reassortant containing the ANDV M segment, and its encoded attachment proteins, indicates that SNV is restricted from causing disease in Syrian hamsters as a result of its S and L segments (33). In fact, S and L segment RNA transcription by the SAS reassortant is identical to SNV and reduced 1 to 2 logs from that of ANDV (33), suggesting that replication differences may restrict SNV from being pathogenic in Syrian hamsters. Consistent with this, SNV did not cause viremia within infected Syrian hamsters, and no S-segment RNA was isolated from peripheral blood mononuclear cells after infection (50). However, it is also possible that SNV and ANDV differ in their ability to regulate other cell or immune response imposed restrictions within Syrian hamsters that both viruses regulate successfully in order to be pathogenic in humans. Although there are many hurdles for a virus to overcome in order to be pathogenic in any species, the use of human and Syrian hamster β3 integrins by ANDV and the function of β3 integrins in regulating vascular permeability provide a compelling role for the involvement of β3 in the hantavirus disease process.

The importance of residues 32 and 33 as determinants of β3 integrin antigenic specificity furthers the potential role of β3 in altering vascular barrier functions during hantavirus infection. The L33P substitution is a naturally occurring αvβ3 integrin polymorphism that differentiates human platelet antigen 1a (HPA-1a) from HPA-1b (24, 51). Interestingly, the L33P substitution is sufficient to direct autoimmune responses to β3 integrins from blood containing a different HPA type, and this immune response to β3 results in two autoimmune diseases, fetomaternal allo-immune thrombocytopenia (FMAIT) and posttransfusion purpura (PTP) (24, 49, 51). FMAIT and PTP patients display vascular permeability and acute thrombocytopenia similar to symptoms of hantavirus-infected HFRS and HPS patients (24, 49, 51). Unfortunately, there are no patient data available on the role of the HPA-1a/1b polymorphism on hantavirus infection or disease. One study has indicated that a polymorphism in the HPA-3b allele (I843S) of αvβ3 integrins results in more severe clinical HFRS disease (29). However, since only 1% of the Asian population is homozygous for the
HPA-1b allele, the study did not determine whether HPA-1 influences hantavirus disease (43).

Thrombocytopenia is another hallmark of patients infected by pathogenic hantaviruses (8, 36, 41, 54); however, neither the role of platelets in hantavirus disease nor the mechanism of hantavirus induced thrombocytopenia has been defined (31, 41). Cosgriff et al. demonstrated that platelets from HFRS patients are defective in platelet activation (aggregation and granule release) and that this defect was not the result of a soluble circulating factor (7). This implies that thrombocytopenia in HFRS patients results from a block in platelet activation rather than from excessive platelet activation. This platelet defect following hantavirus infection, together with an understanding of hantavirus binding to inactive \(\alpha_{IIb}\beta_3\) integrins, suggests that hantaviruses may bind inactive \(\alpha_{IIb}\beta_3\) integrin conformers and prevent platelet activation. Since hantaviruses are cell associated at late times after infection (17), this also suggests that cell surface-displayed hantaviruses could potentially recruit quiescent platelets to the endothelial cell surface and simultaneously prevent platelet activation, reduce the level of activated platelets, and mask the presence of hantavirus-infected cells.

Our findings do not exclude additional immunologic mechanisms which may contribute to hantavirus pathogenesis and vascular permeability (25–28, 32, 34, 44, 46). However, these and other findings suggest that there is a number of means by which hantavirus dysregulation of \(\beta_3\) integrin functions may contribute to vascular permeability and pathogenesis (6, 13–16, 31, 38–40). Pathogenic hantaviruses clearly alter the permeability of infected endothelial cells in a manner consistent with the effects of \(\beta_3\) integrin dysregulation (2, 3, 6, 14, 15, 23, 38–40). Pathogenic hantaviruses selectively inhibit \(\beta_3\) integrin-directed migration (15) and enhance endothelial cell permeability in response to VEGF (14). However, hantavirus regulation of endothelial cell integrin functions is not the result of virus adsorption, since low MOIs (0.1 to 1) are applied to cells, and neither endothelial cell migration nor endothelial cell permeability are altered at early times after infection (14, 15). Inhibited migration and enhanced permeability of endothelial cells is only observed days after infection (14, 15) when hantaviruses reportedly coat the surface of infected cells (17). In fact, pathogenic hantaviruses may remain cell associated through hantavirus-\(\beta_3\) interactions on the surfaces of endothelial cells and thus contribute to \(\beta_3\) dysfunction and enhanced endothelial cell permeability following viral emergence. These findings further suggest that ANDV interactions with \(\beta_3\) integrins could contribute to endothelial cell barrier dysfunction observed in humans and Syrian hamsters after ANDV infection. The studies presented here rationalize the use of the ANDV Syrian hamster HPS disease model to determine whether \(\beta_3\) integrins contribute to hantavirus pathogenesis.

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