Echovirus 1 Interaction with the Human Very Late Antigen-2 (Integrin α2β1) I Domain

**Identification of Two Independent Virus Contact Sites Distinct from the Metal Ion-Dependent Adhesion Site**

The human integrin very late antigen (VLA)-2 (CD49b/CD29) mediates interactions with collagen and is the receptor for echovirus 1. Binding sites for both collagen and echovirus 1 have been mapped to the I domain within the α2 subunit of the VLA-2 α2β1 heterodimer. Although murine VLA-2 interacts with collagen, it does not bind virus. We have used isolated human-murine chimeric I domains expressed as glutathione S-transferase fusion proteins in *Escherichia coli* to identify two groups of amino acids, 199–201 and 212–216, independently involved in virus attachment. These residues are distinct from the metal ion-dependent adhesion site previously demonstrated to be essential for VLA-2 interactions with collagen. Mutations in three metal ion-dependent adhesion site residues that abolish adhesion to collagen had no effect on virus binding. These results confirm that different sites within the I domain are responsible for VLA-2 interaction with extracellular matrix proteins and with viral ligands.

The integrin VLA-2\(^1\) (CD49b/CD29) mediates cell interactions with the extracellular matrix proteins collagen and laminin (1). Human VLA-2 is also the receptor for the human pathogen echovirus 1 (2). VLA-2 is a heterodimer composed of a 150-kDa α2 and a 130-kDa β1 subunit. Within the α2 subunit, the 200-amino acid I domain has sequence similarity to the I or A domains of other I or A domains (12). Because VLA-2 interactions with echovirus 1 are regulated by different mechanisms. In contrast to collagen binding to VLA-2, echovirus 1 interactions do not discriminate between functional forms of the receptor, and are not enhanced by activating antibodies or phorbol esters (9). In addition, whereas VLA-2 interaction with collagen is magnesium-dependent, echovirus 1 attachment occurs in the absence of divalent cations (9).

VLA-2 interactions with collagen and/or echovirus recognize epitopes within the I domain, domain (amino acids 140–349) of the human α2 subunit (4, 5). Monoclonal antibodies that block VLA-2 interactions with collagen and/or echovirus recognize epitopes within the I domain, between amino acids 173 and 259, suggesting that sites required for interactions with both ligands reside within this portion of the molecule (5). Both echovirus 1 (6) and collagen (7, 8) bind to the isolated human VLA-2 I domain expressed in bacteria as a glutathione S-transferase (GST) fusion protein.

Other results suggest that the binding sites for collagen and virus, while both contained within the I domain, are not identical. Although some monoclonal antibodies block VLA-2 interaction with both collagen and virus, others inhibit binding to one ligand or the other (5). Furthermore, a variety of evidence indicates that interactions with collagen and echovirus 1 are regulated by different mechanisms. In contrast to collagen binding to VLA-2, echovirus 1 interactions do not discriminate between functional forms of the receptor, and are not enhanced by activating antibodies or phorbol esters (9). In addition, whereas VLA-2 interaction with collagen is magnesium-dependent, echovirus 1 attachment occurs in the absence of divalent cations (9).

Mutation of any of three aspartate or threonine residues within the VLA-2 I domain (Asp\(^{151}\), Thr\(^{221}\), and Asp\(^{254}\)) abolishes cell adhesion to collagen (5, 7). Mutations at similar positions within the I domain (called by some investigators the A domain) of the β2 integrin CR3 (CD11b/CD18) ablate binding to CR3 ligands and to divalent cations (10, 11). The crystal structure of the CR3 I domain shows that these residues serve to coordinate a magnesium ion, and form what has been termed the metal ion-dependent adhesion site (MIDAS) (12); a similar metal-binding site is evident in the crystal structure of the leukocyte function-associated antigen-1 (CD11a/CD18) I domain (13, 14). A MIDAS motif, consisting of a DXSXS sequence (where X represents any amino acid) plus noncontiguous threonine and aspartate residues, is present in a number of other I or A domains (12). Because VLA-2 interactions with echovirus 1 are relatively cation-independent, it is not clear whether the VLA-2 MIDAS is essential for virus binding.

Although both human and murine VLA-2 bind collagen, only human VLA-2 mediates echovirus 1 attachment and infection (1, 2, 15). Experiments with human-murine chimeras have shown that specific virus attachment to human VLA-2 depends on the human α2 subunit, and on the human I domain (4, 16). We have now identified sites within the I domain, distinct from the MIDAS residues involved in collagen adhesion, responsible for specific echovirus 1 attachment to human VLA-2.

**Experimental Procedures**

**I Domain Fusion Proteins**—A 650-base pair fragment encoding the I domain (amino acids 140–349) was amplified from a murine α2 cDNA...
cloning, using polymerase chain reaction primers that introduced BamHI and EcoRI restriction sites, and inserted into the pGEX-KT GST expression vector (17). Chimeric I domain DNA fragments were created using splice-overlap extension polymerase chain reaction techniques (4) with a modification designed to amplify only products containing mutations introduced in the first reaction (18). The sequence of each subcloned polymerase chain reaction product was verified using an automated Applied Biosystems sequencer. One additional human-murine chimeric I domain, combining human amino acids 140–218 and murine amino acids 219–349, was prepared using a PvuII restriction site present in both murine and human cDNAs.

GST fusion proteins were produced in Escherichia coli and purified on glutathione-Sepharose essentially as described (6). The CR3 I domain-GST fusion protein was provided by P. Rieu and M. A. Arnaout (10). The D151A, T221A, and D254A mutant I domain-GST fusion proteins have been described (7).

Virus Binding Assays—[35S]Methionine-labeled echovirus 1, prepared as described (2), was added either to purified fusion proteins immobilized on glutathione-Sepharose beads (5,000 cpm, 2 μg of protein/aleuqot) or to cell monolayers in 24-well plates (20,000 cpm/well). Samples were incubated for 1 h at room temperature with rocking. Beads or cell monolayers were washed to remove unbound virus and dissolved for liquid scintillation counting. Additional aliquots of fusion proteins immobilized on glutathione-Sepharose beads were boiled in Laemml sample buffer and run on SDS-polyacrylamide electrophoresis gels to confirm equal protein loading (not shown).

Expression and Detection of Mutant VLA-2 on CHO Cells—A chimeric murine α2 construct with human amino acids at positions 199–216 was prepared in the expression vector pFNeo (19). CHO cells were transfected by electroporation (280 V, 960 microfarads in a Bio-Rad Gene Pulser), and selected first in media containing 0.5 mg/ml geneticin, and then for α2 expression by panning two times on collagen-coated plastic dishes as described (15). CHO cells transfected with murine α2 and selected by panning on collagen (15), CHO cells transfected with human α2, and mock-transfected CHO cells were previously described (16). CHO cells expressing human α2 mutants with alanine substitutions at Asp151, Thr221, and Asp254 have also been described (5, 7).

Surface expression of wild-type human α2, and of human α2 with the D151A, T221A, and D254A mutations, was detected by indirect immunofluorescent flow cytometry with monoclonal antibody HAS-4 (20) (provided by F. M. Watt) and fluorescein isothiocyanate-conjugated goat antibody to mouse immunoglobulin obtained from Sigma. The negative control antibody MOPC 195 (mouse IgG2b, κ) was obtained from Sigma. Surface expression of wild-type murine α2 was measured using hamster monoclonal antibody HM2 and the negative control antibody A19-3, with fluorescein isothiocyanate-conjugated mouse antibody to hamster immunoglobulin, all obtained from Pharmingen. Additional monoclonal antibodies were used to test the cell line expressing chimeric murine α2 containing human amino acids 199–216: 12F1 (21) was provided by M. Hemler; 5E8 (22) and GI9 (23) were obtained from the Fifth International Workshop and Conference on Human Leukocyte Differentiation Antigens; and AA10 has been described (2).

RESULTS

The Isolated Human I Domain, but Not the Murine I Domain, Binds Echovirus 1—Echovirus 1 binds to human but not to murine VLA-2 (15), and experiments with human-murine chimeras have indicated that the human I domain is essential for virus binding (4, 6). To confirm that this species selectivity depends on sequences within the I domain itself, we measured virus attachment to the isolated murine and human I domains, produced as GST fusion proteins (Fig. 1). GST alone and a CR3 I domain-GST fusion protein were used as negative controls. As previously observed, virus bound to the human I domain (6). However, no virus bound to the murine I domain, despite its 83% amino acid identity (15) to the human protein.

These results indicate that selective virus attachment to human VLA-2 depends on sequence differences between the human and murine I domains. To localize the essential sequences we constructed a series of chimeric I domains in which the human and murine sequences were interchanged (Figs. 2 and 3) and tested their capacity to bind virus (Fig. 3).

Human Amino Acids 199–216 Are Involved in Virus Binding

We first studied a series of chimeras in which small regions within the human I domain were replaced by murine sequences (chimeras 1–9) (Fig. 3A). A structural model, based on the crystal structure of the CR3 I domain (12), was used to choose clusters of amino acids for replacement that were contained within putative α helices, loops, or β strands. No small sequence replacement abolished virus binding to the human I domain, indicating that the critical sequence differences between human and murine I domains were not confined to any single small region. Replacement of the entire carboxyl-terminal two-thirds of the human I domain (chimera 10) had no effect on virus binding. However, replacement of the aminoterminal portion of the human I domain (chimera 11) reduced binding to background levels.

Two chimeras involving small sequence replacements, chimeras 4 and 5, in which amino acids 199–201 and 205–216 were replaced, respectively, showed somewhat reduced capacity to bind virus (less than 80% of wild-type). An additional chimeric protein (chimera 12), in which amino acids 199–216 were all replaced by murine sequences, bound little or no virus, indicating that this region of the I domain was critical for virus attachment. In a reciprocal experiment, replacement of murine α2 containing human amino acids 199–216 (Fig. 3B; chimera 13), converted the murine I domain into a protein capable of binding virus (100% of wild-type).

To confirm the results obtained with I domain fusion proteins, human amino acids 199–216 were introduced into the murine α2 subunit (1250 amino acids), and expressed on the surface of CHO cells in association with endogenous β1. Chimeric murine α2 containing human amino acids 199–216 was recognized by the anti-human α2 mAb 5E8, but not by anti-α2

![Fig. 1. Echovirus 1 binding to isolated human and murine VLA-2 I domain-GST fusion proteins. Human VLA-2 I domain-GST (human), murine VLA-2 I domain-GST (murine), CR3 I domain-GST fusion protein (CR3 I), or GST protein (GST) bound to glutathione-Sepharose beads were incubated with radiolabeled echovirus 1 as described under “Experimental Procedures.” Results are shown as mean virus bound (in counts per minute) ± S.D. for four samples.](image-url)

| mAb | Specificity | Human | Murine | 199–216 | Mock |
|-----|-------------|-------|--------|---------|------|
| HAS-4 | Human α2 | 89 | <1 | <1 | 2 |
| 5E8 | Human I domain | 70 | <1 | 60 | <1 |
| 12F1 | Human I domain | 90 | <1 | 1 | <1 |
| GI9 | Human I domain | 75 | <1 | 6 | <1 |
| AA10 | Human I domain | 82 | <1 | 1 | <1 |
| HM2α | Murine α2 | <1 | 63 | <1 | 1 |
| MOPC 195 | Negative control | 4 | <1 | 1 | 1 |
| A19-3 | Negative control | <1 | <1 | 1 | 1 |
mAbs 12F1, Gi9, or HAS-4 in flow cytometry experiments (Table I). Although, as previously reported (4, 15), no virus bound to CHO cells expressing murine α2 itself, virus bound at high levels to the VLA-2 chimera containing human amino acids 199–216 (Fig. 4). Thus, the results obtained with chimeric I domain fusion proteins were consistent with those obtained with chimeric VLA-2 expressed on the cell surface.

Human Amino Acids 199–201 and 212–216 Interact with Virus Independently—One interpretation of the results obtained with chimeras 4, 5, and 12, was that echovirus 1 makes two separate contacts with the human I domain, one involving amino acids 199–201, and the other involving amino acids 205–216. Contact at either of these sites might be sufficient for stable virus binding, which, as observed for chimeras 4 and 5, could not be ablated by replacement of either site individually. To test this we produced additional chimeras in which, at each site, murine sequences were replaced by human sequences (Fig. 4B).

Amino acids involved in the MI-DAS are underlined (Asp151, Ser153, Ser155, Thr221, and Asp254) (12). Specific nonconserved amino acids mutated in chimeras numbered 1–9 and 12–17 are indicated by dashed lines below the sequence.

**FIG. 2. Human and murine VLA-2 I domain amino acid sequences.** The amino acid sequence of the human VLA-2 I domain (amino acids 140–349) (31) is shown (h). Nonconserved amino acids in the murine VLA-2 I domain (m) (15) are shown directly below the human sequence. Amino acids involved in the MI-DAS are underlined (Asp151, Ser153, Ser155, Thr221, and Asp254) (12). Specific nonconserved amino acids mutated in chimeras numbered 1–9 and 12–17 are indicated by dashed lines below the sequence.

**FIG. 3. Virus attachment to human-murine chimeras.** The human VLA-2 I domain is represented by the white bar at the top of the figure. The murine VLA-2 I domain is represented by the black bar at the bottom of the figure. Positions of nonconserved amino acids are indicated by short vertical marks on top of the white bar. A, replacement of human sequences. Human sequence segments replaced by murine amino acids are represented by the black bars. The reference number for each chimera is indicated in parentheses to the left of each bar. Binding of radiolabeled echovirus 1 to each chimera is shown to the right of each bar as the percentage of virus bound to the wild-type human I domain, and is calculated from the mean of at least four samples. B, introduction of human sequences. Human sequence segments introduced into the murine I domain in place of murine amino acids are shown in white. Chimera reference numbers and binding results are shown as described above.
echovirus at levels close to the wild-type human I domain (74%), but chimera 17, with human residues 205–208 alone, did not bind virus. These results confirm that the presence of human sequences at either of two sites, one involving residues 199–201, and the other involving residues 212–216, is sufficient for virus binding to occur, and suggest that virus attachment involves independent contacts with each of these sites.

Mutations That Disrupt Collagen Binding to VLA-2 do Not Inhibit Echovirus 1 Binding—VLA-2 interactions with collagen are strictly dependent on divalent cations, and mutations in residues defining the MIDAS abolish adhesion to collagen. In contrast, virus attachment to VLA-2 occurs in the absence of divalent cations. MIDAS residues are conserved between human and murine VLA-2, and cannot be responsible for selective virus attachment to the human, as opposed to the murine, I domain. To determine whether the MIDAS residues are dispensable for virus attachment, we measured virus binding to VLA-2 mutants expressed on CHO cells, in which the MIDAS residues Asp$^{151}$, Thr$^{221}$, and Asp$^{254}$ were replaced by alanine. Each of these mutations abolishes cell adhesion to collagen (5, 7). The D151A, T221A, and D254A mutations did not inhibit echovirus 1 binding to the transfected CHO cells (Fig. 5A). Virus binding to the mutant cell lines was directly proportional to $\alpha_2$ surface expression as determined by flow cytometry. Mutations at Asp$^{254}$ and Thr$^{221}$ (but not Asp$^{151}$) were previously shown to inhibit binding of I domain fusion proteins to collagen (7); however, mutation of the same MIDAS residues had no effect on echovirus 1 binding to isolated I domain-GST

![Echovirus 1 attachment to cell surface murine $\alpha_2$ containing human residues 199–216. CHO cells expressing human $\alpha_2$ (human), chimeric murine $\alpha_2$ containing human residues 199–216 (199–216), murine $\alpha_2$ (murine), or mock-transfected CHO cells (mock) were incubated with radiolabeled echovirus 1 as described under “Experimental Procedures.” Results are shown as mean virus bound (in counts per minute) ± S.D. for four samples.](image)

![Echovirus 1 binding does not depend on MIDAS residues. A, virus attachment to mutant VLA-2 on transfected cells. CHO cells expressing $\alpha_2$ subunits in which the MIDAS residues Asp$^{151}$, Thr$^{221}$, or Asp$^{254}$ are replaced by alanine were incubated with radiolabeled echovirus 1 as described under “Experimental Procedures.” Virus binding results shown represent the mean (in counts per minute) ± S.D. for four samples. B, virus attachment to isolated mutant I domain fusion proteins. Human VLA-2 I domain-GST fusion protein (WT), mutant VLA-2 I domain-GST fusion proteins (D151A, T221A, and D254A), or CR3 I domain-GST (CR3 I) immobilized on glutathione-Sepharose beads were incubated with radiolabeled echovirus 1 as described under “Experimental Procedures.” Results are shown as mean virus bound (in counts per minute) ± S.D. for four samples.](image)

![Location of residues critical for virus binding to the VLA-2 I domain. Two representations of the VLA-2 I domain structure are shown (32): all-atom space-filling model (left) and main chain schematic (right). The view is identical in both cases, looking from one side of the central $\beta$-sheet. Residues implicated in virus attachment are shown in red, and numbered in the schematic. The MIDAS residues are shown in blue, and the magnesium ion, labeled M, in aqua. Secondary structure elements are labeled in the right-hand figure (helices 1, 3–7, and $\alpha C$, and strands A–F).](image)
fusin7ion proteins (Fig. 5B). These results distinguish further between the I domain sites required for interaction with collagen and with virus (Fig. 6).

**DISCUSSION**

Although the human and murine VLA-2 I domains show 83% amino acid identity, echovirus 1 binds only to the human I domain. In the experiments reported here, human-murine chimeric I domains were used to identify sequences responsible for selective virus attachment to the human, as opposed to the murine, I domain. These sequences are distinct from the MIDAS residues essential for interactions with collagen, which we have now shown to be dispensable for virus binding.

Two regions within the human I domain have been identified as critical for echovirus 1 binding. Amino acids 199–201 and 212–216 appear to interact independently with virus; either site is sufficient for virus to bind, and both must be replaced by murine sequences to abolish virus binding. Although these results do not exclude the possibility that amino acids conserved between the human and mouse contact virus, such residues cannot contribute to the species-specificity of the echovirus-VLA-2 interaction. We have also examined echovirus 1 binding to 36 additional CHO cell lines (previously described in Ref. 7) in which single conserved amino acids were replaced by alanine. Although no surface expression of these alanine scanning mutants was highly variable, and could not permit the detection of small alterations in virus binding, no single mutation in a conserved amino acid was found to abolish virus binding to VLA-2. Conversely, because both murine and human VLA-2 bind collagen, it is unlikely that the divergent sequences involved in virus attachment are essential for VLA-2 interactions with collagen.

Three separate mutations within the metal ion-dependent adhesion site of the human VLA-2 I domain, D151A, T221A, and D254A, were studied for their effects on echovirus 1 attachment. Each of these mutations prevents collagen binding to VLA-2 (5, 7), but did not inhibit virus attachment to mutant VLA-2 or isolated I domain-GST fusion proteins. This finding supports previous suggestions (4, 9, 15) that the VLA-2-binding sites for collagen and for echovirus 1 are different.

Several ligand-I domain interactions require an intact MIDAS and may also involve residues on a surface surrounding the MIDAS (24–27). It has been proposed that the magnesium ion bound by the I domain MIDAS may participate in ligand binding by coordinating an acidic residue from a ligand molecule (12). Echovirus 1 binding to the human VLA-2 I domain is unique in that it does not require divalent cations (9) and is not disrupted by mutations in the MIDAS.

The recently determined crystal structure of the human VLA-2 I domain (Ref. 32 and Fig. 6) reveals that residues 199–201 and 212–216 lie in two loops: between strand C and helix 4. This may represent a viral attachment surface, since our results do not rule out a role for other residues lying on this surface that are conserved in murine and human VLA-2. We also note that this surface does not change its structure in the two conformations of the CR3 I domain (28), consistent with the insensitivity of virus attachment to activation state (9).

The viral attachment surface is distinct from the MIDAS surface of the molecule, which lies at the COOH-terminal end of the β-sheet. The two surfaces touch near tyrosine 216, and it is noteworthy that mAb 5E8, which recognizes an epitope that includes tyrosine 216 (29), blocks VLA-2 interactions with both virus and collagen (5). Work on the CR3 I domain suggests that a third surface (the lower surface of the domain at the N-terminal end of the β-sheet) is involved in regulation of ligand binding affinity (30). This surface is also distinct from the viral attachment surface, although the two may touch at the loop preceding helix 3.

The crystal structure of echovirus 1 has also been determined, and is remarkable for depressions on the virus surface at both the 5-fold and 2-fold symmetry axes, either of which may be the site for receptor attachment, and either of which could accommodate insertion of the VLA-2 I domain. It is likely that the essential receptor residues identified here make contacts with viral residues lining one of the surface depressions. Further structural studies, involving the use of both cryoelectron microscopy and x-ray crystallographic techniques, will be required to map these interactions in detail.

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