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High-Efficiency Utilization of the Bovine Integrin $\alpha_v\beta_3$ as a Receptor for Foot-and-Mouth Disease Virus Is Dependent on the Bovine β_3 Subunit

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We have previously reported that *Foot-and-mouth disease virus* (FMDV), which is virulent for cattle and swine, can utilize the integrin $\alpha_v\beta_3$ as a receptor on cultured cells. Since those studies were performed with the human integrin, we have molecularly cloned the bovine homolog of the integrin $\alpha_v\beta_3$ and have compared the two receptors for utilization by FMDV. Both the α_v and β_3 subunits of the bovine integrin have high degrees of amino acid sequence similarity to their corresponding human subunits in the ectodomains (96%) and essentially identical transmembrane and cytoplasmic domains. Within the putative ligand-binding domains, the bovine and human α_v subunits have a 98.8% amino acid sequence similarity while there is only a 93% similarity between the β_3 subunits of these two species. COS cell cultures, which are not susceptible to FMDV infection, become susceptible if cotransfected with α_v and β_3 subunit cDNAs from a bovine or human source. Cultures cotransfected with the bovine $\alpha_v\beta_3$ subunit cDNAs and infected with FMDV synthesize greater amounts of viral proteins than do infected cultures cotransfected with the human integrin subunits. Cells cotransfected with a bovine α_v subunit and a human β_3 subunit synthesize viral proteins at levels equivalent to those in cells expressing both human subunits. However, cells cotransfected with the human α_v and the bovine β_3 subunits synthesize amounts of viral proteins equivalent to those in cells expressing both bovine subunits, indicating that the bovine β_3 subunit is responsible for the increased effectiveness of this receptor. By engineering chimeric bovine-human β_3 subunits, we have shown that this increase in receptor efficiency is due to sequences encoding the C-terminal one-third of the subunit ectodomain, which contains a highly structured cysteine-rich repeat region. We postulate that amino acid sequence differences within this region may be responsible for structural differences between the human and bovine β_3 subunit, leading to more efficient utilization of the bovine receptor by this bovine pathogen.

Foot-and-mouth disease virus (FMDV), an *Aphthovirus* in the *Picornaviridae* family, is the cause of foot-and-mouth disease, a highly infectious disease of domestic livestock. The virus initiates infection by binding to its cellular receptor via an arginine-glycine-aspartic acid (RGD) sequence found within a surface protrusion consisting of the loop between the βG and βH strands (G-H loop) of the capsid protein VP1 (1, 6, 23, 42, 45). While FMDV can utilize other receptors on cultured cells, such as the Fc receptor (7, 44) or heparan sulfate (3, 25, 36, 47), these receptors do not require the RGD sequence (43, 47). We have demonstrated that antibodies to the integrin receptor $\alpha_v\beta_3$ can inhibit adsorption and plaque formation by FMDV (11). Furthermore, we have also shown that the virus, which is virulent for cattle, can infect only cells expressing this integrin receptor and that site-directed mutants of these viruses lacking an RGD sequence are not capable of infecting cells expressing $\alpha_v\beta_3$ (45, 47).

Integrins are heterodimeric molecules, consisting of α and β subunits which interact noncovalently at the cell surface and have a wide species distribution (35). They are involved in extracellular matrix and cell-cell interactions and also serve as signal-transducing receptors (29). A total of 16 α and 8 β subunits have been described, giving rise to 22 different integrins, each with its own ligand-binding specificity, and 7 of which, including $\alpha_v\beta_3$, bind to their natural ligands via an RGD sequence (22, 35). Electron microscopic visualization of integrins reveals a globular structure, presumably the ligand-binding region combining elements of both subunits with two stalk-like structures extending to the cell surface (16, 49).

The $\alpha_v\beta_3$ integrin is one of two receptors within the class of integrins called cytoadhesins (29). The β_3 subunit is found only complexed with one other subunit, α_{IIb}, while the α_v subunit can complex with four additional β subunits (β_1, β_5, β_6, and β_8) (35). Although $\alpha_v\beta_3$ was originally called the vitronectin receptor, it can bind to other ligands (33). While it is clear that both the α and β subunits of integrins structurally contribute to ligand binding (22, 34), there are specific regions of the α_v (41, 57) and β_3 (13, 19, 39, 56, 61, 62) subunits that have been identified as directly interacting with ligands. At least two other picornaviruses can utilize $\alpha_v\beta_3$ to initiate infection, coxsackievirus A9 (CAV9) (53) and echovirus 9 (48). In addition, human adenovirus utilizes integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ to facilitate internalization (64); two hantaviruses, which cause different human disease syndromes, utilize $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ to mediate cellular entry (26, 27); and human parechovirus 1 (formerly echovirus 22) may utilize the $\alpha_v\beta_1$ integrin as a receptor (50).

While previous studies have shown that FMDV can utilize the human (47) and simian (11) homologs of the $\alpha_v\beta_3$ integrin to infect cells, this virus does not cause disease in humans (4). Therefore, we have characterized the bovine homolog of this integrin to determine how it interacts with this bovine pathogen. These studies demonstrate that FMDV utilizes the bovine integrin with much greater efficiency than it utilizes the human

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integrin, and we show that this increased efficiency is due to bovine sequences located within the C-terminal one-third of the β_3 subunit ectodomain.

MATERIALS AND METHODS

cDNA cloning. Single-stranded cDNA was reverse transcribed from bovine lung poly(A)$^+$ RNA (Clontech) using Superscript II reverse transcriptase (Life Technologies) and an oligo(dT)$_{18}$ primer. The bovine α_v cDNA was generated in two pieces by a 30-cycle PCR using *Taq* polymerase (Boehringer Mannheim) and the following sets of primers, which introduce a silent mutation creating a *Bam*HI restriction endonuclease site that could be used to recreate complete α_v coding sequences: 5′CGCGCACCCCGGCGATGGCT3′ plus 5′CCATCGGAT CCGCGATCCATG3′, and 5′GGATCCGATGGCAAACTCCAGGAG3′ plus 5′GGAATTCCTTAAGTTTCTGAGTTTCCTTCACC 3′. The resulting PCR products were ligated into the vector pCR2.1 (Invitrogen) and sequenced. Plasmid DNA containing the 3′ piece of bovine α_v cDNA was ligated to the 5′ bovine α_v cDNA utilizing this synthetic *Bam*HI site. The resulting full-length α_v cDNA fragment was inserted into the vector pcDNA3.1/Zeo(−) (Invitrogen) to create pBovα_vZEO.

The cDNA encoding the mature bovine β_3 subunit was generated by a 30-cycle PCR using Advantage KlenTaq polymerase mix (Clontech) and the following primers, which introduce a silent mutation creating an *Mlu*I site following the predicted N-terminal signal peptide cleavage site: 5′CCACGCGTGGTGTGAG CTCCTG3′ plus 5′GGATCCTAAGGCCCCGGTACGTGATATTG3′. To generate a signal peptide sequence for use with the bovine β_3 coding region, human β_3 cDNA (β_3/pIAP58) (14, 40, 47) was used as a template for 15 cycles of PCR amplification with Advantage KlenTaq polymerase mix and the following primers, which also introduce a silent mutation creating an *Mlu*I site in frame with the bovine open reading frame: 5′CAGATGCGAGCGCGGCCGC3′ plus 5′CGG GATCCTTAAGTGCCCCGGTACGTGATATTG3′. The PCR products were inserted into the pCR-Blunt II-TOPO vector (Invitrogen) and sequenced. The cDNA encoding mature bovine β_3 was ligated to the human signal peptide sequence and inserted into pcDNA3.1/Zeo(−) to create pBovβ_3ZEO. The human α_v-encoding plasmid, pHumα_vZEO, has been described previously (47). The human β_3-encoding plasmid, β_3/pIAP58 (14, 40, 47), was inserted into pcDNA3.1/Zeo(−) to generate pHumβ_3ZEO.

Generation of chimeric β_3 subunits. Chimeric cDNAs for the β_3 integrin were created using a *Kpn*I site shared by pBovβ_3ZEO and pHumβ_3ZEO at codon 136. Plasmid phkbβ_3 contained the first 136 codons of the human β_3 subunit and the remaining codons from the bovine β_3 subunit. Plasmid pbkhβ_3 was the inverse chimera and contained bovine sequences to codon 136 and human sequences for the remainder of the subunit. The chimeric cDNAs phsbβ_3 and pbshβ_3 were created using a similar strategy and a *Sma*I site at codon 488 of pBovβ_3ZEO and pHumβ_3ZEO. The resulting constructs were sequenced around the restriction sites to ensure their identity.

Sequencing. Automated sequencing was performed on an Applied Biosystems 370A sequencer with an XL upgrade, using the ABI Prism Big Dye terminator cycle-sequencing ready reaction kit (Perkin-Elmer) as described by the manufacturer. Sequence analysis was done using the Lasergene analysis software package (DNASTAR Inc.). The nucleotide sequences for the human α_v and β_3 subunits were from GenBank (accession numbers M14648 [60] and M35999 [24], respectively).

Coupled in vitro transcription-translation. Plasmid DNA (1 μg) was used in either a wheat germ extract or rabbit reticulocyte lysate TNT Quick coupled transcription-translation system (Promega) in the presence of [^{35}S]methionine (Amersham) as described by the manufacturer. The resulting protein products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 7.5% gel.

Viruses and cells. FMDV type A_{12}, strain 119ab, was derived from the infectious cDNA clone pRMC$_{35}$ (52). An antigenic variant of type A_{12}, harboring the VP1 sequence present in a bovine tongue tissue-propagated type A_{12} (vRM-SSP), has been described previously (51). The cattle-virulent variant derived from infectious cDNA containing capsid sequences isolated from a vaccine seed stock of type O_1Campos (vCRM8) has also been described previously (54). COS-1 cells were maintained on Dulbecco's minimal essential medium (Life Technologies Inc.) containing 10% calf serum, an additional 2 mM L-glutamine, 1 mM sodium pyruvate, 10 U of penicillin G per ml, 10 U of streptomycin sulfate per ml, and 0.25 μg of amphotericin B per ml.

Transient expression of integrin subunits in COS-1 cells. Cells were plated at a density of 10^5 cells/well on six-well tissue culture dishes the day before transfection. Transfections were performed using 2.0 μg of each integrin-encoding plasmid mixed with the transfection reagent FuGENE 6 (Roche Molecular Biochemicals) as specified by the manufacturer. After incubation at 37°C overnight, the cells were trypsinized and replated onto 2 wells of a 24-well tissue culture dish. After an additional overnight incubation at 37°C, cells in one well of each transfected condition were infected with FMDV and cells in the other well were analyzed for integrin expression by immunohistochemistry (IHC).

Immunohistochemistry. Transfected cells were fixed on ice for 5 min with acetone-methanol (50:50). Fixed cells were rehydrated with minimal essential medium containing 0.2% bovine serum albumin, 50 mM HEPES (pH 7.5), and 1% normal horse serum. The cells were reacted with a 1:500 dilution of the anti-$\alpha_v\beta_3$ monoclonal antibody (MAb) LM609 (MAB1976; Chemicon International Inc.) (17) for 30 min at 37°C. Primary-antibody binding was detected using an alkaline phosphatase avidin-biotin system and biotinylated horse anti-mouse immunoglobulin G (Vectastain Elite ABC kit; Vector Laboratories). Bound phosphatase was visualized using the Vector VIP substrate kit (Vector Laboratories).

Viral replication assays. Transfected and nontransfected COS-1 cells were infected and analyzed as previously described (47). Briefly, cells were infected with various FMDV serotypes at a multiplicity of infection (MOI) of 10 PFU/cell and labeled overnight, beginning at 4 h after infection, with 50 to 75 μCi of [^{35}S]methionine at 37°C. Cell lysates were prepared in 1% Triton X-100, trichloroacetic acid (TCA)-precipitable counts per minute (cpm) were determined, and radioimmunoprecipitation (RIP) was preformed as previously described (5) using anti-FMDV type A_{12} MAb 6EE2 (8) for type A_{12}- and vRM-SSP-infected cells and anti-FMDV type O_1 MAb 10GA4 (59) for vCRM8-infected cultures. Equal amounts of TCA-precipitable CPM were immunoprecipitated and analyzed by SDS-PAGE using a 10% polyacrylamide gel.

Nucleotide sequence accession numbers. Nucleotide sequences for the bovine α_v and β_3 cDNAs have been submitted to GenBank and have been assigned accession numbers AF239958 and AF239959, respectively.

RESULTS

cDNA cloning of the bovine integrin $\alpha_v\beta_3$ subunits. The identification of $\alpha_v\beta_3$ as the receptor for virulent forms of FMDV (47) utilizing human $\alpha_v\beta_3$ cDNAs led us to examine the available integrin subunit sequences. Interspecies comparisons for both the α_v (63) and β_3 (18) subunits have shown that there are differences in the deduced amino acid sequences among the species sequenced to date. For this reason, we thought it important to obtain cDNAs encoding $\alpha_v\beta_3$ from a species that is naturally susceptible to FMDV infection, such as cattle. PCR with cDNA from bovine lung tissue and primers based on known human and mouse integrin sequences (18, 60, 63, 65) generated fragments of the expected sizes for both the α_v and β_3 subunits. These fragments were ligated and inserted into expression vectors that would allow in vitro transcription-translation analysis and eukaryotic expression. The complete coding sequence for the bovine α_v subunit cDNA was 3,144 bp coding for a 1,048-amino acid protein. The encoded protein consists of a 30-residue signal peptide, a 963-residue extracellular ectodomain, a 23-residue transmembrane domain, and a 32-residue cytoplasmic domain. The complete coding sequence for the bovine β_3 subunit cDNA was 2,364 bp coding for a 788-amino-acid protein. Since we were unable to generate a fragment which included the authentic bovine β_3 signal peptide sequence, we removed the coding region for this peptide from the human β_3 cDNA plasmid and ligated it to the remainder of the bovine β_3 cDNA as described in Materials and Methods. Thus, the encoded protein consists of the 26-residue human signal peptide, a 692-residue extracellular ectodomain, a 23-residue transmembrane domain, and a 47-residue cytoplasmic domain.

Coupled in vitro transcription-translation reactions were performed using the cDNA constructs encoding both the human and the bovine α_v and β_3 subunits to determine if the bovine constructs were capable of generating proteins in the same size range as the human subunits. Radiolabeled protein was generated and separated by SDS-PAGE (7.5% polyacrylamide) as described in Materials and Methods. Transcription-translation of the bovine α_v subunit in a rabbit reticulocyte lysate translation system generated a product that was comparable in size, based on migration in a denaturing gel, to the human α_v subunit (Fig. 1a). In contrast, in the rabbit reticulocyte lysate system, the bovine β_3 subunit migrated faster, to an apparent lower molecular weight than the human subunit did (Fig. 1a). Since differences in glycosylation may account for differences in migration on SDS-PAGE, the transcription-translation reactions were repeated utilizing a wheat germ

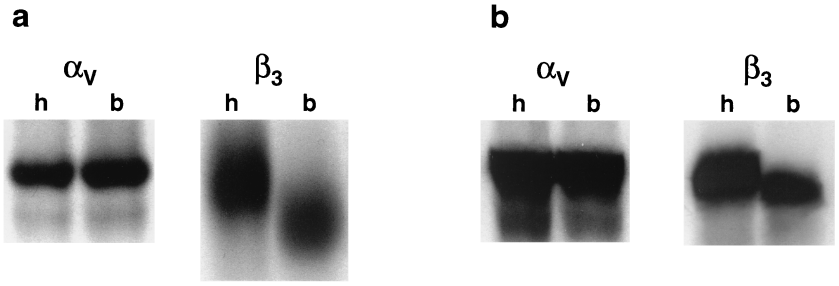

FIG. 1. Transcription-translation of cloned bovine integrin subunits. Plasmids containing sequences representing bovine α_v (pBovα_vZEO), bovine β_3 (pBovβ_3ZEO), human α_v (pHumα_vZEO), or human β_3 (pHumβ_3ZEO) integrin sequences were analyzed for protein expression by coupled in vitro transcription-translation using either a rabbitreticulocyte lysate (a) or wheat germ extract system (b), as described in Materials and Methods. Translation products were analyzed by SDS-PAGE (7.5% polyacrylamide). Bovine and human subunits are indicated by b and h, respectively, above the lanes.

extract. In contrast to the result seen in the rabbit reticulocyte lysate, the bovine and human β_3 subunits synthesized in this system were comparable in size, as were the α_v subunits (Fig. 1b). While O-linked glycosylation occurs in rabbit reticulocyte lysates in the absence of microsomal membranes, this post-translational modification cannot take place in wheat germ extracts in the absence of membranes. In contrast, N-linked glycosylation occurs only in the presence of microsomal membranes in either extract (38, 58). Since we did not add microsomal membranes to either lysate, differences seen between the bovine and human β_3 subunits in the rabbit reticulocyte lysate may have been due to differences in glycosylation. Examination of potential O-linked glycosylation sites in the β_3 subunits revealed differences between human and bovine β_3 subunits which may account for the variation in apparent molecular weight seen in the rabbit reticulocyte lysate system (results not shown).

Sequence comparisons of bovine and human integrin subunits. The cloned bovine subunit cDNAs were sequenced using automated sequencing, as described in Materials and Methods, and compared with the reported human α_v and β_3 subunit sequences (24, 60). Analysis of the two bovine subunit constructs revealed a high degree of sequence similarity to their counterpart human homolog subunits. Alignment of DNA sequence using the Martinez/Needlemen-Wunsch alignment method, with a gap penalty of 1.10 and a gap length penalty of 0.33, showed that the sequence similarities between the bovine and human α_v and β_3 extracellular ectodomains were 93.6 and 90.1%, respectively (Table 1). When the deduced amino acid sequences within this region were compared by Lipman-Pearson alignment (gap penalty of 4 and gap length penalty of 12), the sequence similarity was about 96% for both subunits (Table 1). The transmembrane and cytoplasmic domains of both bovine subunits displayed the highest level of amino acid similarity to their human homologs: the α_v subunits were identical in this domain, and the β_3 subunits had a 99.9% similarity even though the nucleotide sequence similarity was rather low at 88% (Table 1). We also compared sequences which lie within the putative ligand-binding domains of these subunits. The ligand-binding domains of the human α_v and β_3 subunits have been determined using a number of methods, most prominently photoaffinity cross-linking and generation of chimeric receptors with closely related α_v and β_3 subunits. These studies have estimated that the ligand-binding regions for the α_v and β_3 subunits lie between amino acid residues 1 and 340 (41, 57) and between residues 85 and 207 (13, 19, 39, 56, 61, 62), respectively (amino acid residue 1 is the first amino acid after cleavage of the signal sequence). Within this domain, the amino acid sequence similarity between bovine and human sequences was quite high for the α_v subunit (98.8%) but was only 93.5% for the β_3 subunit.

Transient expression of integrin subunits in COS-1 cells. Cells were cotransfected with each integrin subunit using FuGENE 6 as described in Materials and Methods. The cells were grown for 48 h, fixed, and immunostained using the anti-$\alpha_v\beta_3$ MAb LM609. Figure 2 shows that cells expressing either bovine or human $\alpha_v\beta_3$ were stained equally well with this MAb, which reacts only with the heterodimeric $\alpha_v\beta_3$ integrin (17). This confirms previously reported results showing that LM609, which was generated against the human integrin, also reacts with the complete bovine integrin (12, 28, 55). Furthermore, these results indicate that both integrins were expressed to approximately comparable levels in the COS-1 cells. We also examined the expression of the $\alpha_v\beta_3$ integrin in cells transfected with mixed bovine-human subunits. The results in Fig. 2 show that these cultures also expressed the integrin to levels comparable to those seen in either complete bovine or complete human integrin expression. Because integrins can be composed of different combinations of α and β subunits (35), transfections were also done with the individual subunits and cells were analyzed by IHC. In all cases, no staining above the level seen in the control cells was observed (data not shown), indicating that the staining obtained in cells transfected with both subunits was not due to the formation of heterodimers between the transfected subunits and endogenous monkey integrin subunits.

In the experiments reporting FMDV replication in integrin subunit-transfected cells shown below, parallel cultures were

TABLE 1. Nucleotide and encoded amino acid sequence similarities between the human and bovine α_v and β_3 integrin subunits

Subunit	Nucleotide similarity/amino acid similarity[a] in:		
	Ectodomain	Transmembrane and cytoplasmic domains	Ligand-binding domain[b]
α_v	93.6%/95.6%	94.5%/100%	98.1%/98.8%
β_3	90.1%/95.5%	88%/99.9%	91.6%/93.5%

[a] The first number of each pair represents the sequence similarity of nucleotides aligned using the Martinez/Needleman-Wunsch alignment method and determined using a gap penalty of 1.10 and a gap length penalty of 0.33. The second number represents the sequence similarity of amino acids aligned using the Lipman-Pearson alignment method and determined using a gap penalty of 4 and a gap length penalty of 12.

[b] The ligand-binding domains have been defined as amino acid residues 1 to 340 for the α_v subunits (41, 57), and amino acid residues 85 to 207 for the β_3 subunits (13, 19, 39, 56, 61, 62).

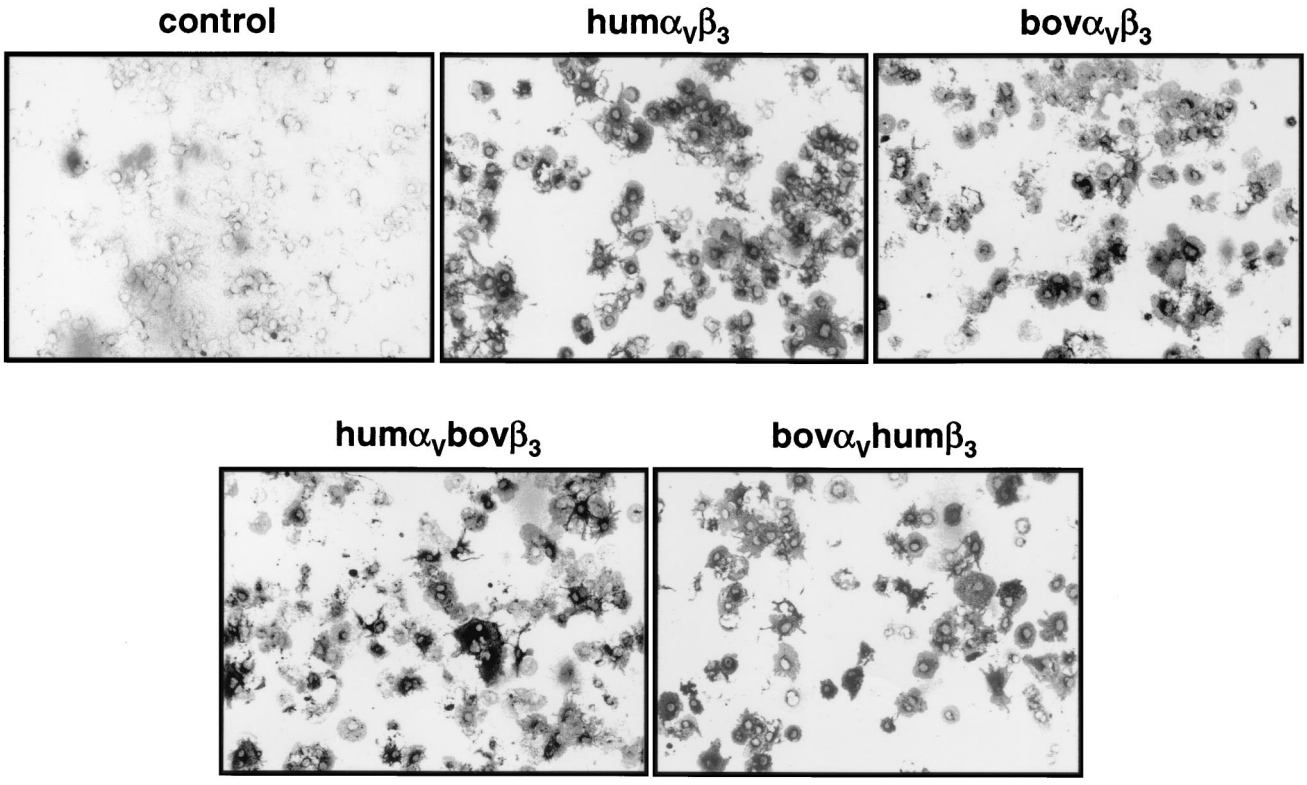

FIG. 2. Analysis of integrin expression in transfected COS-1 cells. Cells were transfected with integrin subunit-encoding plasmids, as shown above each panel, and integrin expression was analyzed by IHC with MAb LM609 48 h after transfection, as described in Materials and Methods.

always examined for integrin expression by IHC and only experiments where the integrin expression in all cultures was qualitatively equivalent are reported. In addition, in some experiments, cells were analyzed for integrin expression using fluorescence-activated cell sorting (FACS), and these results were comparable to those seen using IHC analysis (data not shown).

Replication of FMDV in integrin subunit-transfected cells. COS-1 cells were transfected with complete bovine or human integrin subunit cDNAs or with mixed bovine-human subunits and infected with FMDV as outlined in Materials and Methods. We used three different viruses for these studies: our tissue culture-adapted type A_{12}, an A_{12} variant containing sequences isolated from the tongue of an infected bovine (vRM-SSP) (51), and a highly cattle-virulent variant of type O_1Campos (vCRM8) (54). These viruses utilize only $\alpha_v\beta_3$ as a receptor to infect cultured cells (47). Transfected-infected cells were labeled overnight with [^{35}S]methionine, and lysates were analyzed by RIP and SDS-PAGE as described in Materials and Methods. In all of these assays, equal numbers of TCA-precipitable cpm and equal amounts of protein were immunoprecipitated within each experiment. This normalized the various conditions and allowed us to make semiquantitative comparisons of the levels of viral protein synthesis. The results in Fig. 3 show the presence of viral proteins only in infected cells expressing either bovine or human $\alpha_v\beta_3$, confirming our previous findings obtained in experiments with human integrin-transfected cells (47).

A comparison of the level of viral protein synthesis in cultures expressing the different integrins, however, showed that viral protein synthesis was greater in cultures transfected with the bovine integrin (Fig. 3), even though the bovine and human integrins were expressed to the same level, as determined by IHC. We also examined the level of viral protein synthesis in cells transfected with mixed bovine-human integrin subunits. The results in Fig. 3 show that with all three viruses used, the level of viral protein synthesis was always greater when the bovine β_3 was expressed, regardless of which α_v subunit was transfected. In fact, expression of the bovine β_3 subunit along with the human α_v subunit resulted in a level of viral protein synthesis comparable to that seen with the complete bovine integrin. The differences in the level of viral protein synthesis appeared to be more pronounced when the cells were infected with the laboratory strain A_{12} virus than when they were infected with either of the other two animal-derived viruses. However, it is clear in all cases that viral replication, as measured in this assay, was greater when the bovine $\alpha_v\beta_3$ integrin was used as a receptor. In nontransfected COS-1 cells, there was a very low level of viral protein synthesis in cells infected with the vCRM8 virus (Fig. 3). We are not sure why this occurred; however, it may be the result of a low level of virus which utilizes cell surface heparan sulfate as a receptor (47, 54), either present in the original seed or generated during the overnight incubation.

FMDV infection in cells expressing chimeric bovine-human β_3 subunit receptors. Since the results presented in the previous section indicated that the bovine β_3 subunit was necessary for the higher level of viral protein synthesis seen in transfected-infected cells, we generated chimeric bovine-human β_3 subunits to delineate which portions of the subunit were responsible for this phenomenon. To do this, we took advantage of two unique restriction sites that are conserved in both the bovine and human β_3 cDNAs. A schematic diagram for the chimeric β_3 subunits is shown in Fig. 4a. The first two were created using a *Kpn*I restriction site, which facilitated a recip-

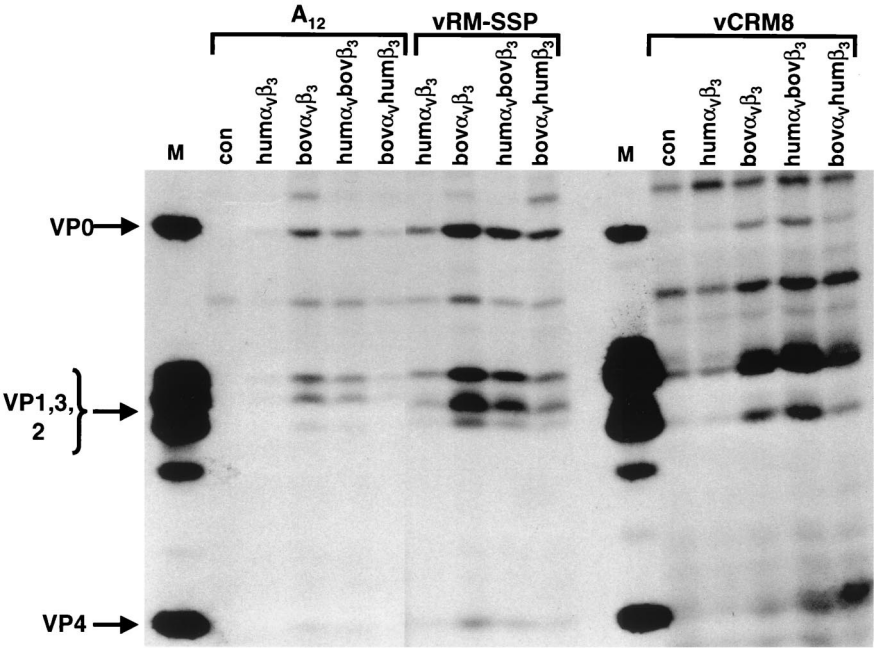

FIG. 3. Analysis of viral protein synthesis in COS-1 cells transfected with integrin subunit cDNAs. Cells were transfected with plasmids encoding human or bovine integrin subunits as shown. Transfected cells were infected with FMDV type A_{12}, vRM-SSP, or vCRM8 at an MOI of 10 PFU/cell. Cells were labeled with [^{35}S] methionine, RIP was performed on cell lysates, and the products were analyzed by SDS-PAGE (10% polyacrylamide). Locations of viral structural proteins are denoted on the left. con, nontransfected cells; M, marker viral proteins from FMDV-infected BHK-21 cells also labeled with [^{35}S]methionine; bov, bovine integrin subunits; hum, human integrin subunits.

rocal swap within the ligand-binding domain at amino acid residue 136. These swaps generated the proteins hkbβ_3, which contains human sequences from the N terminus to codon 136 and bovine sequences for the rest of the subunit, and bkhβ_3, which contains bovine sequences from the N terminus to codon 136 and human sequences for the rest of the subunit. The second set of chimeric β_3 subunits were created using a *Sma*I restriction site, which allowed a reciprocal swap within the C-terminal one-third of the ectodomain at amino acid residue 488, far outside the ligand-binding domain. These swaps generated the proteins hsbβ_3, which contains human sequences from the N terminus to codon 488 and bovine sequences for the rest of the subunit, and bshβ_3, which contains bovine sequences from the N terminus to codon 488 and human sequences for the rest of the subunit.

These chimeras and the wild-type β_3 subunit were cotransfected into COS-1 cells, along with the human or bovine α_v subunit. The resulting cultures were checked for $\alpha_v\beta_3$ expression level by IHC, infected with FMDV type A_{12}, labeled overnight, and analyzed by RIP and SDS-PAGE. The results of transfections with the intact bovine and human β_3 subunits confirmed the importance of the bovine β_3 subunit in increased receptor utilization (Fig. 4b). The results of transfections with the chimeric β_3 subunits showed that the hkbβ_3 or hsbβ_3 chimeras supported replication to the same level as the intact bovine β_3 did. Interestingly, these results suggest that the presence of bovine or human sequences from the N terminus of the β_3 subunit to amino acid residue 488, including the ligand-binding domain, did not influence the level of viral protein synthesis observed. In contrast, the higher levels of viral protein synthesis were observed only when the β_3 subunit contained bovine sequences downstream from codon 488 (hkbβ_3 and hsbβ_3). To rule out any influence of the bovine α_v subunit, we repeated the experiment using the human α_v subunit and obtained similar results to those seen in Fig. 4b (data not shown).

Sequence comparison within the C-terminal region of the β_3 subunit ectodomain. The β_3 subunit has a high cysteine content, as do all integrin β subunits (15). The bovine subunit contains 54 cysteine residues, 30 of which are located within a region of four tandem amino acid repeats near the C terminus of the ectodomain. This is within the region downstream from amino acid residue 488, which appears to be responsible for the observed increase in viral protein synthesis. A comparison of the amino acid sequences of the bovine and human β_3 subunits in this region is shown in Fig. 5. The vertical arrow at amino acid residue 488 represents the location of the *Sma*I restriction endonuclease site, and the horizontal arrows show the four tandem repeats. It can be seen that downstream from residue 488 there are only seven amino acid residues which differ between the bovine and human subunits. However, all of the cysteines are conserved, as they are in the entire subunit, with the exception of one, at residue 503, within the second repeat region, which is an arginine in the bovine integrin. The significance of changes within this region on the ability of the subunit to function as a viral receptor is examined in Discussion.

DISCUSSION

Previous results have implicated integrin $\alpha_v\beta_3$ as a receptor for FMDV by demonstrating inhibition of infection using integrin-specific antibodies (11) and by demonstrating that cells transfected with cloned human integrin subunit cDNAs became susceptible to infection by FMDV (47). Since these studies were performed with cDNAs encoding integrin subunits from a host that is not susceptible to foot-and-mouth disease (see Introduction), we have repeated these studies with molecularly cloned bovine α_v and β_3 cDNAs. The results of these studies indicate that FMDV was able to utilize the bovine integrin more efficiently than it utilized the human homolog, and

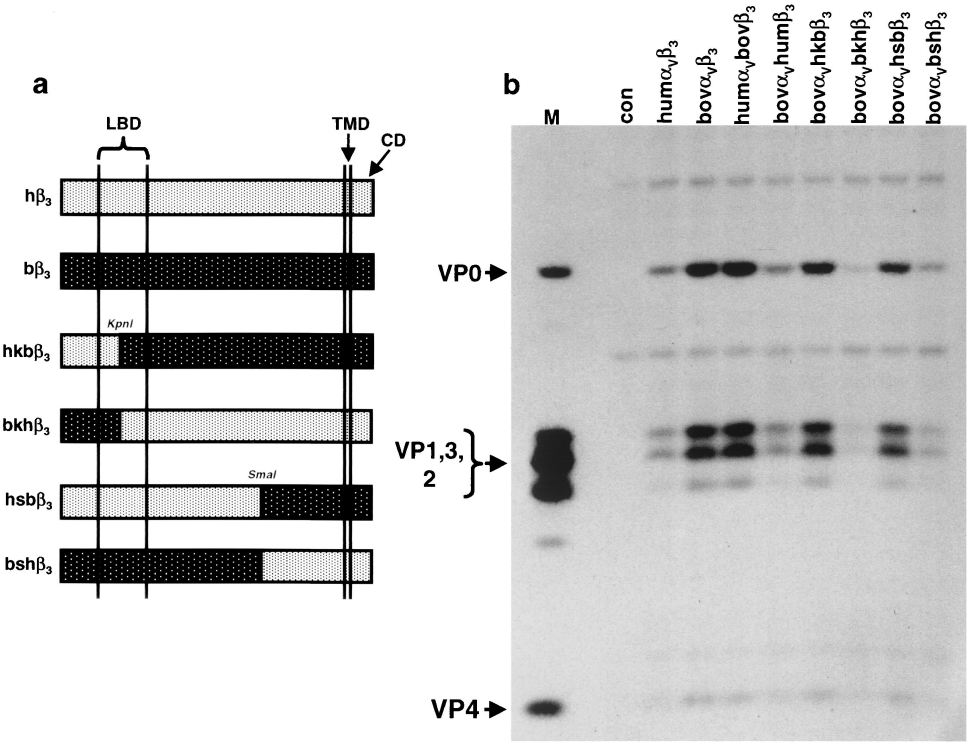

FIG. 4. Analysis of viral protein synthesis in COS-1 cells transfected with bovine-human chimeric β_3 subunits. (a) Schematic diagram of chimeric β_3 subunits, showing the locations of the ligand-binding domain (LBD), transmembrane domain (TMD), and cytoplasmic domain (CD), as well as the locations of the *Kpn*I (codon 136) and *Sma*I (codon 488) sites used to generate them (see Materials and Methods). The white background with the black stipples represents human sequences, and the black background with the white stipples represents bovine sequences. (b) FMDV type A_{12}-infected-radiolabeled cell lysates prepared from cells cotransfected with a bovine α_v subunit and the β_3 subunits shown in panel a were analyzed as described in the legend to Fig. 3 and Materials and Methods.

this increased efficiency appears to correlate with the presence of the bovine β_3 subunit.

We have utilized transient expression of α_v and β_3 subunits of bovine and human origin in COS-1 cells to examine receptor utilization by FMDV. Using this system, we have examined the replication of our laboratory strain of FMDV (type A_{12}), its bovine tongue-derived variant, vRM-SSP (51), and the highly cattle-virulent type O_1Campos variant, vCRM8 (54), which all utilize only the integrin $\alpha_v\beta_3$ as receptor (47). These experiments showed that for these three viruses, the cotransfection of cells with a bovine β_3 subunit and either a bovine or human α_v subunit resulted in the expression of a more efficiently utilized receptor than did the cotransfection of cells with both human subunits or bovine α_v and human β_3 subunits.

To determine the regions of the bovine β_3 subunit that might be responsible for the increased efficiency of use as an FMDV receptor, we generated chimeric bovine-human β_3 subunit cDNAs and tested their efficiency as receptors for type A_{12} FMDV. The results of these studies indicated that bovine sequences downstream from codon 488, outside of the putative ligand-binding domain, appeared to be responsible for the increased efficiency of the bovine receptor (see Results) (Fig. 4).

Comparison of the amino acid sequences of the bovine and human β_3 subunit between codon 488 and the C terminus of the subunit reveals virtually identical transmembrane and cytoplasmic domains, with only seven amino acid changes in the ectodomain (Fig. 5). This region of the subunit is rich in cysteines which contribute to the overall structure of the integrin through disulfide bonding (15). In the mature bovine β_3 subunit, 7% of the amino acids are cysteines (a total of 54 cysteine residues). Thirty of these cysteine residues are within a region of four tandem repeats within the integrin stalk region, known as the cysteine-rich repeats (15, 30, 33, 35). A cysteine residue just upstream of the first cysteine-rich repeat region, at codon 435, forms a disulfide bond with a cysteine near the N terminus, at codon 5, and probably contributes to the formation of the β_3 globular head that interacts with a similar structure on the α subunit to form the complete ligand-binding region (16, 49). The *Sma*I site at codon 488 occurs at the beginning of the second repeat (Fig. 5). Within the cysteine-rich repeat region of all the β subunits, the positions of the cysteines are highly conserved, with seven residues in the first repeat and eight residues in the second through fourth repeats. Similar cysteine-rich repeat regions are also found in laminin B chains and epidermal growth factor (10). Examination of the amino acid sequence comparisons between the bovine and human subunits within this region reveals that the cysteine found at residue 503 in the human subunit has been changed to an arginine in the bovine subunit. Thus, the region of the bovine β_3 subunit that confers the higher efficiency of utilization of the subunit as a receptor for FMDV is missing one cysteine residue.

There are six other amino acid changes within the β_3 region that confers increased receptor efficiency (Fig. 5), and we have not yet determined which of these changes may play a role in receptor efficiency. The loss of the cysteine at residue 503, however, is an intriguing change. The overall structure of the β subunits, based on primary sequence, is conserved among many species, and much of that structure appears to be dependent on the disulfide bonding between cysteine residues (30). Therefore, reports that cysteine 503 in the human β_3 subunit forms a disulfide bond with cysteine 536 in the third repeat region (15) makes the absence of a cysteine at this position

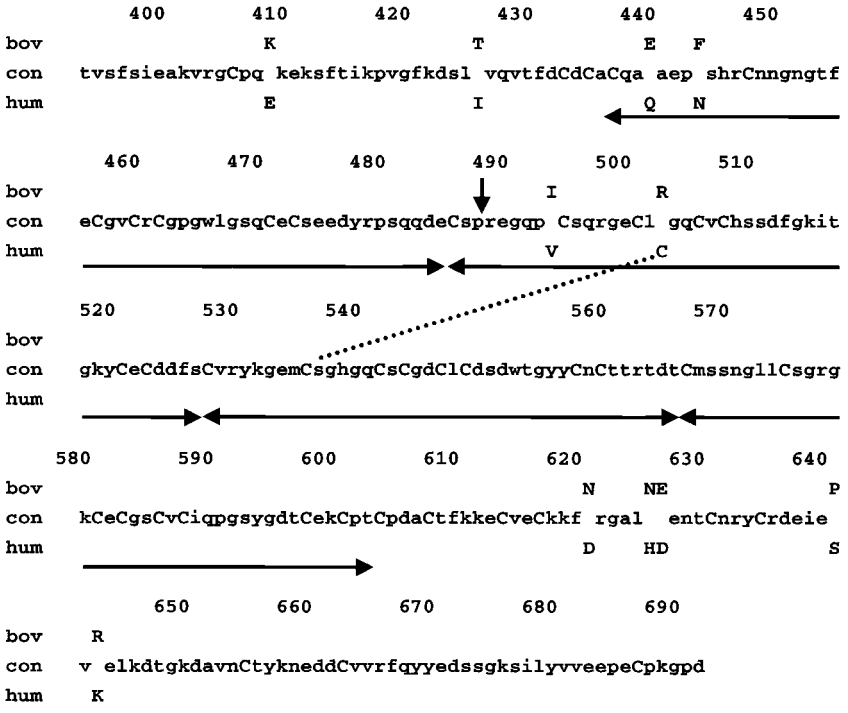

FIG. 5. Comparison of amino acid sequences within the cysteine-rich repeat region between bovine and human β_3 subunits. Deduced amino acid sequences of the bovine and human β_3 subunits from amino acid residue 394 to the last residue in the subunit ectodomain (residue 692) are shown. The conserved sequences (con) are shown in lowercase type, with the exception of the cysteine residues, which are capitalized. Sequences which differ between bovine (bov) and human (hum) subunits are shown in capital letters. The horizontal arrows indicate the location of the four cysteine-rich repeats, and the vertical arrow indicates the location of the *Sma*I site. The dotted line represents the putative disulfide bond assignment for cysteine 503 in the human β_3 subunit (15).

particularly interesting. The cysteine-rich repeat region has been implicated in the modulation of integrin activation and ligand-binding activity. A naturally occurring mutation within this region in the human β_3 subunit is responsible for the activation of both $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ (37), and a MAb which binds to the cysteine-rich repeat region of the β_1 subunit increases the affinity of the $\alpha_5\beta_1$ integrin for its natural ligand, fibronectin (21). In addition, binding of β_3 integrins to their ligands induces conformational changes within the subunit, exposing new epitopes, defined by their ability to bind certain MAbs. These new epitopes are known as ligand-induced binding sites (LIBS). The conformational changes that expose the LIBS can increase the affinity of the β_3 receptor for its ligand (20). Mapping of a number of LIBS has shown that some of them reside within the cysteine-rich repeat region (32).

The experiments reported here are indirect measures of receptor utilization and have not addressed whether the bovine receptor has a higher affinity for the virus or whether the C-terminal region of the ectodomain may be mediating an event subsequent to adsorption, either penetration or eclipse. However, an examination of virus binding in relation to the level of viral protein synthesis observed in transfected-infected cells indicated that cells transfected with bovine subunit cDNA adsorbed higher levels of type A_{12} virus than did cells transfected with human integrin cDNAs (data not shown). Furthermore, analysis of infected cells by IHC using a virus-specific MAb showed that increased numbers of infected cells were present in cultures transfected with the bovine β_3 subunit (not shown).

Finally, it is interesting to speculate, from the standpoint of receptor utilization, why foot-and-mouth disease is limited to cloven-hoofed animals. Results from this and previous studies have shown that FMDV can utilize human and simian $\alpha_v\beta_3$ as a receptor in cell culture (11, 47). However, it is quite clear that the virus replicates to a greater extent in cells expressing the bovine integrin, specifically the cysteine-rich repeat region of the β_3 subunit of that integrin, which has a high degree of structural conservation among all β subunits (30). Thus, it is possible that FMDV evolved into a disease of cloven-hoofed livestock because the structure of their $\alpha_v\beta_3$ receptors resulted in a more advantageous "fit" with the viral surface that would, in turn, lead to much greater viral replication and disease within these species. However, since this integrin probably performs similar functions in a wide variety of species, the structural differences cannot be radically different, as evidenced by the high degree of sequence similarity between the bovine and human integrins. It is also important to note that receptors alone may not necessarily determine FMDV species tropism. Recent results have shown that a type O virus, isolated from an outbreak which occurred only in swine in Taiwan in 1997, contained a deletion in nonstructural protein 3A, which led to restricted growth in bovine cells and attenuation in cattle (9). In the case of poliovirus, which causes disease only in primates, a murine homolog of the poliovirus receptor has been found and is unable to bind the virus (46). This inactivity of the murine homolog has been mapped to a few amino acid differences with the human homolog in the first immunoglobulin domain of the receptor (2, 31). We have transfected COS-1 cells with the murine β_3 subunit (a kind gift from Erich Mackow and Eric Brown) and have found that viral protein synthesis was comparable to that seen with the human receptor (data not shown). We are currently cloning the porcine α_v and β_3 subunits, and it will be interesting to see whether the

changes within the β_3 cysteine-rich region are similar those seen in the bovine integrin.

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