β-h3 is a TGF-β-induced matrix protein known to mediate the adhesion of several cell types. In this study, we found that all four of the fas-1 domains in β-h3 mediate MRC-5 fibroblast adhesion and that this was specifically inhibited by a function-blocking monoclonal antibody specific for the αvβ5 integrin. Using deletion mutants of the fourth fas-1 domain revealed that the MRC-5 cell adhesion motif (denoted the YH motif) is located in amino acids 548–614. Experiments with substitution mutants showed that tyrosine 571, histidine 572, and their flanking leucine and isoleucine amino acids, which are all highly conserved in many fas-1 domains, are essential for mediating MRC-5 cell adhesion.

A synthetic 18-amino acid peptide encompassing these conserved amino acids could effectively block MRC-5 cell adhesion to β-h3. Using HEK293 cells stably transfected with the β integrin cDNA, we confirmed that the αvβ5 integrin is a functional receptor for the YH motif. In conclusion, we have identified a new αvβ5 integrin-interacting motif that is highly conserved in the fas-1 domains of many proteins. This suggests that fas-1 domain-containing proteins may perform their biological functions by interacting with integrins.

β-h3 is an extracellular matrix protein whose expression in several cell types, including fibroblasts, is strongly induced by TGF-β. The gene encoding β-h3 was first identified by Skonier et al. (1), who isolated it by screening a cDNA library made from a human lung adenocarcinoma cell line (A549) that had been treated with TGF-β. The β-h3 protein comprises 683 amino acids containing four homologous internal repeat domains. These domains are homologous to similar motifs in the Drosophila protein fasciclin-1 and thus are denoted fas-1 domains. The fas-1 domain has highly conserved sequences found in secretory and membrane proteins of several species, including mammals, insects, sea urchins, plants, yeast, and bacteria (2).

Mutations in β-h3 have been shown to be responsible for 5q31-linked human autosomal dominant corneal dystrophies. It has a fibrillar structure and interacts with several extracellular matrix proteins such as fibronectin and collagen (3). In addition, β-h3 has been reported to be involved in cell growth and differentiation, wound healing, and cell adhesion (4–9). β-h3 mediates the adhesion of many different cell types, including corneal epithelial cells, chondrocytes, and fibroblasts (8–10). We reported previously that β-h3 mediates corneal epithelial cell adhesion by binding to α3β1 integrin. Two motifs interacting with the α3β1 integrin were located within the second and the fourth fas-1 domains of β-h3. Interestingly, however, we found that these two motifs are not involved in β-h3-mediated fibroblastic cell adhesion. Furthermore, all four fas-1 domains of β-h3 mediate fibroblastic cell adhesion, whereas corneal epithelial cell adhesion is supported by just the second and the fourth fas-1 domains. This suggests that β-h3 has additional motifs that can mediate the adhesion of other cell types. Confirming this, we show in the present study that β-h3 has a motif that promotes fibroblastic cell adhesion by interacting with the αvβ5 integrin on the fibroblast cell surface. This motif is well conserved in many fas-1 domains found in various proteins from different species. Together with our previous findings, these observations suggest that β-h3 mediates cell functions through multiple cell-adhesion motifs that interact with different integrins on various cell types. This also suggests that other fas-1-containing proteins could regulate cell functions by interacting with integrins.

EXPERIMENTAL PROCEDURES

DNA Constructs—Bacterial expression vectors for the wild-type βig-h3 and each fas-1 domain have been described previously (10). βig-h3 n-IV cDNA that encodes amino acids 498–637 (the fourth fas-1 domain) was used to clone several deletion mutant constructs, as follows. Several cDNAs encoding fragments of βig-h3, namely, amino acids 548–637, 498–620, 498–614, 548–620, 548–614, 569–637, and 573–637, were generated by PCR using βig-h3 D-IV cDNA as template. These fragments were cloned into the EcoRV/XhoI sites of the pET-29b(+) vector (Novagen) and were denoted as ΔH1, ΔH2, ΔH2 (6), ΔH1H2, ΔH1H2 (6), 569, and 575–637, respectively. Substitution mutants in which conserved tyrosine, histidine, leucine and isoleucine residues in βig-h3 D-IV were substituted by alanine or serine, were generated by two-step PCR as described previously (11). The mutations were confirmed by DNA sequencing. Recombinant β-h3 protein was induced and purified as described previously (6).

Cell Culture—MRC-5 (human lung fibroblast) cells were cultured at 37 °C in 5% CO2 in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum. Human embryonic kidney (HEK293) cells cotransfected with an empty vector (pCDNA3) or a human integrin β5 expression vector were donated by Dr. Jeffrey Smith (Burnham Institute, San Diego, CA). These stable cell lines were cultured in ...
Identification of the βg-h3 Motif Mediating MRC-5 Cell Adhesion —To identify the αβ5 integrin-interacting motifs in βg-h3, we made five N-terminal- and C-terminal-deleted recombinant proteins of the fourth fas-1 domain, which consists of amino acids 498–637. The proteins were denoted ΔH1, ΔH2, ΔH2 (6), ΔH1H2, and ΔH1H2 (6) (Fig. 2A). ΔH1 and ΔH2 represent the fourth fas-1 domain lacking one of two conserved fas-1 regions known as H1 (amino acids 498–547) and H2 (amino acids 621–637). ΔH1H2 lacks both H1 and H2. ΔH2 (6) and ΔH1H2 (6) are ΔH2 and ΔH1H2 that lack in addition the C-terminal amino acids containing the αβ1 integrin-interacting motif EPDIM. We tested the ability of each protein to promote MRC-5 cell adhesion (Fig. 2A). All, including the smallest mutant protein, ΔH1H2 (6), could mediate MRC-5 cell adhesion (Fig. 2A). Thus, H1 and H2 are not involved and the cell adhesion motif is present within amino acids 548–614.

To further identify the cell-adhesion motif within this fragment, we performed a computer search using Prodom 2001.2 searching for homologies not only among the four βg-h3 domains but also among the fas-1 domains from other proteins. Interestingly, many fas-1 domains, including the four fas-1 domains of βg-h3, contain two amino acid residues, a tyrosine and a histidine, in the domain center (Fig. 2B). In the fourth domain of βg-h3, the tyrosine and the histidine are amino acids 571 and 572, respectively. We assessed whether the tyrosine and histidine are needed for MRC-5 cell adhesion, first by using the two deleted proteins βg-h3 D-IV-569 and βg-h3 D-IV-575. These proteins encode amino acids 569–632 and 575–632, respectively, and thus βg-h3 D-IV-575 lacks the tyrosine and histidine residues. As shown in Fig. 2C, the mutant proteins were both significantly less able to mediate MRC-5 cell adhesion compared with the ΔH1, ΔH2, ΔH2 (6), ΔH1H2, and ΔH1H2 (6) proteins tested earlier (Fig. 2A), suggesting the potential importance of residues 548–569. However, the ability of D-IV-575 to promote MRC-5 cell adhesion was less than that of βg-h3 D-IV-569. Thus, tyrosine 571 and histidine 572 may play an essential role in the cell-adhesion activity of βg-h3. To confirm this, we generated substitution mutant βg-h3 proteins whose tyrosine 571 and/or histidine 571 residues were replaced with alanine (Fig. 2C). Unexpectedly, the single point mutations did not significantly affect cell-adhesion activity. However, the double point mutation significantly inhibited the cell adhesion activity of βg-h3, although not completely. Thus, tyrosine 571 and histidine 572 are clearly required in βg-h3-mediated MRC-5 cell adhesion. However, flanking amino acids may also be needed.

An 18-Amino Acid Sequence Containing the Tyrosine and Histidine Is Required in βg-h3-mediated MRC-5 Cell Adhesion —To confirm that tyrosine 571 and histidine 572 are necessary for MRC-5 cell adhesion, a synthetic peptide corresponding to amino acids 569–574 (YH6) was generated and used to compete cell adhesion to βg-h3. However, this peptide could not inhibit MRC-5 cell adhesion to βg-h3. We then used the larger YH10 peptide corresponding to amino acids 567–576 but it also could not inhibit cell adhesion. However, when we tested YH18, which corresponds to amino acids 563–580, MRC-5 cell adhesion to βg-h3 was almost completely abolished. The control peptide YH18-con, which contains the amino acids of YH18 but in a scrambled sequence, did not affect cell adhesion to βg-h3 (Fig. 3A). We obtained similar results when each of the four fas-1 domains was used as the substrate (Fig. 3B). The competition of cell adhesion by the YH18 peptide was dose-dependent (Fig. 3C). To confirm that the YH18-like sequences of the other three fas-1 domains of βg-h3 are also effective in competing MRC-5 cell adhesion to βg-h3, we synthesized three peptides representing these sequences. As shown in Fig. 3D,
these peptides also inhibited MRC-5 cell adhesion to βig-h3 in a dose-dependent manner. Whereas peptides from D-I, -II, and -III regions showed similar inhibitory activity, the peptide from the D-IV showed slightly higher activity compared with the others. Although all of these peptides were effective in the micromolar range (D-IV peptide inhibited 50% cell adhesion at 200 μM), they displayed far less activity compared with the recombinant domain IV protein which inhibited cell adhesion by 50% between 5 μM and 10 μM (Fig. 3E). This suggests that, although a region of 18 amino acids comprises the basic element required for minimal activity, longer stretches are required for full activity. Taken together, these results suggest that the YH18-like sequences encompassing the conserved tyrosine and histidine residues are essential for MRC-5 cell adhesion, confirming that not only the tyrosine and histidine but also flanking amino acids are minimally required for βig-h3-mediated MRC-5 cell adhesion.

Leucine and Isoleucine Residues Flanking the Tyrosine and Histidine Are Also Required in βig-h3-mediated MRC-5 Cell Adhesion—Because the leucine and isoleucine residues that flank the tyrosine and histidine are also well conserved among the fas-1 domains from different proteins (Fig. 4A), we examined their role in βig-h3-mediated MRC-5 cell adhesion. We constructed a number of substitution mutants of βig-h3 whose tyrosine, histidine, leucine, and isoleucine residues were substituted in various combinations by serine (leucine and isoleucine) or alanine (tyrosine and histidine) (Fig. 4B). These mutants were then used in cell adhesion assays. D-IV-L represents the fourth fas-1 domain whose leucine 565, isoleucine 568, and leucine 569 were replaced with serine. D-IV-R represents...
fourth fas-1 domain whose isoleucine 573, isoleucine 577, and leucine 578 were replaced with serine. D-IV-LAA and D-IV-LAR represents D-IV-L and D-IV-R whose tyrosine 571 and histidine 572 were replaced with alanine. D-IV-LYHR represents the fourth fas-1 domain whose conserved amino acids mentioned above were replaced with serine or alanine. Although D-IV-L, D-IV-R, D-IV-LAA, D-IV-LAR, and D-IV-LYHR show less cell-adhesion activity compared with the fourth fas-1 domain, they still promote some cell adhesion (Fig. 4B). In contrast, the D-IV-LAR mutant, where all conserved amino acids had been substituted, was very poor in mediating MRC-5 cell adhesion. Thus, multiple amino acids are required to form a conformation that mediates MRC-5 cell adhesion to βh3.

The αβ5 Integrin Is a Functional Receptor for βh3—To confirm that βh3 mediates cell adhesion through the αβ5 integrin, we used stably transfected HEK293 cells (β5/293) that express human β5 integrin. HEK293 cells do not normally express the β5 integrin although they do express many β1 integrins, including α1β1, α2β1, α5β1, α6β1, and αvβ1 (12). We confirmed that β5/293 cells express the αβ5 integrin by FACS analysis (data not shown). β5/293 cells strongly adhered to surfaces coated with βh3-WT or vitronectin, unlike pc/293 cells, which are HEK293 cells stably transfected with an empty vector (Fig. 5A). β5/293 cell adhesion to βh3 was specifically inhibited by an antibody recognizing the αβ5 integrin (Fig. 5B) and by the YII18 peptide (Fig. 5C). These results confirm that βh3 mediates cell adhesion by interacting with the αβ5 integrin.

**DISCUSSION**

We previously identified two α3β1 integrin-interacting motifs that are required for βh3-mediated adhesion of corneal epithelial cells (10). However, these motifs are not involved in the βh3-mediated adhesion of mesenchymal cells, including fibroblasts, vascular smooth muscle cells, and osteoblasts. This observation led us to seek the motifs in βh3 that are involved in the βh3-mediated adhesion of fibroblasts. Here we report a new βh3 motif that interacts with the αβ5 integrin and is necessary for the βh3-mediated adhesion of fibroblasts.

Unlike corneal epithelial cells, MRC-5 fibroblasts adhere to all four fas-1 domains of βh3. Furthermore, this adhesion was not blocked by the presence of the α3β1 integrin-interacting peptides EPDIM and NKDL. Thus, MRC-5 cells must adhere to βh3 through an integrin other than the α3β1 integrin. We used several function-blocking integrin-specific antibodies and found that only anti-αβ5 integrin antibody could eliminate MRC-5 cell adhesion to βh3 and the fas-1 domains. We confirmed that MRC-5 cells express the αβ5 integrin on their cell surface. Ohno et al. (9) previously reported that βh3 mediates MRC-5 fibroblast cell adhesion through the α1β1 integrin. However, we consistently found that α1 and β1 integrin-function blocking antibodies did not significantly block MRC-5 cell adhesion to βh3. We confirmed that α1 and β1 integrin function-blocking antibodies used effectively inhibited the adhesion of several cell lines to specific ligands including collagen and fibronectin (data not shown). Thus, it is very unlikely that this discrepancy is due to using different antibodies. Unfortunately, they did not test whether αβ5 function-blocking antibodies could block MRC-5 cell adhesion to βh3.
blocking antibody could inhibit MRC-5 cell adhesion to \( \beta \)-h3. Thus, we are as yet unable to explain the discrepancy clearly.

Interestingly, even though MRC-5 cells and corneal epithelial cells (10) express both the \( \beta \)-h3 and \( \alpha \)-h5 integrins, the two cell lines use only one but not the same integrin type for adhesion to \( \beta \)-h3 (this report and Ref. 10). The choice of the integrin used may depend on the activation state of the integrins in each cell line. There are a variety of intracellular cues that cause cells to modulate the affinity and avidity of their integrins for their extracellular matrix ligands. For example, R-Ras and H-Ras are known to regulate cell adhesion by modulating the affinity of multiple integrins for the extracellular matrix (13, 14). Furthermore, phosphorylation of the cytoplasmic domain of integrins alters the conformation of the integrins and thereby changes their affinity for their ligands (15). We are currently engaged in investigating why MRC-5 and corneal epithelial cells use different integrin types to interact with \( \beta \)-h3.

The finding that the anti-\( \alpha \)-h5 integrin antibody blocks the ability of all the fas-1 domains in \( \beta \)-h3 to mediate MRC-5 cell adhesion suggests that each domain has a motif that interacts with the \( \alpha \)-h5 integrin. We investigated this issue by constructing a series of deletion mutants of the fourth fas-1 domain and assessing their ability to promote MRC-5 cell adhesion. Two regions denoted H1 and H2 are highly conserved in each of the fas-1 domains, but deletion of either or both of these regions did not affect cell adhesion. Because all four fas-1 domains mediate cell adhesion through the same integrin type, it is likely that they all carry the same conserved consensus sequence that facilitates this interaction. We performed sequence analysis of the fas-1 domains of \( \beta \)-h3 and other fas-1-containing proteins and found a tyrosine, a histidine, and many leucine/isoleucine residues flanking the tyrosine and histidine, which are highly conserved. Experiments with deletion and substitution mutants revealed that a motif containing these amino acid residues is essential for the ability of \( \beta \)-h3 to mediate MRC-5 cell adhesion, indicating they are part of the \( \alpha \)-h5 integrin-binding motif in \( \beta \)-h3. Unlike the \( \alpha \)-h5 integrin-interacting motifs NKDIL and EPDIM in \( \beta \)-h3, the cell-adhesion activity of this new motif does not depend on a few crucial amino acid residues because single point mutations of tyrosine and histidine did not significantly affect the cell adhesion activity of \( \beta \)-h3. Even substitution mutations of several of the conserved tyrosine, histidine, leucine, and isoleucine residues could not completely abolish the cell adhesion activity of \( \beta \)-h3. Even substitution mutations of several of the conserved tyrosine, histidine, leucine, and isoleucine residues could not completely abolish the cell adhesion activity of \( \beta \)-h3. 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Even substitution mutations of several of the conserved tyrosine, histidine, leucine, and isoleucine residues could not completely abol
Furthermore, although a peptide of 18 amino acids had activity, it was much less active compared with the recombinant domain IV protein, suggesting that the region of 18 amino acids only comprises the minimal element required for the cell adhesion activity of the fas-1 domain. Amino acids flanking this minimally conserved region could possibly contribute to the conformation of the integrin-binding site, thereby enhancing its activity. It is, however, very hard to predict which amino acids are critical because we could not find any conserved amino acids other than tyrosine, histidine, and leucine/isoleucine among several fas-1 domains although the computer analysis showed a few weakly conserved aliphatic or hydrophobic amino acids were present around the conserved tyrosine and histidine residues. Thus, although the 18-amino acid region comprising the conserved histidine, tyrosine, and leucine/isoleucine residues contains the element required for the cell adhesion activity of the fas-1 domain. Amino acids flanking this minimally conserved region could possibly contribute to the conformation of the integrin-binding site, thereby enhancing its activity. It is, however, very hard to predict which amino acids are critical because we could not find any conserved amino acids other than tyrosine, histidine, and leucine/isoleucine among several fas-1 domains although the computer analysis showed a few weakly conserved aliphatic or hydrophobic amino acids were present around the conserved tyrosine and histidine residues. Thus, although the 18-amino acid region comprising the conserved histidine, tyrosine, and leucine/isoleucine residues contains the

![Image](https://via.placeholder.com/150)

**Fig. 4.** The leucine and isoleucine residues flanking the tyrosine and histidine are also required to mediate MRC-5 cell adhesion to βig-h3. A, comparison of fas-1 domains from several proteins revealing that there are conserved leucine and isoleucine residues flanking the tyrosine and histidine. A computer search was performed using Prodom 2001.2. The conserved leucine/isoleucine residues are shown in bold. BIGOH3, Transforming growth factor beta-induced gene product; OSSF2, osteoblast-specific factor 2; SLL 1735, Cyanobacterium Synechocystis secreted protein MPB70 (a hypothetical 13.9-kDa protein); SLL1483, Cyanobacterium Synechocystis transforming growth factor-induced protein; Midline Fasciclin; Drosophila midline fasciclin; HLC-32, a 32-kDa component of the hyalin layer in the sea urchin. B, effect of substituting the leucine and isoleucine residues as well as the tyrosine and histidine on the cell-adhesion activity of βig-h3. The leucine and isoleucine residues were substituted with serine, and tyrosine and histidine were substituted with alanine. Mutated amino acids are shown in bold. The cell-adhesion assay was then performed.

![Image](https://via.placeholder.com/150)

**Fig. 5.** αvβ5 integrin is a functional receptor for βig-h3. A, adhesion to βig-h3 or vitronectin (VN) by HEK293 cells stably transfected with β5 integrin expression DNA. Plates were coated with BSA, βig-h3-WT or VN, incubated with the cells, and the cells attached to the surface were then quantified. B, the effects of function-blocking integrin antibodies on β5/293 cell adhesion to βig-h3-WT protein. Cells were preincubated with the following function-blocking monoclonal antibodies specific for integrin subunits and then added to the βig-h3-WT precoated wells: α1, FB12; α3, P1B5; αv, P3G8; β1, 6S6; and αvβ5, 1F6. After incubation, the cells attached to the substrates were quantified. C, inhibition of β5/293 cell adhesion to the βig-h3-WT protein by the YH18 peptide. β5/293 cells were preincubated with or without 300 μM synthetic YH18 peptide. After incubation, the cells attached to βig-h3-WT were quantified.
Motifs of βig-h3 Interacting with αββ5 Integrin

basic requirements for cell adhesion, a more extensive region is required for full potentiation of this activity.

The αvβ5 integrin is thought to bind to a number of extracellular matrix proteins, including vitronectin and osteopontin (16, 17). Binding to these ligands appears to be RGD-dependent because peptides containing this tripeptide motif inhibit αvβ5 integrin binding. Although the sequence of the αvβ5 integrin-binding motif contained in YH18 differs from the RGD motif, both peptides may recognize the same site in the αvβ5 integrin because MRC-5 cell adhesion to βig-h3 is inhibited by the RGD peptide (data not shown). Furthermore, MRC-5 cell adhesion to vitronectin is also inhibited by the YH18 peptide (data not shown). Alternatively, the binding of one αvβ5 integrin site may alter the conformation of the other site, thereby inhibiting the interaction.

Because the αvβ3 integrin also interacts with vitronectin and osteopontin in a RGD-dependent manner (18, 19), it is possible that the YH18 peptide could also bind to the αvβ3 integrin. We could not test this notion in the study reported here because MRC-5 cells do not express the αvβ3 integrin. However, it is possible that βig-h3 could interact with the αvβ3 integrin through the YH motif because we have found that adhesion to βig-h3 of some cell types depends both on the YH motif and the αvβ3 integrin (data not shown). In this regard, it is noteworthy that βig-h3 is expressed by endothelial cells and vascular smooth muscle cells (20), both of which are known to use the αvβ3 and αvβ5 integrins for their biological responses to a variety of conditions such as angiogenesis (21) and intimal hyperplasia (22).

In humans, four proteins are known to bear fas-1 domains. βig-h3 and peristin (23) are secretory proteins with four fas-1 domains, and stabilin-1 and stabilin-2 are membrane proteins with seven fas-1 domains (24). Although the biological functions of these proteins are not comprehensively understood, it is possible that all may function in regulating cell-matrix interactions and cell-cell interactions, because all of the fas-1 domains bear potential integrin-interacting YH motifs. These four human proteins are unique among the integrin-interacting proteins in that they bear multiple repeat integrin-interacting motifs. None of the other molecules known to interact with integrins has multiple repeats of motifs that interact with one integrin type. It is of interest to determine the biological meaning of this unique feature of the human fas-1 domain-bearing proteins.

In conclusion, we have identified a new cell-adhesion motif that interacts with the αvβ5 integrin. This motif is present in each of the fas-1 repeat domains of βig-h3 and consists of tyrosine and histidine flanked by several leucine/isoleucine residues. The fact that this motif is well conserved in most of the fas-1 domains present in many disparate proteins suggests that fas-1 domain-containing proteins may regulate various cell functions by interacting with integrins.

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