Identification of Motifs for Cell Adhesion within the Repeated Domains of Transforming Growth Factor-β-induced Gene, βig-h3*

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βig-h3 is a transforming growth factor-β-inducible cell adhesion molecule that has four characteristic homologous repeated domains. We made recombinant βig-h3 proteins, which were highly active in mediating human corneal epithelial (HCE) cell adhesion and spreading. The 2nd and the 4th repeated domains were sufficient to mediate HCE cell adhesion. A sequence analysis showed that aspartic acid (Asp) and isoleucine (Ile) of the 2nd and the 4th domains are highly conserved in many fasciclin 1 homologous (fas-1) domains. Substitution mutational study identified these two amino acids are essential for cell adhesion. Synthetic peptides containing Asp and Ile, NKDIL and EPDIM derived from the 2nd and the 4th domains, respectively, almost completely blocked cell adhesion mediated by not only wild type βig-h3 but also each of the 2nd and the 4th domains. These peptides alone were fully active in mediating cell adhesion. In addition, we demonstrated the functional receptor for βig-h3 is α3β1 integrin. These results, therefore, establish the essential motifs within the 2nd and the 4th domains of βig-h3, which interact with α3β1 integrin to mediate HCE cell adhesion to βig-h3 and suggest that other proteins containing Asp-Ile in their fas-1 domains could possibly function as cell adhesion molecules.

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EXPERIMENTAL PROCEDURES

Generation of Recombinant big-h3 Proteins—Expression plasmids for recombinant big-h3 proteins, big-h3-wild type (WT), and big-h3-ΔRGD were described in the previous report (3) where big-h3-WT and big-h3-ΔRGD were named Hisβ-ε and Hisβ-ε respectively. Each fragment of big-h3 cDNA, encoding amino acids 129–241, 237–377, 368–506, and 498–637, was generated by polymerase chain reaction and cloned into the EcoRV and XhoI sites of pET-28b, named big-h3 D-I, D-II, D-III, and D-IV, respectively. Substitution mutants of big-h3 D-IV were generated by a two-step polymerase chain reaction as described previously (13). The DNA sequences of all mutants were verified. Recombinant big-h3 proteins were induced and purified as described previously (3).

Cell Culture—HCE cells were cultured in Dulbecco’s modified Eagle’s medium with Nutrient Mixture F-12 (Life Technologies, Inc.) supplemented with 15% fetal bovine serum, gentamicin (40 μg/ml), 5 μg/ml insulin, 0.1 μg/ml cholesterol toxin, and 10 ng/ml human epidermal growth factor at 37 °C in 5% CO2. The human erythrocytic cell line, K562, was grown in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and antibiotics.

Cell Adhesion and Spreading Assay—The cell adhesion assay was performed as described previously (14) (4), most often with 96-well microculture plates (Falcon, Becton-Dickinson, Mountain View, CA) were incubated at 37 °C for 1 h and then blocked with PBS containing 0.2% BSA for 1 h at 37 °C. The coated extracellular matrix proteins used were as follows: human plasma vitronectin (Promega), purified human plasma fibronectin (pFN), chicken collagen types I and II (Chemicon International Inc., Temecula, CA), bovine collagen types IV and VI (Chemicon, mouse laminin (Chemicon), and bovine serum albumin (BSA) (Sigma). Cells were trypsinized and suspended in the culture media at a density of 2 × 10^5 cells/ml, and 0.1 ml of the cell suspension was then added to each well of the plates. Cell attachment was analyzed as follows. After incubation for 1 h at 37 °C, unattached cells were removed by rinsing twice with PBS. Attached cells were incubated for 1 h at 37 °C in 50 ml culture medium containing 0.5% nitrophenyl-N-acetyl β-D-glucosaminide (hexosaminidase substrate) and 0.25% Triton X-100. Enzyme activity was blocked by the addition of 50 ml glycine buffer, pH 10.4, containing 5 mM EDTA, and the absorbance was measured at 405 nm in a Multiscan MCC/3420 microplate reader (Titertek Instruments, Inc., Huntville, AL). To determine cell area, 4 × 10^5 cells were applied to substrates in 48-well culture plates. The attached cells were fixed with 8% glutaraldehyde (Sigma) and then stained with 0.25% Crystal Violet (Sigma) in 20% methanol (w/v). Cell area was measured using Image-Pro plus software (Media Cybernetics, Silver Spring, MD).

Experiments were repeated in triplicate with 200 or 300 measurements per site for each experiment. Data are reported as the mean area at each measurement point ± S.E.

Inhibition Assay—Various reagents and synthetic peptides were examined for their ability to prevent cells from adhering to the prepared substrata. Synthetic peptides were synthesized on an automated multiple peptide synthesizer (PE/ABD 433, PE Corp., Norwalk, CT) using standard solid phase procedures. Peptides were purified by reverse phase high performance liquid chromatography. Cell adhesion assay was done as described above. To analyze the divalent cation sensitivity of big-h3-mediated adhesion, cells were suspended at 2 × 10^5 cells/ml Heps-buffered saline (HBS), 150 mM NaCl, 25 mM Heps, pH 7.4, and incubated at 37 °C for 30 min. They were then washed twice in HBS and resuspended in the same buffer. Aliquots of cells (50 μl) were then added to the microculture plate wells and incubated with 50-μl aliquots of HBS containing 1 μM MnCl2, MgCl2, or CaCl2 for 30 min at 37 °C in a humidified atmosphere of 5% CO2. They were then plated on ligand-coated dishes to perform the adhesion assays, as described above. To identify the receptor for big-h3, monoclonal antibodies to different types of integrins (Chemicon) were preincubated individually with HCE in 0.05 ml of incubation solution (2 × 10^5 cells/ml) at 37 °C for 30 min. The preincubated cells were transferred onto plates precoated with big-h3 proteins and then incubated further for 1 h at 37 °C. Attached cells were then quantitated as described above.

Flow Cytometry—For flow cytometry analysis, cells at confluence were detached by gentle treatment with 0.25% trypsin, 0.05% EDTA in PBS, washed, and incubated with the antibodies for 1 h at 4 °C. Cells were then incubated with 10 μg/ml affinity purified fluorescein-labeled secondary antibodies for 1 h at 4 °C and analyzed on the flow cytometer FACScalibur system (Becton Dickinson, San Jose, CA) equipped with a 5-watt argon laser at 488 nm.

Immunoprecipitation—HCE cells or αs-transfected HCE cells were solubilized in 200 mM n-octyl-β-D-glucopyranoside, 1 mM phenylmethylsulfonyl fluoride, 100 mM Tris-HCl, pH 7.4. Immunoprecipitations were carried out by overnight incubation at 4 °C of the immunoadsorbents (antibodies adsorbed onto protein A-Sepharose (Amersham Pharmacia Biotech) with samples of cell lysates. Precipitated proteins were separated on 10% SDS-polyacrylamide gel. After separation of precipitated proteins by SDS-PAGE and transfer to a nitrocellulose membrane (Schleicher & Schuell), blots were incubated for 2 h with either anti-αs or anti-big-h3 polyclonal antibodies, then detected using horseradish peroxidase-conjugated anti-rabbit IgG antibodies (Sigma), followed by enhanced chemiluminescence (ECL) system (NEN Life Science Products).

Antibodies—Anti-integrin monoclonal antibodies utilized were α1 (Fb12), α2 (ASC-1), α3 (PH14), αβ3 (HA6), α5 (CLB701), α6 (PG8), αβ5 (LM609), αβ1 (P1F6), and β1 (12G10) from Chemicon. Polyclonal rabbit anti-α1 (AB1948) antisemur and affinity purified fluorescein-labeled secondary antibodies were also purchased from Chemicon. Polyclonal anti-β3 antisemur against recombinant big-h3 protein was generated in rabbit and described previously (15).

Construction of αs cDNA Expression Vector and Transfection—Full-length cDNA for the human αs subunit (clone 3.10) was purchased from the American Type Culture Collection. A fragment (3.47 kilobases) containing the entire cDNA excised by digestion with XbaI/SalI was cloned into the EcoRI site of the mammalian expression vector pcDNA 3.1+ (Invitrogen, Carlsbad, CA). The αs integrin expression plasmid DNA (1 μg) was transfected into HCE cells using the LipofectAMINE (Life Technologies, Inc.).

RESULTS

β-h3 Supports Cell Adhesion and Spreading Independent of the RGD Motif—For cell adhesion assay, we used two recombinant big-h3 proteins that have been described previously (3). We changed the nomenclature from Hisβ-ε and Hisβ-ε as used in the previous paper (3) to big-h3-WT and big-h3-ΔRGD, respectively (Fig. 1A). β-h3 was previously demonstrated to support the adhesion and spreading of fibroblasts (5, 6), and the RGD motif was proposed not to be necessary for such activity (6). The numbers and surface areas of HCE cells that adhered to big-h3-WT were clearly greater than those that attached to albumin and were comparable to those of cells that adhered to fibronectin (Fig. 1B). The cell adhesion and spreading activities of β-h3 were concentration-dependent (Fig. 1, C and D). Similar results were also obtained with Chinese hamster ovary cells (data not shown). As expected, big-h3-ΔRGD lacking the RGD motif was almost equally effective at supporting cell adhesion and spreading (Fig. 2, A and B). These results confirm that big-h3 supports cell adhesion and spreading independent of the RGD motif.

Cell Adhesion to big-h3 Is Dependent on Integrin αβ3β1 and Divalent Cations—To identify the nature of the cell surface receptor for big-h3, several reagents were used. Cell adhesion to big-h3 was significantly inhibited by big-h3 itself, RGD peptide, and EDTA, and it was partially inhibited by fibronectin and EGTA but not inhibited by RGE peptide. Cell adhesion to fibronectin was also significantly inhibited by fibronectin itself, RGD peptide, and EDTA and partially inhibited by big-h3 and EGTA but not by RGE peptide (Fig. 3A). Then we examined the effects of Mn2+, Mg2+, and Ca2+ on big-h3-mediated cell adhesion. Cell adhesion to big-h3 was strongly promoted by Mn2+, and to a lesser extent by Mg2+, but only marginally by Ca2+ (Fig. 3B). These results suggest that the cell surface receptor for big-h3 could be one of the RGD-dependent integrins, which require divalent cations for interaction with ligands.

To identify the big-h3 receptor, the effects of function-blocking monoclonal antibodies to integrin subunits were examined on the adhesion of HCE cells to the surface coated with big-h3. Adhesion to the big-h3-coated surface was specifically inhibited by antibody to αs subunit but not by antibodies to other α subunits (Fig. 3C). Because the integrin αs subunit is known to
couple with the integrin β₁ subunit, anti-β₁ antibody was also expected to inhibit cell adhesion to β₁h₃. As expected, anti-β₁ antibody significantly blocked cell adhesion (Fig. 3C). To determine whether β₁h₃ interacts with α₃ integrin, we carried out co-immunoprecipitation assays. Immunoblotting with anti-β₁h₃ antiserum showed that β₁h₃ was detected in immunoprecipitates formed by anti-α₃ integrin antibody (Fig. 3D, lanes 2–4). Conversely, α₃ integrin was also detected in immunoprecipitates formed by anti-β₁h₃ antiserum (Fig. 3D, lanes 6–8). α₃ integrin was detected in all four cell lysates (Fig. 3D, lanes 1–4) among which α₃ overexpressed cell lysate showed the highest amount of α₃ integrin in immune complex. Because the basal expression level of β₁h₃ in cell lysate is very low, it was barely detected in immune complex precipitated by anti-β₁h₃ antiserum (Fig. 3D, lane 5). However, when we added recombinant β₁h₃ protein to the culture medium (Fig. 3D, lanes 6 and 8) or to cell lysate (Fig. 3D, lane 7), it was detected in immunoprecipitates. Nothing was detected in immunoprecipitates with nonimmune rabbit serum (data not shown). To determine which integrins are expressed in HCE cell, we performed fluorescence-activated cell sorter analysis. Fig. 4 shows that all the integrins tested were detected on the HCE cell surface although their expression levels varied. The expression level of α₃ integrin was relatively high, whereas that of α₅β₁ was low. All the others had similar expression levels. Taken together, these results suggest integrin α₃β₁ is a specific functional receptor for β₁h₃ in HCE cells.

Each of the 2nd Fas-1 Domains and the 4th Fas-1 Domain Is Sufficient to Mediate Cell Adhesion—In an attempt to identify essential amino acid residues conferring cell adhesion activity of β₁h₃, we first tested whether each repeated domain is capable of mediating cell adhesion. We made four recombinant proteins corresponding to each repeated domain (Fig. 5, A and B) and tested their cell adhesion activities. We found that the 2nd fas-1 domain and the 4th fas-1 domain were equally active compared with the wild type β₁h₃, whereas the 1st fas-1 domain was moderately active and the 3rd fas-1 domain was very weakly active (Fig. 5C). Both 2nd fas-1 and 4th fas-1 domain-mediated cell adhesions were almost blocked by antibodies to α₃ and β₁ integrin subunits (Fig. 5D) suggesting that both 2nd fas-1 and 4th fas-1 domains have essential amino acid residues for interacting with integrin. These results also support that neither H1 nor H2 is mediating cell adhesion activity of β₁h₃ because the 1st and the 3rd domains are not active in cell adhesion although they have H1 or H2.

Two Conserved Amino Acids, Aspartic Acid and Isoleucine, Are Essential for Cell Adhesion—To identify cell adhesion motifs within the 2nd and the 4th domains, we performed a computer search using ProDom Release 99.2 based on homologies not only among the repeated fas-1 domains of β₁h₃ but also among fas-1 domains of other proteins. In many fas-1 domains including the 2nd and the 4th fas-1 domains of β₁h₃, two amino acid residues, aspartic acid and isoleucine near H2, are highly conserved, whereas in some cases including the 1st fas-1 domain of β₁h₃, only aspartic acid is conserved (Fig. 6). Although not shown here, some fas-1 domains such as the 3rd fas-1 domain of β₁h₃ do not have aspartic acid near H2. In order to examine the sequence containing aspartic acid that is needed for cell adhesion, we generated mutated fas-1 proteins of β₁h₃ where each 616 proline, 617 aspartic acid, and 618 isoleucine was replaced with serine, alanine, and serine, respectively (Fig. 7, A and B). D617A (β₁h₃ D-IV-PaI) and I618S (β₁h₃ D-IV-PaI) mutations significantly blocked cell adhesion whereas P616S (β₁h₃ D-IV-sDI) mutation did not affect cell adhesion. Consequently, three amino acids mutation, P616S/D617A/I618S (β₁h₃ D-IV-sDI) also blocked cell adhesion (Fig. 7C). These results support our hypothesis that the 617 aspartic acid is essential for cell adhesion and indicate that the 618 isoleucine is also important for cell adhesion.

Synthetic Peptides, NKDIL and EPDIM, from the 2nd Fas-1 and the 4th Fas-1 Domains Are Sufficient to Mediate HCE Cell Adhesion via α₃β₁ Integrin—To confirm further aspartic acid
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Fig. 2. βig-h3-mediated cell adhesion and spreading are independent of the RGD motif. Plastic culture dishes were coated with 10 μg/ml of each protein, i.e. BSA, plasma FN, βig-h3-WT, or βig-h3-ΔRGD, and were incubated for 1 h at 37 °C. Cells were rinsed, fixed, and stained with crystal violet. Adhesion (A) and spreading (B) of HCE cells were quantified as described under “Experimental Procedures.” The values are the means ± S.D. of triplicate determinations.

and isoleucine are essential for cell adhesion, four synthetic peptides were generated. As shown in Fig. 8A, the first three peptides, KADHH (amino acids 219–223), NKDIL (amino acids 354–358), and EPDIM (amino acids 615–619), correspond to each conserved sequence of the 1st, the 2nd, and the 4th fas-1 domains of βig-h3, respectively. The last one, DEMPI is from the 4th fas-1 but is scrambled and used as a control peptide. We tested whether these four peptides could inhibit βig-h3-mediated cell adhesion. As shown in Fig. 8B, NKDIL from the 2nd fas-1 and EPDIM from the 4th fas-1 were capable of blocking cell adhesion to βig-h3-WT, whereas KADHH from the 1st fas-1 weakly inhibited cell adhesion, and control peptide DEMPI did not affect cell adhesion. Similar results were obtained when we used the 2nd fas-1 and the 4th fas-1 proteins as cell substrata (Fig. 8C). Then we tested whether each peptide itself is capable of mediating cell adhesion. Several different concentrations of each peptide were used as cell substrata and tested for cell adhesion activity. As shown in Fig. 9A, NKDIL and EPDIM were capable of mediating cell adhesion in a dose-dependent manner. KADHH was also capable of mediating cell adhesion in a dose-dependent manner, but the activities were relatively weak. The control peptide was not active in cell adhesion. In the next experiment, we examined whether peptide-mediated cell adhesion was also mediated via α3β1 integrin. Fig. 9B showed that cell adhesion to NKDIL or EPDIM was blocked by antibodies to α3 and β1 integrin subunits. These results suggest that the conserved aspartic acid and isoleucine in the 2nd fas-1 and the 4th fas-1 domains are essential for βig-h3-mediated cell adhesion through α3β1 integrin. To examine whether inhibition of cell adhesion by these two peptides was specific to βig-h3, we tested the effects of peptides on cell adhesion to other adhesion molecules. As is shown in Fig. 10A, NKDIL and EPDIM efficiently blocked cell adhesion not only to βig-h3 but also to laminin, whereas they moderately inhibited cell adhesion to fibronectin and did not affect cell adhesion at all to collagen type I, type II, and vitronectin. These inhibitory effects were dose-dependent (Fig. 10B). These results suggest that NKDIL and EPDIM specifically compete with α3β1 integrin-interacting molecules.

DISCUSSION

Although βig-h3 has been considered to promote cell adhesion and spreading, the interacting cell receptor and the specific motifs of βig-h3 for cell adhesion have not been characterized. In this report, we identified that the functional receptor for βig-h3 is α3β1 integrin and the sequences, NKDIL of the 2nd and EPDIM of the 4th fas-1 domains, are active sites and sufficient to induce cell adhesion through α3β1 integrin. In addition, aspartic acid and isoleucine turned out to be the essential amino acid residues of these motifs.

βig-h3 was first identified by differential screening of a cDNA library made from A549 human lung adenocarcinoma cells treated with transforming growth factor-β (16). Its cell adhesion activity was first reported with human dermal fibroblasts (5) and then with choroidocytes, peritoneal fibroblasts, and human MRC5 fibroblasts (6). Because βig-h3 has an RGD motif at the carboxyl terminus, βig-h3 was thought to mediate cell adhesion through its RGD motif. Ohno et al. (6), however, reported that the RGD motif at the carboxyl terminus of βig-h3 was not necessary for enhancing the spreading of choroidocytes. This result was predictable because the RGD motif was not present in the mature βig-h3 protein as a result of carboxyl-terminal processing, and the mature form was able to inhibit cell adhesion when it was added to the culture medium (1). Our result also supports the RGD motif is not necessary for mediating cell adhesion activity of βig-h3.

Several efforts were made to identify a cell surface receptor for βig-h3. The fact that βig-h3-mediated cell adhesion was blocked by an RGD peptide and EDTA suggests that the surface receptor for βig-h3 could be one of RGD-dependent integrins of which activity requires divalent cations such as Mn2+ and Mg2+. The result of assay using function-blocking antibodies to α1, α2, α4, α5, α6, αv, and β1 integrins suggests that the specific integrin interacting with βig-h3 is α3β1 integrin, which has been known to belong to the RGD- and divalent cation-dependent integrins (12). Indeed, we showed that α3β1 integrin is co-immunoprecipitated by anti-βig-h3 antiserum, and conversely, βig-h3 was also co-immunoprecipitated by anti-α3 integrin antibody indicating that α3β1 integrin is a specific functional receptor for βig-h3 in HCE cells.

fas-1 domain is found in several proteins including βig-h3, periosin, fascilin, HLC-2, and algal-CAM, all of which are known as cell adhesion molecules, but they have different numbers of fas-1 domain (8). It suggests that βig-h3 may not require all four fas-1 domains to mediate cell adhesion and even a single fas-1 domain could mediate cell adhesion. This hypothesis was proved by demonstrating that each of the 2nd and the 4th fas-1 domains was sufficient for cell adhesion activity. However, why were the 1st and the 3rd fas-1 domains not active in mediating cell adhesion? Complete lack of cell adhesion activity of the 3rd fas-1 domain may be due to its less homology with other three domains. Particularly, the region around H2 homologous sequence of the 3rd fas-1 domain is not
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Fig. 3. Identification of HCE cell surface receptor for βig-h3. A, plastic culture dishes were coated with 10 μg/ml of each protein, i.e. pFN, βigh3-WT, or βigh3-ΔRGD. HCE cells were preincubated for 30 min in medium in the absence or presence of 5 mM EDTA, 5 mM EGTA, 100 μg/ml βigh3-WT, 100 μg/ml βigh3-ΔRGD, 1 mM GRGDSP, 1 mM GRGESP, or 100 μg/ml pFN in tubes. B, HCE cells were preincubated for 30 min in Hepes buffer, pH 7.4, containing 150 mM NaCl in the absence or presence of 5 mM CaCl2, MgCl2, or MnCl2 and then transferred to βigh3-WT-coated (10 μg/ml) dishes for cell adhesion assay. C, HCE cells were preincubated with the following function-blocking monoclonal antibodies to integrin subunits at a concentration of 5 μg/ml for 30 min at 37 °C and then added to the precoated wells with 10 μg/ml βigh3-WT: αv, anti-integrin αv subunit antibody (FB12); α5, anti-integrin α5 subunit antibody (PI6); α6, anti-integrin α6 subunit antibody (PI6B); α6, anti-integrin α6 subunit antibody (CLB701); αv, anti-integrin αv subunit antibody (PI3G8); β1, anti-integrin β1 subunit antibody (6C6). After 1 h incubation, cells attached to the substrates were quantified by hexosaminidase assay as described under “Experimental Procedures.” The values are expressed as percentages of the number of cells adhering in the absence of monoclonal antibodies. Each column represents the mean of triplicate assays. D, co-immunoprecipitation of αv integrin and βig-h3. HCE cells and transfectants expressing αv were immunoprecipitated with antibodies to either αv integrin or βig-h3. The immunoprecipitated proteins were separated on 10% SDS-polyacrylamide gels, transferred, and immunoblotted with antibodies to either βig-h3 or αv integrin as described under “Experimental Procedures.” HCE cell extract (lanes 1 and 5), cell extract from HCE cells treated with 2 μg/ml of βig-h3 proteins in the culture medium (lanes 2 and 6), HCE cell extract added with 2 μg/ml of βig-h3 proteins (lanes 3 and 7), and cell extract from HCE cells expressing αv treated with 2 μg/ml of βig-h3 proteins in the culture medium (lanes 4 and 8) were analyzed.

Fig. 4. Analysis of integrins expressed on HCE cell surface. Flow cytometry analysis was performed on HCE cells. Saturating concentrations of the following monoclonal antibodies were used for staining (see “Experimental Procedures”): α1 (6B12), α2 (ASC-1), α3 (PI6H4), α5β1 (HA6), α6 (CLB701), αv (PI6G8), αβ3 (LM609), αβ6 (P16F6), and β3 (12G10). The data are expressed as cell number (y axis) plotted as a function of fluorescence intensity (x axis) and are representative of three separate experiments. Negative control cells were incubated with secondary antibody alone.

well conserved. In contrast, although the 1st fas-1 domain is relatively highly homologous with the 2nd fas-1 and the 4th fas-1 domains, it is not as active as the 2nd and the 4th domains in mediating cell adhesion. These findings suggest that cell adhesion motifs in the 2nd and the 4th domains might be lacking or altered in the 1st domain and the 3rd domain. To answer this question, we first analyzed sequences of each domain of βig-h3 and several other fas-1 domains looking for conserved amino acids other than H1 and H2. In particular, we focused on aspartic acid, which is known to be essential for interacting with integrins. A sequence analysis uncovered that aspartic acid or isoleucine near H2 are highly conserved in many fas-1 domains. It is noteworthy that these two amino acids are not found in the 3rd domain and isoleucine is replaced by histidine in the 1st domain. In fact, mutation of either aspartic acid or isoleucine almost completely blocked the 4th fas-1 domain-mediated cell adhesion. In addition, synthetic peptides, NKDIL and EPDIM from the 2nd and 4th domains, respectively, were efficiently able to block cell adhesion mediated not only by each domain but also by wild type βig-h3, whereas the synthetic peptide KADHH derived from the 1st domain was less efficient to block cell adhesion. These results indicate that both aspartic acid and isoleucine are required for cell adhesion and also give us an answer why the 1st domain is weakly active and the 3rd domain is not active in mediating cell adhesion.

Synthetic peptides, NKDIL and EPDIM, themselves were found to be good substrates for cell adhesion. Like wild type βig-h3, cell adhesion mediated by the 2nd domain, the 4th domain, and two synthetic peptides were also specifically...
blocked by antibodies to \( \alpha_3 \) and \( \beta_1 \) subunits. This implies that \( \beta^3 \)-h3 interacts with \( \alpha_3 \beta_1 \) integrin via two major motifs, where one resides in the 2nd fas-1 domain and the other resides in the 4th fas-1 domain. This finding is different from what Ohno et al. (6) reported. They reported that the cell surface receptor for \( \beta^3 \)-h3 was \( \alpha_1 \beta_1 \) integrin in MRC5 fibroblasts. This discrepancy may not be due to simple differences in surface integrin profiles because we found that both HCE cells and MRC5 cells have not only \( \alpha_3 \) integrin but also \( \alpha_5 \) integrin on their cell surfaces. Actually, MRC5 has more \( \alpha_5 \) integrin than \( \alpha_3 \) integrin (data not shown). Our experiments with MRC5 fibroblasts revealed that their adhesion to \( \beta^3 \)-h3 was incompletely blocked by antibody to \( \alpha_5 \) integrin but, interestingly, was almost completely blocked by \( \alpha_5 \) integrin antibody which Ohno et al. (6) have not tried in their experiment (data not shown). Furthermore, all mutant forms of the 4th fas-1 domain, which failed to mediate corneal epithelial cell adhesion in our experiments, still retain cell adhesion activities for MRC5 fibroblasts (data not shown). These results strongly suggest that \( \beta^3 \)-h3 can mediate cell adhesion through different integrins depending on cell types, and that its interacting domains could be different.

A number of studies have defined multiple ligands for \( \alpha_3 \beta_1 \) integrin, including laminin (17), certain types of collagen (18), fibronectin (18), and nidogen (19). Although there are some conflicting reports that some of these proteins do not support \( \alpha_3 \beta_1 \)-mediated cell adhesion (19, 20) despite apparent P1B5 blocking effects on some of them, \( \alpha_3 \beta_1 \) integrin is considered to respond to a broad spectrum of extracellular ligands (21).
seems to be no conserved binding motif for \(\alpha_3\beta_1\) integrin because no apparent sequence homology is observed among active peptides from thrombospondin (22), laminin (23), and type IV collagen (24), which have been suggested to interact with \(\alpha_3\beta_1\). Our peptides from \(\beta\)-ig-h3 also do not share any sequence homology with the active peptides mentioned above. However, it is interesting to note that the \(\alpha_3\beta_1\)-interacting peptide corresponding to \(\alpha_1(IV)-(531–543)\) has two Asp residues, both of which are important for cell adhesion activity and that the first Asp is flanked by Leu (24, 25). This Asp-Leu residue is reminiscent of our peptides, NKDIL and EPDIM, where Ile replaces Leu. Both Ile and Leu are hydrophobic and have bulky side chains which, together with Asp, are known to be important for interacting with integrins (12). Further investigations, however, are required to identify and prove \(\alpha_3\beta_1\) integrin-interacting motifs more precisely from several ligands.

**FIG. 7.** Effect of substitution mutations of \(\beta\)-ig-h3-domain IV on HCE cell adhesion and spreading. A, diagram of substitution mutant proteins of \(\beta\)-ig-h3 D-IV. Four substitution mutations were made. The lowercase letters indicate substituted amino acids. B, purified recombinant proteins were subjected to 15% polyacrylamide gel electrophoresis. 1st lane, \(\beta\)-ig-h3 D-IV; 2nd lane, \(\beta\)-ig-h3 D-IV-Pal; 3rd lane, \(\beta\)-ig-h3 D-IV-sDI; 4th lane, \(\beta\)-ig-h3 D-IV-PDs; 5th lane, \(\beta\)-ig-h3 D-IV-sas. Molecular mass standards are indicated in kDa on the left of the gel. C, adhesion of HCE cells to mutants of \(\beta\)-ig-h3-domain IV. HCE cells were seeded onto 96-well microculture plates coated with 10 \(\mu\)g/ml of each protein and incubated for 1 h at 37°C. Cell attachment was quantified as described under “Experimental Procedures.”

**FIG. 8.** Effects of synthetic peptides on \(\beta\)-ig-h3-mediated cell adhesion. A, sequence of peptides (bold type) from the 1st, 2nd, and 4th fas-1 domains of \(\beta\)-ig-h3. The composition of amino acids of control peptide is same as peptide from the 4th fas-1 domain, but its order is changed. B, inhibition of HCE cell adhesion by synthetic peptides. Plastic culture dishes were coated with 10 \(\mu\)g/ml BSA or 10 \(\mu\)g/ml \(\beta\)-ig-h3-WT. HCE cells were preincubated for 30 min in medium in the absence or presence of 100 \(\mu\)M each synthetic peptide (DEMPI, KADHH, NKDIL, and EPDIM). C, inhibition of HCE cell adhesion to \(\beta\)-ig-h3 D-II or \(\beta\)-ig-h3 D-IV proteins by synthetic \(\beta\)-ig-h3 peptides. Plastic culture dishes were coated with 10 \(\mu\)g/ml each protein (BSA, \(\beta\)-ig-h3 D-II, and \(\beta\)-ig-h3 D-IV). HCE cells were preincubated for 30 min in medium in the absence or presence of 100 \(\mu\)M each synthetic peptide (DEMPI, KADHH, NKDIL, and EPDIM). After 1 h incubation, cells attached to the substrates were quantified as described under “Experimental Procedures.”
a role for βig-h3 in wound healing in corneal and vascular tissues (4, 26). A role for disturbed morphogenesis has been assigned to βig-h3 missense mutations in families affected with human autosomal dominant corneal dystrophies (7). Given that βig-h3 is highly induced by transforming growth factor-β in several cells and that it functions as a cell adhesion molecule, together with all above reports, we suggest that βig-h3 may play an important role in the regulation of morphogenesis and the maintenance of several tissues in normal and pathological conditions.

In conclusion, we have demonstrated that two motifs, NK-DIL and EPDIM within the 2nd and the 4th fas-1 domains of βig-h3, are sufficient to mediate human corneal epithelial cell adhesion through αβ integrin. We have also identified that two amino acid residues, Asp and Ile, in the context of motifs are essential for cell adhesion activity. These results, therefore, establish the mechanism of βig-h3-mediated cell adhesion and suggest that other proteins containing Asp-Ile near H2 in their fas-1 domains could function as cell adhesion molecules.

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