αVβ6 Is a Novel Receptor for Human Fibrillin-1

COMPARATIVE STUDIES OF MOLECULAR DETERMINANTS UNDERLYING INTEGRIN-RGD AFFINITY AND SPECIFICITY

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Human fibrillin-1, the major structural protein of connective tissue 10–12 nm microfibrils, contains multiple calcium binding epidermal growth factor-like domains interspersed with transforming growth factor β-binding protein-like (TB) domains. TB4 contains a flexible RGD loop that mediates cell adhesion via αVβ3 and α5β1 integrins. This study identifies integrin αVβ6 as a novel cellular receptor for fibrillin-1 with a Kd of ~0.45 μM. Analyses of this interaction by surface plasmon resonance and immunocytchemistry reveal different module requirements for αVβ6 activation compared with those of αVβ3, suggesting that a covalent linkage of an N-terminal calcium binding epidermal growth factor-like domain to TB4 can modulate αV integrin binding specificity. Furthermore, our data suggest α5β1 is a low affinity fibrillin-1 receptor (Kd > 1 μM), thus providing a molecular explanation for the different α5β1 binding pattern distributions seen when human keratinocytes and fibroblasts are plated on recombinant fibrillin fragments versus those derived from the physiological ligand fibronectin. Nonfocal contact distribution of α5β1 suggests that its engagement by fibrillin-1 may elicit a lesser degree and/or different type of intracellular signaling compared with that seen with a high affinity ligand.

Human fibrillin-1, a 350-kDa extracellular matrix glycoprotein, is the major structural component of the 10–12-nm connective tissue microfibrils (1). It has a modular structure dominated by 43 calcium binding epidermal growth factor-like (cbEGF) domains, tandem repeats of which are separated by transforming growth factor β-binding protein-like (TB) domains. Mutations in the fibrillin-1 gene (FBN1) give rise to the connective tissue disease Marfan syndrome and related disorders. In elastic tissues such as arteries, lung, and elastic ligaments, fibrillin microfibrils appear attached to cell membranes. These areas of attachment resemble focal contacts with an abundance of actin microfilaments on the cytoplasmic side of the membrane. Clustering of microfilaments at the cell surface at points of contact with the microfibrils suggest that microfibrillar components interact with cell-surface receptors which in turn serve as a dynamic link between cells and their microenvironment (2, 3).

Fibrillin-1 is one of the microfibrillar proteins shown to mediate cell adhesion through binding to heterodimeric cell surface receptors of the integrin family (4, 5). This binding is at least in part mediated by the TB4 domain that contains an RGD (Arg-Gly-Asp) motif, a minimal integrin binding motif found in a number of extracellular matrix proteins. Fibrillin-integrin interactions are likely to be particularly important in tissues where microfibrils are in close proximity to cells, such as in the elastic lamellae, and may play a role in the assembly of the microfibril network, as has been shown in the case of fibronectin fibrillogenesis (6). Furthermore, the loss of cell-matrix interactions is likely to underlie some of the pleiotropic manifestations of Marfan syndrome.

Until recently integrin αVβ3 was thought to be the major fibrillin-1 receptor in several cell lines, and this interaction has been shown to influence cell spreading, focal contact assembly, cytoskeletal rearrangements, and extracellular matrix deposition (7, 8). Our quantitative studies on αVβ3-mediated interactions with fibrillin-1 identified a high affinity interaction (Kd ~ 0.04 μM) and suggested a previously unrecognized requirement for N-terminal linkage of cbEGF22 to RGD-containing TB4 for activation of αVβ3 (8). Integrin α5β1 has also been shown to bind fibrillin-1 RGD based on inhibition of fibrillin-mediated cell adhesion by α5β1-specific function-blocking antibodies (7). However, the biophysical properties and cellular consequences of this interaction have not been investigated.

In this study we sought to further define the integrin binding selectivity of fibrillin-1 using recombinant TB4-containing domains.
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fragments. Our cellular and biophysical data identify αVβ6 as a novel moderate affinity receptor for fibrillin-1 RGD-containing fragments ($K_d \sim 0.45 \mu M$) and demonstrate that molecular determinants of this interaction are contained within TB4. Thus αVβ3 and αVβ6 each have unique structural requirements for binding to fibrillin-1. In addition, we demonstrate that α5β1 binding to fibrillin-1 gives rise to a low affinity interaction, which contrasts sharply with the high affinity observed for the physiological ligand fibronectin. This may explain the different cellular responses observed when α5β1-expressing cells are plated onto these two substrates. Our data, therefore, provide new insights into the affinity and specificity of fibrillin-integrin interactions and suggest a broader and more diverse involvement of the integrin superfamily in fibrillin-1 function.

EXPERIMENTAL PROCEDURES

Materials—Rabbit polyclonal anti-vinculin and anti-α5 antibodies (Abs) were from Sigma and Chemicon, respectively. Mouse monoclonal Abs (mAbs) LM609 to αVβ3, 10D5 to αVβ6, P1F6 to αVβ5, JBS5 to α5β1, and CS6β to β6 were from Chemicon. Mouse IgG was from Beckman Coulter. Secondary Abs used were fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG, Texas Red-conjugated donkey anti-rabbit IgG, FITC-conjugated donkey anti-rabbit IgG (all from Jackson Immunoresearch Laboratories), and goat anti mouse (Fab')2 (Dako). Full-length soluble recombinant α5β1 was prepared as described previously (9). Recombinant truncated human αVβ6 (10) was expressed in Chinese hamster ovary cells, and the expressed protein was purified by ammonium sulfate precipitation (60% w/v), ion-exchange chromatography (HiTrap Q; eluted with 50–500 mM NaCl gradient), and size exclusion chromatography (Superose 12HR 10/30). Dynamic light scattering (DynaPro) confirmed that αVβ6 integrin is monomeric in the solution. The VB6 and H441 cell lines were a gift from Dr. John Marshall (Cancer Research UK). The human endometrial stromal fibroblasts were provided by Janet Carver (Oxford University).

Expression and Purification of Recombinant Fibrillin-1 Constructs—DNA fragments encoding the wild type sequences of the domain pairs cbEGF22-TB4 (nucleotides 4589–4948, numbering according to Pereira et al. (11)), TB4-cbEGF23 (nucleotides 4712–5074), and the triple construct cbEGF22-TB4-cbEGF23 (nucleotides 4589–5074) were amplified from fibrillin-1 cDNA. Amplified DNA was inserted into pQE30 expression vector (Qiagen) and transformed into Escherichia coli NM554 (pREP4). After Ni$^{2+}$ affinity chromatography, the recombinant His$_6$ fusion proteins were purified and refolded using a previously described redox-shuffling system (12). The D1543A substitution in cbEGF22-TB4-cbEGF23 was introduced by PCR mutagenesis according to standard procedures. For BiACore experiments, wild type domain pairs and D1543A triple construct were modified to contain a C-terminal BirA sequence, which was used for biotinylation and immobilization onto streptavidin sensor chips. The identity of purified products was confirmed by mass spectrometry. All proteins were estimated to be >90% pure, as judged by SDS-PAGE. One-dimensional and two-dimensional NMR analyses were performed to confirm correct folding of wild type fragments. Ca$^{2+}$ binding to the fragments, an important criterion for proper folding, was measured by fluorometric titration (13) or titration with chromophoric chelator (14).

Cell Adhesion Assays—The cell attachment assay was conducted essentially as published previously (15). Briefly, 96-well plates (MaxiSorp) were coated with doubling dilutions of recombinant fibrillin-1 fusion proteins and fibronectin controls in Tris-buffered saline containing 2 mM Ca$^{2+}$ and incubated overnight at 4 °C. VB6 cells were harvested with 0.5% trypsin-EDTA in phosphate-buffered saline (Sigma), neutralized with 1 mg/ml trypsin-inhibitor (Sigma), washed, and resuspended in serum-free Dulbecco’s minimum essential medium. 50 μl of 2 × 10$^5$ cell/ml suspension was added to each well and incubated for 30 min at 37 °C, 5% CO$_2$. Adherent cells were washed once with phosphate-buffered saline (PBS) and fixed in 4% (v/v) glutaraldehyde, 4% (v/v) formaldehyde in PBS. Adherent cells were scored for spread morphology using a phase contrast microscope according to the criteria of Mardon and Grant (15). Results were confirmed by measuring the average surface area of 100 cells per well using a Leica (Milton Keynes, UK) DM IRB inverted microscope equipped with the Openlab imaging software (Improvision, Coventry, UK). Cell attachment was quantified by staining the nuclei with 0.1% crystal violet in 10% ethanol. The dye was solubilized with methanol, and optical density read at 595 nm. For antibody inhibition assay, cells were added to the wells together with serial dilutions of antibodies in 25 μl of phosphate-buffered saline.

Immunocytochemistry—Glass coverslips were coated with 100 μg/ml fibrillin-1 or fibronectin fragments overnight at 4 °C. VB6 cells were prepared in the same way as for cell adhesion assays and replated on fibrillin-1 fragment-coated coverslips for 30 min. After fixation with 3% (w/v) paraformaldehyde, actin was visualized using fluorescein-conjugated phalloidin. Human integrins αVβ6 and α5β1 were visualized using mAbs CS6β and JBS5, respectively, except for the double staining of the two integrins when α5 was labeled with the polyclonal rabbit anti-α5. Assembly of focal adhesions was demonstrated by staining with an antibody to vinculin. Mouse and/or rabbit IgG1 substituted for the primary antibody at 10 μg/ml was used as a negative control. Images were captured using the objective with PL FLUOTAR 100× oil immersion lens with fixed numerical aperture of 1.3 on a Leica DMREBE microscope (Leica) equipped with a Hamamatsu Orca C4742–95 digital camera and analyzed with the OpenLab software (both Improvision).

Flow Cytometry—Cells were prepared as for cell adhesion assays, except that accutase (PAA Laboratories) was used for harvesting cells. Washed and pelleted cells were resuspended in 100 μl of primary antibody diluted in Hanks’ balanced salt solution, 5% normal human serum and incubated for 1 h at 4 °C. The labeling procedure was repeated with secondary antibody (fluorescein isothiocyanate-conjugated goat anti mouse (Fab)$_2$, Dako). After washing, cells were analyzed using a FACscan flow cytometer (Beckman Coulter Epics Altra). Mouse IgG1 was used as a negative control.

Surface Plasmon Resonance (SPR) Studies—Real time biomolecular interaction analysis was performed using a BIACORE 2000 instrument (BiACore, Uppsala, Sweden). All experiments were performed at 25 °C using Tris-buffered saline (25 mM Tris,
Identification of integrins mediating VB6 cell adhesion to fibrillin-1. A, fluorescence-activated cell sorter analysis of integrin expression on human VB6 keratinocytes as carried out using a panel of monoclonal antibodies: anti-αVβ6 (i), anti-α5β1 (ii), anti-αυβ5 (iii), and anti-αVβ5 (iv). The expression level of each integrin heterodimer is indicated by the fluorescence intensity. Numbers indicate the percentage of positively labeled cells above the background labeling with mouse IgG1. Data shown are from one representative experiment of three. B, for inhibition studies, VB6 cell spreading was measured in the presence of function-blocking anti-integrin antibodies. The graph represents dose-dependent spreading of cells on 100 μg/ml cbEGF22-TB4 in the presence of increasing concentrations of anti-αVβ6 (squares), anti-α5β1 (filled circles), anti-αυβ3 (open circles), or anti-αVβ5 (triangles) antibodies. Mouse IgG1 (crosses) was included as a negative control. The data shown represent the mean percentage of spread cells ± S.E. from at least three experiments.

150 mM NaCl, pH 7.4) containing CaCl2, MgCl2, and MnCl2 at 2 mM each as running buffer to ensure that integrins are in the extended conformation optimal for ligand binding (16). Fibrillin-1 fragments were coupled to the surface of CM5 sensor chips (BIAcore) either directly via amine coupling (between 1000 and 2500 RU) or indirectly via streptavidin using a C-terminal biotin tag (1000 and 2500 RU) according to the manufacturer’s instructions. Purified integrin (150 ng) was coated onto microtiter wells followed by blocking of nonspecific binding sites with 1% bovine serum albumin. Biotinylated ligands were added to wells in 50 mM Tris, pH 7.4, 150 mM NaCl containing CaCl2 and MgCl2 at 1 mM each and incubated for 3 h at room temperature. After washing three times with buffer, the bound ligands were quantitated by peroxidase-conjugated streptavidin. Apparent Kd values were calculated from each curve using BIAevaluation 3.0 software steady state fitting mode.

RESULTS

αVβ6 Integrin Is a Novel Receptor for Fibrillin-1—Epithelial cells have been shown to synthesize and deposit fibrillin-1 into the extracellular matrix of human skin (17). We investigated the possibility that epithelial cell-specific αVβ6 integrin may have a role in cell adhesion to fibrillin-1 by testing the capacity of fibrillin-1 fragments to mediate adhesion of the high αVβ6-expressing keratinocyte cell line (VB6) (18). Initially the integrin expression profile of the VB6 cells was determined by flow cytometry (Fig. 1A). This analysis confirmed that a large proportion of VB6 cells (≏80%) express high levels of αVβ6 and α5β1 integrins. Approximately 60% of cells were also found to be αVβ3-positive, whereas very low levels of αVβ3 could be detected on less than 6% of cells. To determine the contribution of each of these receptors to the overall keratinocyte adhesion to fibrillin-1, VB6 cells were allowed to adhere to plates coated with fibrillin-1 cbEGF22-TB4 domain pair in the presence of function-
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FIGURE 3. Quantification of VB6 cell adhesion to fibrillin-1 RGD-containing constructs. Attachment (A) and spreading (B) of VB6 cells on surfaces coated with cbEGF22-TB4 (triangles), TB4-cbEGF23 (squares), cbEGF22-TB4-cbEGF23 (diamonds), or the RGA mutant of cbEGF22-TB4-cbEGF23 (open circles). Results are expressed as percentage of maximum cell attachment (A) or as percentage of cells spread (B). Values represent the mean ± S.E. from three independent experiments. C, phase contrast micrographs of VB6 keratinocytes adherent on fibrillin-1 fragments at a protein coating concentration of 50 μg/ml (~3 μM). Fibronectin domain pair FIII9-10 was used as a positive control for αVβ6-mediated cell attachment and spreading. BSA, bovine serum albumin. Bar, 40 μm.

blocking antibodies (Fig. 1B), cbEGF22-TB4 was chosen as the substrate since preliminary data showed that it contained the molecular determinants necessary for cell attachment and spreading. The antibody against αVβ3 had no detectable effect on VB6 spreading, consistent with its low abundance on these cells. Similarly, the antibody against αVβ5 did not cause any spreading inhibition, suggesting that this integrin may not be able to bind fibrillin-1. At concentrations ≥10 μg/ml, the antibodies against αVβ6 and α5β1 caused ~80 and 20% inhibition of the overall VB6 cell spreading, respectively. These data indicate that αVβ6 integrin is the major receptor for fibrillin-1 on this keratinocyte cell line. Furthermore, the role of this integrin in fibrillin-1-mediated adhesion extends to other αVβ6-expressing cells such as lung-derived H441 adenocarcinoma cells (data not shown).

Module Requirements for Fibrillin-mediated αVβ6 Activation—Because αVβ6 is the most abundant integrin on VB6 cells and the main mediator of their adhesion to fibrillin-1 fragments, this cell line was deemed suitable for investigating the structural requirements of fibrillin-mediated αVβ6 activation. Importantly, given that less than 6% of VB6 cells express low levels of αVβ3, the molecular determinants of αVβ6 activation could be studied in this cell line without any interference from αVβ3 receptor.

To establish whether the αVβ6-fibrillin interface extends beyond TB4 to involve regions in flanking cbEGFs, three partially overlapping RGD-containing fragments cbEGF22-TB4, TB4-cbEGF23, and cbEGF22-TB4-cbEGF23 (Fig. 2) were tested for their ability to induce VB6 cell attachment, spreading, and focal contact formation. Quantification of VB6 adhesion to protein-coated surfaces (Fig. 3) revealed that structure-activity relationships were somewhat different from those observed previously for αVβ3-mediated adhesion of baby hamster kidney and human endometrial stromal fibroblasts (8). TB4-cbEGF23 and cbEGF22-TB4 were found to be equally potent not only in inducing cell attachment but also in triggering cell spreading, with a dose-dependent spreading activity equal to that of cbEGF22-TB4-cbEGF23 (Fig. 3, A and B). A D1543A substitution in the triple domain construct (RGA mutant) abolished the attachment and spreading activity as expected. Phase contrast micrographs in Fig. 3C show the morphology of VB6 keratinocytes on fibrillin-1 and illustrate that there are no significant differences in VB6 attachment or spreading between fibrillin-1 domain pairs. This result suggested that the RGD-containing TB4 domain might be sufficient not only for the initial αVβ6-mediated cell attachment to fibrillin-1 but also for the post-attachment intracellular signaling leading to cell spreading.

To gain further insight into domain requirements for the establishment of intracellular architecture characteristic of well spread cells, VB6 cells adhering to cbEGF22-TB4, TB4-cbEGF23, and cbEGF22-TB4-cbEGF23 were immunofluorescently labeled for αVβ6 integrin and F-actin (Fig. 4). Coverslips...
coated with the fibronectin FIII9-10 domain pair, a known ligand for αVβ6, were used for comparison (10, 19). In VB6 cells cultured on cbEGF22-TB4 and cbEGF22-TB4-cbEGF23, immunofluorescent labeling revealed clusters of αVβ6 integrin colocalized with actin fibers within integrin-rich focal contacts (Fig. 4). Furthermore, adhesion to TB4-cbEGF23 also resulted in αVβ6 recruitment to focal contacts of approximately the same size and number as on cbEGF22-TB4 (Fig. 4). Because previous experiments with baby hamster kidney and human endometrial stromal fibroblast cells indicated a requirement for cbEGF22 in addition to TB4 for αVβ3 recruitment into focal contacts, this result suggests different molecular determinants for αVβ6 activation (8).

**Kinetic Analyses of αVβ6 Binding to Fibrillin-1** — The molecular mechanism underlying fibrillin-1 recognition by αVβ6 integrin was further investigated by monitoring fibrillin-integrin complex formation using SPR. The RGD-containing fragments cbEGF22-TB4, TB4-cbEGF23, and cbEGF-TB4-cbEGF23 (Fig. 2) were immobilized at equimolar levels on the surface of a CM5 sensor chip via primary amine coupling (Fig. 5). The rates of association (k_{on}) and dissociation (k_{off}), obtained by fitting the binding data onto a monoeponential binding model, were almost identical for all three αVβ6-fibrillin-1 complexes with K_d values of ~0.9 μM (Table 1). These SPR results are in good agreement with the observed cellular data since all three fibrillin-1 fragments were able to support recruitment of αVβ6 into focal contacts and the ensuing cell spreading (Figs. 3 and 4).

Because amine coupling of ligands to the sensor surface often leads to their partial inactivation and reduction of the apparent affinity for analyte (20), the kinetics of αVβ6 binding to fibrillin was remeasured with ligands immobilized via streptavidin affinity capture. cbEGF22-TB4, TB4-cbEGF23, and the RGA mutant were expressed as C-terminal BirA fusion proteins and subjected to site-specific biotinylation. Biotinylated FIII9-10 (9) was used as a positive control. Ligands were immobilized at the same density, and binding of soluble αVβ6 was monitored under the same conditions as before. Analysis of sensorgrams confirmed that the k_{on} and the k_{off} of αVβ6 binding to cbEGF22-TB4 and TB4-cbEGF23 are essentially the same, suggesting an equivalent integrin-ligand interface is formed in both cases (Table 1). The overall affinity of the interaction was calculated to be 2-fold higher than that obtained with amine-coupled ligands (K_d ~ 0.45 μM versus 0.9 μM). This difference is most likely due to the partial inactivation of amine-coupled fibrillin fragments and the more native-like presentation of the streptavidin-captured ligand.

Together, these SPR results show that the two RGD-containing domain pairs and triple domain fragment form equally stable complexes with αVβ6 integrin. Furthermore, the data demonstrate that the molecular determinants of the αVβ6/fibrillin-1 interaction are contained within the TB4 domain alone and are independent of the flanking cbEGF domains. To the best of our knowledge, this is also the first report of the K_d value for αVβ6/fibronectin FIII9-10 interaction (~0.18 μM). Given that K_d values for integrin binding to physiological ligands vary from a few nanomolar to high micromolar, the binding of αVβ6 to either fibronectin FIII9-10 or fibrillin fragments can be considered as a medium affinity interaction that is not as high affinity as that observed for αVβ3 (8).

**Integrin α5β1 Is Differentially Distributed in Cells Adhering to Fibrillin-1 and Fibronectin** — The VB6 cell adhesion assay with function blocking antibodies (Fig. 1B) suggested integrin α5β1 in addition to αVβ6 mediates some adhesion to fibrillin-1 and contributes to the intracellular signaling leading to cell spreading. This observation is consistent with an earlier study in which dermal fibroblasts were shown to adhere to recombinant fibrillin fragments and purified microfibrils predominantly via α5β1 (7). However, the signaling events triggered by α5β1 binding to fibrillin-1 have not yet been investigated. To ascertain whether α5β1 assembles into focal contacts upon fibrillin-1 engagement, VB6 cells were immunofluorescently labeled for α5β1 integrin and F-actin after short term culturing on fibrillin-1 fragments and control fibronectin domain pair. Interestingly, the subcellular distribution

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**FIGURE 4. Immunodetection of αVβ6 integrin in human VB6 keratinocytes adherent on wild type fibrillin-1 fragments with the mAb CS/6.** After 30 min of culturing on fibrillin-1 substrates, VB6 cells were simultaneously stained for actin (green), αVβ6 integrin (red) and nuclei (blue). Fibrillin fragment FIII9-10 was used as a positive control for spreading and focal contact formation. Actin filaments terminating in focal contacts enriched in αVβ6 integrin was used as a positive control for spreading and focal contact formation. Actin fibers within integrin-rich focal contacts (Fig. 4). Furthermore, adhesion to TB4-cbEGF23 also resulted in αVβ6 recruitment to focal contacts of approximately the same size and number as on cbEGF22-TB4 (Fig. 4). Because previous experiments with baby hamster kidney and human endometrial stromal fibroblast cells indicated a requirement for cbEGF22 in addition to TB4 for αVβ3 recruitment into focal contacts, this result suggests different molecular determinants for αVβ6 activation (8).

**FIGURE 5. Comparison of the kinetics of αVβ6 binding to wild type fibrillin-1 constructs.** TB4-cbEGF23 and cbEGF22-TB4 domain pairs and the triple domain construct cbEGF22-TB4-cbEGF23 were immobilized on the sensor chip via amine coupling at equimolar levels. Recombinant αVβ6 integrin was injected at 10 μl/min. Sensorgrams shown were acquired with increasing concentrations of αVβ6 integrin (23.9–766 nM), after subtracting the sensogram obtained with the nonspecific fibrillin-1 fragment TB6-cbEGF32 as a reference.
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**TABLE 1**

| Ligand | \(k_{\text{on}}\) \(\times 10^5\) M\(^{-1}\) s\(^{-1}\) | \(k_{\text{off}}\) \(\times 10^{-2}\) s\(^{-1}\) | Apparent \(K_d\) (\(=k_{\text{off}}/k_{\text{on}}\)) μM |
|--------|----------------|----------------|------------------|
| Amine-coupled |
| cbEGF22-TB4 | 0.82 ± 0.02 | 7.00 ± 0.73 | 0.90 ± 0.11 |
| TB4-cbEGF23 | 0.79 ± 0.08 | 7.31 ± 0.52 | 0.91 ± 0.15 |
| cbEGF22-TB4-cbEGF23 | 0.80 ± 0.11 | 7.13 ± 0.38 | 0.88 ± 0.05 |
| Biotinylated |
| cbEGF22-TB4 | 1.86 ± 0.73 | 7.91 ± 1.16 | 0.45 ± 0.11 |
| TB4-cbEGF23 | 1.78 ± 0.97 | 7.94 ± 0.04 | 0.54 ± 0.19 |
| FIII9-10 | 1.65 ± 0.40 | 2.85 ± 0.07 | 0.18 ± 0.05 |

Values represent the mean ± S.D. from three different experiments.

of the integrin on these two substrates was found to be quite different (Fig. 6A). In VB6 cells spread on cbEGF22-TB4, α5β1 showed limited colocalization with actin fibers and was mostly distributed homogeneously over the entire cell surface. A similar diffuse staining pattern was observed on TB4-cbEGF23 and the triple domain construct. In contrast, on FIII9-10, the majority of the integrin was found to colocalize with actin in sharply defined focal contacts at the ends of stress fibers. To confirm the observed distribution patterns, VB6 cells were simultaneously immunolabeled for α5β1 and vinculin, a protein marker of an assembled focal complex. As shown in Fig. 6B on full-length fibronectin and the FIII9-10 domain pair, the integrin is almost completely colocalized with vinculin in prominent focal contacts. However, in cells adherent on fibrillin ligands, integrin remains diffuse and shows minimal colocalization with vinculin. Finally, VB6 cells were double-labeled for α5 and β6 integrins to directly compare their distribution on fibrillin-1 substrates within the same cell (Fig. 6C). In contrast with fibronectin-associated focal contacts, which were found to be both α5- and β6-rich, fibrillin ligands selectively recruited β6 into well defined focal contacts, whereas α5 retained its uniform and indistinct distribution over much of the ventral cell surface.

The non-focal distribution of α5β1 on fibrillin-1 constructs was recapitated in stromal fibroblasts, which like dermal fibroblasts characterized by Sax et al. (7), adhere to fibrillin predominantly via α5β1 with a contribution from αVβ3 (supplemental Fig. 1). This indicates that the fibrillin-induced non-focal distribution of α5β1 is an intrinsic feature of this ligand-receptor complex that does not depend on the degree of α5β1 involvement in the overall adhesion. Taken together, the variation in α5β1 staining pattern and the notable difference in the stromal fibroblast morphology upon adhesion to fibrillin and fibronectin substrates (supplemental Fig. 1C), suggest that these ligands might elicit distinct degrees or different types of intracellular signaling upon α5β1 engagement.

Fibrillin-1 cbEGF22-TB4 Is a Low Affinity Ligand for α5β1 Integrin—The inability of any of the fibrillin-1 RGD-containing fragments to trigger efficient α5β1 clustering in focal contacts suggested that its affinity for fibrillin-1 might be substantially lower than that of αVβ3 or αVβ6. To test this hypothesis, we analyzed α5β1 binding to cbEGF22-TB4 and TB4-cbEGF23 by SPR using αVβ6 as a control analyte. Recombinant full-length α5β1 integrin was injected over fibrillin ligands or FIII9-10 immobilized at equimolar levels via a C-terminal biotin tag. The binding of α5β1 to fibrillin ligands was only marginal compared with the FIII9-10 control, indicating that fibrillin-1 is a weak ligand for this integrin (Fig. 7A). Plotting the near equilibrium binding responses obtained with different concentrations of the integrin shows that at saturation there is an ~30-fold difference between the levels of α5β1 binding to FIII9-10 and cbEGF22-TB4 domain pairs (Fig. 7B). Kinetic analysis of sensorgrams yielded a \(K_d\) of 10.8 ± 2.3 nM for α5β1/FIII9-10 interaction, which is in agreement with the published data (21, 22). However, the kinetics of α5β1-fibrillin-1 complex formation could not be reliably measured by SPR due to its low affinity and the consequent requirement for the large amounts of analyte. We, therefore, sought to characterize the α5β1/fibrillin-1 interaction in reverse orientation by enzyme-linked immunosorbent assay. Integrin was immobilized on the surface of a microtiter plate, and biotinylated fibrillin-1 fragments were added as soluble ligands. Titration curves demonstrated that binding of cbEGF22-TB4 or TB4-cbEGF23 to α5β1 was negligible compared with that of equimolar FIII9-10 (Fig. 7C). An \(EC_{50}\) of 10.7 ± 4.4 nM was obtained for α5β1/FIII9-10 interaction. At a concentration of 1000 nM cbEGF22-TB4, half-maximum binding to α5β1 was still not reached, indicating that the \(K_d\) of α5β1/cbEGF22-TB4 interaction was >1 μM if one assumes simple 1:1 Langmuir binding. These results suggest fibrillin-1 is a low affinity ligand for α5β1 integrin, with the \(K_d\) of this interaction >100-fold higher than that of the α5β1/FIII9-10 interaction.

**DISCUSSION**

This study identifies the epithelial integrin αVβ6 as a novel medium affinity receptor for human fibrillin-1. αVβ6 is normally expressed only at low levels in adult tissue but is rapidly up-regulated in response to injury, inflammation, tumorigenesis, and wound repair and during development. Because fibrillin is known to be secreted by epithelial as well as mesenchymal cells, it is present in the extracellular matrix surrounding cells in which αVβ6 becomes up-regulated (17). The fibrillin-1/αVβ6 interaction is, therefore, likely to play a physiological role in epidermal cell adhesion, and fibrillin-1 joins the list of other known extracellular matrix ligands for αVβ6, including fibronectin, vitronectin, and tenascin (10, 31, 32, 33).

Despite sharing the RGD binding ability, many integrins can nevertheless discriminate between their RGD-containing ligands. In the absence of high resolution structures of integrins in complex with macromolecular ligands, the structural basis for the binding specificity between integrins and their RGD ligands remains poorly understood. In this study we applied an interdisciplinary approach to elucidate the molecular determinants of fibrillin-integrin interactions beyond the known requirement for a solvent-exposed RGD motif in TB4. The dissection of structure-activity relationships using partially overlapping TB4-containing domain pairs as ligands revealed that molecular determinants for αVβ6 binding to fibrillin-1 reside within the RGD-containing TB4. The stability of the αVβ6-fibrillin-1 complex is unaffected by the removal of flanking cbEGF domains, clearly showing that they do not contribute to the αVβ6 binding interface either directly or indirectly. In con-
junction with our previous study, which revealed a requirement for N-terminal linkage of cbEGF22 to TB4 for optimal αVβ3 binding and activation (8), these results demonstrate that αVβ6 and αVβ3 integrins have different structural requirements for fibrillin-1 recognition. Structure-based modeling of the cbEGF22-TB4-cbEGF23-αVβ3 complex (8) shows that, in addition to the proposed role of cbEGF22 in αVβ3 activation, residues just downstream of RGD are likely to interact with the α1 helix and the specificity-determining loop of the β3 I-like domain. These structural elements show very little sequence

FIGURE 6. Subcellular localization of α5β1 integrin in human VB6 keratinocytes upon adhesion to fibrillin-1. VB6 cells were incubated for 30 min on coverslips coated with cbEGF22-TB4, TB4-cbEGF23, or cbEGF22-TB4-cbEGF23 fragment. Coverslips coated with FIII9-10 were used as a positive control. After 30 min cells were fixed, permeabilized, and fluorescently labeled. A, F-actin was stained green with phalloidin, and nuclei were stained blue with 4',6-diamidino-2-phenylindole, and α5β1 integrin is red by indirect fluorescence with the JBS5 mAb. In VB6 cells adhering to FIII9-10, the majority of α5β1 integrin colocalizes with actin in focal contacts at the ends of stress fibers. However, in cells adherent on fibrillin-1 fragments, the integrin has a mostly diffuse distribution over the entire cell surface, showing little colocalization with actin fibers. B, double immunofluorescent labeling of VB6 cells for α5β1 integrin (green) and focal contact marker vinculin (red). Colocalization of these two proteins within focal contacts on full-length fibronectin (Fn) and FIII9-10 produces an orange-yellow color. On fibrillin-1 α5β1 integrin remains diffusely distributed over the entire cell surface, leaving the focal contacts at the cell periphery colored red due to their high vinculin content. C, simultaneous immunofluorescent detection of β6 (green) and α5 (red) integrins with the corresponding antibodies (CSβ6 and AB1928, respectively) in VB6 cells shows that fibronectin-associated focal contacts have an orange-yellow color due to colocalization of integrins within these assemblies. However, a homogenous α5 distribution pattern and selective focal contact recruitment of β6 make these structures predominantly green on fibrillin-1 substrates. Scale bar, 10 μm.
The identity between β3 and β6 subunits, possibly accounting for the weaker affinity of the αVβ6/fibrillin-1 complex.

The role of α5β1 integrin in fibrillin-1 function has been controversial for some time. Earlier cell adhesion assays with function-blocking antibodies, performed on purified microfibrils and recombinant fibrillin-1 fragments, suggested that α5β1 integrin functions as a fibrillin-1 receptor in certain cell lines (7, 26). However, several attempts to demonstrate this interaction in a cell-free system have been unsuccessful (4, 8). Because specific interference of one integrin has been known to influence the behavior of other integrins on the cell (a phenomenon called “integrin cross-talk” (27)), function-blocking studies that use integrin-specific antibodies cannot by themselves be considered as conclusive proof of integrin-ligand interactions. Using a biochemical approach and recombinant integrin, we show for the first time that α5β1 is a low affinity receptor for fibrillin-1.

Immunolocalization of α5β1 integrin in cells spread on fibrillin-1 cbEGF22-TB4 or cbEGF22-TB4-cbEGF23 fragments revealed very limited recruitment of the integrin into focal contacts, giving rise to diffuse staining. Because assembly of integrins into focal contacts depends on the affinity of the integrin-ligand pair (28), the inability of fibrillin ligands to induce efficient α5β1 clustering confirms the low affinity of this interaction at the cell surface. Furthermore, the distinct α5β1 distribution observed in cells adherent on cbEGF22-TB4 and FIII9-10 domain pairs (Figs. 6, A–C, and supplemental Fig. 1 B) raises the possibility that engagement of α5β1 integrin by fibrillin-1 and fibronectin may have distinct cellular consequences. The initial event in outside-in signaling is integrin clustering in the plane of the membrane upon ligand interaction, leading to the assembly of focal complexes and their subsequent maturation into focal contacts (30). The rate of integrin clustering depends on a number of factors including ligand and receptor density, rate of integrin diffusion in membrane, and the lifetime of the ligand-receptor complex (28). All other factors being equal, the lifetime of the receptor-ligand complex, determined by the affinity of the interaction, will be the crucial factor in the initiation and growth of focal contacts. With the affinity of α5β1/cbEGF22-TB4 interaction at least 100-fold lower than that of α5β1/FIII9-10, the dissociation rate of α5β1/cbEGF22-TB4 complex might be too fast to ensure efficient nucleation and growth of focal contacts, giving rise to small and transient integrin clusters. These, although contributing to the overall cell attachment, might trigger a lesser degree or different type of signaling resulting in the gain of

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**FIGURE 7. Binding of α5β1 integrin to fibrillin-1 as measured by SPR (A and B) and enzyme-linked immunosorbent assay (C). A, sensorgrams shown were obtained when recombinant α5β1 integrin was injected over sensor surfaces with equimolar amounts of cbEGF22-TB4 or FIII9-10 immobilized via a C-terminal biotin tag (~1000 RU). The bulk response, obtained in the flow cell with an RGA mutant of cbEGF22-TB4-cbEGF23, was subtracted from all sensorgrams. Sensorgrams obtained with recombinant αVβ6 integrin are included to demonstrate that low levels of α5β1 binding are not due to inactivation of fibrillin upon immobilization. B, the near-equilibrium SPR binding response was plotted versus analyte concentration to obtain the dose-dependent binding curve. The error bars represent S.D. C, solid phase binding assay of biotinylated fibrillin-1 and fibronectin fragments to immobilized α5β1. Soluble cbEGF22-TB4, TB4-cbEGF23, and FIII9-10 (3–1000 nM) were added to wells coated with 150 ng of the integrin. Binding was detected with peroxidase-conjugated streptavidin. The figure shows a representative result from five independent experiments.
specific cellular function such as motility. This would account for the differences in \( \alpha \delta \beta 1 \) distribution (Fig. 6, A–C, and supplemental Fig. 1B) and fibroblast morphology on the two substrates, which was also noted by Bax et al. (7). Although it remains possible that long range effects may modulate \( \alpha \delta \beta 1 \) integrin affinity in full-length fibrillin-1, the similarity of our data with that of Bax et al. (7), where larger fibrillin fragments and microfibrils were utilized, suggests that these effects would be moderate.

It would be interesting to use site-directed mutagenesis to endow cbEGF22-TB4 with higher \( \alpha \delta \beta 1 \) affinity. A model of the \( \alpha \delta \beta 1 \) headpiece predicts an extensive stretch of acidic residues on the top of the \( \alpha / \beta \) interface, where the ligand binding site is located (29). cbEGF22 has five acidic residues (Asp-1512, Asp-1516, Glu-1518, Glu-1552, and Glu-1584) projecting from the same face of the molecule as the RGD loop that are not counterbalanced by neighboring basic residues and might, therefore, cause an electrostatic repulsion from the integrin surface.

The findings described in this study suggest a broader and more diverse involvement of the integrin superfamily in fibrillin-1 function than previously anticipated. Our data show that the RGD motif of fibrillin-1 is recognized by at least three different integrin receptors with a large spectrum of binding affinities and provide novel insights into structural requirements and cellular consequences of these interactions. These results may find application in the understanding of the mouse Tsk phenotype, which is caused by secretion of a fibrillin-1 polypeptide with a duplication of RGD-containing TB4 domain, and also in the advancement of vascular tissue grafts, where the current challenge is to develop a cell-adhesive matrix that supports selective cell adhesion and activity without the risk of thrombosis (23–25).

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