Fibronectin’s Central Cell-binding Domain Supports Focal Adhesion Formation and Rho Signal Transduction*

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Fibroblast adhesion to fibronectin (FN) induces formation of focal adhesions (FAs), structures that have significant effect on cell migration and signaling. FA formation requires actomyosin-based contractility that is regulated by Rho-dependent myosin light chain (MLC) phosphorylation. Previous studies indicated that the FN central cell-binding (and integrin-binding) domain (CBD) is insufficient for FA formation and that the major heparin-binding domain (HepII) facilitates FA formation in a Rho-dependent manner. We describe here conditions under which FN CBD alone is sufficient for FA formation in both human dermal fibroblasts and the FN-null murine fibroblasts. CBD-mediated FA formation is dependent on its surface adsorption and the adhesion activity of the cells. Attachment of FN-null fibroblasts to CBD elicits the same biphasic regulation of Rho activity as seen on intact FN, whereas adhesion to HepII alone does not activate Rho. Activation of Rho requires high levels of integrin occupancy. However, FN or CBD may induce FAs without increased activation of Rho (i.e. the basal level of GTP-Rho induces sufficient phospho-MLC for FA assembly under this condition). In contrast, adhesion to HepII alone does not sustain MLC phosphorylation. Pulse stimulation of cells on CBD or HepII with lysophosphatidic acid elevates Rho GTP loading to the same level, but the lysophosphatidic acid-stimulated MLC phosphorylation is significantly lower in cells on HepII than on CBD. Coating HepII with suboptimal concentrations of CBD induces FAs without increased activation of Rho. Therefore, FN CBD can support FA formation and generate contraction by activating Rho or by facilitating Rho downstream signaling.

Fibronectin (FN) is a multidomain adhesive glycoprotein found in blood and interstitial connective tissue. It interacts with multiple cell surface receptors and plays an important role in the regulation of anchorage-dependent cell growth, cell migration, differentiation, gene expression, tumor development and metastasis, embryogenesis, angiogenesis, and wound healing (1–6). Cell adhesion to the immobilized FN leads to clustering of the integrins and accumulation of multiple cytoskeletal and signaling proteins around the integrin cytoplasmic domain. These protein complexes, named focal adhesions (FAs), form “focal contact” with the matrix via the clustered integrins. Inside the cells, FAs are linked to the contractile actin/myosin filaments (stress fibers) via certain cytoskeletal proteins (reviewed in Ref. 7). Assembly of FAs and the associated actin stress fibers requires the small GTPase Rho (8). Rho enhances phosphorylation of myosin light chain (Mlc), which in turn initiates contraction (9, 10), leading to the assembly of actin stress fibers and FAs (11). The Rho-controlled generation of contraction/tension affects many cellular functions such as cell motility, differentiation, matrix assembly, and cell cycle progression (12–15).

FN consists of two nearly identical polypeptide chains, each of about 220 kDa in size, linked together by disulfide bonds at the C terminus. It has three types of molecular modules named type I, II, and III repeats (16–18) (Fig. 1). The central region spanning roughly from the second to eleventh type III repeats (III2–11) mediates most of the cell adhesion activity of FN, and is thus named the central cell-binding domain (CBD). The adhesion activity was mapped to the fragment III9–10, which contains the RGD sequence in III10 and the synergy site in III9 (19–21). This fragment interacts with cell surface receptors of the integrin family, which are αβ heterodimers (22). C-terminal to the RGD-containing CBD is the major heparin-binding domain (HepII), which spans from III12 to III14. HepII has been reported to interact with the cell surface receptors including syndecan-4 (23, 24), CD44 (25, 26), and “activated” α4β1 integrin (27).

The RGD-containing CBD is required for FA formation but in published studies appears insufficient by itself. The adjacent HepII is required for FA formation (28–33). The requirement for HepII can be bypassed by stimulation with an antibody against syndecan-4, a putative HepII receptor, or by lysophosphatidic acid (LPA), a known activator of Rho (33). It was shown recently that the syndecan-4 null cells appear to have reduced Rho activity (34). These data suggest that the HepII, via the interaction with its receptors (such as syndecan-4), may signal to activate Rho and thereby facilitate the formation of FAs (35). Cell adhesion to FN regulates Rho activity in a

853: LPA, lysophosphatidic acid; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; BSA, bovine serum albumin; GST, glutathione S-transferase; ROCK, Rho-associated kinase; CAPS, 3-(cyclohexylamino)propanesulfonic acid.
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complex pattern. In the absence of serum-soluble factors, Rho activity is slightly inhibited at the early phase of cell adhesion but modestly stimulated at the later phase of adhesion in Swiss 3T3 cells. In the presence of serum, the activation phase is significantly enhanced, indicating a synergistic effect between soluble factors and FN (36–40). Integrin/CBD-mediated Src activation appears responsible for the initial decrease of Rho activity (38), which implies that HepII may be involved in Rho activation.

Rho-associated kinase (ROCK, ROK, or Rho-kinase), one of the Rho effectors, phosphorylates MLC primarily at Ser-19 and to a lesser extent at Thr-18 (41), producing both mono- and diphosphorylated MLC (pMLC<sub>SSS</sub> and ppMLC<sub>TSSS</sub>). ROCK also inhibits myosin phosphate by phosphorylation of its myosin-binding subunit (MBS; also named MYPT1) at Thr-853 and Thr-695 (numbers based on human sequence) (42–45). Therefore, ROCK promotes MLC phosphorylation by direct phosphorylation and by inactivation of myosin phosphatase (46, 47). We have recently found that ppMLC<sub>TSSS</sub>, pMLC<sub>SSS</sub> and phosphorylation of MBS at Thr-853 (pMBST<sub>SSS</sub>) are dependent on Rho and ROCK in human fibroblasts. We also found that Rho signal transduction leading to MLC phosphorylation requires cell adhesion to FN (48). However, the role of CBD and HepII in facilitating Rho signal transduction has not been addressed.

To assess the role of FN CBD and HepII in regulating Rho activity and Rho-induced signal transduction leading to MLC phosphorylation, we constructed recombinant CBD (eighth to eleventh type III repeats; III<sub>8–11</sub>), HepII (12th to 15th type III repeats; III<sub>12–15</sub>), and several derivatives. We report here unexpected findings that a sufficient density of surface adsorbed CBD induces FAs via signaling that is distinct from that of HepII.

EXPERIMENTAL PROCEDURES

General reagents, the Rho activity assay, and detection of MLC phosphorylation by Western blotting were described previously (48). Human plasma FN was purchased from Chemicon International Inc.

Cloning and Purification of the Recombinant Human FN Fragments—The human FN cDNA clone pFH154 was purchased from American Type Culture Collection. Clone pFH1 was obtained from the Japan Health Science Foundation. These clones (16) were used either as PCR templates or as restriction fragments for the cloning of FN fragments (Fig. 1). The pETCH expression vector was constructed by modifying the pET-based plasmid vector (Stratagene) to code for a C-terminal six-histidine affinity tag. The recombinant FN fragments have three extra amino acids (Met-Gly-Ser) at the N terminus and eight extra (Thr-Ser-His-His-His-His-His-His) at the C terminus. In some constructs, the coding sequence of glutathione S-transferase (GST) was inserted at either the N or the C terminus (Fig. 1). Protein expression was induced in the BL21DE3Lys8 strain of <i>Escherichia</i> coli with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside at 30 °C overnight. Protein affinity purification was performed using Ni<sup>2+</sup>-agarose (Qiagen) according to the manufacturer's protocol. After elution with 250 mM imidazole, the protein solution was passed through a rose (Qiagen) according to the manufacturer's protocol. After elution with 250 mM imidazole, the protein solution was passed through a column of HeppII.

CBD induces FAs via signaling that is distinct from that expected findings that a sufficient density of surface adsorbed CBD induces FAs via signaling that is distinct from that of HepII.

Cell Adhesion and Cell Treatment—Adult human dermal fibroblast strain (CF31) isolated from a healthy 31-year-old Caucasian female was purchased from BioWhittaker. Passages 5–12 were used in this study. The FN-null mouse fibroblasts were described previously (33). Cells were maintained in DMEM supplemented with 10% fetal bovine serum (Hyclone). Cells were washed four times with serum-free DMEM pre-equilibrated to 37 °C to initiate serum starvation. For reattachment experiments, cells were detatched in trypsin-EDTA, washed once with serum-free DMEM containing 0.5 mg/ml soybean trypsin inhibitor, and washed once with serum-free DMEM. Cells were then resuspended in serum-free medium containing 1 mg/ml heat-denatured lipid-free BSA (ICN) and plated immediately to the protein-coated plastic dishes. In some instances, cells were maintained in suspension in the 1% agarose-coated dishes for 60 min before being added to the protein-coated dishes.

Confocal Immunofluorescence Microscopy—Cells were fixed with 4% paraformaldehyde/PBS for 10 min, permeabilized with 0.4% Triton X-100/PBS for 4 min, and blocked with 10% normal goat serum in PBS. FAs were stained with an anti-vinculin antibody (9V1313, Sigma), followed by Alexa Fluor® 488-labeled anti-mouse IgG (Molecular Probes, Inc., Eugene, OR). The actin filaments were stained with rhodamine-labeled phalloidin (Molecular Probes). After the staining on a plastic surface, cells were covered with a glass coverslip, and the pictures were taken immediately in the absence of the glycerol-based mounting medium. Image acquisition was performed under a Nikon Eclipse E600 epifluorescence microscope equipped with a PCM2000 laser confocal scanning controller and the SimplePCI image acquisition software. For staining on glass coverslips, images were recorded under a Leica DMI2002 inverted laser confocal scanning microscope.

Enzyme-linked Immunosorbent Assay—Proteins were serial diluted and adsorbed to the 96-well plate at 4 °C overnight. Both tissue culture- and non-treated 96-well plates were from BD Biosciences (Falcon® brand). The wells were blocked with 1% BSA/PBS plus Tween 20 for 60 min and then incubated with the monoclonal antibody against C-terminal His<sub>6</sub> tag (Invitrogen) or MAB1933 anti-FN CBD (Chemicon International Inc.) for 60 min at room temperature. After washing with PBS/Tween 20, horseradish peroxidase-conjugated goat-anti-mouse IgG was added and incubated for 60 min at room temperature. The TMB peroxidase substrate kit (Bio-Rad) was used for color development. The optical densities at 450 nm were measured in the Bio-Rad model 680 microplate reader.

Cell Adhesion Assay—The 96-well plates were coated with 50 μl/well serial diluted proteins at 4 °C overnight. The wells were then blocked experiments, CAPS buffer (pH 11 measured at room temperature) was used to coat proteins. Glass coverslips and Falcon® tissue culture-treated plastic and Falcon®-nontreated Petri plastic dishes were used for protein coating. The coated surface was then blocked with 2% heat-denatured lipid-free BSA for 60 min at room temperature and rinsed three times with PBS before use.

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FIG. 1. Molecular structure of FN and construction of recombinant FN fragments. A, FN is composed of type I (rectangle), type II (oval), and type III (square) repeats. Two extra type III repeats, EDB (B) and EDB (A), are present in cellular FN but not plasma FN. The IHCS region, also named the variable region, can be missing entirely or have up to 120 amino acids due to alternative splicing. The lengths of the human FN cDNA clones, pFH154 and pFH1, are indicated. B, the recombinant FN fragments. The FN fragment spanning from the eighth to eleventh type III repeats (III<sub>8–11</sub>) is constructed to represent HepII. *the, the RGD sequence in III<sub>8</sub>. The adjacent 12th to 15th type III repeats (III<sub>12–15</sub>) is constructed to represent HepII. Most fragments have a C-terminal His<sub>6</sub> tag for the purpose of affinity purification. Some constructs also have a GST tag.

Confocal Immunofluorescence Microscopy—Cells were fixed with 4% paraformaldehyde/PBS for 10 min, permeabilized with 0.4% Triton X-100/PBS for 4 min, and blocked with 10% normal goat serum in PBS. FAs were stained with an anti-vinculin antibody (9V1313, Sigma), followed by Alexa Fluor® 488-labeled anti-mouse IgG (Molecular Probes, Inc., Eugene, OR). The actin filaments were stained with rhodamine-labeled phalloidin (Molecular Probes). After the staining on a plastic surface, cells were covered with a glass coverslip, and the pictures were taken immediately in the absence of the glycerol-based mounting medium. Image acquisition was performed under a Nikon Eclipse E600 epifluorescence microscope equipped with a PCM2000 laser confocal scanning controller and the SimplePCI image acquisition software. For staining on glass coverslips, images were recorded under a Leica DMI2002 inverted laser confocal scanning microscope.

Enzyme-linked Immunosorbent Assay—Proteins were serial diluted and adsorbed to the 96-well plate at 4 °C overnight. Both tissue culture- and non-treated 96-well plates were from BD Biosciences (Falcon® brand). The wells were blocked with 1% BSA/PBS plus Tween 20 for 60 min and then incubated with the monoclonal antibody against C-terminal His<sub>6</sub> tag (Invitrogen) or MAB1933 anti-FN CBD (Chemicon International Inc.) for 60 min at room temperature. After washing with PBS/Tween 20, horseradish peroxidase-conjugated goat-anti-mouse IgG was added and incubated for 60 min at room temperature. The TMB peroxidase substrate kit (Bio-Rad) was used for color development. The optical densities at 450 nm were measured in the Bio-Rad model 680 microplate reader.

Cell Adhesion Assay—The 96-well plates were coated with 50 μl/well serial diluted proteins at 4 °C overnight. The wells were then blocked
His6 fragment (CBD alone), on the other hand, did not induce FAs and actin stress fibers in human fibroblasts attached for 2 hr to the untreated polystyrene (Petri dish) at 10 μg/ml, were able to induce FAs in almost all of the cells (Fig. 2C). Therefore, FN CBD alone is sufficient for FA formation.

**FA Formation on CBD Is Dependent on Surface Adsorption**—
The ability of CBD to induce FAs is not only dependent on its molecular content and concentration and the nature of the adsorbing surface but is also dependent on the coating buffer. We found that the CAPS buffer diminished the ability of GST-III8–11-His6 to induce FAs (data not shown), consistent with the previous observation (32). These results prompted us to investigate the surface adsorption efficiency of CBD under these conditions. Since these recombinant proteins have a C-terminal His6 tag, we used an anti-His6 antibody to study the coating efficiency of these fragments by enzyme-linked immunosorbent assay. Proteins were coated on the same type of plastics (tissue culture-treated versus untreated) from the same manufacturer except in the 96-well format. We found that the coating efficiency of III8–11-His6 was significantly higher on the untreated plastic than on the tissue culture treated plastic (Fig. 3A). GST-His6 adsorbed to the Falcon® tissue culture plastic much more efficiently than III8–11-His6, and the fusion of III8–11-His6 to GST significantly improved surface adsorption (Fig. 3B). To compare III8–11-His6 with the intact FN, we used an antibody against FN CBD (MAB1933) in an enzyme-linked immunosorbent assay. As shown in Fig. 3C, GST-III8–11-His6 had similar surface adsorption efficiency to the intact plasma FN. The CAPS buffer remarkably decreased the coating of GST-III8–11-His6 (Fig. 3D). The high coating efficiencies were consistent with the elevated cell adhesion activities and correlated with the induction of FAs. For example, fusion of III8–11-His6 with GST increased its cell adhesion activity by about 18-fold (Fig. 3A).

**FN CBD Permits FA Formation and Rho Signaling**—
We found that the ability of III8–11-His6 to induce FAs varied under several conditions. First, III8–11-His6 did not induce FAs when coated at low concentrations (5–10 μg/ml) on the Falcon® tissue culture plastic (Fig. 2C). However, a higher concentration (60 μg/ml) of III8–11-His6 induced FAs in about 50% of the cells (Fig. 2C). Second, GST-III8–11-His6 induced FAs in almost all of the cells when coated at 10 μg/ml on the Falcon® tissue culture plastic (Fig. 2C). Fusion of GST at the C terminus (III8–11-GST-His6) showed the same effect, whereas coating III8–11-His6 at 5–10 μg/ml with GST-His6, as separate molecules did not induce FAs (data not shown). Third, III8–11-His6, when immobilized to the untreated polystyrene (Petri dish) at 10 μg/ml, were able to induce FAs in almost all of the cells (Fig. 2C). Therefore, FN CBD alone is sufficient for FA formation.

**MLC Phosphorylation Is Sustained by CBD but Not HepII**—As described in the Introduction, formation of FAs requires cellular contractility, which is regulated by MLC phosphorylation. We reported recently that adhesion to FN, but not poly-L-lysine, induced Rho/ROCK-dependent phosphorylation of MLC (48). To determine which parts of FN are required to induce MLC phosphorylation, we used GST-III8–11-His6 or GST-III12–15-His6 to coat the Falcon® tissue culture plastic. When the cells were detached and plated directly on GST-III8–11-His6-coated plastic dishes under the serum-free condition, there was an initial decline of both pMLC819 and ppMLCT18/S19 levels followed by a gradual rebound to reach the basal level after about 80–120 min (Fig. 4). It should be noted that although the phospho-MLC signals diminished significantly after detachment (48), the signals could still be clearly visualized after extended exposure. The level of phospho-MLC in detached cells (time 0) is similar to that in starved adherent cells (48), which is sufficient to maintain FAs (Fig. 2A). The recovery of pMLC819 was more readily detected and occurred

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**RESULTS**

**FN CBD Is Able to Support FA Formation**—We first characterized the assembly of FAs and stress fibers in the human dermal fibroblasts. FAs and stress fibers were reduced in number and size after serum withdrawal but were still clearly seen after 20 hr of starvation (Fig. 2A). FAs were reattached to the glass coverslips coated with either intact FN or the recombinant III8–11-His6 fragment (containing both CBD and HepII) under serum-free conditions (Fig. 2B). The III8–11-His6 fragment (CBD alone), on the other hand, did not induce FAs (Fig. 2B). These results are consistent with previous reports (28, 31–33). However, we unexpectedly found that the GST-tagged III8–11-His6 induced strong FAs that were indistinguishable from the ones induced by the intact plasma FN (Fig. 2B).

Since the biochemical analyses of Rho activity and Rho signal transduction are all carried out using FN domains coated on plastic dishes, we further stained FAs on the plastic surface.

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**FIG. 2. Assembly of FAs and stress fibers in human dermal fibroblasts.** A, confocal microimages of FAs and stress fibers in human fibroblasts before and after serum starvation. B, the vinculin-containing FAs and actin stress fibers in human fibroblasts attached for 2 hr to glass coverslips coated with 30 μg/ml intact FN, 10 μg/ml III8–11-His6, 10 μg/ml III8–11-His6, 60 μg/ml III8–11-His6, and 10 μg/ml GST-III8–11-His6. C, FA and stress fiber staining in human fibroblasts attached for 2 hr to the Falcon® tissue culture-treated plastic coated with 30 μg/ml intact FN, 10 μg/ml III8–11-His6, 60 μg/ml III8–11-His6, and 10 μg/ml GST-III8–11-His6, or to the untreated plastic (Petri dish) coated with 10 μg/ml III8–11-His6 (III8–11-His6/Petri) with 2% heat-denatured lipid-free BSA for 60 min at room temperature and rinsed with PBS before adding the cells. Cells were suspended in serum-free DMEM for 60 min as described above and added to the wells (1.2 × 104/well) for 30 min before being fixed with 1% glutaraldehyde for 10 min. Cells were rinsed with water, air-dried, and stained with freshly made 0.1% crystal violet in 200 mM boric acid for 20 min. After rinsing, the plates were dried, and the dye absorbed by the cells was extracted using 10% acetic acid for 30 min. The optical densities at 590 nm were measured in the Bio-Rad model 680 microplate reader.

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*FN CBD Permits FA Formation and Rho Signaling*
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FIG. 3. Analyses of CBD surface adsorption by enzyme-linked immunosorbsent assay and the cell adhesion assay. A, adsorption of GST-III8–11-His6 and GST-III12–15-His6 to Falcon®-nontreated (Petri) and tissue culture-treated 96-well plates measured using an anti-FN antibody. B, effect of coating efficiency on cell adhesion activity. FN, GST-III8–11-His6, and III8–11-His6 were serial diluted and coated in the coating buffers (PBS versus CAPS) on the adsorption of GST-III8–11-His6 to the Falcon® tissue culture plastic detected using an anti-FN antibody. C, comparison of protein adsorption to the Falcon® tissue culture plastic using an anti-FN antibody. D, effect of coating buffers (PBS versus CAPS) on the adsorption of GST-III8–11-His6 to the Falcon® tissue culture plastic detected using an anti-FN antibody. E, effect of coating efficiency on cell adhesion activity. FN, GST-III8–11-His6, and III8–11-His6 were serial diluted and coated in the Falcon® tissue culture-treated 96-well plates. Cells were treated as described under "Experimental Procedures." All data points are the average from triplicates or quadruplicates with error bars (S.E.) included in the curve (some are too small to be seen).

FIG. 4. Regulation of MLC phosphorylation by FN, GST-III8–11-His6, and GST-III12–15-His6. A, human fibroblasts were detached with trypsin/EDTA, washed twice, and plated in serum-free medium to the dishes coated with 30 µg/ml FN, GST-III8–11-His6, or GST-III12–15-His6. Cells were then collected at the indicated times for a Rho activity assay. The results are summarized (average ± S.E.; n = 3) relative to time 0. B, effect of serum starvation on Rho activity in stably adherent human fibroblasts. C, effect of matrix on Rho activity. FN CBD permits FA formation and Rho signaling.

FIG. 5. Regulation of Rho activity in human dermal fibroblasts. A, human fibroblasts were detached with trypsin/EDTA, washed twice, and plated in serum-free medium to the dishes coated with 30 µg/ml FN, GST-III8–11-His6, or GST-III12–15-His6. Cells were then collected at the indicated times for a Rho activity assay. B, the results are summarized (average ± S.E.; n = 3) relative to time 0. C, effect of serum starvation on Rho activity in stably adherent human fibroblasts. The results show that the CBD, but not HepII, is able to function similarly to intact FN in inducing MLC phosphorylation.

Effect of CBD and HepII on Rho Activity—Since MLC phosphorylation is dependent on Rho in these cells, we measured the GTP-Rho level under the same conditions. When the cells were detached and plated immediately on the coated dishes under serum-free conditions, a rapid reduction of GTP-Rho level was detected (Fig. 5, A and B). This is possibly due to the combined effect of detachment-stimulated Rho GTP loading (48) and serum withdrawal, since serum starvation resulted in a rapid reduction of GTP-Rho level in stably attached cells (Fig. 5C). The decrease of GTP-Rho was significantly faster on FN or GST-III8–11-His6 than on GST-III12–15-His6, but the GTP-Rho level was similar at the end of the 2-h incubation regardless of the proteins coated (Fig. 5, A and B). The slower decline of Rho activity similar to cells on GST-III12–15-His6 also observed with cells on poly-L-lysine (data not shown), suggesting that it is an effect due to the lack of integrin-induced signals. These data show that the relatively lower levels of phospho-MLC in cells attached to HepII are not accompanied by a reduction of Rho activity in human dermal fibroblasts.

The published studies of FA formation on FN fragments usually employed cells that were detached and plated directly. To be comparable with those studies, we analyzed MLC phosphorylation and Rho activity under the same conditions. However, the effect of matrix on Rho activity was traditionally measured after the cells were suspended for 60 min in serum-free medium when Rho activity was at a relatively stable level. Under this assay condition, the human dermal fibroblasts used in this study, like the FAK+/+ cells described previously (37), did not significantly activate Rho when attached to FN (data not shown). This makes it difficult to assess the role of individual domains on Rho activity. Furthermore, the cells spread significantly on HepII after being suspended for 60 min (data earlier than that of ppMLCThr18/Ser19, possibly due to the preferential phosphorylation of Ser-19 by ROCK (41). This time course of phospho-MLC induction by GST-III8–11-His6 was very similar to that induced by FN. In contrast, cell adhesion to recombinant HepII (GST-III12–15-His6) showed a gradual decrease of both ppMLCThr18/Ser19 and ppMLCThr18/Ser19 (Fig. 4). These results show that the CBD, but not HepII, is able to function similarly to intact FN in inducing MLC phosphorylation.
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Fig. 6. Regulation of Rho activity in FN-null mouse fibroblasts. A, FN-null cells were suspended for 60 min in serum-free medium and plated on the Falcon® tissue culture-treated dishes coated with either 20 μg/ml FN, 20 μg/ml GST-III8–11-His6, or 20 μg/ml GST-III12–15-His6 for the indicated times. Cells were then collected for Rho activity assay. One representative experiment is shown in the right panel with error bars indicating the S.E. B, FN-null cells were detached, resuspended in serum-free medium, and seeded in the 96-well tissue culture-treated plates coated with serial diluted GST-III8–11-His6, GST-III12–15, or GST-His6. Cells were fixed after a 30-min incubation, and the attached cells were quantified. All data points are the average from triplicates with error bars (S.E.) included in the curve. C, regulation of Rho by GST-III8–11 in FN-null cells. Cells were treated as described in A, except the dishes were coated with 20 μg/ml GST-III8–11. Cells attached to FN (20 μg/ml) for 90 min were included as a positive control. One representative experiment is shown in the left panel. The summary (average ± S.E.) from three experiments is shown in the right panel relative to time 0 (suspended cells; gray bar).

Fig. 7. Activation of Rho by CBD is dose-dependent in FN-null cells. A, Rho activity was assayed in FN-null cells that were suspended for 60 min (Sus) and then plated for 2 h to the dishes coated with either 20 μg/ml GST-III8–11-His6, 5 μg/ml GST-III11–11-His6, 80 μg/ml III8–11-His6, 20 μg/ml III8–11-His6, or 5 μg/ml III11–11-His6. B, FN-null cells were suspended for 60 min in serum-free medium and plated in the Falcon® tissue culture-treated dishes coated with 10 μg/ml III8–11-His6 for the indicated times. Cells were then collected for Rho activity assay. One representative experiment is shown in the top panel. The summary from three experiments is shown in the lower panel, with error bars indicating the S.E. C, comparison of the cell adhesion activity between the human fibroblasts (CF31) and the FN-null fibroblasts. III8–11-His6 was serial diluted and coated in the Falcon® tissue culture-treated 96-well plates. Cells were processed as described under “Experimental Procedures.” All data points are the average from quadruplicates. The error bars (S.E.) are included in the curve, but most are too small to be seen. D, FN-null cells were suspended for 60 min and then plated on coverslips coated with 20 μg/ml FN, 20 μg/ml GST-III8–11-His6, 80 μg/ml III8–11-His6, or 10 μg/ml III8–11-His6 for 2 h. Cells were fixed and stained for FAs and stress fibers.
whether the high adhesion activity affects FA formation, we performed a side-by-side comparison in a single experiment using the same batch of III₈₋₁₁-His₆ (the cell adhesion activity of III₈₋₁₁-His₆ could vary from batch to batch). We found that whereas human fibroblasts did not form FAs on 80 μg/ml III₈₋₁₁-His₆ (Fig. 2B), the FN-null cells formed strong FAs even on 10 μg/ml III₈₋₁₁-His₆ (Fig. 7D). These results further confirm that the CBD alone is sufficient for FA formation. They also show that the matrix-induced Rho activation may be related to a higher degree of integrin occupancy and that FAs can be formed without increased Rho activation.

Supporting Rho Signal Transduction by CBD—That the level of phospho-MLC, but not GTP-Rho, is lower on HepII in human fibroblasts suggests that HepII may be unable to effectively support Rho signaling. To test this hypothesis, we compared the effect of GST-III₁₂₋₁₅-His₆ and GST-III₈₋₁₁-His₆ on LPA-stimulated MLC phosphorylation. Serum or LPA was able to stimulate a significant increase of MLC phosphorylation in a Rho- and ROCK-dependent manner in attached human fibroblasts (48). In human fibroblasts attached to GST-III₈₋₁₁-His₆, LPA stimulated robust MLC phosphorylation that is similar to the level of phospho-MLC, but not GTP-Rho, is lower on HepII in human fibroblasts (48). In human fibroblasts attached to GST-III₈₋₁₁-His₆, LPA stimulated robust MLC phosphorylation that is similar to that seen on FN. MLC phosphorylation stimulated by LPA was limited in cells attached to GST-III₈₋₁₁-His₆ (Fig. 8). On both substrates, Rho activity was increased by LPA to a similar level (Fig. 8). These results support the notion that CBD, not HepII, is able to effectively support Rho downstream signaling leading to MLC phosphorylation, which may account for FA assembly on CBD under basal level of Rho activity.

HepII Enhances FA Assembly without Increased Rho Activation—The above data showed that HepII can be dispensable for FA formation. We also showed that CBD alone, but not HepII alone, can activate Rho. However, HepII has been documented to facilitate FA formation on CBD (when CBD was coated insufficiently to induce FA by itself). To investigate whether this process involves Rho activation, we first examined the effect of GST-III₁₂₋₁₅-His₆ on FA formation in the FN-null cells that were attached to a low concentration of III₈₋₁₁-His₆. We found that cells spread poorly on 2.5 μg/ml III₈₋₁₁-His₆, and only 18% (9 of 50) cells formed very weak FAs. To avoid competitive inhibition of III₈₋₁₁-His₆ adsorption by HepII, we added HepII after the adsorption of III₈₋₁₁-His₆ was completed. Because the coating of HepII is for a shorter time (2 h instead of overnight), we used GST-III₁₂₋₁₅-His₆ instead of III₁₂₋₁₅-His₆, since the GST fusion is more adsorptive. Co-coating with GST-III₁₂₋₁₅-His₆ did enhance the assembly of FAs, consistent with the previous observations (28, 32, 33). GST-III₁₂₋₁₅-His₆ adsorbed at 1 μg/ml increased the FA-containing cells to 39% (25 of 63); the effect reached a maximum (65%, 38 of 58) at 3 μg/ml but declined to 9.6% (5 of 52) at 10 μg/ml (Fig. 9A). Adding soluble GST-III₁₂₋₁₅-His₆ to cells attached to 2.5 μg/ml III₈₋₁₁-His₆ had
little effect on FAs (20%, 10 of 50). Co-coating 3 μg/ml GST-III_{12-15}-His_{6} notably increased cell spreading and the appearance of hair-like actin microspikes and lamellipodia, consistent with the previous observation (Fig. 9A; also see Ref. 32). Unlike the FAs formed on the intact FN or GST-III_{12-15}-His_{6} (Fig. 7D), which were more abundant, localized across the cell body, and associated with actin stress fibers, these FAs were mainly localized within lamellipodia, and the actin filaments were less well organized (Fig. 9A). Consistent with this less contractile phenotype, RhoA activity was not noticeably elevated when GST-III_{12-15}-His_{6} was co-coated at a range of concentrations (Fig. 9B). Therefore, the effect of HepII on FA formation appears modest and is not accompanied by a global increase of Rho activity.

**DISCUSSION**

We show in this report that, rather than requiring both CBD and HepII from FN, as reported previously, CBD alone is sufficient to induce FA formation. The key difference between our study and the previous literature is that we have controlled for the poor adsorptive capacity of CBD. We found that the CBD is minimally adsorptive on certain plastic surfaces and possibly also on glass, compared with the intact FN. Hence, when CBD and FN are adsorbed on a surface from solutions containing equal molar concentrations, CBD adsorbs to the surface at a much lower density compared with the intact FN. Many studies used CBD to investigate its function. To be comparable with the intact FN, an “equal molar” concentration of FN CBD was often used. Results obtained with CBD in this fashion may therefore need reevaluation. We also found that it is important to study morphology and biochemistry on the same surface. This is because the adsorption of CBD and the resulting morphology can be remarkably different on various surfaces. For example, III_{12-15}-His_{6} does not induce FAs in human fibroblasts when coated on glass coverslips but is able to induce FAs to various degrees on surfaces of different plastic surfaces.

It was noted previously that the recombinant III_{12-15} fragment has low adhesion activity when adsorbed directly to plastic surface but has much higher activity when presented using a noninhibitory antibody preabsorbed to plastic (21). The adsorption of III_{12-15} is significantly lower than III_{12-15}. The antibody may be immobilized more efficiently than III_{12-15}; thus, precoating antibody may increase the surface density of III_{12-15}. In this study, GST fusion at either the N or C terminus of CBD has the same effect on FA formation, indicating that the effect of GST fusion may not involve conformational change of CBD. Furthermore, simply increasing CBD (without GST) concentration could induce FA on the same surface (Falcon® tissue culture-treated plastic). In addition, limited unfolding of CBD under increasing temperatures failed to induce FA formation when coated on glass coverslips. Under the conditions described in this study, enhanced FA formation on CBD or cell adhesion activity is accompanied by a parallel increase of surface adsorption. Therefore, FA formation on CBD appears mostly dependent on its surface density. Further studies will be required to exclude the alternative hypothesis that conformational variations account for some of the differences.

We demonstrate that CBD, not HepII, is able to activate Rho. This provides further support for the induction of FAs by CBD. We previously described biphasic regulation of Rho activity by cell adhesion to FN in Swiss 3T3 cells (36). The activation of Rho at the 60–120-min time period was later found to be variable with the cell types or the amount of FN used. For example, activation of Rho is not readily detectable in the FAK+/+ cells (37), or it can be entirely missing in CHO cells that are β3 integrin-deficient (50). Furthermore, in cells that exhibit Rho activation, decreasing FN coating concentration results in reduced Rho activation (49). In this study, we show that Rho activation by CBD is dose-dependent, cell type-specific, and correlates with a higher cell adhesion activity. Taken together, these observations suggest that the adhesion intensity (or the degree of integrin occupancy), controlled by the level of both substratum and the receptor, determines Rho activity.

This integrin-induced Rho activation is not, however, necessary for FA assembly. In human dermal fibroblasts where Rho is not noticeably activated by intact FN or in FN-null cells attached to a lower dose of CBD when Rho is not activated, FAs and stress fibers are still assembled. It appears that the initial assembly of FA requires only the basal level of Rho activity, whereas the cells that are capable of forming stronger adhesions could further activate Rho to increase contraction. In this model, FAs may function as mechanosensors to facilitate the balance between adhesion and contraction (12, 51). Stronger contractile force may be necessary for tail detachment in more adhesive cells during migration.

In the absence of additional Rho activation, CBD is able to induce FAs by supporting Rho signal transduction leading to MLC phosphorylation. HepII alone has much lower capacity. We previously determined that FN is required for ROCK phosphorylation of MLC. We have attempted to assess whether HepII is able to mediate this effect. However, cells expressing activated ROCK (or activated Rho) could no longer attach to HepII (data not shown). Using LPA to stimulate Rho-dependent signaling, we found that phosphorylation of MLC is lower on HepII. Whether ROCK activation is compromised in cells attached to HepII is not clear. A final proof requires a valid ROCK kinase assay. However, establishing such an assay continues to be a challenge. We found that the available antibodies against the endogenous ROCK (Transduction Laboratories) do not immunoprecipitate the kinase. This is consistent with an earlier report, which showed that the epitope in the kinase domain is not readily accessible in native condition (52). The same report also indicated that some antibodies against ROCK may artificially stimulate its activity during immunoprecipitation, indicating the complexity of the in vitro kinase assay.

Our data indicate that sufficient integrin occupancy can induce FA formation, and that HepII appears dispensable, since it could be replaced with a nonadhesive fusion partner, GST. However, our data do not exclude the role of HepII in FA formation. In fact, coating CBD and HepII as separate molecules enhances FA formation. This is consistent with the previous observation that mixing III_{12-15} with III_{12} induced stress fiber formation (32). We did not, however, observe the effect of soluble HepII on FA formation. This discrepancy from the literature may in part be due to our vigorous blocking of the noncoated surface with 2% heat-denatured BSA, because we found that soluble HepII was only effective when the glass coverslips were not blocked after coating III_{12-15}-His_{6} (data not shown). Nevertheless, the effect of HepII is only apparent when the CBD surface adsorption is suboptimal or, in other words, when integrin occupancy is insufficient. Consistent with this notion, the HepII receptor syndecan-4 can be dispensable for FA formation under certain conditions (53, 54). How HepII-syndecan-4 interaction facilitates the formation of FAs is unclear at present. Our results show that HepII alone does not stimulate Rho activity or MLC phosphorylation. In the presence of low concentrations of CBD, HepII induces assembly of FAs to some degree without elevating Rho activity. It therefore appears that HepII may cooperate with CBD in a Rho-indepen-

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2 X.-D. Ren, unpublished data.
dent fashion. There are several lines of evidence supporting this notion. First, HepII was able to enhance cell spreading, a process that is usually antagonized by Rho. Second, higher concentrations of HepII actually diminish FAs (Fig. 9A). Third, although LPA could induce FAs on low concentrations of CBD, expression of activated Rho does not produce the same effect.2 Therefore, the lack of FAs on low concentrations of CBD may not be solely due to insufficient Rho activity. It should be noted that LPA, in addition to being a Rho activator, could activate Rac to promote cell spreading (55). It is also interesting to note that FA formation requires both Rho and protein kinase C activation by phorbol 12-myristate 13-acetate (56), an agent that promotes cell spreading. Therefore, signaling by HepII/syndecan-4 may initially require some basal signals from CBD/integrins. For example, integrin-mediated production of phosphtidylinositol, 4,5-bisphosphate, either Rho-dependent or Rho-independent (57–61), may be required for syndecan-4 to activate protein kinase Ca (62), which in turn may enhance FA formation. Much work still remains to be done to confirm this hypothesis.

In summary, we report here that FN CBD alone is able to induce FA formation, Rho activation, and/or Rho signaling to MLC phosphorylation. The permissive role of CBD (integrins) in Rho signal transduction leading to MLC phosphorylation plays a critical role in FA formation. Which integrin(s) is involved, how integrins activate Rho, and which Rho exchange factor(s) is involved are under investigation. It is of interest to note that there have been two conflicting reports regarding which β integrin is involved in Rho activation (50, 63). It appears that overexpressing the missing integrin, whether β1 or β3, is able to activate Rho. It should be pointed out that the increase of the effective concentration of αβ1 (or αβ3) is more important than the level of β1 or β3 itself. Taking β1 as an example, it not only complexes with α5 but also with some other α subunits. Therefore, in cells that already have β1, a significant increase of αβ1 requires a quite remarkable level of exogenous β1. Nevertheless, these results are consistent with our model that increasing the adhesion activity to FN could activate Rho.

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