The Angiogenic Factors Cyr61 and Connective Tissue Growth Factor Induce Adhesive Signaling in Primary Human Skin Fibroblasts*

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The angiogenic inducers cysteine-rich angiogenic protein 61 (Cyr61) and connective tissue growth factor (CTGF) are structurally related, extracellular matrix-associated heparin-binding proteins. Both can stimulate chemotaxis and promote proliferation in endothelial cells and fibroblasts in culture and induce neovascularization in vivo. Encoded by inducible immediate early genes, Cyr61 and CTGF are synthesized upon growth factor stimulation in cultured fibroblasts and during cutaneous wound healing in dermal fibroblasts. Recently, we have shown that adhesion of primary human fibroblasts to immobilized Cyr61 is mediated through integrin α6β1 and cell surface heparan sulfate proteoglycans (HSPGs) (Chen, N., Chen, C.-C., and Lau, L.F. (2000) J. Biol. Chem. 275, 24953–24961), providing the first demonstration of an absolute requirement for HSPGs in integrin-mediated cell attachment. We show in this study that CTGF also mediates fibroblast adhesion through the same mechanism and demonstrate that fibroblasts adherence to immobilized Cyr61 or CTGF induces distinct adhesive signaling responses consistent with their biological activities. Compared with fibroblast adhesion to fibronectin, laminin, or type I collagen, cell adhesion to Cyr61 or CTGF induces 4) more extensive and prolonged formation of filopodia and lamellipodia, concomitant with formation of integrin α6β1-containing focal complexes localized at leading edges of pseudopods; 2) activation of intracellular signaling molecules including focal adhesion kinase, paxillin, and Rac with similar rapid kinetics; 3) sustained activation of p42/p44 MAPKs lasting for at least 9 h; and 4) prolonged gene expression changes including up-regulation of MMP-1 (collagenase-1) and MMP-3 (stromelysin-1) mRNAs and proteins sustained for at least 24 h. Together, these results establish Cyr61 and CTGF as bona fide adhesive substrates with specific signaling capabilities, provide a molecular basis for their activities in fibroblasts through integrin α6β1, and HSPG-mediated signaling during attachment and indicate that these proteins may function in matrix remodeling through the activation of metalloproteinases during angiogenesis and wound healing.

The recent emergence of the CCN family of angiogenic regulators has called attention to their functional versatility and mechanisms of actions (1). This family of secreted proteins consists of six members: Cyr61, CTGF1, Nov, WISP-1, WISP-2, and WISP-3 (1, 2), with the first three members of the family identified providing the acronym CCN. These structurally conserved proteins share four modular domains with sequence similarities to insulin-like growth factor-binding proteins, von Willebrand factor type C repeat, thrombospondin type 1 repeat, and growth factor cysteine knots (1, 3). Each of these domains is encoded by a separate exon, suggesting that CCN genes arose through exon shuffling of preexisting genes to form proteins with multiple functional domains. Cyr61 and CTGF are both encoded by immediate early genes and are coinduced by serum, bFGF, platelet-derived growth factor, and TGF-β1 in fibroblasts (4–7). Cyr61 and CTGF share 45% amino acid sequence identity (4, 8), and both proteins bind heparin, associate with the ECM, and exhibit remarkable functional versatility (9, 10). Purified Cyr61 and CTGF mediate cell adhesion, stimulate cell migration, and augment growth factor-induced DNA synthesis (10–12). Cyr61 and CTGF can promote chondrogenic differentiation, consistent with their expression in prechondrogenic mesenchyme during embryogenesis (13–15). Both Cyr61 and CTGF stimulate chemotaxis in endothelial cells through an integrin α6/β1-dependent pathway and induce neovascularization in vivo (16, 17).

Expression of Cyr61 in tumor cells enhances tumorigenicity by increasing tumor size and vascularization (16), whereas expression of CTGF has been correlated with both systemic and localized fibrotic diseases (18–20). Both proteins are also coinduced in granulation tissues during cutaneous wound healing (19, 21). Thus, Cyr61 and CTGF may participate in wound repair by acting as angiogenic inducers upon endothelial cells and by acting as chemotactic, proliferative, and matrix remodeling factors upon fibroblasts.

Through what mechanism(s) might Cyr61 and CTGF act to execute such a diversity of biological functions? Inasmuch as CTGF was first identified as a growth factor (8), it has been tempting to postulate that it might function as a classical growth factor, although a cell surface receptor for CTGF that resembles a classical growth factor receptor has not been identified to date. By contrast, both Cyr61 and CTGF share char-

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‡ The abbreviations used are: CTGF, connective tissue growth factor; bFGF, basic fibroblast growth factor; BSA, bovine serum albumin; ECM, extracellular matrix; FAK, focal adhesion kinase; HSPG, heparan sulfate proteoglycan; IMDM, Iscove's modified Dulbecco's medium; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; mAb, monoclonal antibodies; PBS, phosphate buffered saline; TGF-β1, transforming growth factor-β1; WISP, Wnt-inducible secreted protein; PAGE, polyacrylamide gel electrophoresis.
The culture was maintained in IMDM (Life Technologies, Inc.) with 10% fetal bovine serum (Intergen, Purchase, NY) at 37 °C with 5% CO₂ and used for experiments before passage 8. Cell adhesion assays were carried out largely as described (10, 25). Briefly, 96-well microtiter plates (Becton Dickinson, Franklin Lakes, NJ) were coated with test proteins diluted in PBS (37 mM NaCl, 2.7 mM KC1, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.3) at 50 μg/well, incubated at 4 °C for 16 h, and blocked with 1% BSA at room temperature for 1 h. 1064SK fibroblasts were plated at 2–3 x 10⁵ cells/dish in 4 ml of 0.5% BSA-IMDM. Dishes and media were warmed at 37 °C, which helped to ensure consistent results with respect to cell adhesion and spreading within 5–15 min after plating. After incubation, cells were washed and lysed in a buffer (50 mM Tris-Cl, pH 7.5, 135 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 2 mM EDTA, 50 mM NaF, 2 mM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride); immunoprecipitation and immunoblotting were carried out according to standard protocols (31). To determine total FAK or paxillin in each sample, blots were stripped in a buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM β-mercaptoethanol) at 60 °C and then reprobed with mAbs against FAK or paxillin.

To study protein secreted by cells adhered on various substrates (as in Fig. 8), the conditioned media were collected after 24 h of cell incubation. The media were first centrifuged to remove cellular debris. Protein in the media was concentrated using Centricron YM-10 (molecular mass cut-off 10 kDa). An equal amount of conditioned media was loaded on SDS-PAGE and analyzed by immunoblotting with monoclonal antibodies against human MMP-1 (clone 41–1ES), MMP-2 (clone 42–5D11), and MMP-3 (clone 55–2A4). p24/p44 MAPK Activation—1064SK fibroblasts were serum-starved for 24 h, harvested, and plated on 35-mm dishes precoated with proteins as described above. Total cell lysates were prepared and applied on SDS-PAGE, and immunoblotting was carried out using rabbit polyclonal antibodies against the dually phosphorylated active form of p42/p44 MAPK (Thr202/Tyr204) at a 1:500 dilution as suggested by the manufacturer (Promega, Madison, WI).

Rac Activation—1064SK fibroblasts were serum-starved for 24 h, harvested, and plated on plastic dishes precoated with various proteins as described above. The Rac activation assay was done using Rac activation kit according to the manufacturer’s protocol (Upstate Biotechnology, Inc.).

RNA Analysis—1064SK fibroblasts were serum-starved 24 h, mildly trypsinized, and replated on protein-coated 100-mm plastic dishes in serum-free IMDM as described above. After incubation at 37 °C for various times, total cellular RNA was isolated and subjected to RNA blot analysis using various [³²P]dCTP-labeled cDNA probes (32). Human MMP-1 and MMP-2 full-length cDNA clones were obtained from the American Type Culture Collection. MMP-3 probe was generated by reverse transcriptase-polymerase chain reaction with a primer pair corresponding to human MMP-3 DNA nucleotides 1493–1521 and 1736–1763. The blots were washed at high stringency (0.2× SSC, 0.1% SDS at 65 °C) and analyzed by a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

RESULTS

Adhesion of Primary Human Skin Fibroblasts to CTGF Requires both Integrin α₂β₁ and Cell Surface HSPGs—We previously showed that adhesion of primary human skin fibroblasts to Cyr61 is mediated through both integrin α₂β₁ and cell surface HSPGs (27). Given that CTGF is also a heparin-binding...
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Fig. 1. Adhesion of human skin fibroblasts to CTGF requires cell surface HSPGs. A, washed 1064SK fibroblasts were detached with 2.5 mM EDTA and resuspended in serum-free IMDM at 2.5 x 10^5 cells/ml. 50 μl of cell suspension was plated on each microtiter well coated with the indicated amounts of CTGF. After incubation at 37 °C for 30 min, adherent cells were fixed and stained with methylene blue. Extracted dye was quantified by absorbance at 620 nm. Where indicated, soluble heparin (2 μg/ml) was added to the plating medium. B, cells were treated with 40 mM sodium chlorate or with sodium chlorate plus 10 mM sodium sulfate, for 24 h before plating on wells coated with CTGF (1 μg/ml), vitronectin (VN, 0.4 μg/ml), or fibronectin (FN, 2 μg/ml), and adhesion experiments were performed as above. C, cells were treated with 2 units/ml heparinase 1 or 2 units/ml chondroitinase ABC at 37 °C for 30 min, washed, and then plated on wells coated with CTGF (1 μg/ml), fibronectin (FN, 2 μg/ml), or vitronectin (VN, 0.4 μg/ml). Data shown for all panels are mean ± S.D. of triplicate determinations and are representative of three experiments.

protein with strong sequence homology with Cyr61, we surmised that CTGF may also support fibroblast adhesion through a similar mechanism. To test this hypothesis, we prepared microtiter wells coated with purified recombinant CTGF onto which primary human skin fibroblasts were allowed to adhere under serum-free conditions. Fibroblast adhesion to CTGF was dose-dependent and saturable and was completely abrogated by the presence of 2 μg/ml heparin in the plating medium (Fig. 1A). These results suggest that occupancy of the CTGF heparin-binding site by soluble heparin may prevent its interaction with cell surface HSPGs, thus inhibiting cell adhesion. To test this possibility, we cultured human fibroblasts in the presence of sodium chlorate, an inhibitor of 3-phosphoadenosine 5'-phosphosulfate synthesis, to block sulfation of proteoglycans (29). Adhesion to CTGF was inhibited under this condition, whereas adhesion of the same cells to fibronectin and vitronectin was unaffected (Fig. 1B). The inhibitory effects of sodium chlorate on cell adhesion to CTGF was reversed by the inclusion of 10 mM Na$_2$SO$_4$ in the culture medium, verifying that this inhibitory effect is mediated through a sulfation block (29). To substantiate the finding that cell surface sulfated proteoglycans are required for cell adhesion to CTGF, fibroblasts were treated with heparinase I, an enzyme that targets highly sulfated heparan sulfate proteoglycans (33, 34). Heparinase I-treated cells were unable to adhere to CTGF, whereas the same treated cells adhered to fibronectin or vitronectin indifferently (Fig. 1C). Treatment of fibroblasts with chondroitinase ABC had no effect. These results show that CTGF, like Cyr61, requires cell surface HSPGs to mediate adhesion of fibroblasts.

Fibroblast adhesion to CTGF was inhibited by the presence of EDTA or Ca$^{2+}$ in the assay media, but not by Mg$^{2+}$ (Fig. 2A). In control experiments, cell adhesion to type I collagen showed the same divalent cation sensitivity as to CTGF, whereas cell adhesion to vitronectin was not inhibited by Ca$^{2+}$, as previously observed (27). This divalent cation sensitivity of fibroblasts adhesion to CTGF is consistent with the involvement of an integrin receptor. To help distinguish the specific integrin involved, we examined the inhibitory effects of RGD-containing peptides. The peptide GRGDSP was unable to inhibit fibroblast adhesion to CTGF even when present at 2 mM, a concentration that abrogated cell adhesion to either fibronectin or vitronectin, ligands of the integrin α$_5$β$_1$ and the α$_V$ integrins, respectively (Fig. 2B). The control peptide GRGESP was unable to inhibit cell adhesion to any substrate. Thus, adhesion of fibroblasts to CTGF is unlikely to be mediated through the major RGD-sensitive integrins in these cells, namely the α$_V$ integrins and integrin α$_5$β$_1$.

To define the specific integrin that mediates cell adhesion to CTGF, we tested the ability of a panel of function-blocking mAbs to inhibit adhesion. Preincubation of cells with mAbs against integrin subunits α$_1$, α$_2$, α$_3$, or α$_4$ or the integrins α$_5$β$_1$ (JB5) and α$_5$β$_2$ (LM609) had no effect on fibroblast adhesion to CTGF (data not shown). By contrast, mAb against integrin α$_5$ subunit (GoH3) completely abrogated cell adhesion to CTGF, whereas adhesion to fibronectin was not affected (Fig. 2C). Adhesion to laminin, a known ligand for integrin α$_5$β$_1$, was only minimally affected. This is most likely because skin fibroblasts utilize α$_5$β$_2$, rather than α$_5$β$_1$, as the major receptor for laminin (35). Likewise, mAb against the integrin β$_1$ subunit strongly inhibited cell adhesion to CTGF but not to vitronectin (Fig. 2D). Similar inhibitory effect was observed in adhesion to type I collagen, a known substrate for β$_1$ integrins. Together, these results show that adhesion of human skin fibroblasts to CTGF, like adhesion to Cyr61, requires both integrin α$_5$β$_1$ and cell surface HSPGs.

Fibroblast Adhesion to Cyr61 or CTGF Induces Extensive and Prolonged Formation of Filopodia and Lamellipodia—Interaction of integrins and their adhesive ligands leads to intracellular signaling, resulting in a range of cellular responses including cytoskeletal reorganization, focal adhesion complex formation, and cell spreading (36, 37). Fibroblasts adhered to matrix proteins such as fibronectin or laminin are known to transiently form filopodia, lamellipodia, and pseudopod extensions indicative of cell motility, although these structures usually retreat within 30 min after plating, both in reports by others and in our own observations (38).

To understand the signaling responses as cells adhere to Cyr61 and CTGF, we have examined the morphological changes and signaling responses in cells adhered to these proteins. More than 90% of cells attached to and spread on cover-
slips coated with Cyr61 or CTGF within 10 min after plating, as was the case when cells were allowed to adhere to fibronectin or laminin, whereas cells plated on coverslips coated with BSA never spread even after 1 h of incubation (Fig. 3). Adherent fibroblasts were fixed with 3% paraformaldehyde and stained with rhodamine-conjugated phalloidin to reveal their actin cytoskeleton structures. Fluorescence microscopy showed that 30 min after plating, fibroblasts adhered to Cyr61 or CTGF had numerous extended pseudopods (Fig. 3). Arrays of actin filaments existed inside the cells, and microspikes of actin filaments protruded from the cell surface in actin-containing membrane ruffling, typical of filopodia and lamellipodia (39, 40). These structures became even more prominent at later times, and by 60 min after plating, cells displayed complex contours due to their abundance. In contrast, filopodia and lamellipodia formed in fibroblasts plated on fibronectin or laminin are fewer in number and more transient in nature, giving the cells a smoother overall shape by 30–60 min after plating (Fig. 3). Thus, fibroblast adhesion to Cyr61 or CTGF resulted in cytoskeleton changes and cell spreading including formation of filopodia and lamellipodia, consistent with the chemotactic activities of both proteins (11, 17).

**Formation of Focal Complexes upon Adhesion to Cyr61 or CTGF**—Ligation of integrins to their adhesive substrates can lead to their association with actin cytoskeleton through their intracellular cytoplasmic domains. Depending on the nature of F-actin organization, integrins may be clustered into focal adhesions or focal complexes. Focal adhesions are large integrin-containing protein complexes at the end of prominent actin stress fibers, whereas focal complexes are smaller, integrin-containing protein complexes at the tips of filopodia and lamellipodia (41). To investigate whether focal adhesions or focal complexes are formed upon adhesion to Cyr61 or CTGF, human skin fibroblasts were plated on coverslips coated with various proteins. Since both Cyr61 and CTGF mediate fibroblasts adhesion through integrin α6β1, the resultant focal complexes should include this integrin. Consistent with this expectation, indirect immunofluorescence microscopy revealed that fibroblasts adhered to either Cyr61 or CTGF form focal complexes that contain both integrin α6 and β1 subunits (Fig. 4A). Moreover, α6β1 integrin was localized in needle head-shaped structures at the tips of filopodia and lamellipodia characteristic of focal complexes. Cell adhesion to fibronectin, which binds integrin α5β1, resulted in focal adhesions that included integrin β1 but not integrin α6, as expected. Likewise, cells adhered to laminin, which interacts with integrins α5β1 and α6β1, formed focal adhesions involving both integrins α6 and β1 (42–44). Immunofluorescence with control IgG gave only weak and diffused background staining and did not reveal any discrete structure (Fig. 4A).

Associated with focal adhesions and focal complexes are numerous signaling proteins including FAK, and structural proteins including paxillin and vinculin (41, 45, 46). When cells adhered to fibronectin or laminin, paxillin and talin are found in typical focal adhesion structures distributed over the cell bodies, as previously reported (30, 44, 47) (Fig. 4B). When cells adhered to Cyr61 or CTGF, however, both paxillin and talin were also localized at the tips of filopodia and lamellipodia characteristic of focal complexes.

Focal complexes are sites of intracellular signaling and are rich in protein kinases and tyrosine-phosphorylated proteins (36, 37). Immunofluorescence staining with mAbs against phosphotyrosine revealed a pattern of numerous needle head-shaped protein complexes in cells adhered to Cyr61 or CTGF similar to the pattern of paxillin and talin localization (Fig. 4B). As expected, tyrosine-phosphorylated proteins were detected in protein complexes in cells adhered to fibronectin or laminin. Together, these data show that fibroblast adhesion to Cyr61 or CTGF is mediated through integrin α6β1 and cell surface HSPGs, resulting in cell spreading and actin cytoskeleton reorganization, as well as formation of focal complexes involving integrin α6β1, paxillin, and talin at the leading edges of resultant filopodia and lamellipodia.

**Cell Adhesion to Cyr61 or CTGF Activates FAK, Paxillin, and Rac**—Of the many protein kinases activated by integrins and localized in focal complexes, FAK plays a central role in inte-
To examine whether Cyr61 and CTGF can activate FAK, human skin fibroblasts were allowed to adhere on plastic dishes coated with either protein for various durations and harvested. FAK was immunoprecipitated from the resulting cell lysates with mAb against FAK and analyzed by immunoblotting with mAb recognizing phosphotyrosine residues. Increased levels of tyrosine phosphorylation on FAK were observed in cells adhered on either Cyr61 or CTGF as compared with cells plated on poly-L-lysine (Fig. 5A), indicating activation of FAK. The induction occurred within 30 min and was maintained for 1 h at a level comparable with those induced by other ECM proteins such as fibronectin, laminin, or type I collagen (Fig. 5A). These data indicate that Cyr61 and CTGF are both capable of activating FAK signaling, a property that is consistent with their ability to support cell adhesion and spreading.

To establish that FAK signaling pathway is activated by Cyr61 and CTGF, we tested whether paxillin, a major substrate of FAK, is tyrosine-phosphorylated upon cell adhesion to either protein. Paxillin is a structural protein having multiple tyrosine phosphorylation sites and plays integral roles in the

**Fig. 3.** Human skin fibroblast adhesion to Cyr61 and CTGF induces actin cytoskeleton reorganization. 1064SK fibroblasts were washed with PBS and harvested with 2.5 mM EDTA and 0.025% trypsin. The enzyme was neutralized immediately, and cells were resuspended in serum-free IMDM at 5 × 10⁶ cells/ml. Cells were plated on coverslips precoated with 50 μg/ml each of Cyr61, CTGF, fibronectin, or laminin at 10⁴ cells/cm² of coverslip area. Cells were incubated at 37 °C for either 30 min or 1 h, and adherent cells were fixed with 3% paraformaldehyde in PBS followed by permeabilization with 0.5% Triton X-100 in PBS. Actin filaments were stained with rhodamine-conjugated phalloidin. Data shown are typical of three experiments. Bar, 20 μm.

**Fig. 4.** Cyr61 and CTGF induce formation of focal adhesion complexes in fibroblasts. A. 1064SK fibroblasts were collected and resuspended in serum-free IMDM. Cells were plated on coverslips precoated with Cyr61, CTGF, fibronectin, or laminin (50 μg/ml each) at 10⁴ cells/cm² of coverslip and incubated at 37 °C for 30 min. Adherent cells were fixed, permeabilized, and stained with mAb against integrin α₆ (clone 4F10) or β₁ subunit (clone DE9) at 20 μg/ml. For control, normal mouse IgG (20 μg/ml) was used in place of primary antibody. B, cells adhered on the indicated substrates were fixed, permeabilized, and stained with mAb against phosphotyrosine (clone PY-20), paxillin, or talin at 20 μg/ml. Data shown are representative of three experiments. Bar, 20 μm.
FIG. 5. Fibroblasts adhesion to Cyr61 or CTGF leads to activation of FAK and paxillin. Serum-starved and washed 1064SK fibroblasts were detached with 2.5 mM EDTA plus 0.025% trypsin in PBS and resuspended in serum-free IMDM at 5 × 10^6 cells/ml. 4 ml of cell suspension was plated on each 100-mm plate coated with 10 μg/ml each of poly-l-lysine (B, Cyr61, CTGF, fibronectin (FN), laminin (LN), or type I collagen (Col.I). Cells were incubated at 37 °C for either 30 min or 1 h, lysed, and immunoprecipitated with mAb against either FAK or paxillin as described under “Materials and Methods.” A, FAK was immunoprecipitated from lysates, electrophoresed on 6% SDS-PAGE, and immunoblotted with mAb (clone 4G10) against phosphotyrosine (Pi-Tyr). The blot was then stripped of antibodies and reblotted with mAb (clone 4G10) against phosphotyrosine immunoprecipitated from lysates, electrophoresed on 6% SDS-PAGE, and immunoblotted sequentially with anti-phosphotyrosine mAb and anti-paxillin mAb. Data shown are representative of at least three independent experiments.

assembly and regulation of integrin-mediated signaling complexes (37). Once tyrosine-phosphorylated, paxillin is recruited into focal adhesions or focal complexes and binds to various signaling protein molecules including FAK, Src, and Csk (49–51). In experiments similar to those performed to examine FAK activation, we found that paxillin was also tyrosine-phosphorylated upon fibroblasts adhesion to Cyr61 or CTGF (Fig. 5B). The time course of paxillin phosphorylation was similar to that of FAK activation, and the level was also comparable with that induced by other ECM proteins tested, including fibronectin, laminin, and type I collagen (Fig. 5B). This result is consistent with localization of paxillin in focal complexes upon cell adhesion to Cyr61 or CTGF (Fig. 4B). Thus, fibroblast adhesion to Cyr61 or CTGF activates the FAK signaling pathway.

The Rho family GTPase Rac regulates actin dynamics, leading to formation of lamellipodia and clustering of ligand-bound integrins into focal complexes (52, 53). Of the effector protein kinases regulated by Rac, p21-activated kinases bind directly to the active form of Rac, leading to autophosphorylation and kinase activation (54). To study if Rac is activated in cells adhered on Cyr61 or CTGF, an affinity precipitation assay using PDB (p21 binding domain from human PAK-1)-glutathione S-transferase fusion protein was employed in a pull-down assay to capture activated Rac from cell lysates (55). A similar approach has been used to show that 3T3 fibroblast adhesion to fibronectin promotes transient activation of Rac and more prolonged activation of Rho (56, 57), consistent with the actin cytoskeletal organization and lamellipodia formation that follow adhesion (58, 59). As shown in Fig. 6, the levels of activated Rac captured by the pull-down assay were minimal 5–10 min after replating, during which cell spreading had just begun to occur. By 30 min after plating and when most cells were actively spreading, Rac was activated to maximal levels that were maintained through 60 min after plating and then declined thereafter (Fig. 6). The level of activated Rac at the zero time point was higher than during the first 5–10 min after replating (Fig. 6), which may be due to perturbation of actin cytoskeleton by the physical events of detaching and attaching cells. A similar pattern of Rho activation was also reported when Swiss 3T3 cells were detached and replated on fibronectin-coated surfaces (57). Together, these results are consistent with the role of Rac in cell spreading and in the formation of lamellipodia.

Sustained Activation of MAPKs Induced by Cell Adhesion to Cyr61 or CTGF—MAPKs are known to be transiently activated by integrin-mediated signaling upon cell adhesion to matrix proteins such as collagen, fibronectin, vitronectin, and laminin (60–62). To address whether cell adhesion to Cyr61 or CTGF can activate MAPKs, serum-starved human skin fibroblasts were allowed to adhere to these proteins or control ECM proteins for various durations in serum-free medium. Cell lysates were electrophoresed and immunoblotted with phoshospecific antibodies against p42/p44 MAPKs. When fibroblasts adhered to fibronectin, laminin, or type I collagen, p42/p44 MAPK activation was rapid and transient, reaching maximal levels by 15 min and declining thereafter to background levels within 1 h (Fig. 7A). In contrast, p42/p44 MAPK activation in cells adhered to Cyr61 or CTGF was maintained at a high level for at least 9 h after cell plating. The same blots were stripped and reprobed with antibodies against p42/p44 MAPKs, and the results indicated that the total amount of MAPK proteins was unchanged (Fig. 7B). Thus, fibroblasts adhered to Cyr61 or CTGF exhibit strong and prolonged activation of p42/p44 MAPKs observed 3–9 h after plating, in sharp contrast to the rapid and transient activation in cells plated on fibronectin, laminin, or type I collagen (Fig. 7). This sustained activation of MAPKs upon fibroblast adhesion to Cyr61 or CTGF appears to be unique among immobilized matrix substrates and may reflect distinct signaling capabilities mediated through integrin αβ1.

Adhesion of Human Skin Fibroblasts to Cyr61 or CTGF Induces Expression of MMP-1 and MMP-3—Expression of both Cyr61 and CTGF is highly induced in granulation tissues during cutaneous wound healing (21, 63), leading to the possibility that Cyr61 and CTGF may act to facilitate matrix degradation and remodeling. To test this hypothesis, serum-starved primary fibroblasts were harvested and allowed to adhere on immobilized Cyr61 or type I collagen in serum-free medium for various durations, and steady-state levels of MMP-1 and MMP-2 mRNAs were analyzed by RNA blots. As shown in Fig. 8A, MMP-1 mRNA level was minimal 2 h after replating but increased to a high level between 6 and 12 h after replating on Cyr61 or collagen. By 24 h after plating, MMP-1 mRNA level in cells plated on Cyr61 was 5-fold higher than cells plated on type I collagen. By contrast, MMP-2 mRNA level remained the same under all conditions. We next examined if CTGF can also lead
to elevated MMP-1 mRNA level in fibroblasts by acting as an adhesion substrate. As shown in Fig. 8B, cells adhered to either CTGF or Cyr61 displayed 5–7-fold higher steady levels of MMP-1 mRNA 24 h after plating compared with cells adhered to fibronectin, laminin, or type I collagen. Likewise, MMP-3 mRNA levels were 7–9-fold higher in cells adhered to either Cyr61 or CTGF compared with cells adhered to fibronectin, laminin, or type I collagen (Fig. 8B). It is noteworthy that in normal diploid fibroblasts, MMP-1 and MMP-3 genes are regulated coordinately by growth stimuli, whereas MMP-2 expression is inducible by phorbol ester but not responsive to growth factors (64).

To show that MMP-1 and MMP-3 protein synthesis is actually induced upon cell adhesion to Cyr61 or CTGF, we examined the conditioned media of cells plated on various substrates 24 h after plating by immunoblotting. As shown in Fig. 8C, both MMP-1 and MMP-3 are readily detected in conditioned media when human fibroblasts were plated on Cyr61 or CTGF, but none was detectable when these cells were plated on fibronectin, laminin, or collagen. The level of MMP-2 protein in the conditioned media remains the same in cells plated on all substrates, consistent with the levels of mRNAs expressed in these cells (Fig. 8B). Since serum-starved normal skin fibroblasts express minimal levels of MMP-1 and MMP-3 (65) (Fig. 8, A and C), we conclude that adhesion of fibroblasts to Cyr61 or CTGF results in the up-regulation of these metalloproteinases at the levels of both mRNA and protein synthesis (Fig. 8).

**DISCUSSION**

Although a multitude of activities has been described for Cyr61 and CTGF, the mechanisms of their actions have not been elucidated. Results in this study present the first conclusive evidence that Cyr61 and CTGF can function through adhesive signaling. The adhesion of fibroblasts to immobilized Cyr61 or CTGF, in the absence of any other stimulus, is sufficient to induce distinct cellular responses consistent with the activities of both proteins and different from those induced by other known matrix ligands. Based on the high degree of homology among members of the CCN family, we anticipate that other proteins in this family may also function through adhesive signaling.

Several unexpected findings emerged through examination of Cyr61 and CTGF as ECM-associated adhesive ligands. First, the absolute requirement for cell surface HSPGs for cell attachment to Cyr61 or CTGF appears to be unique. Although increasing evidence indicates that HSPGs can function with integrins as coreceptors in cell and matrix interactions (66, 67), HSPGs thus far have been observed to influence only the cell spreading phase of adhesion following cell attachment (27, 68, 69). Second, fibroblast adhesion to Cyr61 and CTGF induces...
persistent formation of filopodia and lamellipodia with focal complexes rather than focal adhesions. Third, cell adhesion to Cyr61 or CTGF induces signaling responses with unusual kinetics, including sustained activation of MAPKs and prolonged induction of MMP-1 and MMP-3 expression. Together, these findings provide new insight into the biological functions of Cyr61 and CTGF as well as a mechanistic interpretation of these activities through integrin-mediated signaling.

Both Cyr61 and CTGF have been shown to support the attachment phase of cell adhesion through integrin receptors, and the specific integrins involved appeared to be cell type-dependent. Thus, Cyr61 and CTGF support the attachment of endothelial cells through integrin α6β1 (17, 25), blood platelets through integrin αMβ2 (26), and fibroblasts through integrin α6β1 and cell surface HSPGs (27) (Figs. 1 and 2). The present study extends these observations to document the cellular responses as a result of cell attachment to Cyr61 and CTGF. One of the most prominent responses upon integrin engagement to Cyr61 or CTGF is actin cytoskeleton reorganization, leading to cell spreading and formation of filopodia and lamellipodia (Fig. 3). Whereas fibroblast adhesion to matrix proteins such as fibronectin and laminin also results in formation of filopodia and lamellipodia, these structures are transient in nature, and they largely retract within 30 min after plating. By contrast, these pseudopods persist for at least 1 h after cell plating on Cyr61 or CTGF and in fact grew more prominent with time (Fig. 3). In agreement with recent studies indicating that Rac is a critical activator for lamellipodia (41, 45), cell adhesion on Cyr61 and CTGF also leads to activation of Rac (Fig. 6). Lamellipodia are required for cell motility, while filopodia are indispensable for migration toward a chemgradient (70, 71). Inasmuch as the chemotactic activities of soluble Cyr61 and CTGF have been previously documented in a Boyden chamber assay (16, 17), these observations suggest that both proteins may function as haptotactic factors when tethered to the ECM to stimulate cell migration within tissues.

Concomitant with cell spreading and actin cytoskeleton reorganization, fibroblasts adhered to Cyr61 or CTGF formed integrin α6β1-containing focal complexes localized to the leading edges of filopodia and lamellipodia (Fig. 4). Similar patterns of focal complexes can be discerned by immunostaining for paxillin and talin, consistent with the known association of paxillin with focal adhesion complexes, and with the demonstrated direct interaction between talin and the cytoplasmic domains of engaged integrins (72). Furthermore, both FAK, a tyrosine kinase that plays central roles in integrin signaling, and its substrate paxillin were activated by tyrosyl phosphorylation when cells were adhered to Cyr61 or CTGF (Fig. 5). Together, these data show that fibroblast adhesion to Cyr61 or CTGF is mediated through integrin α6β1 and cell surface HSPGs, leading to formation of integrin α6β1-containing focal complexes, cytoskeleton reorganization, and cell spreading with the formation of lamellipodia and filopodia. These morphological changes are accompanied by signaling events including the activation of FAK, paxillin, and Rac. These observations provide compelling evidence that Cyr61 and CTGF function as bona fide adhesive signaling molecules.

The activities of MAPKs are central to a variety of cell functions, in particular cell proliferation, differentiation, and gene expression (73). Cells in suspension have no contact with solid surface and therefore are round in shape, and MAPK activity is down-regulated. Once reattached, cell spreading occurs and MAPKs are transiently activated. In the present study, we employed this cell detachment-reattachment scheme to test if Cyr61 and CTGF will also lead to MAPK activation. Remarkably, cell adhesion to Cyr61 and CTGF caused a marked and sustained activation of p42/p44 MAPKs in skin fibroblasts lasting for at least 9 h, compared with the transient activation observed when cells were plated on type I collagen, fibronectin, or laminin (Fig. 7). To our knowledge, this sustained activation of MAPK by Cyr61 and CTGF is unique among adhesive substrates in fibroblasts. This prolonged activation of MAPK following adhesion is consistent with the ability of Cyr61 and CTGF to enhance proliferation through augmentation of growth factor-induced DNA synthesis (10, 11).

There exist conflicting reports about whether integrin α6β1 can activate MAPK. Cross-linking of the mAb GoH3 to integrin α6β1 in fibroblasts kept in suspension did not induce MAPK (74). However, expression of integrin α6a and α6b subunits separately in the P388D1 mouse macrophage cell line, which normally does not express endogenous α6 integrins (75), showed activation of MAPK only in α6a-expressing cells but not in α6b-expressing cells when both were plated on laminin-1 (76). One of the major differences between these studies is the agents used to activate integrin. While the former study used mAb that binds to the extracellular domain of integrin α6, the other employed a natural α6β1 ligand that also binds to cell surface HSPGs. It has been observed that a heparin-binding peptide derived from thrombospondin-1 can synergize with T-cell receptor to enhance activation of MAPKs (77). It is possible that integrin α6β1 requires costimulation of cell surface HSPGs to activate MAPKs and that Cyr61 and CTGF may sustain more prolonged activation of MAPKs by virtue of their dual affinity for integrin α6β1 and HSPGs.

Aside from Cyr61 and CTGF, natural ligands for integrin α6β1 include laminin, invasin (78), and fertilin (79). Invasin is a bacterial protein involved in invasion of mammalian tissues, and fertilin is a sperm surface protein involved in the binding of sperm to egg. Since neither invasin nor fertilin is an endogenous substrate, these proteins are not likely to play a role in wound repair. The presence of laminin is restricted to the basement membrane of epidermis and associated with vascular structures in dermis (80, 81). Given its confined presence in the basement membrane, laminin is unlikely to have important roles in the majority of skin fibroblasts in the granulation tissue. In contrast, both Cyr61 and CTGF are induced in granulation tissues during cutaneous wound repair and are likely to be the principal adhesion ligands of integrin α6β1 during wound healing. In addition, unlike laminin, which interacts with a multitude of integrins, Cyr61 and CTGF appear to utilize only integrin α6β1 in adhesion of dermal fibroblasts, thus providing a unique paradigm for studying integrin α6β1-mediated adhesive signaling in a natural context, free of interference from other interacting integrins.

As adhesive substrates, proteolytic fragments of fibronectin containing the cell binding domain have been observed to induce MMP-1 and MMP-3 through integrin α6β1 (82). Other ECM substrates, such as vitronectin, laminin, type I collagen, and intact fibronectin, either have no effect on MMP-1 and MMP-3 expression or induce their expression only transiently (82, 83) (Fig. 8). Fibroblast adhesion to Cyr61 or CTGF, on the other hand, induces a more prolonged activation of MMP-1 and MMP-3 lasting for at least 24 h, resulting in accumulation of MMP-1 and MMP-3 proteins in the conditioned media (Fig. 8, B and C). Since CTGF expression has been associated with fibrosis and subcutaneous injection of CTGF induces granulation tissue (12), our finding that both Cyr61 and CTGF can up-regulate MMP gene expression may appear paradoxical. However, the induction of metalloproteinases is indeed consistent with a role for these proteins in matrix remodeling of granulation tissue during wound healing, where the degrada-
tion of a provisional matrix and the synthesis of new matrix must occur simultaneously (84, 85). Moreover, degradation of the ECM can promote the migration of endothelial cells in angiogenesis.

Several lines of evidence support the roles of Cyr61 and CTGF in wound healing: 1) expression of Cyr61 and CTGF genes are minimal in normal dermis and becomes highly induced in dermal fibroblasts in granulation tissue during wound repair; 2) both Cyr61 and CTGF are angiogenic inducers and may recruit new blood vessels to supply nutrients to sites of wound healing; and 3) Cyr61 and CTGF promote chemotaxis and proliferation in fibroblasts. Encoded by immediate early genes, Cyr61 and CTGF are induced by serum growth factors implicated in wound healing, including bFGF and TGF-β1 (4, 7). It is interesting to note that in fibroblasts TGF-β1 suppresses the expression of MMP-1 and MMP-3 (86, 87), whereas it is interesting to note that in fibroblasts TGF-β1 suppresses the expression of MMP-1 and MMP-3 (86, 87), whereas it is interesting to note that in fibroblasts TGF-β1 suppresses the expression of MMP-1 and MMP-3 (86, 87), whereas it is interesting to note that in fibroblasts TGF-β1 suppresses the expression of MMP-1 and MMP-3 (86, 87), whereas it is interesting to note that in fibroblasts TGF-β1 suppresses the expression of MMP-1 and MMP-3 (86, 87), whereas it is interesting to note that in fibroblasts TGF-β1 suppresses the expression of MMP-1 and MMP-3 (86, 87), whereas it is interesting to note that in fibroblasts TGF-β1 suppresses the expression of MMP-1 and MMP-3 (86, 87), whereas.

Thus, although it has been suggested that CTGF can mediate some of the activities of TGF-β1 (89, 90), their effects on gene expression are not identical. With respect to induction of MMP-1 and -3, the activities of Cyr61 and CTGF closely parallel those of bFGF instead of TGF-β1. The interplay between the actions of bFGF, TGF-β1, and the induced effectors, Cyr61 and CTGF, is likely to be complex and requires to be investigated.

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