The angiogenic inducer Cyr61 is an extracellular matrix-associated heparin-binding protein that can mediate cell adhesion, stimulate cell migration, and enhance growth factor-stimulated DNA synthesis in both fibroblasts and endothelial cells in culture. In vivo, Cyr61 induces neovascularization and promotes tumor growth. Cyr61 is a prototypic member of a highly conserved family of secreted proteins that includes connective tissue growth factor, nephroblastoma overexpressed, Elm-1/WISP-1, Cop-1/WISP-2, and WISP-3. Encoded by an immediate early gene, Cyr61 synthesis is induced by serum growth factors in cultured fibroblasts and in dermal fibroblasts during cutaneous wound healing. We previously demonstrated that Cyr61 mediates adhesion of vascular endothelial cells and activation-dependent adhesion of blood platelets through direct interaction with integrins αvβ3 and αIIbβ3, respectively. In this study, we show that the adhesion of primary human skin fibroblasts to Cyr61 is mediated through integrin αvβ1 and cell surface heparan sulfate proteoglycans (HSPGs), which most likely serve as co-receptors. Either destruction of cell surface HSPGs or prior occupancy of the Cyr61 heparin-binding site completely blocked cell adhesion to Cyr61. A heparin-binding defective mutant of Cyr61 was unable to mediate fibroblast adhesion through integrin αvβ1 but still mediated endothelial cell adhesion through integrin αvβ3, indicating that endothelial cell adhesion through integrin αvβ3 is independent of the heparin-binding activity of Cyr61. These results identify Cyr61 as a novel adhesive substrate for integrin αvβ3 and provide the first demonstration of the requirement for HSPGs in integrin-mediated cell attachment. In addition, these findings suggest that Cyr61 might elicit disparate biological effects in different cell types through interaction with distinct integrin receptors.

Analyses of the angiogenic inducer Cyr61 have revealed a multifunctional protein with a remarkable diversity of activities. Originally identified in mouse fibroblasts as a secreted, cysteine-rich protein encoded by a growth factor-inducible immediate early gene, Cyr61 was found to be an extracellular matrix-associated, heparin-binding protein that is capable of diverse functions in different cell types (1–3). Purified recombinant Cyr61 protein mediates cell adhesion, stimulates cell migration, and augments growth factor-induced DNA synthesis in both fibroblasts and endothelial cells (4, 5). Cyr61 promotes chondrogenesis in mouse limb bud mesenchymal cells in micromass cultures, in agreement with its expression in condensing prechondrocytic mesenchyme of both neuroectodermal and mesodermal origins during embryogenesis (6, 7). Cyr61 has pro-angiogenic activities in culture and induces neovascularization in vivo, consistent with its localization in angiogenic cell types during development (8). Furthermore, expression of cyr61 enhances the tumorigenicity of human tumor cells in immunodeficient mice by increasing tumor size and vascularization (8).

Cyr61 is a prototypic member of the CCN protein family, which also includes connective tissue growth factor (CTGF), nephroblastoma overexpressed, Elm-1/WISP-1, Cop-1/WISP-2, and WISP-3 (3, 9). These highly conserved proteins share four modular domains with sequence similarities to insulin-like growth factor-binding proteins, von Willebrand factor, thrombospondin, and growth factor cysteine knots. The biochemical and cellular activities of Cyr61 and CTGF are very similar in vitro, and both proteins induce angiogenesis in vivo (8, 10). Both cyr61 and CTGF are co-induced in fibroblasts by growth factors implicated in tissue repair and matrix remodeling, including fibroblast growth factor, platelet-derived growth factor, and transforming growth factor-β1 (1, 11, 12). Consistent with these observations, both cyr61 and CTGF are induced in granulation tissue during cutaneous wound healing (13, 14). Thus, Cyr61 and CTGF may participate in wound healing by acting as angiogenic factors upon endothelial cells, and by acting as chemotactic, mitogenic, and matrix remodeling factors upon fibroblasts.

Cells perceive vitally important information from their three-dimensional tissue contexts through adhesion receptors. These receptors support various aspects of cellular functions, including proliferation, differentiation, migration, and survival (15–17). One of the activities of Cyr61 as an extracellular matrix-associated protein is to support cell adhesion (4, 5). Although devoid of an RGD sequence motif, Cyr61 is a ligand of, and binds directly to, the adhesion receptors integrins αvβ3 and αIIbβ3 (18, 19). A number of the activities of Cyr61 can be attributed to its interaction with integrin receptors. For example, endothelial cell adhesion and migration to Cyr61 are dependent on integrin αvβ3, and activation-dependent adhesion of blood platelets to Cyr61 is mediated through interaction with integrin αIIbβ3 (8, 18, 19). To understand the mechanism of Cyr61 action in dermal fibroblasts, we sought to identify the
cellular adhesion receptor(s) through which Cyr61 may function. In this study, we show that both integrin αβ1 and cell surface HSPGs are indispensable for the adhesion of normal human skin fibroblasts to Cyr61. A heparin-binding defective Cyr61 mutant protein is unable to support fibroblast adhesion, yet retains the ability to support endothelial cell adhesion through integrin αβ3. These findings identify Cyr61 as a novel adhesion substrate for integrin αβ3, underscore the importance of HSPGs in adhesion through this integrin, and demonstrate the utilization of distinct adhesive receptors for Cyr61 in different cell types.

**EXPERIMENTAL PROCEDURES**

**Antibodies, Peptides, and Reagents—**Function-blocking mAbs against various integrins were purchased from Chemicon, Inc., including JB1A (anti-β1), ACS-3 (anti-β1), FB12 (anti-αv), P1E6 (anti-αv), P1B5 (anti-αv), P1H4 (anti-αv), P1D6 (anti-αv), NKI-G0H3 (anti-αv), P3G8 (anti-αv), and LM609 (anti-αv). Polyclonal anti-Cyr61 antibodies were raised in rabbits and affinity purified as described (2). Synthetic peptides GRGDSP and GRGESP were purchased from Life Technologies, Inc. Heparin (sodium salt; from porcine intestinal mucosa), chondroitin sulfate A, C, and decorin were from Sigma. Chondroitin sulfate IIb, III, and cell suspension of 1064SK (ATCC CRL-2076, passage 2) was kept in Dulbecco's modified minimal essential medium (4.5 mg/ml glucose, Life Technologies, Inc.) containing 1% bovine serum albumin as described (18). Protein under study was diluted to the desired concentration in phosphate-buffered saline, applied to 96-well microtiter plates (50 μl/well), and blocked with 5% nonfat dried milk at room temperature for 1 h. Where indicated, reagents (EDTA, heparin, peptides, etc.) were mixed with cells prior to plating. Antibodies were incubated with cells at room temperature for 30 min before plating. To each well 50 μl of cell suspension was plated, and after incubation at 37 °C for 48 h, cells were then detached and plated for cell adhesion assays as described above (22). To show the specificity of sulfation blockage, 10 mS sodium sulfate was included in the culture medium together with sodium chloride. For enzymatic digestion of cell surface glycosaminoglycans, harvested cells were washed, resuspended in Dulbecco's modified minimal essential medium, and treated with heparinase I (EC 4.2.2.7; 2 units/ml, Sigma) or chondroitinase ABC (EC 4.2.2.4; 2 units/ml, Sigma) at 37 °C for 30 min. Cells were spun down, washed, and plated for adhesion assays as above.

**RESULTS**

**Primary Human Skin Fibroblast Adhesion to Cyr61 Is Mediated through Integrin αβ1**—We have previously shown that immobilized Cyr61 can support the adhesion of endothelial cells, epithelial cells, 3T3 fibroblasts, as well as blood platelets (4, 5, 19). In view of the potential involvement of Cyr61 in cutaneous wound healing, we examined the ability of Cyr61 to mediate cell adhesion in normal human skin fibroblasts. Microtiter wells were coated with purified recombinant Cyr61 protein, onto which 1064SK primary human foreskin fibroblasts were allowed to adhere under serum-free conditions. Adhesion of 1064SK cells to Cyr61 was dose-dependent and saturable (Fig. 1A). Furthermore, affinity purified anti-Cyr61 antibodies blocked cell adhesion to Cyr61 but not to vitronectin (Fig. 1B), indicating that the ability to mediate fibroblast adhesion is an intrinsic property of the Cyr61 protein. We previously showed that Cyr61 is a ligand of the integrins αβ1 and α3β1, and mediates the adhesion of endothelial cells and platelets, respectively, through these integrins (18, 19). Therefore, we sought to determine whether adhesion of human skin fibroblasts to Cyr61 is also mediated through an integrin receptor. Preincubation of fibroblasts with LM609, a mAb specific for integrin αβ1 (23), had no effect on fibroblast adhesion to Cyr61 (data not shown). Thus, integrin αβ1 is unlikely to be involved in fibroblast adhesion to Cyr61. We next examined the effects of divalent cations in Cyr61-mediated cell adhesion. Fibroblast adhesion to Cyr61 was completely blocked by 2.5 mM EDTA and was restored by the addition of 5.0 mM MgCl2 (Fig. 1C). The presence of MgCl2, but not MnCl2, was able to reverse the inhibitory effect of Ca2+ on cell adhesion to Cyr61, suggesting that Mn2+ may bind the Cyr61 adhesion receptor with higher affinity than Ca2+ (Fig. 1C). This divalent cation sensitivity profile of Cyr61-mediated cell adhesion is consistent with and indicative of an integrin being the adhesion receptor for Cyr61 (24, 25).

A number of integrins expressed on fibroblasts recognize the RDG sequence motif in their protein ligands and are sensitive to inhibition by RGD-containing peptides (26). The peptide GRGDSP, but not the control peptide GRGESP, completely abolished 1064SK cell adhesion to vitronectin at concentrations above.
and the fibronectin receptor (integrin α5β1), this observation indicated that fibroblast adhesion to Cyr61 is unlikely to be mediated through either one of the αv integrins or integrin αβ1. To investigate this notion further, we challenged 1064SK cells by preincubation with function-blocking mAbs against the αv and αβ integrin subunits. Whereas cell adhesion to vitronectin and fibronectin was blocked by mAbs against αv and α5, respectively, these antibodies had no effect on cell adhesion to Cyr61 (Fig. 2B). These results excluded the possibility that either one of the αv integrins or integrin αβ1 may function as the Cyr61 adhesion receptor.

The β1 integrins constitute the major types of integrins expressed in skin fibroblasts, although β2 and β3 integrins are also expressed at low levels. Because the only β2 integrin in fibroblasts is α5β2, this integrin can be excluded inasmuch as fibroblast adhesion to Cyr61 was insensitive to RGD-containing peptides and was not inhibited by the mAb LM609. Likewise, anti-β3 mAb did not block adhesion to Cyr61, thus excluding the β4 integrins (data not shown). By contrast, inhibitory anti-β1 mAb (JB1A) effectively blocked adhesion to Cyr61 by >80%. In comparison, adhesion to type I collagen, which binds to several β1 integrins (α5β1, α5β3, and αβ5), was inhibited about ~70% by the anti-β1 mAb (Fig. 3A). Adhesion to vitronectin, which is mediated through αv integrins (α5β3 and αβ5), was unaffected. These results indicate that adhesion of fibroblasts to Cyr61 is mediated through a β1 integrin other than α5β1, consistent with the RGD insensitivity and Ca2+ sensitivity of this process.

To identify the specific β1 integrin that mediates fibroblast adhesion to Cyr61, we tested the inhibitory effects of function-blocking mAbs against various integrin α subunits. Anti-integrin α5, αv, or α5 mAbs or a combination of mAbs against α1, αv, α5, and αv had no effect on Cyr61-mediated cell adhesion (Fig. 3B). In control experiments, whereas mAbs against α1, α2, and α5 exhibited little to modest inhibition of fibroblast adhesion to collagen as previously reported (25), a mixture of the three antibodies conferred nearly complete inhibition (Fig. 3B). In contrast to the lack of inhibition by mAbs against α1, αv, and α5, mAb against integrin αv(Go13) blocked 1064SK fibroblast adhesion to Cyr61 by >90%, although having no effect on adhesion to fibronectin (Fig. 3C). Adhesion to laminin, a known ligand for integrin αβ1, was only partially blocked by ~30%. This is most likely because integrin αβ1, rather than α5β1, serves as the major laminin receptor in human skin fibroblasts (25). Together, these biochemical and immunological data demonstrate that 1064SK fibroblast adhesion to Cyr61 is mediated through integrin αvβ1.

**Adhesion of Human Skin Fibroblasts to Cyr61 Requires Cell Surface HSPGs—**Because Cyr61 is known to bind heparin with high affinity (2), we endeavored to determine the role of heparin binding in the activities of Cyr61. We added various amounts of soluble heparin to suspensions of 1064SK fibroblasts prior to plating onto either Cyr61- or fibronectin-coated wells (Fig. 4A). Remarkably, as little as 1–10 ng/ml heparin was sufficient to elicit a detectable inhibition of cell adhesion to Cyr61, whereas 0.1 μg/ml heparin completely inhibited Cyr61-mediated fibroblast adhesion. In comparison, the addition of up to 10,000 times more heparin had little or no effect on fibroblast adhesion to fibronectin, itself also a heparin-binding protein. Similar inhibitory effects were observed when heparin was added onto Cyr61-coated microtiter wells and washed before cells were permitted to adhere, suggesting that heparin can bind to immobilized Cyr61 and block cell adhesion (data not shown). The same inhibitory effect was observed with low molecular mass heparin (~3 kDa), thus arguing against the possibility that heparin binding might prevent Cyr61 from inter-
Fig. 2. Fibroblast adhesion to Cyr61 is independent of fibronectin and vitronectin receptors. Cell adhesion assays were performed with 1064SK fibroblasts plated on microtiter wells coated with either Cyr61 (2 μg/ml), type I collagen (Col.I, 2 μg/ml), vitronectin (VN, 0.5 μg/ml), or fibronectin (FN, 1 μg/ml). A, GRGDSP or GRGESP peptide (2 mM) was added to the cell suspension, and cell adhesion assay was performed. B, cells were preincubated with 40 μg/ml mAbs against integrin α5 or integrin αV where indicated prior to adhesion assay. Data shown for all panels are mean ± S.D. of triplicate determinations and are representative of three experiments.

Fig. 3. Integrin αβ1 mediates human skin fibroblast adhesion to Cyr61. Adhesion assays were performed with 1064SK fibroblasts, which were preincubated with indicated mAbs against various integrin subunits at room temperature for 30 min and then plated on microtiter wells coated with either Cyr61 (2 μg/ml), vitronectin (VN, 0.5 μg/ml), fibronectin (FN, 1 μg/ml), type I collagen (Col.I, 2 μg/ml), or laminin (LN, 5 μg/ml) as indicated. A, where indicated (open bar), cells were preincubated with mAb (50 μg/ml) against the integrin β1 subunit. B, cells were preincubated with 40 μg/ml mAbs against integrin α1, α2, α3, and α6 or treated with a mixture of α1, α2, and α3 antibodies (40 μg/ml each). C, cells were preincubated with mAb (20 μg/ml) against integrin α6 subunit. Data shown for all panels are mean ± S.D. of triplicate determinations and are representative of three experiments.
were treated with 40 mM sodium chlorate as above, either in the three experiments.

were plated on microtiter wells coated with Cyr61 (2 µg/ml), increasing amounts of heparin were included in the presence or absence of 10 mM Na$_2$SO$_4$. Data shown for all panels are formed.

cell suspension as indicated before plating and adhesion assay performed. B, cells were cultured in medium containing the indicated amount of sodium chloride for 24 h, washed, harvested, and then plated on Cyr61 (2 µg/ml), fibronectin (FN, 1 µg/ml), type I collagen (Col I, 2 µg/ml), vitronectin (VN, 0.5 µg/ml), or laminin (LN, 5 µg/ml). C, cells were treated with 40 mM sodium chloride as above, either in the presence or absence of 10 mM Na$_2$SO$_4$. Data shown for all panels are mean ± S.D. of triplicate determinations and are representative of three experiments.

To distinguish between these possibilities, we asked whether damaging the integrity of cell surface glycosaminoglycans can affect fibroblast adhesion to Cyr61. 1064SK fibroblasts were cultured in the presence of sodium chlorate, an inhibitor of 3-phosphoadenosine 5'-phosphosulfate synthesis, to block sulfation of proteoglycans (Fig. 4B). Adhesion to Cyr61 was inhibited in a dose-dependent manner when cells were treated with increasing amounts of sodium chloride, and >80% inhibition was achieved at 40 mM sodium chloride. By contrast, adhesion of the same cells to fibronectin, collagen, vitronectin, or laminin was not significantly affected (Fig. 4B). The inhibitory effect of sodium chloride on cell adhesion to Cyr61 was reversed by the inclusion of 10 mM Na$_2$SO$_4$ in the culture medium (Fig. 4C), thus verifying that this inhibitory effect is mediated through a sulfation block (22).

Cell surface-sulfated proteoglycans include heparan sulfate and chondroitin sulfate proteoglycans. To test whether chondroitin sulfate proteoglycans might play a role in Cyr61-mediated adhesion, we examined the effect of various chondroitin sulfates (Fig. 5A). The presence of chondroitin sulfate A, B, or the small chondroitin sulfate proteoglycan decorin, but not chondroitin sulfate C, blocked Cyr61-mediated cell adhesion. However, the concentrations of chondroitin sulfates needed for this inhibition were 2–4 orders of magnitude higher than the effective concentration of heparin (compare 0.1 µg/ml heparin with 1 mg/ml chondroitin sulfate A), suggesting that the affinity of Cyr61 for chondroitin sulfate is relatively weak compared with that for heparan sulfate.

To confirm the importance of cell surface sulfated proteoglycans on fibroblast adhesion to Cyr61, we treated 1064SK fibroblasts with heparinase I and chondroitinase ABC. As shown in Fig. 5B, heparinase I treatment rendered cells unable to adhere to Cyr61, but had no effect on cell adhesion to vitronectin or fibronectin. In contrast, chondroitinase ABC had no effect on cell adhesion to Cyr61, indicating that cell surface chondroitin sulfates do not contribute significantly to human skin fibroblast adhesion to Cyr61. Together, these results show that the presence of cell surface HSPGs are necessary for fibroblast adhesion to Cyr61 and that Cyr61 must be available to bind heparan sulfate to mediate cell adhesion through integrin $\alpha_v\beta_1$.

Adhesive Properties of Heparin-binding Defective Cyr61 Mutants—Although cell surface heparan sulfates are essential for Cyr61-mediated adhesion of fibroblasts (Figs. 4 and 5), whether Cyr61 must interact with heparan sulfate proteoglycans through its heparin-binding activity remained to be determined. To address this question, we created mutant Cyr61 proteins that are deficient in heparin binding. Two putative heparin-binding motifs could be recognized within the Cyr61 carboxyl-terminal domain that conformed to the consensus XB-BBXB sequence for heparin binding, where B denotes basic amino acid residues such as lysine or arginine (27; Fig. 6A).

We performed site-directed mutagenesis to replace the lysine and arginine residues in these heparin-binding motifs with alanine, although maintaining the integrity of the cysteine residues to avoid disruption of any potential disulfide bonds. Two Cyr61 mutants, H1 and H2, were thus created, each altering one of the heparin-binding motifs (Fig. 6A). In addition, both motifs were mutated in another Cyr61 variant, DM. All three mutations were created in the context of the full-length Cyr61, and the full-length mutant proteins were expressed and purified from the baculovirus system in insect cells (“Experimental Procedures”). The recombinant mutant and wild type Cyr61 proteins were bound to a heparin-Sepharose column and eluted with increasing concentrations of salt. Whereas wild type Cyr61 eluted from a heparin-Sepharose column at 0.8 to 1.0 M NaCl as previously reported (2), H1 and H2 eluted at lower salt...
Site-directed mutagenesis created aa substitutions as shown by the B mutations in H1 and H2, resulting in the sequence as shown.

with anti-Cyr61 antibodies. SDS-polyacrylamide gel electrophoresis followed by immunoblotting chloride. An equal amount of eluate from each fraction was analyzed on eluted with buffer containing the indicated concentration of sodium CL-6B heparin-Sepharose column. After washing, bound protein was Sepharose column; FT WT amount of wild type (underlined (1) includes two heparin-binding motifs at the

Wild type (WT) H1 and H2 were able to support adhesion through integrin α6β1 and HSPGs as wild type Cyr61 (data not shown). Importantly, fibroblast adhesion to H1 and H2 was blocked by mAb against the integrin subunit α6, confirming that cell adhesion to these mutants is mediated through integrin α6β1 as wild type Cyr61 (Fig. 7B). These results suggest that the integrin α6β1-binding sites of Cyr61 are distinct from the heparin-binding sites mutated in H1 and H2. The ability of H1 and H2 to support integrin α6β1-mediated cell adhesion is consistent with their ability to bind heparin, albeit at a lower affinity than wild type Cyr61 (Fig. 6B).

By contrast, DM was unable to support 1064SK fibroblast adhesion at any concentration tested, indicating that the intrinsic heparin-binding activity of Cyr61 is essential for mediating fibroblast adhesion (Fig. 6B). In a similar cell adhesion assay, DM was able to support adhesion of HUVECs (Fig. 8), as demonstrated for the wild type Cyr61 protein (4, 18). Furthermore, HUVEC adhesion to the Cyr61 mutant DM was also mediated through integrin α6β1 like the wild type, because this adhesion was inhibited by agents that disrupt α6β1-ligand interaction such as EDTA, RGD-containing peptide, and the anti-α6 mAb LM609. These results show that the Cyr61 mutant DM is conformationally intact and is able to interact with integrin α6β1. Cyr61 interaction with integrin α6β1 is independent of its ability to bind heparin, whereas its interaction with integrin α6β1 to mediate fibroblast adhesion is absolutely dependent on its binding to cell surface HSPGs.

DISCUSSION

The major findings of this study are that human skin fibroblasts adhere to Cyr61 through integrin α6β1, and this adhesion process requires the interaction of Cyr61 with cell surface HSPGs. These observations identify both the adhesion receptor for Cyr61 and a novel adhesive substrate for integrin α6β1 and demonstrate the importance of HSPGs in cell adhesion through this integrin. Furthermore, whereas the heparin-binding activity of Cyr61 is crucial for α6β1-mediated fibroblast adhesion, it is not required for α6β1-mediated adhesion of endothelial cells. These results show a differential requirement for HSPGs in the interaction of Cyr61 with different integrins and suggest that Cyr61 may utilize and signal through different receptors in different cell types, thereby eliciting disparate responses.

The conclusion that human dermal fibroblast adhere to Cyr61 through integrin α6β1 is supported by both biochemical

concentration of mutant protein (2.5–5.0 μg/ml) compared with wild type Cyr61 (1.5–2 μg/ml). Moreover, fibroblast adhesion to both H1 and H2 exhibited the same characteristics as adhesion to wild type Cyr61 with regard to sensitivities to EDTA, divalent cations, RGD-containing peptides, and heparin, suggesting that H1 and H2 supported adhesion through integrin α6β1 and HSPGs as wild type Cyr61 (data not shown). Importantly, fibroblast adhesion to H1 and H2 was blocked by mAb against the integrin subunit α6, confirming that cell adhesion to these mutants is mediated through integrin α6β1 as wild type Cyr61 (Fig. 7B). These results suggest that the integrin α6β1-binding sites of Cyr61 are distinct from the heparin-binding sites mutated in H1 and H2. The ability of H1 and H2 to support integrin α6β1-mediated cell adhesion is consistent with their ability to bind heparin, albeit at a lower affinity than wild type Cyr61 (Fig. 6B).

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must be available and competent to interact with cell surface heparan sulfate proteoglycan for it to support fibroblast adhesion.

Increasing evidence now indicates that HSPGs can function as co-receptors with integrins in cell-matrix interactions (28, 29). For example, syndecans not only act as co-receptors for fibroblast growth factors (22, 29) they also cooperate with integrins in cell adhesion to result in Rho-dependent assembly of focal adhesion plaques and actin stress fibers (30, 31). Induction of β-casein expression by laminin in mammary epithelial cells is mediated through both integrin α6β1 and cell surface HSPGs, again indicating a role in signal transduction for the cooperation between integrins and HSPGs (32). To our knowledge, however, the present study provides the first demonstration of an absolute requirement of HSPGs for integrin-mediated cell attachment. Although adhesion to other heparin-binding extracellular matrix molecules such as fibronectin and thrombospondin also involves cell surface HSPGs, these interactions contribute to adhesion but are not essential for the attachment of cells to substrates (33, 34; also see Fig. 4B).

The specific sequence in Cyr61 that interacts with integrin α6β1 is currently unknown. Aside from laminin-1, integrin α6β1 is known to bind invasin (35), a bacterial protein involved in the invasion of mammalian tissues, and fertilin (36), a sperm surface protein of the ADAM family involved in the binding of sperm to egg. However, there is no clearly discernable sequence similarity between the integrin-binding fragments or peptides derived from these protein ligands and Cyr61 (37–40). Furthermore, three peptides that can bind integrin α6β1 have been identified through a nonsaturating phage display library screening, and these peptides do not share sequence similarity with any of the known protein ligands of integrin α6β1 or with Cyr61 (41). These findings suggest that integrin α6β1 may recognize a broad range of sequences or may recognize structural or conformational features presented by its protein ligands.

Because the Cyr61 mutants H1 and H2 still mediated α6β1-dependent fibroblast adhesion (Fig. 7), it follows that the sequences altered in H1 and H2 are not essential for binding to this integrin. The mutant DM, which combined alterations in H1 and H2, lost the ability to mediate cell adhesion concomitant with the complete loss of heparin-binding activity. These results support the conclusion that the heparin-binding activity of Cyr61 is indispensable for integrin α6β1-mediated fibroblast adhesion. However, endothelial cell adhesion to Cyr61 through integrin α6β1 was unaffected by the heparin-binding status of Cyr61, suggesting fundamental differences between the interaction of Cyr61 with integrins α7β3 and α6β1 (Fig. 8).

Given the high degree of sequence homologies (−40–50% aa
sequence identity) among members of the CCN protein family (3, 9), we anticipate that other CCN proteins might also exhibit similar properties with respect to fibroblast adhesion. Members of this protein family share four conserved structural domains, each encoded by a separate exon (3, 42). The heparin-binding motifs lie (43, 44). Based on our results, we can hypothesize that Cop-1/WISP-2 would be unable to support integrin αβ1-mediated fibroblast adhesion, even though it can mediate adhesion of osteoblasts (45). It is also possible that Cop-1 may counteract the activities of other CCN proteins in a dominant negative manner (46). These possibilities can be experimentally tested to help unravel the distinct functions of each CCN protein.

 Whereas Cyr61 has multiple functions, its roles in fibroblasts are likely related to granulation tissue formation during cutaneous wound healing. The Cyr61 gene is transcriptionally activated in cultured fibroblasts by growth factors implicated in wound healing, including fibroblast growth factor, platelet-derived growth factor, and transforming growth factor-β (1, 12, 11). In addition, cyr61 expression level is minimal in normal dermis and becomes highly induced in dermal fibroblasts in granulation tissue during wound healing (14). A protein closely related to Cyr61, CTGF, is also induced in granulation tissues during wound repair (13). Expression of CTGF has been associated with many fibrotic diseases (47), and subcutaneous injection of CTGF into NIH Swiss mice results in the formation of granulation tissue (48). Furthermore, CTGF regulates collagen and fibronectin gene expression in fibroblasts, suggesting that it may play a role in matrix remodeling in wound healing (13, 48). Thus, considering the biochemical and functional similarities of Cyr61 and CTGF (3, 4), these proteins may play roles in wound healing by inducing angiogenesis upon endothelial cells and stimulating chemotaxis, proliferation, and matrix remodeling upon fibroblasts. In this context, it is interesting to note that whereas laminin is a major component of the basement membrane, its relatively low abundance in the stromal extracellular matrix suggests that its role in wound healing may be limited. Cyr61 may serve as the major adhesive substrate for integrin αβ1 in fibroblasts during dermal wound repair.

 Targeted disruption of the integrin α and β genes share the same phenotype, neonatal death due to epidermolysis bullosa, suggesting that this phenotype is primarily because of the lack of integrin αβ1 in hemidesmosomes between epithelial cells and the basement membrane rather than because of the lack of integrin αβ1 (49, 50). Examination of the α knockout mice also revealed abnormality in the lamination of the developing cerebral cortex and retina (51) where Cyr61 is also expressed (5, 14). Comparison of the expression patterns of Cyr61 and integrin α in mouse embryonic development shows that they are also co-localized in such tissues as the skin epidermis, the nervous system (ganglia, nerves, and spinal cord), and the cardiovascular system (heart and endothelia), suggesting that the potential interaction between Cyr61 and integrin αβ1 may play a role in the development of these organs and tissues (5, 52). Integrin αβ1 has also been implicated in regulating tumor invasion and metastasis (53–55), whereas Cyr61 can promote tumor growth and vascularization (8). It is thus possible to envisage an interaction between Cyr61 and integrin αβ1 during tumorigenesis. The possibility that Cyr61 plays a role in development, wound healing, and tumorigenesis through unique signaling events resulting from its interactions with integrin αβ1 and HSPGs is intriguing and merits further investigation.

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