Pro-angiogenic Activities of CYR61 (CCN1) Mediated through Integrins αvβ3 and α6β1 in Human Umbilical Vein Endothelial Cells*

Shr-Jeng Leu‡, Stephen C.-T. Lam§, and Lester F. Lau‡¶

From the Departments of ¶Molecular Genetics and §Pharmacology, University of Illinois College of Medicine, Chicago, Illinois 60607-7170

CYR61 (CCN1) is an extracellular matrix-associated protein of the CCN family, which also includes CTGF (CCN2), NOV (CCN3), WISP-1 (CCN4), WISP-2 (CCN5), and WISP-3 (CCN6). Purified CYR61 induces neovascularization in corneal implants, and Cyr61-null mice suffer embryonic death due to vascular defects, thus establishing that CYR61 is an important regulator of angiogenesis. Aberrant expression of CYR61 is associated with breast cancer, wound healing, and vascular diseases such as atherosclerosis and restenosis. In culture, CYR61 functions through integrin-mediated pathways to promote cell adhesion, migration, and proliferation. Here we show that CYR61 can also promote cell survival and tubule formation in human umbilical vein endothelial cells. Furthermore, we have dissected the integrin receptor requirements of CYR61 with respect to its pro-angiogenic activities. Thus, CYR61-induced cell adhesion and tubule formation occur through interaction with integrin αvβ3 in early passage endothelial cells in which integrins have not been activated. By contrast, in endothelial cells in which integrins are activated by phorbol ester or vascular endothelial growth factor, CYR61-promoted cell adhesion, migration, survival, growth factor-induced mitogenesis, and endothelial tubule formation are all mediated through integrin αvβ3. These findings indicate that CYR61 is an activation-dependent ligand of integrin αvβ3 and an activation-independent ligand of integrin αvβ3, and that these integrins differentially mediate the pro-angiogenic activities of CYR61. These findings help to define the mechanisms by which CYR61 acts as an angiogenic regulator, provide a molecular interpretation for the loss of vascular integrity and increased apoptosis of vascular cells in Cyr61-null mice, and underscore the importance of CYR61 in the development and homeostasis of the vascular system.

Angiogenesis, the formation of new vessels by spraying from pre-existing ones, is critical for embryonic development, pregnancy, and placenta as well as wound healing and tissue repair (1). Imbalance in angiogenesis can underlie or exacerbate a variety of diseases such as rheumatoid arthritis, diabetic retinopathy, and cancer (2). Angiogenesis begins with degradation of the basement membrane that surrounds the parent vessel, followed by endothelial cell migration, proliferation, and assembly into tubular structures that convey blood supply to target tissues. These processes are regulated by the coordinated interaction of endothelial cells with both angiogenesis-inducing factors and components of the extracellular matrix (3, 4).

CYR61 is a secreted, extracellular matrix-associated angiogenic regulator of the CCN protein family (5, 6), which includes six members: CYR61, CTGF,¹ NOV, WISP-1, WISP-2, and WISP-3 (7–9). Members of the CCN family are organized into four distinct structural domains with sequence similarities to insulin-like growth factor-binding proteins, von Willebrand factor type C repeat, thrombospondin type I repeat, and carboxy-terminal domains of extracellular matrix proteins such as von Willebrand factor and mucins. Encoded by a growth factor-inducible immediate-early gene, CYR61 is a cysteine-rich matricellular protein that supports cell adhesion and induces adhesion signaling (10–12). Furthermore, CYR61 stimulates endothelial cell migration and enhances growth factor-induced DNA synthesis in culture (10) and induces angiogenesis in vivo (13).

The essential nature of CYR61 as a regulator of vascular development has been established through gene targeting studies in mice (14). Cyr61-null mice suffer embryonic death due to vascular defects that include undervascularization of the placental labyrinth and loss of vascular integrity in the embryo. Large vessels of CYR61-deficient mice show a disorganized basal lamina, paucity of smooth muscle cells that normally comprise the vessel wall, and vascular cells undergoing apoptosis (14). Consistent with the angiogenic activity of CYR61, its expression is elevated in healing wounds (15, 16). Furthermore, overexpression of Cyr61 promotes tumor growth and vascularization (13, 17) and is associated with human breast cancer (17–19).

Mechanistically, CYR61 acts as a non-RGD-containing ligand of integrin receptors (8). Integrins are heterodimeric cell-surface receptors capable of transducing extracellular signals and functions to regulate cell adhesion, motility, proliferation, survival, and differentiation (20, 21). Thus, extracellular ligand binding to integrins can transduce "outside-in signaling" similar to a growth factor receptor. Conversely, intracellular molecules that interact with the integrin cytoplasmic domains can also induce "inside-out signaling," thereby "activating" the integrin to adopt a conformational change that results in en-

¹ The abbreviations used are: CTGF, connective tissue growth factor; VEGF, vascular endothelial growth factor; BSA, bovine serum albumin; PMA, phorbol 12-myristate 13-acetate; mAb, monoclonal antibody; LIBS, ligand-induced binding site; HUVEC, human umbilical vein endothelial cell; TUNEL, terminal deoxynucleotidyltransferase-mediated biotinylated UTP nick end labeling.
hanced affinity for ligands (22). Recent studies indicate that integrin αβ3, a prominent integrin in angiogenic endothelial cells, can be activated either through serial passage in culture or by stimulation with agonists such as growth factors and phorbolester tumor promoters. Activation can also be accomplished by binding of monoclonal antibodies or mAbs to the extracellular domains of integrins, thereby inducing a conformational change (22–24).

In this study, we have uncovered two additional pro-angiogenic activities for CYR61 that have not been previously described, namely the promotion of endothelial cell survival and tubule formation. To understand the mechanisms of CYR61 actions, we have identified the integrin receptors mediating each of five pro-angiogenic activities: endothelial cell adhesion, migration, proliferation, survival, and tubule formation. We show that integrin αβ3 mediates CYR61-supported cell adhesion and tubule formation in unactivated endothelial cells. By contrast, when integrins are activated by stimulation of endothelial cells with agonists, CYR61 promotes cell adhesion, migration, proliferation, survival, and tubule formation through integrin αβ3.

MATERIALS AND METHODS

Proteins, Antibodies, Peptides, and Reagents—Recombinant murine CYR61 protein was purified from serum-free insect cell-conditioned medium using the baculovirus expression system (Invitrogen) as described (10). Rat type I collagen, vitronectin, laminin, Matrigel, and VEGF were purchased from Collaborative Biomedical (Bedford, MA). BSA, heparin (sodium salt, from porcine intestinal mucosa), PMA, and control normal mouse IgG were obtained from Sigma. PMA was prepared as 2 mM stock solutions in MeSO. GRGDSP and GRGESP synthetic peptides and function-blocking mAbs against integrin β3 (clone P4C10) were from Invitrogen. Function-blocking mAbs against integrin αvβ3 (clone LM609, azide-free) and integrin αvβ5 (clone CuBS, azide-free) were from Chemicon International, Inc. (Temecula, CA) and Immuno- tech (Marseille, France), respectively. The affinity-purified integrin β3-activating anti-LIBS-6 mAb was kindly provided by Dr. Mark Ginsberg (Scirps Research Institute, La Jolla, CA).

Cell Culture and Adhesion Assay—Primary HUVECs were maintained at 37°C with 5% CO2 in Medium 200 containing 2% serum and endothelial growth supplements according to the manufacturer's instructions (Cascade Biologics). Cells were used in passages 5–10 for all experiments. HUVEC adhesion assays were carried out essentially as described (10). Briefly, 96-well microtiter plates (BD Biosciences) were coated with test proteins diluted in phosphate-buffered saline at 50 μg/ml and then incubated at 4°C for 16 h, followed by blocking with 1% BSA at room temperature for 1 h. Subconfluent HUVECs were washed twice with phosphate-buffered saline containing 1 mM EDTA and 0.1% glucose and harvested by incubation in the same buffer for 15 min at room temperature. Cells were washed and resuspended in serum-free basal medium containing 0.2% BSA and 10 μM HEPES (pH 7.2) at 7 × 10^5 cells/ml. Where indicated, cells were stimulated with PMA, VEGF, or Mn2+ for 30 min to activate integrin receptors. EDTA, peptides, or function-blocking mAbs were mixed with cells for an additional 30 min prior to plating. The cell suspension was plated at 50 μl/well and allowed to adhere to protein-coated wells at 37°C for 20 min, followed by washing twice with phosphate-buffered saline. Adherent cells were fixed with 10% Formalin and stained with methylene blue. Adhesion was quantified by dye extraction and measurement of absorbance at 620 nm as described (10). Vehicle buffers containing MeSO (ranging from 0.001 to 0.005%) had no effect on cell adhesion.

Cell Migration Assay—Cell migration was monitored as described (26). Using Transwell chambers (Corning Costar) with tissue culture-treated filter membranes separating the upper and lower chambers. The lower surfaces of polycarbonate filters were coated with BSA or treated filter membranes separating the upper and lower chambers. (25) using Transwell chambers (Corning Costar) with tissue culture-

Thymidine Incorporation Assay—DNA synthesis was assessed as described (10) with minor modifications. Briefly, subconfluent HUVECs were plated on 24-well plates (3 × 10^5 cells/plate) in serum-free medium for 16 h and starved in serum-free basal medium with 0.5% BSA for 24 h. Cells were then either left untreated or incubated with mAbs in serum-free basal medium for 1 h as indicated prior to their removal and addition of test proteins. [3H]Thymidine (5 μCi/ml), CYR61, or VEGF was simultaneously added to the wells in medium containing 0.5% charcoal-stripped serum (Sigma). After 48 h of incubation, cells were washed with phosphate-buffered saline and fixed with 10% trichloroacetic acid. DNA was extracted in 0.1 N NaOH, and thymidine incorporation was measured using a scintillation counter.

Measurement of Apoptosis—Subconfluent HUVECs were serum-starved for 24 h and harvested. Cells were suspended in serum-free medium with 0.2% BSA and stimulated with PMA or the integrin β3-activating anti-LIBS-6 mAb for 1 h. Glass coverslips (18 × 18 mm) were coated with poly-L-lysine, vitronectin, CYR61, or laminin for 16 h at 4°C and then blocked with 1% BSA. Cells were seeded on the glass coverslips at low cell density (10,000 cells/cm²) and allowed to adhere to these different matrix coatings, and challenged with serum-free medium containing 0.2% BSA. After 20 h of incubation, cells were fixed in 4% paraformaldehyde for 30 min, and apoptosis was monitored by TUNEL assays using an in situ cell death detection kit (Roche Molecular Biochemicals). Prior to analysis of apoptotic nuclei, cells were lightly counterstained with hematoxylin, and total cell numbers were examined. The total numbers of cells attached to different matrix coatings, and stained with propidium iodide were counted. The extent of apoptosis was represented as a percentage of apoptotic cells versus the total; at least 500 cells were counted from random fields in each slip.

Endothelial Tube Formation—The formation of capillary-like tubules by HUVECs was evaluated using a three-dimensional collagen gel as described (26) with modifications. Briefly, cells were harvested and resuspended in serum-free basal medium with 0.2% BSA. A mixture of type I collagen gels, CYR61 protein stocks (0.5 mg/ml) or vehicle buffer (containing 0.75 nM NaCl and 50 mM phosphate buffer), and neutralization buffer (260 mM NaHCO3, 200 mM HEPES, and 50 mM NaOH) was mixed at a ratio of 7:2:1 by volume and kept on ice to prevent gelation. Cold collagen mixtures (250 μl) were put in 24-well plates, and gelation occurred at 37°C for 1 h. Unstimulated or PMA or CYR61 or VEGF-stimulated HUVECs were inoculated on the wells at 125,000 cells/cm² and cultured at 37°C. After 16 h of incubation, the medium was removed, and a second collagen gel mixture of identical components was overlaid on the cells. After 10 min of gel polymerization at 37°C, 0.5 ml of supplemented serum-free medium was added to each well. Formation of apparent endothelial tubules was scored 16–20 h thereafter.

RESULTS

Differential Utilization of Integrins αβ3 and αβ6 as Adhesion Receptors for CYR61 in HUVECs—CYR61 is a ligand of integrin αβ3 and supports HUVEC adhesion through interaction with this integrin (27). To investigate the effects of integrin activation on CYR61 action, we examined the characteristics of adhesion of unstimulated early passage HUVECs to CYR61 compared with cells stimulated to activate integrins. As shown in Fig. 1A, unstimulated early passage HUVECs adhered to CYR61. However, only a minor fraction of this adhesion was inhibited by agents that disrupt integrin αβ3 function such as GRGDSP peptide (24% inhibition) or LM609 (19% inhibition). Neither the control peptide GRGESP nor normal mouse IgG had any effect on cell adhesion to CYR61, whereas EDTA abolished cell adhesion completely (Fig. 1A). These results show that in naive early passage HUVECs, integrin αβ3 is not the principal adhesion receptor for CYR61. Another cation-dependent receptor is required for adhesion to CYR61.
Washed HUVECs resuspended in serum-free medium were plated on BSA. Cells were either left unstimulated (H9262 mouse IgG for another 30 min before plating. Cells were allowed to adhere to microtiter wells at 37 °C for 20 min. Adherent cells were fixed, stained with methylene blue, and quantified by absorbance at 620 nm.

**Fig. 1. Effects of HUVEC activation on adhesion to CYR61.** Washed HUVECs resuspended in serum-free medium were plated on microtiter wells coated with CYR61 (15 μg/ml) and blocked with 1% BSA. Cells were either left unstimulated (A) or stimulated with 100 nM PMA (B) or 10 ng/ml VEGF (C) for 30 min to activate integrin receptors. As indicated, cells were also incubated with vehicle buffer (No Add), 10 mM EDTA, 0.2 mM GRGDSP peptide (RGDS), 0.2 mM GRGESP peptide (RGES), 25 μg/ml anti-integrin α6β1, mAb LM609, or 100 μg/ml normal mouse IgG for another 30 min before plating. Cells were allowed to adhere to microtiter wells at 37 °C for 20 min. Adherent cells were fixed, stained with methylene blue, and quantified by absorbance at 620 nm. Data are means ± S.D. of triplicate determinations and are representative of four separate experiments.

Treatment of endothelial cells with agonists such as the phorbol ester PMA, the divalent cation Mn2+, and the angiogenic growth factor VEGF can rapidly increase integrin α6β1 activity via enhancement of ligand binding affinity without changing the receptor expression profile (28, 29). When HUVECs were stimulated with PMA to activate integrin α6β1, total cell adhesion to CYR61 was significantly increased (∼1.6-fold) (Fig. 1B). This PMA-dependent enhancement of cell adhesion to CYR61 was inhibited by GRGDSP peptide or mAb LM609, indicating that this cell adhesion is mediated through integrin α6β1. Similarly, following stimulation with the angiogenic growth factor VEGF (10 ng/ml), HUVEC adhesion to CYR61 was increased by −2-fold (Fig. 1C). This increment was also blocked by GRGDSP peptide or mAb LM609, but not by the control peptide GRGESP or by normal mouse IgG. In addition to these two agents that activate integrins through inside-out signaling, we observed that Mn2+ (1 mM), which can activate integrins by binding to the extracellular domain and inducing a conformational change (30), also enhanced HUVEC adhesion to CYR61 (data not shown). Together, these results indicate that activation of endothelial cell integrins by PMA, VEGF, or Mn2+ greatly enhances cell adhesion to CYR61 through integrin α6β1. The diversity of these agonists indicates that it is activation of integrins, rather than other cellular events, that is responsible for the enhanced cell adhesion. However, a residual level of HUVEC adhesion to CYR61 remained even in the presence of α6β1 antagonists (e.g., RGD-containing peptides or mAb LM609), again suggesting that another receptor might also contribute to endothelial cell adhesion to CYR61 (27).

In light of the recent finding that fibroblasts and smooth muscle cells adhere to CYR61 through integrin α6β1 (31, 32), we explored the possible involvement of integrin α6β1 in mediating HUVEC adhesion to CYR61. As shown in Fig. 2A, unstimulated HUVEC adhesion to CYR61 was effectively inhibited (−80%) by mAb against the integrin α6 (GoH3) or β1 (P4C10) subunit. By contrast, mAb against integrin α6β1 (LM609) minimally inhibited adhesion (−20%). These results indicate that integrin α6β1 is the primary adhesion receptor for CYR61 in naive HUVECs. Notably, a combination of LM609 and GoH3 or of LM609 and P4C10 completely abrogated cell adhesion to CYR61, indicating that integrins α6β1 and α6β3 together serve as the adhesion receptors for CYR61 in HUVECs.

In fibroblasts and smooth muscle cells, adhesion to CYR61 requires both integrin α6β1 and heparin sulfate proteoglycans serving as co-receptors, and occupancy of the CYR61 heparin-binding site by soluble heparin abolishes its ability to support cell adhesion (31, 32). Likewise, we found that soluble heparin diminished CYR61-supported HUVEC adhesion by −80%, similar to inhibition by mAb GoH3 or P4C10 (Fig. 2A). Administration of a combination of soluble heparin and GRGDSP peptide, which inhibit cell adhesion mediated through integrins α6β1 and α6β3, respectively, abolished adhesion completely. These results further support the conclusion that HUVEC adhesion to CYR61 is mediated through both integrins α6β1 and α6β3. Nonetheless, integrin α6β1 functions in cooperation with heparin sulfate proteoglycans to serve as the principal adhesion receptor in unstimulated HUVECs.

Activation of integrin α6β1 by PMA or VEGF stimulation of HUVECs strongly enhanced the affinity of this integrin for CYR61, rendering α6β1, instead of α6β3, the principal adhesion receptor (Fig. 2, B and C). After activation by either agonist, mAb against integrin α6β1 (LM609) inhibited cell adhesion by −75%, whereas mAbs against integrin α6β3 (GoH3 and P4C10) inhibited cell adhesion only by 15–20%. The combined administration of LM609 and P4C10 or of GRGDSP peptide and heparin completely abrogated cell adhesion to CYR61. Together, these results show that integrin α6β1 is the principal adhesion receptor for CYR61 in unstimulated HUVECs. Upon stimulation, integrin α6β3 becomes the major receptor for CYR61, and integrin α6β1 retreats to play an auxiliary role.

**HUVEC Migration to CYR61 Is Mediated through Activated Integrin α6β1**—It was previously shown that CYR61 induces integrin α6β1-dependent chemotaxis in human microvascular endothelial cells (13). Inasmuch as integrin α6β1 acts as another receptor for CYR61 in HUVECs as described above, we
sought to determine the role of this integrin in HUVEC migration. Cell migration was evaluated using a modified Boyden chamber assay. Where indicated, prior to plating, cells were preincubated with vehicle buffer (No Add), RGDS peptide (0.2 mM), RGES peptide (0.2 mM), LM609 (25 μg/ml), GoH3 (25 μg/ml), PAC10 (1:50 ascites), heparin (1 μg/ml), or control normal mouse IgG (100 μg/ml). Cells were placed in the upper chamber, and cells that migrated to the lower chamber were counted in 10 high power fields as described under “Materials and Methods.” Results are expressed as numbers of migrated cells/field. Data are means ± S.D. of triplicate determinations and are representative of four separate experiments.

Fig. 3. Activated integrin αβ₃ mediates HUVEC migration to CYR61. Migration of HUVECs to CYR61 was measured using Transwell chambers. Where indicated, prior to plating, cells were preincubated with vehicle buffer (No Add), RGDS peptide (0.2 mM), RGES peptide (0.2 mM), LM609 (25 μg/ml), GoH3 (25 μg/ml), PAC10 (1:50 ascites), heparin (1 μg/ml), or control normal mouse IgG (100 μg/ml). Cells were placed in the upper chamber, and cells that migrated to the lower chamber were counted in 10 high power fields as described under “Materials and Methods.” Results are expressed as numbers of migrated cells/field. Data are means ± S.D. of triplicate determinations and are representative of four separate experiments.

Fig. 2. Differential adhesion of endothelial cells to CYR61 through integrins αβ₆ and αβ₃. Unstimulated (A), PMA-activated (B), or VEGF-stimulated (C) HUVECs were plated on microtiter wells precoated with CYR61 (15 μg/ml) and blocked with 1% BSA. Where indicated, HUVECs were preincubated with vehicle buffer (No Add), GoH3 (25 μg/ml), PAC10 (1:50 ascites), LM609 (25 μg/ml), normal mouse IgG (100 μg/ml), heparin (1 μg/ml), or RGDS peptide (0.2 mM). Cell adhesion was measured as described in legend to Fig. 1. Data are means ± S.D. of triplicate determinations and are representative of four separate experiments.

of migration toward CYR61. (The background level of migrated cells was 14 ± 3 cells/field on BSA-coated membrane versus 47 ± 6 cells/field on CYR61-coated membrane.) Either GRGDSP peptide or LM609 blocked CYR61-induced cell migration, suggesting that the cell migration activity is mediated through a low level of activated integrin αβ₃. However, anti-integrin αβ₆ (GoH3) and anti-integrin β₁ (P4C10) mAbs or soluble heparin had no effect (Fig. 3B). Stimulation with PMA greatly enhanced HUVEC migration toward CYR61 (285 ± 24 cells/field in PMA-stimulated cells versus 47 ± 6 cells/field in unstimulated cells) (Fig. 3, A and B). This enhanced cell migration was obliterated by integrin αβ₃ antagonists (GRGDSP peptide and LM609), but not by integrin αβ₁ antagonists (GoH3 and PAC10), and soluble heparin also had no effect. These data show that HUVEC migration toward CYR61 is dependent upon activated integrin αβ₃, whereas integrin αβ₁ plays no role in this process.

CYR61 Enhances VEGF-induced DNA Synthesis through Activated Integrin αβ₃—Although CYR61 is not mitogenic by itself, it enhances growth factor-induced DNA synthesis in fibroblasts and endothelial cells (27, 33). To understand the mechanism of CYR61 action, we tested the effect of CYR61 on HUVEC mitogenesis. As expected, CYR61 added alone to HUVECs had no effect on DNA synthesis (Fig. 4), and PMA alone or in combination with CYR61 also did not stimulate DNA synthesis (data not shown). DNA synthesis was induced when HUVECs were stimulated with 5 ng/ml VEGF, which is suboptimal as determined in a dose-response titration (data not shown). However, the presence of CYR61 was able to induce an
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Fig. 4. CYR61 enhances VEGF-induced DNA synthesis through integrin α6β1. After serum starvation, HUVECs were pretreated with anti-integrin α6β1 mAb LM609 (25 μg/ml), anti-integrin α6 mAb GoH3 (25 μg/ml), or control normal mouse IgG (25 μg/ml). Cells were then treated with VEGF (5 ng/ml) and/or CYR61 (5 μg/ml) in the presence of [3H]thymidine, incorporation of which was measured 48 h thereafter. Data are means ± S.D. of triplicate determinations and are representative of three experiments.

Additional 2-fold enhancement of DNA synthesis over VEGF treatment alone (Fig. 4). This CYR61-enhanced DNA synthesis (but not DNA synthesis induced by VEGF alone) was completely abolished by LM609. By contrast, GoH3 had no effect on CYR61-enhanced or VEGF-induced DNA synthesis. These data show that CYR61 enhances VEGF-induced HUVEC DNA synthesis through ligation to activated integrin α6β3, and not integrin α6β1.

CYR61 Promotes Endothelial Cell Survival through Integrin α6β3—A number of angiogenic factors can protect endothelial cells from apoptotic death, although the effect of CYR61 on endothelial cell survival is unknown. To investigate this question, we plated HUVECs on poly-L-lysine, vitronectin, laminin, or CYR61. Cells were either left unstimulated or stimulated with PMA. Additionally, a class of mAbs against integrins called anti-LIBS antibodies recognize ligand-induced binding sites that become exposed when integrins have assumed the activated conformation upon ligand binding. At sufficiently high concentrations, some anti-LIBS antibodies can bind to integrins and force them to assume the activated conformation in the absence of ligand. The integrin β3-activating anti-LIBS-6 mAb is one such antibody (34) and was employed as an alternative and specific means to activate β3 integrins (Fig. 5). After incubation, apoptosis was analyzed using a TUNEL assay. Cells plated on poly-L-lysine were attached through non-integrin-mediated electrostatic interactions, and the majority of these cells (>90%) underwent apoptosis even in the presence of PMA or anti-LIBS-6 mAb stimulation (Fig. 5). Unstimulated HUVECs attached to vitronectin, a well characterized extracellular matrix ligand capable of conferring anti-apoptotic signals (35, 36), showed a minimal apoptotic response (~20%); PMA or anti-LIBS-6 mAb pretreatment of cells did not provide further protection against apoptosis. Previous studies showed that laminin, a ligand of several integrins including α6β1, failed to sustain endothelial cell survival even in a defined medium containing a high dose of basic fibroblast growth factor (25 ng/ml) due to the inability of laminin to engage integrin receptors that transduce anti-apoptotic signals (37). Consistent with these observations, a large fraction of HUVECs were apoptotic (~65%) when plated on laminin, irrespective of the activation state (Fig. 5). When unstimulated HUVECs adhered to CYR61, a high apoptotic rate (~60%) was observed. By contrast, treatment of HUVECs with PMA or anti-LIBS-6 mAb to activate integrin α6β3 resulted in significant enhancement of cell survival, thereby reducing the fraction of apoptotic cells to ~25%. These results are consistent with the interpretation that in unstimulated HUVECs, cells adhere to CYR61 through integrin α6β1, which is unable to transduce cell survival signals. When HUVECs are stimulated to activate integrin α6β3, however, cells attached to CYR61 are protected from apoptotic death through engagement of integrin α6β3.

CYR61 Induces Tube Formation in HUVECs through Integrins α6β3 and α6β1—Although CYR61 has been shown to induce angiogenesis in corneal micropocket implants, its ability to induce tubule formation in endothelial cells has not been demonstrated. We utilized a collagen gel assay to assess the ability of purified CYR61 to induce endothelial cell differenti-
Fig. 6. CYR61 induces integrin αβ₁-dependent endothelial tubule formation in unstimulated HUVECs. HUVECs were plated on 24-well plates precoated with type I collagen gels (2 mg/ml) in the absence (A) or presence (B–H) of purified CYR61, and a second layer of gel was overlaid on the attached cells as described under "Materials and Methods." Tubule formation was assessed 16–20 h thereafter. A, the collagen gel was formulated with CYR61 buffer. B–E, the collagen gels were formulated with 25, 50, 75, and 100 μg/ml CYR61, respectively, with endothelial tubules becoming evident at 25–50 μg/ml CYR61 (indicated by arrows in B and C). F, the presence of LM609 (40 μg/ml) failed to inhibit tubule formation induced by CYR61 (100 μg/ml) in the collagen gel. G and H, addition of GoH3 (40 μg/ml) and P4C10 (1:50 ascites), respectively, effectively blocked tubule formation. Results are representative of three separate experiments, each performed in duplicate (magnification ×100).

Fig. 7. CYR61 induces integrin αβ₁-dependent endothelial tubule formation in PMA-activated HUVECs. Tubule formation of HUVECs was examined in type I collagen gel (2 mg/ml) with (+) or without (−) CYR61 (25 μg/ml) as described in the legend to Fig. 6. HUVECs were harvested and resuspended in serum-free medium and then stimulated with PMA (5 nM) or vehicle buffer (No Add). Unstimulated HUVECs showed no tubule formation in the absence of CYR61 (A), and 25 μg/ml CYR61 barely induced any tubule formation (B). PMA-stimulated HUVECs displayed tubular structures (C), which became much more extensive in the presence of CYR61 (D). Addition of LM609 (40 μg/ml) to cell suspensions prior to plating inhibited induction of endothelial tubules (E and F), whereas addition of GoH3 (40 μg/ml) had little effect on tubule formation (G and H). Results are representative of three separate experiments (magnification ×200).

CYR61 barely induced any tubule formation in unstimulated cells (Fig. 7B), whereas PMA stimulation greatly enhanced the tubule formation response (Fig. 7D). Administration of LM609 inhibited endothelial morphogenesis in PMA-treated HUVECs in both the presence and absence of CYR61. By contrast, GoH3 or control normal mouse IgG had little effect (Fig. 7, E–H) (data not shown). Together, these data show that CYR61 induces endothelial tubule formation through integrin αβ₁ in unstimulated HUVECs, but that it induces more extensive endothelial morphogenesis when integrin αβ₃ has been activated.

DISCUSSION

The primary findings of this study provide new insights into the angiogenic actions of CYR61, an essential regulator of mammalian vascular development. Functionally, we have documented two novel activities of CYR61, establishing its ability to promote vascular endothelial cell survival and tubule formation. Mechanistically, we have identified the cell-surface receptors mediating each of five pro-angiogenic activities of CYR61. In agonist-stimulated endothelial cells in which integrins are activated, CYR61-promoted cell adhesion, migration, survival, growth factor-induced mitogenesis, and endothelial tubule formation are all mediated through integrin αβ₁. By contrast, CYR61-induced cell adhesion and tubule formation occur through interaction with integrin αβ₃ in early passage endothelial cells in which integrins have not been activated. These findings indicate that CYR61 is an activation-dependent ligand...
of integrin \( \alpha_\beta_3 \) and an activation-independent ligand of integrin \( \alpha_\beta_1 \), provide a mechanistic interpretation for certain phenotypes in Cyr61-null mice, and suggest a role for Cyr61 in vessel development and maintenance.

For new vessels to form, endothelial cells must migrate, proliferate, and differentiate into tubule structures. Consistent with these requisite cellular processes, Cyr61 has been shown to promote endothelial cell adhesion, migration, and proliferation in culture (10) and to induce angiogenesis in corneal implants (13). Data presented herein show that Cyr61 also promotes endothelial cell survival (Fig. 5) and formation of endothelial tubules (Figs. 6 and 7). Together, these findings demonstrate that in a purified form, Cyr61 can act directly upon endothelial cells to promote each of the requisite cellular steps of angiogenesis. Cyr61 is also known to up-regulate the expression of other potent angiogenic inducers such as VEGF-A and VEGF-C in fibroblasts (15). Thus, the angiogenic actions of Cyr61 can be both direct and indirect.

During embryonic development, Cyr61 expression is notable in the cardiovascular system, especially in vascular endothelial cells and in smooth muscle cells surrounding the larger vessels (16, 23). The importance of Cyr61 in vascular development has been established by mutational analysis via gene targeting in mice. Cyr61-null mice suffer undervascularization of the placental labyrinth and loss of vascular integrity in large vessels, resulting in embryonic death (14). In particular, large vessels in Cyr61-null embryos display a disorganized basal lamina with vascular cells undergoing apoptosis. This finding is consistent with the observation that by acting as an adhesion substrate, Cyr61 can provide an anti-apoptotic function to endothelial cells (Figs. 5). Enhanced cell survival is critical for endothelial cells in the angiogenic process as they undergo migration, mitogenesis, and differentiation. It is interesting to note that WISP-1, a closely related protein of the CCN family, can promote cell survival through activation of the Akt pathway (38).

Cyr61 acts as a non-RGD-containing ligand of integrin receptors, which mediate many of its activities. The utilization of integrins by Cyr61 is cell type- and function-specific. For example, cell adhesion to Cyr61 in platelets and monocytes is mediated through integrins \( \alpha_\beta_3 \) and \( \alpha_\beta_2 \), respectively, whereas cell adhesion and migration in smooth muscle cells are mediated through integrin \( \alpha_\beta_1 \) (32, 39, 40). Remarkably, Cyr61 promotes fibroblast adhesion, migration, and proliferation through integrins \( \alpha_\beta_1 \), \( \alpha_\beta_3 \), and \( \alpha_\beta_2 \), respectively (31, 41). In this study, we have shown that Cyr61 can selectively utilize either integrin \( \alpha_\beta_2 \) or \( \alpha_\beta_3 \) in endothelial cells to mediate cell adhesion, depending on the activation state of the cell. Together, these findings demonstrate that Cyr61 interacts with distinct integrins in a cell type-, function-, and activation state-specific manner to mediate disparate biological activities.

Integrins are capable of transducing extracellular signals into the cell via ligand binding in outside-in signaling, analogous to signal transduction by growth factor receptors (20, 21). It has been recognized that integrins are also capable of inside-out signaling, whereby interaction of intracellular molecules with the cytoplasmic domains of the integrin subunits can lead to a conformational change in the extracellular domains, resulting in an “activated” conformation with increased ligand binding affinity (22). Inside-out signaling has been particularly well established for integrins \( \beta_2 \) and \( \beta_3 \), and these integrins can be activated by either physiological (e.g. growth factors and cytokines) or nonphysiological (e.g. antibodies and Mn\(^{2+}\)) agents. In this study, we have shown that Cyr61 is another example of an activation-dependent ligand of integrin \( \alpha_\beta_3 \). It is notable that other ligands such as fibrinogen can bind integrin \( \alpha_\beta_3 \) in an activation-independent manner (30). \( \beta_1 \) integrins also undergo activation, as demonstrated by the existence of anti-LIBS antibodies against the \( \beta_1 \) subunit (22). However, activation does not appear to alter the affinity of integrin \( \alpha_\beta_1 \) for Cyr61.

A wealth of evidence indicates that integrins play important roles in angiogenesis, and in particular, signaling through \( \alpha_\beta_3 \) integrins has long been implicated in angiogenic events (3). A number of angiogenic regulators can bind directly to integrins, which mediate at least some of their activities (43). VEGF- and basic fibroblast growth factor-induced angiogenesis has been shown to require integrins \( \alpha_\beta_1 \) and \( \alpha_\beta_3 \), respectively, in angiogenesis assays using rabbit corneal pocket implants and chick chorioallantoic membrane (3). Furthermore, integrins have been known to associate with growth factor receptors, and cross-talk between the two receptor systems is evident (21, 43). Notably, VEGF receptors can associate with \( \alpha_\beta_3 \) integrins and can function to activate integrin \( \alpha_\beta_3 \) strongly (28, 44). Thus, it is surprising that extensive vasculosclerosis, angiogenesis, and organogenesis can occur in mice that lack all \( \alpha_\beta_3 \) integrins, indicating that the roles of \( \alpha_\beta_3 \) integrins are nonessential in the early phases of mammalian development (45). All \( \alpha_\beta_3 \)-null mice develop normally to embryonic day 9.5, although 80% die in mid-gestation due to deficient placentalization, and the remaining 20% are born alive, but suffer intracerebral and intestinal hemorrhages. Interestingly, pathological angiogenesis is clearly abnormal in integrin \( \beta_1 \) and \( \beta_3 \) knockout mice, suggesting that molecular requirements may differ for developmental versus pathological angiogenesis (46).

Integrin \( \alpha_\beta_1 \) is the principal adhesion receptor for Cyr61 in unactivated endothelial cells, and Cyr61 induces endothelial tubular networks in both collagen gels and Matrigel (Fig. 6) (data not shown). Matrigel, a basement membrane extract, can support a basal level of endothelial tubule formation without additional angiogenic factors and can promote tumor growth in mice when co-injected with tumor cells (47). Although the signals that promote tubule formation in Matrigel are not well defined, it was found to be dependent upon integrin \( \alpha_\beta_1 \), rather than integrin \( \alpha_\beta_3 \) (48–50). Given that Cyr61 can induce endothelial tubule formation via integrin \( \alpha_\beta_1 \), we explored the possible presence of CCN family members in Matrigel. Interestingly, we found that CTGF can be detected by immunoblotting in the range of 2–5 \( \mu \)g/ml, depending on the batch of Matrigel (data not shown). Inasmuch as the cellular and angiogenic activities of Cyr61 and CTGF are very similar (39), this concentration of CTGF is likely close to the active range. Thus, endothelial tubule formation observed in Matrigel in the absence of additional factors may be due, at least in part, to the activities of CTGF acting through integrin \( \alpha_\beta_3 \).

Although it is notable that \( \beta_3 \)-null mice fail to develop a vasculature (51), it is unclear which \( \beta_3 \) integrin plays the most critical roles in vascular development. Although the precise role of integrin \( \alpha_\beta_3 \) in angiogenesis is currently unknown, our data indicate that integrin \( \alpha_\beta_3 \) may participate in the angiogenic process. To date, only a few ligands are known for integrin \( \alpha_\beta_3 \); these include laminin, Cyr61, CTGF, and a collagen fragment known as tumstatin (11, 31, 52). In addition, the bacterial protein invasin and the sperm surface protein fertilin also bind integrin \( \alpha_\beta_1 \), although these proteins are not expected to play a role in developmental angiogenesis (53, 54). Tumstatin is a 28-kDa fragment of the type IV collagen \( \alpha_3 \) chain that inhibits angiogenesis, displays both anti-angiogenic and pro-apoptotic activities, and binds both integrins \( \alpha_\beta_2 \) and \( \alpha_\beta_3 \) (52). Two RGD-independent integrin \( \alpha_\beta_3 \)-binding sites in tumstatin confer anti-angiogenic activities, although the role of
integrin αβ3 in tumstatin function is unknown (55). Interestingly, whereas short-term stimulation of endothelial cells by angiogenic cytokines such as fibroblast growth factor-β leads to acute activation of integrins, prolonged treatment leads to down-regulation of integrin αβ3 and up-regulation of integrin αβ1 (42). Thus, it is tempting to speculate that during the early phase of vessel sprouting, activated endothelial cells may utilize integrins to mediate the angiogenic response. In the late phases of angiogenesis and upon withdrawal of inducing signals, maintenance of vessel integrity may occur via integrin αβ1. The possibility that integrins αβ3 and αβ1 may be selectively utilized to play distinct roles in vessel regression and maintenance during different phases of angiogenesis is intriguing and merits further investigation.

Acknowledgment—We are grateful to Dr. Mark Ginsberg for the generous gift of the anti-LIBS-6 antibody.

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11. Acknowledgment—We are grateful to Dr. Mark Ginsberg for the generous gift of the anti-LIBS-6 antibody.

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Shr-Jeng Leu, Stephen C.-T. Lam and Lester F. Lau

J. Biol. Chem. 2002, 277:46248-46255.
doi: 10.1074/jbc.M209288200 originally published online October 2, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M209288200

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