Adhesion of Human Umbilical Vein Endothelial Cells to the Immediate-Early Gene Product Cyr61 Is Mediated through Integrin $\alpha_v\beta_3$*

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Cyr61 is a member of a family of growth factor-inducible immediate-early gene products thought to act cooperatively with the activities of growth factors. Upon synthesis, Cyr61 is secreted and is predominantly incorporated into the extracellular matrix. Recently, we demonstrated that Cyr61 promotes cell adhesion and migration and augments growth factor-induced DNA synthesis (Kireeva, M. L., Mo, F.-E., Yang, G. P., and Lau, L. F. (1996) Mol. Cell. Biol. 16, 1326–1334). In the present study, we investigated possible candidate receptor(s) on human umbilical vein endothelial cells (HUVECs) mediating adhesion to Cyr61. Under both serum-containing and serum-free conditions, adhesion of HUVECs to Cyr61 was dose-dependent, saturable, and abolished by affinity-purified anti-Cyr61 antibodies. Cell adhesion to Cyr61 was divalent cation-dependent and specifically inhibited by the peptide RGDS and LM609, a monoclonal antibody against integrin $\alpha_v\beta_3$. Furthermore, purified $\alpha_v\beta_3$ bound directly to an affinity matrix of Cyr61-coupled Sepharose 4B, and this interaction was specifically blocked by anti-Cyr61 antibodies. Additionally, in a solid phase binding assay, soluble Cyr61 bound to immobilized $\alpha_v\beta_3$ in a dose-dependent manner, and half-saturation binding occurred at approximately 5 nM Cyr61. As expected, the interaction of Cyr61 with immobilized $\alpha_v\beta_3$ was blocked by RGDS and LM609. In sum, these results identified Cyr61 as a novel ligand for $\alpha_v\beta_3$ and indicate that the adhesion of HUVECs to Cyr61 is mediated through interaction with this integrin. The possibility that integrin $\alpha_v\beta_3$ functions as a signaling receptor for Cyr61 accounts for most if not all activities that can be ascribed to Cyr61 to date and suggests a mechanism of action discussed herein.

One of the primary cellular responses to the actions of polypeptide growth factors is the rapid and transient activation of a set of immediate-early genes, the expression of which is thought to be involved in mediating the biological responses of the growth factors (1, 2). Among the immediate-early genes identified in fibroblasts is Cyr61 (3), which encodes a secreted cysteine-rich and heparin-binding protein associated with the extracellular matrix (ECM)¶ or with the cell surface (4). In a recent study, we reported the purification of recombinant Cyr61 and its biological activities in cell culture systems. Thus, Cyr61 promotes cell adhesion, stimulates cell migration, and augments growth factor-induced DNA synthesis (5). Fisp12, a closely related protein also encoded by an immediate-early gene in fibroblasts (6), has indistinguishable activities (7). During embryogenesis, Cyr61 exhibits a temporally restricted and tissue-specific distribution that closely associates with the development of cartilage and the circulatory system (8). Consistent with this expression pattern, Cyr61 promotes the differentiation of mouse limb bud mesenchymal cells into chondrocytes in micromass cultures (9). Taken together, these findings suggest that Cyr61 functions as a downstream regulator of growth factor actions, possibly by acting as an ECM-associated signaling molecule to regulate physiological processes involving cell adhesion, migration, proliferation, and differentiation.

The biological activities of Cyr61 suggest that it might interact with a cell surface receptor. The ability of Cyr61 to mediate cell adhesion and a strict requirement for divalent cations in this process (5) are consistent with the possible interaction of Cyr61 with one of the divalent cation-dependent cell adhesion receptors of the integrin, selectin, or cadherin families (10–13). Selectins and cadherins are primarily involved in cell-cell interactions. Integrins, on the other hand, are a large family of heterodimeric adhesion receptors involved in both cell-cell and cell-ECM interactions (11). In addition to serving as cell adhesion receptors, integrins also play an essential role in cell motility (14, 15). Furthermore, integrin occupancy and clustering induces signaling cascades that intersect with growth factor-induced signaling processes that regulate cell proliferation and differentiation (16–21). Based on these considerations, we hypothesized that Cyr61 might interact with an integrin receptor, thereby promoting cell adhesion and migration as well as augmenting growth factor-induced DNA synthesis. In the present study, by examining the effect of inhibitors of integrin $\alpha_v\beta_3$ on cell adhesion and in vitro protein binding assays, we investigated the possibility that adhesion of HUVECs to Cyr61 is mediated through direct interaction of Cyr61 and integrin $\alpha_v\beta_3$.

MATERIALS AND METHODS

Antibodies and Peptides—The anti-$\alpha_v\beta_3$ monoclonal antibody LM609 (22) was a generous gift of Dr. D. A. Cheresh (Scripps Research Institute, La Jolla, CA). The monoclonal antibodies LM142 (anti-$\alpha_v$) and JB55 (anti-$\alpha_v\beta_3$) were purchased from Chemicon (Temecula, CA). Production and characterization of polyclonal anti-$\beta_3$ antibodies have been

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1 The abbreviations used are: ECM, extracellular matrix; HUVEC, human umbilical vein endothelial cell; PBS, phosphate-buffered saline; BSA, bovine serum albumin.
Cyr61 Interacts with Integrin \( \alpha_v \beta_3 \)

**RESULTS**

Adhesion of HUVECs to Cyr61 Is Dependent on Integrin \( \alpha_v \beta_3 \).—In a previous report (5), we showed that HUVECs, suspended in a serum-containing medium, adhere to immobilized Cyr61 in a divalent cation-dependent manner, suggesting that the observed adhesion is mediated through a divalent-cation-dependent adhesion receptor. However, inasmuch as serum contains several adhesive proteins, it remains a possibility that cell adhesion to Cyr61 may not be mediated through a direct interaction between Cyr61 and cell surface receptor(s). To exclude this possibility, we examined the adhesion of HUVECs to purified Cyr61 under serum-free conditions. In this study, to obliterate the possibility of serum protein contamination, Cyr61 was purified from serum-free conditioned medium harvested from S9 insect cells and maintained in serum-free SF900-II medium.

The \( \alpha_v \beta_3 \) integrin was purified from HUVEC lysate as described (23, 25). Briefly, \( 10^6 \) cells were lysed in 1 ml of PBS, pH 7.5, containing 1 mM CaCl\(_2\), 1 mM MgCl\(_2\), 0.5 mM phenylmethyleneisulfonil fluoride, and 100 mM octylglucoside. The lysate was passed four times through a 0.5-ml column of GRGDS-activated Sepharose 4B. The column was washed with 10 ml of lysis buffer, and bound \( \alpha_v \beta_3 \) was subsequently eluted with 2 ml of RGDS (1 mM) in the same buffer. The purified receptor was dialyzed extensively against PBS, pH 7.5, containing 1 mM CaCl\(_2\), 1 mM MgCl\(_2\), 0.5 mM phenylmethyleneisulfonil fluoride, and 5 mM octylglucoside to remove the RGDS peptide. Figure 2B shows that the RGDS eluate contained two silver-stained proteins with molecular masses of 160 and 90 kDa. Immunoblotting with subunit-specific antibodies confirmed their identities as the \( \alpha_v \) and \( \beta_3 \) integrin subunits, respectively.

**Cell Adhesion Assay**—Adhesion of HUVECs in serum-containing complete medium to Cyr61 immobilized onto microtiter wells was performed as described (5, 7). In some experiments, HUVECs were harvested with 2 mM EDTA in PBS (20 min, room temperature), washed twice with F12K serum-free medium containing 7 mg/ml BSA (Sigma) and resuspended in the same medium at 2.5 \( \times \) 10\(^6\) cells/ml. Where indicated, EDTA, inhibitory peptides, and antibodies were preincubated with the cells for 15 min before plating. After incubation at 37 °C for 20 min, the wells were washed with PBS, and adherent cells were fixed and stained with methylene blue. HUVEC adhesion was quantitated by dye extraction and measurement of absorbance at 620 nm.

**Binding of Integrin \( \alpha_v \beta_3 \) to Cyr61-coupled Sepharose**.—Purified Cyr61 protein (0.6 mg/ml) or human plasma vitronectin (1 mg/ml; Collaborative Biomedical Research) was coupled to cyanogen bromide-activated Sepharose 4B (hydrated bead volume, 100 \( \mu \)l, Pharmacia) using standard procedures as recommended by the manufacturer. After blocking nonspecific sites with 1% BSA, the affinity matrices were equilibrated with TBS-OG (50 mM Tris, pH 7.5, 150 mM NaCl containing 1 mM CaCl\(_2\), 1 mM MgCl\(_2\), 0.1 mM phenylmethyleneisulfonil fluoride, and 5 mM octylglucoside). Purified integrin \( \alpha_v \beta_3 \) (250 ng in 50 mM TBS-OG) was incubated with 20 \( \mu \)l of Cyr61- or vitronectin-coupled Sepharose beads overnight at 4 °C. Where indicated, anti-Cyr61 or anti-human vitronectin antiserum were incubated with the ligand-coupled Sepharose for 2 h at 4 °C prior to the addition of integrin \( \alpha_v \beta_3 \). Following extensive washing with TBS-OG, bound integrin was extracted with nonreducing SDS gel loading buffer, resolved by SDS-polyacrylamide gel electrophoresis, and detected by immunoblotting with anti-\( \beta_3 \) polyclonal antibodies.

**Solid Phase Binding Assay of Cyr61 to Integrin \( \alpha_v \beta_3 \)**.—The binding of Cyr61 to immobilized \( \alpha_v \beta_3 \) was measured using previously described methods with slight modifications (26, 27). Briefly, microtiter wells (Pro-Blend plates, Falcon) were coated with purified \( \alpha_v \beta_3 \) (1 \( \mu \)g/ml, 50 \( \mu \)l/well) overnight at 4 °C, and then blocked with 2% BSA for 2 h at room temperature. After four washes with PBS, pH 7.5, containing 1 mM CaCl\(_2\), 1 mM MgCl\(_2\), and 5 mM octylglucoside, Cyr61 or vitronectin was added and incubated for 3 h at room temperature. In inhibition studies, EDTA, blocking peptides and antibodies were preincubated with the immobilized integrin for 1 h before the addition of ligands to wells containing the inhibitors. Bound ligands were detected by specific polyclonal antisera diluted in PBS, pH 7.5, containing 1 mM CaCl\(_2\), 1 mM MgCl\(_2\), and 5 mM BSA (anti-Cyr61, 1:500; anti-vitronectin, 1:1000) followed by horseradish peroxidase-conjugated secondary antibody (1:20,000). The color reaction was developed using the horseradish peroxidase immunoassay kit (Bio-Rad), and the absorbance at 450 nm was measured.
RGES, specifically blocked HUVEC adhesion to Cyr61 (Fig. 3B). These results indicated that cell adhesion to Cyr61 is mediated through an RGD-sensitive integrin in HUVECs. Therefore, we examined the inhibition of HUVEC adhesion to Cyr61 by antibodies against the \( \alpha_v\beta_3 \) and \( \alpha_5\beta_1 \) integrins. Fig. 3C shows that LM609 (22), an anti-\( \alpha_v\beta_3 \) monoclonal antibody, inhibited HUVEC adhesion to Cyr61 by >80%. As expected, we found that LM609 also blocked cell adhesion to vitronectin but not to fibronectin. In contrast, JBS5, an anti-\( \alpha_5\beta_1 \) monoclonal antibody, had no effect on HUVEC adhesion to Cyr61, whereas it effectively blocked cell adhesion to fibronectin (Fig. 3D). Taken together, these results indicated that under serum-free conditions, the adhesion of HUVECs to Cyr61 is mediated specifically by integrin \( \alpha_v\beta_3 \).

To further characterize HUVEC adhesion to Cyr61, we tested the ability of vitronectin, a physiological ligand of \( \alpha_v\beta_3 \), to inhibit this process. When cell adhesion was allowed to proceed for 15 min, soluble vitronectin (50 \( \mu \)g/ml) significantly inhibited HUVEC adhesion to Cyr61 and to vitronectin by 55 and 65%, respectively (Fig. 4A). These findings are consistent
with the suggestion that Cyr61 and vitronectin compete for binding to the same integrin receptor on HUVECs. However, when cell adhesion was allowed to proceed for 30 min, soluble vitronectin failed to inhibit HUVECs from adhering to Cyr61, and inhibition of adhesion to vitronectin was reduced to 38% (Fig. 4B). The diminution of inhibition under the longer incubation period was likely due to the nonequilibrium nature of \( \alpha_v \beta_3 \)-mediated adhesion to immobilized ligands (28). Thus, the adhesion of HUVECs to Cyr61 may result in a stabilized interaction between Cyr61 and \( \alpha_v \beta_3 \). These results showed that under serum-free conditions, HUVECs are capable of adhering to Cyr61 via \( \alpha_v \beta_3 \) even in the presence of soluble vitronectin.

Because serum contains several RGD-containing ligands for integrin \( \alpha_v \beta_3 \) in addition to vitronectin, we examined whether HUVEC adhesion to Cyr61 in the presence of serum is also mediated by this integrin. In these experiments, HUVECs were resuspended in complete F12K medium containing 10% fetal bovine serum and allowed to adhere to microtiter wells coated with Cyr61. Under these conditions, RGDS inhibited HUVEC adhesion to Cyr61 in a dose-dependent manner, whereas a control peptide with the reverse sequence SDGR had no effect (Fig. 5A). Furthermore, in control samples, much higher concentrations of RGDS were required to inhibit \( \alpha_5 \beta_1 \)-mediated cell adhesion to fibronectin. This may be due to the difference in affinities of short RGD peptides for the \( \alpha_v \beta_3 \) and \( \alpha_5 \beta_1 \) integrins (29). Likewise, LM609, at concentrations of 0.1 and 1 \( \mu \)M, completely blocked HUVEC adhesion to Cyr61 and vitronectin (Fig. 5B). The specificity of inhibition caused by LM609 was demonstrated by the lack of effect of an irrelevant IgG and by the failure of LM609 to inhibit cell adhesion to fibronectin. Collectively, these results indicate a direct involvement of integrin \( \alpha_v \beta_3 \) in HUVEC adhesion to Cyr61 both in the presence of serum and under serum-free conditions.

\[ \text{Cyr61 Interacts with Purified Integrin } \alpha_v \beta_3 \]

The observation that RGDS and LM609 block HUVEC adhesion to Cyr61 suggests that integrin \( \alpha_v \beta_3 \) may act as a cell surface receptor for Cyr61. To assess this possibility, we sought to determine whether Cyr61 interacts with \( \alpha_v \beta_3 \) in purified systems. Accordingly, we examined direct binding of \( \alpha_v \beta_3 \) to an affinity matrix onto which Cyr61 was coupled. As controls, vitronectin- or glycine-coupled matrices were used. In these experiments, purified \( \alpha_v \beta_3 \) was incubated with the affinity matrices and washed extensively, and bound integrin was extracted and analyzed by immunoblotting with anti-\( \beta_3 \) antibodies. As shown in Fig. 6, \( \alpha_v \beta_3 \) bound to both Cyr61- or vitronectin-coupled matrices.
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EDTA, an RGD-containing peptide, and the anti-αvβ3 monoclonal antibody LM609; and 2) purified integrin αvβ3 bound specifically to an Cyr61-coupled affinity matrix, and conversely, purified Cyr61 interacted directly with immobilized αvβ3 in a solid phase receptor binding assay.

Integrin αvβ3 binds to a broad spectrum of both RGD-containing and non-RGD-containing ligands (26, 31, 32). The primary sequence of Cyr61 does not contain the RGD recognition motif present in αvβ3 ligands such as vitronectin, fibronectin, fibrinogen, thrombospondin, and osteopontin (31). In addition to Cyr61, at least two other αvβ3 ligands also do not contain an RGD sequence: a cell surface molecule from the immunoglobulin superfamily CD31/PECAM-1 (32) and metalloproteinase MMP-2 (26). However, RGD peptides block the interaction between integrin αvβ3 and these proteins. Similar inhibition of Cyr61 binding to αvβ3 by RGDS was also observed in the present study. The inhibitory effect of RGD peptides may be due to conformational changes of αvβ3 upon RGD occupancy, thus masking other binding sites in the receptor for these non-RGD-containing proteins.

The ability of Cyr61 to mediate HUVEC adhesion was observed under both serum-free and serum-containing conditions (Figs. 2–5). Within a short adhesion period (15 min), vitronectin was capable of inhibiting HUVEC adhesion to Cyr61, consistent with competition of these proteins for binding to the same receptor. However, inhibition was not observed when cell adhesion was allowed to proceed for a longer period (30 min) (Fig. 4). This is likely due to the nonequilibrium nature of cell adhesion to immobilized substrates mediated through αvβ3 (28). The observation that HUVECs adhered to Cyr61 via αvβ3 when challenged with 50 μg/ml vitronectin (Fig. 4) or 10% serum (Fig. 5) indicate that Cyr61 may function as a physiologic adhesion substrate even in the presence of RGD-containing αvβ3 ligands.

Although it is currently unclear how Cyr61 interacts with αvβ3, studies employing phage display libraries have revealed sequences other than RGD capable of binding to the integrins αvβ3 and αvβ1 (33–35). Furthermore, data from studies on disintegrin and phage display libraries indicate that certain peptide sequences bind integrins with substantially higher affinity when cyclized via disulfide bonding (33–39). In this regard, the processed, secreted Cyr61 contains 38 cysteine residues, thereby providing the possibility of creating cyclized sequences that interact with αvβ3. Identification of the recognition sequence within Cyr61 would be essential for our understanding of the ligand diversity of the αvβ3 integrin, as well as the structure-function relationship of the newly discovered Cyr61 family of growth-related proteins.

The inducible expression of Cyr61 by growth factors suggests that Cyr61 may function as a transient and localized signaling molecule regulating biological processes in which cell adhesion plays a role. Cell migration, proliferation, and differentiation are examples of such processes. Although we have established that the cell adhesive properties of Cyr61 are mediated through interaction with integrin αvβ3, whether other activities of Cyr61 are also manifested through the same receptor is not yet known. It is tempting, however, to speculate that the chemo- tactic and proliferative activities of Cyr61 may also be mediated through the αvβ3 integrin. Although these processes involve much more complex cellular responses than adhesion, they are completely consistent with the capabilities of integrins to induce these signaling events. In this regard, it is widely accepted that cell adhesion receptors are crucial for cell migration (10, 15), and the regulation of integrin affinity to proteins in the ECM is likely a driving force of cell migration (40, 41). Therefore, the modulation of the affinity of integrin αvβ3 to-

Fig. 6. Binding of integrin αvβ3 to affinity matrices. Cyr61 (61), vitronectin (VN), or glycine (C) was coupled to cyanogen bromide-activated Sepharose 4B, and purified integrin αvβ3 was added to the coupled Sepharose beads. Anti-vitronectin antibodies, anti-Cyr61 antibodies, or buffer alone (no add) were incubated with the affinity matrices where indicated prior to the addition of integrin. After washings, the bound protein was extracted with nonreducing SDS gel loading buffer, electrophoresed on SDS-10% polyacrylamide gel, and immunoblotted with polyclonal anti-β3 antibodies. Lane L represents the total amount of integrin loaded onto the Sepharose beads. Positions of molecular weight markers are indicated on the right.

Seharose but not to the control glycine-coupled matrix. As expected, preincubation of the affinity matrices with anti-vitronectin antibodies blocked αvβ3 binding to vitronectin but not to Cyr61. Likewise, anti-Cyr61 antibodies blocked αvβ3 binding to Cyr61 but not to vitronectin. These results demonstrated a specific interaction between integrin αvβ3 and Cyr61.

To further investigate the interaction between Cyr61 and αvβ3, we developed a solid-phase receptor binding assay. Purified αvβ3 was coated onto microtiter wells, and the binding of soluble Cyr61 to the immobilized receptor was detected by ELISA using polyclonal anti-Cyr61 antibodies. Fig. 7A shows that Cyr61 bound saturably to immobilized αvβ3, and half-saturation occurred at approximately 0.2 μg/ml (5 nM) Cyr61. To demonstrate the specificity of the interaction between Cyr61 and αvβ3, inhibition studies were performed. As shown in Fig. 7B, the binding of Cyr61 to immobilized αvβ3 was blocked by the divalent cation chelator EDTA, as well as by the αvβ3 antagonists RGDS and LM609. Taken together, results from these protein binding assays are consistent with those obtained from the cell adhesion assays, and they demonstrate that Cyr61 interacts directly with integrin αvβ3.

DISCUSSION

Cyr61, encoded by a growth factor-inducible immediate-early gene, belongs to an emerging family of extracellular signaling proteins in which members (e.g. Cyr61 and Fisp12) have been shown to promote cell adhesion, migration, and proliferation (5, 7). Sequence analysis of Cyr61 and other members of this protein family revealed four conserved domains sharing homology with 1) insulin-like growth factor-binding proteins, 2) von Willebrand Factor type C domain, 3) heparin-binding proteins, and 4) the C-terminal domains of some types of collagens and mucins (30). To date, a cell surface receptor for these proteins has not been identified. In this study, we investigated the mechanism by which Cyr61 mediates HUVEC adhesion and identified integrin αvβ3 as a cell surface receptor for Cyr61. This conclusion is based on both functional and biochemical evidence: 1) HUVEC adhesion to Cyr61 was inhibited by...
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ward Cyr61 and other ligands in the ECM may account for the chemotactic activity of Cyr61. The participation of integrins in modulation of growth factor-mediated signaling is also well established (16, 17). For example, regulation of cell cycle progression by \( \beta_1 \) integrins (42–44) and integrin \( \alpha_v \beta_3 \) (45, 46) have been reported. Consistent with the observation that Cyr61 is able to augment basic fibroblast growth factor-induced and platelet-derived growth factor-induced DNA synthesis (5), integrin- and growth factor-induced signaling cascades have been shown to intersect and converge (16, 17, 47).

The finding that Cyr61 interacts with integrin \( \alpha_v \beta_3 \) also provides insights into its possible biological functions. Although Cyr61 is a secreted protein, it is tightly associated with the ECM and the cell surface upon secretion and is thus likely to act locally in either an autocrine or a paracrine fashion (4). During development, Cyr61 is highly expressed in the placenta, most notably in the trophoblastic giant cells and trophoblasts of the ectoplacental cone (8). \( \alpha_v \beta_3 \) is the only integrin found to be expressed on the apical surface of trophoblasts and on the uterine epithelium during the period of receptivity for implantation (48, 49), and \( \alpha_v \beta_3 \) continues to be expressed in the trophoblastic giant cells and ectoplacental cone in later stages (49, 50). Because Cyr61 is likely to be deposited from the trophoderm onto the neighboring matrix, it can be hypothesized that interaction between Cyr61 and \( \alpha_v \beta_3 \) may help to mediate attachment of the embryo to the endometrium through cell-matrix interactions. Furthermore, in the post-implantation embryo, Cyr61 interaction with \( \alpha_v \beta_3 \) may mediate attachment to the decidua. In addition, because Cyr61 has been shown to promote chondrogenesis to form the embryonic skeleton (9), the presence of both Cyr61 (8, 9) and \( \alpha_v \beta_3 \) (51) in chondrocytes suggests that their interaction may play a role in this process.

In the circulatory system, Cyr61 and \( \alpha_v \beta_3 \) are coexpressed in vascular smooth muscle cells (8, 52) and in microvascular endothelial cells (53). In HUVECs, we previously demonstrated that Cyr61 mediates cell adhesion and augments basic fibroblast growth factor-induced DNA synthesis (5, 54). Because basic fibroblast growth factor, which induces angiogenesis, activates the synthesis of both Cyr61 (3, 55) and \( \alpha_v \beta_3 \) (53), these observations suggest that Cyr61 may be involved in angiogenesis during wound repair. In this regard, \( \alpha_v \beta_3 \) has been shown to mediate angiogenesis in chicken chorioallantoic membrane (56) and basic fibroblast growth factor-induced neovascularization in rabbit cornea (57). Our finding that Cyr61 is a ligand for \( \alpha_v \beta_3 \) is consistent with its potential role in growth factor-induced angiogenesis.

In sum, we demonstrated that integrin \( \alpha_v \beta_3 \) serves as an adhesion receptor for Cyr61 on HUVECs. Although we cannot exclude the possibility that Cyr61 may also bind to other cell surface molecules, such as heparan sulfate proteoglycans and growth factor receptors, the interaction of Cyr61 with integrin \( \alpha_v \beta_3 \) may account for most, if not all, of the ascribed physiological activities of Cyr61 to date. Thus, Cyr61 and other members of its family may represent a novel class of signaling molecules, providing additional functional links between the growth factor-dependent and matrix-dependent signaling events.

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