MOLECULAR AND CELLULAR BIOLOGY, Apr. 1996, p. 1326–1334 Vol. 16, No. 4
0270-7306/96/$04.00+0


# Cyr61, a Product of a Growth Factor-Inducible Immediate-Early Gene, Promotes Cell Proliferation, Migration, and Adhesion

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Received 14 September 1995/Returned for modification 27 October 1995/Accepted 28 December 1995

***cyr61* was first identified as a growth factor-inducible immediate-early gene in mouse fibroblasts. The encoded Cyr61 protein is a secreted, cysteine-rich heparin-binding protein that associates with the cell surface and the extracellular matrix, and in these aspects it resembles the Wnt-1 protein and a number of known growth factors. During embryogenesis, *cyr61* is expressed most notably in mesenchymal cells that are differentiating into chondrocytes and in the vessel walls of the developing circulatory system. *cyr61* is a member of an emerging gene family that encodes growth regulators, including the connective tissue growth factor and an avian proto-oncoprotein, Nov. *cyr61* also shares sequence similarities with two *Drosophila* genes, *twisted gastrulation* and *short gastrulation*, which interact with *decapentaplegic* to regulate dorsal-ventral patterning. In this report we describe the purification of the Cyr61 protein in a biologically active form, and we show that purified Cyr61 has the following activities: (i) it promotes the attachment and spreading of endothelial cells in a manner similar to that of fibronectin; (ii) it enhances the effects of basic fibroblast growth factor and platelet-derived growth factor on the rate of DNA synthesis of fibroblasts and vascular endothelial cells, although it has no detectable mitogenic activity by itself; and (iii) it acts as a chemotactic factor for fibroblasts. Taken together, these activities indicate that Cyr61 is likely to function as an extracellular matrix signaling molecule rather than as a classical growth factor and may regulate processes of cell proliferation, migration, adhesion, and differentiation during development.**

The actions of polypeptide growth factors on responsive cells include the rapid and transient activation of a set of immediate-early genes, the expression of which is thought to mediate the biological responses to the growth factors (15, 22). The spectrum of genes activated in this manner is complex, and many encode previously unknown proteins. Among those characterized to date, a number of immediate-early genes encode transcription factors (43), while others encode phosphatases (7, 10, 25, 30) and putative kinases (11, 29, 42). Other immediate-early genes encode proteins without clearly recognizable structural domains or homologies to proteins of well-characterized functions. Understanding the biochemical activities and biological function of these novel genes will likely require extensive characterization of their gene products.

*cyr61* is an immediate-early gene that is transcriptionally activated by serum growth factors in fibroblasts (27). It encodes a protein with biochemical but not sequence similarities to those of the mouse protein Wnt-1 (46). Both Cyr61 and Wnt-1 are secreted, cysteine-rich heparin-binding proteins; both are not found in the conditioned medium of expressing cultured cells but are associated with the extracellular matrix (ECM) and the cell surface upon secretion (4, 37, 46). Some of these properties are also shared with a number of known growth factors. In situ hybridization analysis has shown that expression of *cyr61* during mouse embryogenesis is closely correlated with the differentiation of mesenchymal cells into chondrocytes; in addition, *cyr61* is expressed in the vessel walls of the developing circulatory system (26). Taken together, these data suggest that Cyr61 might be involved in cell-cell or cell-matrix communications, or alternatively, Cyr61 might act in a manner similar to that of known growth factors (46).

These views have been fortified by the discovery that *cyr61* is a member of an emerging gene family encoding regulators of growth, including a growth factor. To date this gene family includes three distinct members (*cyr61*/*CEF-10*, *fisp12*/*CTGF*, and *Nov*) whose encoded proteins share a high degree of sequence homology, the presence of a secretory signal, and complete conservation of 38 cysteine residues (2, 26). First, the chicken homolog of Cyr61, CEF-10, is encoded by a *src*-inducible gene (36). Fisp12, encoded by another growth factor-inducible immediate-early gene identified in mouse fibroblasts, shares 65% amino acid sequence identity with Cyr61 (32). The human homolog of Fisp12 was identified as a gene encoding a connective tissue growth factor (CTGF); its activity is found in the conditioned medium of human umbilical vein endothelial (HUVE) cells (3). Another distinct family member, Nov, is encoded by an avian gene overexpressed in myeloblastosis-associated virus-induced nephroblastomas (19). Expression of a truncated *nov* sequence encoding a protein with the N terminus deleted transforms chicken embryo fibroblasts, whereas expression of the full-length sequence does not. Thus, the *cyr61* gene family includes one encoding a growth factor, CTGF, and a proto-oncogene, *nov*. In addition to the above family members, *cyr61* also shares sequence similarities with two recently identified *Drosophila* genes, *twisted gastrulation* (23) and *short gastrulation* (13). Both of these genes interact with *decapentaplegic* to control dorsal-ventral patterning. These observations strengthen the hypothesis that Cyr61 function may be important in development.

Despite the above clues, it has been difficult to define specific activities of Cyr61 by the expression of *cyr61* in cultured cells. Constitutive expression of *cyr61* in NIH 3T3 cells did not evoke a specific change in cell morphology, although clonal cell lines transfected with a *cyr61*-expressing vector saturated at a lower cell density than the nontransfected parent (45). The

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analysis of the biological function of Cyr61 has been hindered heretofore by difficulties in preparing sufficient quantities of purified proteins in a biologically active form (45). Similar difficulties have been encountered in the purification of other highly cysteine-rich proteins. In this paper, we describe the purification of recombinant Cyr61 protein in a biologically active form. We found that despite a high degree of homology to CTGF and biochemical properties that resemble those of known growth factors, purified Cyr61 protein exhibits effects on cells that are distinct from those of known growth factors. First, Cyr61 does not have detectable mitogenic activity by itself, but rather, it enhances the mitogenic effect of growth factors on fibroblasts and endothelial cells. Second, while Cyr61 is chemotactic like many growth factors, it also promotes cell attachment and spreading, activities that are not attributed to classical growth factors. Moreover, Cyr61 is localized to the ECM upon secretion (46) and is expressed in a highly regulated manner during embryogenesis (26). Taken together, these effects indicate that Cyr61 is likely to function as an ECM signaling molecule rather than as a classical growth factor and may regulate processes of cell proliferation, migration, adhesion, and differentiation during development.

## MATERIALS AND METHODS

**Purification of recombinant Cyr61.** Conditioned media of Sf9 insect cells infected with a baculovirus (39) driving the synthesis of Cyr61 were used as a source for purification. Sf9 cells, Grace's media with supplements, transfer plasmid pBlueBac2, linear wild-type *Autographa californica* nuclear polyhedrosis virus DNA, and cationic liposomes were from the MaxBac kit (Invitrogen). A fragment of the murine *cyr61* cDNA (27) encompassing the entire coding region (nucleotides 56 to 1560) was cloned into the pBlueBac2 vector at the *Nhe*I and *Bam*HI sites. Transfer vector along with target *Autographa californica* nuclear polyhedrosis virus DNA was delivered into cells by liposome-mediated transfection. Recombinant virus was plaque purified and amplified through three passages of Sf9 cell infection (39).

Sf9 cells in monolayer cultures grown to a subconfluent density in a complete medium were infected with 10 PFU of recombinant virus per cell, incubated for 16 h, and fed with serum-free Grace's medium. The conditioned medium was collected 48 h postinfection and cleared by centrifugation at 5,000 × *g* for 5 min. The conditioned medium was chilled to 4°C, adjusted to 50 mM MES (morpholineethanesulfonic acid [pH 6.0]), 2 mM EDTA, and 1 mM PMSF (phenylmethylsulfonyl fluoride), and applied to a Sepharose S (Sigma) column at 4°C (5-ml column for 500 ml of medium). The column was washed with a buffer (50 mM MES [pH 6.0], 2 mM EDTA, 0.5 mM PMSF) containing 150 mM NaCl, and bound proteins were subsequently eluted with a linear gradient of NaCl (0.2 to 1 M) in the same buffer. The column fractions were analyzed by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–10% PAGE) followed by Coomassie brilliant blue staining or Western blotting (immunoblotting). Western blots were probed with affinity-purified anti-Cyr61 antibodies as described previously (46) and stained with enhanced chemiluminescence detection reagents (Amersham). Fractions containing Cyr61 were combined and adjusted to pH 7.5 with 1 M Tris (pH 8.5)–10% glycerol (final concentration) before storage in aliquots at −70°C. Protein concentration was determined by the modified Lowry method with a Bio-Rad protein assay kit ($D_C$) with bovine serum albumin (BSA) as a control.

**Limited proteolysis.** Purified Cyr61 was digested with trypsin or chymotrypsin (sequencing grade; Boehringer-Mannheim) in the storage buffer at a 1:20 protease/Cyr61 weight ratio. For digestion with chymotrypsin, $CaCl_2$ was added to 10 mM. NIH 3T3 cell lysate was prepared from serum-stimulated cells as described (46) and digested in radioimmunoprecipitation assay buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% deoxycholate) without PMSF at a protease/protein ratio of 1:200. Proteolysis was carried out at 37°C for various durations, and the reaction was stopped by the addition of SDS-PAGE sample buffer followed by immediate boiling. Cyr61 proteolytic fragments were separated by SDS–12% PAGE and detected by Western blotting.

**Heparin binding assay.** The heparin binding assay for Cyr61 (46) was modified for the purified protein. Two hundred microliters of a 50% (vol/vol) slurry of heparin-Sepharose CL 6B beads (Pharmacia) was added to 100 μl of Cyr61 solution in radioimmunoprecipitation assay buffer and incubated for 1 h. The protein was eluted with increasing concentrations of NaCl in radioimmunoprecipitation assay buffer.

**Assays for cell attachment and spreading.** Immunological 96-well plates (Falcon) were coated with 50 μl of 0.1% BSA in phosphate-buffered saline (PBS) with or without Cyr61 or fibronectin at 4°C. Two hours after coating was started, nondiluted immune or preimmune antisera (30 μl per well) or affinity-purified anti-Cyr61 antibodies (27) were added. Where indicated, the coating mixture was adjusted to 10 mM dithiothreitol or 100 mM HCl (see Fig. 4). After 16 h of incubation the coating solution was removed, and the well surface was blocked with 1% BSA in PBS for 1 h at room temperature before HUVE cells were plated in a complete medium at $5 \times 10^3$ to $5 \times 10^4$ cells per well. Cycloheximide was added at 100 μg/ml immediately before plating and monensin was added at 1 μM 14 h before plating, where indicated. After a 2-h incubation at 37°C, the wells were washed with PBS and attached cells were fixed and stained with methylene blue (28). The attachment efficiency was determined by quantitative dye extraction and measurement of the extract $A_{650}$ as previously described (28). An $A_{650}$ value of 0.5 corresponded to the attachment of $6 \times 10^3$ cells.

Cell spreading was studied in a similar experimental system. Polystyrene petri dishes were coated with 2 ml of a 10-μg/ml solution of Cyr61 or fibronectin in PBS with 0.1% BSA and treated as described above. Cells ($7 \times 10^6$) were plated into each dish and incubated for 2 h, and cell spreading was analyzed by microscopy at a magnification of ×100.

**Thymidine incorporation assay.** NIH 3T3 cells were plated on 24-well plates at $3 \times 10^4$ cells per well and grown in Dulbecco minimal essential medium (DMEM) with 10% fetal bovine serum (FBS) (Intergen) for 3 to 4 days and incubated with medium containing 0.2% FBS for the following 48 h. Platelet-derived growth factor (PDGF-BB) or basic fibroblast growth factor (bFGF) (Gibco BRL), Cyr61 protein or protein storage buffer, and [$^3$H]thymidine (1-μCi/ml final concentration; ICN) were added simultaneously in fresh DMEM containing 0.2% FBS. After 18 to 20 h of incubation, cells were washed with PBS and fixed with 10% trichloroacetic acid. DNA was dissolved in 0.1 N NaOH and the thymidine incorporation was counted. HUVE cells (CRL 1730) were grown in F12K medium (Gibco BRL) containing 10% FBS, 100 μg of heparin per ml, and 30 μg of endothelial cell growth supplement (Collaborative Biomedical Products) per ml. Thymidine, bFGF, and Cyr61 or human plasma fibronectin (Gibco BRL) were added to fresh F12K medium containing 100 μg of heparin per ml and 10% FBS without endothelial cell growth supplement. Cells were incubated for 48 to 72 h and treated as described above.

The thymidine incorporation assay on the HUVE cells attached to Cyr61 and fibronectin was performed on cells plated as described for the cell attachment assay (see above) in F12K medium with heparin and without growth supplement. Two hours after plating, the media with nonattached cells were removed and replaced with fresh media containing 5 μCi of [$^3$H]thymidine per ml and 10 ng of bFGF per ml, 5 μg of Cyr61 per ml, or 25 μg of fibronectin per ml, as indicated. The cells were incubated for 48 h and then fixed and lysed as described above for standard mitogenic assays.

**Chemotaxis assay.** The chemotactic response of NIH 3T3 cells was examined by using the modified Boyden chamber (14). Purified Cyr61 protein was serially diluted in DMEM containing BSA (0.2 mg/ml) and added to the lower well of the chamber. The lower well was then covered with a collagen-coated polycarbonate filter (8-μm pore diameter; Nuclepore). Cells ($6 \times 10^4$) were then loaded into the upper well. After 5 h of incubation (10% $CO_2$, 37°C), the filter was removed and the cells were fixed and stained with Giemsa-Wright stain (Harleco). Cells from the upper surface of the filter were then removed by wiping with a tissue swab. The chemotactic response was determined by counting the total number of migrating cells detected in 10 randomly selected microscopic fields (magnification, ×400) on the lower surface.

## RESULTS

**Purification and characterization of recombinant Cyr61 protein.** Previously we found that Cyr61 synthesized in bacterial expression systems formed insoluble protein aggregates, whereas mammalian cells expressing *cyr61* suffered an apparent growth disadvantage that resulted in their loss through successive passages (45). In contrast, we found that Cyr61 could be produced in the soluble form by using the baculovirus expression system. Infection of Sf9 cells with a recombinant baculovirus driving the synthesis of Cyr61 resulted in protein production within 24 h. Although Cyr61 in Sf9 cell lysates also formed insoluble aggregates as in bacterial cell extracts, approximately 10% of the Cyr61 synthesized was secreted into the medium in a soluble form. Therefore, serum-free conditioned media of recombinant-virus-infected Sf9 cells were used as a source for Cyr61 purification, which was accomplished by cation-exchange chromatography on a Sepharose S column (Fig. 1). The pooled fractions of Cyr61, eluted at 0.6 to 0.7 M NaCl as a distinct broad peak, contained Cyr61 of at least 90% purity as judged by SDS-PAGE. This purification procedure was repeated at least five times with similar results; the typical yield was 3 to 4 mg of protein from 500 ml of conditioned medium.

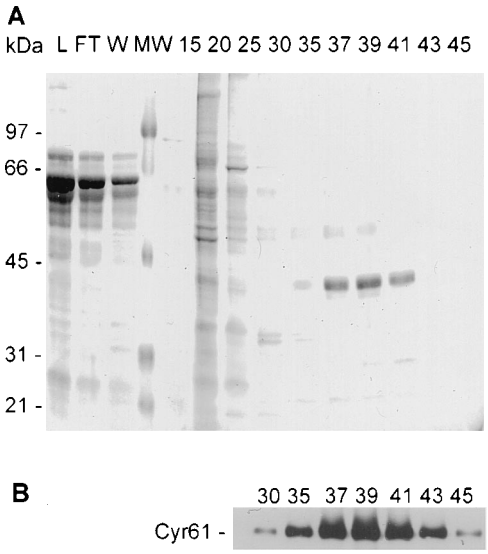


FIG. 1. Purification of recombinant Cyr61. Cyr61 was purified from conditioned medium of Sf9 cells programmed for its synthesis through a Sepharose S column. (A) Coomassie brilliant blue-stained SDS–10% polyacrylamide gel of various fractions of the purification. L, serum-free conditioned medium of the Sf9 cells producing Cyr61; FT, flowthrough fraction of the column; W, wash fraction of the column containing elution buffer with 150 mM NaCl; MW, molecular weight markers. Numbering of other lanes corresponds to column fractions eluted with the salt gradient (0.2 to 1.0 M NaCl). Molecular masses of markers are on the left. The bulk of the 40-kDa Cyr61 protein was eluted from fractions 35 to 43. (B) Western blot analysis of the indicated column fractions probed with affinity-purified anti-Cyr61 antibodies.

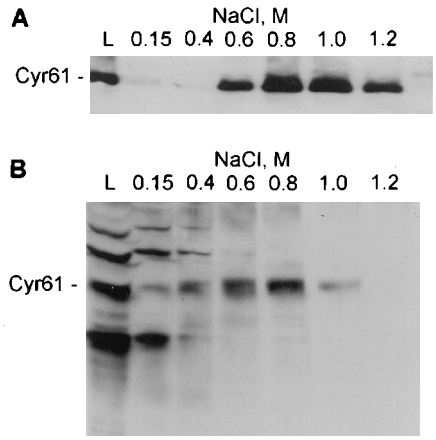


FIG. 3. Heparin binding activity of Cyr61. Purified recombinant Cyr61 protein (A) or serum-stimulated 3T3 cell lysate (B) was loaded onto a heparin-Sepharose column (lanes L). Proteins were eluted from the column with increasing concentrations of salt as indicated and were analyzed by SDS-PAGE followed by Western blotting with anti-Cyr61 antibodies as probe.

To ascertain whether recombinant Cyr61 purified from Sf9 cells is structurally and functionally similar to the endogenous protein of mouse fibroblasts, two sets of experiments were performed. First, purified recombinant Cyr61 and a lysate of serum-stimulated 3T3 cells were subjected to limited proteolysis with either trypsin or chymotrypsin, and their digestion products were compared (Fig. 2). Partial trypsin digestion of both the recombinant protein and cell lysate resulted in two Cyr61 fragments of approximately 21 and 19 kDa. Likewise, partial digestion of both preparations with chymotrypsin produced a stable 23-kDa fragment. These results are consistent with the interpretation that the purified recombinant Cyr61 is folded into a tertiary structure similar to that of the endogenous native protein in fibroblast lysates, thus exposing identical or neighboring sites for proteolytic cleavage.

Another criterion we used to assess the properties of recombinant Cyr61 is its ability to bind heparin, an attribute previously demonstrated for Cyr61 from serum-stimulated fibroblasts (46). We observed that recombinant Cyr61 purified by ion-exchange chromatography as described above bound quantitatively to heparin-Sepharose at 0.15 M NaCl and was eluted at 0.8 to 1.0 M NaCl, similar to Cyr61 from serum-stimulated fibroblasts (Fig. 3). Thus, the heparin binding activity remains intact in the purified recombinant Cyr61. Taken together, these results indicate that the purified recombinant Cyr61 is structurally and functionally similar to Cyr61 in serum-stimulated 3T3 fibroblasts and is thus appropriate for analysis.

**Cyr61 promotes cell attachment and spreading.** Since Cyr61 is a secreted protein associated with the ECM and the cell surface, we hypothesized that Cyr61 might mediate cell-ECM or cell-cell interactions. We thus carried out cell attachment assays to test this possibility. Nontissue culture dishes were coated with BSA, fibronectin, or Cyr61. HUVE cells were plated on these dishes, and the relative number of attached cells 2 h after plating was quantified.

As has been described previously, HUVE cells attached poorly to dishes treated with BSA alone but adhered well to dishes coated with fibronectin (Fig. 4A) (12). We found that Cyr61-coated surfaces also supported HUVE cell attachment in a dose-dependent manner, similar to fibronectin-coated surfaces. Cyr61 also promotes the attachment of NIH 3T3 cells, though less effectively than fibronectin for this cell line (data not shown). Although we present data on Cyr61-mediated cell

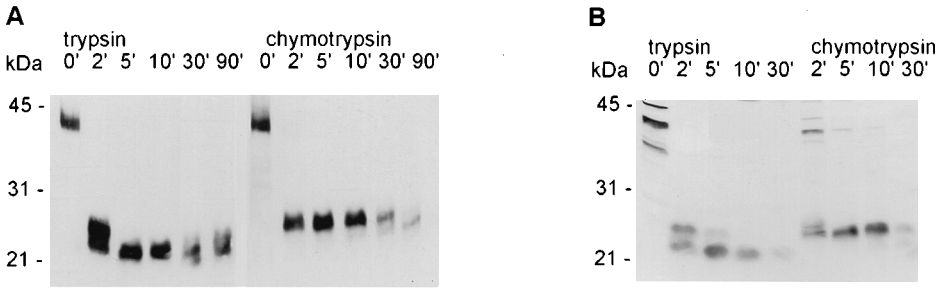


FIG. 2. Limited proteolysis of Cyr61. Partial proteolytic digestion with trypsin or chymotrypsin of either purified recombinant Cyr61 protein (A) or serum-stimulated 3T3 cell lysate (B) for the indicated periods. Digestion products were analyzed by SDS-PAGE followed by Western blotting with the anti-Cyr61 antibodies as probes. Molecular masses of marker proteins are on the left.

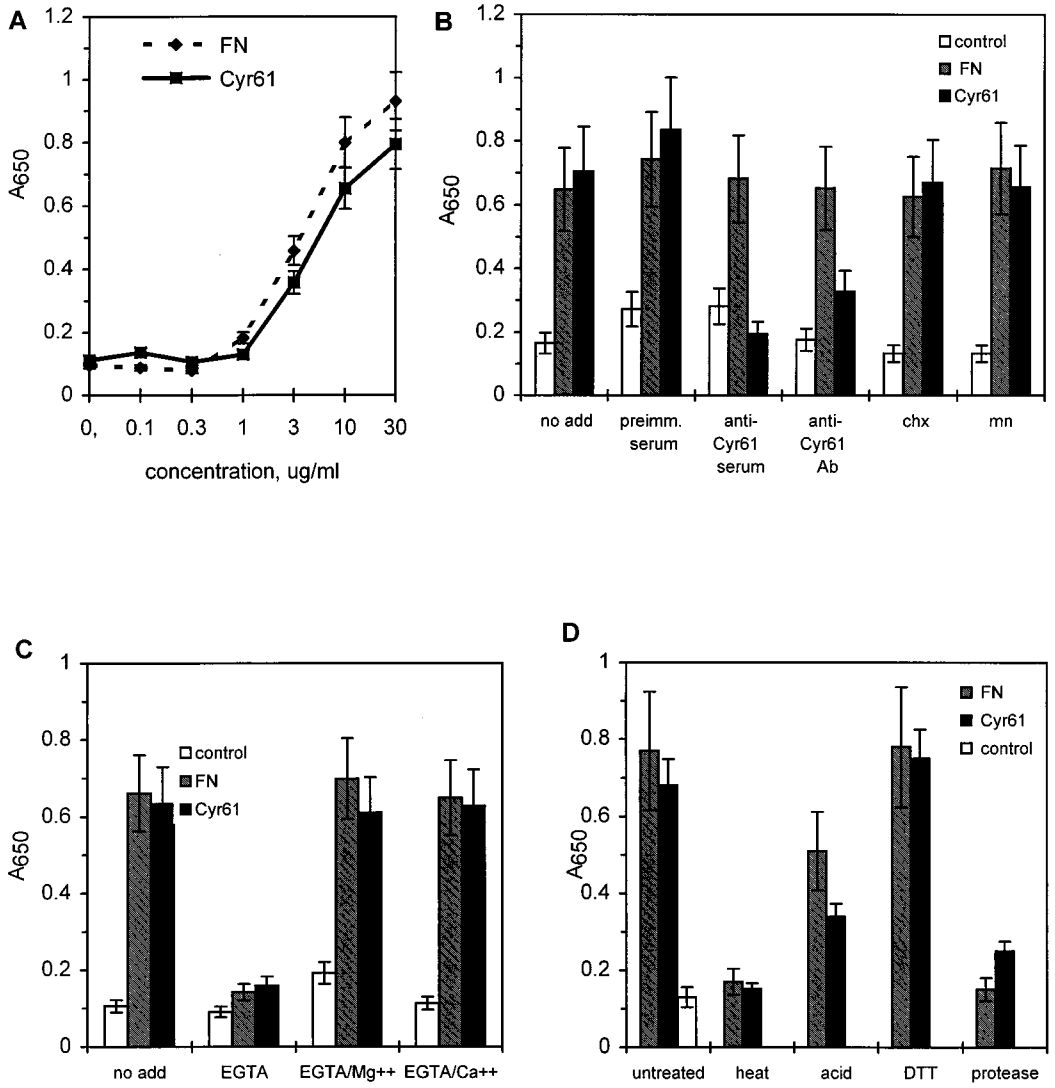


FIG. 4. Effects of Cyr61 on the attachment of HUVE cells. (A) Cell attachment on plates coated with various concentrations of Cyr61 or fibronectin as indicated. The relative number of attached cells 2 h after plating was assessed by staining the cells with methylene blue and determining the $A_{650}$. (B) Cyr61-specific promotion of HUVE cell attachment. HUVE cells were plated on BSA-coated plates (control), fibronectin (FN)- or Cyr61-coated plates and their attachment 2 h after plating was measured as described for panel A. The effects of preimmune serum, anti-Cyr61 immune serum, affinity-purified anti-Cyr61 antibodies, cycloheximide (chx; 100 μg/ml), and monensin (mn; 1 μM) on HUVE cell attachment are shown. (C) Effect of divalent cations on cell attachment. HUVE cells were plated in the presence of 5 mM EGTA, 5 mM EGTA plus 6 mM $MgSO_4$, or 5 mM EGTA plus 10 mM $CaCl_2$. The plates were coated with Cyr61 or fibronectin (10 μg/ml) and treated as described above. (D) Characterization of Cyr61 activity. HUVE cells were plated on either control plates, fibronectin-coated plates, or Cyr61-coated plates, and cell attachment was measured as above. The effects of heat (75°C for 5 min), 10 mM dithiothreitol (DTT), 0.1 M HCl, and 12-h chymotrypsin treatment at the enzyme/protein ratio 1:20 on the ability of Cyr61 to promote cell attachment were measured. Values are means for three parallel experiments ± standard deviations.

attachment quantified 2 h after plating, cell attachment can be observed by microscopy as early as 30 min after plating (data not shown).

The adhesion of HUVE cells on Cyr61-coated surfaces was specifically inhibited by anti-Cyr61 antiserum and by affinity-purified anti-Cyr61 antibodies but not by preimmune serum (Fig. 4B). In contrast, attachment of cells to fibronectin-coated dishes was not affected by either the anti-Cyr61 antiserum or affinity-purified anti-Cyr61 antibodies. These results show that enhancement of cell adhesion is a specific activity of the Cyr61 protein. Furthermore, the Cyr61-mediated cell attachment was insensitive to cycloheximide or monensin treatment (Fig. 4B), indicating that Cyr61 does not act through the induction of de novo synthesis of ECM components or stimulation of fibronectin or collagen secretion (41) and probably acts directly. The Cyr61-mediated attachment of HUVE cells was completely abolished by the presence of EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid]; however, attachment was restored by the addition of $CaCl_2$ or $MgSO_4$ to the medium (Fig. 4C). These results indicate that the interaction between Cyr61 and its cell surface receptor requires divalent cations.

Since Cyr61 is a cysteine-rich protein (38 cysteines in the 355-amino-acid secreted portion), it may potentially be held in a rigid structure by the formation of multiple disulfide bonds. Surprisingly, the ability of Cyr61 to mediate cell attachment was not affected by treatment with 10 mM dithiothreitol for 16 h but was inactivated after heating at 75°C for 5 min, after incubation in 100 mM HCl, or upon extensive digestion with chymotrypsin (Fig. 4D). These results indicate that Cyr61 is a

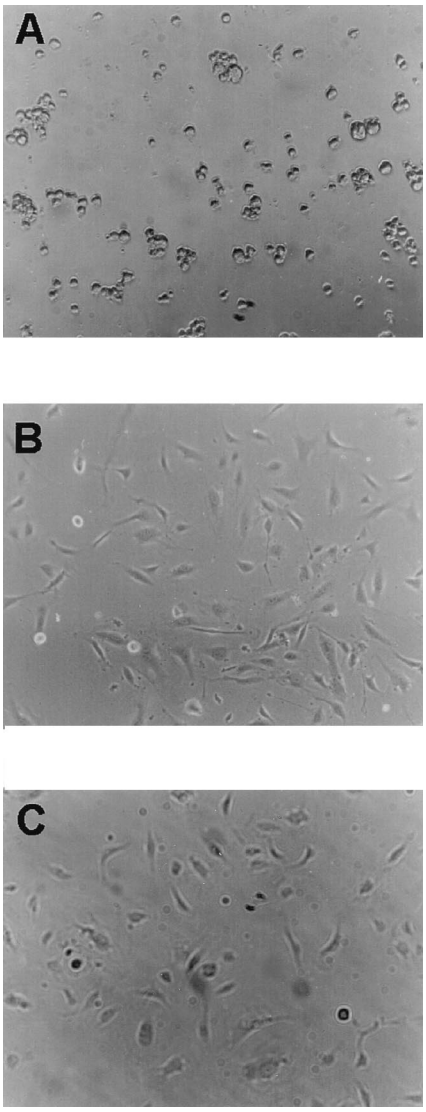


FIG. 5. Cyr61-dependent cell spreading. HUVE cells were plated on plastic surfaces coated with BSA (A), fibronectin (B), or Cyr61 (C) as described in Materials and Methods, and photomicrographs were taken 2 h after plating. Magnification, ×100.

heat- and acid-labile protein whose active conformation is not sensitive to reducing agents.

In addition to promoting the attachment of cells to the substratum, Cyr61 also promotes cell spreading in a manner similar to that observed for fibronectin (Fig. 5). The efficient attachment and spreading of cells on Cyr61-coated substrates suggests that Cyr61 may interact with a signal-transducing cell surface receptor, leading to a cascade of cytoskeletal rearrangements and possible formation of focal contacts.

**Cyr61 enhances growth factor-induced DNA synthesis.** Since Cyr61 is homologous to CTGF, a mitogenic growth factor (3), we hypothesized that it might also promote cell proliferation and thus tested this possibility. We found that Cyr61, when added by itself to cultures of NIH 3T3 or HUVE cells, had no effect on the rate of DNA synthesis (Fig. 6). This lack of effect was not due to rapid degradation of Cyr61, since it was stable for at least 24 h after addition to the culture medium as detected by Western blotting (data not shown). Moreover, in these experiments we tested a broad range of purified Cyr61 concentrations, from 10 ng/ml up to 5 μg/ml (Fig. 6 and data not shown). We therefore conclude that Cyr61, by itself, does not act as a mitogen to the cells tested under our experimental conditions.

Strikingly, we found that Cyr61 does have the ability to enhance the effect of other growth factors on the rate of DNA synthesis. When NIH 3T3 fibroblasts (Fig. 6A) or HUVE cells (Fig. 6B) were treated with a nonsaturating dose of either bFGF (Fig. 6A and B) or PDGF-BB (Fig. 6A), the addition of Cyr61 significantly increased thymidine incorporation compared with cells treated with the growth factors alone. This effect of Cyr61 was dose dependent and required a minimal concentration of 0.5 to 1.0 μg of recombinant protein per ml for either cell type. Significantly, the enhancement of DNA synthesis by Cyr61 can be inhibited by the addition of specific anti-Cyr61 antiserum (Fig. 6C).

**Promotion of growth factor-induced DNA synthesis and cell attachment are distinct activities of Cyr61.** The ability of Cyr61 to mediate cell adhesion raises the possibility that the observed Cyr61-dependent enhancement of growth factor-induced DNA synthesis might be explained by an increase in the number of cells attached upon Cyr61 treatment. To differentiate between a cell attachment effect and true mitogenic stimulation, we plated HUVE cells on fibronectin- or Cyr61-coated surfaces. After 2 h, when most cells were firmly attached and spreading had occurred, nonadherent cells were removed by washing with PBS. Soluble bFGF, Cyr61, and fibronectin were then added to the cells in fresh medium and their effects on DNA synthesis were assessed. No difference between the basal and bFGF-induced levels of DNA synthesis was observed for cells plated on either Cyr61- or fibronectin-coated dishes. However, addition of Cyr61 to the medium increased the growth factor-induced thymidine incorporation in cells plated on either Cyr61- or fibronectin-coated substrates (Fig. 7). In contrast, plasma fibronectin added to the medium did not have such an effect. Furthermore, Cyr61, but not fibronectin, stimulated bFGF-induced DNA synthesis in cells plated on tissue culture dishes (Fig. 7C). Therefore, in cells that are already firmly attached, Cyr61 clearly stimulates growth factor-induced DNA synthesis in a manner that fibronectin does not. This enhancement of cell proliferation is an activity of Cyr61 distinct from its ability to promote cell adhesion.

**Cyr61 is a chemotactic factor.** Since CTGF has been shown to be a chemoattractant for NIH 3T3 cells (3), it is possible that Cyr61 might also act as a chemotactic factor. Indeed, we have previously observed that Cyr61 partially purified from serum-stimulated NIH 3T3 cells by using heparin-Sepharose chromatography acts as a chemoattractant for fibroblasts (45) (data not shown). However, it was difficult to exclude other interpretations in experiments with partially purified cell extracts. This problem was obviated in experiments with highly purified recombinant Cyr61 protein. We observed that NIH 3T3 cells responded to Cyr61 as a chemotactic factor in a dose-dependent manner in the Boyden chamber assay (Fig. 8). Thus, we conclude that Cyr61 can act as a chemotactic factor for fibroblasts. The optimal concentration for the chemotactic activity of Cyr61 is 1 to 5 μg/ml in this assay; this concentration range is consistent with those of known chemotactic activities of other ECM molecules, such as thrombospondin (5 to 50 μg/ml for endothelial cells) (40) and fibronectin (1 to 30 μg/ml) (5, 6), as determined by using similar Boyden chamber assays.

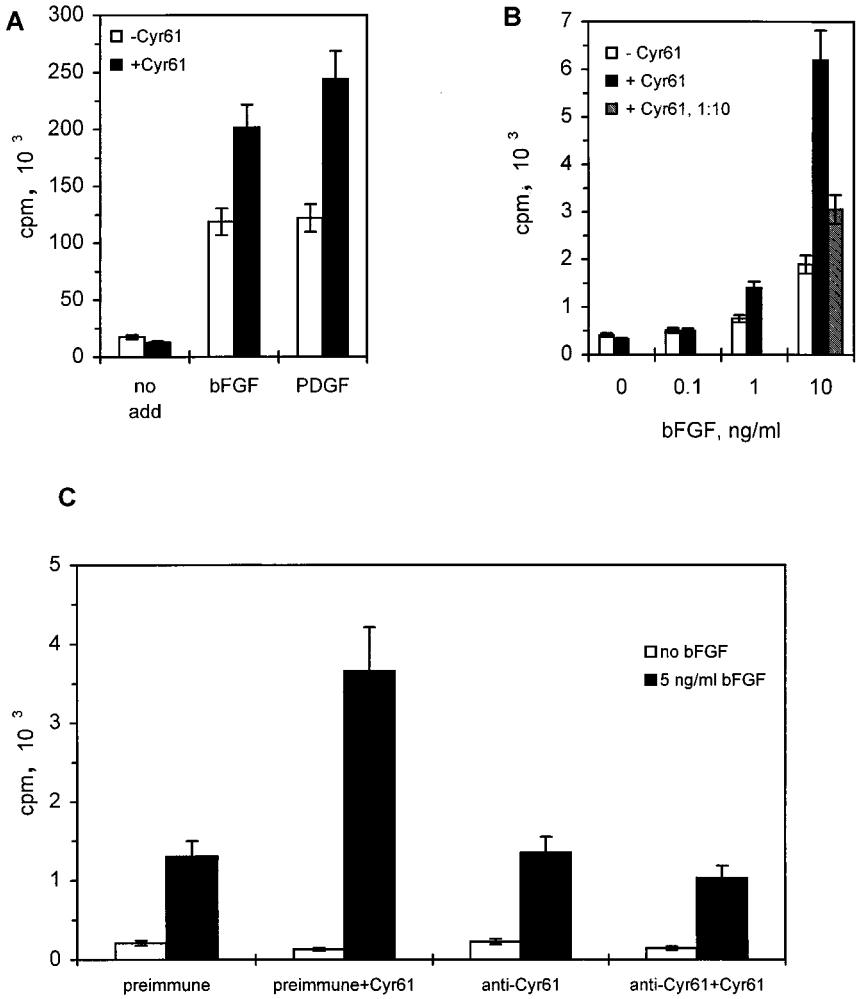


FIG. 6. Effect of Cyr61 on growth factor-induced DNA synthesis. (A) NIH 3T3 fibroblasts were either unstimulated or stimulated with bFGF at 15 ng/ml or PDGF at 30 ng/ml. Cyr61 was added to 5 μg/ml where indicated. (B) HUVE cells were either unstimulated or stimulated with the indicated amounts of bFGF. Cyr61 was added to 5 μg/ml (+Cyr61) or 0.5 μg/ml (+Cyr61, 1:10). (C) HUVE cells were treated as described for panel B, but Cyr61 or Cyr61 storage buffer was preincubated for 1 h at room temperature with anti-Cyr61 antiserum or preimmune serum before addition to the medium. In these experiments Cyr61 was added to 4 μg/ml and antisera were added to 3%. [$^3$H]thymidine incorporation was measured 18 h after stimulation of NIH 3T3 fibroblasts and 72 h after stimulation of HUVE cells. Values are the means of three parallel experiments ± standard deviations. The experiments were repeated at least three times with different preparations of Cyr61 with similar results.

## DISCUSSION

Cyr61 has been identified as an immediate-early-gene-encoded protein with biochemical similarities to Wnt-1 and some growth factors and sequence homology to a growth factor and a proto-oncoprotein (26, 27, 46). However, because of the lack of purified Cyr61, it has been difficult to discover the specific activities of this protein. In this report, we describe the purification of the Cyr61 protein and demonstrate that it promotes cell proliferation, migration, and adhesion. These activities, taken together with the tissue-specific and temporally restricted pattern of expression of the *cyr61* gene during embryogenesis (26), suggest that Cyr61 may play important regulatory roles in growth and differentiation during development.

The discovery of the activities of Cyr61 relied upon purification of recombinant Cyr61 protein in a biologically active form. The purified recombinant Cyr61 is indistinguishable from the endogenous protein, since it retained both the heparin binding activity of Cyr61 from 3T3 cells (Fig. 3) and a tertiary structure similar to the endogenous protein as probed by limited proteolysis (Fig. 2). The activities we describe herein are bona fide properties of the Cyr61 polypeptide by reason of the following arguments. (i) Cyr61 was purified to near homogeneity from serum-free conditioned medium of insect cells programmed for its synthesis; thus, it is unlikely that the preparation would be contaminated with other growth-related proteins active in mammalian cells. (ii) The combination of activities we ascribe to Cyr61 is novel and cannot be attributed to contamination by any known protein. (iii) Both the cell adhesion and DNA synthesis enhancement activities of Cyr61 can be inhibited by antibodies specific for the Cyr61 polypeptide. (iv) The chemotaxis activity confirms what has been observed for Cyr61 partially purified from serum-stimulated 3T3 cells by heparin-Sepharose chromatography (45). Furthermore, this Cyr61 activity in 3T3 cells has the same characteristics as the recombinant protein as determined by the adhesion assay, i.e., acid and heat lability but dithiothreitol insensitivity (45) (Fig. 4).

The combination of activities of Cyr61 indicates that it is likely to act as an ECM signaling molecule rather than as a classical growth factor. Since Cyr61 does not act as a mitogen

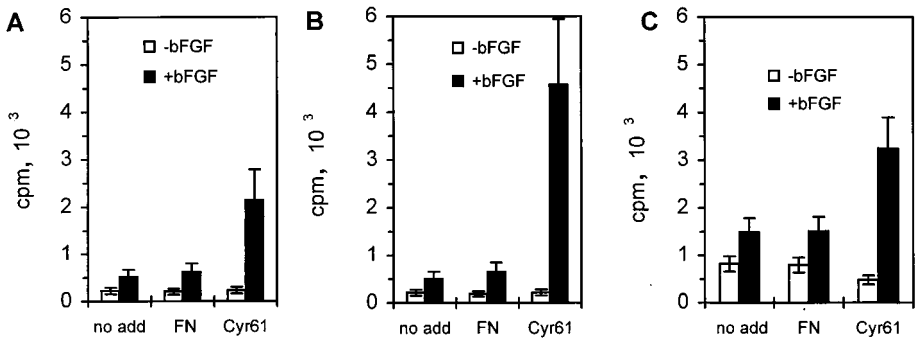


FIG. 7. Cyr61 enhancement of mitogenesis is an activity distinct from cell adhesion. The effects of soluble Cyr61 and fibronectin (FN) on growth factor-induced mitogenesis were assessed on HUVE cells already firmly attached on Cyr61- or fibronectin-coated plates. Cells were plated on surfaces coated with Cyr61 (10 μg/ml) (A) and human plasma fibronectin (10 μg/ml) (B) and on a tissue culture dish (C). After cell attachment and the removal of nonadherent cells, cells were either unstimulated or stimulated with bFGF (10 ng/ml). Cells were simultaneously treated with Cyr61 (5 μg/ml) or fibronectin (25 μg/ml) added to the medium where indicated. [$^3$H]thymidine incorporation assays were then carried out.

by itself, it cannot be considered a growth factor per se (Fig. 6). On the other hand, the ability of Cyr61 to enhance the mitogenic effect of other growth factors is consistent with similar activities exhibited by several other ECM and cell surface-associated molecules. For example, a secreted bFGF-binding protein (9), the basal lamina protein perlecan (1), and human immunodeficiency virus type 1 Tat protein (12) promote bFGF-induced cell proliferation and angiogenesis; thrombospondin activates a latent form of transforming growth factor-β (34); and a secreted growth-potentiating factor from vascular smooth muscle cells is required for an efficient activation of epidermal growth factor- or thrombin-induced DNA synthesis in these cells (24). Thus, some ECM molecules can regulate cell proliferation in conjunction with other growth factors. Moreover, no known growth factor can mediate cell adhesion in a manner similar to that of Cyr61 (Fig. 4 and 5), whereas both growth factors and ECM signaling molecules have been known to have chemotactic activities (Fig. 8). Cyr61 is chemotactic in the 50 to 100 nM range, which is in the range of the active concentrations of other ECM molecules such as thrombospondin (10 to 100 nM) (40) and fibronectin (10 to 100 nM) (5, 6). In contrast, many growth factors are chemotactic at much lower concentrations (38). Taken together with the ECM association of Cyr61 upon secretion, the activities of Cyr61 to promote cell proliferation, migration, and adhesion are consistent with its role as a matrix signaling molecule that mediates cell-cell and cell-matrix communication (46).

Since Cyr61-mediated cell adhesion occurs quickly with a time course similar to that of adhesion by fibronectin (data not shown) and is insensitive to cycloheximide, which blocks de novo protein synthesis, and to monensin, which blocks ECM protein secretion (41) (Fig. 4), it is likely that Cyr61 mediates cell adhesion directly. The divalent-cation dependence of Cyr61-mediated adhesion (Fig. 4C) suggests that Cyr61 might work through interaction with a $Ca^{2+}$-dependent adhesion molecule on the cell surface. The ability of Cyr61 to promote DNA synthesis, chemotaxis, and cell adhesion argues that Cyr61 might interact with a specific cell adhesion molecule, resulting in signal transduction and a myriad of intracellular biological responses. Among these responses might be the activation of an ancillary mitogenic signaling pathway that, though insufficient to cause mitogenesis by itself, can nevertheless augment the effects of growth factors. Recent discoveries that link focal adhesion to the activation of components in mitogenic pathways, including protein kinase C and mitogen-activated protein kinases, are supportive of this view (8, 20).

It was reported that CTGF is a PDGF-immunoreactive molecule that possesses mitogenic activity on NIH 3T3 cells and competes with PDGF for binding to a cell surface receptor (3, 17). Although CTGF is not the human ortholog of mouse Cyr61, they are closely related family members that share 45% sequence identity and complete conservation of all 38 cysteines (26). It is thus surprising that we have not detected any mitogenic activity intrinsic to the Cyr61 polypeptide; as discussed above, Cyr61 is more likely an ECM signaling molecule than a growth factor. These observations raise the intriguing possibility that two closely related molecules may work in completely different ways: CTGF as a growth factor and Cyr61 as an ECM signaling molecule. On the other hand, it might be possible to interpret the reported findings by suggesting that CTGF increased the activity of trace amounts of PDGF or other growth factors that might be present in the CTGF preparations par-

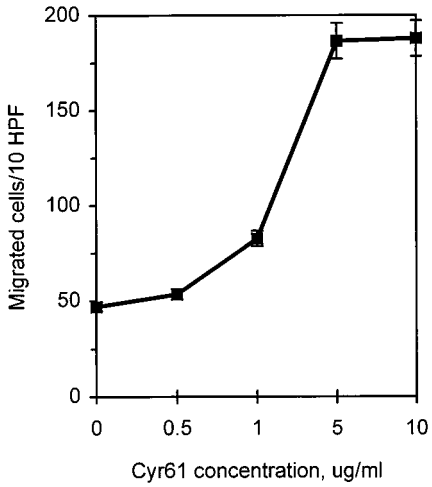


FIG. 8. Chemotactic response of NIH 3T3 cells to purified Cyr61. The migration of NIH 3T3 cells across a 8-μm-pore-size membrane in a Boyden chamber was monitored as a function of various amounts of Cyr61 protein used as a chemoattractant. Cell migration was quantified by counting the number of migrated cells in 10 randomly chosen high-power fields (HPF). Data are average numbers of migrated cells in duplicate experiments. Error bars indicate standard deviations. The experiment was repeated three times with similar results.

tially purified from serum-containing conditioned media of HUVE cells (3). It will be interesting to identify the receptors specific for CTGF and Cyr61 and to compare their mechanisms of action, as these lines of investigation may reveal specific features of the mechanisms of action of these two closely related molecules.

Given the high degree of sequence homology and conservation of all 38 cysteine residues among proteins of the Cyr61 family, it is tempting to speculate that (i) there may be other members of this protein family as yet unidentified; (ii) members of the family may have similar or overlapping activities; and (iii) members of the family might have distinct tissue-specific expression patterns and may function in different cell types and in different tissues. Analysis of the sequence homology among the five known sequences of this family revealed four conserved domains that are related to other proteins (2): domain I shares homology with the low-molecular-weight insulin-like growth factor-binding proteins; domain II corresponds to a region in von Willebrand factor; domain III resembles some heparin-binding proteins; and domain IV is related to the *Drosophila* gene *slit* (31), which is involved in development of the midline glia and might mediate protein-protein interactions. These structural domains also correspond to the intron-exon boundaries (21, 32) and may reflect a result of gene fusion through evolution. Further evidence for this notion comes from the recent identification of two *Drosophila* genes involved in dorsal-ventral patterning: *twisted gastrulation* (23), which has sequence similarity with domain I of Cyr61, and *short gastrulation* (13), which is functionally and structurally homologous to the *Xenopus* gene *chordin* (16, 33) and has sequence similarity to domain II of Cyr61. These observations suggest that members of the Cyr61 family are results of domain fusion through metazoan evolution, culminating in the convergence of several functional domains in a single polypeptide in avian and mammalian species.

It is interesting that the mammalian homologs of many *Drosophila* gastrulation-specific genes participate in the formation of cartilage and bone; these include bone morphogenetic protein 1 (35), bone morphogenetic protein 2, and other transforming growth factor β-related proteins (44). The intrinsic similarity of these processes is that both require extensive cell movement and clustering (23). The ability of Cyr61 to promote cell migration and adhesion and the expression of the *cyr61* gene during the differentiation of chondrocytes in the mouse embryo are consistent with these intriguing links (26). In addition, the ability of Cyr61 to promote proliferation, migration, and adhesion in fibroblasts and endothelial cells suggests possible roles for Cyr61 in wound healing. Indeed, the closely related CTGF is found to be expressed during wound healing (18). On this basis, we propose that Cyr61 might function as a regulatory ECM signaling molecule that mediates cell-cell and cell-ECM interactions, cooperates with the actions of growth factors, and promotes cell growth, migration, adhesion, and differentiation. Thus, Cyr61 may represent a potentially important family of novel regulators of growth and development. These hypotheses can be specifically tested in future investigations.

## ACKNOWLEDGMENTS

We thank our colleagues C. C. Chen for helpful suggestions and S. Lam and C. Yeung for a critical reading of the manuscript.

This work was supported by a grant from the National Institutes of Health (R01 CA46565-08). L.F.L. is an Established Investigator of the American Heart Association.

## REFERENCES

1. **Aviezer, D., D. Hecht, M. Safran, M. Eisinger, G. David, and A. Yayon.** 1994. Perlecan, basal lamina proteoglycan, promotes basic fibroblast growth factor-receptor binding, mitogenesis, and angiogenesis. Cell **79:**1005–1013.
2. **Bork, P.** 1993. The modular architecture of a new family of growth regulators related to connective tissue growth factor. FEBS Lett. **327:**125–130.
3. **Bradham, D. M., A. Igarashi, R. L. Potter, and G. R. Grotendorst.** 1991. Connective tissue growth factor: a cysteine-rich mitogen secreted by human vascular endothelial cells is related to the SRC-induced immediate early gene product CEF-10. J. Cell Biol. **114:**1285–1294.
4. **Bradley, R. S., and A. M. C. Brown.** 1990. The proto-oncogene *int*-1 encodes a secreted protein associated with the extracellular matrix. EMBO J. **9:**1569–1575.
5. **Carsons, S., B. B. Lavietes, and H. S. Diamond.** 1989. Role of fibronectin in rheumatic diseases, p. 327–361. *In* D. F. Mosher (ed.), Fibronectin. Academic Press, San Diego, Calif.
6. **Carsons, S., B. B. Lavietes, H. S. Diamond, and S. G. Kinney.** 1985. The immunoreactivity, ligand, and cell binding characteristics of rheumatoid synovial fluid fibronectin. Arthritis Rheum. **28:**601–612.
7. **Charles, C. H., H. Sun, L. F. Lau, and N. K. Tonks.** 1993. The growth factor-inducible immediate-early gene 3CH134 encodes a protein-tyrosine-phosphatase. Proc. Natl. Acad. Sci. USA **90:**5292–5296.
8. **Clark, E. A., and J. S. Brugge.** 1995. Integrins and signal transduction pathways: the road taken. Science **268:**233–239.
9. **Czubayko, F., R. V. Smith, H. C. Chung, and A. Wellstein.** 1994. Tumor growth and angiogenesis induced by a secreted binding protein for fibroblast growth factor. J. Biol. Chem. **269:**28243–28248.
10. **Diamond, R. H., D. E. Cressman, T. M. Laz, C. S. Abrams, and R. Taub.** 1994. PRL-1, a unique nuclear protein tyrosine phosphatase, affects cell growth. Mol. Cell. Biol. **14:**3752–3762.
11. **Donohue, P. J., G. F. Alberts, Y. Guo, and J. A. Winkles.** 1995. Identification by targeted differential display of an immediate early gene encoding a putative serine/threonine kinase. J. Biol. Chem. **270:**10351–10357.
12. **Ensoli, B., R. Gendelman, P. Markham, V. Fiorelli, S. Colombini, M. Raffeld, A. Cafaro, H.-K. Chang, J. N. Brady, and R. C. Gallo.** 1994. Synergy between basic fibroblast growth factor and HIV-1 Tat protein in induction of Kaposi's sarcoma. Nature (London) **371:**674–680.
13. **Francois, V., M. Solloway, J. W. O'Neill, J. Emery, and E. Bier.** 1994. Dorsal-ventral patterning of the *Drosophila* embryo depends on a putative negative growth factor encoded by the *short gastrulation* gene. Genes Dev. **8:**2602–2616.
14. **Grotendorst, G. R.** 1987. Spectrophotometric assay for the quantitation of cell migration in the Boyden chamber chemotaxis assay. Methods Enzymol. **147:**144–152.
15. **Herschman, H. R.** 1991. Primary response genes induced by growth factors and tumor promoters. Annu. Rev. Biochem. **60:**281–319.
16. **Holley, S. A., P. D. Jackson, Y. Sasai, B. Lu, E. M. De Robertis, F. M. Hoffmann, and E. L. Ferguson.** 1995. A conserved system for dorsal-ventral patterning in insects and vertebrates involving *sog* and *chordin*. Nature (London) **376:**249–253.
17. **Igarashi, A., D. M. Bradham, H. Okochi, and G. R. Grotendorst.** 1992. Connective tissue growth factor. J. Dermatol. **19:**642–643.
18. **Igarashi, A., H. Okochi, D. M. Bradham, and G. R. Grotendorst.** 1993. Regulation of connective tissue growth factor gene expression in human skin fibroblasts and during wound repair. Mol. Biol. Cell **4:**637–645.
19. **Joliot, V., C. Martinerie, G. Dambrine, G. Plassiart, M. Brisac, J. Crochet, and B. Perbal.** 1992. Proviral rearrangements and overexpression of a new cellular gene (*nov*) in myeloblastosis-associated virus type 1-induced nephroblastomas. Mol. Cell. Biol. **12:**10–21.
20. **Juliano, R. L., and S. Haskill.** 1993. Signal transduction from the extracellular matrix. J. Cell Biol. **120:**577–585.
21. **Latinkic, B. V., T. P. O'Brien, and L. F. Lau.** 1991. Promoter function and structure of the growth factor-inducible immediate early gene *cyr61*. Nucleic Acids Res. **19:**3261–3267.
22. **Lau, L. F., and D. Nathans.** 1991. Genes induced by serum growth factors, p. 257–293. *In* P. Cohen and J. G. Foulkes (ed.), Molecular aspects of cellular regulation, vol. 6. Hormonal regulation of transcription. Elsevier, Amsterdam.
23. **Mason, E. D., K. D. Konard, C. D. Webb, and J. L. Marsh.** 1994. Dorsal midline fate in *Drosophila* embryos requires *twisted gastrulation*, a gene encoding a secreted protein related to human connective tissue growth factor. Genes Dev. **8:**1489–1501.
24. **Nakano, T., K.-I. Higashino, N. Kikuchi, J. Kishino, K. Nomura, H. Fujita, O. Phara, and H. Arita.** 1995. Vascular smooth muscle cell-derived, Gla-containing growth-potentiating factor for $Ca^{++}$-mobilizing growth factors. J. Biol. Chem. **270:**5702–5705.
25. **Noguchi, T., R. Metz, L. Chen, M.-G. Mattéi, D. Carrasco, and R. Bravo.** 1993. Structure, mapping, and expression of *erp*, a growth factor-inducible gene encoding a nontransmembrane protein tyrosine phosphatase, and effect of ERP on cell growth. Mol. Cell. Biol. **13:**5195–5205.
26. **O'Brien, T. P., and L. F. Lau.** 1992. Expression of the growth factor-inducible immediate early gene *cyr61* correlates with chondrogenesis during mouse embryonic development. Cell Growth Differ. **3:**645–654.
27. **O'Brien, T. P., G. P. Yang, L. Sanders, and L. F. Lau.** 1990. Expression of

*cyr61*, a growth factor-inducible immediate-early gene. Mol. Cell. Biol. **10:** 3569–3577.
28. **Oliver, M. H., N. K. Harrison, J. E. Bishop, P. J. Cole, and G. J. Laurent.** 1989. A rapid and convenient assay for counting cells cultured in microwell plates: application for assessment of growth factors. J. Cell Sci. **92:**513–518.
29. **Pelech, S. L., and J. S. Sanghera.** 1992. Mitogen-activated protein kinases: versatile transducers for cell signaling. Trends Biochem. Sci. **17:**233–238.
30. **Rohan, P. J., P. Davis, C. A. Moskaluk, M. Kearns, H. Krutzsch, U. Siebenlist, and K. Kelly.** 1993. PAC-1: a mitogen-induced nuclear protein tyrosine phosphatase. Science **259:**1763–1766.
31. **Rothberg, J. M., J. R. Jacobs, C. S. Goodman, and S. Artavanis-Tsakonas.** 1990. *slit*: an extracellular protein necessary for development of midline glia and commissural axon pathways contains both EGF and LRR domains. Genes Dev. **4:**2169–2187.
32. **Ryseck, R.-P., H. Macdonald-Bravo, M.-G. Mattei, and R. Bravo.** 1991. Structure, mapping, and expression of *fisp-12*, a growth factor-inducible gene encoding a secreted cysteine-rich protein. Cell Growth Differ. **2:**225–233.
33. **Sasai, Y., B. Lu, H. Steinbeisser, D. Geissert, L. K. Gont, and E. M. De Robertis.** 1994. Xenopus *chordin*: a novel dorsalizing factor activated by organizer-specific homeobox genes. Cell **79:**779–790.
34. **Schultz-Cherry, S., S. Ribeiro, L. Gentry, and J. E. Murphy-Ullrich.** 1994. Thrombospondin binds and activates the small and large forms of latent transforming growth factor-beta in a chemically defined system. J. Biol. Chem. **269:**26775–26782.
35. **Shimell, M. J., E. L. Ferguson, S. R. Childs, and M. B. O'Connor.** 1991. The Drosophila dorsal-ventral patterning gene *tolloid* is related to human bone morphogenetic protein 1. Cell **67:**469–480.
36. **Simmons, D. L., D. B. Levy, Y. Yannoni, and R. L. Erikson.** 1989. Identification of a phorbol ester-repressible v-*src*-inducible gene. Proc. Natl. Acad. Sci. USA **86:**1178–1182.
37. **Smolich, B. D., J. A. McMahon, A. P. McMahon, and J. Papkoff.** 1993. *Wnt* family proteins are secreted and associated with the cell surface. Mol. Biol. Cell **4:**1267–1275.
38. **Sporn, M. B., and A. B. Roberts.** 1990. Peptide growth factors and their receptors. Springer-Verlag, Berlin.
39. **Summers, M., and G. E. Smith.** 1987. Manual of methods for baculovirus vectors and insect cell culture procedures. Tex. Agric. Exp. Stn. Bull. **1555:** 1–55.
40. **Taraboletti, G., D. Roberts, L. A. Liotta, and R. Giavazzi.** 1990. Platelet thrombospondin modulates endothelial cell adhesion, motility, and growth: a potential angiogenesis regulatory factor. J. Cell Biol. **111:**765–772.
41. **Ucida, N., H. Smilowitz, P. W. Ledger, and M. L. Tanzer.** 1980. Kinetic studies of the intracellular transport of procollagen and fibronectin in human fibroblasts. J. Biol. Chem. **255:**8638–8644.
42. **Webster, M. K., L. Goya, Y. Ge, A. C. Maiyar, and G. L. Firestone.** 1993. Characterization of *sgk*, a novel member of the serine/threonine protein kinase gene family which is transcriptionally induced by glucocorticoids and serum. Mol. Cell. Biol. **13:**2031–2040.
43. **Williams, G. T., A. S. Abler, and L. F. Lau.** 1992. Regulation of gene expression by serum growth factors, p. 115–162. *In* G. S. Stein and J. B. Lian (ed.), Molecular and cellular approaches to the control of proliferation and differentiation. Academic Press, Orlando, Fla.
44. **Wozney, J. M., V. Rosen, A. J. Celeste, L. M. Mitsock, M. J. Whitters, R. W. Kriz, R. M. Hewick, and E. A. Wang.** 1988. Novel regulators of bone formation: molecular clones and activities. Science **242:**1528–1534.
45. **Yang, G. P.** 1993. Ph.D. thesis. The University of Illinois at Chicago, Chicago.
46. **Yang, G. P., and L. F. Lau.** 1991. Cyr61, product of a growth factor-inducible immediate early gene, is associated with the extracellular matrix and the cell surface. Cell Growth Differ. **2:**351–357.