Purification and Characterization of Novel Heparin-binding Growth Factors in Uterine Secretory Fluids

IDENTIFICATION AS HEPARIN-REGULATED M₁, 10,000 FORMS OF CONNECTIVE TISSUE GROWTH FACTOR*

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Uterine growth factors are potential effector molecules in embryonic growth signaling pathways. Pig uterine luminal flushings contained a heparin-binding growth factor (HBGF) that required 0.8 M NaCl for elution from heparin columns and was termed HBGF-0.8. This factor, which was heat- and acid-labile and of M₁, 10,000 as assessed by gel filtration, stimulated DNA synthesis in fibroblasts and smooth muscle cells but not endothelial cells. Two forms of HBGF-0.8, termed HBGF-0.8-P1 and HBGF-0.8-P2, exhibited differential heparin-binding properties. SDS-polyacrylamide gel electrophoresis showed that each form of HBGF-0.8 migrated with an apparent Mᵡ of 10,000 under reducing conditions. Amino acid sequencing revealed the N-terminal sequence EEN-IKGK/KXIRPKI for HBGF-0.8-P1 and ENIKGK/KXIRT for HBGF-0.8-P2. These sequences corresponded, respectively, to residues 247–262 and 248–259 of the 349-residue predicted primary translation product of porcine connective tissue growth factor (pCTGF). 10-kDa CTGF-mediated fibroblast DNA synthesis was modulated by exogenous heparin, and CTGF-immunoreactive proteins of 10, 16, and 20 kDa were present in unfractionated uterine luminal flushings. These data reveal the identity of a novel growth factor in uterine fluids as a highly truncated form of CTGF and show that the N-terminal two-thirds of the CTGF primary translation product is not required for mitogenic activity or heparin binding.

Uterine secretions contain a variety of chemical constituents including ions, vitamins, proteins, and carbohydrates that are proposed to sustain the growth and viability of the early embryo during the preimplantation period and, in species that exhibit noninvasive placental development, of the conceptus after attachment of the extraembryonic membranes to the uterine epithelium (1). Polypeptide growth factors and cytokines are emerging as a class of uterine proteins that may be involved in uterine-embryo growth signaling pathways either through their secretion into the uterine lumen or their localization at the implantation site (2–5). Studies in a variety of species have suggested that epidermal growth factor (EGF),¹ heparin-binding EGF-like growth factor (HB-EGF), insulin-like growth factors-I and -II (IGF-I, IGF-II), acidic and basic fibroblast growth factors (aFGF, bFGF), pleiotrophin (PTN), leukemia inhibitory factor, colony-stimulating factor-1, and transforming growth factor-α may be among the uterine growth-regulatory molecules involved in these processes (2, 6–12). The pig is a useful model for these studies because the embryos of this species produce extensive extraembryonic membranes, exhibit a high incidence (approximately 30%) of mortality around the time of blastocyst expansion and attachment, produce a diffuse noninvasive placenta, and appear highly dependent on uterine secretory components throughout gestation (1, 13–15). Here we describe the characterization and purification of a principal heparin-binding growth factor (HBGF) in pig uterine secretions that is mitogenic for fibroblasts and smooth muscle cells in vitro. The N termini of two microheterogeneous forms of this factor are 100% conserved with residues 247–262 and 248–259 of the 349-residue predicted primary translational products of human and porcine connective tissue growth factor (hCTGF, pCTGF). These data reveal the identity of a novel uterine HBGF and provide new insights into structural and functional properties of CTGF.

MATERIALS AND METHODS

Animals

Uteri were collected at random from slaughterhouse pigs that were approximately 8 months or less in age. Each uterine horn was flushed with cold (4 °C) phosphate-buffered saline (PBS) as described (8) to collect uterine luminal components. These studies involved the use of 323 individual pools of uterine luminal flushings (ULF) (384 liters total volume) obtained from approximately 9,680 pigs; growth factor characterization studies were performed on 1-liter pools of ULF obtained from about 30 individual uteri, and growth factor purification was performed

¹ The abbreviations used are: EGF, epidermal growth factor; HB-EGF, heparin-binding EGF-like growth factor; PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; aFGF, acidic FGF; bFGF, basic FGF; PTN, pleiotrophin; IGF, insulin-like growth factor; CTGF, connective tissue growth factor; hCTGF, human CTGF; pCTGF, pig CTGF; HBGF, heparin-binding growth factor; PTN, pleiotrophin; CCN, CTGF/cyc61/nao; ULF, uterine luminal flushing(s); FPLC, fast protein liquid chromatography; HPLC, high performance liquid chromatography; BCEC, bovine capillary endothelial cells; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; CT, C-terminal; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; CAPS, 3-(cyclohexylamino)-propanesulfonic acid.

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on 4-liter pools of ULF obtained from up to 120 animals. ULF were clarified by centrifugation at 13,500 × g for 30 min at 4 °C, and the supernatant was passed through glass wool.

For Western blot analysis of crude ULF samples, pigs (n = 4) of mixed breed (Duroc, Landrace, Yorkshire) were sacrificed on day 16 of the estrous cycle (day 0 representing the first day of estrus). ULF from both horns of individual animals were pooled and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting as described below.

**Growth Factor Purification from ULF**

*Cation Exchange Chromatography*—1- or 4-liter samples of clarified ULF supernatant were applied at 4 °C to a Bio-Rex 70 cation exchange column (5 × 6 cm; Bio-Rad) that had previously been equilibrated in PBS, 0.2 mM NaCl. After sample application, the column was washed with 500 ml of PBS, 0.2 mM NaCl, and bound proteins were eluted using a 500-ml gradient of 0.2-2 mM NaCl in PBS. The flow rate was 3.5 ml/min throughout, and fractions of 10 ml were collected during treatment of the column with the NaCl gradient. Fractions demonstrating mitogenic activity for Balb/c 3T3 fibroblasts were selected for further use. All subsequent chromatographic steps were performed at room temperature.

**Heparin Affinity Chromatography**—Biologically active fractions containing the 0.3-0.6 mM NaCl eluate from the Bio-Rex column were pooled, diluted 3-fold with 20 mM Tris-HCl (pH 7.4) containing 0.1% CHAPS, passed through a 0.45-μm membrane filter, placed in a siliconized polypropylene vessel, and applied with a peristaltic pump to an EconoPac heparin column (0.7 × 36 cm; Bio-Rad) at 2 ml/min. The heparin column was then washed with 50 ml of 20 mM Tris-HCl buffer, 0.2 mM NaCl, 0.1% CHAPS (pH 7.4) and developed at 1 ml/min with a 40-ml gradient of 0.2-2 mM NaCl in 20 mM Tris-HCl, 0.1% CHAPS (pH 7.4) using a fast protein liquid chromatography (FPLC) system (Pharmacia Biotech Inc.). Fractions (1 ml) were collected into siliconized tubes during NaCl gradient elution and tested for 3T3 cell mitogenic activity.

To perform a second heparin affinity purification step, biologically active fractions containing the 0.8 mM NaCl eluate from the EconoPac heparin affinity FPLC of ULF were applied at 0.5 ml/min to a TSK G2000 SW FPLC column (30 cm × 8 mm, 10-μm particle size, M, 500–100,000 fractionation range; Tosohaas) equipped with a SW guard column (4 cm × 8 mm, 10 μm; Tosohaas). Proteins were eluted with PBS containing 0.3 mM NaCl. Fractions of 200 μl were collected and tested for their ability to stimulate DNA synthesis in 3T3 cells. Column calibration was performed using EGF (6,000 Da), lactalbumin (14,200 Da), trypsin inhibitor (20,100 Da), and ovalbumin (45,000 Da).

**SDS-PAGE, Silver Staining, and Western Blotting**—SDS-PAGE was performed under reducing conditions using 18% polyacrylamide minigels as described (8). Silver staining of proteins was performed as described (9). SDS-PAGE and Western blotting were performed on (i) HPLC-purified growth factors, (ii) 8 μl of unfractonated ULF, or (iii) 100 μl of ULF after passage through 20-μl beds of heparin-Sepharose in the presence of 10 mM Tris-HCl, 0.5 mM NaCl (pH 7.4) and subsequent extraction of the heparin beads with SDS-PAGE sample buffer. Gels were blotted and blocked as described (8) and incubated with a 1:1,000 dilution of rabbit anti-pCTGF (247–280) peptide antiserum (rabbit A; see below). Immunoreactive bands were visualised using alkaline phosphatase-conjugated goat anti-rabbit IgG followed by nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate chromogenic substrates.

**CTGF Peptide Synthesis and Characterization**—Eighteen synthetic peptides spanning the entire 103 C-terminal residues of CTGF were synthesized and received as a cleaved PepSet™ from Chiron Mimitope, Victoria, Australia. All peptides were designed with acetylated N termini and amidated C termini except CTGF(247–255) and CTGF(247–260), which were synthesized with free N-terminal amines, and CTGF(326–349) and CTGF(339–349), which were synthesized with C-terminal amines.

All peptides contained one or no Cys residues; Cys292 in CTGF(285–292) and Cys325 in CTGF(318–328) were replaced with Ser to prevent intrachain disulfide bridging to Cys292 or Cys325 within the respective peptides. Heparin-binding properties were determined using an adaptation of the method of Baird et al. (20). Briefly, 37.5 nmol of each peptide was absorbed in duplicate to nitrocellulose using a dot-blot apparatus. The blots were blocked for 30 min with 10 mM Tris-HCl, 0.15 μM NaCl, 0.1% bovine serum albumin (pH 7.4) and then incubated for 3 h at room temperature in this solution containing 10 μg/ml of PHBepharin (NEN Life Science Products). The blots were washed four times with 10 mM Tris-HCl, 0.15 mM NaCl, and individual dots were mixed with scintillation fluid for counting of 3H.

**CTGF Antibody Production**—A four-branched multiple antigenic CTGF(247–280) peptide comprising the sequence EEIKKKGKCRTRP (residues 247–280) was produced on a Synergy 432A peptide synthesizer and purified by reverse-phase HPLC using a C18 column (0.46 × 25 cm; Rainin Instruments) that was developed with a 90-μl 5–95% acetonitrile gradient in water, 0.1% trifluoroacetic acid. Fractions containing the purified peptide were pooled, evaporated to dryness, and reconstituted in sterile water. Two New Zealand White rabbits (rabbits A and B), which had been bled to collect preimmune serum, were injected subcutaneously with 1 mg of peptide in Freund's complete adjuvant, followed 3 weeks later by an intramuscular injection...
of 250 μg of peptide in Freund's incomplete adjuvant. Animals were bled 7 days later for collection of antiserum. Reactivity of the antisera was validated by Western blotting and immunoprecipitation. Preimmune serum was pooled and subjected to EconoPac heparin affinity chromatography, and the biologically active fractions containing the 0.3–0.6 M NaCl eluate were pooled and applied to a TSK G2000 SW FPLC column as described under "Materials and Methods." Fractions were tested for their ability to stimulate DNA synthesis in 3T3 cells at 40 μl/ml. The figure shows the elution position of protein standards that were used to calibrate the column.

CATGGAAGAGACATTAAGAAGGG-3’ and 3’-CCCTCTGATCCCTACTTTAA-3’ and 3’-CCCTCAGCCGGCCGCACC-5’, respectively. The probe was used to screen 10^6 plaques, two of which showed reproducible hybridization and were isolated using a Rapid Excision Kit (Stratagene). Two ~5.0-kilobase-pair pBluescript SK pig CTGF clones, termed pBSK-pCTGF1 and pBSK-pCTGF2, were obtained and used for initial sequencing reactions. pBSK-pCTGF1 was then fully sequenced by a combination of manual and automated dideoxy terminator sequencing (21). Sequence data were obtained from both strands of DNA.

RESULTS

Characterization and Purification of an Atypical HBGF in ULF—Ion exchange chromatography of ULF showed the presence of cationic growth factor activity for Balb/c 3T3 cells that was eluted from Bio-Rex 70 columns by 0.3–0.6 M NaCl (data not shown). Although we have previously shown that constitutive growth factors in this cationic extract include PDGF, HB-EGF, bFGF, and PTN (2, 8, 12), heparin affinity chromatography revealed the presence of an additional unidentified HBGF that required 0.8 M NaCl for elution from an EconoPac heparin column and, in terms of the amount of bioactivity recovered from the column, appeared to be a principal cationic HBGF in ULF for 3T3 cells (Fig. 1A). The elution position of this factor (hereafter termed HBGF-0.8) from heparin affinity columns was clearly distinct from PDGF, HB-EGF, bFGF, and PTN (2, 8, 12). Stability tests performed by exposing HBGF-0.8 to pH 1.5 for 2 min, 56 °C for 5 min, or 0.3 ng/ml bFGF (data not shown). HBGF-0.8 was also mitogenic activity of HBGF-0.8 was highly susceptible to acid or heat inactivation (data not shown). Gel filtration FPLC showed that HBGF-0.8 was of M_r ~10,000 under nonreducing and non-denaturing conditions (Fig. 2). When compared with other 3T3 cells mitogens, the level of maximum induction of [3H]thymidine incorporation by HBGF-0.8 was comparable with that of calf serum or purified PDGF or bFGF rather than that of weaker mitogens such as IGF or EGF (Table I). Further, the 3T3 cell mitogenic activity of HBGF-0.8 was synergistically enhanced by the addition of a maximal amount of EGF but was less than the activity of HBGF-0.8 (data not shown). HBGF-0.8 was also mitogenic for smooth muscle cells and produced a level of stimulation that exceeded that of a maximal amount of EGF but was less than that of bFGF (Table I). HBGF-0.8 lacked mitogenic activity for endothelial cells when tested alone or in the presence of 100 μg/ml heparin (Table I).

Fractions that contained HBGF-0.8 activity (i.e. fractions 15–18; see Fig. 1A) were pooled, diluted, and subjected to a second cycle of heparin affinity FPLC using a TSK heparin column to achieve a final M_r > 300,000 (Fig. 2B). Two rounds of purification resulted in up to 1.4 × 10^10 plaque-forming units/ml.
Connective Tissue Growth Factor in Uterine Secretions

TABLE I

| Cell type                  | Treatment                   | [3H]Thymidine incorporation (mean ± S.D.) |
|----------------------------|-----------------------------|------------------------------------------|
| Balb/c 3T3 fibroblasts     | None                        | 428 ± 18                                 |
|                            | 20% calf serum              | 123,820 ± 7,470                          |
|                            | 30 ng/ml IGF-1              | 4,412 ± 170                              |
|                            | 30 ng/ml EGF                | 11,550 ± 101                             |
|                            | 10 ng/ml bFGF               | 73,853 ± 3,122                           |
|                            | 30 ng/ml PDGF-AB            | 110,110 ± 7,077                          |
|                            | 20 μl HBGF-0.8              | 114,730 ± 3,200                          |
|                            | 3 ng/ml EGF                | 680 ± 341                                |
|                            | 3 ng/ml bFGF               | 1,343 ± 378                              |
|                            | 3 ng/ml bFGF + 10 μg/ml heparin | 3,082 ± 374                           |
|                            | 15 μg/ml HBGF-0.8          | 1,709 ± 403                              |
| Vascular smooth muscle cells | None                       | 316 ± 84                                 |
|                            | 100 μg/ml heparin           | 240 ± 52                                 |
|                            | 3 ng/ml heparin            | 2,865 ± 276                              |
|                            | 3 ng/ml heparin + 100 μg/ml heparin | 1,840 ± 4                              |
|                            | 3 ng/ml aFGF               | 603 ± 46                                 |
|                            | 3 ng/ml aFGF + 100 μg/ml heparin | 2,232 ± 236                           |
|                            | 20 μl HBGF-0.8             | 243 ± 4                                  |
|                            | 20 μl HBGF-0.8 + 100 μg/ml heparin | 195 ± 12                               |
| Capillary endothelial cells | None                       |                                        |
|                            | 30 ng/ml IGF-1              |                                        |
|                            | 30 ng/ml EGF                |                                        |
|                            | 10 ng/ml bFGF               |                                        |
|                            | 30 ng/ml PDGF-AB            |                                        |
|                            | 20 μl HBGF-0.8              |                                        |
|                            | 3 ng/ml EGF                |                                        |
|                            | 3 ng/ml bFGF               |                                        |
|                            | 3 ng/ml bFGF + 10 μg/ml heparin |                                        |
|                            | 15 μg/ml HBGF-0.8          |                                        |

5PW column. As shown in Fig. 1B, HBGF-0.8 was again eluted by 0.8 M NaCl (fractions 31–36) but was resolved as two peaks of mitogenic activity that had distinct heparin-binding properties. These activity peaks were termed HBGF-0.8 peak 1 (HBGF-0.8-P1) and HBGF-0.8 peak 2 (HBGF-0.8-P2) and were present in fractions 31–34 and fractions 35 and 36, respectively (Fig. 1B).

HBGF-0.8-P1 and -P2 were adjusted to 10% acetonitrile, 0.1% trifluoroacetic acid and individually subjected to C₈ reverse-phase HPLC. As shown in Fig. 3, the elution positions of HBGF-0.8-P1 and -P2 were determined by bioassay of aliquots of fractions containing the column eluate after they had been evaporated and reconstituted in PBS, demonstrating that there was sufficient activity in the purified HBGF-0.8 samples to permit their detection and further characterization despite prolonged (approximately 30–40-min) exposure to pH 2 during the HPLC step. Silver-stained SDS-PAGE analysis of the fractions containing either HBGF-0.8-P1 or -P2 revealed the presence of a 10-kDa band under reducing conditions, the levels of which were directly correlated with the levels of mitogenic activity over the peak fractions (Fig. 3). This and other HPLC separations proved conclusively that the bioactivity was attributable to the 10-kDa moiety only. Second step HPLC purification of HBGF-0.8-P2 resulted in the isolation of a single 10-kDa protein that co-purified with Balb/c 3T3 cell mitogenic activity. The unassigned residue in cycle 10 of HBGF-0.8-P1 and cycle 9 of HBGF-0.8-P2 corresponded to Cys256 of hCTGF and Cys 255 of mouse CTGF (28–30).

To verify that the partial sequences of HBGF-0.8-P1 and -P2 were actually present in the pCTGF molecule, a full-length pCTGF cDNA was isolated by hybridization screening of a pig endometrial cDNA library using a32P-labeled hCTGF probe. Analysis of the individual purification steps showed that 0.5–1.1 μg of HBGF-0.8-P1 or -P2 were each purified from 342 mg of crude ULF protein and that 10–22 activity units for HBGF-0.8-P1 or P2 were recovered after the first HPLC step as compared with 66,666 units in 1 liter of starting material (Table II). It should be noted that the apparent low recovery of HBGF-0.8 activity was attributable to (i) a major contribution by IGF, EGF, PDGF, bFGF, HB-EGF, and PTN to the overall 3T3 cell mitogenic activity of the crude and partially purified samples (2, 8, 9, 12, 25–27) and (ii) acid lability of HBGF-0.8 mitogenic activity during the HPLC separation step(s). Although alternative strategies were attempted to recover purified growth factors of higher specific activity, it was not possible to avoid the use of either reverse-phase HPLC or trifluoroacetic acid for ion pairing without compromising the purity of the final product. While, in terms of their biological activity, recovery of HBGF-0.8-P1 and -P2 was somewhat compromised, structural characterization of the proteins was readily achieved, since they retained sufficient activity to be unequivocally attributable to a single, homogenous 10-kDa band in SDS-polyacrylamide gels, and adequate quantities of each protein were isolated from several liters of ULF (Fig. 3, Table II).

Fractions containing the HPLC-purified growth factors were pooled, dried, and subjected to preparative SDS-PAGE, after which proteins in the gel were transferred to a polvinyldene difluoride membrane, and the position on the blots of HBGF-0.8-P1 or -P2 proteins was determined by staining with Coomassie R250. Each 10-kDa band was excised and submitted for N-terminal microsequencing with the result that a 16-residue sequence was obtained for HBGF-0.8-P1 with an undetermined residue at position 10, and a 12-residue sequence was obtained for HBGF-0.8-P2 with an undetermined residue at position 9 (Table III). These data showed that HBGF-0.8-P1 and -P2 were N-terminally identical except for the presence of an additional Glu residue at the N terminus of HBGF-0.8-P1. A search of GenBank revealed that these sequences aligned perfectly with predicted internal sequences of hCTGF and mouse fisp-12 (also termed βIG-M2), the murine homologue of CTGF (28–30). The unassigned residue in cycle 10 of HBGF-0.8-P1 and cycle 9 of HBGF-0.8-P2 corresponded to Cys²⁵⁶ of hCTGF and Cys²⁵⁵ of fisp-12 (Table III).

To verify that the partial sequences of HBGF-0.8-P1 and -P2 were actually present in the pCTGF molecule, a full-length pCTGF cDNA was isolated by hybridization screening of a pig endometrial cDNA library using a32P-labeled hCTGF probe. The cloned pig CTGF cDNA was determined to be 1.51 kilobase pairs, with an open reading frame of 1,047 base pairs (data not shown). The primary translational product of pCTGF is pre-proCTGF, which is predicted to comprise 323 amino acids and to
CTGF exist in uterine fluid in vivo. Taken together, these data suggested that ULF contained nondetectable levels of 38-kDa CTGF and that 10-kDa CTGF was most likely identified in these studies due to its natural occurrence in utero.

Heparin-binding Properties of HBGF-0.8—Previous studies have shown that heparin modulates receptor binding and biological activity of several HBGFs including bFGF, HB-EGF, and amphiregulin (31–37). Since HBGF-0.8 exhibited strong affinity for heparin, we examined the effect of this glycosaminoglycan on the mitogenic activity of HBGF-0.8. As shown in Fig. 5, the activity of a high stimulatory dose of HBGF-0.8 was significantly potentiated by 1–3 μg/ml heparin but was inhibited by 30–100 μg/ml heparin. The same heparin doses had no effect on basal or calf serum-stimulated DNA synthesis in 3T3 cells (Fig. 5).

The presence of an additional acidic Glu residue at the N terminus of HBGF-0.8-P1 was correlated with the lower heparin affinity of this molecule as compared with HBGF-0.8-P2, suggesting that the N terminus of HBGF-0.8 may be part of a heparin-binding domain. To test the heparin-binding properties of the N-terminal region as well as other portions of the CTGF molecule, the ability of 18 peptides spanning the entire C-terminal 103 residues of hCTGF to bind [3H]heparin was investigated. Table IV shows that the highest level of heparin binding was obtained for peptides containing residues 247–260, 274–286, and 305–328. Although the functional significance of heparin binding of these domains requires further investigation, none of the peptides had HBGF-0.8 agonist or antagonist activity in the 3T3 cell DNA synthesis assay (data not shown).

DISCUSSION

Although the presence of CTGF-like activity in uterine flushings was initially demonstrated several years ago (2, 8, 38), its identification and structural characterization was not possible until the development of the purification protocol described herein, which allowed for its separation from other unrelated HBGFs such as PDGF, bFGF, HB-EGF, and PTN (2, 8, 12). The data presented in this report show that a fibroblast and smooth muscle cell growth factor in uterine secretory fluids is a CTGF protein that is heparin-binding and substantially N-terminally truncated in comparison with the predicted CTGF primary translation product. CTGF belongs to a recently recognized immediate early gene family, members of which are transcribed within minutes of stimulation by growth factors such as

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**FIG. 3.** C8 reverse-phase HPLC and SDS-PAGE of HBGF-0.8-P1 and -P2. HBGF-0.8 was purified from 4 liters of ULF obtained from 87 uteri by cation exchange chromatography, EconoPac heparin affinity FPLC, and TSK heparin affinity FPLC. TSK heparin column fractions that contained HBGF-0.8-P1 (A) or HBGF-0.8-P2 (B) were pooled and applied to a C8 reverse phase HPLC column (0.46 × 25 cm; 5 μm). After application of each sample, the column was washed with 10% acetonitrile in water, 0.1% trifluoroacetic acid from 0 to 10 min and then treated with a 10–90% acetonitrile gradient in water from 10 to 146 min. The flow rate was 1 ml/min, and 0.5-ml fractions were collected 20 min after the samples were injected. 80 μl of selected fractions were dried and redissolved in 25 μl of 10 mM Tris-HCl (pH 7.4), and 10 μl were tested for their ability to stimulate 3T3 cell DNA synthesis. The insets show silver-stained SDS-PAGE analysis of successive reactive fractions from each HPLC separation. 10 μl of the appropriate reconstituted fractions were mixed with 10 μl of 2 × SDS-PAGE sample buffer containing 50 mM dithiothreitol and electrophoresed in an 18% polyacrylamide minigel for 1 h at 200 V. Proteins in the gel were visualized with silver staining as described (19). The arrow indicates the 10-kDa protein that co-eluted with vitamin activity.
that produced loaded with one-fourth of the amount of HPLC-purified HBGF-0.8-P2 beads with SDS-PAGE sample buffer (lanes A–F, pig 4-5 (day 14); lanes G–J, pig 64-6 (day 14); lanes K–R, pig 12-6 (day 16)). Lanes A and B were loaded with one-fourth of the amount of HPLC-purified HBGF-0.8-P2 that produced ~40,000 cpm (3H]thymidine incorporation when added to 3T3 cells in 200 µl of medium. Lanes A, C–F, and K–N were incubated with a 1:1,000 dilution of rabbit preimmune serum. Lanes B, G–J, and O–R were incubated with a 1:1,000 dilution of rabbit anti-pCTGF-(247–262) peptide antiseraum. Immunoreactive bands were visualized using alkaline phosphatase-conjugated goat anti-rabbit IgG followed by nitro blue tetrazolium/5-bromo-4-chloro-3-indoly phosphate chromogenic substrates. The bands at ~14 and ~40 kDa (lanes G–J) appear to be nonspecific, since they were also detected by preimmune serum (lanes C–F). They were also removed by heparin purification of the samples (lanes O–R). Lanes C, G, K, and O, pig 64-6 (day 14); lanes D, H, L, and P, pig 79-6 (day 14); lanes E, I, M, and Q, pig 4-5 (day 14); lanes F, J, N, and R, pig 12-6 (day 16). Specific immunoreactive proteins are arrowed.

FIG. 4. CTGF Western blot analysis of unpurified ULF. SDS-PAGE and Western blotting were performed directly on 8 µl of uterine flushings from four nonpregnant pigs, representing 0.7–2.3 µl of undiluted fluid (i.e. 0.028–0.035% of the total intrinsic uterine fluid volume) (lanes C–J) or on the equivalent of 100 µl of flushings after passage through 20-µl beds of heparin-Sepharose in the presence of 10 mM Tris-HCl, 0.5 mM NaCl (pH 7.4) and subsequent extraction of the heparin beads with SDS-PAGE sample buffer (lanes K–R). Lanes A and B were incubated with a 1:1,000 dilution of rabbit preimmune serum (lanes A, B) or the purified proteins, Western blotting showed that 10-kDa forms of CTGF in their immunoreactivity, chromatographic behavior, and/or mitogenic activity.

Although CTGF mitogenic and chemotactic activities were reported to correlate with the presence of 36–39-kDa proteins in human endothelial cell conditioned medium (28), these proteins were not individually isolated, tested for biological activity, or structurally characterized. Also, while the mouse CTGF gene product has been characterized as a nonglycosylated 35-kDa protein that is rapidly produced in and secreted by NIH 3T3 cells after serum stimulation (29), its intrinsic growth factor activity has not been reported. The susceptibility of CTGF to acid inactivation suggests that 10-kDa HBGF-0.8 proteins are likely to be more potent in their native state than after purification (i.e. ED50 of 25 ng/ml for twice HPLC-purified HBGF-0.8-P1; see Table II). However, even in comparison with the purified proteins, Western blotting showed that 10-kDa CTGF is present in uterine secretions at levels that are predicted to be highly mitogenic, which is strongly suggestive of a physiological role for this protein in utero. Native hCTGF, partially purified on an PDGF immunoaffinity column, was reported to stimulate DNA synthesis in NIH 3T3 cells with an ED50 of ~10 ng/ml (28). A similar ED50 was shown for the

dium from cultured mouse or human fibroblasts, suggesting that production of low-mass CTGF is common to several species and biological systems. The relationship between the predicted pCTGF primary translational product and the truncated uterine CTGF proteins is shown schematically in Fig. 6. We have yet to determine whether our lack of detection of 38-kDa CTGF reflects its actual absence from ULF or its immediate processing after secretion or is a result of differences between the 38- and 10-kDa forms of CTGF in their immunoreactivity, chromatographic behavior, and/or mitogenic activity.
stabilized by association with heparan sulfate proteoglycans and residues 255–329 of mouse CTGF, has been termed the CT module, which spans residues 256–330 of hCTGF and pCTGF module II dimerization/receptor binding domain (40). The fourth, indicated by our data, since the CT module is entirely conserved in the pCTGF molecule (Fig. 6). The fourth (1108–1129) of mCTGF, and residues 256–260 may act cooperatively with the proposed C terminus as in the primary translational product. The presence of this motif in the truncated forms of CTGF identified in this study suggests that additional heparin-binding domains exist in the C-terminal 102 residues of CTGF. The N terminus of HBGF-0.8-P1 and -P2 have yet to be established, although the apparent molecular masses of the HBGF-0.8 proteins are consistent with the same or similar C terminus as in the primary translational product. Domains within the primary translational product include the signal peptide (SP) and four structural modules that show structural similarity to an IGF binding region (module I), a von Willebrand factor type C repeat (module II), a sulfated glycosaminoglycan binding motif (module III), and a C-terminal (CT) dimerization/receptor binding domain (module IV) (40).

Recently, 38-kDa hCTGF was reported to bind to heparin-Sepharose, although the salt concentration required for its elution was not stated (43). However, the 41-kDa cyr61 gene product showed peak elution from heparin-agarose with 0.8 M NaCl (44), a salt concentration identical to that required for elution of 10-kDa CTGF from immobilized heparin. Based on sequence homology with thrombospondin, properdin, TRAP, f-spondin, UNC5, and several complement and cieumoporozole proteins, Bork (40) proposed a binding motif (WSX3X3XCG) for sulfated glycosaminoglycates between residues 206 and 214 of hCTGF. However, the absence of this motif in the truncated forms of CTGF identified in this study suggests that additional heparin-binding domains exist in the C-terminal 102 residues of CTGF. The N terminus of HBGF-0.8-P1 and -P2 may be involved in heparin-binding, since the two proteins displayed differential binding to heparin yet differ only by a single N-terminal Glu; the lack of this acidic residue may facilitate the binding of heparin to basic residues located in the N-terminal region. There is a very high proportion (50%) of basic amino acids (K, R) in the 17-residue region CTGF-(251–267), a composition that is well conserved in the corresponding regions of all CCN family members. Residues 250–255 (IKKGGK) of pCTGF conform to a proposed heparin recognition consensus sequence (XBBXXB, where a basic residue is strongly favored at the B position and a nonbasic residue is usually, but not absolutely, found at the X position) that occurs in other heparin-binding proteins (45). While this sequence may account, at least partly, for the binding of [3H]heparin to CTGF-(247–260), the lack of binding of [3H]heparin to CTGF-(247–255) suggests that residues 256–260 may act cooperatively with the proposed consensus sequence in binding heparin. Our data further highlights a potential contribution from other regions (residues 274–286 and 305–328) to the overall net heparin-binding characteristics of 10-kDa CTGF. While these domains require further study, the heparin dependence of HBGF-0.8 bioactivity suggests that CTGF-heparin interactions are functionally significant. Although 10 μg/ml heparin was recently shown to stimulate the mitogenic activity of partially purified recombinant 38-kDa hCTGF (43), our finding of a biphasic effect of heparin on CTGF activity has not previously been described and suggests that the heparin:CTGF ratio is an important determinant of net CTGF mitogenic activity. Additionally, cyr61 is present in the extracellular matrix, where it may be stabilized by association with heparan sulfate proteoglycans (44).

The predicted sequences of all CCN members conform to four distinct structural modules that span the entirety of each molecule, suggesting that all of the family members are mosaic proteins (40). Based on our cDNA analysis, these modules are fully conserved in the pCTGF molecule (Fig. 6). The fourth module, which spans residues 256–330 of hCTGF and pCTGF and residues 255–329 of mouse CTGF, has been termed the C-terminal (CT) module and is also present in a variety of unrelated extracellular mosaic proteins including slit, Ndp, and PDGF-B (40, 46, 47). Further, it has been suggested that the CT module may also be involved in receptor binding (40), a possibility that is supported by our data, since the CT module is entirely con-

| Peptide domain | Sequence | [3H]Heparin bound (mean ± S.D.) | cpm/μg |
|---------------|---------|-------------------------------|--------|
| None          |         |                               | 11 ± 0.2 |
| CTGF-(247–255)| EENIKGGK*|                               | 10 ± 0.3 |
| CTGF-(247–280)| EENIKGGKCI RTTP*               | 858 ± 1 |
| CTGF-(257–279)| IRTPKISKPIKFELSGL            | 70 ± 12 |
| CTGF-(259–275)| TPKISKPIKFELS GCTS           | 124 ± 3 |
| CTGF-(274–283)| TSMKTYRAFK                    | 388 ± 12 |
| CTGF-(274–286)| TSMKTYRAFKCGV                | 1108 ± 119 |
| CTGF-(285–291)| GVCIDGR                      | 7 ± 0.3 |
| Sea²²² CTGF-(285–292)| GVCIDGSR | 8 ± 0.4 |
| CTGF-(293–306)| CTHRTTTLVF EK                | 9 ± 1.1 |
| CTGF-(294–306)| TPHRTTLVF EK                 | 11 ± 0.4 |
| CTGF-(305–322)| FKCPDEVMKRMNMF IKT          | 237 ± 22 |
| CTGF-(308–322)| PGDEVMKRMNMF IKT            | 71 ± 2 |
| CTGF-(318–324)| MFIKTCA                      | 475 ± 116 |
| Sea²²² CTGF-(318–328)| MFIKTCAHYN | 601 ± 40 |
| CTGF-(322–328)| ACHYN                        | 9 ± 1 |
| CTGF-(326–349)| HYNCPCDIFESLYY RYMGDMA³¹⁶ | 10 ± 1 |
| CTGF-(330–340)| PGGNDIESLY                  | 10 ± 0.5 |
| CTGF-(339–349)| LYRMYGDMA³¹⁶                | 9 ± 0.5 |

* Free N-terminal amine.
³ Acid C terminus.

FIG. 6. Relationship between HBGF-0.8 proteins and pCTGF primary translational product. HBGF-0.8-P1 and -P2 are shown aligned relative to one another and to the pCTGF primary translational product. The question mark indicates that the C termini of HBGF-0.8-P1 and -P2 have yet to be established, although the apparent molecular masses of the HBGF-0.8 proteins are consistent with the same or similar C terminus as in the primary translational product. Domains within the primary translational product include the signal peptide (SP) and four structural modules that show structural similarity to an IGF binding region (module I), a von Willebrand factor type C repeat (module II), a sulfated glycosaminoglycan binding motif (module III), and a C-terminal (CT) dimerization/receptor binding domain (module IV) (40).
tained within the truncated uterine CTGF protein.

In conclusion, we have shown that (i) heparin-binding forms of CTGF of M, 10,000, 16,000, and 20,000 are present in uterine flushings; (ii) 10-kDa CTGF corresponds to two microheterogeneous N-terminally truncated forms of the primary translational product, both of which are biologically active; (iii) mitogenic activity of 10-kDa CTGF is heparin-dependent; and (iv) receptor-binding and heparin-binding domains of CTGF lie in the C-terminal 103 residues of the molecule. Since CTGF is a uterine secretory growth factor, it may play a role in growth and remodeling of the endometrium and, during pregnancy, growth and development of the placental membranes. Future work will involve investigations of the physiological role of CTGF in female reproductive tract function and embryogenesis, as well as detailed structure-function studies of CTGF and determination of the mechanism of production of 10-kDa CTGF.

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