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The insulin-like growth factor (IGF)-dependent IGF binding protein-4 protease secreted by human fibroblasts is pregnancy-associated plasma protein-A

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ABSTRACT **Proteolytic cleavage of the six known insulin-like growth factor binding proteins (IGFBPs) is a powerful means of rapid structure and function modification of these important growth-regulatory proteins. Intact IGFBP-4 is a potent inhibitor of IGF action *in vitro,* and cleavage of IGFBP-4 has been shown to abolish its ability to inhibit IGF stimulatory effects in a variety of systems, suggesting that IGFBP-4 proteolysis acts as a positive regulator of IGF bioavailability. Here we report the isolation of an IGF-dependent IGFBP-4-specific protease from human fibroblast-conditioned media and its identification by mass spectrometry microsequencing as pregnancy-associated plasma protein-A (PAPP-A), a protein of unknown function found in high concentrations in the maternal circulation during pregnancy. Antibodies raised against PAPP-A both inhibited and immunodepleted IGFBP-4 protease activity in human fibroblast-conditioned media. Moreover, PAPP-A purified from pregnancy sera had IGF-dependent IGFBP-4 protease activity. PAPP-A mRNA was expressed by the human fibroblasts and osteoblasts, and PAPP-A protein was secreted into the culture medium. In conclusion, we have identified an IGF-dependent IGFBP protease and at the same time assigned a function to PAPP-A. This represents an unanticipated union of two areas of research that were not linked in any way before this report.**

Insulin-like growth factors (IGFs) are essential polypeptides with potent anabolic and mitogenic actions both *in vivo* and *in vitro* (1, 2). IGF bioactivity is modulated by distinct high-affinity IGF binding proteins (IGFBPs), six of which have been identified to date (3, 4). Adding another layer of refinement to this growth regulatory system has been the recent discovery that IGFBPs can undergo limited proteolysis with consequent modification of IGFBP structure and function, and hence IGF action (5). Currently, there is active investigation into identifying, classifying, and ascertaining the biological significance of IGFBP proteases in a variety of biological systems.

We have characterized previously IGFBP-4-specific metalloprotease activity secreted by normal human fibroblasts in culture (6, 7). Incubation in human fibroblast-conditioned medium (HFCM) under cell-free conditions results in cleavage of IGFBP-4 (24 kDa nonreduced, 32 kDa reduced) in the midportion of the molecule producing distinct fragments of 18 and 14 kDa. The defining feature of this IGFBP-4 proteolytic reaction is its absolute dependence on IGFs for functional activity. Only very low concentrations of IGFs are needed, and, in general, IGF-II is more potent than IGF-I in activating proteolysis (6). Similar IGF-dependent IGFBP-4 proteolysis has been described in cultures of normal human osteoblasts (8, 9), vascular smooth muscle cells (10), endometrial stromal cells (11), decidual cells (12), and granulosa cells (13), as well as in ovarian follicular fluid (14), but in none of these systems was the responsible enzyme identified. This lack of identification has made delineation of a physiological role for IGFBP-4 proteolysis extremely difficult. Nonetheless, based on *in vitro* data showing increased bioavailability of IGFs with IGFBP-4 proteolysis in the various cell systems, it has been suggested that the IGF-dependent IGFBP-4 protease plays an important role in local proliferative responses such as wound healing, bone remodeling, and atherosclerotic plaque development, as well as in several aspects of human reproduction (5–15).

As a first step toward defining this IGF regulatory mechanism, we purified the IGF-dependent IGFBP-4 protease from HFCM. Herein we describe this enzyme purification and its identification as pregnancy-associated plasma protein-A (PAPP-A).

MATERIALS AND METHODS

HFCM. Human fibroblasts from a normal male donor (GM03652, Coriell Institute, Camden, NJ), were grown to confluency in T75 flasks, washed twice with DMEM, and then changed to 15 ml of 50:50 Waymouth's medium:DMEM containing 100 units/ml penicillin, 100 μg/ml streptomycin/4 mM glutamine/0.1% BSA for 6 hr at 37°C. The cells were again washed and changed to 10 ml of the serum-free medium and incubated for 72 hr at 37°C. This HFCM was placed in a sterile conical tube and centrifuged at 1,500 $\times$ *g* for 30 min at 4°C to remove cellular debris, decanted into another sterile conical tube, and stored at −30°C.

IGFBP-4 Protease Assay. IGFBP-4 proteolysis was assayed, as described previously (6–8), by incubating sample overnight at 37°C with 2 mM $CaCl_2$/50 mM Tris (pH 7.5)/10,000 cpm of [^{125}I]IGFBP-4 in the absence and presence of 5 nM IGF-II in a total volume of 25 μl. Proteins were separated by reducing 15% SDS/PAGE and were visualized by autoradiography.

For some experiments, PAPP-A polyclonal antibody (16) or nonspecific rabbit IgG was added to the assay mixture (Fig. 2*a*) or was used in conjunction with protein G plus protein A-agarose (Oncogene Science) to immunoprecipitate IGFBP-4 protease activity before assay (Fig. 2*b*).

Purification. HFCM (800 ml) in six 130- to 150-ml batches, were passed over a 25-ml bed volume of iminodiacetic acid immobilized to Sepharose 6B (Sigma) loaded with Zn^{+2} and

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Abbreviations: IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; HFCM, human fibroblast-conditioned medium; PAPP-A, pregnancy-associated plasma protein-A; proMBP, proform of major basic protein.

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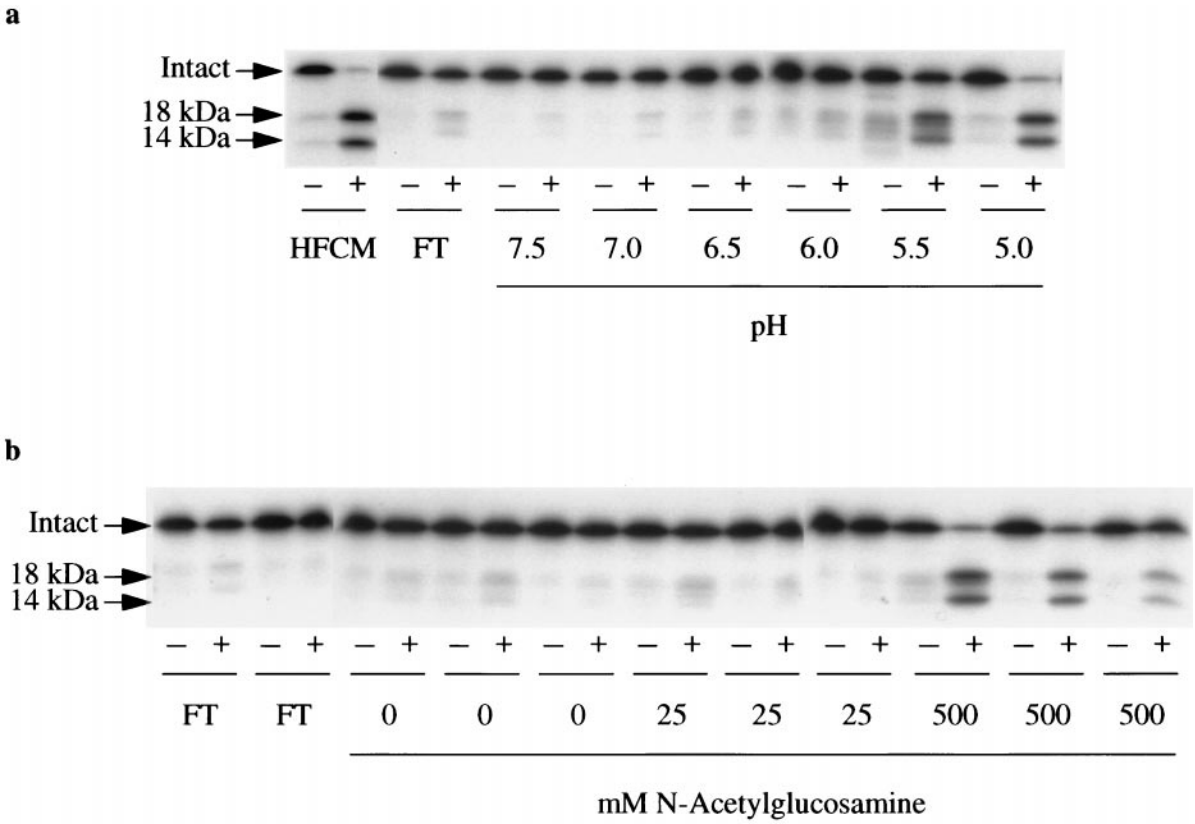

FIG. 1. Purification of the IGF-dependent IGFBP-4 protease from HFCM. (*a*) Metal-chelating affinity chromatography. Bound proteins were eluted with a descending stepwise pH gradient, and 50 μl aliquots of each of these fractions were dialyzed and assayed for IGFBP-4 protease activity. IGF-dependent IGFBP-4 protease activity is defined as the loss of intact 24-kDa [^{125}I]IGFBP-4 and the appearance of 18- and 14-kDa radiolabeled fragments (denoted by arrows) in the presence (+), but not in the absence (-), of 5 nM IGF-II. HFCM, starting material; FT, flowthrough. (*b*) Wheat-germ agglutinin chromatography. Metal-chelating affinity chromatography fraction eluting at pH 5.0 was adjusted to pH 7.4 and immediately passed over a wheat-germ agglutinin column. Non- and weakly bound proteins came out in the flowthrough (FT) or were eluted with three washes of 0 mM and three washes of 25 mM *N*-acetylglucosamine. IGF-dependent IGFBP-4 protease activity was eluted with three washes of 500 mM *N*-acetylglucosamine.

equilibrated with 50 mM Tris/50 mM NaCl, pH 7.4. Bound proteins were eluted sequentially with 50 ml each of a 0.5 pH unit stepwise decreasing gradient. The pH 5.0 fraction was immediately adjusted to pH 7.4 and passed over a 1-ml bed volume wheat germ agglutinin column equilibrated with 20 mM Tris/100 mM NaCl (pH 7.5) at 4°C. Bound proteins were eluted with 15 ml each of 50 mM Tris/100 mM NaCl (pH 7.5) alone and then with 25 mM and 500 mM *N*-acetylglucosamine. Five-ml fractions were collected and assayed for IGFBP-4 protease activity. Fractions from the six chromatography runs were pooled, concentrated by ultrafiltration, and electrophoresed through a 5% acrylamide SDS/PAGE gel under reducing conditions. The SDS/PAGE gel was silver stained, and four bands at 400, 230, 200, and 175 kDa that correlated to IGFBP-4 protease activity were excised for mass spectrometric analysis.

Mass Spectrometry Sequencing. The excised bands were subjected to in-gel trypsin digestion (17). Soluble fragments were recovered from the digestion, then resolved and characterized by microcolumn high-performance liquid chromatography (18) and automated tandem mass spectrometry. Microelectrospray columns were constructed from 360-μm o.d. × 100-μm i.d. fused silica capillary with the column tip tapered to a 5- to 10-μm opening (19). The microcolumns were packed with POROS 10 R2 (PerSeptive Biosystems, Framingham, MA) to a length of 10–15 cm. The mobile phase used for gradient elution consisted of (*i*) 0.5% acetic acid and (*ii*) acetonitrile/water 80:20 vol/vol containing 0.5% acetic acid. The gradient was linear from 0 to 40% over 30 min and from 40 to 60% over 5 min. The mass spectrometer used was a Finnigan-MAT (San Jose, CA) LCQ equipped with a microelectrospray ionization source. Tandem mass spectra were acquired during the entire gradient run automatically as previously described (20). The protein sequence and nucleotide sequence databases were searched directly with tandem mass spectra by using the computer program SEQUEST (Finnigan, San Jose, CA) (21). Each sequence returned by SEQUEST was verified by inspecting the fit of the amino acid sequence to the corresponding tandem mass spectrum.

Northern Blot Analysis. Following previously published protocols (22), poly(A)-tailed mRNA (10 μg) was electrophoresed through a 1.5% agarose gel and transferred to nylon membrane (Hybond-N, Amersham Pharmacia). The membrane was prehybridized for 6 hr and then hybridized overnight at 43°C with 10^7 cpm of ^{32}P-labeled PAPP-A cDNA probe corresponding to nucleotide 10–2365 (GenBank accession no. X68280). The membrane was washed three times, dried, and exposed to film.

Western Blot Analysis. Samples were run on 8% Tris/tricine gels and blotted onto polyvinylidene difluoride membrane (Millipore). The membrane was blocked in 2% Tween 20 and washed with 50 mM Tris/500 mM NaCl/0.1% Tween 20/1% fetal bovine serum, pH 9.0. The blots were then incubated with monoclonal PAPP-A antibodies 234–2 and 234–5 (23). The secondary antibodies were peroxidase-conjugated anti(mouse-IgG) P260 (Dako). Blots were developed by using enhanced chemiluminescence (ECL, Amersham).

ELISA. PAPP-A antigen was measured by using a standard sandwich ELISA. The capture antibody was polyclonal anti-PAPP-A/proMBP, and detection was done with anti-PAPP-A monoclonal antibodies 234–2 and 234–5 (23) followed by

Table 1. Protein identification by means of mass spectrometry

Protein molecular mass, kDa	Protein identity	Peptides identified
175	Human α-2-macroglogulin	854–863 QTVSWAVTPK
		935–945 LPPNVVEESAR
		1264–1273 YGAATFTRTG
	Human thrombospondin 1	61–83 IEDANLIPPVPDDKFQDLVDAVR
		217–228 FVFGTTPEDILR
		289–299 TIVTTLQDSIR
		1086–1099 NALWHTGNTPGQVR
		1148–1164 LGLFVFSQEMVFFSDLK
	Human collagen	1063–1084 SGDRGETGPAGPAGPVGPVGAR
	Bovine α-1-antichymotrypsin isoform pHHK1	37–58 GTSVDGHSLASSNTDFAFSLYK
		321–329 DILSQLGIK
		331–346 IFTSDADFSGITDDHK
	Human soares testis NHT cDNA clone 727252 5′	46–57 KLEEGTKQQWQG
	Sommer *Pristionchus Pristionchus pacificus*	32–48 EVCNKLIPDSIGKDIEK
	Human ribonuclease 6	124–139 GCLCLALLCLGGADKR
	Caenorhabditis elegans cDNA clone yk182d6	68–75 FLIQVIHK
200	Human pregnancy-associated plasma protein-A	110–116 ADLELPR
		133–143 SPAVITGLYDK
		190–209 SYLPGQWVYLAATYDGQFMK
		373–387 EQVDFQHHQLAEAFK
		1071–1087 TISYPYSQLAQTTFWLR
		1180–1195 SFDNFDPVTLSSCQRG
	Rat hemiferrin	74–91 DQTVIQNTDGNNNEAWAK
	Bovine transferrin	230–243 HSTVFDNLPNPEDR
230	Human laminin γ-1 chain	1122–1135 NTIEETGNLAEQAR
		1407–1423 EAQQALGSAAADATEAK
	Human laminin β-1 chain	1509–1530 NFLTQDSADLDSIEAVANEVLK
		1597–1611 EALEEAEKAQVAAEK
400	Human collagen α-1	1–89 TLIPSTFFR
		117–123 VIYLGLR
		190–214 SSQALAFESSAGIFMGNAGATGLER
		215–229 FTGSLQQLTVHPDPR
		1243–1251 AAGLLSTYR
		1344–1360 TADTAVTGLASPLSTGK

Peptides from bovine serum albumin were identified in each of the four analyzed bands and have been omitted for brevity.

peroxidase-conjugated anti(mouse-IgG) P260. Highly purified PAPP-A/proMBP, prepared as described previously (16), was used for calibration.

RESULTS AND DISCUSSION

Purification of the IGF-dependent IGFBP-4 protease was monitored with a specific bioassay, i.e., cell-free degradation, of [^{125}I]IGFBP-4 into 18- and 14-kDa radiolabeled fragments in the presence, but not the absence, of added IGF-II (6, 7). This approach had the clear advantage of ensuring that we were purifying our specific enzyme of interest. We obtained highly purified IGF-dependent IGFBP-4 protease from 800 ml of HFCM by a combination of zinc chelate and lectin affinity chromatography (Fig. 1). The final fraction containing the IGF-dependent IGFBP-4 protease activity was further analyzed by SDS/PAGE, which revealed four high molecular mass bands at 400, 230, 200, and 175 kDa. The proteins in these bands were identified by tandem mass spectrometry microsequencing (Table 1). All of the peptides represented known proteins or proteins deduced from known cDNA sequences. An extensive literature search involving a comparison of the characteristics of the IGF-dependent IGFBP-4 protease from HFCM and the proteins identified by tandem mass spectrometry revealed only one match. This candidate protein was PAPP-A. PAPP-A is one of four proteins originally isolated in 1974 from normal human pregnancy serum (24). Although knowledge of PAPP-A's biological function was lacking, particular interest in PAPP-A had developed with its suggested clinical utility as an index of placental function and a first-trimester screen for Down's Syndrome (25, 26). In addition, placental PAPP-A "knock-out" in humans appears to be associated with Cornelia de Lange syndrome, a condition involving incomplete fetal development and subsequent deformities (27). PAPP-A and the IGF-dependent IGFBP-4 protease from HFCM are similar in that they are both high molecular weight glycosylated proteins that bind Zn^{2+} (7, 16). Furthermore, amino acid sequence derived from cloned cDNA encoding PAPP-A reveals a specific Zn^{2+} binding motif (HEXXHXXGXXH) and a Met-turn further carboxyl terminal found only in the metzincin family of metalloproteases (28–31). In pregnancy, circulating PAPP-A is complexed to the proform of eosinophil major basic protein (proMBP) in a disulfide-bridged 2:2 heterotetramer denoted PAPP-A/proMBP (32). In nonreducing SDS/PAGE, PAPP-A/proMBP migrates with a molecular mass of ≈500 kDa. In reducing SDS/PAGE, the molecular mass of PAPP-A monomer is ≈200 kDa (32). Like PAPP-A, proMBP is synthesized in the placenta during pregnancy (33) and its role in pregnancy is unknown.

Identification of the IGF-dependent IGFBP-4 protease as PAPP-A was verified with further biochemical analyses. Anti-PAPP-A/proMBP polyclonal IgG, but not nonspecific rabbit IgG, inhibited IGF-dependent IGFBP-4 protease activity in HFCM in a dose-dependent manner (Fig. 2*a*). At a 1:50 titer of PAPP-A antibody, IGFBP-4 proteolysis was completely inhibited; a 1:500 titer inhibited 84% of the protease activity, whereas a 1:5,000 titer inhibited 15% of IGF-induced IGFBP-4 protease activity in these cell-free assays. In other experiments, we used anti-PAPP-A/proMBP polyclonal IgGs to immunode-

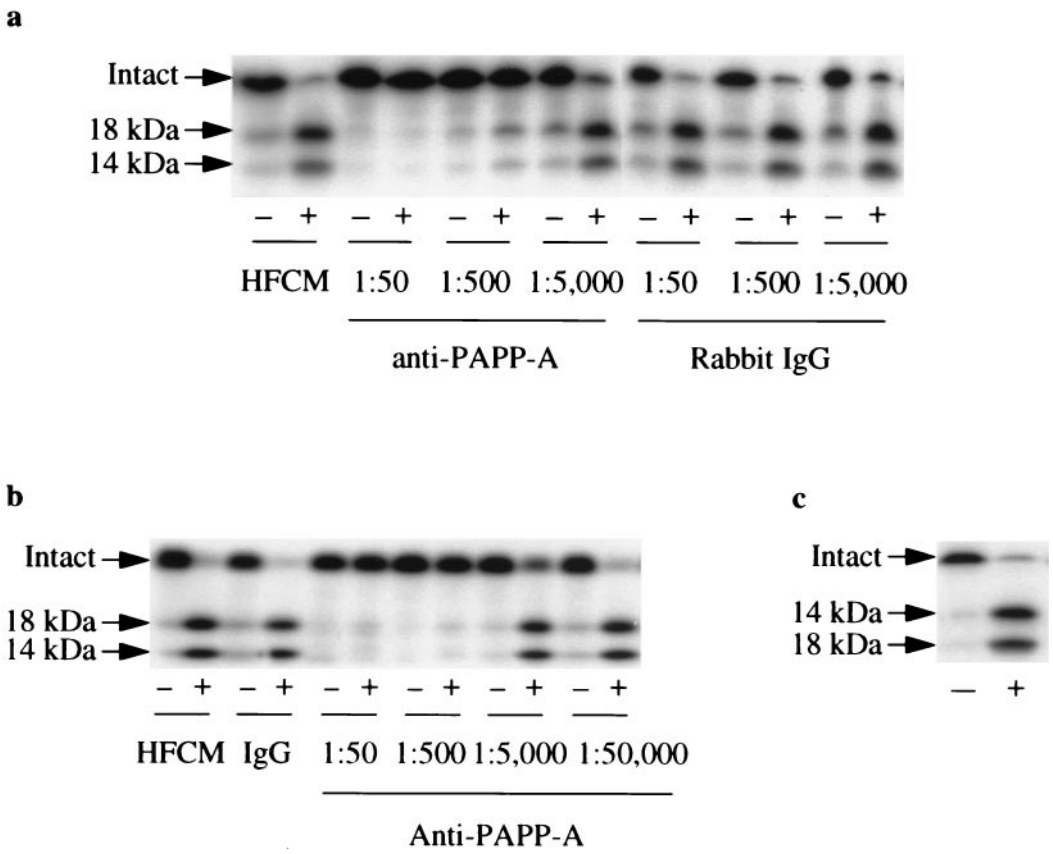

FIG. 2. Confirmation of IGF-dependent IGFBP-4 protease as PAPP-A. (*a*) Inhibition of IGFBP-4 protease activity in HFCM by PAPP-A/proMBP antibodies. HFCM was assayed for IGFBP-4 protease activity, as described in Fig. 1 and in *Materials and Methods*, in the presence of indicated titer of either PAPP-A/proMBP polyclonal antibody (anti-PAPP-A) or nonspecific rabbit IgG. (*b*) Immunodepletion of IGFBP-4 protease activity from HFCM by PAPP-A antibodies. HFCM was precleared with a 1:50 titer of nonspecific rabbit IgG. The precleared fractions were immunodepleted with indicated titer of PAPP-A/proMBP polyclonal antibody and assayed for IGFBP-4 protease activity. (*c*) IGF-dependent IGFBP-4 protease activity of purified PAPP-A. PAPP-A/proMBP (0.5 μg) purified from pregnancy sera was assayed for IGFBP-4 protease activity in the absence (-) and presence (+) of 5 nM IGF-II.

plete specifically and completely IGFBP-4 protease activity from the medium (Fig. 2*b*). Moreover, PAPP-A/proMBP that had been purified from serum of pregnant women (16) exhibited IGF-dependent IGFBP-4 protease activity (Fig. 2*c*). In cell-free assay, PAPP-A/proMBP alone had no effect on [^{125}I]IGFBP-4, but addition of IGF-II initiated proteolysis into radiolabeled fragments of 18 and 14 kDa, identical to what is seen with HFCM. Similar results were obtained with four different preparations of highly purified PAPP-A/proMBP (data not shown).

PAPP-A is most highly expressed in the syncytiotrophoblast of the placenta, which is the main source of circulating PAPP-A in pregnancy (33). However, PAPP-A has been detected in serum from nonpregnant as well as pregnant women and in preovulatory follicular fluid and has been immunolocalized to secretory endometrium, vascular endothelium, and actively proliferating fetal and adult tissues (25, 34, 35). These results were all based on polyclonal antisera that react with both PAPP-A and proMBP (32) and to an unknown extent with other proteins. In Fig. 3*a*, we demonstrate un-

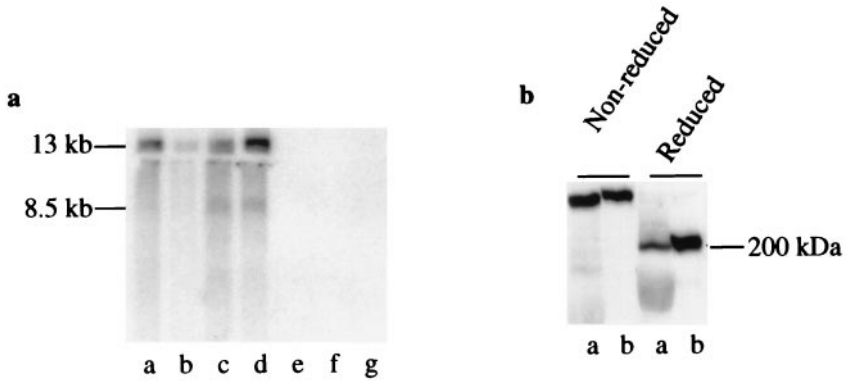

FIG. 3. Expression and secretion of PAPP-A. (*a*) Northern analysis. Poly-A tailed mRNAs (10 μg) from cultured human cells were probed with ^{32}P-labeled PAPP-A cDNA. The lanes are: a, adult fibroblasts; b, adult osteoblasts; c, fetal osteoblasts; d, marrow stromal cells; e, MG63 osteosarcoma cells; f, U2 osteosarcoma cells; g, TE85 osteosarcoma cells. (*b*) Western immunoblot. a, HFCM (10 μl of 30$\times$ concentrate corresponding to 0.05 μg PAPP-A by ELISA) and b, PAPP-A/proMBP purified from pregnancy serum (0.05 μg) were separated by SDS/PAGE under both nonreducing and reducing conditions, transferred to membrane, and immunoblotted with PAPP-A-specific monoclonal antibodies.

equivocally PAPP-A expression in cultured human fibroblasts and bone cells by Northern blot analysis. Human fibroblasts expressed PAPP-A transcripts at ≈13 and 8.5 kilobases, similar to that detected by Northern blot analysis of placental mRNA (28, 29). The results from Northern blotting also show PAPP-A mRNA expression by normal human osteoblasts from adult and fetal sources and by osteoprogenitor cells, but not by several osteosarcoma cell lines. These findings are in agreement with those of previous studies showing IGF-dependent IGFBP-4 protease activity in medium conditioned by normal human osteoblasts but not by transformed osteoblastic cells (36). Not only is PAPP-A mRNA expressed by human fibroblasts, but PAPP-A protein is, in fact, secreted by cultured human fibroblasts. By ELISA, HFCM was found to contain 0.18 ± 0.02 μg/ml PAPP-A ($n = 4$). For comparison, term pregnancy serum contains ≈25 μg/ml PAPP-A. Western blotting analysis showed that HFCM reacted with monoclonal antibodies specific for PAPP-A (Fig. 3*b*). In nonreducing SDS/PAGE, PAPP-A from HFCM migrates with a molecular mass of ≈400 kDa, somewhat faster than PAPP-A/proMBP isolated from pregnancy serum but slower than PAPP-A monomer seen at ≈200 kDa in reducing SDS/PAGE. Expression of proMBP by fibroblasts was not detected by ELISA or Northern blotting (data not shown). Thus, PAPP-A in HFCM may form homodimers, although this remains to be determined.

The IGF-dependent IGFBP-4 protease/PAPP-A is a new member of the metzincin family of metalloproteases (30, 31), and it is clear that this enzyme is unique among other multifunctional proteases capable of degrading IGFBP-4 (37, 38). The identification of PAPP-A as the IGF-dependent IGFBP-4 protease has immediate ramifications for placental function and fetal development. In addition, the identification of the IGF-dependent IGFBP-4 protease as PAPP-A and the availability of pure protein and associated molecular tools now allows determination of the mechanism underlying its IGF dependence and the biological role of localized IGF-dependent IGFBP-4 proteolysis in such diverse systems as wound healing, bone remodeling, atherosclerosis, and follicular development.

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