Studies on regulation of IGF (insulin-like growth factor)-binding protein (IGFBP) 4 proteolysis by pregnancy-associated plasma protein-A (PAPP-A) in cells treated with phorbol ester

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PAPP-A (pregnancy-associated plasma protein-A) is produced by hSFs (human skin fibroblasts) and hOBs (human osteoblasts) and enhances the mitogenic activity of IGFs (insulin-like growth factors) by degradation of IGFBP-4 (insulin-like growth factor-binding protein 4). PKC (protein kinase C) activation in these cells led to reduction in IGFBP-4 proteolysis. This study was undertaken to determine the mechanism by which activation of PKC suppresses IGFBP-4 proteolysis. Treatment of hSFs/hOBs with TPA (PMA; 100 nM) reduced IGFBP-4 proteolysis without significantly decreasing the PAPP-A level in the CM (conditioned medium). Immunodepletion of the proform of eosinophil major basic protein (proMBP), a known PAPP-A inhibitor, from CM of TPA-treated cells (TPA CM) failed to increase IGFBP-4 proteolytic activity. Transduction of hSFs with proMBP retrovirus increased the concentration of proMBP up to 30 ng/ml and led to a moderate reduction in IGFBP-4 proteolysis. In contrast, TPA treatment blocked IGFBP-4 proteolysis but failed to induce a detectable amount of proMBP in the CM. While proMBP overexpression led to the formation of a covalent proMBP–PAPP-A complex and reduced the migration of PAPP-A on SDS/PAGE, TPA

treatment dose- and time-dependently increased the conversion of a ≈ 470 kDa PAPP-A form (PAPP-A₄₇₀) to a ≈ 400 kDa PAPP-A form (PAPP-A₄₀₀). Since unreduced PAPP-A₄₀₀ co-migrated with the 400 kDa recombinant PAPP-A homodimer and since PAPP-A monomers from reduced PAPP-A₄₇₀ and PAPP-A₄₀₀ co-migrated on SDS/PAGE, conversion of PAPP-A₄₇₀ to PAPP-A₄₀₀ is unlikely to be caused by proteolytic cleavage of PAPP-A. Consistent with the data showing that the increase in the ratio of PAPP-A₄₀₀/PAPP-A₄₇₀ is correlated with the extent of reduction in IGFBP-4 proteolysis, partially purified PAPP-A₄₀₀ exhibited a 4-fold reduction in IGFBP-4 proteolytic activity compared with PAPP-A₄₇₀. These data suggest that a novel mechanism, namely conversion of PAPP-A₄₇₀ to the less-active PAPP-A₄₀₀, could account for the TPA-induced suppression of PAPP-A activity.

Key words: insulin-like growth factor (IGF), insulin-like growth factor-binding protein 4 (IGFBP-4), osteoblast, post-translational modification, pregnancy-associated plasma protein-A (PAPP-A), protease.

INTRODUCTION

The IGFs (insulin-like growth factors) are mitogenic polypeptides that play an important role in promoting cell differentiation, proliferation and survival in a variety of biological systems [1–8]. These actions of IGFs are regulated by IGFBPs (IGF-binding proteins), which have a high affinity and specificity for the IGFs [1–8]. Among the six identified high-affinity IGFBPs, IGFBP-4 is produced by a variety of cell types, and is a consistent inhibitor of IGF action [1–8]. The bioavailability of IGFBP-4 depends not only on the amount of IGFBP-4 synthesized but also on the rate of its degradation by an IGF-II-dependent-specific protease now known as the PAPP-A (pregnancy-associated plasma protein-A) [9]. Studies from our laboratory and others have demonstrated that PAPP-A represents the primary IGFBP-4 protease produced by a number of cell types, including hOBs (human osteoblasts) [10] and hSFs (human skin fibroblasts) [9], and is present in various body fluids such as hPS (human pregnancy serum) [11], follicular fluids [12] and amniotic fluids [13].

The activity of IGFBP-4 protease has been shown to be regulated by a number of agents [14–20]. In this regard, Conover et al. first reported that treatment of normal hSFs with PKC (protein kinase C) activators [TPA (PMA) or β -PDD (β -phorbol 12,13-didecanoate)] dose- and time-dependently reduced the IGFBP-4 proteolytic activity in the CM (conditioned medium) [15]. A direct addition of TPA to collected CM had no effect on IGFBP-4 proteolysis, suggesting that the TPA effect was a cell-mediated event. These studies also demonstrated that the TPA-induced reduction of IGFBP-4 proteolytic activity requires *de novo* protein synthesis [15]. Subsequently, the TPA-induced blockage of IGFBP-4 proteolysis was confirmed in hOB cultures [20]. In addition to TPA treatment, SV-40 (Simian virus 40) transformation of hOBs also induced a decrease in IGFBP-4 proteolytic activity in the CM [20]. The hSF CM containing high IGFBP-4 protease activity was unable to cleave IGFBP-4 after it was incubated with the immortalized hOBs. On the other hand, the IGFBP-4 proteolytic activity in hSF CM was not affected when the hSF CM was incubated with the immortalized hOBs

Abbreviations used: β -PDD, β -phorbol 12,13-didecanoate; CM, conditioned medium; hOB, human osteoblast; hPS, human pregnancy serum; hSF, human skin fibroblast; IGF, insulin-like growth factor; IGFBP, IGF-binding protein; PAPP-A, pregnancy-associated plasma protein-A; PAPP-A₄₇₀, ≈ 470 kDa active PAPP-A form; PAPP-A₄₀₀, ≈ 400 kDa less-active PAPP-A form; PKC, protein kinase C; proMBP, proform of eosinophil major basic protein; SV-40, Simian virus 40; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum.

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that were pre-treated with a protein synthesis inhibitor. Although these studies provided initial evidence that TPA treatment or cell transformation may have induced an IGFBP-4 protease inhibitor, further definitive studies were not possible as the identity of the IGFBP-4 protease was not known until 1999 [9].

Recently, Chen et al. reported that proMBP (proform of eosinophil major basic protein) contributes to the decrease in IGFBP-4 proteolysis in the CM of hSFs treated with β -PDD (a different PKC activator) [21]. This conclusion was based on the following observations: (i) β -PDD treatment or SV-40 transformation of hSFs reduced IGFBP-4 proteolytic activity without a significant effect on PAPP-A production, (ii) β -PDD treatment or SV-40 transformation of hSFs increased production of proMBP and (iii) co-culturing of hSFs with ovarian cancer cells which over-express proMBP led to a loss of IGFBP-4 proteolytic activity in the CM. Although these studies provided ample indirect evidence that proMBP might play a role in mediating the activation of PKC-induced decrease in IGFBP-4 proteolysis, it is not known whether induction of proMBP constitutes the only mechanism by which PKC activators induce reduction in IGFBP-4 proteolysis.

The main objective of this study is to determine if there are additional mechanisms besides the regulation of proMBP which could mediate suppression of IGFBP-4 proteolysis induced by PKC activation. Our studies revealed that PAPP-A produced by hOB/hSFs exists as two forms based on their electrophoretic migration pattern on SDS/PAGE, a ≈ 470 kDa active PAPP-A form (PAPP-A₄₇₀) and a ≈ 400 kDa less-active PAPP-A form (PAPP-A₄₀₀). Treatment with TPA dose- and time-dependently enhanced the conversion of the PAPP-A₄₇₀ to PAPP-A₄₀₀ with a parallel decrease in PAPP-A catalytic activity. Since the binding of proMBP to PAPP-A reduced, rather than enhanced, the migration of PAPP-A, the TPA-induced suppression of IGFBP-4 proteolysis is mediated by an additional novel mechanism besides the known regulation of proMBP production.

MATERIALS AND METHODS

Materials

DMEM (Dulbecco's modified Eagle's medium) was purchased from Life Technologies. FCS (fetal calf serum) was purchased from Hyclone (Logan, UT, U.S.A.). The hSFs from normal male adult donors (GM03440) were purchased from the Human Genetic Mutant Cell Repository (Coriell Institute, Camden, NJ, U.S.A.). Normal hOBs were isolated from vertebral bone specimens as described in [22]. The $6 \times$ His-tagged recombinant human IGFBP-4 ($6 \times$ His-IGFBP-4) was prepared as described previously [23]. Recombinant human PAPP-A was kindly provided by Dr C. Oxvig (Department of Molecular Biology, University of Aarhus, Aarhus, Denmark) [24]. Purified polyclonal anti-human PAPP-A IgG (catalogue no. A0230) and normal IgG (catalogue no. X0936) produced in rabbits were purchased from DAKO Corp. (Carpinteria, CA, U.S.A.). Purified mouse monoclonal antibody raised against the mature peptide of human proMBP (MBP; catalogue no. CBL 419) was purchased from Cymbus Biotechnology (Chandlers-Ford, Hants, U.K.). The monoclonal proMBP antibodies (J163-15E10 and J14-8A2) were provided by Dr H. Kita (Mayo Clinic, Rochester, MN, U.S.A.). The recombinant proMBP standards used in the radioimmunoassay is prepared as described [25]. Affi-Gel Heparin Gel was purchased from Bio-Rad (Hercules, CA, U.S.A.). All peroxidase-conjugated secondary antibodies were purchased from Pierce Chemical Co. (Rockford, IL, U.S.A.). Other chemicals and reagents were of reagent grade and were obtained from Sigma (St. Louis, MO, U.S.A.).

Cell culture

hSFs or hOBs were cultured in DMEM containing 10 % FCS and antibiotics. At confluence, cells were washed twice with DMEM and incubated with DMEM/0.01 % BSA supplemented with or without effectors for the indicated times and concentrations as indicated in the Figure legends. At the end of the incubation period, the CM was collected, centrifuged at 10 000 *g* for 10 min to remove cell debris and stored at -20°C until use.

Preparation of mammalian proMBP expression plasmid

The full-length human proMBP cDNA was obtained by reverse transcriptase PCR using total RNAs isolated from human osteoblasts with a procedure described in our previous studies [13]. The proMBP cDNA was cloned in frame with the FLAG coding sequence in the mammalian expression vector, pFLAG-CMV-1 (Sigma). Subsequently, the FLAG coding sequence was deleted to generate the plasmid expressing proMBP peptide without a FLAG tag. Finally, this construct was used as the template to obtain a PCR fragment containing a 5' *SalI* site, a Kosac GCCGCC sequence, the pretrypsin sequence, the full-length proMBP cDNA sequence and a 3' *BamHI* site. This PCR fragment was cloned into a carrier vector, released using *SalI* and *BamHI* restriction enzymes, and cloned into a retroviral vector that was developed at Musculoskeletal Disease Center, J. L. Pettis Memorial Veterans' Medical Center, Loma Linda, CA, U.S.A. [24]. This retroviral expression construct led to expression of the full-length proMBP peptide, Ala¹-Tyr²⁰⁷ (amino acid numbering does not include the signal peptide).

Overexpression of proMBP in HEK-293T cells

HEK-293T cells were seeded in 10 cm dishes (3×10^6 cells/dish) in DMEM/10 % FCS. After overnight incubation, cells were transiently transfected with 20 μg of proMBP/pFLAG plasmid or empty vector plasmid using the standard calcium phosphate precipitation method. After overnight incubation, cells were carefully washed with DMEM twice and incubated in DMEM/0.01 % BSA for an additional 24 h. CM was collected, centrifuged to remove cell debris and stored at -20°C prior to use.

Retroviral transduction of hSFs

proMBP or β -gal retrovirus was prepared by transiently transfecting HEK-293T cells with the test plasmid and the VSV-G (a viral envelope protein) plasmid as described in [26]. The virus titre of the unconcentrated CM has been determined to be $\approx 1 \times 10^7$ transforming units/ml. Retroviral transduction of hSFs was performed as described in [26]. Briefly, hSFs were seeded in six-well plates (100 000 cells/well) in DMEM/10 % FCS. After overnight incubation, cells were incubated for 10 h with various doses of retrovirus in the presence of polybrene. After changing the medium, cells were cultured for an additional 48 h prior to addition of effectors.

ProMBP radioimmunoassay

The proMBP concentration in the CM was measured by a specific radioimmunoassay. Briefly, 100 μl of PBS containing 10 μg /ml of a proMBP monoclonal antibody (J14-8A2) was added to each well in 96-well microtitre plates. After an overnight incubation at 4°C , the plates were washed with a 0.1 M phosphate buffer (pH 7.4) containing 1 % Tween-20. The plates were then

blocked with 200 μ l/well of 0.1 M phosphate buffer (pH 7.4) containing 0.5 % FCS, 0.1 % NaN_3 , 0.01 M EDTA and 1 % protamine sulphate. After 1 h incubation at room temperature, the plates were washed with the phosphate buffer. To reduce and alkylate samples, 50 μ l of sample or proMBP standards was incubated for 1 h at room temperature with 130 μ l of Tris/EDTA (0.33 M Tris, 0.12 M NaCl and 0.01 M EDTA, pH 8.0) and 20 μ l of 0.1 M dithiothreitol. Then, 20 μ l of 0.02 M iodoacetamide was added to each reaction and incubation was allowed for additional 20 min. Following this, 100 μ l of reduced and alkylated samples/standards was added to each well. After an overnight incubation at 4 °C, the plates were washed three times with 0.1 M phosphate buffer. Then 100 μ l of the second proMBP monoclonal antibody (J163-15E10), labelled with ^{125}I , was added to each well. After a 3 h incubation at room temperature, plates were washed four times and radioactivity in each well was measured with a γ counter.

PAPP-A ELISA

PAPP-A ELISA was conducted with a commercial kit according to the manufacturer's instructions (Diagnostic Systems Laboratories, Webster, TX, U.S.A.). Since the manufacturer was unable to provide a conversion of the 'units' to the actual amount of PAPP-A, we used recombinant human PAPP-A (kindly provided by Dr C. Oxvig) [24] as standards (0–80 ng). In these assays, 10 μ l of 30-fold-concentrated CM or partially purified PAPP-A samples was used. The background readings were determined using 10 μ l of DMEM/0.1 % BSA. This assay was able to detect less than 2 ng of PAPP-A.

Immunodepletion of proMBP

Either 30-fold concentrated hSF CM (150 μ l) or 250 μ l of straight CM from HEK-293T cells transfected with proMBP expression plasmids was incubated with 50 μ l of 70 % Protein A–agarose for 1 h. The supernatant was collected and incubated for 2 h with either control IgG or MBP antibody (10 μ g for hSF CM and 15 μ g for HEK-293T CM). Then, supernatant was incubated for 1 h with 100 μ l of 70 % Protein A–agarose. All incubations were performed at 4 °C in a rotating mixer. The supernatant was then collected for immunoblot analysis and IGFBP-4 protease assays.

Ligand-binding blot and Western immunoblot analyses

^{125}I -IGF-II Western ligand-blot analyses of IGFBPs were performed as described previously [11]. Concentrated CM was resolved by SDS/PAGE (either a 5 or 10 % gel) under reducing or non-reducing conditions. The proteins were transferred to a Transblot nitrocellulose membrane (Bio-Rad) and subjected to immunoblot analysis as described previously [11]. Briefly, membranes were washed in double-distilled water for 15 min and blocked with 12 ml of washing buffer (20 mM Tris, 138 mM NaCl and 0.1 % Tween-20, pH 7.4) containing 1 % dry skimmed milk (blocking buffer) for 1 h. The blocked membranes were then incubated with 12 ml of blocking buffer containing primary antibody (32 μ g of PAPP-A antibody, 4 μ g of MBP antibody or 10 μ g of proMBP antibody) for 2 h. After washing the membranes with washing buffer three times, the membranes were incubated for 1 h with 20 ml of diluted secondary antibody in blocking buffer (20 000-fold diluted goat anti-rabbit IgG for PAPP-A detection; 2500-fold diluted goat anti-mouse IgG for proMBP detection). The membranes were washed five times with

20 ml of washing buffer for 5 min each time. All incubations were performed at room temperature with gentle shaking. Finally, each membrane was incubated with 12 ml of SuperSignal West Pico chemiluminescent substrate (Pierce Chemical Co.) for 5 min and exposed to X-ray film for 5 s–10 min depending on the signal strength.

Partial purification of two forms of PAPP-A

Normal hSFs were seeded in 10 cm dishes with DMEM/10 % FCS. Upon 100 % confluence, cells were washed with DMEM twice and incubated in DMEM/0.01 % BSA supplemented with 100 nM TPA or vehicle. After a 24 h incubation, CM was collected and centrifuged to remove cell debris. Straight TPA CM or control CM (15 ml) was applied to a heparin affinity column (1 ml of resin). The CM was reloaded to the column four additional times to increase the binding of PAPP-A to heparin. After washing the column with 20 ml of 50 mM Tris buffer (pH 7.5) containing 50 mM NaCl, bound proteins were eluted with 10 ml of 1 M NaCl in 50 mM Tris buffer (pH 7.5). The 1 M NaCl fraction was supplemented with BSA (0.05 %) and concentrated with Centricon filtration unit (5 kDa cut) to 1 ml. To reduce salt concentration, concentrated samples were diluted with 10 ml of DMEM and re-concentrated to final volume of 0.5 ml. Samples were stored at –20 °C prior to use.

Quantification of IGFBP-4 proteolysis by partially purified PAPP-A

The amount of IGFBP-4 remaining uncleaved in the IGF-II ligand blot was quantified as described previously [23]. Briefly, the area of the membrane corresponding to the intact ^{125}I -hybridized IGFBP-4 band was marked and excised. Three individual areas of membranes containing no labelled band were cut and used to obtain background radioactivity. The radioactivity in each cut membrane piece was quantified by γ counting. The specific IGFBP-4 protease activity of PAPP-A was calculated using the following formula and expressed as the amount of IGFBP-4 (ng) cleaved by 1 ng of PAPP-A for an incubation period of 20 h at 37 °C in a DMEM buffer:

$$\text{PAPP-A activity (ng of IGFBP-4 cleared/ng of PAPP-A)} = \frac{\text{IGFBP-4 added (ng)} - \text{IGFBP-4 uncleaved (ng)}}{\text{amount of PAPP-A added (ng)}}$$

RESULTS

TPA-induced suppression of IGFBP-4 proteolysis is mediated through PAPP-A

Since PAPP-A is now known to be a potent IGFBP-4 protease, we first determined if the effect of TPA treatment on IGFBP-4 proteolysis is mediated through regulation of the synthesis and/or activity of PAPP-A in these cells. Figure 1 shows that treatment of normal hOBs or hSFs with 100 nM TPA for 48 h reduced the proteolysis of the IGFBP-4 exogenously added to the CM. Since addition of the PAPP-A antibody to CM of vehicle-treated hSFs and hOBs blocked IGFBP-4 proteolysis, PAPP-A is the predominant IGFBP-4 protease produced by these cells. Therefore, TPA-induced inhibition of IGFBP-4 proteolysis is unlikely to be caused by inhibition of the activity or synthesis of other low-abundance non-specific IGFBP-4 proteases produced by these cells under defined conditions.

Figure 1 Effect of PAPP-A antibody on IGFBP-4 proteolytic activity in CM from hSFs or hOBs treated with or without TPA

hSFs or hOBs were seeded in six-well plates in DMEM/10% FCS. Upon confluence, cells were washed twice with serum-free DMEM and then incubated with 2 ml of DMEM/0.01% BSA containing 100 nM TPA or vehicle (2 μ l of 0.0125% DMSO). After a 48 h incubation, CM was collected, centrifuged to remove cell debris and stored at -20°C prior to use. In (A) 8 μ l of 20-fold hOB CM was incubated with 100 ng of hIGFBP-4 and 50 ng of IGF-II in the presence vehicle (2 μ g BSA), control IgG (2 μ g) or anti-PAPP-A IgG (2 μ g). In (B) 40 μ l of pooled straight CM from hSFs was used to digest IGFBP-4 as described in (A). After a 20 h incubation at 37°C , the digested samples were subjected to Western ^{125}I -IGF-II ligand blotting.

Figure 2 Treatment of hOBs and hSFs with TPA did not significantly decrease production of PAPP-A

Either hOBs or hSFs were seeded in six-well plates in DMEM/10% FCS ($n=3$). Upon confluence, cells were washed with DMEM twice and incubated with DMEM/0.01% BSA supplemented with 100 nM TPA or vehicle. After an additional 36 h incubation, CM was collected and concentrated with an Amicon centrifugal device (5 kDa cut-off) by 40-fold. PAPP-A ELISA was conducted with a commercial kit according to the manufacturer's instruction (Diagnostic Systems Laboratories) using 10 μ l of concentrated CM and a pooled third-trimester hPS as a standard according to the manufacturer's instructions. The data are expressed as mean $\pm$ S.E.M. ($n=3$). PAPP-A concentration was not significantly different between control CM and TPA CM ($P > 0.05$).

TPA-induced reduction in IGFBP-4 proteolysis is not primarily caused by a reduction in PAPP-A production

To determine whether TPA treatment suppresses IGFBP-4 proteolysis by decreasing the production of PAPP-A, we compared the level of PAPP-A in the CM collected from TPA-treated with that in vehicle-treated hOBs or hSFs. TPA treatment of hOBs did not significantly reduce PAPP-A concentration in the CM (Figure 2). A marginal reduction ($\approx 30\%$) in PAPP-A concentration in the CM of TPA-treated hSFs was observed in this experiment. Although a reduction in PAPP-A production was frequently observed in TPA-treated hSFs, especially when the cell density was lower, the extent of reduction in PAPP-A production was much lower than the extent of reduction in IGFBP-4 proteo-

Figure 3 Immunodepletion of proMBP did not affect PAPP-A activity in TPA CM

Control CM and TPA CM from hSF cultures were prepared as described for Figure 1. HEK-293T CM containing recombinant proMBP was prepared as described in the Materials and methods section. Then 150 μ l of 30-fold concentrated hSF CM or 250 μ l of straight HEK-293T CM was incubated with 50 μ l of 70% Protein A-agarose for 1 h. The supernatant was collected and incubated for 2 h with either control IgG or MBP antibody (10 μ g for hSF CM and 15 μ g for HEK-293T CM). Then the supernatant was incubated for 1 h with 100 μ l of 70% Protein A-agarose. All incubations were performed at 4°C in a rotating mixer. The supernatant was then collected for immunoblot analysis and IGFBP-4 protease assays. In (A) 70% of immunodepleted HEK-293T CM was concentrated, applied to SDS/PAGE (5% gel), and subjected to Western immunoblot analysis with the MBP antibody to ensure that proMBP was completely depleted using this protocol. In (B) IGFBP-4/IGF-II mix was incubated with 10 μ l of unconcentrated immunodepleted hSF CM for 20 h at 37°C and then subjected to IGF-II ligand-blot analysis.

lysis (Figure 1). Thus TPA-induced suppression of IGFBP-4 proteolytic activity is not primarily mediated through the regulation of PAPP-A synthesis.

Immunodepletion of proMBP from CM collected from TPA-treated cells does not increase IGFBP-4 proteolysis

As proMBP has been shown to be a PAPP-A inhibitor present in hPS [27], we evaluated if immunodepletion of proMBP from TPA CM would affect IGFBP-4 proteolysis. Since either free or PAPP-A-bound proMBP was not detected in the TPA CM under our experimental conditions by the Western immunoblot analysis with a polyclonal antibody raised against the mature peptide of proMBP (MBP), we evaluated whether this antibody is effective in binding proMBP under native conditions using CM of HEK-293T cells overexpressing proMBP. Figure 3 shows that proMBP was detected in the HEK-293T CM after immunodepletion with the control IgG but not the MBP antibody, demonstrating that the MBP antibody preparation is useful in immunodepletion of proMBP. Since both HEK-293T CM and hSF CM were subjected to immunodepletion at the same time under identical conditions, the hSF CM treated with MBP antibody should contain little or no proMBP. Results from IGFBP-4 protease assays revealed that IGFBP-4 proteolysis was not altered by proMBP depletion in either control CM or TPA CM (Figure 3).

Figure 4 Level of proMBP in TPA CM is not adequate to inhibit the activity of PAPP-A

The hSFs were seeded in six-well plates, transduced with proMBP or β -gal retrovirus preparation (see the Materials and methods section) and then treated with 100 nM TPA or vehicle for 48 h. CM was collected and subjected to PAPP-A and proMBP immunoblot analyses and IGFBP-4 protease assays. In (A) concentrated CM (400 μ L straight CM) was resolved by SDS/PAGE (5 or 10% gel) under non-reducing conditions and subjected to immunoblot analysis with a monoclonal proMBP antibody (see the Materials and methods section). In (B) IGFBP-4/IGF-II mix (200 ng of IGFBP-4/50 ng of IGF-II) was incubated with 50 μ L of straight CM for 20 h and subjected to IGF-II ligand-blot analysis. In (C) the membrane used for PAPP-A immunoblot analysis in (A) (5% gel) was treated with 0.2 M NaOH for 20 min, washed with TBS buffer, and subjected to immunoblot analysis with the PAPP-A antibody (see the Materials and methods section). Similar results were obtained from a second independent experiment.

TPA treatment increased whereas proMBP overexpression reduced the rate of migration of PAPP-A on SDS/PAGE gels under non-reducing conditions

To further evaluate if proMBP plays a significant role in mediating the TPA-induced inhibition of PAPP-A activity, we determined whether TPA-induced proMBP production under our experimental conditions is sufficient to inhibit PAPP-A activity. The hSFs were first transduced with β -gal or proMBP retrovirus and then treated with 100 nM TPA for 48 h. Overexpression of proMBP in hSFs dose-dependently increased the levels of free proMBP (Figure 4A) and proMBP-PAPP-A complex (Figure 4B). In contrast, no proMBP in either free or bound form was detected in the TPA CM (Figures 4A and 4B). Results from proMBP radioimmunoassay using 10-fold-concentrated CM samples revealed that CM of the proMBP-transduced cells contained up to 30 ng/ml proMBP whereas proMBP concentrations in the TPA CM were below the detection limit of the assay. Results from protease assays revealed that TPA CM containing undetectable amount of proMBP exhibited even less IGFBP-4 proteolytic activity compared with the CM collected from transduced cells overexpressing large amount of proMBP (Figure 4B). Thus the TPA-induced reduction in IGFBP-4 proteolysis is not primarily due to an increased proMBP production by the TPA-treated cells.

An intriguing observation from these experiments was that an immunoreactive PAPP-A doublet was visualized when the samples were resolved by a low-percentage SDS/PAGE gel (5% acrylamide) after prolonged separation (Figure 4C). Using the recombinant human PAPP-A (400 kDa) as one of the molecular-mass standards, the molecular masses of the two forms in

Figure 5 TPA treatment of hSFs and hOBs increased conversion of the PAPP-A₄₇₀ form to the PAPP-A₄₀₀ form and decreased IGFBP-4 proteolysis

hOBs or hSFs were seeded in six-well plates and treated with 100 nM TPA for 48 h as described in Figure 1. In (A) concentrated CM (hSF, 600 μ L of straight CM equivalent; hOB, 1.5 ml of straight CM equivalent) resolved by SDS/PAGE (5% gel) and subjected to PAPP-A immunoblot analysis. In (B) IGFBP-4 (200 ng) and IGF-II (50 ng) mix was incubated for 20 h at 37 °C with 50 μ L of hSF CM or 5 μ L of 15-fold hOB CM. Digested samples were subjected to IGF-II ligand-blot analysis. Similar results were obtained from at least three independent experiments.

the PAPP-A doublet were calculated to be 400 ± 1 kDa ($n = 3$) and 474 ± 3 kDa ($n = 3$). The intensity of the 474 kDa PAPP-A form, designated PAPP-A₄₇₀, was dramatically reduced with a parallel increase in the intensity of a 400 kDa PAPP-A form, designated PAPP-A₄₀₀, in the TPA CM (Figure 5). In contrast, overexpression of proMBP inhibited IGFBP-4 proteolysis and reduced the electrophoretic migration of PAPP-A. Treatment of hOBs with TPA also led to the conversion of PAPP-A₄₇₀ to PAPP-A₄₀₀ and reduction in IGFBP-4 proteolysis (Figure 5). In the control CM, a faint band was observed between the PAPP-A₄₇₀ and the PAPP-A₄₀₀. This band may represent a proteolytic fragment of the PAPP-A₄₇₀. Examination of the data obtained from multiple experiments revealed that TPA treatment reproducibly induced conversion of PAPP-A₄₇₀ to PAPP-A₄₀₀, although the extent of conversion varied. However, the conversion of the PAPP-A₄₇₀ to PAPP-A₄₀₀ was found to always be accompanied with a decrease in IGFBP-4 proteolysis.

To evaluate the correlation between the conversion of PAPP-A₄₇₀ to PAPP-A₄₀₀ and reduction in IGFBP-4 proteolysis, we performed TPA dose-response and time-course experiments using hSFs. Treatment of hSFs with TPA dose-dependently enhanced the conversion of PAPP-A₄₇₀ to PAPP-A₄₀₀ and decreased IGFBP-4 proteolysis (Figure 6). At a TPA concentration of 10 ng/ml, conversion of PAPP-A₄₇₀ to PAPP-A₄₀₀ and a reduction in IGFBP-4 proteolysis nearly reached a plateau. Results from the time-course experiment revealed that conversion of PAPP-A₄₇₀ to PAPP-A₄₀₀ and reduction in IGFBP-4 proteolysis required at least 6 h of TPA exposure (results not shown). The time-course and dose-response of TPA treatment on IGFBP-4 proteolysis are similar to those reported previously [15].

Partially purified PAPP-A₄₀₀ exhibits reduced IGFBP-4 proteolytic activity compared with the partially purified PAPP-A₄₇₀

To obtain further evidence that the conversion of the PAPP-A₄₇₀ to PAPP-A₄₀₀ contributed to the TPA-induced reduction in IGFBP-4 proteolytic activity, we prepared partially purified PAPP-A from the CM of hSF cultures treated or not with TPA and subsequently evaluated the IGFBP-4 proteolytic activity of the partially purified enzymes. We chose to use heparin-affinity

Figure 6 TPA treatment of hSFs dose-dependently enhanced conversion of the PAPP-A₄₇₀ form to the PAPP-A₄₀₀ form and inhibited IGFBP-4 proteolysis

hSFs were seeded in six-well plates and treated with TPA for 48 h at indicated doses. In (A) concentrated CM (equivalent to 600 μ l CM) was resolved by SDS/PAGE (5% gel) and subjected to PAPP-A immunoblot analysis. In (B) IGFBP-4 (200 ng) and IGF-II (80 ng) mix was incubated for 20 h at 37 °C with 50 μ l of straight CM followed by IGF-II ligand-blot analysis.

chromatography because it has been previously used as a major step to purify biologically active recombinant PAPP-A [24,27]. In addition, heparin-affinity chromatography involves the use of native buffers containing no reagents which could inhibit PAPP-A activity, thereby maximally reducing potential artifacts. While the recovery of the PAPP-A from TPA CM was nearly 100%, the recovery of PAPP-A from control CM was $\approx$ 50% (Figure 7A). Since no significant amount of PAPP-A was detected in the unbound fractions, the reason for the decrease in PAPP-A recovery from the control CM is not clear but could be caused by variation in the quality of the filtration device, which occasionally leaks.

Results from immunoblot analysis revealed that partially purified PAPP-A from the control CM and TPA CM contained primarily the PAPP-A₄₇₀ and the PAPP-A₄₀₀ form, respectively, as observed in the straight CM (Figure 7A). The partially purified PAPP-A₄₀₀ co-migrated with the recombinant human PAPP-A homodimer (Figure 7A), the molecular mass of which had previously been determined to be $\approx$ 400 kDa [24,27].

Next, we compared the IGFBP-4 proteolytic activities of PAPP-A₄₇₀ and PAPP-A₄₀₀. Figure 7(B) shows that the IGFBP-4 proteolytic activity of PAPP-A₄₇₀ was higher than that of PAPP-A₄₀₀ at each dose. No IGFBP-4 proteolytic activity was observed in the heparin-unbound fractions (Figure 7B), which contained little PAPP-A (Figure 7A). To more precisely determine the specific activity of PAPP-A₄₇₀ and PAPP-A₄₀₀, IGFBP-4 was incubated with estimated amounts of PAPP-A₄₇₀ (2.5 ng) or PAPP-A₄₀₀ (10 ng; Figure 7C) which was expected to cleave 40–50% IGFBP-4 based on the results from the dose–response experiment (Figure 7B). It was found that the partially purified PAPP-A₄₀₀ exhibited an $\approx$ 4-fold reduction in specific IGFBP-4 proteolytic activity compared with the partially purified PAPP-A₄₇₀ (Figure 7D).

PAPP-A monomers derived from PAPP-A₄₇₀ and PAPP-A₄₀₀ co-migrate on SDS/PAGE gel

The co-migration of the non-reduced PAPP-A₄₀₀ with the 400 kDa recombinant PAPP-A homodimer (Figure 7A) suggests that PAPP-A₄₀₀ is not a proteolytic product of intact PAPP-A homodimer. To further rule out this possibility, we compared

Figure 7 Partially purified PAPP-A₄₀₀ form co-migrated with the recombinant hPAPP-A and exhibited reduced IGFBP-4 proteolytic activity compared with the partially purified PAPP-A₄₇₀ form

CM from hSF cultures treated with TPA (TPA CM) or vehicle (control CM) was prepared and subjected to heparin–agarose affinity chromatography (see the Materials and methods section). PAPP-A concentration was measured as described in the Materials and methods section. In (A) concentrated straight CM (equivalent to 400 μ l), heparin-affinity purified (heparin-bound) PAPP-A (15 μ l; equivalent to 450 μ l of straight CM), heparin-affinity unbound fraction (equivalent to 450 μ l of straight CM), and recombinant human PAPP-A standard were separated by SDS/PAGE (5% gel) under non-reducing conditions and subjected to PAPP-A immunoblot analysis (see the Materials and methods section). In (B) IGFBP-4 (200 ng)/IGF-II (50 ng) mix was incubated at 37 °C for 20 h with various doses of partially purified PAPP-A and subjected to IGF-II ligand-blot analysis. In (C) IGFBP-4 (200 ng)/IGF-II (50 ng) mix was incubated at 37 °C for 20 h with 2.5 ng of partially purified PAPP-A₄₇₀ ($n=3$) or 10 ng of partially purified PAPP-A₄₀₀ ($n=3$) and subjected to IGF-II ligand-blot analysis. In (D) the specific activity of the partially purified PAPP-A₄₇₀ form or PAPP-A₄₀₀ form (ng of IGFBP-4 cleaved/ng of PAPP-A; means $\pm$ S.E.M., $n=3$) in the experiment described in (C) was determined as described in the Materials and methods section. The specific activity of PAPP-A₄₇₀ form was 4-fold that of the PAPP-A₄₀₀ form ($P<0.001$).

the migration of PAPP-A monomer derived from PAPP-A₄₇₀ versus PAPP-A₄₀₀. Control CM or TPA CM collected from hSF cultures was subjected to PAPP-A immunoblot analysis under both reducing and non-reducing conditions. Straight CM, instead of partially purified PAPP-A, was used in these experiments because PAPP-A proteolytic fragments may not be co-purified with the intact PAPP-A. Under non-reducing conditions, TPA

Figure 8 Treatment of hSFs with TPA did not alter migration of the PAPP-A monomer

hSFs were seeded in 10 cm dishes in DMEM/10% FCS. Upon confluence, cells were washed with DMEM twice and incubated in DMEM/0.002% BSA supplemented with 100 nM TPA or vehicle. After a 36 h incubation, CM was collected, concentrated and subjected to PAPP-A immunoblot analysis. SDS/PAGE gels (5% acrylamide; 8 cm $\times$ 10 cm $\times$ 0.15 cm) were pre-run for 15 min at 120 V prior to sample loading. Concentrated CM was resolved by SDS/PAGE at 4 °C under non-reducing (equivalent to 400 μ l of straight CM) and reducing conditions (equivalent to 5 ml of straight CM, incubated with 10% β -mercaptoethanol) respectively. Third-trimester hPS was included as a positive control. To achieve maximal separation, gels were run until the 120 kDa molecular-mass marker reached the gel front in this experiment (no immunoreactive bands below 120 kDa were detected in the same samples in a separate immunoblot; results not shown). Proteins were transferred to nitrocellulose membranes and subjected to Western immunoblot analysis with the polyclonal PAPP-A antibody. Similar results were obtained from analysis of different batches of CM.

CM contained primarily the PAPP-A₄₀₀ whereas control CM contained primarily the PAPP-A₄₇₀ (Figure 8). Under reducing conditions, the major PAPP-A species in control and TPA CM co-migrated as a 200 kDa band as observed in hPS-positive control; thus, this band represented the PAPP-A monomer. Similar results were obtained from analysis of hOB CM (results not shown). The PAPP-A monomer derived from PAPP-A₄₇₀ and PAPP-A₄₀₀ exhibited a similar, if not identical, molecular mass on SDS/PAGE. These results, together with the determined molecular mass of non-reduced PAPP-A₄₀₀ ($\approx$ 400 kDa) [24,27], strongly suggest that the conversion of PAPP-A₄₇₀ to PAPP-A₄₀₀ is unlikely to be caused by proteolytic cleavage of PAPP-A itself.

DISCUSSION

Activation of PKC in hSFs or hOBs has been shown to reduce IGFBP-4 proteolysis dramatically [15,20,21]. However, the mechanism underlying this regulation of IGFBP-4 proteolysis has not been resolved. In this study, we have shown for the first time that PAPP-A (the major IGFBP-4 protease produced by hSFs and hOBs) exists as two forms, a 470 kDa PAPP-A form (PAPP-A₄₇₀) and a 400 kDa PAPP-A form (PAPP-A₄₀₀), based on their electrophoretic migration pattern. PKC activation in hSFs and hOBs enhanced the conversion of PAPP-A₄₇₀ to PAPP-A₄₀₀ accompanied with a parallel decrease in IGFBP-4 proteolysis. These data have provided strong evidence for a newly identified mechanism which participates in the regulation of PAPP-A activity.

Among the demonstrated regulators of IGFBP-4 proteolysis [14–20], PKC activators exert the most dramatic effect in that IGFBP-4 proteolysis in the CM of treated cells is essentially blocked [15]. Since PAPP-A has now been determined to be the

primary IGFBP-4 protease produced by both hSFs and hOBs [9,10], several potential mechanisms could be proposed to explain the reduction of IGFBP-4 activity in the CM from TPA-treated cells: (i) reduction in PAPP-A production/secretion; (ii) induction of a protease which cleaves, and thus inactivates PAPP-A; (iii) induction of a protein which binds to and inactivates PAPP-A and (iv) reduction in synthesis or increase in degradation of a PAPP-A activator protein. Of these potential mechanisms, the TPA-induced inhibition of IGFBP-4 proteolysis appears to be not due to reduction in PAPP-A production based on the findings from Chen et al. [21] and our findings from this study (Figure 2).

Since the activity of PAPP-A is reduced by at least 100-fold after it forms a covalent complex with proMBP, as occurs in hPS [27], it is possible that activation of the PKC pathway induces the production of proMBP, which in turn inhibits PAPP-A activity. In support of this idea, Chen et al. have reported that treatment of hSFs with PKC activator increased proMBP production [21]. In addition, co-culturing of hSFs with cells overexpressing proMBP reduced IGFBP-4 proteolytic activity in the CM [21]. Although this study provided strong evidence that proMBP inhibits the IGFBP-4 proteolytic activity of PAPP-A, it is not evident that induction of proMBP synthesis is the only mechanism by which PKC activation decreases IGFBP-4 proteolysis.

In this study, we failed to detect proMBP in the CM of TPA- or vehicle-treated hSFs by Western immunoblot analysis with either a MBP antibody (Figure 3) or a monoclonal proMBP antibody (Figure 4), although these antibodies were effective in reacting with the recombinant proMBP-positive control. Moreover, the concentration of proMBP in 10-fold-concentrated TPA CM was below the detection limit of a proMBP radioimmunoassay. These results are inconsistent with those reported by Chen et al. [21]. There are a number potential explanations for the observed differences in proMBP production between the two studies: (i) the proMBP assay employed in the two studies is different; (ii) the intrinsic differences in the hOB and hSF preparations used in the two studies could contribute to the observed difference and (iii) there may be differences in cell culture conditions. In any case, our findings that overexpression of proMBP decreased PAPP-A activity are consistent with those of Chen et al. [21] and suggest that PAPP-A activity is subject to regulation by activation in proMBP production. Our findings also provide evidence for regulation of PAPP-A activity by a novel mechanism that does not involve proMBP. In this regard, our data revealed that proMBP immunodepletion from TPA CM failed to increase the IGFBP-4 proteolytic activity. Since TPA CM immunodepleted with control IgG or MBP antibody did not show difference in the amount of PAPP-A (results not shown), the lack of an increase in IGFBP-4 proteolytic activity in proMBP-free TPA CM is not caused by co-immunodepletion of PAPP-A with the MBP antibody. These data provided the first line of evidence that TPA treatment may suppress IGFBP-4 proteolysis by an additional mechanism that is different from inhibition of PAPP-A activity by proMBP.

Our novel finding that PAPP-A exists as two forms, PAPP-A₄₇₀ and PAPP-A₄₀₀, and that TPA treatment enhanced the conversion of PAPP-A₄₇₀ to PAPP-A₄₀₀, further supports the idea that PAPP-A activity is subject to regulation by an additional novel mechanism besides proMBP. Our data from dose-response and time-course experiments suggest that conversion of PAPP-A₄₇₀ to PAPP-A₄₀₀ contributed to the decrease in IGFBP-4 proteolysis. This contention is further supported by the data showing that partially purified PAPP-A containing mainly the PAPP-A₄₀₀ form exhibited a 4-fold-reduced IGFBP-4 proteolytic activity compared with the partially purified PAPP-A containing more PAPP-A₄₇₀ than PAPP-A₄₀₀. Importantly, the conversion of the PAPP-A₄₇₀ to the less-active PAPP-A₄₀₀ is independent of the proMBP modification of

PAPP-A since we have demonstrated that the binding of proMBP to PAPP-A reduced, rather than enhanced, the migration of PAPP-A (Figures 3 and 4). Taken together, our data have provided strong evidence that a novel mechanism, namely conversion of PAPP-A₄₇₀ to PAPP-A₄₀₀, accounts for the suppression of PAPP-A activity induced upon PKC activation.

There are a number of possible mechanisms which could cause the conversion of PAPP-A₄₇₀ to PAPP-A₄₀₀. First, the auto-proteolytic cleavage of PAPP-A [24] may have been enhanced in the cells treated with TPA. Alternatively, TPA treatment may have induced synthesis/activity of another proteolytic enzyme which cleaves PAPP-A. Second, TPA treatment may have led to formation of a less glycosylated PAPP-A₄₀₀ since PAPP-A is known to be heavily glycosylated [28]. Third, PAPP-A₄₇₀ but not PAPP-A₄₀₀ may contain a PAPP-A accessory protein. Activation of the PKC pathway may lead to a reduction in the synthesis of this protein or increased degradation of this protein. Our results support the third hypothesis mentioned above based on the following reasons: first, under non-reducing conditions the PAPP-A₄₀₀ but not the PAPP-A₄₇₀ co-migrated with the 400 kDa recombinant hPAPP-A. Second, the PAPP-A monomers derived from PAPP-A₄₇₀ and PAPP-A₄₀₀ co-migrated with the 200 kDa PAPP-A monomer derived from both hPS and recombinant human PAPP-A. Future studies are needed to confirm, identify and functionally analyse this potential PAPP-A accessory protein.

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