Pregnancy-associated Plasma Protein-A Regulates Myoblast Proliferation and Differentiation through an Insulin-like Growth Factor-dependent Mechanism

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Pregnancy-associated plasma protein-A (PAPP-A), a member of the metalloproteinase superfamily, is an important regulator of mammalian growth and development. However, the role of PAPP-A and its mechanism of action in various cellular processes remain unknown. In this study, we have investigated the role of PAPP-A in skeletal myogenesis using C2C12 myoblasts. Recombinant PAPP-A was purified from the conditioned medium of HT1080 cells overexpressing PAPP-A. Treatment of C2C12 myoblasts with PAPP-A increased their proliferation in a dose- and time-dependent manner. Addition of exogenous PAPP-A also increased the myotube formation and the activity of creatine kinase in C2C12 cultures. Transient overexpression of the full-length PAPP-A (1–1547), but not truncated protease-inactive N-terminal PAPP-A (1–920) or C-terminal PAPP-A (1100–1547), significantly enhanced the proliferation of C2C12 myoblasts. In vitro and in situ experiments demonstrated that PAPP-A cleaves insulin-like growth factor-binding protein (IGFBP)-2, but not IGFBP-3, in the conditioned medium of C2C12 myoblasts. Overexpression of PAPP-A led to degradation of the IGFBP-2 produced by C2C12 myoblasts and increased free IGF-I concentrations without affecting total IGF-I concentrations. Addition of protease-resistant IGFBP-4 completely abolished the PAPP-A-induced proliferation of C2C12 myoblasts. Our results demonstrate that 1) PAPP-A increases the proliferation and differentiation of myoblasts, 2) the stimulatory effect of PAPP-A on myogenesis is governed by its proteolytic activity, and 3) PAPP-A promotes skeletal myogenesis by increasing the amount of free IGFs via specific degradation of IGFBP-2 produced by myoblasts.

Myogenesis is a complex phenomenon that involves the proliferation of myoblasts followed by their morphological, biochemical, and molecular modifications, resulting in the formation of multinucleated myotubes (1–3). Among the few factors that have been shown to promote the myogenic program, the insulin-like growth factors (IGFs)1 and II potentially stimulate proliferation and differentiation of myogenic cells (4). The physiological significance of IGFs in skeletal muscle development has been strongly supported by genetic studies that show that targeted disruption of the Igf-I gene in mice led to a dramatic decrease in both mass and function of skeletal muscles (5, 6). Conversely, overexpression of IGF-I in mice significantly increased muscle mass (7), and Igf-1 gene transfer to muscles via adenoviral vectors blocked age-related muscle degeneration (8). Furthermore, muscle-specific expression of Igf-1 has been shown to counteract muscle loss in mdx mice, the mouse model of Duchenne muscular dystrophy (9).

The actions of IGFs in vitro and in vivo are modulated by IGF-binding proteins (IGFBPs), which have high affinity and specificity for the IGFs (10, 11). Although some IGFBPs may potentiate IGF action in certain biological systems (10, 12), most of the known IGFBPs inhibit the myogenic function of IGFs (4, 13–17). This suggests that in the rate of the synthesis or degradation of the inhibitory IGFBPs may play a pivotal role in the regulation of the myogenic actions of IGFs.

Recent studies from our group and others have demonstrated that PAPP-A is a major protease that degrades IGFBP-4 (18–21). PAPP-A also cleaves IGFBP-5 into fragments with reduced IGF binding activity (22). Additionally, it has been shown that PAPP-A present in bovine and porcine follicular fluid can cleave IGFBP-2 (23). These in vitro studies demonstrate that PAPP-A serves as a proteolytic enzyme for selected IGFBPs, namely, IGFBP-2, IGFBP-4, and IGFBP-5. Recently, the physiological significance of PAPP-A has been strongly supported by a study by Conover et al. (24) that showed that PAPP-A and Igf-II knock-out mice exhibit similar phenotypes, characterized by a 40% reduction in birth weight and impaired bone development. Based on these in vitro and in vivo findings, we have proposed that PAPP-A may enhance the bioavailability of IGFs (the amount of free IGFs) by degrading selected IGFBPs, subsequently releasing IGFs from IGFBP complexes to act on target tissues.

Although significant progress has been made toward biochemical characterization and identification of in vivo function of PAPP-A, the role and mechanisms of action of PAPP-A in various cellular processes, including myogenic differentiation, have not been determined. In addition to the protease domain, PAPP-A contains several other functional domains, such as the five complement control protein (CCP1–5) modules and two Lin12-Notch repeats (25). However, the roles of these additional functional domains regarding PAPP-A function remain enigmatic. Using C2C12 cells (a mouse myoblast cell line), we have investigated the potential role, mechanism of action, and functional determinants of PAPP-A in myogenesis. Our data show that the stimulatory effect of PAPP-A on proliferation and differentiation of myoblasts is caused by increased IGF bioavailability, which occurs as a consequence of IGFBP-2 degradation. Furthermore, our results suggest that the-
teolytic activity of PAPP-A is required for the induction of the myogenic program.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Invitrogen. The His$_6$-tagged recombinant IGFBP-4 and amino acid 121–142-deleted IGFBP-4 peptides were prepared as previously described (26). Purified polyclonal anti-human PAPP-A IgG and normal IgG produced in rabbits were purchased from DAKO Corp. (Carpinteria, CA). Heparin-agarose was purchased from Bio-Rad. M2 FLAG antibody, M2 FLAG antibody-agarose, FLAG peptide, horse serum, and fetal calf serum (FCS) were from Sigma. Mouse monoclonal MF20 antibody specific to myosin heavy chain-fast twitch (MyHCf) protein was obtained from the Developmental Studies Hybridoma Bank of the University of Iowa. Creatine kinase assay kit was obtained from Stanbio Laboratory (Boerne, TX). Recombinant human IGF-I and IGF-II were purchased from GroPep. Other chemicals and reagents were of reagent grade and were obtained from Sigma.

Cell Culture—C2C12 and HT1080 cell lines were obtained from American Type Culture Collection (Rockville, MD). Cells were grown at 37 °C in a CO$_2$ incubator in DMEM containing 10% FCS. Differentiation of C2C12 myoblasts was induced by replacing the medium with differentiation medium (DM) (2% heat-inactivated horse serum in DMEM) for 96 h. All culture media were also supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin.

PAPP-A Expression Plasmids—Plasmids expressing the full-length PAPP-A-(1–1547), C-terminal PAPP-A-(1100–1547), and N-terminal PAPP-A-(1–920) were constructed in the pFLAG-CMV-1 vector (Sigma) as described previously (26). When transfected into mammalian cells, these plasmids led to expression of N-terminal FLAG epitope-tagged PAPP-A peptides.

Purification of Recombinant Human PAPP-A—HT1080 cells were seeded in 6-well plates and co-transfected with linearized pcDNA3.1 vector (0.2 μg) and PAPP-A-(1–1547)/pFLAG plasmid DNA (2 μg). After 48 h, the cells were trypsinized and selected in the presence of G418 (400 μg/ml). Expression of recombinant PAPP-A in G418-resistant HT1080 cells was confirmed by Western blot analysis of the conditioned medium (CM), using either PAPP-A or FLAG antibodies. Serum-free CM (200–400 ml) was collected from cultured HT1080/PAPP-A cells, centrifuged to remove cellular debris, adjusted to pH 7.5, and applied to a heparin-agarose affinity column (1 ml of resin). After washing the column with 30 ml of 50 mM Tris-Cl (pH 7.5) containing 300 mM NaCl, bound proteins were eluted with 50 mM Tris-Cl (pH 7.5) containing 1 M NaCl. Eluted proteins were concentrated with an Amicon filtration unit (10 kDa cutoff), diluted with 50 mM Tris-Cl (pH 7.5) to 150 mM NaCl, and applied to a M2 FLAG monoclonal antibody-agarose column (1 ml of resin). After washing the column with 30 ml of Tris-Cl (pH 7.5) containing 150 mM NaCl, bound proteins were eluted with 5 ml of 50 mM Tris-Cl (pH 7.5) containing 150 mM NaCl and 100 μg/ml FLAG peptide (Sigma). FLAG peptides (<1 kDa) in the purified PAPP-A were removed by an Amicon filtration device (100 kDa cutoff) with extensive washing with phosphate-buffered saline (PBS). Purified PAPP-A was quantitated by Bradford reagents using bovine serum albumin as standard and stored at −75 °C in aliquots.

Proliferation Assay—Proliferation of C2C12 myoblasts was measured using AlamarBlue dye, which measures the metabolic activity of live cells, as described previously (27). C2C12 myoblasts were seeded in DMEM/10% FCS in 24-well plates (10,000 cells/well). After 12–24 h of incubation, the cells were washed once with DMEM and starved in serum-free DMEM for an additional 12–24 h prior to addition of effectors in DMEM containing 5% FCS unless specified. After an appropriate length of incubation, the CM was removed, and 0.5 ml (for 24-well plates) or 1 ml (for 6-well plates) of 10% AlamarBlue (BioSource International, Camarillo, CA) in phenol red-free DMEM was added to each well. After 1–2 h of incubation, 0.2-ml reaction from each well was used to determine fluorescence at the optimal excitation and emission wavelengths of 546 and 590 nm, respectively. The proliferation of myoblasts was also confirmed by measuring the total protein content in cell lysates.

Myogenic Index Determination—As a morphological parameter of muscle differentiation, the myogenic index is defined as the number of nuclei residing in the cells containing three or more nuclei, divided by the total number of nuclei in hematoxylin-stained cells. Cells were washed twice in PBS, fixed with 3.7% formaldehyde in PBS for 10 min, and permeabilized with 0.1% Triton X-100 in PBS for 5 min. Cells were then stained with hematoxylin for 20 s followed by washing in running water. Distribution of nuclei in myoblasts and myotubes was measured by counting the nuclei at 7–10 different locations selected randomly using an inverted microscope with counting grid (Olympus).

Immunocytochemistry—Expression of MyHCf was examined by immunocytochemistry. C2C12 myoblasts were grown in a 24-well plate and were allowed to differentiate into myotubes. The cells were then fixed with 3.7% paraformaldehyde in PBS for 10 min, permeabilized with 0.1% Triton X-100 in PBS for 5 min. Cells were then stained with hematoxylin for 20 s followed by washing in running water. Distribution of nuclei in myoblasts and myotubes was measured by counting the nuclei at 7–10 different locations selected randomly using an inverted microscope with counting grid (Olympus).
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**CREATINE KINASE ASSAY**—Creatine kinase activity was measured to assess myogenic differentiation biochemically. Following treatment with recombinant PAPP-A, cells were washed twice in cold PBS and lysed in lysis buffer A (50 mM Tris-Cl (pH 8.0), 200 mM NaCl, 50 mM NaF, 1 mM dithiothreitol, 0.3% IPEGAL). Lysates were centrifuged for 5 min at 14,000 rpm, and the supernatant collected was used immediately for creatine kinase assay. Protein content in the samples was measured using Bradford reagent. Creatine kinase activity was measured using a spectrophotometrically based kit (Stanbio Laboratory, Boerne, TX). Specific creatine kinase activity was calculated after correction for total protein and expressed as units/mg protein.

**Miscellaneous Procedures**—Western immunoblot analysis of PAPP-A using either FLAG antibody or PAPP-A antibody was performed as described previously (26, 28).[^1] [IGF-II ligand blot analysis was conducted as described (21). Free IGF-I concentrations in the CM samples were determined by a commercial kit using 50 μl of CM (Diagnostic Systems Laboratories, Webster, Texas). Total IGF-I concentrations were measured by radioimmunoassay (29).

**Statistical Analysis**—Results are expressed as mean ± S.E. and statistically analyzed by Student’s t-test or analysis of variance. A value of p < 0.05 was considered statistically significant.

**RESULTS**

Here we have investigated the role and mechanisms by which PAPP-A modulates skeletal myogenesis. Because the major muscle differentiation steps can be reproduced in vitro with the mouse myoblast cell line C2C12 (30, 31), we used C2C12 myoblasts to investigate the role of PAPP-A in myogenesis.

**Purification of Recombinant PAPP-A Peptide**—Development of an efficient procedure to produce and purify recombinant PAPP-A peptide is crucial to study cellular responses. We employed the two-step purification protocol (described under “Experimental Procedures”) that has been shown to remove both contaminating proteins and PAPP-A proteolytic fragments, as heparin antibody and FLAG antibody bind to the distal C-terminal region and N terminus of recombinant PAPP-A, respectively (26, 32). Assessment of PAPP-A purity by Coomassie Blue staining revealed the presence of a major single band (Fig. 1A) with the expected molecular mass (~400 kDa). Immunoblot analyses using either polyclonal PAPP-A antibody (Fig. 1B) or monoclonal FLAG antibody (Fig. 1C) demonstrate that the purified PAPP-A peptide contains insignificantly low amounts of PAPP-A proteolytic fragments.

**PAPP-A Stimulates Proliferation of C2C12 Myoblasts**—Because C2C12 myoblasts cultured at low cell density in serum-free medium underwent severe apoptosis and did not proliferate, the stimulatory effect of PAPP-A on cell proliferation was less pronounced under serum-free conditions (data not shown). In contrast, as shown in Fig. 1D, PAPP-A was unable to cleave the protease-resistant IGFBP-4 analog (PR-BP4) lacking a broad sequence (amino acids 121–142) containing the cleavage site (Met-135-Lys-136) (33). Consistent with previous published reports (21, 22), purified recombinant PAPP-A peptide also effectively cleaved IGFBP-5 (data not shown).

**PAPP-A Promotes Differentiation of C2C12 Myoblasts into Myotubes**—The effect of PAPP-A on differentiation of C2C12 myoblasts was examined. C2C12 myoblasts were incubated in DM (DMEM/2% heat-inactivated horse serum) with or without 100 ng/ml PAPP-A. Myoblasts cultured in growth medium (DMEM/10% FCS) were included as a negative control for myogenic differentiation. After 96 h, myotube formation in C2C12 cultures was studied by immunostaining...
Overexpression of PAPP-A in C2C12 myoblasts enhanced their proliferation, evident by increased specific creatine kinase activity (Fig. 4D).

Proteolytic Activity of PAPP-A Is Required for Its Stimulatory Effect on Myoblast Proliferation—Studies were performed to investigate whether the myogenic activity of PAPP-A is determined by its proteolytic activity or is due to other potential functional domains (25). Cell proliferation was compared in C2C12 cells transfected with either the plasmid expressing the full-length PAPP-A-(1–1547) or the N-terminal PAPP-A-(1–920), which exhibits at least two orders of magnitude reduction in IGFBP-4 proteolytic activity (26). Immunoblot analysis with FLAG antibody revealed a dose-dependent increase in PAPP-A production with increasing amounts of PAPP-A/pFLAG plasmids (Fig. 5A). In our previous studies, nonreduced PAPP-A-(1–920) peptide overexpressed in 293T cells was detected as a primary band at 120 kDa and a minor band at >500 kDa (26). Although only the >500-kDa band was detected in the CM of transfected C2C12 cells (Fig. 5A), reduced PAPP-A-(1–920) was detected as a major band of 140 kDa (Fig. 5B) as reported in our previous studies (26). Overexpression of the full-length PAPP-A dose-dependently stimulated C2C12 cell growth, whereas overexpression of the PAPP-A-(1–920) peptide had no significant effect (Fig. 5C). Addi-
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FIGURE 5. Effect of overexpression of mutant PAPP-A on the proliferation of C2C12 myoblasts. C2C12 myoblasts were transfected with either full-length (amino acids 1–1547) or truncated (amino acids 1–920) PAPP-A plasmid as described in Fig. 4A. Empty vector DNA was used to adjust the total amount of DNA to 2 μg/well. 40 μl of CM was treated without (A) or with (B) reducing agent β-mercaptoethanol and subjected to immunoblot analysis with FLAG antibody. C, proliferation of transfected C2C12 myoblasts was determined in 5% fetal calf serum medium using AlamarBlue assay as described under “Experimental Procedures.” The data presented here show that overexpression of full-length PAPP-A, but not truncated PAPP-A, significantly increased the proliferation of C2C12 myoblasts (*, p < 0.05 versus vector alone).

PAPP-A Degradates IGFBP-2 Present in the Culture Supernatants of C2C12 Myoblasts—To understand the mechanisms responsible for increased myogenesis in response to PAPP-A, we tested the hypothesis that degradation of IGFBPs in cultured myoblasts by PAPP-A increases the bioavailability of IGFs. IGF-II ligand blot analysis of serum-free CM collected from C2C12 myoblasts revealed the presence of a predominant 30-kDa murine IGFBP-2 (Fig. 6A, mIGFBP-2). It has been reported that mIGFBP-2 is the predominant IGFBP produced by C2C12 myoblasts that are treated with 100 ng/ml PAPP-A (pooled from three independent experiments) or transfected with PAPP-A expression plasmid (pooled from two independent experiments) (21). Consistent with our previously published report (21), IGFBP-3 (mainly originating from the added bovine serum) was also supported by the finding that the 30-kDa IGFBP-2 (bIGFBP-2) and the 30-kDa IGFBP as murine IGFBP-2 (mIGFBP-2) were cleaved in the presence of 100 ng/ml PAPP-A (Fig. 6A, lane 5 versus lane 3, lane 6 versus lane 4, and lane 7 versus lane 5, respectively). The levels of mIGFBP-2 or bIGFBP-2 in the CM of C2C12 myoblasts transfected with either PAPP-A-(1–920) or PAPP-A-(1100–1547) were not affected. The majority of the mIGFBP-2 and bIGFBP-2 were cleaved in the CM of C2C12 myoblasts transfected with full-length proteolytically active PAPP-A-(1–1547) plasmid (Fig. 6C, lanes 1 and 2 versus lane 3). Consistent with our previously published report (21), IGFBP-3 (mainly originating from the added bovine serum) was not degraded by PAPP-A.

PAPP-A Increases the Free IGF Concentration in C2C12 Cultures—The ability of PAPP-A to increase free IGF-1 concentrations through enhancing degradation of the IGFBP-2 produced by the myoblasts was investigated. The effect of PAPP-A on proliferation was determined in C2C12 myoblasts cultured in the presence of 5% bovine CS that contained a significantly lower amount of the 34-kDa bovine IGFBP-2 (bIGFBP-2) as compared with 5% bovine FCS (Fig. 6C, lane 1 versus lane 2).
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FIGURE 7. PAPP-A overexpression increases free IGF-I concentration primarily through enhancing degradation of the IGFBP-2 produced by C2C12 myoblasts. C2C12 cells were seeded in 5% CS in 6-well plates at a density of 80,000 cells/well. After 24 h of incubation, cells were transfected with 1 μg of pFLAG empty vector DNA or PAPP-A-(1–1547)/pFLAG plasmids in 1 ml of DMEM containing 5% CS. After 24 h of incubation, medium was replaced with 2 ml of DMEM containing 5% CS. CM samples were collected after an additional 48 h of incubation for determination of IGFBP proteolysis and IGF-I concentrations. A, overexpression of PAPP-A significantly increased cell proliferation as determined by the AlamarBlue assay. B, 40 μl of CM were subjected to IGF-II ligand blot analysis. Overexpression of PAPP-A caused a nearly complete degradation of the mIGFBP-2 fragment (indicated by arrow) produced by C2C12 myoblasts. C, 50 μl of CM was used to determine free IGF-I concentrations by a commercial kit (Diagnostic Systems Laboratories, Webster, Texas). Total IGF-I concentrations were determined by radioimmunoassay (29). Similar results were obtained in another independent experiment. *, p < 0.05 versus vector control (n = 6).

Consistent with the results from experiments using 5% bovine FCS, overexpression of PAPP-A significantly increased cell proliferation in the presence of 5% CS (Fig. 7A) and caused a complete degradation of the mIGFBP-2 endogenously produced by the C2C12 myoblasts (Fig. 7B). Free IGF-I in the CM of vector plasmid-transfected cells was below the sensitivity of the assay (<0.1 ng/ml). The concentration of free IGF-I in the CM of C2C12 cells overexpressing PAPP-A was increased to 1.3 ± 0.1 ng/ml, which is ~10% of the total IGF-I (Fig. 7C). A similar increase in free IGF-I concentration was found in the CM of cells treated with recombinant PAPP-A in either DMEM/5% CS or DMEM/5% FCS (data not shown). It should be noted that the free IGF-I enzyme-linked immunosorbent assay measures truly free IGF-I. The IGF-I loosely bound to the N-terminal IGFBP-2 proteolytic fragments may not be detected as free IGF-I but can be released to interact with its receptors more quickly than IGF-I bound to the intact high affinity IGFBP-2.

To exclude the possibility that increase in free IGF-I concentration is due to increased IGF-I production in response to PAPP-A treatment, we also estimated the total IGF-I (free IGF-I + IGF-I bound to IGFBPs in the culture medium) in the same samples. PAPP-A overexpression significantly increased the total IGF-I concentration in the CM by ~30%. However, no significant difference was observed after normalizing for the cell numbers in control and PAPP-A-transfected cultures (Fig. 7C). In addition, quantitative real-time PCR analysis revealed that treatment of C2C12 cells with PAPP-A did not affect the level of Igf-I mRNA (data not shown). These data suggest that PAPP-A increases free IGF-I concentration through degradation of IGFBP-2 endogenously produced by the C2C12 myoblasts.

PAPP-A Enhances C2C12 Myoblast Proliferation via an IGF-dependent Mechanism—To further confirm that PAPP-A promotes myoblast proliferation by increasing the bioavailability of IGFs, we investigated whether inhibition of the activity of IGFs could block cell proliferation induced by PAPP-A. We have previously shown that the PR-BP4 (protease-resistant IGFBP-4) analog binds to IGFs (IGF-I and IGF-II) with similar affinity as exhibited by the wild-type IGFBP-4 but cannot be cleaved by the IGFBP-4 protease produced by osteoblasts (33, 41). More importantly, this mutant IGFBP-4 peptide cannot be cleaved by PAPP-A (Fig. 1D). Thus, this IGFBP-4 mutant can serve as an ideal IGF inhibitor in the presence of PAPP-A. C2C12 myoblasts were treated with PAPP-A either alone or in combination with PR-BP4 peptide, and the cellular proliferation was measured. Interestingly, PAPP-A-induced myoblast proliferation was completely abolished by the PR-BP4 (Fig. 8). It should be noted that PR-BP4 at the same concentration did not inhibit cell proliferation induced by des-1–3 IGF-I (lacking the first 3 amino acids), which has very low binding affinity with IGFBPs (data not shown).
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DISCUSSION

Using exogenous recombinant PAPP-A or ectopic expression of PAPP-A in C2C12 myoblasts, we have studied the role and mechanism by which PAPP-A affects myogenesis. Our data clearly show that PAPP-A induces the proliferation and differentiation of C2C12 myoblasts. Increased myogenesis in response to PAPP-A is intrinsic to its proteolytic activity. Furthermore, the results of our in vitro experiments suggest that PAPP-A promotes C2C12 myoblast proliferation by degradation of IGFBP-2, the major IGFBP produced by C2C12 myoblasts. Based on the results of this study, we propose a model summarizing the mode of action of PAPP-A in skeletal myogenesis (Fig. 9).

Myogenesis is a developmental program that generates and regenerates skeletal muscle (3). Intensive research in the last decade has led to significant understanding of the complexity of specification and differentiation of skeletal muscle cells in mammals. Myogenic differentiation is regulated by both positive- and negative-acting factors (42). Serum is a major source of IGFs exit in the form of IGFBPs. Data suggest that IGFBP-2 produced by myoblasts and present in FCS exogenously added bovine serum, and a 34-kDa bIGFBP-2 originating from exogenously added bovine CS that contains very low levels of the bIGFBP-2 (Fig. 6, left panel). Based on the abundance of IGFBP-2 in the CM of C2C12 cultures, the majority of IGFs would be expected to exist in the IGFI-IGFBP-2 complex, which is biologically inactive. Indeed, there is essentially no free IGF-I in control C2C12 cultures (Fig. 7C).

Although proliferation and differentiation are both necessary for myogenesis, they are intrinsically opposing pathways, because most factors that stimulate proliferation inhibit differentiation and vice versa (4, 48). IGFs are rare growth factors, because they are able to stimulate both proliferation and differentiation of myoblasts (4). Our finding that the dual biological functions of IGFs in myogenesis are reproduced by PAPP-A treatment implicates that PAPP-A acts on myoblasts via an IGF-dependent mechanism. This contention is strongly supported by three lines of evidence. First, PAPP-A fragments with extremely low or no IGF-binding activity (26) are unable to stimulate myoblast proliferation (Fig. 5). Second, PAPP-A overexpression led to significant levels of free IGF-I, which was essentially absent in untreated C2C12 cultures (Fig. 7C). Third, biological activity of PAPP-A on myoblasts was completely abolished by a potent IGF inhibitor, protease-resistant IGFBP-4 analog (Fig. 8), which should neutralize all of the free IGFs from the system.

Studies demonstrate that addition of exogenous IGFBP-2 inhibits proliferation or differentiation of myoblasts induced by exogenous IGFs (51). Consistent with these in vitro data, it has been reported recently that serum IGFBP-2 concentrations are negatively correlated with skeletal muscle strength, physical activity, and bone quality in the elderly (52, 53). Based on our in vitro findings that PAPP-A promotes myogenesis via an IGF-dependent mechanism that involves degradation of IGFBP-2 (Fig. 9), it is conceivable that PAPP-A may play an important role in the regulation of myogenesis in vivo. Future studies involving targeted overexpression of PAPP-A in a skeletal muscle or local intramuscular injection of recombinant PAPP-A peptide are needed to define the in vivo role of PAPP-A in skeletal myogenesis.

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accounts for >80% of the total IGF binding activity and can be effectively cleaved by PAPP-A (Figs. 6 and 7). The importance of endogenously produced IGFBP-2 by myoblasts in mediating the effects of PAPP-A is supported by the data that PAPP-A overexpression significantly increased proliferation of C2C12 myoblasts in the presence of 5% bovine CS that contains very low levels of the bIGFBP-2 (Fig. 6C, left panel). Based on the abundance of IGFBP-2 in the CM of C2C12 cultures, the majority of IGFs would be expected to exist in the IGFI-IGFBP-2 complex, which is biologically inactive. Indeed, there is essentially no free IGF-I in control C2C12 cultures (Fig. 7C).
