Quantitative Analysis of Insulin-like Growth Factor-Modulated Proteolysis of Insulin-like Growth Factor Binding Protein-4 and -5 by Pregnancy-Associated Plasma Protein-A[†]

Claus Gyrup and Claus Oxvig*

Department of Molecular Biology, University of Aarhus, Gustav Wieds Vej 10C, DK-8000 Aarhus C, Denmark

Received October 27, 2006; Revised Manuscript Received December 20, 2006

ABSTRACT: The metzincin metalloproteinase pregnancy-associated plasma protein-A (PAPP-A, pappalysin-1, EC 3.4.24.79) specifically cleaves insulin-like growth factor binding protein (IGFBP)-4 and -5. Regulation of insulin-like growth factor (IGF) bioavailability through cleavage of these inhibitory binding proteins is an important mechanism for the control of growth and development of vertebrate cells. Although proteolysis of IGFBP-4 and -5 by PAPP-A has been extensively studied in many systems, quantitative analyses have been lacking. We have characterized the cleavage of its natural substrates, IGFBP-4 and -5, in the absence and presence of IGF-I or -II and determined the kinetic parameters (K_m and k_{cat}) for the different combinations of IGFBP and IGF. The rate of IGFBP-4 proteolysis is dramatically increased upon addition of IGF-I or -II. Kinetic analysis revealed that IGF-II was a more potent activator of IGFBP-4 proteolysis than IGF-I. Proteolysis of IGFBP-5 is slightly inhibited by IGF, and we find that IGF-I and -II display a similar degree of inhibition of IGFBP-5 cleavage. We show that the mechanism of IGF-modulated proteolysis of IGFBP-4 and -5 involves changes in both the recognition of substrate (K_m) and the turnover rate (k_{cat}). In addition, we have devised a novel method of revealing potential consequences of substrate modification for kinetic analysis, and we have used this method to establish that there is no apparent difference in the behavior of radiolabeled IGFBP-4 and -5 compared to the behavior of the unmodified protein substrates. We also propose experimental conditions for the proper analysis of IGFBP proteolysis, and we demonstrate their usefulness by quantitatively evaluating the effect of inhibitory compounds on the rate of proteolysis. Finally, we have compared PAPP-A to other proteinases thought to have IGFBP-4 or -5 as a substrate. This emphasizes the potential of PAPP-A to specifically and efficiently function as a regulator in the IGF system.

The insulin-like growth factors (IGF-I[1] and -II) are approximately 7.5 kDa polypeptides with auto- and paracrine effects on cell proliferation, migration, and differentiation (*1*). IGF-I and -II exert their activities by binding to the IGF-1 receptor (IGF1R), which initiates an intracellular signaling cascade (*2*). Binding to IGF1R is modulated by six IGF binding proteins, IGFBP-1−6. All IGFBPs have higher affinities for IGF-I and IGF-II (10^{-9}−10^{-11} M) (*3*) than does IGF1R (10^{-8}−10^{-9} M) (*4*), thereby sequestering IGF from the receptor (*1*, *5*). Specific proteolysis of the IGFBPs represents the principal regulatory mechanism of IGF bioavailability, generating IGFBP fragments with weakened affinity for IGF and thus promoting binding to IGF1R. The study of IGFBP proteolysis is relevant because the IGFs are involved in both normal physiology and human disease, e.g., cancer (*6*, *7*) and cardiovascular disease (*8*, *9*), and therefore, strategies for the direct inhibition of IGF signaling are being developed (*10*, *11*). However, specific inhibitors of growth promoting proteolytic activity may represent a valuable alternative, in particular because such inhibition of IGF receptor stimulation is unlikely to interfere with insulin signaling.

One of the most studied IGFBP proteinases is pregnancy-associated plasma protein-A (PAPP-A). PAPP-A is a protein of 1547 residues (*12*) and belongs to the metzincin superfamily of metalloproteinases (*13*−*17*). PAPP-A specifically cleaves IGFBP-4 (*18*), IGFBP-5 (*19*), and IGFBP-2 (*20*), thereby releasing sequestered IGF. While the extent of proteolysis of IGFBP-4 and -2 is increased many fold upon binding of IGF, the cleavage of IGFBP-5 appears to be slightly impaired by the presence of IGF (*19*).

PAPP-A is found in the circulation of normal individuals at a very low concentration, but the level is found to increase throughout pregnancy (*21*). In the circulation of pregnant women, PAPP-A is present in a 2:2 complex with the pro form of eosinophil major basic protein (proMBP) (*22*), which is proteolytically inactive (*23*). Recently, it has been found that patients with acute coronary syndromes (ACS) show an elevated level of PAPP-A antigen and that this correlates with the presence of unstable atherosclerotic plaques (*24*). Furthermore, PAPP-A seems to be implicated in follicle selection (*25*) as well as wound healing (*26*).

[†] This work was supported by grants from the Novo Nordic Foundation and the Danish Natural Science Research Council.

* To whom correspondence should be addressed: Department of Molecular Biology, University of Aarhus, Gustav Wieds Vej 10C, DK-8000 Aarhus C, Denmark. Phone: +45 8942 5060. Fax: +45 8942 5068. E-mail: co@mb.au.dk.

[1] Abbreviations: IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; PAPP-A, pregnancy-associated plasma protein-A.

10.1021/bi062229i CCC: $37.00
Published on Web 01/24/2007

There are several candidate proteinases for IGFBP-4 and/or -5 (*27*−*31*); however, kinetic analysis has not been carried out for any of these, nor has the effect of IGF or other binding partners on the rate and specificity been quantitatively studied. Furthermore, most reports on proteolytic activity against IGFBP-4 or -5 were based on nonphysiological enzyme-to-substrate ratios and extended incubation times, and the in vivo relevance of the enzymes studied is therefore uncertain. Kinetic analysis of PAPP-A has not been performed, neither with natural substrates nor with low-molecular weight chromogenic substrates, currently not available. We have therefore determined the kinetic parameters for its cleavage of IGFBP-4 and -5 in the presence and absence of IGF-I or -II. The kinetic analysis reported here represents the first study of a proteinase of the pappalysin family, and it will support the development of models for PAPP-A function in vivo.

MATERIALS AND METHODS

Expression, Purification, and Labeling of Proteins. Human IGFBP-4 and IGFBP-5 were recombinantly expressed in HEK293T cells. Briefly, serum-free media from a population of stably transfected cells were harvested, and IGFBP was purified as previously described (*19*). Human IGFBP-2 was produced in a similar manner (L. S. Laursen and C. Oxvig, unpublished observations). Medium containing recombinant human PAPP-A was harvested from a stable population of HEK293T cells transfected with PAPP-A cDNA (*23*). Concentrations of purified proteins were determined by quantitative amino acid analysis (*32*). IGFBP (5−20 μg) was radiolabeled using the Chloramine-T method (*33*). The concentration of radiolabeled IGFBP was determined using a direct capture ELISA. In brief, unlabeled IGFBP and radiolabeled IGFBP were added to a Maxisorp Microplate (Nunc). A monoclonal antibody against the *c-myc* epitope (9E10) followed by peroxidase-conjugated anti-mouse IgG (P0260, DAKO) was used for detection. The ELISA was developed using *O*-phenylenediamine (OPD, S2045, DAKO) according to the manufacturer's instructions, and the enzymatic reaction was terminated by adding 0.5 M H_2SO_4. Dilution series of unlabeled IGFBP-4 and -5 were used to establish standard curves. Ulilysin was activated as described previously (*34*) and used at a final concentration of 40 ng/mL. Plasmin (American Diagnostica) was used at a concentration of 10 ng/mL. Trypsin (Worthington) was used at a concentration of 100 ng/mL. mAbs 234-5 and 234-10 (*21*), mAb PA-1A (V. Rodacker and C. Oxvig, unpublished observations), and polyclonal anti-PAPP-A (*35*) antibodies were used.

Proteolytic Assays. All reactions were carried out in 50 mM Tris, 100 mM NaCl, and 1 mM $CaCl_2$ at pH 7.5. Radiolabeled IGFBP (*19*) was preincubated with or without IGF (Diagnostic Systems Laboatories) at a 5−10-fold molar excess for 15 min before the reaction was initiated to allow complete formation of the IGFBP−IGF complex. The reaction was initiated by adding the proteinase to the reaction mixture followed by incubation at 37 °C. Aliquots were removed at individual time points, and the reaction was stopped by the addition of SDS loading buffer supplemented with 100 mM EDTA. The samples were separated by nonreducing SDS−PAGE (10−20% Tris-glycine) (*36*). Following electrophoresis, gels were dried and exposed to a storage phosphor screen (Molecular Dynamics) for a minimum of 20 h. The screens were scanned on a Typhoon Trio scanner (GE Healthcare) and quantified using the ImageQuant software package (GE Healthcare). The degree of cleavage ([cleaved]) in each lane was determined by relating the intensity of the cleaved fragments (I^{cleaved}) to the sum of the intensities of the intact protein (I^{intact}) and cleaved fragments in each lane after subtraction of background (eq 1). [S] is the initial concentration of substrate.

$$[\text{cleaved}] = \frac{I^{\text{cleaved}}}{I^{\text{cleaved}} + I^{\text{intact}}}[\text{S}] \quad (1)$$

Determination of Kinetic Parameters. Purified, unlabeled IGFBP in the presence or absence of a minimum of a 5-fold molar excess of IGF was used to make dilution series from approximately 5 μM to 40 nM. Radiolabeled IGFBP (20 nM) was added to the reaction mixture as a tracer to follow the cleavage of the unlabeled substrate. Reactions were initiated by adding the proteinase PAPP-A (160 pM) followed by incubation at 37 °C. At individual time points, aliquots were removed and analyzed by SDS−PAGE and autoradiography. Reaction rates were calculated by linear approximation of progress curves. The resulting data were analyzed by nonlinear regression using Sigmaplot 8.02 and Enzyme Kinetics Module version 1.1 (Systat Software Inc.). Kinetic parameters were obtained by fitting the standard Michaelis−Menten equation (eq 2) to the data.

$$V = \frac{V_{\text{max}}[\text{S}^*]}{K_{\text{m}} + [\text{S}^*]} \quad (2)$$

To obtain specificity constants ($k_{\text{cat}}/K_{\text{m}}$) of ulilysin, trypsin, and plasmin toward IGFBP-4 and -5, and of PAPP-A toward IGFBP-2, initial rates were measured at substrate concentrations of 10 and 20 nM (i.e., >10-fold lower than the K_{m} of cleavage of IGFBP-5 by PAPP-A) and enzyme concentrations of 1 nM (ulilysin), 4 nM (trypsin), 0.1 nM (plasmin), or 1.5 nM (PAPP-A). The linear approximation of eq 2 at substrate concentrations below K_{m} (eq 3) was fitted to the data.

$$\frac{V}{[\text{E}^0]} = \frac{k_{\text{cat}}}{K_{\text{m}}}[\text{S}^*] \quad (3)$$

Analysis of Effects upon Radiolabeling of the Substrate. To determine if substrate radiolabeling influences proteolysis by PAPP-A, we used a strategy based on competive inhibition. The labeled substrate was defined as the signal-generating substrate (S*) and the unlabeled substrate as an inhibitory silent substrate (S^0). Substitution of these definitions ([S] = [S^*] and [I] = [S^0]) in the general equation for competitive enzyme inhibition, in which K_{i} is the dissociation constant for inhibitor binding, yields eq 4.

$$V = \frac{V_{\text{max}}[\text{S}^*]}{K_{\text{m}}(1 + [\text{S}^0]/K_{\text{i}}) + [\text{S}^*]} \quad (4)$$

A restraint on [S*] and [S^0] was introduced (eq 5). Rearrangement of eq 5 and substitution into eq 4 yield eq 6. Simulations were carried out using this equation.

$$[S^*] + [S^0] = K \quad (5)$$

$$V = \frac{V_{max}[S^*]}{K_m[1 + (K - [S^*])/K_i] + [S^*]} \quad (6)$$

Importantly, if the inhibitor has no interactions with the proteinase ($K_i \rightarrow \infty$), eq 6 approximates the standard Michaelis−Menten equation (eq 2). In the case that the two substrates are apparently equal ($K_m = K_i$), eq 6 reduces to eq 7.

$$V = \frac{V_{max}}{K_m + K}[S^*] \quad (7)$$

When plotted, eq 7 yields a straight line through the origin and a slope of $V_{max}/(K_m + K)$. For analysis of binding proteins IGFBP-4 and -5, the concentration of labeled and unlabeled IGFBP was measured with an ELISA and adjusted to approximately 0.3 μM. Labeled IGFBP was serially diluted in buffer containing the same concentration of unlabeled IGFBP to satisfy the restraint in eq 5. IGF-I or -II was added to the reaction mixture in excess (2 μM) to ensure saturation. The reaction was initiated by the addition of PAPP-A (160 pM).

RESULTS

Proteolysis of IGFBP-4 and -5 by PAPP-A. Proteolysis of IGFBP-4 and -5 by PAPP-A was analyzed using purified recombinant proteins expressed in HEK293T cells. The substrates and the enzyme were quantified by amino acid analysis. Initially, it was assumed that substrate cleavage was not affected by iodination, and trace amounts of radiolabeled substrate were added to the reaction mixture to allow quantification. The degree of cleavage was determined by densitometry following separation by SDS−PAGE and autoradiography using a phosphorimager (Figure 1A−D). From the progress curves (Figure 1E,F), linearity was observed under conditions of less than 20% substrate cleavage. Similar experiments with human IGFBP-2 in the presence of IGF showed less efficient cleavage of this substrate compared to the cleavage of both IGFBP-4 and -5. In the presence of IGF, the k_{cat}/K_m value for the PAPP-A−IGFBP-2 complex was approximately 25-fold lower than that for the PAPP-A−IGFBP-4 complex (data not shown). Further kinetic analysis of IGFBP-2 was not carried out.

Proteolytic Susceptibility of IGFBP to PAPP-A in the Presence of IGF. To determine the effects of IGF on the proteolysis, the proteolytic susceptibility of the substrates

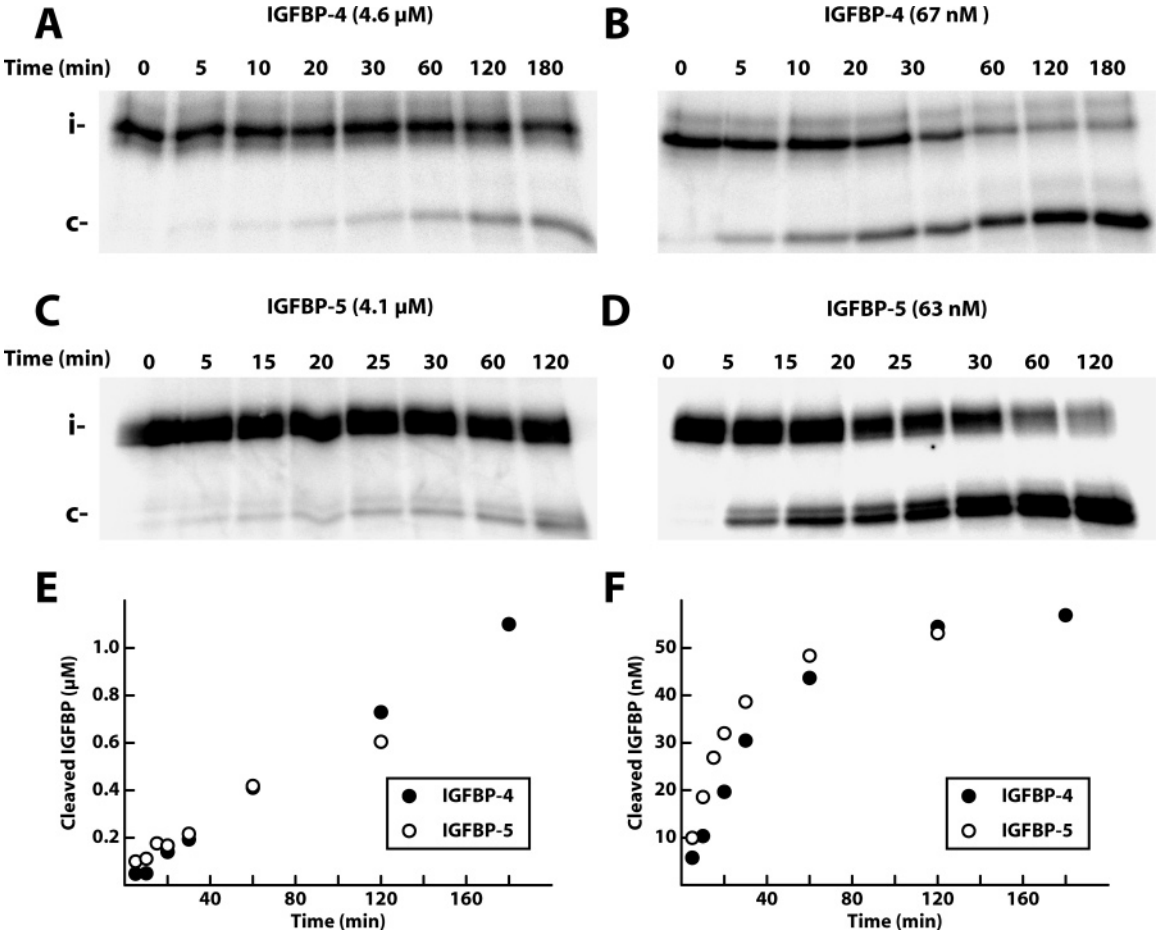

FIGURE 1: Progress curves for the specific cleavage of IGFBP-4 and -5 by PAPP-A. Proteolysis of radiolabeled IGFBP-4 (A and B) and IGFBP-5 (C and D) was monitored by SDS−PAGE followed by autoradiography at two different substrate concentrations. The cleavage of intact (i) IGFBP-4 and -5 results in fragments (c) that comigrate on SDS−PAGE, as indicated. To allow visualization and quantification of cleavage, trace amounts (20 nM) of iodinated IGFBP-4 and -5, respectively, were added to the reaction mixtures. The degree of cleavage was quantified by densitometry as described in Materials and Methods and plotted as a function of time (E and F). Cleavage of IGFBP-4 was performed in the presence of IGF-I (A and B), and cleavage of IGFBP-5 was performed in the absence of IGF (C and D). The concentrations of IGFBP-4 and IGF-I were 4.6 and 25 μM (A) and 67 and 350 nM (B), respectively. The concentrations of IGFBP-5 were 4.1 μM (C) and 63 nM (D). PAPP-A was used at a concentration of 160 pM.

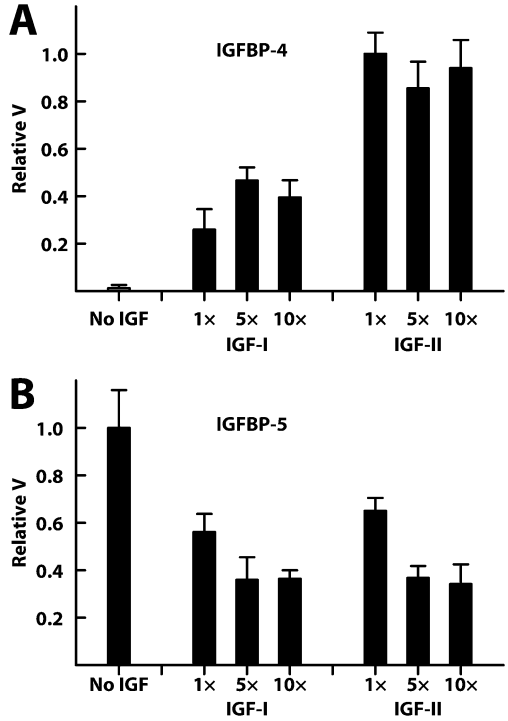

FIGURE 2: Proteolytic susceptibility of IGFBP-4 and -5 in the absence and presence of IGF. IGFBP-4 (20 nM) (A) or IGFBP-5 (20 nM) (B) was incubated with no IGF and increasing amounts (up to 10-fold molar excess) of IGF-I or -II, respectively. The initial rates of cleavage (V) were determined and normalized to the highest value. Each column is the average of three independent experiments. Standard deviations are given.

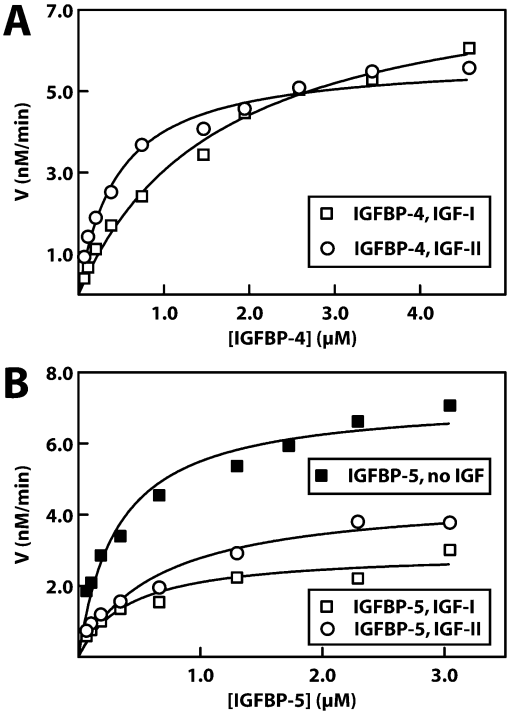

FIGURE 3: Michaelis−Menten plots for the concentration-dependent cleavage of IGFBP-4 and -5 in the absence or presence of IGF-I or -II. Initial rates for the proteolysis of IGFBP-4 and -5 in the absence (IGFBP-5 only) or presence of IGF-I or -II were measured at substrate concentrations from 70 nM to 5 μM. The Michaelis−Menten equation was fitted to the data to obtain the kinetic constants K_m and k_{cat} (Table 1). The best fit to the data is represented as a solid line.

was analyzed using increasing amounts of IGF-I and -II (Figure 2). As previously observed (*19*), the activity of PAPP-A against IGFBP-4 without IGF is almost absent. In the presence of IGF-I or -II, the initial rates dose-dependently increased as more IGF was added, reaching saturation at a molar excess of 5-fold IGF over IGFBP (Figure 2A). The reaction rate of IGFBP-4 proteolysis is significantly increased by the binding of either IGF-I or -II, with IGF-II being a more potent activator of the IGFBP-4 substrate (Figure 2A). In contrast to that of IGFBP-4, the proteolysis of IGFBP-5 does not depend on bound IGF. From the experiment with IGFBP-5 (Figure 2B), it is apparent that the initial rates are slightly reduced in the presence of IGF-I or -II. In further contrast to IGFBP-4, no difference in the effects of IGF-I or -II on the reaction rates was observed. These experiments quantitatively demonstrate that IGF-I and -II modulate the substrate behavior of IGFBP-4 and -5. In subsequent experiments, a molar excess of at least 5-fold IGF over IGFBP was used, unless otherwise specified.

Enzyme Kinetic Analysis of Cleavage of IGFBP by PAPP-A. The kinetic analysis of cleavage of IGFBP-4 and -5 by PAPP-A was carried out using the procedure described above. From individual progress curves, initial rates were determined and plotted as a function of substrate concentration (Figure 3). The standard Michaelis−Menten equation (eq 3) was fitted to the data that were obtained, and kinetic parameters were calculated (Table 1). Kinetics of IGFBP-4 cleavage were analyzed only in the presence of IGF-I and -II, as the proteolysis rate of IGFBP-4 in the absence of IGF could not be reliably measured (Figure 3A). It was estimated that in the absence of IGF, the initial rate of cleavage of

Table 1: Kinetic Parameters for the Proteolysis of IGFBP-4 and -5 by PAPP-A

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})
IGFBP-4			
IGF-I	1.63 ± 0.32	0.89 ± 0.08	0.55 ± 0.12
IGF-II	0.45 ± 0.11	0.64 ± 0.04	1.44 ± 0.36
IGFBP-5			
no IGF	0.31 ± 0.06	0.75 ± 0.04	2.38 ± 0.45
IGF-I	0.41 ± 0.11	0.31 ± 0.03	0.76 ± 0.22
IGF-II	0.64 ± 0.14	0.47 ± 0.04	0.75 ± 0.18

IGFBP-4 by PAPP-A was 40-fold (IGF-I) to 100-fold (IGF-II) lower (data not shown). For the cleavage of IGFBP-4 with bound IGF-I or -II, K_m values of 1.63 ± 0.32 and 0.45 ± 0.11 μM, respectively, were found. k_{cat} was found to be 0.89 ± 0.08 s^{-1} with IGF-I and 0.64 ± 0.04 s^{-1} with IGF-II, yielding apparent second-order rate constants (k_{cat}/K_m) of 0.55 × 10^6 M^{-1} s^{-1} for IGF-I and 1.44 × 10^6 M^{-1} s^{-1} for IGF-II. Thus, IGFBP-4 with bound IGF-II is a better substrate for PAPP-A with the k_{cat}/K_m being approximately 3-fold higher than with IGF-I.

For the proteolytic cleavage of IGFBP-5 without bound IGF, K_m and k_{cat} was determined to be 0.32 ± 0.06 μM and 0.75 ± 0.04 s^{-1}, respectively, yielding a k_{cat}/K_m of 2.38 × 10^6 M^{-1} s^{-1}. IGFBP-5 in the absence of IGF is therefore a better substrate than IGFBP-4 with either IGF-I or -II bound. The value of K_m for the cleavage of IGFBP-5 was found to be 0.41 ± 0.11 μM with IGF-I and 0.64 ± 0.14 μM with IGF-II, and k_{cat} was found to be 0.31 ± 0.03 and 0.47 ± 0.04 s^{-1} for IGF-I and -II, respectively. The second-order rate constants for IGF-I and -II were 0.76 × 10^6 and 0.75 ×

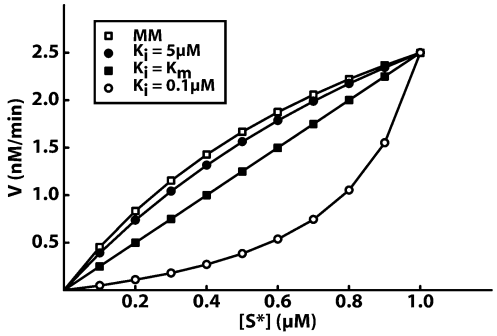

FIGURE 4: Simulations of the possible adverse effects of using a modified protein substrate. Simulated V(S*) curves are shown for different values of K_i (cf. eq 6). Data simulations were carried out with the following parameters: $K_m = 1$ μM, $V_{max} = 5$ nM/min, and $K = 1$ μM (simulated from [S*] = 0 to [S*] = K). The situation in which the silent substrate has no interaction with the enzyme is shown (□). In this situation, K_i reaches ∞ and the curve approximates the Michaelis−Menten curve (eq 6 approximates eq 2). Three different values of K_i were used to illustrate the effect of altered substrate interaction: $K_i = 5$ μM (●), $K_i = K_m = 1$ μM (■), and $K_i = 0.1$ μM (○).

10^6 M^{-1} s^{-1}, respectively. Interestingly, a difference between IGF-I and -II in the modulation of proteolytic susceptibility was observed for IGFBP-4, but not for IGFBP-5. The kinetic parameters are summarized in Table 1.

Iodination of IGFBP-4 and IGFBP-5 Does Not Affect Susceptibility toward PAPP-A. As mentioned in the analysis given above, it was assumed that radiolabeled IGFBP and unlabeled IGFBP are equally good PAPP-A substrates. The recently published structures of the N-terminal part of IGFBP-4 (*37*) and -5 (*38*) in complex with IGF-I show that indeed Tyr-49 of IGFBP-4 and Tyr-50 of IGFBP-5 are engaged in hydrogen bonding to Asp-11 of IGF-I. Since it is well-established that neither IGF-I nor IGF-II binds to PAPP-A (*19*, *39*) and that the modulatory effect of IGF on IGFBP proteolysis is a consequence of binding of IGF to IGFBP, the use of radiolabeled IGFBP as a tracer in kinetic assays could lead to erroneous results.

To address the potential difference in substrate behavior of labeled versus unlabeled IGFBP, substrate cleavage was analyzed using an approach based on competitive inhibition enzyme kinetics. The competitive inhibition of the iodinated signal-generating substrate in the presence of a silent substrate at a total concentration of K (eq 5) is expected to result in a straight line (eq 7) through the origin with a slope of $V_{max}/(K_m + K)$, provided they are equally good substrates. To visualize the potential error of using a tracer, we simulated data describing different scenarios using kinetic parameters similar to those determined for PAPP-A (Figure 4). In case modification of the substrate enhances the substrate, an upward curvature starting at the origin and ending at [S*] = K is expected. If, on the other hand, modification compromises substrate functionality, the resulting curve will be downward. These three scenarios are all plotted together (Figure 4). For comparison, a simulated Michaelis−Menten curve based on the same kinetic parameters is also shown.

IGFBP-5 was analyzed in the absence of IGF (Figure 5A). Evidently, the points lie on a straight line through the origin with no tendency of curving. The hypothetical cases where K_i deviates from K_m by a factor of 0.1 or 10 are also shown. Likewise, IGFBP-5 (Figure 5B) and IGFBP-4 (Figure 5C) were analyzed in the presence of IGF-I and -II. In all cases,

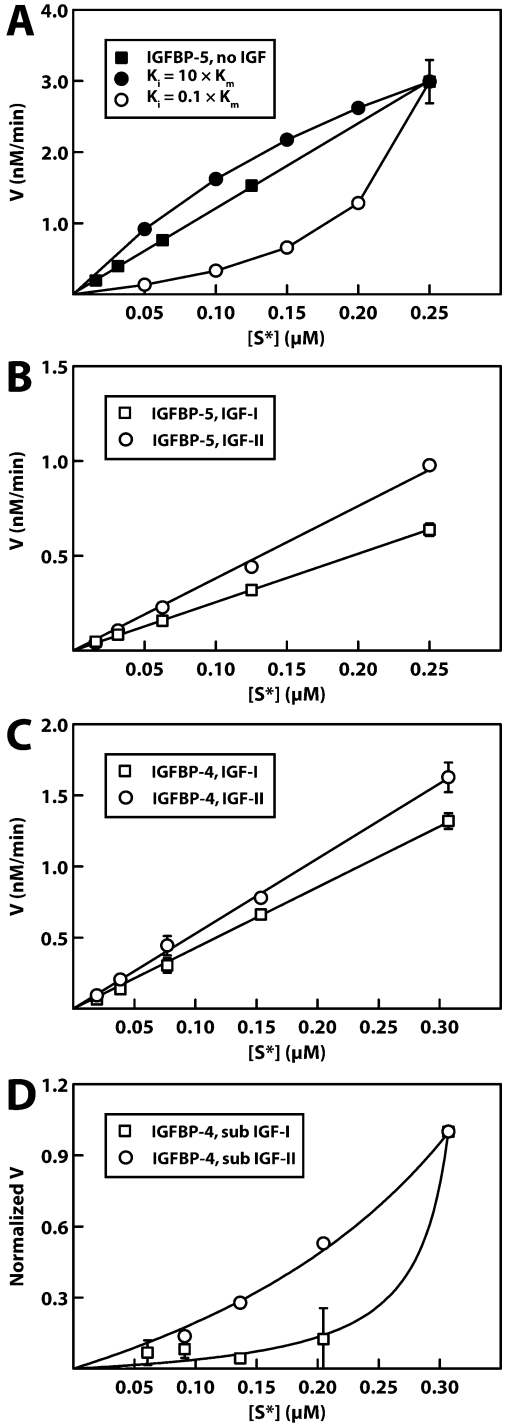

FIGURE 5: Substrate recognition or proteolysis is not affected by iodination of IGFBP-4 or -5. Potential differences between iodinated and unmodified IGFBP were evaluated. (A) IGFBP-5 was analyzed as described in Materials and Methods. Equation 6 was fitted to the data obtained with the parameters K (0.25 μM) and V_{max} (7.24 nM/min) kept constant. The kinetic constants obtained from this initial fit were used to simulate the situation in which $K_i = 0.1K_m$ and $K_i = 10K_m$. (B) Analysis of IGFBP-5 with a K of 0.25 μM and the determined V_{max} values (Table 1). (C) Analysis of IGFBP-4 with a K of 0.31 μM and the determined V_{max} values (Table 1). (D) Analysis of IGFBP-4 in the presence of nonsaturating amounts (0.1 μM) of IGF-I and -II. Standard deviations are given for all plotted curves.

data points were on a straight line through the origin with no detectable deviations from linearity.

To further characterize the effects of radiolabeling, we reasoned that if the binding of IGF to radiolabeled IGFBP-4

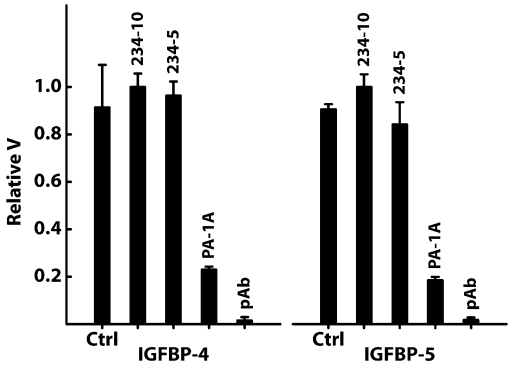

FIGURE 6: Effect of inactivating compounds on PAPP-A proteolytic activity. Initial rates of cleavage of IGFBP-4 (10 nM) and IGFBP-5 (10 nM) by PAPP-A measured in the presence of different antibodies (10 μg/mL). IGF-II (100 nM) was added to the reaction mixtures with IGFBP-4. Four antibodies were used, as indicated: mAb 234-10 (an irrelevant mouse antibody), mAb 234-5 and mAb PA-1A (mouse antibodies against PAPP-A), and pAb (polyclonal anti-PAPP-A). Reaction rates were measured in triplicate and normalized to the highest rate for IGFBP-4 and -5 separately.

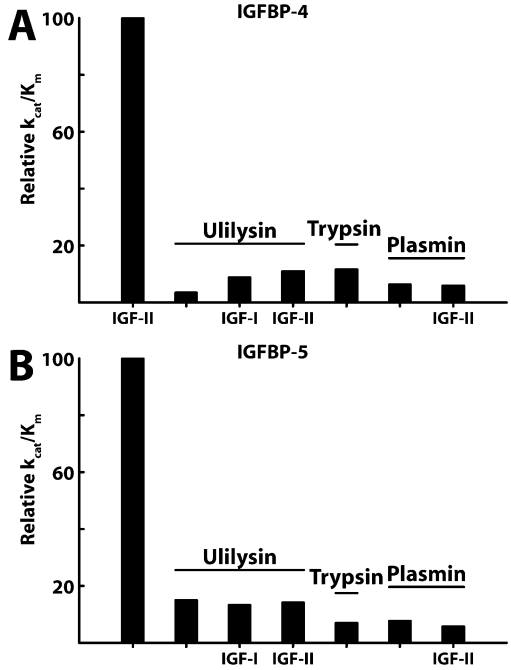

FIGURE 7: Comparison of PAPP-A with other proteinases reportedly able to cleave IGFBPs. The apparent second-order rate constant (k_{cat}/K_m) for the cleavage of IGFBP-4 (A) or IGFBP-5 (B) was determined for PAPP-A, ulilysin, trypsin, and plasmin. The analyses were performed under conditions (2 h) where only one peptide bond of each substrates appeared to be cleaved with each enzyme, as judged by SDS−PAGE analysis. After extended incubation, trypsin and plasmin cleave both substrates at more than one site. For comparison, the results are normalized to the values of PAPP-A. The presence of IGF-I or -II in some reaction mixtures is indicated.

or -5 was impaired, the addition of sub-equimolar amounts of IGF to the mixture of labeled and unlabeled IGFBP would yield a distribution of IGF between the two proteins in favor of the unlabeled IGFBP. Under conditions where IGFBP-4 was not saturated with IGF-I or -II, downward curvatures were observed for both IGF-I and -II, demonstrating a reduced level of binding to IGFBP-4 (Figure 5D). For the analysis of IGFBP-5, the curvatures were less pronounced, but there was a slight tendency toward an upward curvature, in agreement with the inhibiting effect of IGF on IGFBP-5 proteolysis, indicating a reduced level of binding of IGF to radiolabeled IGFBP-5 (data not shown).

Effect of Different Inactivating Compounds on PAPP-A Proteolytic Activity. In biological systems, inhibitors of PAPP-A activity may be present. Evaluation of such compunds is relevant and should be done according to our knowledge of the kinetic parameters to avoid misinterpretation of the results. For the routine analysis of PAPP-A activity against IGFBP-4 and -5 contained in biological samples, we suggest that substrate concentrations be kept below K_m (from 100 to 10 nM) to ensure a linear correlation with the rate of proteolysis. Furthermore, time course experiments, rather than end point assays, should always be carried out and used for comparison. In end point assays, the error levels are significantly higher when proteinases with large differences in the progress of the proteolytic reaction are compared. We analyzed the effect of selected antibodies on the proteolysis of IGFBP-4 and -5 by PAPP-A, illustrating that the effect of inhibitory compounds is readily determined using this assay (Figure 6).

PAPP-A Is a Highly Specific and Efficient Proteinase Compared to Other Proteinases Reportedly Able To Cleave IGFBPs. To evaluate the specificity and efficiency of PAPP-A against the natural substrates IGFBP-4 and -5, we compared the apparent second-order rate constants for proteolysis of these two substrates by PAPP-A, plasmin, trypsin, and ulilysin (Figure 7). Initial rates were measured at two different substrate concentrations below K_m to achieve pseudo-first-order kinetics. In contrast to common belief, we found that PAPP-A cleaves both IGFBP-4 and -5 much more efficiently than ulilysin, trypsin, and plasmin, probably by means of a higher affinity for the substrates rather than a higher k_{cat}. Interestingly, the archaebacterial enzyme uliysin, which also belongs to the family of pappalysins (*34*), shows a pattern of IGF-modulated proteolysis of IGFBP-4 with no IGF < IGF-I < IGF-II, similar to the cleavage of IGFBP-4 by PAPP-A.

DISCUSSION

By means of its proteolytic activity against the inhibitory binding proteins IGFBP-4 and -5, PAPP-A functions in vivo in complicated regulatory networks that control the availability of free IGF and ultimately the stimulation of the IGF receptor. Models of IGFBP proteolysis must take into account, for example, binding affinities between IGFs and the different IGFBPs present in the same biological system, concentrations of network components, and the balance between proteolytic enzymes and potential inhibitors. To allow the development of such models, knowledge of enzyme−substrate interactions, causing IGF release, is required. We have derived the kinetic parameters for cleavage of its natural substrates IGFBP-4 and -5 by PAPP-A in the presence and absence of their binding partners, IGF-I and -II (Table 1). This is the first quantitative characterization of an important regulatory proteinase of the IGF system and of the founding family member of the pappalysin family (*17*).

Time course experiments for the proteolysis of IGFBP-4 and -5 were performed. The two substrates are both cleaved at a single site, and the progress is linear within proteolysis of the first 20% (Figure 1). This allowed the determination of kinetic parameters by steady-state kinetic analysis. The

influence of IGF-I or -II on the rate of proteolysis was also analyzed (Figure 2). The rate of proteolysis of IGFBP-4 is dramatically increased in the presence of IGF-I or -II, presumably because IGFBP-4 undergoes a conformational change upon binding of IGF which causes it to become a much better PAPP-A substrate. However, the ability of IGF-I and -II to induce such conformational change in IGFBP-4 differs with IGF-II being a more potent activator of IGFBP-4 proteolysis than IGF-I. Kinetic analysis demonstrates that the difference in IGF-modulated proteolysis of IGFBP-4 lies in the recognition of substrate (Figure 3). These results support a model in which binding of IGF-I or -II to IGFBP-4 induces distinct conformations which are differently recognized by PAPP-A. However, although it has not been possible to demonstrate an interaction between PAPP-A and IGF in the absence of IGFBP (*19*), we cannot exclude the possibility that IGF while in complex with IGFBP-4 contributes to the binding surface recognized by active PAPP-A.

Preliminary kinetic analysis of the IGF-dependent cleavage of human IGFBP-2 by PAPP-A showed relatively slow cleavage of this substrate compared to IGFBP-4 (k_{cat}/K_m approximately 25 fold lower), and further kinetic analysis was not performed. However, the pair of PAPP-A and IGFBP-2 may still be physiologically relevant, as myoblast proliferation and differentiation have been shown to involve these two proteins (*40*).

Chemical modification of the surface of IGFBP engaged in the interaction with IGF potentially affects the recognition of IGFBP by PAPP-A. To address this question, we developed a method for the analysis of potential discrepancies between modified substrates and their unmodified counterparts based on measurement of reaction rates at different ratios of radiolabeled to unlabeled substrates. Simulations of different scenarios, in which two apparently identical substrates interact equally or differently with an enzyme, were made to evaluate the method (Figure 4). These simulations revealed that even small differences in substrate recognition would be detected by this method. Such analysis is particularly relevant for IGFBP-4, because a conserved tyrosine residue at position 49 is known to be involved in hydrogen bonding with Asp-11 of IGF-I in the published crystal structure and is likely to react with I_2 upon iodination. The effect of iodination on IGF binding was previously analyzed for bovine IGFBP-2 and was found to lower the affinity of IGF for IGFBP-2 by a factor of 8. Likewise, it was found that iodination of bovine IGFBP-2 in the presence of IGF-I or -II protects Tyr-60 from reaction with I_2 (*41*, *42*). Using the developed method, we demonstrated that the substrate properties of radiolabeled IGFBP-4 could not be distinguished from unlabeled IGFBP-4 (Figure 5). This suggested that either Tyr-49 is not involved in the binding of IGF or the rate-accelerating effect of IGF on IGFBP-4 proteolysis is not mediated through interactions with this residue. At sub-equimolar levels of IGF, cleavage analysis of IGFBP-4 showed a reduced rate of proteolysis (Figure 4D). This indicates that iodination of Tyr-49 of IGFBP-4 may inhibit the binding of IGF to IGFBP-4, while not affecting substrate modulation. The method described here can easily be employed in other systems, relevant for not only enzyme kinetic studies but also binding kinetics in general, e.g., receptor−ligand interactions. Examples of iodination causing altered properties of proteins are numerous (*43*−*45*).

Many proteolytic enzymes have been analyzed by means of low-molecular weight chromogenic substrates with the advantage of convenient detection and high sensitivity, but no such substrate has been found for PAPP-A. However, kinetic analysis using artificial substrates may not properly reflect the cleavage of natural protein substrates (*46*), in particular, for PAPP-A which appears to interact with substrate residues distant from the cleavage site (*47*). In fact, a peptide spanning the cleavage site of IGFBP-4 must include 15 amino acids on its N-terminal side to function as a PAPP-A substrate (*47*), emphasizing the relevance of analyzing physiological substrates.

Although most enzymes have been characterized using chromogenic substrates, some kinetic studies are based on natural protein substrates. For example, the metalloproteinase ADAMTS-13 cleaves a single site in the A2 domain of the von Willebrand factor (VWF) with a specificity constant of $\sim 7 \times 10^4$ M^{-1} s^{-1} (*48*), 10−30 times lower than that for cleavage of IGFBP by PAPP-A. Another example is the cleavage of collagen types I−III by human skin fibroblast collagenase (MMP-1) (*49*), for which the specificity constants range from 1.4×10^2 to 1.1×10^5 M^{-1} s^{-1}. Thus, the efficiency with which PAPP-A cleaves its natural substrates (k_{cat}/K_m ranges from 5.5×10^5 to 2.4×10^6 M^{-1} s^{-1}) is comparable to or better than those of many other physiological pairs of a proteinase and a substrate.

We directly compared the cleavage of IGFBP-4 and -5 by PAPP-A with three other proteinases (Figure 7), two of which have previously been reported to cleave the IGFBPs (*34*, *50*, *51*). Ulilysin, a metzincin metalloproteinase from *Methanosarcina acetivorans* whose sequence is homologous to that of the proteolytic domain of PAPP-A, was recently cloned and crystallized and was found to be able to cleave IGFBP-2−6 (*34*). Plasmin has been suggested as a physiological proteinase of both IGFBP-4 (*51*) and IGFBP-5 (*50*). Comparison of the second-order rate constant for proteolysis of IGFBP-4 and -5 (Figure 7) indicates that PAPP-A represents a proteolytic enzyme optimized for the cleavage of both of these substrates. Interestingly, ulilysin, which represents the prototype of a pappalysin proteolytic domain, also displays IGF-modulated proteolysis of IGFBP-4, suggesting that conserved structural features in the proteolytic domain are responsible for substrate recognition of the IGFBP-4−IGF complex.

In conclusion, analysis of the cleavage of the natural substrates IGFBP-4 and -5 by PAPP-A represents the first example of kinetic analysis within the pappalysin family of metalloproteinases. Efficient and specific substrate cleavage suggests that PAPP-A is optimized for the cleavage of these substrates and further substantiates the roles of PAPP-A in the IGF system.

ACKNOWLEDGMENT

We thank F. Xavier Gomis-Rüth for providing ulilysin and S. Fedosov for helpful discussion.

REFERENCES

1. Jones, J. I., and Clemmons, D. R. (1995) Insulin-like growth factors and their binding proteins: Biological actions, *Endocr. Rev. 16*, 3−34.

2. Nakae, J., Kido, Y., and Accili, D. (2001) Distinct and overlapping functions of insulin and IGF-I receptors, *Endocr. Rev. 22*, 818−835.
3. Wong, M. S., Fong, C. C., and Yang, M. (1999) Biosensor measurement of the interaction kinetics between insulin-like growth factors and their binding proteins, *Biochim. Biophys. Acta 1432*, 293−301.
4. Forbes, B. E., Hartfield, P. J., McNeil, K. A., Surinya, K. H., Milner, S. J., Cosgrove, L. J., and Wallace, J. C. (2002) Characteristics of binding of insulin-like growth factor (IGF)-I and IGF-II analogues to the type 1 IGF receptor determined by BIAcore analysis, *Eur. J. Biochem. 269*, 961−968.
5. Sitar, T., Popowicz, G. M., Siwanowicz, I., Huber, R., and Holak, T. A. (2006) Structural basis for the inhibition of insulin-like growth factors by insulin-like growth factor-binding proteins, *Proc. Natl. Acad. Sci. U.S.A. 103*, 13028−13033.
6. LeRoith, D., and Roberts, C. T., Jr. (2003) The insulin-like growth factor system and cancer, *Cancer Lett. 195*, 127−137.
7. Pollak, M. N., Schernhammer, E. S., and Hankinson, S. E. (2004) Insulin-like growth factors and neoplasia, *Nat. Rev. Cancer 4*, 505−518.
8. Bayes-Genis, A., Conover, C. A., and Schwartz, R. S. (2000) The insulin-like growth factor axis: A review of atherosclerosis and restenosis, *Circ. Res. 86*, 125−130.
9. Delafontaine, P., Song, Y. H., and Li, Y. (2004) Expression, regulation, and function of IGF-1, IGF-1R, and IGF-1 binding proteins in blood vessels, *Arterioscler. Thromb. Vasc. Biol. 24*, 435−444.
10. De Meyts, P., and Whittaker, J. (2002) Structural biology of insulin and IGF1 receptors: Implications for drug design, *Nat. Rev. Drug Discovery 1*, 769−783.
11. Surmacz, E. (2003) Growth factor receptors as therapeutic targets: Strategies to inhibit the insulin-like growth factor I receptor, *Oncogene 22*, 6589−6597.
12. Kristensen, T., Oxvig, C., Sand, O., Moller, N. P., and Sottrup-Jensen, L. (1994) Amino acid sequence of human pregnancy-associated plasma protein-A derived from cloned cDNA, *Biochemistry 33*, 1592−1598.
13. Bode, W., Gomis-Ruth, F. X., and Stockler, W. (1993) Astacins, serralysins, snake venom and matrix metalloproteinases exhibit identical zinc-binding environments (HEXXHXXGXXH and Met-turn) and topologies and should be grouped into a common family, the 'metzincins', *FEBS Lett. 331*, 134−140.
14. Gomis-Ruth, F. X. (2003) Structural aspects of the metzincin clan of metalloendopeptidases, *Mol. Biotechnol. 24*, 157−202.
15. Stocker, W., Grams, F., Baumann, U., Reinemer, P., Gomis-Ruth, F. X., McKay, D. B., and Bode, W. (1995) The metzincins: Topological and sequential relations between the astacins, adamalysins, serralysins, and matrixins (collagenases) define a superfamily of zinc-peptidases, *Protein Sci. 4*, 823−840.
16. Boldt, H. B., Overgaard, M. T., Laursen, L. S., Weyer, K., Sottrup-Jensen, L., and Oxvig, C. (2001) Mutational analysis of the proteolytic domain of pregnancy-associated plasma protein-A (PAPP-A): Classification as a metzincin, *Biochem. J. 358*, 359−367.
17. Oxvig, C., Overgaard, M. T., and Sottrup-Jensen, L. (2004) Pappalysin-1 (pregnancy-associated plasma protein-A), in *Handbook of Proteolytic Enzymes* (Barrett, A. J., Rawlings, N. D., and Woessner, J. F., Eds.) pp 754−757, Academic Press, London.
18. Lawrence, J. B., Oxvig, C., Overgaard, M. T., Sottrup-Jensen, L., Gleich, G. J., Hays, L. G., Yates, J. R., III, and Conover, C. A. (1999) The insulin-like growth factor (IGF)-dependent IGF binding protein-4 protease secreted by human fibroblasts is pregnancy-associated plasma protein-A, *Proc. Natl. Acad. Sci. U.S.A. 96*, 3149−3153.
19. Laursen, L. S., Overgaard, M. T., Soe, R., Boldt, H. B., Sottrup-Jensen, L., Giudice, L. C., Conover, C. A., and Oxvig, C. (2001) Pregnancy-associated plasma protein-A (PAPP-A) cleaves insulin-like growth factor binding protein (IGFBP)-5 independent of IGF: Implications for the mechanism of IGFBP-4 proteolysis by PAPP-A, *FEBS Lett. 504*, 36−40.
20. Monget, P., Mazerbourg, S., Delpuech, T., Maurel, M. C., Maniere, S., Zapf, J., Lalmanach, G., Oxvig, C., and Overgaard, M. T. (2003) Pregnancy-associated plasma protein-A is involved in insulin-like growth factor binding protein-2 (IGFBP-2) proteolytic degradation in bovine and porcine preovulatory follicles: Identification of cleavage site and characterization of IGFBP-2 degradation, *Biol. Reprod. 68*, 77−86.
21. Qin, Q. P., Christiansen, M., Oxvig, C., Pettersson, K., Sottrup-Jensen, L., Koch, C., and Norgaard-Pedersen, B. (1997) Double-monoclonal immunofluorometric assays for pregnancy-associated plasma protein A/proeosinophil major basic protein (PAPP-A/proMBP) complex in first-trimester maternal serum screening for Down syndrome, *Clin. Chem. 43*, 2323−2332.
22. Oxvig, C., Sand, O., Kristensen, T., Gleich, G. J., and Sottrup-Jensen, L. (1993) Circulating human pregnancy-associated plasma protein-A is disulfide-bridged to the proform of eosinophil major basic protein, *J. Biol. Chem. 268*, 12243−12246.
23. Overgaard, M. T., Haaning, J., Boldt, H. B., Olsen, I. M., Laursen, L. S., Christiansen, M., Gleich, G. J., Sottrup-Jensen, L., Conover, C. A., and Oxvig, C. (2000) Expression of recombinant human pregnancy-associated plasma protein-A and identification of the proform of eosinophil major basic protein as its physiological inhibitor, *J. Biol. Chem. 275*, 31128−31133.
24. Bayes-Genis, A., Conover, C. A., Overgaard, M. T., Bailey, K. R., Christiansen, M., Holmes, D. R., Jr., Virmani, R., Oxvig, C., and Schwartz, R. S. (2001) Pregnancy-associated plasma protein A as a marker of acute coronary syndromes, *N. Engl. J. Med. 345*, 1022−1029.
25. Conover, C. A., Faessen, G. F., Ilg, K. E., Chandrasekher, Y. A., Christiansen, M., Overgaard, M. T., Oxvig, C., and Giudice, L. C. (2001) Pregnancy-associated plasma protein-a is the insulin-like growth factor binding protein-4 protease secreted by human ovarian granulosa cells and is a marker of dominant follicle selection and the corpus luteum, *Endocrinology 142*, 2155.
26. Chen, B. K., Leiferman, K. M., Pittelkow, M. R., Overgaard, M. T., Oxvig, C., and Conover, C. A. (2003) Localization and regulation of pregnancy-associated plasma protein a expression in healing human skin, *J. Clin. Endocrinol. Metab. 88*, 4465−4471.
27. Ghosh, M., Shanker, S., Siwanowicz, I., Mann, K., Machleidt, W., and Holak, T. A. (2005) Proteolysis of insulin-like growth factor binding proteins (IGFBPs) by calpain, *Biol. Chem. 386*, 85−93.
28. Nakamura, M., Miyamoto, S., Maeda, H., Ishii, G., Hasebe, T., Chiba, T., Asaka, M., and Ochiai, A. (2005) Matrix metalloproteinase-7 degrades all insulin-like growth factor binding proteins and facilitates insulin-like growth factor bioavailability, *Biochem. Biophys. Res. Commun. 333*, 1011−1016.
29. Overgaard, M. T., Boldt, H. B., Laursen, L. S., Sottrup-Jensen, L., Conover, C. A., and Oxvig, C. (2001) Pregnancy-associated plasma protein-A2 (PAPP-A2), a novel insulin-like growth factor-binding protein-5 proteinase, *J. Biol. Chem. 276*, 21849−21853.
30. Busby, W. H., Jr., Nam, T. J., Moralez, A., Smith, C., Jennings, M., and Clemmons, D. R. (2000) The complement component C1s is the protease that accounts for cleavage of insulin-like growth factor-binding protein-5 in fibroblast medium, *J. Biol. Chem. 275*, 37638−37644.
31. Zheng, B., Clarke, J. B., Busby, W. H., Duan, C., and Clemmons, D. R. (1998) Insulin-like growth factor-binding protein-5 is cleaved by physiological concentrations of thrombin, *Endocrinology 139*, 1708−1714.
32. Sottrup-Jensen, L. (1993) Determination of halfcystine in proteins as cysteine from reducing hydrolyzates, *Biochem. Mol. Biol. Int. 30*, 789−794.
33. Hunter, W. M., and Greenwood, F. C. (1962) Preparation of iodine-131 labelled human growth hormone of high specific activity, *Nature 194*, 495−496.
34. Tallant, C., Garcia-Castellanos, R., Seco, J., Baumann, U., and Gomis-Ruth, F. X. (2006) Molecular analysis of ulilysin, the structural prototype of a new family of metzincin metalloproteases, *J. Biol. Chem. 281*, 17920−17928.
35. Oxvig, C., Sand, O., Kristensen, T., Kristensen, L., and Sottrup-Jensen, L. (1994) Isolation and characterization of circulating complex between human pregnancy-associated plasma protein-A and proform of eosinophil major basic protein, *Biochim. Biophys. Acta 1201*, 415−423.
36. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature 227*, 680−685.
37. Siwanowicz, I., Popowicz, G. M., Wisniewska, M., Huber, R., Kuenkele, K. P., Lang, K., Engh, R. A., and Holak, T. A. (2005) Structural basis for the regulation of insulin-like growth factors by IGF binding proteins, *Structure 13*, 155−167.
38. Zeslawski, W., Beisel, H. G., Kamionka, M., Kalus, W., Engh, R. A., Huber, R., Lang, K., and Holak, T. A. (2001) The

interaction of insulin-like growth factor-I with the N-terminal domain of IGFBP-5, *EMBO J. 20*, 3638−3644.
39. Qin, X., Byun, D., Lau, K. H., Baylink, D. J., and Mohan, S. (2000) Evidence that the interaction between insulin-like growth factor (IGF)-II and IGF binding protein (IGFBP)-4 is essential for the action of the IGF-II-dependent IGFBP-4 protease, *Arch. Biochem. Biophys. 379*, 209−216.
40. Kumar, A., Mohan, S., Newton, J., Rehage, M., Tran, K., Baylink, D. J., and Qin, X. (2005) Pregnancy-associated plasma protein-A regulates myoblast proliferation and differentiation through an insulin-like growth factor-dependent mechanism, *J. Biol. Chem. 280*, 37782−37789.
41. Hobba, G. D., Lothgren, A., Holmberg, E., Forbes, B. E., Francis, G. L., and Wallace, J. C. (1998) Alanine screening mutagenesis establishes tyrosine 60 of bovine insulin-like growth factor binding protein-2 as a determinant of insulin-like growth factor binding, *J. Biol. Chem. 273*, 19691−19698.
42. Hobba, G. D., Forbes, B. E., Parkinson, E. J., Francis, G. L., and Wallace, J. C. (1996) The insulin-like growth factor (IGF) binding site of bovine insulin-like growth factor binding protein-2 (bIGFBP-2) probed by iodination, *J. Biol. Chem. 271*, 30529−30536.
43. Fuchs, H., and Gessner, R. (2002) Iodination significantly influences the binding of human transferrin to the transferrin receptor, *Biochim. Biophys. Acta 1570*, 19−26.
44. Kuo, B. S., Nordblom, G. D., and Wright, D. S. (1997) Perturbation of epidermal growth factor clearance after radioiodination and its implications, *J. Pharm. Sci. 86*, 290−296.
45. Lee, P. H., Nguyen, T. M., Chung, N. N., Schiller, P. W., and Chang, K. J. (1995) Tyrosine-iodination converts the *δ*-opioid peptide antagonist TIPP to an agonist, *Eur. J. Pharmacol. 280*, 211−214.
46. Inoue, I., Rohrwasser, A., Helin, C., Jeunemaitre, X., Crain, P., Bohlender, J., Lifton, R. P., Corvol, P., Ward, K., and Lalouel, J. M. (1995) A mutation of angiotensinogen in a patient with preeclampsia leads to altered kinetics of the renin-angiotensin system, *J. Biol. Chem. 270*, 11430−11436.
47. Laursen, L. S., Overgaard, M. T., Nielsen, C. G., Boldt, H. B., Hopmann, K. H., Conover, C. A., Sottrup-Jensen, L., Giudice, L. C., and Oxvig, C. (2002) Substrate specificity of the metalloproteinase pregnancy-associated plasma protein-A (PAPP-A) assessed by mutagenesis and analysis of synthetic peptides: Substrate residues distant from the scissile bond are critical for proteolysis, *Biochem. J. 367*, 31−40.
48. Zanardelli, S., Crawley, J. T., Chion, C. K., Lam, J. K., Preston, R. J., and Lane, D. A. (2006) ADAMTS13 substrate recognition of von Willebrand factor A2 domain, *J. Biol. Chem. 281*, 1555−1563.
49. Welgus, H. G., Jeffrey, J. J., and Eisen, A. Z. (1981) The collagen substrate specificity of human skin fibroblast collagenase, *J. Biol. Chem. 256*, 9511−9515.
50. Campbell, P. G., and Andress, D. L. (1997) Plasmin degradation of insulin-like growth factor-binding protein-5 (IGFBP-5): Regulation by IGFBP-5-(201−218), *Am. J. Physiol. 273*, E996−E1004.
51. Remacle-Bonnet, M. M., Garrouste, F. L., and Pommier, G. J. (1997) Surface-bound plasmin induces selective proteolysis of insulin-like-growth-factor (IGF)-binding protein-4 (IGFBP-4) and promotes autocrine IGF-II bio-availability in human colon-carcinoma cells, *Int. J. Cancer 72*, 835−843.

BI062229I