ADAM-9 Is an Insulin-like Growth Factor Binding Protein-5 Protease Produced and Secreted by Human Osteoblasts†

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ABSTRACT: IGF binding protein-5 (BP-5) is an important bone formation regulator. Therefore, elucidation of the identity of IGF binding protein-5 (BP-5) protease produced by osteoblasts is important for our understanding of the molecular pathways that control the action of BP-5. In this regard, BP-5 protease purified by various chromatographic steps from a conditioned medium of U2 human osteosarcoma cells migrated as a single major band, which comigrated with the protease activity in native PAGE and yielded multiple bands in SDS−PAGE under reducing conditions. N-Terminal sequencing of these bands revealed that three of the bands yielded amino acid sequences that were identical to that of α2 macroglobulin (α2M). Although α2M was produced by human osteoblasts (OBs), it was not found to be a BP-5 protease. Because α2M had been shown to complex with ADAM proteases and because ADAM-12 was found to cleave BP-3 and BP-5, we evaluated if one of the members of ADAM family was the BP-5 protease. On the basis of the findings that (1) purified preparations of BP-5 protease from U2 cell CM contained ADAM-9, (2) ADAM-9 is produced and secreted in high abundance by various human OB cell types, (3) purified ADAM-9 cleaved BP-5 effectively while it did not cleave other IGFBPs or did so with less potency, and (4) purified ADAM-9 bound to α2M, we conclude that ADAM-9 is a BP-5 protease produced by human OBs.

Insulin-like growth factors (IGFs)[1] I and II are growth factors which have both mitogenic and metabolic actions which participate in the growth, survival, and differentiation of a number of cell types and tissues (*1*, *2*). IGFs are unique among growth factors in that they can act both systemically as a hormone and locally as an autocrine/paracrine factor (*1*−*4*). Although liver-derived IGF-I contributes to the main circulating source of IGF-I, it is now known that many tissues, including brain, muscle, and bone, produce IGF-I (*1*−*5*). The importance of IGFs in the growth and maintenance of various tissues is evident from studies using mice lacking functional IGFs or their signaling type I IGF receptor (*6*−*8*). For example, the bone density of mice lacking functional IGF-I is reduced by 60% compared to corresponding control mice, thus providing direct evidence for a role for IGF-I in regulating bone density (*8*).

The functions of IGFs depend not only on the amount of IGF produced but also on the level of IGF binding proteins (IGFBPs). IGFBPs exert the traditional functions of binding proteins whereby they modulate the half-life and activity of IGFs (*9*, *10*). In addition, some of the IGFBPs have been shown to act via mechanisms independent of IGFs (*11*−*13*). Of the six high-affinity IGFBPs produced by osteoblasts, IGFBP-5 has several distinct features that suggest it is a key component of the IGF system in bone: (1) IGFBP-5 is the most abundant IGFBP stored in bone where it is bound to hydroxyapatite and extracellular matrix proteins, binding which provides a mechanism to fix IGFs in bone for subsequent release in a regulatable manner (*14*, *15*); (2) IGFBP-5 has consistently been shown to stimulate bone formation parameters in vitro and in vivo (*16*−*18*); (3) IGFBP-5 shows considerable changes in clinical disease states and correlates with changes in bone formation (*3*, *19*−*21*); and (4) IGFBP-5 can also function as a growth factor in addition to its role as a traditional binding protein as evident from recent studies using IGF-I knockout mice (*13*, *22*).

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[1] Abbreviations: ADAM, a disintegrin and metalloprotease; α2M, α2 macroglobulin; CM, conditioned medium; FPLC, fast protein liquid chromatography; IGF, insulin-like growth factor; IGFBP-5, IGF binding protein-5; MMP, matrix metalloprotease; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

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Past studies in our laboratories and others have shown that the rate of IGFBP-5 degradation by protease is one of the key control mechanisms regulating the effective concentration of IGFBP-5 in local body fluids (*23*−*25*). In this regard, we have previously found that human osteoblasts in culture produce an IGFBP-5-specific protease which is subject to regulation by key osteoregulatory agents (*26*, *27*). Although IGFBP-5 has been shown to be cleaved by MMP-2 produced by mouse osteoblasts (*28*), the IGFBP-5-specific protease produced by human osteoblasts is not MMP-2 since this protease degrades multiple IGFBPs. Recent studies have also shown that complement component C1s produced by smooth muscle cells and the pregnancy-associated plasma protein-A2 found in pregnancy serum cleave IGFBP-5 specifically (*29*, *30*). However, on the basis of the fragmentation patterns, cleavage site, and inhibitor profile, the IGFBP-5-specific protease produced by human osteoblasts appears to be different from those of previously known proteases capable of degrading IGFBP-5 (*23*, *26*, *27*). Therefore, we undertook studies to purify and characterize the IGFBP-5-specific protease present in the CM of human osteoblasts. In these studies, it was found that ADAM-9, a disintegrin and metalloprotease, is an IGFBP-5 protease produced and secreted by human osteoblasts in culture.

MATERIALS AND METHODS

Materials. Purified α2M and antibody to α2M were purchased from Sigma Chemicals (St. Louis, MO). Native α2M purified from plasma was a kind gift from Dr. Steven L. Gonias (Charlottesville, VA). Anti-human ADAM-9 antibodies raised in rabbits were purchased from R&D Systems (Stillwater, MN). Anti-human ADAM-10 and ADAM-17 antibodies raised in rabbits were purchased from Chemicon International, Inc. (Temecula, CA). Rabbit anti-ADAM-12 polyclonal antibody was a kind gift from Dr. Ulla M. Wewer (Copenhagen, Denmark). Recombinant human IGFBP-2 and IGFBP-6 were purchased from Austral Biologicals (San Ramon, CA) and GroPep (Adelaide, Australia), respectively. Recombinant human IGFBP-3 was a kind gift from Dr. A. Sommers (Celtrix, CA). Recombinant human IGFBP-4 was expressed in *Escherichia coli* and purified as described previously (*31*). Recombinant human IGFBP-5 was a kind gift from Drs. Carola Dony and Kurt Lang (Roche Pharmaceuticals, Penzberg, Germany).

Cell Culture. U2 (human oseosarcoma), H-35 (rat hepatoma), and HEPG2 (human hepatoma) cells were purchased from the American Type Culture Collection (Rockville, MD). Untransformed normal human osteoblasts derived from calvaria, vertebra, and rib (*32*) were a generous gift of Dr. Thomas A. Linkhart (Loma Linda, CA). The cells were grown to confluence in 10 cm culture dishes (Falcon Labware, Fairfield, NJ) at 37 °C in Dulbecco's minimum essential medium (Mediatech, Inc., Herndon, VA) supplemented with 10% bovine serum (Hyclone, Logan, UT). Medium was changed to serum-free DMEM at approximately 80% confluence by removing serum-containing medium, rinsing cells with phosphate-buffered saline (PBS), and replacing with serum-free DMEM. After 4 h, medium was removed, and fresh serum-free DMEM was added. After 48 h incubation, CM was aspirated, and fresh medium was added for subsequent collection. No more than three CM collections were performed per dish. CM was filtered to remove cellular debris and stored frozen at −70 °C until use.

Purification of IGFBP-5 Protease. CM (4 L) from U2 cells which produce abundant IGFBP-5 protease (*23*) was concentrated using an Amicon ultrafiltration system with a YM10 membrane (10 kDa) and subjected to ammonium sulfate precipitation (60% saturation). The protein precipitate was resuspended in 35 mL of 10 mM HEPES buffer, pH 7.4, 30% saturated with ammonium sulfate, and applied to a phenyl-Sepharose column (Pharmacia, Piscataway, NJ). The flow-through was applied once more to the column to maximize recovery. Proteins were eluted in stepwise elutions of decreasing ammonium sulfate concentration (30%, 20%, 10%, 0%) with a minimum of 10 column volumes for each elution. The strongly bound proteins containing IGFBP-5 protease activity were subsequently eluted with approximately 50 column volumes of 10 mM HEPES containing 0.1% Triton X-100. The active fractions containing IGFBP-5 protease activity were pooled and incubated with immobilized iminodiacetic acid−agarose (Pierce, Rockford, IL) pretreated with $ZnCl_2$ according to manufacturer's instructions. After overnight incubation at 4 °C, the slurry was poured into a column, and unbound proteins were eluted by washing the beads with 10 column volumes of 10 mM HEPES, 50 mM NaCl, and 0.01% Brij 35, pH 7.4 (buffer A). The bound proteins were eluted with 5 column volumes of buffer A containing 20 mM EDTA and 5 column volumes of buffer A containing 50 mM EDTA. The 20 mM EDTA fractions containing IGFBP-5 protease were pooled, concentrated using a Centricon centrifugal filtration device with a 3 kDa cutoff membrane, and applied to Superose-6 gel filtration column using the fast protein liquid chromatography (FPLC) system (Pharmacia, Piscataway, NJ). Proteins were eluted by 10 mM HEPES buffer at a flow rate of 0.5 mL/min. An aliquot of each fraction was assayed for IGFBP-5 protease activity, and the active fractions were used for determination of purity.

Gel Electrophoresis. IGFBP-5 protease purified by the above-mentioned chromatographic steps was applied to native PAGE using a 6% gel in duplicate lanes. One lane was used for the visualization of proteins by silver staining. The second lane was used for extracting bands that corresponded to stained bands in order to assay for protease activity. The unstained protein bands were excised from the gel, diced using a razor blade, and transferred to an Eppendorf microfuge tube. Proteins were extracted in 50−200 μL of 10 mM HEPES and 0.1% Triton X-100, pH 7.4, depending on the size of the gel slice, using a manual homogenizer. Quickly freezing the samples at −70 °C for 30 min and then rehomogenizing the gel mush increased protein recovery. Samples were then centrifuged for 10 min at 10000*g* to pellet the gel; the supernatant containing the protein was recovered for each gel slice and used for protease assay.

The purified IGFBP-5 protease fraction was also applied onto SDS−polyacrylamide gel (6%) in the presence of β-mercaptoethanol (3%) and electrophoresed as described previously (*33*). Appropriate protein markers were run in a parallel lane to determine the molecular weight. The proteins were then transferred to a ProBlott sequencing membrane in Tris−glycine buffer with 10% methanol for 1.5 h at 20 V

at 4 °C. After several washes of the membrane with doubly distilled water to remove residual glycine, the membrane was stained with 0.25% Coomassie Blue in 40% methanol and 10% acetic acid. The stained bands were excised from the membrane and subjected to N-terminal amino acid sequence analysis.

Amino Acid Sequence Analysis. Sequencing analysis was performed using an Applied Biosystems Model 470A vapor phase sequencer (Protein Micro Analytical Laboratory, Beckman Institute, California Institute of Technology, Pasadena, CA) as described previously (*34*) and in http://www.its.caltech.edu/~ppmal/. The protein sequences were compared with existing protein sequences using the "tblastn" program of advanced BLAST search, which compares the protein query sequence against the nucleotide sequence database dynamically translated into all reading frames.

RT−PCR of ADAM cDNA Fragments. Total RNA isolated from U2 cells using the Trizol reagent (GIBCO) was used as a template for reverse transcription/polymerase chain reaction (RT−PCR). The first strand cDNA synthesis was prepared using oligo(dT) primer as outlined in the cDNA cycle kit for the RT−PCR user manual (Invitrogen). The first cDNA strand synthesized was used as a template for amplifying cDNA fragments using specific gene primers for metalloproteases as recommended in the cDNA cycle kit manual. For 620 bp ADAM-9 cDNA amplification, the forward primer, 5′ GCTGTCTTGCCACAGACCCG 3′, and the reverse primer, 5′ ATAGGCTTCATCAGGCTTTGGAA 3′, were used. For 566 bp ADAM-10 cDNA amplification, a pair of primers was purchased from R&D Systems Inc. For 829 bp ADAM-12 cDNA amplification, the forward primer, 5′ GTAGCCACACCAGGATAGAG 3′, and the reverse primer, 5′ CCAGAAGACAGAGGCATCAT 3′, were used. For 784 bp ADAM-17 cDNA amplification, the forward primer, 5′ GCAGCTGGAGTCCTGTGCATGTAAT 3′, and the reverse primer, 5′ GACCAGCATCTGCTAAGTCA 3′, were used. For 994 bp ADAM-TS1 cDNA amplification, the forward primer, 5′ AGCGTATCTTGCCAGTAACC 3′, and the reverse primer, 5′ ACCTGCTACCCCTCAGCAGC 3′, were used. The PCR products obtained were purified and confirmed by sequencing and were then used as probes for Northern analysis.

Northern Blot Analysis. Total RNA from untransformed normal human osteoblasts as well as transformed human osteosarcoma cell lines was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA). Total RNA (10−20 μg) was loaded on a 1.2% agarose gel, and the gel was blotted using standard techniques after electrophoresis (*35*). The cDNA fragments for ADAM-9, -10, -12, and -17 and ADAM-TS1 were randomly ^{32}P-labeled using a commercial kit (BioLabs, New England) for Northern hybridization. Probes were hybridized at 42 °C in the presence of 50% formamide using standard protocols.

Preparation of CM and Cell Extracts for Immunoblotting. Human osteosarcoma cells and normal human osteoblasts were cultured in 12 cm culture dishes as described previously (*36*). Up on 70−80% confluency, serum-free media were added and incubated for 48 h for CM collection (*36*). After the media were removed, cells were rinsed with PBS and scraped off the plate with lysis buffer (1% Triton X-100, 1 mM EDTA in PBS containing protease inhibitors). After homogenization in a polytron homogenizer, the cell extracts were centrifuged for 30 min at 27000*g* at 4 °C. The cell lysates were incubated with concanavalin A−Sepharose (100 μL/mL of extract) for 1 h at 4 °C to enrich for glycoproteins. After three washes with lysis buffer, the bound glycoproteins were eluted with 200 μL of 1× electrophoresis buffer and used for immunoblotting. Culture media samples were concentrated 10-fold using Centricon with 10000 molecular weight cutoff and used for immunoblotting.

Western Immunoblotting. Concentrated CM samples, cell lysates, or purified proteins were loaded onto 10% SDS−polyacrylamide gels and electrophoresed as described previously (*37*). Appropriate protein markers were run in parallel lanes to determine the molecular weight. The proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH), blocked for 1 h with 5% nonfat dry milk, and incubated with appropriate dilution of antiserum to ADAM protease or α2M. After extensive washing with PBS to remove unbound antibodies, membranes were incubated with horseradish peroxidase conjugated anti-rabbit or anti-goat IgG (1:1000 dilution; Zymed Laboratories, San Francisco, CA). Antigen−antibody reactions were visualized using ECL chemiluminescence reagents as recommended by the manufacturer (Amersham Life Sciences, Arlington Heights, IL).

Recombinant Expression and Purification of ADAM-9. *Pichia pastoris* clones containing a pPICZα expression vector (*38*) with a cDNA insert encoding protease and metalloprotease domains (corresponding to residues 30−412) were used for expression of ADAM-9 protein. To express ADAM-9, *P. pastoris* transformants containing the ADAM-9 insert (*38*) were cultured in 500 mL of medium (BMGY) containing 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, and 1% glycerol for 24 h to an OD_{578} of 2−5 at 28 °C. The cells were collected and resuspended in 250 mL of BMGY medium containing 0.5% methanol instead of glycerol. Expression was carried out for 3−4 days at 28 °C with vigorous shaking, and culture medium was collected and used for ADAM-9 purification. Briefly, culture medium was centrifuged at 8000*g*, and the supernatant was dialyzed against 50 mM Tris-HCl buffer (pH 8.5) and incubated with Q-Sepharose Fast Flow prequilibrated with the same buffer. After the column was washed with 3 bed volumes of 50 mM Tris-HCl, pH 8.5, the bound proteins were eluted with a stepwise increase in NaCl concentration from 200 to 700 mM. An aliquot of each fraction was used for evaluation of protein concentration (*39*) and IGFBP-5 protease activity (*40*). The purity of ADAM-9 in the purified fraction was evaluated by SDS−PAGE followed by sliver staining and Western immunoblotting using ADAM-9 antibodies.

IGFBP-5 Protease Assay. Fractions from various chromatographic columns were assessed for IGFBP-5 protease activity by incubating a 1−10 μL sample with 200−500 ng of recombinant human IGFBP-5 for 4−16 h at 37 °C in 10 mM HEPES, pH 7.4. After incubation, 5 μL of 5× sample loading buffer with 1% SDS and β-mercaptoethanol was added to the samples, and the resultant sample was subjected to SDS−PAGE followed by silver staining. Proteolytic activity was determined by the disappearance of the intact IGFBP-5 protein band (29 kDa) and the appearance of smaller molecular weight IGFBP-5 fragments. In an alternate assay, the products of a reaction were analyzed by SDS−

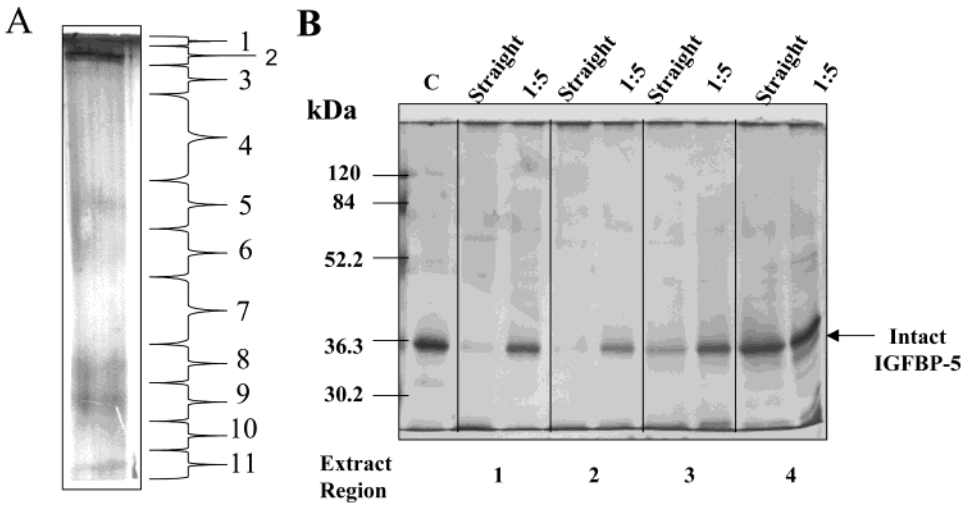

FIGURE 1: Protein (panel A) and activity (panel B) of the purified IGFBP-5 protease preparation from a representative native PAGE. IGFBP-5 protease purified as described in Materials and Methods was subjected to native 6% PAGE under nonreducing conditions and subjected to silver stain (A) or activity determination. Regions of corresponding unstained duplicate gel slices were excised as shown in panel A and extracted with 100 μL of HEPES and 0.1% Triton X-100, pH 7.4. 20 μL of neat or one-fifth diluted gel extracts were incubated with 500 ng of IGFBP-5 for 2 h at 37 °C. Intact IGFBP-5 was separated from IGFBP-5 fragments by SDS−PAGE and visualized by silver stain. "C" denotes IGFBP-5 control in which IGFBP-5 was incubated with buffer. Gel region 2 which contained the major protein band contained most of the activity. Gel regions 5−11 did not contain measurable IGFBP-5 protease activity (data not shown).

PAGE under nonreducing conditions with immunoblotting using specific anti-IGFBP-5 antiserum as described (*40*, *41*). The specificity of IGFBP-5 protease was evaluated by incubating the purified protease with other IGFBPs.

RESULTS

Purification of IGFBP-5 Protease. IGFBP-5 protease is produced by untransformed normal human osteoblasts and several human osteosarcoma cell lines. We chose to use the human U2 osteosarcoma cell line as the source for the purification of IGFBP-5 protease, as it is considerably easier to grow large numbers of U2 osteosarcoma cells than human osteoblasts for CM collection. This approach was justified by preliminary studies that revealed that IGFBP-5 protease produced by U2 cells is similar to that of normal human osteoblasts (*27*). We optimized a purification scheme (data not shown) for the isolation of the IGFBP-5-specific protease based, in part, on the findings from the inhibitor profile, as well as other empirically determined properties such as strong hydrophobicity and large molecular mass under native conditions. The first step involved concentration of IGFBP-5 protease by ammonium sulfate precipitation. Because a high concentration (60% saturation) of ammonium sulfate was required to precipitate the majority of IGFBP-5 protease activity (data not shown), it was determined that the protease was relatively hydrophobic. This observation led to the use of hydrophobic interaction chromatography, in which ammonium sulfate precipitated proteins were resuspended in 30% ammonium sulfate, added to phenyl-Sepharose beads, and eluted stepwise using 30%, 20%, 10%, and 0% ammonium sulfate. Little or no activity eluted in the ammonium sulfate eluted fractions. The majority of activity eluted when the beads were washed with 10 mM HEPES buffer, pH 7.4, in the presence of nonionic detergent 0.1% Triton X-100 (data not shown). The fraction containing protease activity was directly added to Zn^{2+}-conjugated IDA agarose beads, since we have found that Zn^{2+} inhibited IGFBP-5 proteolysis. After the unbound proteins were washed with 10 mM HEPES, the bound IGFBP-5 protease was eluted with 20 mM EDTA and used for a final purification step using a FPLC Superose-6 gel filtration column. Under these conditions, IGFBP-5 protease activity comigrated with the major protein peak and eluted in the void volume as a high molecular weight protein (data not shown).

Native PAGE. To determine the purity of the IGFBP-5 protease and to evaluate if the protease comigrated with the major protein band, Superose-6-purified protease was subjected to duplicate lanes of a 6% native polyacrylamide gel. One lane was stained with silver to visualize the proteins. The corresponding unstained lane was cut into slices, and the proteins were extracted and used to assay for protease activity. Figure 1A shows a single major band of high molecular weight which migrated very little into the native gel, a finding consistent with the gel filtration data. Figure 1B shows that the majority of IGFBP-5 protease activity was recovered in the top two gel slices. No measurable IGFBP-5 protease activity was seen in gel slices 5−11 (data not shown). These data suggested that the BP-5 protease comigrated with the major protein band in native PAGE.

N-Terminal Amino Acid Sequencing. Because many of the known proteases are much smaller than 100 kDa (*42*−*44*), we considered the possibility that IGFBP-5 protease may form a complex with other protein(s) and exist as a high molecular weight complex under native conditions. Therefore, we subjected the purified protease fraction to SDS−PAGE in the presence of a reducing agent (β-mercaptoethanol). The proteins were then transferred to ProBlott membrane and stained briefly with Coomassie Blue R-250. Figure 2 shows that the high molecular weight band, observed under native conditions, resolved into six bands under reducing conditions in the presence of SDS. N-Terminal sequencing of each of the bands revealed that three of the bands (180, 120, and 90 kDa) yielded amino acid sequences that were identical to the amino-terminal sequence of α2M, while the remaining three bands did not yield

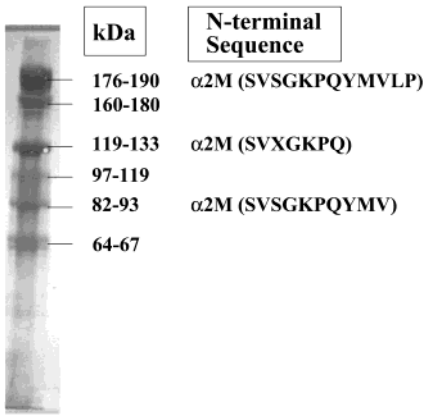

FIGURE 2: SDS−PAGE separation of IGFBP-5 protease and N-terminal amino acid squencing of SDS−PAGE protein bands. Purified IGFBP-5 protease was incubated in the presence of reducing agent (5% β-mercaptoethanol), separated by 6% SDS−PAGE, transferred to ProBlott membrane, and stained with Coomassie Blue R-250. Each band was excised and sequenced. Estimated molecular masses of bands and their sequence similarity to known proteins are shown. The 160, 97, and 64 kDa bands did not yield reliable sequence information of sufficient length to determine if they matched known sequences.

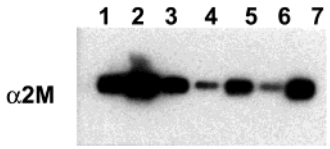

FIGURE 3: α2M production by various cell types. Serum-free culture media collected from human osteosarcoma cells (lane 1 = U2, lane 4 = high ALP SaOS-2, lane 5 = MG63, lane 6 = low ALP SaOS-2), liver hepatoma cells (lane 2 = HEPG2, lane 3 = H-35), and 20 ng of purified α2M (lane 7) were subjected to Western immunoblot analysis using α2M antibody. These data show that some of the human osteosarcoma cells express α2M to a level comparable to that of liver cell types.

reliable sequence information of sufficient length for comparison with known sequences in the NCBI database.

Evidence That α2 Macroglobulin (α2M) Is Not the IGFBP-5 Protease. Because the major protein in the active IGFBP-5 protease fraction turned out to be α2M, we next evaluated if α2M exhibits IGFBP-5 protease activity. Figure 3 shows that although α2M is produced by various human OB cell types to levels comparable to those produced by liver cell types, which represent the major source of circulating levels of α2M, the levels of α2M in the OB and liver cell CM did not correlate with IGFBP-5 protease activity. For example, SaOS-2 cells expressed abundant IGFBP-5 protease but produced little α2M. On the other hand, HEPG2 cells produced large amounts of α2M but exhibited little IGFBP-5 proteolytic activity in the CM (data not shown). Figure 4 shows that purified α2M exhibited IGFBP-5 proteolytic activity only at extremely high concentrations and this activity could not be blocked by antibody generated against α2M (data not shown). The weak IGFBP-5 proteolytic activity of α2M is likely to be due to the presence of IGFBP-5 proteases in the native purified preparations of α2M since a variety of proteases including ADAMs are known to bind to α2M.

ADAM-9 Is Present in Purified IGFBP-5 Protease Preparations. In our subsequent studies to identify the IGFBP-5 protease, we focused our efforts on the ADAM family of

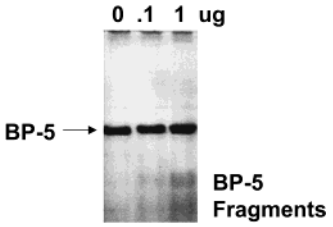

FIGURE 4: IGFBP-5 proteolysis by α2M. 500 ng of IGFBP-5 was incubated with various concentrations of α2M overnight at 37 °C prior to SDS−PAGE and silver staining for visualization of IGFBP-5 and its fragments. Proteolysis against IGFBP-5 was seen only with high doses [1 μg or higher (not shown)].

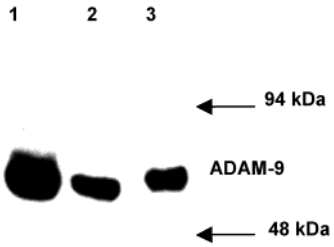

FIGURE 5: Purified preparations of IGFBP-5 protease contain ADAM-9. A 20 μL aliquot of IGFBP-5 protease purified by NH_4-SO_4 precipitation + phenyl-Sepharose (lane 1) + zinc agarose (lane 2) + FPLC gel filtration (lane 3) was subjected to SDS−PAGE followed by Western immunoblot analysis. A 60 kDa protein was recognized by ADAM-9 antibodies in purified preparations of IGFBP-5 protease.

proteases on the basis of the recent findings that ADAM-12 was identified as an interacting partner for IGFBP-3 using a yeast two-hybrid screen of human placental cDNA library (*45*) and that ADAM-12 cleaved not only BP-3 but also IGFBP-5 but did not cleave other IGFBPs (*46*). Because some of the ADAM proteases, including ADAM-12, have been shown to bind α2M and are known to be produced by mouse bone cells (*47*−*49*), and because much of the IGFBP-5 proteolysis in the CM of human OBs was inhibited by 1,10-phenanthroline (inhibits ADAM activity) but not by matrix metalloprotease inhibitors such as TIMP-1 and TIMP-2 (data not shown), we considered the possibility that the IGFBP protease produced by OBs was an ADAM protease. If an ADAM family member contributes to the proteolysis of IGFBP-5 in the CM of human OBs, we would expect our highly purified preparations of IGFBP-5 protease to contain ADAM. Figure 5 shows data from Western immunoblot analysis which reveal that the FPLC gel filtration fraction containing IGFBP-5 protease purified by four chromatographic steps contained significant amounts of ADAM-9. In contrast, ADAM-10, ADAM-12, or ADAM-17 could not be detected in the purified IGFBP-5 protease fraction (data not shown). Since our purified fraction contained ADAM-9, we expected the amino acid sequence of one of the bands in SDS−PAGE to match that of ADAM-9. In this regard, we found that SDS−PAGE of the purified fraction under reducing conditions yielded a band of 64−67 kDa (Figure 2), the size of which is similar to that of the major ADAM-9 band detected in osteoblast cell CM and in the purified fractions. However, the sequence of this band did not yield a reliable sequence of sufficient length (i.e., produced multiple signals with low yield) to convincingly demonstrate that one of these sequences corresponded to ADAM-9.

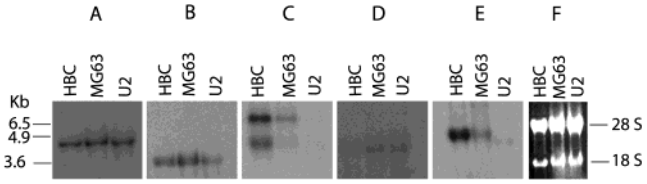

FIGURE 6: Northern blot analysis of total RNA extracted from various human osteoblast cell preparations using cDNAs for various ADAMs as probes. 20 μg of RNA from normal human osteoblasts derived from calvaria (HBC) and human osteosarcoma cells (MG63 and U2) was probed with cDNAs for ADAM-9 (A), -10 (B), -12 (C), and -17 (D) and ADAM-TS1 (E). 18 and 28 S ribosomal RNA bands are shown in panel F to indicate uniformity in loading. Normal human osteoblasts produce multiple members of the ADAM family.

ADAM-9 Is Produced and Secreted by Human Osteoblasts. We evaluated expression levels of various ADAMs (ADAM-9, ADAM-10, ADAM-12, ADAM-17, and ADAM-TS1), which are known to contain an active proteolytic domain and are, therefore, considered to be potentially active metalloproteases (*45*, *47*, *48*) in human OBs. The relative expression of various members of the ADAM family was evaluated by Northern blot analysis in U2 and MG63 human osteosarcoma cells and in untransformed normal human osteoblasts derived from calvaria. This comparison revealed evidence that ADAM-9 was consistently expressed at high levels compared to other ADAM members in the three human osteoblast cell types tested. Figure 6 shows that ADAM-9 mRNA (approximately 4 kb) is expressed in all three human OB cell types under serum-free conditions while the expression of ADAM-10, ADAM-12, ADAM-17, and ADAM-TS1 is variable in the three human OB cell types tested. For example, U2 cells which produce IGFBP-5 protease in high abundance showed only weak expression of ADAM-12, ADAM-17, and ADAM-TS1. Previous studies have shown that IGFBP-5 protease is secreted in the CM of various human osteoblast cell types (*23*, *26*). Because many of the ADAMs, including ADAM-9, have been shown to be membrane bound and function as sheddases (release membrane proteins), we evaluated if any of these ADAM family members are secreted as soluble proteins. Figure 7 shows a Western immunoblot analysis of cell extracts and serum-free CM samples from U2 and MG63 human osteosarcoma cells and in untransformed normal human osteoblasts derived from rib using antibodies to ADAM-9, ADAM-10, ADAM-12, and ADAM-17. In these studies, it was found that, although cell extracts of three human osteoblast cell types contained one or more ADAM proteins, CM preparations contained ADAM-9 in significant levels but not any of the other ADAM proteins tested. A 65 kDa major band was detected in the CM of all three human osteoblast cell types that produce IGFBP-5 protease. In addition, two minor bands of approximately 80 and 90 kDa were also visible in the CM. The intensity of all three bands was much higher in the CM compared to cell extracts in all three cell types. The multiple bands may represent different glycosylated forms or alternatively processed forms (*50*, *51*). In contrast, ADAM-10, ADAM-12, and ADAM-17 were present in much higher abundance in cell extracts compared to CM, which contained little or no measurable levels of ADAM-10, -12, and -17 (Figure 7). The production and secretion of ADAM-9 in high abundance was also confirmed using several human osteosarcoma cell types and normal human osteoblasts derived from different skeletal sites (Figure 8). These data suggest that, of the four members of the ADAM family (ADAM-9, -10, -12, and -17) which are known to contain an active proteolytic domain, ADAM-9 is secreted in much higher abundance compared to the other three ADAMs by human osteoblast cell types.

Recombinant ADAM-9 Cleaves IGFBP-5. To provide direct evidence that ADAM-9 is an IGFBP-5 protease, we expressed the metalloprotease domain of ADAM-9 (residues 30−412) as described recently (*38*). Culture media from the *P. pastoris* ADAM-9 clone, but not the control clone, exhibited a band of anticipated molecular mass (30 kDa) as performed by silver stain and Western immunoblot (Figure 9A). To determine if recombinant ADAM-9 cleaves IGFBP-5, various concentrations of purified recombinant ADAM-9 were incubated with 500 ng of IGFBP-5, and proteolysis was evaluated. Figure 9B shows that purified recombinant ADAM-9 cleaves IGFBP-5 in a dose-dependent manner. In contrast to IGFBP-5, ADAM-9 did not cleave or cleaved other IGFBPs with much less potency (Figure 10).

Recombinant ADAM-9 Binds to α2M. Because purified IGFBP-5 protease existed as a complex by binding to α2M, we expected ADAM-9 to bind to α2M if it were the IGFBP-5 protease. Figure 11 shows that purified recombinant ADAM-9 formed a complex with purified α2M. This binding appears to be relatively specific since purified albumin did not bind to ADAM-9. In terms of the significance of α2M binding to proteases, it is known that α2M inhibits a variety of proteases via a steric trapping mechanism (*52*). To determine if α2M alters the potency of ADAM-9 to cleave IGFBP-5, ADAM-9 was incubated with or without purified native α2M prior to IGFBP-5 proteolysis. Figure 12 shows that the addition of α2M did not prevent the degradation of IGFBP-5 by ADAM-9.

DISCUSSION

We and others have reported previously that human osteoblasts secrete an IGFBP-5 protease that cleaves IGFBP-5 into fragments that bind IGF with more than 100-fold reduced affinity compared to intact IGFBP-5 (*9*). This proteolytic activity has been shown to be a metalloprotease and is relatively specific to IGFBP-5 (*23*). The factors that regulate the activity of this protease have the potential to regulate the activity of IGFBP-5 and thereby regulation of bone formation in health and diseases based on the following: (1) IGFBP-5 has been shown to increase bone formation via a mechanism independent of IGFs (i.e., IGFBP-5 is a growth factor), and (2) several lines of indirect evidence support the fact that IGFBP-5 functions as a physiologic regulator of bone formation and when deficient in serum is a risk factor in the development of hip fracture (*21*, *53*). Since the molecular identity of IGFBP-5 protease produced by osteoblasts is critical for studies on the regulation and role of this protease in regulating the IGFBP-5 molecular pathway, we focused our efforts to purify and characterize the IGFBP-5-specific protease produced by human osteoblasts. These studies led to the exciting finding that ADAM-9 is an IGFBP-5 protease produced by human osteoblasts. To our knowledge, our data provide the first evidence that ADAM-9 is secreted by human osteoblasts, degrades IG-

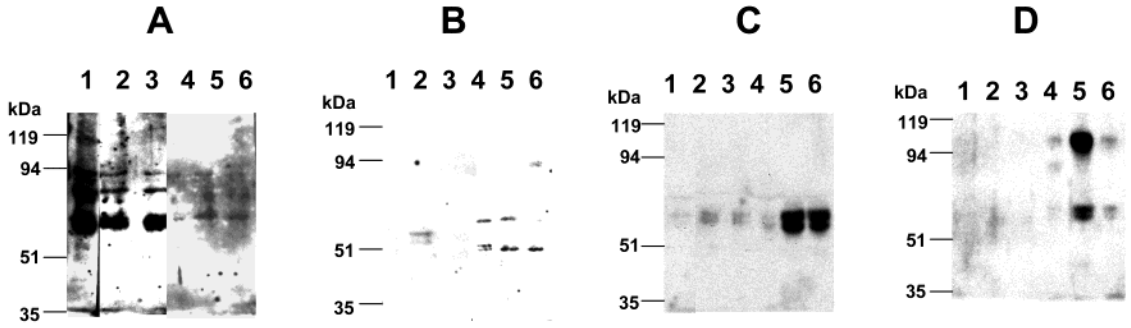

FIGURE 7: Western immunoblot analysis of CM and cell lysates for various ADAMs. Serum-free CM samples and cell lysates were prepared for U2 human osterosarcoma cells, MG63 human osteosarcoma cells, and normal human osteoblasts derived from calvaria as described in Materials and Methods. 20 μL samples were subjected to SDS−PAGE and proteins transferred to nitrocellulose membrane. The membranes were then probed with antibody to ADAM-9 (A), ADAM-10 (B), ADAM-12 (C), and ADAM-17 (D). Lanes 1−3 represent CM samples while lanes 4−6 represent cell lysates. Lanes 1 and 4 represent U2 cells, lanes 2 and 5 represent MG63 cells, and lanes 3 and 6 represent normal human osteoblasts derived from calvaria.

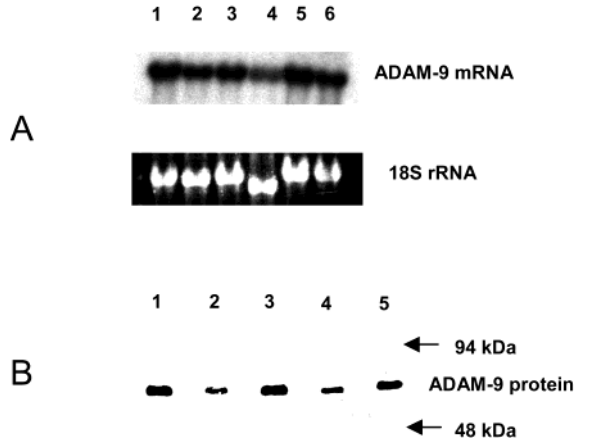

FIGURE 8: Expression of ADAM-9 mRNA (A) and protein (B) in human osteoblast cell types. In panel A, 10 μg of RNA from normal human osteoblasts (lanes 1 and 3 = osteoblasts derived from calvaria; lane 2 = osteoblasts derived from rib) and human osteosarcoma cells (lane 4 = MG63; lane 5 = SaOS-2; lane 6 = U2) was loaded per lane, and the blot was probed with ^{32}P-labeled ADAM-9 cDNA probe. The probe detected a 4 kb band in all cell types. 18 S rRNA band is shown to indicate uniform loading. In panel B, 10 μL of concentrated CM (50×) was applied to SDS−PAGE, transferred to nitrocellulose membrane, and subjected to Western immunobloting using ADAM-9 antibodies. CM from U2 (lane 1), low ALP SaOS-2 (lane 2), normal human osteoblasts derived from rib (lane 3), and calvaria (lanes 4 and 5) exhibited a band of approximately 60 kDa recognized by ADAM-9 antibodies but not by nonspecific sheep IgG (data not shown).

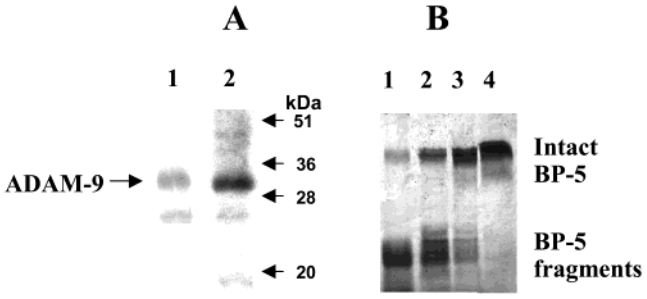

FIGURE 9: IGFBP-5 proteolysis by recombinant ADAM-9. Culture medium from *P. pastoris* with and without pPICZα expression vector containing a cDNA insert for ADAM-9 was subjected to Q-Sepharose. The bound protein was eluted with a gradient of NaCl, and aliquots of the bound fraction were used for Western immunoblotting using ADAM-9 antibodies and for protein estimation by BCA assay. Panel A shows silver staining (lane 1) and Western immunoblotting (lane 2) of the 0.5 M NaCl eluted fraction containing ADAM-9. In panel B, 10 ng (lane 1), 1 ng (lane 2), or 0.1 ng (lane 3) of the 0.5 M NaCl eluted fraction containing ADAM-9 or buffer control (lane 4) was subjected to proteolysis against IGFBP-5 (500 ng) for 4 h at 37 °C. The amount of intact and fragment forms of IGFBP-5 was visualized by silver staining after SDS−PAGE separation.

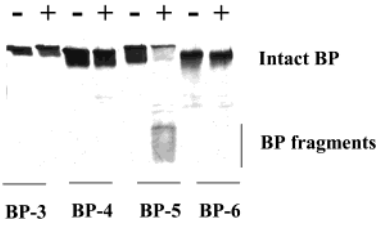

FIGURE 10: Protease assays on the four members of the high-affinity IGFBPs. 500 ng each of IGFBP-3, -4, -5, and -6 was incubated with 10 ng of Q-Sepharose-purified recombinant ADAM-9 for 4 h at 37 °C. The amount of intact and fragment forms of IGFPs was visualized by silver staining after SDS−PAGE separation.

FBP-5 with high potency, and thereby may regulate the actions of this important protein.

The ADAM family includes nearly 40 proteins and contains a number of domain structures, including metalloproteinase, disintegrin, cysteine-rich, EGF-like, and transmembrane (*54*, *55*). The ADAM protease domain is well conserved among all members of the family with a typical reprolysin-type Zn-binding signature (HExxHxxGxxHD) and a critically conserved methionine, which forms the "met turn", a structural feature common to all metzincins (*54*, *55*). Unlike other zinc protease families, ADAM proteins have three distinguishing features. First, the intact zinc binding site is absent in several ADAMs including 2−7, 11, 14, 18, 22, and 29, and therefore, these ADAMs are not considered zinc proteases. Second, the metalloprotease domain is not retained in several mature ADAMs (e.g., ADAM-1 and -2). Since cleaved ADAM protease domains have not been identified, it remains to be established whether these protease domains have additional functions. Third, many of the ADAMs are not known to be secreted (*49*, *54*−*56*). Because many of the ADAMs has been discovered because of cloning or genetic analysis, little is known regarding their physiological substrate or their functions.

The proteins of the ADAM family are widely distributed in many organs, tissues, and cells (*47*−*58*). Inoue et al. (*47*) reported evidence for expression of four members of the ADAM family (ADAM-9, -12, -15, and -19) in mouse bone

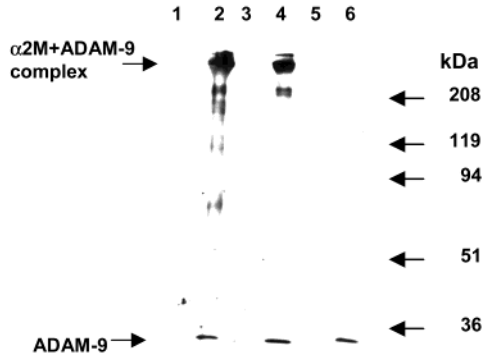

FIGURE 11: Western blot analysis of ADAM-9 binding to α2M. 1 μg of α2M from Sigma Chemicals (lanes 1 and 2), 1 μg of native α2M from Dr. Steven Gonias (lanes 3 and 4), or 1 μg of BSA (lanes 5 and 6) was incubated without (lanes 1, 3, and 5) or with (lanes 2, 4, and 6) recombinant ADAM-9 (100 ng) for 1 h at 4 °C. ADAM-9 was visualized after SDS−PAGE separation by Western immunoblot analysis. α2M from two different sources but not BSA formed a complex with ADAM-9.

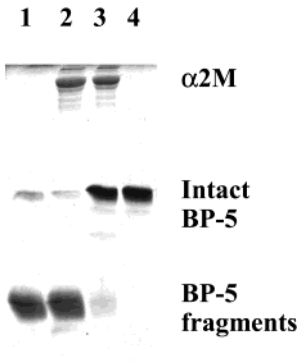

FIGURE 12: Effect of α2M on proteolysis of IGFBP-5 by ADAM-9. 10 ng of recombinant ADAM-9 was incubated without (lane 1) or with 500 ng of α2M (lane 2) for 1 h at 4 °C prior to the addition of 500 ng of IGFBP-5. After 4 h incubation at 37 °C, the samples were subjected to SDS−PAGE to separate the intact and fragment forms of IGFBP-5 and visualized by silver stain. Lane 3 contains IGFBP-5 that was incubated with α2M but not ADAM-9. Lane 4 contains IGFBP-5 that was incubated with buffer control. Intact IGFBP-5 was degraded in the presence of ADAM-9 which was not affected by the addition of α2M.

cells. Our data provide the first evidence that ADAM-9, also known as MDC (metalloproteinase/disintegrin/cysteine-rich) or meltrin-γ, is expressed by various human osteoblast cell types. Besides bone cells, ADAM-9 is also expressed in a number of other tissues including kidney, muscle, lung, and ovary (*47*, *51*, *56*). In many of these tissues, ADAM-9 is anchored to the membrane and not secreted. In contrast, we found that abundant ADAM-9 is secreted by human osteoblasts. Further studies are needed to evaluate whether secretion of ADAM-9 is specific to osteoblasts or other cell types besides osteoblasts that secrete ADAM-9.

Several lines of evidence suggest that ADAM-9 is an IGFBP-5-specific protease produced by human osteoblasts. These include the following: (1) ADAM-9 is expressed in high levels in various human osteoblast cell types that produce IGFBP-5 protease. In contrast, several other ADAMs, which contain an active proteolytic domain, are weakly expressed or not expressed at all in different human osteoblast cell types known to produce IGFBP-5 protease. (2) ADAM-9 is detected as a 65 kDa major band and 80 and 90 kDa minor bands in the CM samples of human osteoblasts. In contrast to ADAM-9, which is present in high abundance in CM samples compared to cell extracts, ADAM-10, ADAM-12, and ADAM-17 are present in cell extracts with little or no protein in CM samples. (3) ADAM-9 is present in highly purified IGFBP-5 protease preparations. In contrast to ADAM-9, ADAM-12, which is known to cleave IGFBP-5 in addition to IGFBP-3, could not be detected in purified IGFBP-5 protease preparations. (4) Purified recombinant ADAM-9 cleaves IGFBP-5 at a higher potency than that of other related IGFBPs. In this regard, the amount of ADAM-9 needed to cleave 500 ng of IGFBP-5 is extremely low (1 ng) and comparable to other specific proteases known to cleave IGFBPs (*29*, *46*, *59*). In any case, it is important to show in future studies that the IGFBP-5 protease produced by human osteoblasts cleaves IGFBP-5 at the same site as ADAM-9 in order to convincingly demonstrate that ADAM-9 is the major IGFBP-5 protease.

Although our findings demonstrate that ADAM-9 is an IGFBP-5 protease produced by osteoblasts, the issue of whether any of the other known IGFBP-5 proteases also contribute to IGFBP-5 proteolysis in osteoblast cell CM can only be speculated at this time. In this regard, it has recently been shown that ADAM-12 cleaves IGFBP-5 in addition to IGFBP-3 (*46*). However, ADAM-12 does not appear to be a major protease in human osteoblast cell CM since little or no ADAM-12 could be detected by Western immunoblot analysis of CM from various human osteoblast cell types. Although ADAM-TS1 is known to be expressed by some osteoblast cell types (*60*), U2 cells which produce abundant IGFBP-5 protease expressed little ADAM-TS1 mRNA (Figure 6), thus suggesting that ADAM-TS1 is unlikely to be the major IGFBP-5 protease. Previous studies have also shown that mouse osteoblasts secrete MMP-2 capable of cleaving IGFBP-5 (*28*). Because TIMP-2, tissue inhibitor of MMP-2, did not block IGFBP-5 proteolysis in the CM of human osteoblasts, MMP-2 is unlikely to be the major IGFBP-5 protease produced by human osteoblasts. Furthermore, MMP-2 cleaves other IGFBPs besides IGFBP-5 (*61*). Busby et al. (*29*) have shown that complement C1r and C1s actively cleaved IGFBP-5 in fibroblast cell CM. Recently, it has also been shown that PAPP-A, a predominant IGFBP-4-specific protease in human pregnancy serum, could also cleave IGFBP-5 but with a lesser potency (*40*, *62*). Furthermore, more recently, PAPP-A2 has been shown to cleave IGFBP-5 but not IGFBP-4 (*30*). The issue of whether any of these other proteases also contribute to the degradation of IGFBP-5 in bone under certain physiological or pathological conditions remains to be determined.

The data currently available on the substrates for ADAM proteases reveal that ADAM proteases could cleave a wide variety of substrates ranging from extracellular matrix proteins to growth factors. For example, ADAM-10 purified from bovine kidney can cleave type IV collagen (*63*). ADAM-17, also known as TACE (TNFα converting enzyme), is involved in Notch1 processing (*64*). ADAM-9 has been shown to cleave insulin-B-chain as well as fibronectin (*38*, *56*). Our findings demonstrate that ADAM-9 cleaves IGFBP-5 at higher potency compared to other related IGFBPs. The issue of whether ADAM-9, as well as other ADAMs with active proteolytic domains, could cleave multiple substrates in different cell types remains to be established.

Another major finding in this study relates to the existence of ADAM-9 as a complex by binding to α2M. In terms of the significance of α2M binding to ADAM-9, α2M is generally considered to be a typical member of the proteinase inhibitor family, capable of inhibiting proteinases from all classes by a steric trapping mechanism (*65*). Upon cleavage of a peptide bond in the bait region by the proteinase, α2M traps the proteinase by a major conformational change of the tetrameric α2M structure. This conformational change exposes the recognition sites for the α2M receptor, thereby allowing for the specific elimination of the α2M−proteinase complexes. Since α2M did not inhibit the activity of ADAM-9 to degrade IGFBP-5, the significance of the binding of α2M to ADAM-9 can only be speculated. In this regard, it is possible that α2M binding to ADAM-9 may protect ADAM-9 from degradation or may trap ADAM-9 to maintain sufficient concentrations of ADAM-9 in the vicinity of osteoblasts.

Additional studies are required to answer the following questions: (1) How is ADAM-9 secreted? In the case of ADAM-12, alternate splicing of the mRNA results in a shorter form that is secreted as a soluble protein (*54*). An alternate possibility is that the membrane-bound ADAM-9 could be cleaved by another protease to release the soluble form. (2) Does full-length recombinant ADAM-9 cleave IGFBP-5 with a potency similar to that of the 30−412 form containing only the metalloprotease domain (*54*, *55*)? In this regard, since our purified IGFBP-5 protease fraction containing 65 kDa ADAM-9 was very active in cleaving IGFBP-5, it is likely that the full-length recombinant ADAM-9 cleaves IGFBP-5 with high potency. (3) What is the physiological significance of ADAM-9 produced by human osteoblasts? On the basis of the findings that ADAM-9 is an IGFBP-5 protease and that IGFBP-5 is an important regulator of bone formation, we predict that ADAM-9 cleaves IGFBP-5 and, thereby, regulates the bioactivity of IGFBP-5 in response to osteoregulatory agents. Thus, ADAM-9 could be an important player in the regulation of IGFBP-5 and, thereby, the bone formation pathway. As discussed earlier, ADAM-9 has other functional domains in addition to the metalloproteinase domain. These other functional domains could have other functions not related to IGFBP-5 proteolytic function. In this regard, ADAM-9 has been proposed to function as an adhesion molecule by interacting with $\alpha_v\beta_5$ integrin in myeloma cells (*66*). In terms of the need for ADAM-9 to exert multiple functions in addition to being a protease, the recent finding that the number of genes in human genome is much less than predicted underscores the need for the diversification of gene function to account for human complexity.

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