Heparan Sulfate Regulates ADAM12 through a Molecular Switch Mechanism

The disintegrin and metalloproteases (ADAMs) are emerging as therapeutic targets in human disease, but specific drug design is hampered by potential redundancy. Unlike other metzincins, ADAM prodomains remain bound to the mature enzyme to regulate activity. Here ADAM12, a protease that promotes tumor progression and chondrocyte proliferation in osteoarthritic cartilage, is shown to possess a prodomain/catalytic domain cationic molecular switch, regulated by exogenous heparan sulfate and heparin but also endogenous cell surface proteoglycans and the polyanion, calcium pentosan polysulfate. Sheddase functions of ADAM12 are regulated by the switch, as are proteolytic functions in placental tissue and sera of pregnant women. Moreover, human heparanase, an enzyme also linked to tumorigenesis, can promote ADAM12 sheddase activity at the cell surface through cleavage of the inhibitory heparan sulfate. These data present a novel concept that might allow targeting of ADAM12 and suggest that other ADAMs may have specific regulatory activity embedded in their prodomain and catalytic domain structures.

Heparan sulfate proteoglycans, which consist of a core protein with one or more covalently attached heparan sulfate glycosaminoglycans (HSGAGs), are among the most important modulators of protein function at the cell surface (1). Two major groups of cell surface heparan sulfate proteoglycans are the transmembrane syndecans and glycosyl phosphatidylinositol-anchored glypicans (2). Many proteins interact specifically with glycosaminoglycans (GAGs) through saccharide sequences of well defined length and structure (3), whereas the degree of specificity may vary according to the protein and the biological context (4). The structure and specificity of HSGAGs at the cell surface is altered during tumor development primarily by matrix-degrading endoglycosidases and proteases (5). Remodeling of HSGAGs facilitates tumor cell proliferation, angiogenesis, invasion, and metastasis (6). Similar mechanisms are responsible for trophoblast invasion in human pregnancy (7).

ADAM12 (a disintegrin and metalloprotease 12) has been determined to have a role in both cancer and pregnancy (8). ADAM12 is highly expressed by malignant tumor cells (9) and is associated with the progression and spread of human cancers (10, 11). Most recently, we and others showed that ADAM12 regulates tumor progression in gene-modified mice models (12, 13). Unlike other ADAMs, ADAM12 is selectively overexpressed in osteoarthritic cartilage and enhances IGF-I-mediated chondrocyte proliferation by degradation of insulin-like growth factor–binding protein (IGFBP)-5 (14).

ADAM12 is expressed as two splice variants, the transmembrane ADAM12-L (long form) and the secreted ADAM12-S (short form). The prototypical ADAM12-L contains extracellular prodomain (P); catalytic (C), disintegrin (D), cysteine-rich (CR), and epidermal growth factor-like (EGF) domains; a class I transmembrane region; and a cytoplasmic tail (Fig. 1A). ADAM12-S contains the five extracellular domains and a unique stretch of 33 amino acids at the C terminus. Both ADAM12 splice variants are processed between the prodomain and catalytic domain by a furin-like protease, and the mature form subsequently translocates to the plasma membrane or extracellular space. ADAM12 is an active metalloprotease, cleaving IGFBP-3 and -5 and mediating ectodomain shedding of pro-EGF (15, 16). The catalytic domain is responsible for the metalloprotease activity of ADAM12 and contains three zinc-binding histidines and a catalytic glutamic acid in the conserved HEXXHXXGXXH motif (17).

ADAM prodomains are not homologous to any other proteins and may elicit novel functions (18). The prodomain of ADAM12 remains noncovalently associated with the mature enzyme both under physiological conditions and in vitro (19). ADAM8, -9, -10, -15, -17, and -33 are also processed intracellularly, and their prodomains appear to be present following secretion (20–25). The prodomains of ADAM10 and -17 act as inhibitors of mature enzymes in vitro (21, 26) and are required...
ADAM12 Regulation by HSGAGs

for both maturation and transport to the cell surface (27). Altogether, previous work has not identified the fate and exact function of the ADAM prodomains, but data suggest that they are probably not restricted to a single general mechanism.

Shedding of pro-EGF is mediated by ADAM8, -10, -12, and -17, and this redundancy is typical of ADAM substrates (16). Specificity based on mechanistic insight is crucial in drug development, as illustrated by the disappointing outcomes of recent clinical trials of inhibitors of matrix metalloproteases for cancer treatment (28).

Here we show that the noncovalently associated prodomain and the catalytic domain of ADAM12 in concert form a novel molecular switch critical for the regulation of the ADAM12 proteolytic activity by HSGAGs. ADAM12-mediated proteolysis is specifically regulated by this mechanism in vitro and under physiological conditions. The identified mechanism represents a new avenue for specific therapeutic targeting of ADAM metalloproteases for treating human disease.

EXPERIMENTAL PROCEDURES

Materials—Recombinant ADAM17 was purchased from R&D Systems. TNF-α-based fluorescent peptide substrate (dabcyl-LAQAhomoPhersK(5FAM)-NH₂) was from BioZyme, Inc. Heparin beads (TSK gel heparin 5PW) were from Tosoh Bioscience. Heparin oligosaccharides were prepared as described (29). Heparin, heparan sulfate, dermatan sulfate, l-chondroitin 4- and 6-sulfate, completely desulfated heparin, and IGF-I were purchased from Sigma. The monoclonal mouse antibody 10E4 (anti-heparan sulfate) and bacterial heparinase were from Seikagaku. The ADAM12 polyclonal antibodies rb116 (ADAM12-S-specific), rb122 (cysteine-rich domain-specific), rb128 (catalytic domain-specific), and rb132 (prodomain-specific) were described previously (30). A monoclonal mouse antibody (mAb-11) recognizing the ADAM12 cysteine-rich domain was generated using ADAM12-S as the antigen, essentially as described (31). Antibodies for detection of heparanase (mouse clone HP3/17), and IGFBP-3 (polycrimal goat) were from Cell Sciences and Diagnostic Systems, respectively.

Expression Constructs and Protein Purification—All expression constructs used for transfection and transformation of eukaryotic cells or Escherichia coli are described in supplemental Table 1. Transfection of HEK 293 EBNA cells and purification of ADAM12-S and deletion mutants were performed as described previously (15, 19, 32). C-terminally histidine-tagged ADAM12-P, ADAM12-D, and ADAM12-CR were expressed in E. coli, refolded, and purified as described (33). The NusA/heparin-binding EGF (HB-EGF)/GFP fusion protein was purified by standard methods using nickel chromatography and gel filtration. CHO cell lines (CHO K1 and GAG-deficient CHO 761) were transfected by pcDNA3 constructs using FuGENE (Roche Applied Science), and HEK 293 EBNA cells were transfected by pCEP4 constructs using Lipofectamine (Invitrogen).

Binding of ADAM12-S to Heparin-Sepharose—A 1-ml heparin-Sepharose column (GE Healthcare) was equilibrated in buffer A (25 mM Tris-HCl, pH 7.5, 50 mM NaCl) and loaded with 10 ml of serum-free conditioned medium from HEK 293 EBNA cells transiently expressing ADAM12-S. The column was washed extensively in buffer A and eluted with a gradient of NaCl from 0 to 2 M in buffer A.

Surface Plasmon Resonance Analysis—Streptavidin (in sodium acetate buffer, pH 4.5) was immobilized onto two N-ethyl-N’-(diethylaminopropyl)-carbodiimide/N-hydroxy-succinimide-activated flow cells of a CM4 sensor chip (BIAcore), as described (34). Biotinylated 6-kDa heparin was then injected on one of them, with the other serving as a negative control surface. Remaining free activated groups were blocked by injection of 1 M ethanolamine, pH 8.5. Binding assays were conducted in 10 mM Hepes, 150 mM NaCl, pH 7.4. Sensorsgrams shown correspond to signals obtained after online subtraction of the negative control. Data were analyzed using the BIAeval 3.1 software.

Heparin Beads Approach Analysis—Heparin beads in PBS were activated with N-ethyl-N’-(diethylaminopropyl)-carbodiimide/N-hydroxy-succinimide for 15 min, inactivated with β-mercaptopoethanol (15 mM final concentration), rinsed, and then incubated with purified ADAM12-S (20 mg) for 2 h at room temperature as described (35). After quenching of free activated groups using 1 M Tris, pH 7.5, beads were washed with PBS, 2 M NaCl to remove unbound material and then incubated for 15 min in PBS, 2 M urea at 60 °C for protein denaturation and digested overnight with thermolysin (53 μl) at 50 °C, under agitation. Released peptides were removed by extensive washing with PBS, 2 M NaCl, 15 mM β-mercaptoethanol, 1% Triton X-100. Heparin beads were rinsed in water and packed on a Biobrene-treated filter, and peptides cross-linked to heparin beads were sequenced using an Edman degradation automated protocol.

ADAM12 Proteolytic Activity Assays—ADAM12 activity was assayed essentially as described using S-carboxymethylated transferrin (cmTF), the TNF-α-based fluorescent peptide substrate (32, 36), or a substrate containing 82 residues of HB-EGF between E. coli NusA and GFP constructed to test the proposed cleavage of HB-EGF by ADAM12 (37). The exact assay conditions for each experiment are specified in the figure legends. All experiments were performed in triplicate.

Staining of Cells and Quantitation of Fluorescence—CHO or ARH (see Ref. 38) cells used for the experiments were transfected with ADAM12-PC cDNAs cloned into pcDNA3, or purified ADAM12 was added to the media of cells in culture. Living cells were incubated with ADAM12 antibodies for 1 h at 4 °C before fixation in 4% paraformaldehyde as described (33). To avoid influence from intracellular ADAM12, permeabilization by detergents was not performed. After fixation, the suspended cells were centrifuged for 2 min in a Cytospin centrifuge (Shandon). After washing in PBS, both the adherent and suspended cells were incubated with an appropriate AlexaFluor-conjugated secondary antibody for 30 min at room temperature, mounted in Dako fluorescent mounting medium (Dako A/S), and examined using an inverted confocal microscope (LSM510 meta; Carl Zeiss, Inc.) or an inverted Zeiss Axiosvert 200. The images were processed using the Axiomvision program (Carl Zeiss). The fluorescence intensity on the cell surface was quantitated using the Profile Display in the Axiomvision Program on the LSM510 metami-
ADAM12 Regulation by HSGAGs

croscope. The biological experiments were repeated three times with three analytical replicates.

**Immunocapture ADAM12-S Proteolytic Activity Assay—** Ninety-six-well Maxisorp plates (Nunc) were coated with monoclonal antibody mAb-11 at 250 μg/ml in NaHCO	extsubscript{3} pH 9.6 (2 h at room temperature). Next, wells were washed four times in PBS-T (PBS containing 5% Tween 20) and incubated for 1 h at room temperature with either serum-free conditioned medium from HEK 293 EBNA cells transfected with ADAM12-S constructs or serum samples. Wells were washed four times in PBS-T and four times in assay buffer (20 mM Tris, pH 8).

ADAM12-S activity was measured by adding 10 μM TNF-α-based peptide substrate to the wells, and fluorescence was quantitated at 1-min intervals at excitation and emission wavelengths of 485 and 530, respectively. All experiments were performed in triplicate.

**Assay for Pro-EGF Shedding—** Two days after transfection, CHO cells were starved for 2 h in serum-free medium essentially as described (16). The conditioned media (supernatants) were collected, and cell lysates were prepared using PBS containing 1% Triton X-100 and complete protease inhibitor mixture (Roche Applied Science). All transfections were performed in triplicate. Cell lysates or supernatants were mixed in a 1:1 ratio with 2 mg/ml p-nitrophenyl phosphate (Sigma) in a 96-well plate. The samples were incubated at 37 °C, and absorbance was monitored at 405 nm (A	extsubscript{405}) once every hour. The activity in supernatants and lysates was calculated from the A	extsubscript{405}/min enzyme velocity, and ADAM12 pro-EGF shedding activity was expressed as the ratio between this value in supernatants and lysates. The mean value of the ratio for supernatant/lysate was rescaled as -fold induction as compared with the mock control (Fig. 1C) or plotted directly (Fig. 1D). All activity measurements were performed in triplicate.

**Staining of Tissue Sections and Tissue Extraction—** Cryostat sections of human placenta tissue at term were air-dried for 15 min and preincubated for 1 h at 37 °C in PBS alone or PBS containing heparinase I–III at 5 units/ml or heparin at 10 μg/ml. Sections were stained with antibodies recognizing heparanase and the ADAM12-S–specific 33-amino-acid stretch (rb116). Normal mouse or rabbit serum was used as control.

**Assay for IGFBP-3 Degradation by ADAM12—** Human serum was diluted 10-fold in 20 mM Tris-HCl, pH 8, and incubated with 500 nM ADAM12 enzyme in the presence of GAGs and/or heparinase I–III, as indicated.

**RESULTS**

ADAM12-mediated Ectodomain Shedding Is Regulated by HSGAGs—Wild-type CHO K1 and GAG-deficient CHO 761 cells (39) were transfected with ADAM12-Δcyt (lacking the cytoplasmic tail) or ADAM12-S in combination with the pro-EGF-alkaline phosphatase (AP) reporter construct, and pro-EGF shedding assayed (Fig. 1, A and B). Transfection of CHO K1 cells with ADAM12-Δcyt increased pro-EGF shedding 3-fold compared with the mock control, whereas ADAM12-S inefficiently shed pro-EGF (Fig. 1C). ADAM12-Δcyt is efficiently expressed at the cell surface of CHO K1 cells, whereas ADAM12-L (including the cytoplasmic tail) is only weakly expressed. ADAM12-L was therefore not used in the shedding experiments. Interestingly, transfection of CHO 761 cells with ADAM12-Δcyt increased pro-EGF shedding by 7-fold compared with mock controls (Fig. 1C). Cotransfection of ADAM12-Δcyt and human heparanase stimulated ADAM12-Δcyt-mediated pro-EGF shedding in CHO K1 cells, whereas shedding of pro-EGF by ADAM12-Δcyt in CHO 761 cells was inhibited by heparin (Fig. 1D). Western blotting showed that ADAM12-Δcyt was expressed at similar levels in CHO K1 and CHO 761 cells when cotransfected with human heparanase or treated with heparin (Fig. 1E). Furthermore, fluorescence-activated cell sorting analysis revealed that ADAM12-Δcyt was present in similar concentrations on the cell surface of both CHO K1 and CHO 761 transfected cells (supplemental Fig. 1). These experiments indicated that heparin and heparanase regulated ADAM12-Δcyt-mediated shedding of pro-EGF from the cell surface.

ADAM12 Interacts with HSGAGs by Its Prodomain and Catalytic Domain—To investigate the regulatory mechanism of pro-EGF shedding by ADAM12, we used heparin-Sepharose column chromatography, as well as surface plasmon resonance analysis. Purified recombinant ADAM12-S bound to heparin-Sepharose and was eluted between 200 and 1000 mM NaCl (Fig. 2A). Surface plasmon resonance analysis also demonstrated that purified ADAM12-S bound to heparin (Fig. 2B and supplemental Fig. 2A). The interaction appeared to be complex, because the dissociation constant (K	extsubscript{d}) could not be calculated by fitting the data to simple models of ligand–receptor interaction. Interestingly, the interaction between ADAM12-S and heparin could be dissociated by EDTA, suggesting a dependence on divalent metal ions bound to ADAM12-S (data not shown).

Both ADAM12-PCD and ADAM12-PC also bound to heparin (Fig. 2B). Sensorgrams of the ADAM12-PC domain interaction with heparin were fitted to a 1:1 Langmuir binding model with a dissociation constant of ~76–78 nM (supplemental Fig. 2B). Recombinant ADAM12-P bound heparin with an affinity similar to ADAM12-PCD and ADAM12-PC, whereas the cysteine-rich (CR) domain and disintegrin (D) domains did not bind heparin (Fig. 2B).

The heparin interaction sites on purified ADAM12-S were mapped using a previously described “beads approach” based on the formation of cross-linked complexes of the protein of interest with heparin beads, the proteolytic digestion of these complexes, and the subsequent identification of the heparin binding peptides by N-terminal sequencing (35). By this method, three overlapping heparin-binding peptides in the prodomain and one in the catalytic domain were identified (Fig. 2C).

The specificity of ADAM12-PC binding to GAGs was assessed by interaction and competition assays using surface plasmon resonance analysis (supplemental Fig. 2C). Heparan sulfate inhibited ADAM12-PC binding to the heparin surface as efficiently as heparin. Heparin is characterized by high levels of sulfation and is a stronger polyanion than heparan sulfate (4). The similar efficiency indicated that the interaction involved specific determinants on heparan sulfate, whereas the increased ionic potential of heparin appeared less important. l-Chondroitin 4- or 6-sulfates did not interfere substantially with heparin binding to ADAM12-PC. Dermatan sulfate partially inhibited ADAM12-PC binding to heparin.
However, substantial binding of ADAM12-PC directly to a dermatan sulfate surface was not observed (data not shown), indicating that the interaction between ADAM12-PC and dermatan sulfate was weak. Heparin oligosaccharides of defined molecular weight were tested for the ability to compete for binding of heparin to ADAM12-PC (supplemental Fig. 2D). Oligosaccharides of at least 16 monosaccharides were efficient competitors for heparin binding, suggesting that the structural binding site involves several amino acid residues.

HSGAGs Inhibit the Proteolytic Activity of ADAM12—We next investigated the functional relevance of the binding of HSGAGs to ADAM12 using three different protease activity assays. The substrates included cmTF, NusA/HB-EGF/green fluorescent protein (GFP), and a tumor necrosis factor (TNF)-α-based fluorescent peptide. These substrates did not themselves bind heparin (data not shown). In particular, the heparin-binding domain of HB-EGF was not included in the NusA/HB-EGF/GFP chimeric protein. Cleavage of cmTF by ADAM12-S and ADAM12-PC was inhibited by heparin in a dose-dependent manner, and the inhibition could be reversed by incubation with heparinase I-III from *Flavobacterium heparinum* (Fig. 3, A and B).

To ensure that the inhibition was not a substrate-specific effect, the NusA/HB-EGF/GFP and fluorescent TNF-α-based substrates (36) were tested. NusA/HB-EGF/GFP was cleaved within the HB-EGF-encoding region by ADAM12-S, and the cleavage was inhibited by heparin in a dose-dependent manner, and the inhibition could be reversed by incubation with heparinase I-III from *Flavobacterium heparinum* (Fig. 3, A and B).

Both ADAM12-S and ADAM17 proteolytic activities are highly affected by salts like NaCl (32, 40). However, the observation that heparin inhibited ADAM12-S but not ADAM17 cleavage of the TNF-α-based substrate indicated that inhibition...
was specific and not mediated by sulfate ions or sodium counterions contributed by heparin (Fig. 3D).

Consistent with the interaction data, heparan sulfate inhibited the ADAM12-S degradation of cmTF with efficiency similar to that of heparin, whereas 1-chondroitin 4- or 6-sulfates, dermatan sulfate, and completely desulfated heparin were unable to inhibit the activity (data not shown). Furthermore, a substantial inhibition of ADAM12-S proteolysis of both cmTF and the TNF-α-based substrate could be achieved with heparin oligosaccharides containing eight (dp8) or more saccharide units (Fig. 3E). These results showed that the proteolysis of three different substrates by ADAM12-S could be inhibited by relatively short HSGAG structures.

Binding of ADAM12-PC to the Cell Surface Is Regulated by HSGAGs and Heparanase—When CHO 761 and CHO K1 cells were transfected with ADAM12-PC, conditioned media from the two cell lines displayed similar ADAM12 expression levels (Fig. 4A). Immunofluorescent surface staining of the same transfected cells demonstrated a 3-fold reduction in the ADAM12-PC binding to the cell surface in GAG-deficient CHO 761 cells as compared with CHO K1 cells, suggesting that ADAM12-PC was bound to a GAG at the cell surface after secretion (Fig. 4A).

We generated mutants within the ADAM12 sequence corresponding to the heparin-binding peptides identified by the heparin “beads approach” (Fig. 2C). ADAM12-PMut represented a mutant harboring the six mutations K158A, R163A, K165A, K170A, K171A, and K173A in the prodomain. ADAM12-CMut was mutated at R229A, K232A, and K236A in the catalytic domain. ADAM12-PMutCMut contained all nine mutations. Western blotting of supernatants from transfected CHO K1 cells with antibodies specific for the ADAM12 prodomain revealed that ADAM12-P, ADAM12-PMut, ADAM12-PMutC, and ADAM12-PMutCMut were expressed in similar extracellular concentrations in transfected CHO K1 cells (Fig. 4B). However, ADAM12-PMut and ADAM12-PMutCMut were not detected at the cell surface of CHO K1 cells following transfection, indicating that the residues in the prodomain and catalytic domain that had been mutated were responsible for binding to the cell surface (Fig. 4, B and C).

Next, recombinant ADAM12-PC was added directly to CHO K1 cells that had been transfected with vector only or human heparanase. Immunostaining showed that ADAM12-PC bound to the cell surface of mock-transfected cells, but not to CHO K1 cells transfected with human heparanase (Fig. 4D). Collectively, these data indicate that binding of ADAM12-PC
to the cell surface could be regulated by heparanase and HSGAGs. Consistent with these data, ADAM12-PC bound to the surface of ARH-77 B lymphoid cells expressing only syndecan 1, 2, or 4 but not to control ARH-77 cells that virtually lack cell surface proteoglycans (supplemental Fig. 3). From these experiments, it appeared that ADAM12-PC binding to the cell surface did not require a particular core protein, only the presence of heparan sulfate.

**Specific Residues in the Prodomain and Catalytic Domain Form a Heparan Sulfate-sensitive Switch to Regulate the Protease Activity of ADAM12**—The data presented above demonstrated that HSGAGs bound to the prodomain and catalytic domain of ADAM12 and inhibited proteolytic activity. A novel immunocapture assay was developed to measure ADAM12 proteolytic activity in complex samples. Monoclonal antibodies specific for the ADAM12 cysteine-rich domain were coated on a 96-well plate to capture ADAM12-S from the conditioned media of transfected cells (Fig. 5A). The proteolytic activity of captured ADAM12-S was measured by cleavage of the TNF-α-based fluorescent peptide substrate. Proteolytic activity of ADAM12-S in the conditioned media from HEK 293 EBNA cells transfected with ADAM12-S, ADAM12-S (PMutC), ADAM12-S (PMutC), and ADAM12-S (PMutC) was compared. All constructs were efficiently expressed (Fig. 5B), and neither the specific proteolytic activity nor the binding to the monoclonal antibody used in the capture assay was affected by the mutations, indicating that the overall conformation of the proteins was unchanged (data not shown). Wild-type ADAM12-S protease activity was efficiently inhibited by heparin (Fig. 5C). Similarly, ADAM12-S (PCMut) protease activity was inhibited by heparin. However, the proteolytic activity of ADAM12-S (PMutC) was not inhibited by heparin, and ADAM12-S (PMutC) was weakly inhibited by heparin when compared with the wild type protease. Immunoprecipitation experiments showed that the prodomain was bound to mature ADAM12-S as well as the three mutants (Fig. 5D). Quantitation of Western blots indicated that the relative stoichiometry of prodomain to mature ADAM12-S was similar for wild type and mutant proteins (data not shown).

Based on these data, we suggest that the heparin-binding region of the prodomain and catalytic domain formed a structural molecular switch that interacted with HSGAGs to regulate the proteolytic activity of ADAM12-S and that the same mechanism was responsible for the inhibition of ADAM12-Dcyt cleavage of pro-EGF (Fig. 1).

**ADAM12 Expressed in Pregnancy Is Proteolytically Active and Regulated by the HSGAG Switch**—ADAM12-S is present in pregnancy serum at a concentration of 12 μg/ml at term but undetectable in serum from nonpregnant individuals (41). Interestingly, ADAM12-S is a potential candidate in prenatal screening for Downs syndrome (41, 42). The proteolytic activity and regulation of ADAM12-S in human pregnancy serum and in tissue extracts from placenta were investigated using the immunocapture assay (Fig. 5A). The proteolytic activity of ADAM12-S was highly elevated in term pregnancy serum as compared with nonpregnancy serum, where it could not be detected (Fig. 6A). Heparin inhibited, whereas heparinase pre-
treatment activated, the ADAM12-S protease activity in pregnancy serum (Fig. 6B). The activation by bacterial heparinase suggested that at least some of the ADAM12-S in pregnancy serum was associated with a HSGAG that inhibited cleavage of the TNF-α-based substrate by ADAM12-S.

Immunostaining of human placenta tissue localized ADAM12-S to the apical borders of the trophoblasts (Fig. 6C). Treatment of tissue sections with heparin or bacterial heparinase before immunostaining removed ADAM12-S from the trophoblasts, indicating that ADAM12-S was bound to the cell surface through interaction with HSGAGs. Interestingly, ADAM12-S co-localized with heparinase at the apical borders of the trophoblasts (Fig. 6C). Extracts prepared from placental tissue contained ADAM12-S proteolytic activity that was inhibited by heparin (Fig. 6D). Placenta extracts prepared in the presence of bacterial heparinase showed increased ADAM12 protease activity, indicating that proteolytically active ADAM12-S was bound to trophoblasts by HSGAGs and was released by treating tissue with heparinase (Fig. 6D).

ADAM12-S Cleavage of IGFBP-3 Is Promoted by HSGAG Binding to ADAM12-PC—IGFBP-3 binds IGF-1 in human serum and can be detected by immunoblotting as an ~40-kDa
intact form. In pregnancy serum, the 40-kDa form is degraded by proteolysis, resulting in increased IGF-I bioavailability (43) (Fig. 7A). An ADAM-like protease has been proposed to degrade IGFBP-3 in pregnancy serum (44). We have previously shown that ADAM12 binds and cleaves purified IGFBP-3 in vitro (15, 45) but did not determine whether ADAM12 degrades IGFBP-3 in serum. Recombinant ADAM12-S added to nonpregnancy serum did not degrade IGFBP-3 (Fig. 7B). However, when heparin was added to nonpregnancy serum in addition to ADAM12-S, the degradation of IGFBP-3 was promoted (Fig. 7B). A similar pattern was observed when purified ADAM12-PC was used. Notably, the ADAM12 catalytic site mutant (E351Q) did not degrade IGFBP-3 (Fig. 7B), and bacterial heparinase counteracted the cleavage of IGFBP-3 promoted by heparin (Fig. 7C), confirming the specificity of the ADAM12 proteolytic activity and heparin induction.

From these observations, it was concluded that serum IGFBP-3 degradation by ADAM12-PC could be promoted by the binding of heparin. Furthermore, we showed that ADAM12-PC proteolysis of IGFBP-3 was promoted by heparan sulfate but not chondroitin 4- or 6-sulfates or dermatan sulfate (supplemental Fig. 4, A and B).

Activation of IGFBP-3 degradation by ADAM12-PC required heparin oligosaccharides of at least 14 residues and was more efficient with dp16 and dp18 oligosaccharides (Fig. 7D). Therefore, the efficient promotion of IGFBP-3 cleavage by ADAM12-PC required a longer HSGAG (dp16–18) than inhibition of ADAM12-PC cleavage of cmTF and the TNF-α-based substrate (dp8–10) (Fig. 3E). Promotion of ADAM12-PC cleavage of IGFBP-3 was unaffected by IGF-I (supplemental Fig. 4C). Furthermore, ADAM12-PC MutCMut was unable to cleave serum IGFBP-3 in the presence of heparin, indicating that the basic residues in the prodomain and catalytic domain were involved in the promotion (Fig. 7E). IGFBP-3 encodes a well characterized heparin binding region (46). IGFBP-3 with the heparin binding region (residues 242–259) replaced by the corresponding nonheparin binding region of IGFBP-4 (residues 206–223) was not degraded by ADAM12-PC (Fig. 7F). Because promotion of a number of interactions by heparin was previously reported to be accompanied by conformational changes (47), we used CD spectroscopy to analyze the secondary structure of ADAM12-PC upon the addition of heparin. Following heparin addition, changes in the ellipticity could not be observed (Fig. 7G).

Altogether, the results in Fig. 7 demonstrated that binding of a HSGAG to both ADAM12 and IGFBP-3 was required to promote efficient cleavage of IGFBP-3. Based on the data, we suggest that a bridging mechanism similar to that shown in Fig. 7H regulates ADAM12-S cleavage of IGFBP-3.
Osteoarthritis-improving Calcium Pentosan Polysulfate Regulates ADAM12 Proteolytic Activity—Calcium pentosan polysulfate (CaPPS) is a polyanion with weak antithrombotic effects that has been suggested as an osteoarthritis treatment (48). It was recently reported that ADAM12 is up-regulated in osteoarthritis and degrades IGFBP-5 to increase IGF-I bioavailability and stimulate chondrocyte proliferation (14). However, IGFBP-3 degradation was not reported to be increased. Incubation of ADAM12-S with increasing doses of CaPPS inhibited the degradation of cmTF (Fig. 8A). However, CaPPS promoted ADAM12-S degradation of the TNF-α-based substrate as well as ADAM12-PC degradation of IGFBP-3 (Fig. 8C). Cleavage of the TNF-α-based substrate by ADAM17 was unaffected by CaPPS (Fig. 8B). From these data, we conclude that ADAM12 degradation of cmTF and IGFBP-3 are similarly regulated by heparin and CaPPS. CaPPS promotes ADAM12 degradation of the TNF-α-based substrate, whereas heparin is an inhibitor, indicating that ADAM12-mediated proteolysis is regulated by a structure-dependent HSGAG switch that may influence CaPPS treatment of osteoarthritis.

DISCUSSION

Here we present data to propose a novel mechanism for specific regulation of the ADAM12 protease activity by HSGAGs...
both in vitro and under physiological conditions. These conclusions are supported as follows. First, the association of ADAM12-PC with the cell surface was mediated by HSGAGs. Second, HSGAGs inhibited ADAM12-S proteolysis of three different substrates in vitro (cmTF, 82 residues of HB-EGF, and a TNF-α-based peptide) as well as membrane-bound ADAM12 ectodomain shedding of pro-EGF. Interestingly, degradation of serum IGFBP-3 by recombinant ADAM12 was induced by HSGAGs. Third, both cell surface binding and regulation of proteolytic activity was mediated by HSGAG binding to specific clusters of basic residues in the ADAM12 prodomain and catalytic domain.

Mechanism of HSGAG-regulated ADAM12 Proteolysis—The interaction between HSGAGs and ADAM12-PC seems to be reversible, suggesting that it is a molecular switch. HSGAGs that were bound to ADAM12-S could be removed by heparinase treatment, which resulted in proteolytic activation. In contrast, essentially irreversible interactions with HSGAGs have been reported for fibroblast growth factor-2 and matrix metalloprotease-7, which both require 2 M NaCl for elution from heparin-Sepharose (4, 49). We suggest that the affinity of ADAM12-S for heparin is moderate under physiological conditions, because elution from heparin-Sepharose started as soon as 200 mM NaCl. The potency of binding and regulation of the ADAM12 proteolytic activity by heparin and heparan sulfate was similar, indicating a specific interaction with the HSGAG. In contrast to heparin, CaPPS increased the degradation of the TNF-α-based substrate by ADAM12-S (Fig. 8B). This demonstrates that the HSGAG binding site is a molecular switch that responds by promoting or inhibiting substrate proteolysis by interaction with specific anionic structures.

The HSGAG switch of ADAM12-PC binds to cell surface heparan sulfate proteoglycans, and this interaction results in down-regulated shedding of pro-EGF. In this respect, ADAM12 shedding of pro-EGF bears similarity to the shedding of amyloid precursor protein in Alzheimer disease that is mediated by the transmembrane secretase, BACE1, which has been shown to be inhibited by GAGs (50). Inhibition was mediated by binding of GAGs to BACE1 but also to the substrate. GAG binding has been suggested to sequester amyloid precursor protein away from the protease. However, HSGAGs are unable to bind and sequester pro-EGF, because a HSGAG binding site is absent.

Cleavage of pro-EGF by membrane-bound ADAM12 and of cmTF, an 82-residue portion of HB-EGF, and a TNF-α-based peptide substrate by ADAM12-S was inhibited by HSGAGs and
promoted by human heparanase. However, cleavage of IGFBP-3 in serum by ADAM12-S was selectively promoted in the presence of HSGAGs (Fig. 7). A similar proteolytic complex activation has been found in other systems. Formation of protease-antithrombin-heparin ternary complexes inhibits factor Xa and thrombin by a mechanism that includes bridging of the serpin antithrombin to the protease by binding to a specific pentasaccharide sequence in heparin (51). Binding of heparin to antithrombin mediates a conformational change in the serpin that generates an exosite for interaction with factor Xa but not thrombin (52). Furthermore, heparin bound to antithrombin interacts with an exosite on both thrombin and factor Xa and mediates formation of the ternary complex (53). A heparin binding site on IGFBP-3 has previously been described and characterized (54).

Our investigations of IGFBP-3 cleavage suggested that the presence of heparin or heparinase respectively promoted or disrupted the interaction between ADAM12 and IGFBP-3. From the data, we propose that the heparin binding region of IGFBP-3 and the switch in ADAM12-PC are bridged by HSGAGs (Fig. 7, E, F, and H). Binding of heparin to IGFBP-3 has been shown to displace IGF-I (55). However, the heparin-induced cleavage of IGFBP-3 by ADAM12-S was independent of IGF-I (supplemental Fig. 4C). Our experiments showed that an HSGAG of at least 14 monosaccharide units was required to induce ADAM12-S cleavage of IGFBP-3 (Fig. 7D). However, inhibition of ADAM12-S proteolysis of cmTF required only 8–10 monosaccharide units (Fig. 3E). This difference indicated that formation of the proposed IGFBP-3-ADAM12-S-HSGAG ternary complex required a longer oligosaccharide than inhibition of ADAM12-S cleavage of cmTF. We hypothesized that ADAM12 may change its conformation when binding heparin; however, we were unable to detect changes in the secondary structure of ADAM12-PC in the presence of heparin (Fig. 7G).

Conservation of the HSGAG Switch—Many proteins interacting with heparin and HSGAGs at the cell surface contain clusters of basic amino acids that form binding sites for saccharide polyanions (56). This was demonstrated here for ADAM12-PC, where mutation of basic residues abolished binding to HSGAGs at the cell surface. We suggest that the basic residues responsible for binding of HSGAGs are exposed at the surface of the prodomain and catalytic domain, where they form a structural molecular switch. Three-dimensional structural studies of the ADAM prodomains are under way, and we have recently proposed a secondary structure composed of β-strands (18). The identified HSGAG binding motif in the ADAM12 prodomain was located just after the last predicted β-strand. Conservation of the implicated residues (Lys158, Arg163, Lys165, Lys170, Lys171, and Lys173) was not revealed when aligning the sequences of the PC domains of 18 different human ADAMs (supplemental Fig. 5). However, among the ADAM12 genes of 13 sequenced species, there is strong conservation of Lys158, Arg163, and Lys165, whereas Lys170, Lys171, and Lys173 are less conserved (supplemental Fig. 6). Therefore, based on sequence analysis, binding of HSGAGs to the basic residues in the prodomain is not a universal feature of the ADAMs. However, the residues in the catalytic domain (Arg229, Arg232, and Lys236) are highly conserved among ADAM12 sequences from different species and less well conserved among the ADAMs.

Altogether, our data have identified a HSGAG switch mechanism. The conservation of the switch among different ADAMs and species remains to be experimentally investigated. Understanding and identifying similar specific switch mechanisms in other ADAMs is essential for the development of targeted therapies for ADAM metalloproteases.

Physiological Implications of ADAM12 Regulation by HSGAGs—ADAM12 shedding of pro-EGF releases the growth factor EGF, and ADAM12-S proteolysis of IGFBP-3 releases IGF-I. Both processes are regulated by HSGAGs and human heparanase through a switch in ADAM12-PC that therefore

FIGURE 8. Calcium pentosan polysulfate regulates ADAM12 proteolytic activity. Degradation of cmTF (A) and the TNF-α-based substrate (B) by ADAM12-S (A) and ADAM12-S and ADAM17 (B) was measured in the presence of increasing doses of CaPPS. C, Western blot (WB) showing degradation of serum IGFBP-3 by ADAM12-PC in the presence of CaPPS and heparin.
CaPPS may promote ADAM12 degradation of IGFBP-3 and ADAM12 proteolytic activity. According to the heparan sulfate but lack of effect of galactosaminoglycans on highly chondroitin sulfate-rich environment. This is consistent lage and degrades IGFBP-5 but not IGFBP-3 in chondrocyte showed that ADAM12 is overexpressed in osteoarthritic carti-
and chondrocyte proliferation in transgenic mice (65). Others the proteolytic activity of ADAM12-S stimulates bone growth
liferation in articular cartilage (64). We showed previously that and IGFBPs are major anabolic regulators of chondrocyte pro-
growth factors are intimately related to cancer cell proliferation
IGF and EGF by ADAM12 is regulated by HSGAGs. These
Here we have described how the release of the growth factors IGF and EGF by ADAM12 is regulated by HSGAGs. These growth factors are intimately related to cancer cell proliferation and metastasis (62, 63).
In addition to their importance in cancer and pregnancy, IGF and IGFBPs are major anabolic regulators of chondrocyte prol-
er brings in articular cartilage (64). We showed previously that the proteolytic activity of ADAM12-S stimulates bone growth and chondrocyte proliferation in transgenic mice (65). Others showed that ADAM12 is overexpressed in osteoarthritic cartil-
age and degrades IGFBP-5 but not IGFBP-3 in chondrocyte cultures (14). In this case, ADAM12 would be functional in a highly chondroitin sulfate-rich environment. This is consistent with the current data showing specific effects of heparin and heparan sulfate but lack of effect of galactosaminoglycans on ADAM12 proteolytic activity. According to the in vitro results presented here, treatment of patients with the heparan analog CaPPS may promote ADAM12 degradation of IGFBP-3 and stimulate chondrocyte proliferation in osteoarthritic cartilage.

Acknowledgments—We kindly thank Israel Vlodavsky (Bruce Rappaport Faculty of Medicine, Technion, Israel) for providing the plasmid for expression of human heparanase, Shigeki Higashiyama (Department of Biochemistry and Molecular Genetics, Ehime University, Ehime, Japan) for providing the plasmid for expression of pro-EGF-AP, Hideaki Nagase (Kennedy Institute of Rheumatology Division, Imperial College London, London, UK) for providing CaPPS, and Zulfiya Sukhova and Jean-Pierre Andrieu for technical assistance.

REFERENCES
1. Tumova, S., Woods, A., and Couchman, J. R. (2000) Int. J. Biochem. Cell Biol. 32, 269–288
2. Couchman, J. R. (2003) Nat. Rev. Mol. Cell. Biol. 4, 926–937
3. Baman, R., Sasisekharan, V., and Sasisekharan, R. (2005) Chem. Biol. 12, 267–277
4. Kreuger, J., Spillmann, D., Li, J. P., and Lindahl, U. (2006) J. Cell Biol. 174, 323–327
5. Sasisekharan, R., Shriver, Z., Venkataraman, G., and Narayanasami, U. (2002) Nat. Rev. Cancer 2, 521–528
6. Li, J. P. (2008) Anticancer Agents Med. Chem. 8, 64–76
7. Cohen, M., and Bischof, P. (2007) Glycobiol. 176, 2093–2101
8. Kveiborg, M., Albrechtsen, R., Couchman, J. R., and Wewer, U. M. (2008) Int. J. Biochem. Cell Biol. 40, 1685–1702
9. Iba, K., Albrechtsen, R., Gilpin, B. J., Loechel, F., and Wewer, U. M. (1999) Am. J. Pathol. 154, 1489–1501
10. Förhlich, C., Albrechtsen, R., Dyrskjet, L., Rudkær, L., Ørntoft, T. F., and Wewer, U. M. (2006) Clin. Cancer Res. 12, 7359–7368
11. Roy, R., Wewer, U. M., Zurakowski, D., Pories, S. E., and Moses, M. A. (2004) J. Biol. Chem. 279, 51323–51330
12. Kveiborg, M., Förhlich, C., Albrechtsen, R., Tischler, V., Dietrich, N., Holck, P., Kronesvist, P., Rank, F., Mercurio, A. M., and Wewer, U. M. (2005) Cancer Res. 65, 4754–4761
13. Peduto, L., Reuter, V. E., Sehara-Fujisawa, A., Shaffer, D. R., Scher, H. I., and Blobel, C. P. (2006) Oncogene 25, 5462–5466
14. Okada, A., Mochizuki, S., Yatabe, T., Kimura, T., Shiomi, T., Fujita, Y., Matsumoto, H., Sehara-Fujisawa, A., Iwamoto, Y., and Okada, Y. (2008) Arthritis Rheum. 58, 778–788
15. Loechel, F., Fox, J. W., Murphy, G., Albrechtsen, R., and Wewer, U. M. (2000) Biochem. Biophys. Res. Commun. 278, 511–515
16. Horiuchi, K., Le Gall, S., Schulte, M., Yamaguchi, T., Reiss, K., Murphy, G., Toyama, Y., Hartmann, D., Saftig, P., and Blobel, C. P. (2007) Mol. Biol. Cell 18, 176–188
17. Loechel, F., Gilpin, B. J., Engvall, E., Albrechtsen, R., and Wewer, U. M. (1998) J. Biol. Chem. 273, 16993–16997
18. Sorensen, H. P., Jacobsen, J., Niebø, S., Poulsen, F. M., and Wewer, U. M. (2008) Protein Expression Purif. 61, 175–183
19. Wewer, U. M., Mörgelin, M., Holck, P., Jacobsen, J., Lydolph, M. C., Johnsen, A. H., and Albrechtsen, R. (2006) J. Biol. Chem. 281, 9418–9422
20. Schlamom, U., Wildeboer, D., Webster, A., Antropova, O., Zeuschner, D., Knight, C. G., Docherty, A. J., Lambert, M., Skelton, L., Jocksch, H., and Bartsch, J. W. (2002) J. Biol. Chem. 277, 48210–48219
21. Moss, M. L., Bomar, M., Liu, Q., Sage, H., Dempsey, P., Lenhart, P. M., Gillispie, P. A., Stoeck, A., Wildeboer, D., Bartsch, J. W., Palmisano, R., and Zhou, P. (2007) J. Biol. Chem. 282, 35712–35721
22. Roghani, M., Becherer, J. D., Moss, M. L., Atherton, R. E., Erdjument-Bromage, H., Arribas, J., Blackburn, R. K., Weskamp, G., Tempst, P., and Blobel, C. P. (1999) J. Biol. Chem. 274, 3531–3540
23. Lum, L., Reid, M. S., and Blobel, C. P. (1998) J. Biol. Chem. 273, 26236–26247
24. Garlisi, C. G., Zhou, J., Devito, K. E., Tian, F., Zha, F. X., Liu, J., Shah, H., Wan, Y., Motasim Billah, M., Egan, R. W., and Umland, S. P. (2003) J. Biol. Chem. 278, 3531–3540
25. Schlondorff, J., Becherer, J. D., and Blobel, C. P. (2000) Biochem. J. 347, 131–138
26. Gonzales, P. E., Solomon, A., Miller, A. B., Leesnitzer, M. A., Sagi, I., and Milla, M. E. (2004) J. Biol. Chem. 279, 31638–31645
27. Leonard, J. D., Lin, F., and Milla, M. E. (2005) Biochem. J. 387, 797–805
28. Coussens, L. M., Fingleton, B., and Matrisian, L. M. (2002) Science 295, 2387–2392
29. Vives, R. R., Goedger, S., and Pye, D. A. (2001) Biochem. J. 354, 141–147
30. Gilpin, B. J., Loechel, F., Mattei, M. G., Engvall, E., Albrechtsen, R., and Wewer, U. M. (1998) J. Biol. Chem. 273, 157–166
31. Kristensen, J., Sperling-Petersen, H. U., Mortensen, K. K., and Sorensen, H. P. (2005) Int. J. Biochem. Mol. Biol. 37, 212–217
32. Jacobsen, J., Visse, R., Sorensen, H. P., Engblad, J. J., Brew, K., Wewer, U. M., and Nagase, H. (2008) Biochemistry 47, 537–547
