Proteolysis of the hyalectans (aggrecan, versican, brevican) in vivo appears to result from the activity of ADAMTS4 (aggrecanase-1, herein referred to as an hyalectanase). To examine the mode of activation of ADAMTS4, a human chondrosarcoma cell line, JJ012, has been stably transfected with the full-length c-DNA for human ADAMTS4. The cells synthesized a high molecular weight form of the enzyme (p100), which in serum-free culture was processed to three truncated forms, p75, p60, and p50. Treatment of the p100 form with recombinant furin indicated that the p75 form is generated by the removal of the prodomain by a furin-like activity. Analysis with domain-specific antisera showed that the p60 and p50 forms are generated by C-terminal truncation of the p75 form. The appearance of the p60 and p50 forms in culture medium was prevented by inclusion of a furin inhibitor, inhibitors of glycosylphosphatidylinositol synthesis, glucosamine, a hydroxamate-based matrix metalloproteinase (MMP) inhibitor, and TIMP-1, but not by AEBSF (4-(2-aminoethyl)benzenesulfonamide) or E64. Only medium samples containing the p60/p50 forms exhibited aggrecanase activity, and isolation of the p75, p60, and p50 forms by preparative SDS-PAGE showed that only p60 and p50 were active in aggrecanase and versicanase assays. Pig synovium and human cartilages also contained ADAMTS4 in the p75, p60, and p50 forms. We suggest that in vivo production of proteolytically active ADAMTS4 requires not only removal of the prodomain by a furin-like activity but also MMP-mediated removal of a portion of the C-terminal spacer domain.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Shriners Hospital for Children, 12502 North Pine Dr., Tampa, FL 33612-9499. Tel.: 813-972-2250; Fax: 813-975-7127; E-mail: jsandy@shctampa.usf.edu.

‡ The abbreviations used are: ECM, extracellular matrix; MMP, matrix metalloproteinase; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; DMEM, Dulbecco’s modified Eagle’s medium; GPI, glycosylphosphatidylinositol.

Activation of the Proteolytic Activity of ADAMTS4 (Aggrecanase-1) by C-terminal Truncation*

Received for publication, August 3, 2001, and in revised form, January 8, 2002
Published, JBC Papers in Press, January 16, 2002, DOI 10.1074/jbc.M107443200

Gui Gao‡, Jennifer Westling‡, Vivian P. Thompson‡, Troy D. Howell‡, Paul E. Gottschall§, and John D. Sandy‡§
From the ‡Center For Research in Skeletal Development and Paediatric Orthopaedics, Shriners Hospital for Children and the §Department of Pharmacology and Therapeutics, University of South Florida, Tampa, Florida 33612

Aggrecan, versican, neurocan, and brevican are components of the extracellular matrix (ECM) in a wide range of tissues. They are all members of the family of large aggregating proteoglycans (1), which are characterized by an N-terminal globular domain that binds to hyaluronan. They have therefore been included, along with related species such as link protein and CD44, in the molecular grouping termed hyaladherins (2). At the same time they are all synthesized with a C-terminal globular domain that is related structurally to selectins, consisting of a C-type lectin domain flanked by epidermal growth factor and complement regulatory protein domains. Because of this structural feature they have also been given the family name of lecticans (3). In an attempt to accommodate the functionality of both the N-terminal and C-terminal globular domains, and also to indicate their proteoglycan nature, the group has also been termed the hyalectans (4).

Proteolytic degradation of the hyalectans in the ECM appears to result from the activity of a subgroup of the ADAMTS family of metalloproteinases, all of which exhibit some degree of glutamyl-endopeptidase activity for specific Glu-X bonds (where X is most often Ala or Gly) in these glycosaminoglycan-substituted substrates. Thus, ADAMTS1, -4, and -5 exhibit “aggrecanase” activity (5–7), ADAMTS1 and -4 exhibit “versicanase” activity (8), and ADAMTS4 exhibits “brevicanase” activity (9). Among this group, ADAMTS4 appears to exhibit the highest level of activity for each substrate, and we propose here the term “hyalectanase” to describe this activity. Consistent with their common substrate specificity, ADAMTS1, -4, and -5 exhibit quite a high degree of sequence homology and in this regard are quite distinct from a second subgroup, ADAMTS2, -3, and -14, all of which are procollagen N-proteinases.

Although 14 members of the ADAMTS family have now been cloned, and some have been expressed and purified, there has been no information generated on the mode of activation of these proteinases. Active ADAMTS4 (aggrecanase-1) was initially purified as a 62-kDa doublet protein from bovine nasal cartilage explants (10) and was subsequently cloned (6) as an 837-residue protein containing a prodomain (residues 1–207), a furin cleavage site (residues 208–212), a catalytic domain (residues 213–440), a disintegrin-like motif (residues 441–462), a thrombospondin-1-like motif (residues 463–547), a Cys-rich domain (residues 548–694), and a C-terminal spacer domain (residues 695–837) (see diagram of domains in Fig. 3). Expression of the recombinant protein (6) generated an active 64-kDa species with the same N terminus as the natural protein, at phenylalanine 213, confirming that removal of the prodomain by a furin-like activity is required for generation of an active form of the enzyme. Expression of an inactive mutant lacking the TSP-1 motif and the identification of substrate binding activity within the TSP-1 region at GGWGPWGPWGD (residues 521–532) (11) provided strong evidence that the TSP-1 motif is necessary for activity, together suggesting that the species spanning residues 213–547 is the minimum requirement for an active form of the enzyme. However, the precise C terminus of the active 62–64 kDa proteinase was not determined (6), and so the extent to which the Cys-rich domain (residues 548–694) and the C-terminal spacer (residues 695–837) modulate the proteolytic activity of the proteinase is unknown. In this regard it is interesting to note that the C-terminal
Activation of Hyalectanase Activity of ADAMTS4

hemopexin-like domain of the matrix metalloproteinase (MMP) family modulates both the proteolytic activity and inhibitor binding properties of these proteinases (12).

To investigate the potential role of the C-terminal Cys-rich and spacer domains of ADAMTS4, we have established a stably transfected human chondrosarcoma cell system, which in serum-free culture processes recombinant ADAMTS4 to generate multiple forms. Structural analysis and activity assays for these forms has now shown that in addition to removal of the prodomain by a furin-like activity, C-terminal processing within the spacer domain is required to generate species that can degrade aggrecan and versican. Importantly, analysis of ADAMTS4 in extracts of cartilage and synovium has shown that these C-terminally truncated species are also the dominant forms of the enzyme in normal tissues.

**EXPERIMENTAL PROCEDURES**

**Materials**—citrate buffer (20 mM Tris, 10 mM CaCl2, 100 mM NaCl), pH 7.5 (buffer 1); these were dissolved in bottled water (Invitrogen) and supplemented with sodium bicarbonate (4.7 g/liter), ascorbic acid (50 mg/liter), gentamycin (50 mg/liter), and 10% fetal calf serum (HyClone). At about 80% confluence, the growth medium was replaced, and the cells (about 7 × 10^6 cells) were washed twice with catalytic medium (23000 DMEM powder (Invitrogen), 1000 mM EDTA, 150 mM NaCl, and 0.5% Nonidet P-40) (40). The cell lysates were centrifuged at 14,000 × g for 15 min at 4 °C. The concentration was 10-fold by freezing lyophilization and stored at −80 °C. Cells were removed from the dish in cold phosphate-buffered saline by gentle pipetting and shaking and then pelleted by centrifugation immediately lysed in 40 μl of buffer (50 mM Tris-Cl, pH 8.0, 5 mM EDTA, 150 mM NaCl, and 0.5% Nonidet P-40) (40). The ECL detection system was from Amersham Biosciences, Inc.

**Cell Culture and Treatment for ADAMTS4 Processing**—Cells were maintained in the serum-free medium for 4 days. Both the medium and ECM were harvested using the same methods as for JJ012 cells. The medium was solubilized in the buffer at −80 °C.

**Isolation, Analysis, and Assay of ADAMTS4 Truncated Forms from Proteins**—Proteins (30 μg) were run on SDS-PAGE gels (20 μl/lane) was fractionated on 6% SDS-PAGE Mini-gels (Bio-Rad) under nonreducing conditions. The position of the individual ADAMTS4 forms was determined by Western analysis of the outer lanes with anti-VMAH, and the gel was sliced into 2 mm × 5 cm “bands” with a razor blade. The individual gel slices were crushed and rocked in 200 μl of 20 mM Tris, 10 mM CaCl2, 100 mM NaCl, 2% Triton X-100, pH 7.5, at 4 °C for 20 h to elute the proteins for the purity check. Portions of the eluates were taken for Western analysis of ADAMTS4 with anti-VMAH. For determination of the activity of individual SDS-PAGE-separated species, the freshly prepared gel bands (about 5 cm × 2 mm) were washed in 2.5% Triton X-100 for 1 h at 4 °C to remove SDS, and after a brief wash rinse the gels were added to 200 μl of assay buffer containing either rat chondrosarcoma aggrecan (500 ng/ml) or the recombinant β-GAG domain of human versican as described previously (8). After incubation at 37 °C for 20 h the gel was removed, and the digested aggrecan was dehydrated as described previously (14). Portions of digests were taken for Western analysis as described (8) with anti-NITEGE (aggrecan neoepitope) and anti-DPEAAE (versican neoepitope).

**Western Analysis with recombinant preparations of ADAMTS5 and ADAMTS4**

**Preparation of Cells, Medium, and Extracellular Matrix**—Serum-free conditioned medium from JJ012 cells was collected by centrifugation at 14,000 × g for 15 min at 4 °C. The medium was concentrated 10-fold by freezing lyophilization and stored at −80 °C. Cells were removed from the dish in cold phosphate-buffered saline by gentle pipetting and shaking and then pelleted by centrifugation and immediately lysed in 40 μl of buffer (50 mM Tris-Cl, pH 8.0, 5 mM EDTA, 150 mM NaCl, and 0.5% Nonidet P-40) (40). The cell lysates were centrifuged at 14,000 × g for 30 min at 4 °C, and the supernatant was stored at −80 °C. ECM, which remained attached to the dish, was washed twice with cold phosphate-buffered saline to remove residual cells; then 1 ml of lysis buffer (as above) was added, and the ECM was solubilized in the buffer for storage at −80 °C.

**Activation of Hyalectanase Activity of ADAMTS4**

Cells were centrifuged at 14,000 × g for 30 min at 4 °C, and the supernatant was stored at −80 °C. ECM, which remained attached to the dish, was washed twice with cold phosphate-buffered saline to remove residual cells; then 1 ml of lysis buffer (as above) was added, and the ECM was solubilized in the buffer for storage at −80 °C.

**Materials**—citrate buffer (20 mM Tris, 10 mM CaCl2, 100 mM NaCl), pH 7.5 (buffer 1); these were dissolved in bottled water (Invitrogen) and supplemented with sodium bicarbonate (4.7 g/liter), ascorbic acid (50 mg/liter), gentamycin (50 mg/liter), and 10% fetal calf serum (HyClone). At about 80% confluence, the growth medium was replaced, and the cells (about 7 × 10^6 cells) were washed twice with catalytic medium (23000 DMEM powder (Invitrogen), 1000 mM EDTA, 150 mM NaCl, and 0.5% Nonidet P-40) (40). The cell lysates were centrifuged at 14,000 × g for 15 min at 4 °C. The concentration was 10-fold by freezing lyophilization and stored at −80 °C. Cells were removed from the dish in cold phosphate-buffered saline by gentle pipetting and shaking and then pelleted by centrifugation and immediately lysed in 40 μl of buffer (50 mM Tris-Cl, pH 8.0, 5 mM EDTA, 150 mM NaCl, and 0.5% Nonidet P-40) (40). The cell lysates were centrifuged at 14,000 × g for 30 min at 4 °C, and the supernatant was stored at −80 °C. ECM, which remained attached to the dish, was washed twice with cold phosphate-buffered saline to remove residual cells; then 1 ml of lysis buffer (as above) was added, and the ECM was solubilized in the buffer for storage at −80 °C. Cells were removed from the dish in cold phosphate-buffered saline by gentle pipetting and shaking and then pelleted by centrifugation and immediately lysed in 40 μl of buffer (50 mM Tris-Cl, pH 8.0, 5 mM EDTA, 150 mM NaCl, and 0.5% Nonidet P-40) (40). The cell lysates were centrifuged at 14,000 × g for 30 min at 4 °C, and the supernatant was stored at −80 °C. ECM, which remained attached to the dish, was washed twice with cold phosphate-buffered saline to remove residual cells; then 1 ml of lysis buffer (as above) was added, and the ECM was solubilized in the buffer for storage at −80 °C. Cells were removed from the dish in cold phosphate-buffered saline by gentle pipetting and shaking and then pelleted by centrifugation and immediately lysed in 40 μl of buffer (50 mM Tris-Cl, pH 8.0, 5 mM EDTA, 150 mM NaCl, and 0.5% Nonidet P-40) (40). The cell lysates were centrifuged at 14,000 × g for 30 min at 4 °C, and the supernatant was stored at −80 °C. ECM, which remained attached to the dish, was washed twice with cold phosphate-buffered saline to remove residual cells; then 1 ml of lysis buffer (as above) was added, and the ECM was solubilized in the buffer for storage at −80 °C.
Preparation of ADAMTS4 from Cartilage and Synovium—Normal full-depth human tibial cartilage was supplied by Dr. Ada Cole of Rush University, Chicago, IL. Normal human femoral notch cartilage was supplied by Dr. Fred Nelson, San Diego Naval Base. Synovium was obtained from the hock joint of 12-week-old pigs and either extracted fresh or maintained (200 mg wet wt per ml) in catabolic medium for 4 days with daily medium changes. For Western analysis, conditioned medium was combined and concentrated 10-fold by lyophilization, and cultures were terminated after 24 h. Portions of lysed cells, ECM, and medium (see “Experimental Procedures” for details of preparation) were taken for Western analysis with an antisera to ADAMTS4 (anti-VMAH). The analysis shown is typical of three experiments and was obtained with samples that represented 5% of the product in each compartment in a single culture plate. The – lanes are products of wild-type cells, and the + lanes are products of ADAMTS-4 transfectants. Purified recombinant ADAMTS-4 was used to generate the standard curve shown in the top panels (means ± S.E. of three separate analyses), which relates integrated pixel density (i.p.d.) to ng of ADAMTS4 protein with 1-min film exposure. For this purpose, standard bands were analyzed with Scion Image software as integrated pixel densities (with background subtraction) using the Measure function. For unknowns, all gels were internally calibrated with at least two standards, and films were exposed for different times up to 5 min with quantitation within the linear range of response (up to 180 integrated pixel density units).

RESULTS

Expression of ADAMTS4 by Stably Transfected Human Chondrosarcoma Cells—Near confluent 3-day cultures of human chondrosarcoma cells stably transfected with ADAMTS4 and the nontransfected controls were transferred to serum-free medium. After 1 day, the medium, ECM, and cell pellet fractions were prepared for Western analysis with anti-VMAH (Fig. 1, bottom panels). In contrast to the wild-type cells, which produced essentially undetectable ADAMTS-4 (lanes labeled with a –), the transfected cells (lanes labeled with a +) produced immunoreactive ADAMTS4, which was present as a p100 species (cells only), p75 species (in all fractions but highly enriched in the ECM), p60 species (mostly in the medium with traces in the ECM), and traces of a p50 species (ECM and medium). Quantitation from the standard curve shown at top (see figure legend for details) indicated that these cells produced a total (all species) of about 5 μg of enzyme protein/plate (about 7 × 10^9 cells at termination) over the 4-day culture period, the majority of which was present as the p75 form in the ECM.

ADAMTS4 Processing by Human Chondrosarcoma Cells—To examine the origins of the multiple species generated in this system, we next extended the period of serum-free culture and terminated plates at 1, 2, 3, and 4 days (Fig. 2). Analysis of cells, ECM, and medium showed the p100 form in the cells only and a time-dependent processing of the p75 form, which was partially depleted from the ECM over the 4 days and concomitantly enriched in the medium, along with increasing amounts of p60 and p50. Quantitation from the standard curve (Fig. 1) showed that the 4-day medium contained the p75, p60, and p50 forms at concentrations of about 350, 250, and 100 ng/ml, respectively.

To analyze these forms further, we next probed portions of the 4-day medium with antisera to the catalytic domain (anti-VMAH), the Cys-rich region (anti-YNHR), and the FLAG tag on the C terminus of the expressed protein (see diagram in Fig. 3). Each species exhibited a characteristic reactivity pattern. The p100 species reacted weakly with anti-VMAH and quite strongly with anti-YNHR and anti-FLAG, whereas the p75 reacted strongly with all three antisera. The p60 reacted weakly with anti-YNHR, strongly with anti-VMAH, and not at all with anti-FLAG, whereas the p50 reacted weakly with anti-VMAH, strongly with anti-YNHR, and not at all with anti-FLAG. The reactivity pattern shown (Fig. 3) and the relative sizes of the species suggest a structural model in which the p100 represents the full-length protein (residues 1–837) and the p75 represents the form generated by removal of the prodo-
activity in this conversion to the p75 form, consistent with the involvement of a furin-like VMAH (Fig. 4) showed that furin digestion converted the p100 recombinant furin followed by Western analysis with anti- without processing. Incubation of medium with and without nosamine are potent metabolic inhibitors of glycosylphosphatidylinositol anchor synthesis, which like glucosamine are known to block interleukin-1-induced aggrecanase activity (16); MMP inhibitor II and TIMP-1 are effective inhibitors of extracellular MMP-dependent processing; AEBSF and E64 are broad spectrum inhibitors of serine and cysteine proteinases respectively, without the capacity to enter cells.

The yield and distribution of the p100, p75, p60, and p50 forms of ADAMTS4 in each culture condition are shown in Fig. 5. Three different patterns were observed. In control cells (lane C) and in cells treated with either E64 (lane E) or AEBSF (lane A), the distribution was essentially identical, showing that the processing of ADAMTS4 in this system was not affected by broad spectrum inhibitors of serine or cysteine proteinases. In these cultures, all of the p100 originally in the cells appears to have been converted to the p75 form, which is found in the cells (top panel) and abundantly in the ECM (middle panel). In addition, a portion (perhaps 50%) of the p75 associated with the cells and ECM is released to the medium (bottom panel) as the p75 form (major), the p60 form (major), and a small amount of the p50 form. The simplest explanation of this process is that under control conditions the p100 (full-length) form is secreted in the p75 form and that this intermediate is processed in the ECM to the p60/50 forms, which are released to the medium. A small proportion of the unprocessed p100 was also released into the medium in all cultures.

In a second pattern, exhibited by cells treated with TIMP-1 (lane T) or the hydroxamate-based MMP inhibitor (lane MMP), the relative abundance of the forms and their distribution at 3 days was quite different to control. There was a partial inhibition of conversion of the p100 to the p75 form, less p75 accumulated in the ECM, and essentially no p75 was released into the medium. In addition there was total inhibition of the appearance of the p60/p50 forms in the medium. Therefore, the overall effect of the metalloproteinase inhibitors was complete inhibition of the conversion of p75 to p60/50 along with a marked inhibition of the conversion of p100 to p75.

In a third pattern, cells treated with either 2-deoxyfluorogluco- case (lane 2DFG), glucosamine (lane G), mannosamine (lane M), or decanoyl-RVKR (lane dec) showed even greater changes relative to controls. In these cultures there was almost total inhibition of the conversion of the p100 form to other forms, so that very little p75 was generated in any compartment, and there was, predictably, total inhibition of the formation of the p60/p50 species. It therefore appears that whereas conversion of the p100 to the p75 was inhibited by decanoyl-RVKR and therefore requires a furin-like activity, this activity alone may not be sufficient for efficient removal of the prodomain. Thus inhibitors of GPI anchor formation (mannosamine and 2-deoxyfluoro- glucose), as well as glucosamine, blocked C-terminal truncation but also effectively blocked removal of the prodomain in these cells.

**Inhibition of C-terminal Processing Blocks the Appearance of Active ADAMTS4 in the Medium**—When medium from each of the cultures described in Fig. 5 was taken for aggrecanase assay, it was found that only those samples that contained the C-terminally truncated p60/p50 forms (lanes C, E, and A) were active (Fig. 6). Indeed, semi-quantitation of the p60/50 forms in these samples by Western analysis showed that the active media contained about 250 and 100 ng/ml of these forms, respectively. Accordingly, the aggrecanase assay (see “Experimental Procedures” for details) showed that the 3 ml of medium collected from the C, E, and A cultures contained about 30 units of activity (see “Experimental Procedures” for definition of unit), whereas inhibitor-treated cultures (lanes 2DFG, G, M, dec, MMP, and T on Fig. 6) were totally inactive. The results clearly confirmed that the p100 proform is inactive, because it...
Fig. 3. Structural analysis of ADAMTS4 species present in chondrosarcoma cell cultures. Portions of medium collected after 4 days of culture in serum-free medium were studied by Western analysis with anti-VMAH, anti-YNHR, and anti-FLAG. The locations of the epitopes are shown in the schematic, which also illustrates the domain structure and the residue numbers at the domain borders. Analysis is shown at low (L) and high (H) film exposure for anti-VMAH and anti-YNHR.

Fig. 4. Furin-mediated conversion of the p100 form of ADAMTS4 to the p75 form. Portions of medium from ADAMTS4 stably transfected VA13 cells (CONT) were incubated without (−) and with (+) recombinant furin, and the products were analyzed with an antiserum to ADAMTS4 (anti-VMAH).

was present in all samples, and also suggested that processing to the p75 form, and most importantly subsequent formation of the p60/50 forms, is necessary for the generation of active ADAMTS4 (aggrecanase) in this system.

Only the p60 and p50 Truncated Forms of ADAMTS4 Exhibit Aggrecanase and Versicanase Activity—The results of the experiment shown in Fig. 6 suggested that C-terminal truncation to the p60 and/or p50 forms is required for generation of the aggrecanase activity of the expressed ADAMTS4 in this system. To test this directly, portions of day 4 medium containing major immunoreactive species at 125, 75, 60, and 50 kDa were dialyzed and concentrated for preparative SDS-PAGE (see “Experimental Procedures”). The p100 species was apparently at very low abundance in these particular media samples, and the nature of the p125 species shown in Fig. 7 is unknown. The individual proteins were effectively separated as shown by Western analysis of the gel-separated fractions (Fig. 7, top). The gel slices containing these species were next assayed for aggrecanase activity (Fig. 7, bottom left) and versicanase activity (Fig. 7, bottom right). These assays use anti-neoeptope antisera, which are nonreactive with the intact substrates, which for native aggrecan migrates at more than 250 kDa (bottom left panel) and for intact recombinant β-GAG substrate (8) migrates at about 50 kDa (bottom right panel). The results showed clearly that for both substrates the high majority of the proteolytic activity was associated with gel slices 3 and 4, which contained the p60 and p50 species, respectively. In contrast gel slices 1 (p125/p100 form) and 2 (p75 form) exhibited a trace amount of aggrecanase but no detectable versicanase activity. This result provided an explanation for the aggrecanase activity data shown in Fig. 6 and fully support the idea that C-terminal truncation is essential for the generation of proteolytically active forms of ADAMTS4.

Truncation of ADAMTS4 Occurs in Normal Joint Tissues in Vivo—To examine the extent to which C-terminal truncation is a feature of ADAMTS4 processing in normal tissues, we studied the immunoreactive forms present in fresh normal pig synovial membrane, medium from explanted membrane, and fresh normal human tibial and femoral articular cartilages (Fig. 8). In the synovial membrane (lane 1) there was evidence for a proform (about p125) and both the product of furin-like activity (p75) and the C-terminally cleaved product (p60). After explant culture, the tissue appeared to release into the medium (lane 2) abundant p100 form along with some p75 and p50 forms. In articular cartilages (lanes 3–6) there were trace amounts of extractable proforms (p125 and p100), relatively abundant p75 and p60 forms, but apparently no p50 form. A schematic depicting the proposed domain structures for the p100, p75, p60, and p50 forms is shown at the bottom of Fig. 8.

DISCUSSION

The results presented here with human chondrosarcoma cells can most readily be explained by a model for ADAMTS4 processing (see Fig. 8, lower panel) in which the initial full-length 837-residue intracellular product (p100) is processed by removal of the prodomain (residues 1–212) to produce the secreted and matrix-associated product (p75). This product accumulates in the cells and ECM during growth of the cells in serum-containing medium (Fig. 1). On removal of the serum (Fig. 2) it appears that a second proteolytic event is initiated, and this results in at least two further cleavages (within the region of the spacer domain represented by residues 694–837) with the formation of the p60 and p50 forms. Interestingly these most truncated forms appear almost exclusively in the medium, showing that they no longer associate with the ECM, much as was shown by C-terminal deletion of recombinant ADAMTS1 (19).

This model of ADAMTS4 processing therefore appears to be similar to that described in recent studies of a number of other ADAMTS family members. Expression of recombinant ADAMTS1 by 293T cells generated a p87 form, which was processed at the furin site, and a p65 form, which was generated by subsequent C-terminal processing (20). The position of this C-terminal proteolysis was identified as very close to residue 744, which is in the same region identified in the present work
for ADAMTS4 truncation. Whether such processing alters the proteolytic activity of ADAMTS4 has not been determined. However, in earlier work with murine ADAMTS1 (19) it was shown that the C-terminal region was responsible for binding of the protease to ECM, whereas mutants consisting only of the catalytic, disintegrin, and TSP domain (C-terminal at residue 615) exhibited proteolytic activity against the bait region of α2-macroglobulin.

In a similar study with ADAMTS12 (21) expressed by COS7 cells, the initial recombinant product was shown to be processed by furin to remove the prodomain and subsequently processed within the C-terminal region. The authors concluded that upon synthesis, ADAMTS12 is subjected to furin-mediated cleavage followed by an intracellular maturation process leading to the generation of a fragment containing the N-terminal region of the molecule (including the metalloproteinase, disintegrin-like, Cys-rich, and TSP-1 domains) and a C-terminal fragment containing the spacer-2 and the four additional TSP-1 domains characteristic of ADAMTS12. Significantly, in relation to the present study, the processing was partially inhibited by the metalloproteinase inhibitor BB94, and the processing site appeared to be in the Cys-rich/spacer region, which could again make it equivalent to the process described here for ADAMTS4.

The present study therefore provides new insights into an important possible function of such C-terminal processing for the ADAMTS proteins, which is the generation of proteolytically active forms. This finding poses the central question of how C-terminal truncation is mediated in vivo. Interestingly, in human chondrosarcoma cells expressing high levels of recombinant ADAMTS4, the processing and activation of ADAMTS4 was inhibited by a range of additives (Fig. 5). Perhaps most revealing is the finding that both MMP inhibitors (TIMP-1 and Calbiochem MMP inhibitor II) were effective blocking agents, whereas neither AEBSF nor E64 had any effect. Thus it appears that a TIMP-1-sensitive matrix metalloproteinase, but not an extracellular serine or cysteine proteinase, is involved in this process in this cell system. In addition, although the metalloproteinase inhibitors (Fig. 5) completely blocked conversion of p75 to p60/50, they also partially (maybe by 50%) blocked the conversion of p100 to p75. This suggests that the C-terminal and N-terminal proteolytic events are functionally linked and that direct inhibition of C-terminal processing might secondarily interfere with the removal of the prodomain. In keeping with this interpretation, inhibition of GPI anchor formation was accompanied by a complete absence of p60/50 formation and also by essentially complete inhibition of the formation of p75, suggesting that the unidentified GPI-anchored component is required for C-terminal truncation and also maybe for removal of the prodomain. It should be noted that 5 mM glucosamine was also found to block C-terminal processing, enzyme activation, and prodomain removal (Figs. 5 and 6) in a manner consistent with its capacity to block interleukin-1-induced aggrecanolyysis (17). This effect, however, is likely to be mediated by a depletion in cellular ATP levels and an inhibition of vacuolar acidification (16) much as was observed on inhibition of aggrecan degradation with baclofen A1 (22).
Our finding that only the C-terminally truncated forms of ADAMTS4 could degrade aggrecan and versican (Figs. 6 and 7) suggests that the C-terminal region of the protein (approximately residues 700–837), when bound to the catalytic domain, exerts an intramolecular inhibitory effect on this protease. Whether this region has inhibitory activity after proteolytic removal is unclear and must await purification and N-terminal analysis of the C-terminal fragments. In this regard it may be relevant that mammalian papilin is inhibitory to ADAMTS2 and it contains a “cassette” that includes regions with homology to the C-terminal domains of the ADAMTS family (23).

Activation of C-terminal truncation of an inactive proproteinase has recently been reported for the mammalian cysteine endopeptidase, legumain, and in this case the cleavage is autoproteolytic at a specific Asn–Asp bond (24). In this regard, it seems unlikely that the activation observed here in culture with ADAMTS4 is autoproteolytic because it was effectively blocked by 125 nM TIMP-1, which has been found to be ineffective as an inhibitor of ADAMTS4 activity (10). On the other hand we have found that incubation of purified 75-kDa recombinant ADAMTS4 can result in the accumulation of a lower molecular weight form that comigrates with p60, so that autoproteolytic activation might also be possible with ADAMTS4. Identification of the C-terminal cleavage site(s) and the metalloproteinase(s) responsible for cleavage in vivo may offer new opportunities for therapeutic control of ADAMTS4 activity in disease states such as osteoarthritis, where its activity appears to be excessive (14).

The more general physiological importance of these findings will require further analysis of ADAMTS4 molecular forms in tissues such as cartilage (25), aorta (8), and spinal cord (26) where ADAMTS4 and maybe other hyalectans, such as ADAMTS5 and ADAMTS1, appear to be involved in the proteolysis of the other hyalectans, versican and brevican. In this regard, it is significant that ADAMTS4 extracted from normal joint tissues (Fig. 8) is present in both the p60 and p50 forms, clearly suggesting that C-terminal truncation is a normal event in vivo and therefore that it likely plays an important role in controlling ADAMTS activity in hyalectan-rich tissues of this kind.

Acknowledgments—We thank Dr. Joel Block of Rush University, Chicago, for kindly providing the original wild-type JJ012 cell line. We also thank Dr. Sunee Apte for providing anti-HRRA to the C-terminal of ADAMTS4. Dr. Michael Pratta for the anti-VMAH to the catalytic domain of ADAMTS4, Dr. John Mort for anti-NITEGE, and Drs. Dieter and Maria Zimmermann for recombinant versican β-GAG domain.

REFERENCES

1. Harding, T. E., Ewins, R. J., and Muir, H. (1976) Biochem. J. 157, 127–143
2. Toole, B. P. (1990) Curr. Opin. Cell Biol. 2, 839–844
3. Yamaguchi, Y. (2000) Cell. Mol. Life Sci. 57, 276–289
4. Iouzo, R. V., and Murdoch, A. D. (1996) FASEB J. 10, 598–614
5. Kim, K., Okada, Y., Kawashima, H., Nakamura, H., Miyazak, M., Okano, H., and Matsuhashid, K. (2000) FEBS Lett. 478, 241–245
6. Tortorella, M. D., Burn, T. C., Pratta, M. A., Abbazide, I., Holliss, J. M., Liu, R., Rosenfeld, S. A., Copeland, R. A., Decicco, C. P., Wynn, R., Rockwell, A., Yang, F., Duke, J. L., Solomon, K., George, H., Bruckner, R., Nagase, H., Itoh, Y., Ellis, D. M., Ross, H., Wiswall, B. H., Murphy, K., Hillman, M. C., Jr., Holliss, G. F., Arner, E. C., et al. (1999) Science 284, 1664–1666
7. Abbazide, I., Liu, R. Q., Yang, F., Rosenfeld, S. A., Ross, O. H., Link, J. R., Ellis, D. M., Tortorella, M. D., Pratta, M. A., Holliss, J. M., Wynn, R., Duke, J. L., George, H. J., Hillman, M. C., Jr., Murphy, K., Wiswall, B. H., Copeland, R. A., Decicco, C. P., Bruckner, R., Nagase, H., Itoh, Y., Newton, R. C., Mapinda, R. L., Traskos, J. M., Burn, T. C., et al. (1999) J. Biol. Chem. 274, 23443–23450
8. Sandy, J. D., Westling, J., Kenagy, R. D., Iruela-Arispe, M. L., Verscharen, C., Rodriguez-Mazeanque, J. C., Zimmerman, D., Lemire, J. M., Fischer, J. W., Wight, T. N., and Clowes, A. W. (2001) J. Biol. Chem. 276, 13372–13378
9. Matthews, R. T., Gary, S. C., Zerillo, C., Pratta, M., Solomon, K., Arner, E. C., and Hochfeld, S. (2000) J. Biol. Chem. 275, 22695–22703
10. Arner, E. C., Pratta, M. A., Traskos, J. M., Decicco, C. P., and Tortorella, M. D. (1999) J. Biol. Chem. 274, 6594–6601
11. Tortorella, M., Pratta, M., Liu, R. Q., Abbazide, I., Ross, H., Burn, T., and Arner, E. (2000) J. Biol. Chem. 275, 25791–25797
12. Murphy, G., and Knauper, V. (1997) Matrix Biol. 16, 511–518

2 G. Gao, J. Westling, V. P. Thompson, T. D. Howell, P. E. Gottschall, and J. D. Sandy, unpublished data.
Activation of Hyalectanase Activity of ADAMTS4

13. Scully, S. P., Berend, K. R., Toth, A., Qi, W. N., Qi, Z., and Block, J. A. (2000) Clin. Orthop. Relat. Res. 376, 291–303
14. Sandy, J. D., Flannery, C. R., Neame, P. J., and Lohmander, L. S. (1992) J. Clin. Invest. 89, 1512–1516
15. Sandy, J. D., and Verscharen, C. (2001) Biochem. J. 358, 615–626
16. Sandy, J. D., Thompson, V., Verscharen, C., and Gamett, D. (1999) Arch. Biochem. Biophys. 367, 258–264
17. Sandy, J. D., Gamett, D., Thompson, V., and Verscharen, C. (1998) Biochem. J. 335, 59–66
18. Denault, J. B., Claing, A., D’Orleans-Juste, P., Sawamura, T., Kido, T., Masaki, T., and Leduc, R. (1995) FEBS Lett. 362, 276–280
19. Kuno, K., and Matsushima, K. (1998) J. Biol. Chem. 273, 13912–13917
20. Rodriguez-Manzaneque, J. C., Michanowski, A. B., Dufour, E. K., Leduc, R., and Iruela-Arispe, M. L. (2000) J. Biol. Chem. 275, 33471–33479
21. Cal, S., Arguelles, J. M., Fernandez, P. L., and Lopez-Otin, C. (2001) J. Biol. Chem. 276, 17902–17908
22. Yocum, S. A., Lopresti-Morrow, L. L., Gabel, C. A., Milici, A. J., and Mitchell, P. G. (1995) Arch. Biochem. Biophys. 316, 827–835
23. Kramerova, I. A., Kawaguchi, N., Fessler, L. I., Nelson, R. E., Chen, Y., Kramerev, A. A., Kusche-Gullberg, M., Kramer, J. M., Ackley, B. D., Sieron, A. L., Prockop, D. J., and Fessler, J. H. (2000) Development 127, 5475–5485
24. Chen, J. M., Fortunato, M., and Barrett, A. J. (2000) Biochem. J. 352, 327–334
25. Flannery, C. R., Little, C. B., Hughes, C. E., and Caterson, B. (1999) Biochem. Biophys. Res. Commun. 260, 318–322
26. Lemons, M. L., Sandy, J. D., Anderson, D. K., and Howland, D. R. (2001) J. Neurosci. 21, 4772–4781