Procollagen II Amino Propeptide Processing by ADAMTS-3

INSIGHTS ON DERMATOSPARAXIS

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The amino and carboxyl propeptides of procollagens I and II are removed by specific enzymes as a prerequisite for fibril assembly. Null mutations in procollagen I N-propeptidase (ADAMTS-2) cause dermatosparaxis in cattle and the Ehlers-Danlos syndrome (dermatosparactic type) in humans by preventing proteolytic excision of the N-propeptide of procollagen I. We have found that procollagen II is processed normally in dermatosparactic nasal cartilage, suggesting the existence of another N-propeptidase(s). We investigated such a role for ADAMTS-3 in Swarm rat chondrosarcoma RCS-LTC cells, which fail to process the procollagen II N-propeptide. Stable transfection of RCS-LTC cells with bovine ADAMTS-2 or human ADAMTS-3 partially rescued the processing defect, suggesting that ADAMTS-3 has procollagen II N-propeptidase activity. Human skin and skin fibroblasts showed 30-fold higher mRNA levels of ADAMTS-2 than ADAMTS-3, whereas ADAMTS-3 mRNA was 5-fold higher than ADAMTS-2 mRNA in human cartilage. We propose that both ADAMTS-2 and ADAMTS-3 process procollagen II, but ADAMTS-3 is physiologically more relevant, given its preferred expression in cartilage. The findings provide an explanation for the sparing of cartilage in dermatosparaxis and, perhaps, for the relative sparing of some procollagen I-containing tissues.

Collagens consist of the major structural proteins of the extracellular matrix (ECM) and exist in both fibril-forming (e.g. collagens I–III, V, and XI) and nonfibrillar forms (1, 2). Molecules belonging to both categories are homotrimERIC (e.g. collagen II) or heterotrimeric (e.g. collagen I) assemblies of specific α chains, each the product of a single gene (1, 2). The molecular types of collagen, as well as the specific supramolecular aggregates they form, are often tissue-specific and provide a specialized function. For example, collagen I, the principal collagen of skin, is arranged in randomly oriented bundles in the dermis but in parallel bundles in tendons. Collagen II, a specific component of cartilage ECM, is arranged in an open meshwork that traps proteoglycans and facilitates resistance to compression.

The synthesis, secretion, and assembly of collagens into specific supramolecular aggregates is a complex, multistep process (3, 4). Fibrillar collagens I–III are synthesized as a soluble procollagen monomer comprising a long triple helical "collage nous" region with smaller polypeptide extensions (propeptides) at the amino and carboxyl ends (4). Removal of the propeptides by specific enzymes, the N- and C-propeptidases (propeptidases), is a prerequisite for the correct assembly of collagens I and II into growing fibrils (3, 4). The procollagen C-propeptidase is identical to bone morphogenetic protein-1 and processes all three of these fibrillar collagens (5). Biochemically distinct N-propeptidases with specificity for procollagens I and II or procollagen III are known (6). The bovine and human procollagen I N-propeptidases have been cloned (7, 8). This enzyme (designated ADAMTS-2, EC 3.4.24.14), is a zinc-containing, calcium-dependent metalloendopeptidase belonging to the recently described ADAMTS family (9, 10).

ADAMTS2 mutations cause the recessively inherited connective tissue disorder dermatosparaxis in animals and the Ehlers-Danlos syndrome (EDS)-VIIC or dermatosparactic type (Mendelian Inheritance in Man (OMIM) number 225410) in humans (8, 11–18). These disorders present clinically with severe fragility of skin. Their molecular hallmark is the presence of irregular, thin, branched collagen fibrils in the dermis that appear "hieroglyphic" in cross-section and contain collagen I with an intact N-propeptide, termed N-collagen I (19). Sim-

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RACE, rapid amplification of DNA ends; X-gal, 5-bromo-4-chloro-3-indolyl-b-D-galacto-
side; PAGE, polyacrylamide gel electrophoresis.

2 The approved nomenclature is used for human and mouse genes. ADAMTS2 and ADAMTS3 are human genes, adamts2 and adamts3 are the corresponding mouse orthologs. The protein products of the respective genes are designated ADAMTS-2 and ADAMTS-3. Suggested trivial names for the protein products of ADAMTS2 and ADAMTS3 are procollagen N-propeptidase 1 (PCNP1) and procollagen N-propeptidase 2 (PCNP2).
ilar findings have been described very recently in ad
tams-2 transgenic knockout mice (20).
Among the various members of the ADAMTS family (cur
cently 19 gene products), the overall domain organization and
amino acid sequence of ADAMTS-2 is most similar to that of
ADAMTS-3 (10). ADAMTS3 cDNA was originally cloned from
human brain and named the KIAA0366 gene3 (21). The
KIAA0366 cDNA was incomplete at the 5' end, and the transla
tion start codon had not been identified (21). Through molecu
lar cloning described herein, we now provide the complete
primary structure of ADAMTS-3.

Substrates for ADAMTS-3 have not been previously identi
fied. In light of the new data presented here, showing that
procollagen II in dermatosparactic cartilage is completely pro
cessed, we investigated a role for ADAMTS-3 in procollagen II
processing. The model system we used to test this hypothesis
was the Swarn rat chondrosarcoma-derived cell line, RCS-
LTC. In monolayer culture, these cells deposit an ECM con
taining collagens II, IX, and XI. However, the collagen is orga
nized into thin filaments instead of fibrils (22). RCS-LTC cells fail to process procollagen II beyond the stage of pN-collagen II,
although the amino acid sequence of the N-propeptidase cleav
age site in RCS-LTC procollagen II is normal (23). RCS-LTC
pN-collagen II is, however, processed in vitro by addition of
conditioned medium from cultures of chick chondrocytes (23).
This suggested that RCS-LTC chondrocytes either fail to ex
press procollagen II N-propeptidase or lack a soluble, essential
cofactor.

In this article, we demonstrate that transfection of RCS-LTC
cells with ADAMTS3 or ADAMTS2 results in conversion of
some of the pN-collagen II to a fully processed form. The results estab
lish that N-propeptidase deficiency is responsible, at least in
part, for defective collagen processing in RCS-LTC cells. We fur
ther demonstrate that steady-state mRNA levels of ADAMT
S-3 and ADAMTS-2 are different in normal human skin
and in skin fibroblasts than in cartilage, with ADAMTS-3 being
expressed at higher levels than ADAMTS-2 in cartilage. To
gether, these data suggest that ADAMTS-3, not ADAMTS-2, is the
major procollagen II N-propeptidase.

EXPERIMENTAL PROCEDURES
Cloning of ADAMTS-3—The previously reported 5774-base pair
KIAA0366/ADAMTS3 cDNA (21) was extended further in the 5'
direction by RACE using the Marathon34 system, and MarathonTM human
fetal brain cDNA (reagents from CLONTECH, Palo Alto) as template,
especially as described previously (10). PCR was done with nested
ADAMTS3-specific antisense oligonucleotide primers 5'-TCAAGGGCT
TGCAGTGCGACACTTC-3' and 5'-GGGAGCCTGTTCCTACAGCTGAT
CTC-3' and with nested adapter primers at the 5' end of the template.
The RACE products were cloned and sequenced as described previously (10).

Generation of ADAMTS3 and ADAMTS2 Expression Constructs—To
generate a cDNA construct for expression of full-length ADAMTS-3, we first deleted the 5' end of the KIAA0366 cDNA (in pBluescript II SK+;
(Stratagene, La Jolla, CA), provided by Dr. Takahiro Nagase of the
Kazusa DNA Institute). The deleted segment extended from the 5' NotI cloning (i.e. vector) site up to a unique internal AccI site at nucleotide
position 598 (KIAA0366 sequence enumeration). We replaced this frag
ment with a PCR-derived fragment of ADAMTS3 cDNA extending from
the 5'-untranslated sequence to just downstream of the AccI site.
 Briefly, PCR was performed with Advantage PCR reagents (CLON
TECH, Palo Alto, CA), using the RACE cDNA clone as template, the
forward primer 5'-AATCTGAAAGGTCAATCTTATTGAGTGAGTGAG
TGTCGT-3' (XhoI site underlined) and reverse primer 5'-AGCCGTGGTCTTAG
CTGACT-3'. The resulting amplion was digested with XhoI and AccI (at
the internal AccI site) and cloned into the SalI-AccI-restricted KIA
A0366 cDNA. This introduced the authentic ADAMTS3 ribosome-binding
sequence, translation start codon, and complete signal peptide into the
KIAA0366 cDNA. This insert encoding full-length ADAMTS-3 was ex
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ated from pBluescript with Xhol and NotI and ligated into the corresponding sites in pcDNA 3.1 (+)-myc-His A (Invitrogen, San
Diego, CA). In this mammalian expression construct, ADAMTS-3 is not in frame with the C-terminal myc and polyhistidine tags. For ex
pression of ADAMTS-2, three overlapping bovine cDNA clones that have been stored previously were used (with NotI, BclI, EcoR1, and KpnI) and assembled to generate a construct encoding full-length bovine ADAMTS-2. The ADAMTS2 cDNA was inserted into the NotI/XbaI sites of the pcDNA3 expression vector (Invitrogen, San
Diego, CA) using an XbaI adapter.

Isolation of RNA—Skin samples obtained from the forearm of healthy volunteers were used to isolate dermal fibroblasts which were stored in liquid nitrogen until use (Laboratoire de Biologie des Tissus
Conjonctifs, Sart-Tilman, Belgium, Ethics Committee Approval F94/14/
1871). Dermal fibroblasts grown from skin explants were cultivated in Dulbecco's minimum essential medium supplemented with 10% fetal
bovine serum. For Northern analysis, total RNA was purified from skin
samples pulverized in liquid nitrogen or from dermal fibroblasts in
culture, after solubilization of homogenates and cells in 0.1 M Tris-HCl,
ph 7.5, 5 M guanidinium thiocyanate, 1% ß-mercaptoethanol. These
extracts were centrifuged at 100,000 x g for 18 h on a cesium chloride
(CsCl) cushion (0.01 M EDTA, ph 7.5, 5.7 M CsCl). mRNA was subse
quently purified using Poly(A)Tract mRNA Isolation System (Promega
Beneux B.V.), according to the manufacturer's instructions. For quan
titation, cDNA was reverse transcribed from fibroblast RNA using retro
transcription from the respective clones using the Strip-EZ RNA kit (Am
bion, Austin, TX).

Quantitative Reverse Transcriptase-PCR (RT-PCR) Analysis—Total
RNA (2.5 µg) was reverse-transcribed with the SuperScript First
Strand Synthesis System for RT-PCR (Life Technologies, Inc.) using oligo(dT) as a primer. Real time PCR was performed in an ABI Prism
7700 Sequence Detector using SYBR Green PCR Core Reagents (Ap
plied Biosystems, Foster City, CA). In this system, continuous, auto
mated quantitation of the PCR product is performed by measuring the
fluorescence generated by the binding of SYBR Green to double
stranded DNA. PCR amplifications were performed in triplicate for
skin fibroblast RNA and in quadruplicate for cartilage RNA, along with
parallel measurements of GAPDH cDNA (an internal control). Data
were analyzed according to the comparative Ct method (Applied Bi
osystems protocols) and represented after normalization to GAPDH
levels. To confirm these data, PCR products were also sequenced on a
2.0% agarose gel, visualized with UV light through a SYBR Green filter,
and photographed. The following primers were used for amplification at
a concentration of 300 nM: ADAMTS2, 5'-TGGGAGACACTTG
TG3' (forward) and 5'-TCGTCGTCGAGGATTTAGG-3' (reverse); ADAMTS3, 5'-TCATGTTGGAGTCCAAATTGCA-3' (forward) and
5'-G-CAAAAGAAGAACAGCAGGCG-3' (reverse); GAPDH, 5'-CCACCTGCC
AAGCTGTCAGGTG-3' (forward) and 5'-AAAGTGGAGAGGCTTGGTCG
-3' (reverse). As an additional control, RT-PCR was also performed in the absence of template.

Northern Analysis of ADAMTS2 and ADAMTS3 Expression—A com
mercially available adult human multiple tissue Northern blot and a
mouse embryo Northern blot (CLONTECH Inc. Palo Alto, CA) were
hybridized as per manufacturer's instructions using ExpressHybTM hyb
ridization fluid (CLONTECH, Palo Alto, CA). The following cDNA
 probes were used after random-priming labeling with [α-32P]dCTP: a
fragment containing nucleotides 946–1379 of human ADAMTS-2
cloned in pCR4-TOPO (for human multiple tissue Northern blot); a
1.1 kb HindIII fragment from the KIAA0366/ADAMTS3 cDNA (for
human multiple tissue Northern blot); the insert of IMAGE clone
1246561, available with GenBankTM accession number AA832579 (mouse ADAMTS-2 probe for mouse embryo Northern blot); the
insert of IMAGE clone 727026, available with GenBankTM accession number AA0402760 (mouse ADAMTS-3 probe for mouse embryo
Northern blot). Exposure of the blots to x-ray film was for 3–7 days.

Poly(A)2 RNA (0.8 µg) from human skin or human skin fibroblasts
was electrophoresed on a formaldehyde-agarose gel and blotted to
Hybond N+ nylon membrane (Amersham Pharmacia Biotech). cDNA
probes for human ADAMTS2 and ADAMTS3 were generated by tran
scription using the respective clones using the Strip-EZ RNA kit (Am
bion) or a T3 RNA polymerase as per manufacturer's in
structions. Prehybridization (1 h) and hybridization (18 h) were
performed at 65 °C in 0.2 x NaHPO4 (pH 7.2), 1 mM EDTA, 1% bovine
serum albumin, 7% SDS, and 20% formamide. Stringency washes were
carried out at 65 °C in 40 mM NaHPO4, 1 mM EDTA, and 1% SDS.

Cell Culture and Stable Transfection of RCS-LTC Chondrocytes—
Monolayer cultures of RCS-LTC cells were maintained in Dulbecco's
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minimum essential medium containing 4.5 g/liter glucose (Life Technologies, Inc.) and 10% fetal bovine serum (HyClone Labs, Logan, UT) at 37 °C in 5% CO₂. Culture medium was changed every other day, and confluent cultures were sub-cultured every 2 weeks as described previously (24, 25). LipofectAMINE Plus (Life Technologies, Inc.) was used for Hs68-LTC transfections. Cells were transfected at a density of 1 × 10⁶ cells/well in 6-well plates (Falcon, Franklin Lakes, NJ). After 24 h, the wells were rinsed with Opti-MEM (Life Technologies, Inc.) and transfected with the human ADAMTS3 or bovine ADAMTS2 cDNA constructs (1 µg of DNA/well) as per the manufacturer's instructions. As a control for efficiency of transfection and to provide a negative control for procollagen II processing, cells were separately transfected with pcDNA3.1/Myc-His (+) lacZ encoding the Escherichia coli lacZ gene. Mock transfections were performed without cDNA as a control for transfection and cell survival. Geneticin (G418 sulfate) was used to select cells following sub-culture as described. For the last day of culture, the presence of 1 mg/ml geneticin was confirmed. The human ADAMTS3 gene was previously described for the RCS-LTC cell line, but in the continued subculture, geneticin was used to select stable transfectants as well as untransfected RCS-LTC chondrocytes. Cells were selected in media supplemented with 1 mg/ml G418 sulfate (geneticin, Life Technologies, Inc.). After 2 weeks in culture, the chondrocytes from the mock transfections did not survive selection. Geneticin-resistant ADAMTS3, ADAMTS2, and lacZ transfected chondrocytes were expanded and maintained as pools in serial monolayer culture as described previously for the RCS-LTC cell line but in the continued presence of 1 mg/ml geneticin.

β-Galactosidase expression in the lacZ stable transfectants was detected histochemically by staining the cells with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal, Life Technologies Inc.). After 2 weeks in culture, transfected cells were selected in media supplemented with 1 mg/ml G418 sulfate (geneticin, Life Technologies, Inc.). After 2 weeks in culture, the chondrocytes from the mock transfections did not survive selection. Geneticin-resistant ADAMTS3, ADAMTS2, and lacZ transfected chondrocytes were expanded and maintained as pools in serial monolayer culture as described previously for the RCS-LTC cell line but in the continued presence of 1 mg/ml geneticin.

Collagen in this extract was separated by reducing SDS-PAGE and stained histochemically by staining the cells with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal, Life Technologies Inc.). After 2 weeks in culture, transfected cells were selected in media supplemented with 1 mg/ml G418 sulfate (geneticin, Life Technologies, Inc.). After 2 weeks in culture, the chondrocytes from the mock transfections did not survive selection. Geneticin-resistant ADAMTS3, ADAMTS2, and lacZ transfected chondrocytes were expanded and maintained as pools in serial monolayer culture as described previously for the RCS-LTC cell line but in the continued presence of 1 mg/ml geneticin.

RESULTS

Molecular Cloning of Full-length ADAMTS-3 and Comparison with ADAMTS-2—By using 5'-RACE, we obtained a novel 720-bp pair cDNA clone encoding the 5'-untranslated region, the translation start codon, signal peptide, and pro-domain of ADAMTS-3. The novel 5' sequence obtained has not been previously described and is deposited in GenBank™ with accession number AF247668. When incorporated into the KIAA0366 sequence, we were therefore able to identify the open reading frame and conceptual translation product of ADAMTS3. The predicted start codon is the 5'-most methionine codon (ATG) in the open reading frame (Fig. 1B) and is preceded by an in-frame stop codon, 26 nucleotides upstream. It is located within the context of a suitable Kozak consensus sequence for translation (contains purine at position -3 with respect to the A of the ATG codon, and G at position +4) (25). The predicted ADAMTS3 protein (1205 amino acids) is comparable in length to human and bovine ADAMTS2 (1211 and 1205 amino acids, respectively) (7, 8). The predicted mature (furin-processed) forms of these proenzymes are also of comparable length, 957 residues (ADAMTS-3) and 953 residues (bovine and human ADAMTS-2) long. ADAMTS3 predicts a full-length protein of molecular mass 153.6 kDa and a furin-processed form of 107.5 kDa, although it is likely that post-translational modification of ADAMTS-3 will increase its mass (see below).

ADAMTS-3 and ADAMTS-2 have a similar domain structure (Fig. 1A) and an overall sequence identity of 61% (Fig. 1B). From N to C terminus, each of these enzymes consists of the following domains (with percent sequence identity in parentheses): signal peptide, pro-domain (37%), catalytic domain (85%), disintegrin-like domain (77%), central thrombospondin type I (TS) repeat (63%), cysteine-rich domain (67%), spacer domain (56%), and three additional TS repeats (64%), followed by an essentially unique C-terminal extension (Fig. 1A).

The putative ADAMTS-3 furin-processing site is at a location identical to the downstream of two furin consensus sites found in ADAMTS-2 (Fig. 1B). ADAMTS-3 and ADAMTS-2 catalytic domains have complete sequence identity (but for one amino acid) over 69 amino acids which includes the zinc-binding active site (Fig. 1B). ADAMTS3 and ADAMTS2 each have eight consensus sequences (Asn-X-(Ser/Thr)) for potential N-linked glycosylation (Fig. 1, A and B). Of these, four are conserved absolutely between ADAMTS-2 and ADAMTS-3, two conserved sites being in the pro-domain, and one site each in the catalytic domain and the second TS domain. One site in both ADAMTS-2 and ADAMTS-3 has the sequence Asn-Pro-Ser. It has been shown that the presence of proline between Asn and Ser/Thr inhibits N-glycosylation (26), and it is possible that this particular site may not be modified. In contrast to ADAMTS-2, which contains the potential cell-binding sequence CVRGEC, ADAMTS-3 has the sequence CVRGEC at the corresponding location (Fig. 1B).

The C-terminal extensions of these two molecules are of comparable length (184 and 191 amino acids in ADAMTS-2 and ADAMTS-3, respectively) but show little sequence similarity other than a highly conserved PLAC (protease and LACunin) domain (83% identity, Fig. 1B) (27). The PLAC domain was first described in an insect protein, lacunin (27), which contains all the ancillary domains of ADAMTS, as well as other domains and can therefore be considered a complex ADAMTS-like protein. The PLAC domains of human ADAMTS-3 and bovine ADAMTS-2 each contain six cysteine residues (Fig. 1B). In a human ADAMTS-2 sequence published previously (8) (GenBank™ accession number AJ003125), one of these cysteines (at position 1090, Fig. 1B) was substituted by serine. However, it is likely that this represents a sequence variation or error since there is a cysteine at this position in three independent ADAMTS2 expressed sequence tag sequences (GenBank™ accession numbers AJ1417257, AJ624388, and AI089232).

Differential Tissue-specific Expression of ADAMTS2 and ADAMTS3—adams2 and adams3 were both expressed during mouse embryogenesis. adams2 expression was noted in mouse

4 D. R. Eyre, unpublished data.
FIG. 1. A, domain organization of ADAMTS-3 and ADAMTS-2. Key for the domains is shown at the bottom. Amino acid identity (in percent) between ADAMTS-3 and ADAMTS-2 is shown for each domain (except the C-terminal domain). a.a., amino acids. B, alignment of the primary structures of ADAMTS-3 and ADAMTS-2 using the single-letter amino acid code. The likely furin-processing site for generation of mature ADAMTS-3 and ADAMTS-2 is indicated by the flat arrowhead. Another consensus furin-processing site in ADAMTS-2 is indicated by an arrowhead. The sequence encoded by the RACE clone is overlined. TS repeats are underlined and indicated by numbers. The zinc-binding histidine triad is enclosed in a box. The boundaries of a region of complete amino acid sequence identity in the catalytic domain are indicated by the vertical arrows. The N termini of the disintegrin-like (Dis) and spacer domains are indicated. The cysteine-rich domain extends from TS repeat 1 to the start of the spacer domain. Potential cell-binding RG(D/E) sequences are indicated by the thick overline. The PLAC domains are shown by the dashed underline. Potential sites for N-linked glycosylation are indicated by stars.
embryos at 7, 15, and 17 days but not at 11 days. Two mRNA species (7.8 and 4.0 kb) were detected (Fig. 2). A single *adams3* mRNA species (~7.2 kb in size) was also detected in mouse embryos at 7, 15, and 17 days but not at 11 days of gestation (Fig. 2A, bottom panel). Expression of *ADAMTS2* and ADAMTS3 was restricted among eight normal human adult tissues examined by Northern analysis (Fig. 2B). In human placenta, lung, and liver, two *ADAMTS2* transcripts were present, migrating at ~7.8 and 4.4 kb similar to those described previously (10). The previously described 2-kb transcript that encodes a truncated form of ADAMTS-2 was not detected on these blots (10). The highest expression of *ADAMTS3* was noted in placenta with lower level expression in lung, brain, and heart, with a single mRNA species migrating at ~7.0 kb.

In skin samples and in skin fibroblasts, Northern analysis with equivalent amounts of cRNA probes for *ADAMTS2* or *ADAMTS3* demonstrated a differential prevalence of steady-state mRNA levels. Fig. 3 shows autoradiograms generated by 1 (ADAMTS2) and 18 h of exposure (ADAMTS-3). Based on the different exposure times, a substantially stronger signal is evident with an *ADAMTS2* probe than with *ADAMTS3* in skin and skin fibroblasts. All three previously reported *ADAMTS2* transcripts (7.0, 4.5, and 2.0 kb) as well as several other minor transcripts were seen in skin fibroblasts, whereas only the 4.5- and 2.0-kb mRNAs were found in skin. With the *ADAMTS3* probe, we identified a 4.5-kb band plus 2.3-kb band in skin but...
one 4.5-kb band in skin fibroblasts. The discrepancy in number and size of these ADAMTS-3 bands with those evident in multiple tissue Northern blots (Fig. 2) is presently unexplained, but it is possible that, like ADAMTS2, ADAMTS3 also generates multiple transcripts in a tissue-specific fashion. It was also noted that, whereas ADAMTS3 signal is stronger in skin than in skin fibroblasts, the reverse was true for ADAMTS2 (Fig. 3).

To obtain a numerical comparison of relative mRNA levels, we performed quantitative RT-PCR analysis of ADAMTS2 and ADAMTS3 mRNA in cultured skin fibroblasts and human cartilage. The data demonstrate considerably higher steady-state ADAMTS2 mRNA levels in human skin fibroblasts relative to ADAMTS3 levels (mean, 30.6-fold; range, 20.41–45.84-fold ADAMTS2 over ADAMTS3). In contrast, quantitative RT-PCR analysis of RNA from human fetal cartilage demonstrates an approximately 5-fold higher steady-state level of ADAMTS3 mRNA compared with ADAMTS2 (mean, 4.86-fold; range, 2.89–7.88-fold ADAMTS3 over ADAMTS2).

Procollagen II Is Completely Processed in Dermatosparactic Cartilage—Dermatosparactic animals have no functioning ADAMTS-2 (10). Despite this, Coomassie Blue staining of collagen extracted from dermatosparactic nasal cartilage demonstrated α1 chains migrating to the expected position of α1(II) chains from control cartilage (Fig. 4a). This was confirmed by Western blot analysis of these extracts using a monoclonal antibody that recognizes an epitope in the triple helical domain of α1(II) (Fig. 4b), showing that essentially all of the immunoreactive collagen was fully processed. Some pN-collagen I was visible in the extract from dermatosparactic cartilage (Fig. 4a).

ADAMTS3 and ADAMTS2 Process Procollagen II in Transfected RCS-LTC Cells—The efficiency of RCS-LTC transfection was monitored by β-galactosidase staining. lacZ-transfected cells also served as a negative control for analysis of procollagen processing. Cells stably transfected with lacZ showed dark blue staining (representing about 10% of the population), whereas no staining was seen in the ADAMTS3 and ADAMTS2 stably transfected populations as expected. A similar efficiency of transfection and/or expression was assumed for the ADAMTS3- and ADAMTS2-transfected cells as for the lacZ-transfected cells.

To determine if ADAMTS-3 was capable of enzymatically removing the N-propeptide of procollagen II, lysates of ADAMTS-3, ADAMTS2-, and lacZ-transfected RCS-LTC chondrocytes were blotted, and procollagen II and collagen II were identified using monoclonal antibody 1C10. The results show some processing of pN-collagen II to mature collagen II in the ADAMTS2- and ADAMTS3-transfected cells (Fig. 5, lanes 3 and 4) but none in lacZ-transfected cells (Fig. 5, lane 5) or in untransfected cells (Fig. 5, lane 6). Following N-propeptide excision, the α1(II) chains migrate faster than the pN-collagen II chains (e.g. Fig. 5, lane 3) and at a position similar to the naturally processed α1(II) chain (Fig. 5, lane 2) or pepsin-collagen II (Fig. 5, lane 1).

**DISCUSSION**

**Unsolved Enigmas in Dermatosparaxis and EDS-VIIC**—Procollagen I processing in dermatosparaxis is most deficient in skin, although mature skin has some processed collagen (28). Many collagen I-containing dermatosparactic tissues such as tendon, ligament, sclera, and aorta show the presence of significant amounts of fully processed collagen I (12, 28). None of these tissues, nor bone, which relies on collagen I for its mechanical strength, have been noted to be fragile (13). Very recently, adams2 knockout mice have been reported to have significant amounts of processed collagen in skin (20).

These anomalies were attributable to the presence of residual pN-collagen processing activity, due to either the incompleteness of the genetic defect or to compensation by another enzyme (13, 28). The demonstration that the causative mutations were functionally null favored the existence of one or more additional procollagen N-propeptidases (8). The presence of processed procollagen I in many dermatosparactic tissues, including skin, suggested that this putative alternative propeptidase(s) might be regulated differently from ADAMTS-2 in skin and other tissues or that it may not be as efficient in procollagen I processing as ADAMTS-2 (28).

**Procollagen II Processing Is Normal in Dermatosparaxis**—ADAMTS-2 can process procollagen II (29, 30). However our finding of processed procollagen II in dermatosparaxis supported the existence of an enzyme other than ADAMTS-2 that actually removes the N-propeptide of collagen II in vivo. Although EDS-VIIC patients are of short stature, they do not have chondrodysplasia or premature arthritis (11, 13, 15–19). Failure of procollagen II processing comparable to that of collagen I in dermatosparactic skin might be expected to cause a severe chondrodysplasia, given the critical role of collagen II in the structural stability of cartilage matrix (31, 32). Our studies thus provide an explanation for the absence of cartilage fragil-
ity and/or chondrodysplasia in dermatosparaxis or EDS-VIIC.

The presence of procollagen I in dermatosparactic nasal cartilage (Fig. 4) but not in normal cartilage may be explained by differences in composition of these cartilages, the inclusion of perichondrium in the extract, or by up-regulation of collagen I gene expression, which has been previously noted in dermatosparaxis (33). In contrast to our data, the recently described adams2 knockout mice retain some unprocessed collagen II in their cartilage (20).

ADAMTS-2 and ADAMTS-3 Comprise of a Structurally and Functionally Distinct Subfamily of ADAMTS Proteases but Are Regulated Differently—ADAMTS-2 and ADAMTS-3 are the only two members of the ADAMTS family to have three C-terminal TS domains and the only published members of the ADAMTS family to have a substantial C-terminal extension downstream of these TS domains. The location of the PLAC domain within this C-terminal extension is also unique, because in other ADAMTS family members where it is present, such as in ADAMTS-7B and ADAMTS-10, it is usually at the C-terminal end of the protein.

A number of sequence hallmarks are unique to this PCNP subfamily of ADAMTS enzymes as follows. (a) The pro-domain contains only two cysteines, in contrast to other ADAMTS enzymes, which usually contain three. (b) The catalytic domain contains six cysteines as opposed to eight for the other ADAMTS, where the usual arrangement is five cysteines upstream of the zinc-binding site and three downstream. In the PCNP subfamily, only three cysteines occur upstream of the zinc-binding sequence; the three downstream cysteines, however, are at absolutely conserved positions with regard to other ADAMTS enzymes. This arrangement of cysteines suggests that the catalytic domain of this subfamily may be structurally different from that of the other ADAMTS enzymes. (c) The sequence of the zinc-binding triad (HETGHLGMEHD) in this subfamily is unique in having threonine in the 3rd position (underlined), whereas all other ADAMTS enzymes have a hydrophobic residue with a long side chain (leucine or isoleucine) at this position. (d) The spacer domains of the ADAMTS family vary in length and sequence. Within the PCNP subfamily, they are significantly similar to each other (56% amino acid identity).

On the basis of domain and amino acid sequence homology, this subfamily appears to contain no more than three members as determined by a search of the complete human genome sequence (Celera Genomics, Rockville, MD, and GenBank™ at NCBI). A third, equally closely related member of this family is located on human chromosome 10 (encoded by genomic sequences with GenBank™ accession numbers AC0069538, AC016043, and AC007484). We previously mapped ADAMTS3 to human chromosome 4, distinct from the ADAMTS2 locus on human chromosome 5 (10). Thus, although these are the most closely related enzymes within the ADAMTS family, they are not clustered within the human genome.

Whereas the similarities in the catalytic domains of this subfamily suggest similar catalytic mechanisms, differences in their ancillary domains (i.e. the TS, disintegrin-like, cysteine-rich, and spacer domains) may affect substrate preferences, intermolecular interactions, or compartmentalization in ECM. For example, ADAMTS-1 ancillary domains are responsible for ECM binding (34), and the TS domains of aggrecanase-1 (ADAMTS-4) are required for binding to native aggrecan (35). A splice variant of ADAMTS2 that generates a short form of ADAMTS-2 lacking the ancillary domains is functionally inactive in procollagen I processing (8).

Transfection with ADAMTS2 and ADAMTS3 Leads to Procollagen II Processing in RCS-LTC Cells—We have taken a genetic approach to identify a function for ADAMTS-3 and to determine the nature of the underlying processing defect in RCS-LTC cells. Transfection of these cells with ADAMTS2 and ADAMTS3, but not with lacZ (a negative control), results in processing of some pN-a1(II) to a1(II). N-propeptide removal was detected by the co-migration of the processed form and pepsin-treated RCS-LTC collagen II (pepsin removes the propeptides) compared with collagen II extracted from human cartilage. Our results suggest that in quantitative terms the activity of ADAMTS-3 is roughly equivalent to that of ADAMTS-2. One caveat in our studies is that we have not performed experiments with purified procollagen II and recombinant ADAMTS-3. However, given the fact that ADAMTS-2 is an established procollagen I/II-processing enzyme (29, 36) and that there is a high degree of similarity of ADAMTS-3 to ADAMTS-2, it is very likely that ADAMTS-3 directly processes procollagen II.

There are several possible explanations for the persistence of pN-collagen II in ADAMTS2- or ADAMTS3-transfected cells. The transfected cells were maintained as a pooled population rather than as clonally selected lines, so that there may be transfected cells that do not express the construct at all or do so at low levels. In support of this possibility, only a small proportion of lacZ-transfected cells expressed β-galactosidase activity. We believe it unlikely that cells not containing the cDNA constructs survived because control, untransfected cells subjected to genetin selection pressure were killed after 2 weeks. Other possibilities include an inadequate access of enzymes to pN-collagen II, an excess of procollagen II over the amount of enzyme required for processing, or that the culture conditions were not optimized for maximal processing.

Previous studies have shown that procollagen I processing in dermatosparactic fibroblasts is enhanced by including dextran sulfate (37, 38) or polyethylene glycol (38) in the culture medium. However, despite the increased efficiency, processing was still incomplete (37, 38). In previous studies in which culture medium conditioned by chick chondrocytes was added to pN-collagen II synthesized by RCS-LTC cells, we were unable to get complete processing to collagen II even after 18 h of incubation in vitro (23). A final possibility, which we consider to be unlikely, is that there may be some species specificity in the enzyme-substrate interactions that may affect the efficiency of processing. Our studies used bovine ADAMTS-2 and human ADAMTS-3 against rat collagen II. The procollagen II aminopropeptidase site is identical in a number of species, including rat and human (39). Furthermore, ADAMTS-2 is shown to have essentially similar activity against procollagen I from unrelated species. We therefore believe that effects resulting from species specificity are likely to be negligible.

Failure of RCS-LTC cells to produce a functional processing enzyme could result from a structural mutation in ADAMTS-2 or ADAMTS-3 or because of transcriptional repression of these genes in RCS-LTC cells. We have been unable to amplify ADAMTS2 or ADAMTS3 mRNA in RCS-LTC cells by RT-PCR suggesting the latter mechanism (data not shown; PCR was done using mouse primers, since rat sequences are currently not available for these two genes. However, rat and mouse show considerable genetic identity, about 96–98%, and these data may thus be valid.).

Is ADAMTS-3 Responsible for Procollagen II Processing in Dermatosparaxis?—We propose that, on the basis of either substrate preference for procollagen II in vivo or of a higher expression than ADAMTS-2 in cartilage, ADAMTS-3 is the principal collagen II N-propeptidase in vivo. Data from our

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5 S. Apte, unpublished data.
studies that provide a rationale for this speculation are as follows. (a) Collagen II is processed in dermatosparactic cartilage despite a null mutation in ADAMTS2. (b) pN-collagen II is equally processed in RCS-LTC cells transfected with ADAMTS-2 or ADAMTS-3. (c) The ratio of ADAMTS3 to ADAMTS2 mRNA in human cartilage is about 5:1.

More definitive proof is required: for example, the identification of ADAMTS3 mutations in a human chondrodysplasia or from the targeted inactivation of adams3 in mice and analysis of the phenotype in cartilage. Such studies may uncover other functions for ADAMTS-2 in male fertility (20). It is also possible that other enzymes of the ADAMTS family or other families contribute substantially to collagen N-propeptide processing in tissues other than skin. Finally, it is possible that the existence of procollagen N-proteinases(s) such as ADAMTS-3 can be exploited in the treatment of dermatosparactic patients. Before this can be done, it will be necessary to demonstrate procollagen I processing activity by ADAMTS-3 and to identify factors that up-regulate it in skin fibroblasts.

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REFERENCES
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