Punctin (ADAMTSL-1) is a secreted molecule resembling members of the ADAMTS family of proteases. Punctin lacks the pro-metalloprotease and the disintegrin-like domain typical of this family but contains other ADAMTS domains in precise order including four thrombospondin type I repeats. Punctin is the product of a distinct gene on human chromosome 9p21-22 and mouse chromosome 4 that is expressed in adult skeletal muscle. His-tagged punctin expressed in stably transfected High-Five™ insect cells was purified to apparent homogeneity by Ni-chromatography of conditioned medium. The NH₂-terminus is not blocked and has the sequence EEDRD and so forth as determined by Edman degradation, demonstrating signal peptidase processing. Recombinant epitope-tagged punctin has a calculated mass of 59,991 Da but exhibits major molecular species of 61970 ± 6 Da and 62131 ± 5 Da as measured by liquid chromatography electrospray mass spectrometry. Punctin is a glycoprotein based on carbohydrate staining and liquid chromatography electrospray mass spectrometry glycopeptide analysis. Glycosylation occurs at a single N-linked site as demonstrated by altered electrophoretic migration of punctin expressed in the presence of tunicamycin A. Punctin contains disulfide bonds based on antibody accessibility and electrophoretic migration under reducing versus nonreducing conditions. Rotary shadowing demonstrates that punctin is hatchet-shaped having a globular region attached to a short stem. In transfected COS-1 cells, punctin is deposited in the cell substratum in a punctate fashion and is excluded from focal contacts. Punctin is the first member of a novel family of ADAMTS-like proteins that may have important functions in the extracellular matrix.

Metalloproteases responsible for extracellular (ECM) turnover have a modular structure. Matrix metalloproteinases (MMPs) (1), a disintegrin-like and metalloprotease (ADAMs) (2), and proteases of the ADAMTS family (3, 4) are composed of characteristic domains arranged in a precise order that is the hallmark of each family. These enzymes are structurally and functionally bipartite consisting of an enzymatic domain attached to nonenzymatic or ancillary domains. The ancillary domains localize these proteases to substrates, the cell surface, or to the ECM. The ancillary domains of the gelatinases MMP-2 and MMP-9 are among the best studied of the substrate-binding domains. The fibronectin type II domains of the gelatinases are involved in binding to gelatin and some collagens as well as to fibronectin and heparin as in the case of MMP-2 (5, 6). The gelatin-binding domain of MMP-2 binds the matricellular proteins thrombospondin-1 (TSP1) and TSP2 (7). Although neither is a substrate for MMP-2, the interaction may mediate the clearance of MMP-2 and affect cell-adhesive properties (8). The MMP-2 hemopexin domain interacts with the carboxyl terminus of the tissue inhibitor of metalloproteases-2, facilitating pro-MMP-2 activation by membrane-type MMPs (1, 5, 6, 9). The MMP-2 hemopexin domain also interacts with a chemokine called monocyte chemoattractant protein-3, which allows its processing by the catalytic domain (10). The disintegrin domains of ADAMs such as ADAM-15 are implicated in cell-cell adhesion (2, 11, 12), and the ancillary domains of ADAMTS-1 are required for its binding to the ECM (13). In some ADAMs, the zinc-binding active site is nonfunctional, suggesting that they do not function as proteases at all but may instead have a primary role in adhesion via their ancillary domains (2).

With this background, it is conceptually possible that gene products containing only the ancillary domains of ADAMTS may have specific functions in cell-cell or cell-matrix interactions or may regulate ADAMTS proteases. We have identified an ADAMTS-like (ADAMTSL) molecule named punctin,²

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¹ The abbreviations used are: ECM, extracellular matrix; ADAMTSL, a disintegrin-like and metalloprotease domain with thrombospondin type I motifs like; ADAMTS, a disintegrin-like and metalloprotease domain with thrombospondin type I motifs; ADAM, a disintegrin-like and metallopeptase; MS, mass spectrometry; EST, expressed sequence tag; EC-ESMS, liquid chromatography-electrospray mass spectrometry; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MMP, matrix metalloprotease; ORF, open reading frame; PBS, phosphate-buffered saline; RACE, rapid amplification of cDNA ends; TSP, thrombospondin; TS, thrombospondin type I domain; HexNAc, N-acetylatedhexosamine; NeuAc, N-acetylnuraminic acid.

² Apparent gene symbols ADAMTSL1 and ADAMTS1 indicate human and mouse orthologs, respectively. The corresponding protein product of these genes, ADAMTSL-1, is designated by the trivial name punctin because of its punctate distribution beneath transfected cells.
which is the product of a gene distinct from any in the ADAMTS family and is composed of ADAMTS ancillary domains alone. We have purified and characterized recombinant punctin produced in insect cells, visualized it by electron microscopy, and demonstrated that it is a glycoprotein and a component of the ECM.

**EXPERIMENTAL PROCEDURES**

cDNA Cloning and Sequence Analysis—Using BLAST programs from the National Center for Biotechnology Information, we scanned the data base of ESTs using the protein sequences of ADAMTS previously cloned by us (4, 14) and identified a human EST (GenBank™ accession number AA482392 encoded by IMAGE clone 752797). The EST predicted a polypeptide with a similarity to the carboxyl half of cognate ADAMTS members but with no identities in GenBank™ or other protein and nucleotide data bases. Using nested oligonucleotide primers based on the sequences at the 5′ and 3′ ends of the IMAGE clone insert and human skeletal muscle cDNA (Marathon cDNA, CLONTECH, Palo Alto, CA) as the template, we performed RACE and extended the cDNA at 5′ and 3′ ends by PCR essentially as described previously (4, 14).

Northern Blot Analysis—Multiple tissue Northern blots from adult human and mouse tissues (CLONTECH, Palo Alto, CA) were hybridized with [32P]CTP-labeled punctin probe, a 1200-bp cDNA fragment from the 5′ end of the punctin coding sequence, followed by autoradiographic exposure for 7 days.

Chromosomal Mapping and Genomic Arrangement—To determine the chromosomal location of *Adamts11*, we analyzed a panel of DNA samples from an interspecific cross that has been characterized for over 1260 genetic markers throughout the mouse genome (15). Markers can be seen on the worldwide web (www.informatics.jax.org/searches/cross/suggest.shtml) by entering "DNA Mapping Panel Data Sets" from the mouse genome data base and then selecting the "Seldin cross" and "Chromosome." Initially, DNA from the two parental mice, (C3H/HeJ-glдв and C3H/HeJ-glдв × Mus spretus F1), were digested with various restriction endonucleases and hybridized with the *Adamts11* cDNA probe (IMAGE clone 2076907 with GenBank™ accession number AB767755) to determine restriction fragment length variants for haplotype analyses. Gene linkage was determined by segregation analysis. Gene order was determined by analyzing all haplotypes and minimizing crossover frequency among all genes that were determined to be within a linkage group. This method resulted in the determination of the most probable gene order. To define the locus for *ADAMTS11*, the human punctin cDNA sequence was used for BLAST searches of the human genome (Celera Sciences, Rockville, MD).

**Generation and Characterization of Anti-punctin Antiserum—**The peptide (NH₂-C-{[G]YYPEN[K]P(KPQ]E]-OH) located in the third TS do- main of punctin (Fig. 1B) was synthesized using Fmoc (N-(9-fluorenyl) methoxycarbonyl) chemistry, purified by reverse-phase high-pressure liquid chromatography, and molecular weight was confirmed by MS (API, Micromass, Manchester, UK). A cysteine (C) residue was included at the NH₂ terminus for coupling to keyhole limpet hemocyanin. Peptide-keyhole limpet hemocyanin conjugate was dialyzed in PBS and used for immunization. Two New Zealand White male rabbits (7–8 pounds) were immunized with the conjugate (≈200 µg/injection/rabbit, multiple intramuscular and subcutaneous sites) at biweekly intervals for 8 weeks. After an initial injection in Freund’s complete adjuvant, subsequent injections were given in incomplete adjuvant. Antibody titer was measured by enzyme-linked immunosor- bent assay using free peptide.

Immune sera were tested by Western blot analysis of extracts from COS-1 cells transiently transfected with punctin cDNA (see below). Although antiserum from both rabbits (antisera 4112 and 4113) gave qualitatively similar results, the best signal/noise ratio was obtained with antisera 4113. Affinity-purified antibodies were prepared by column chromatography of antisera 4113 using the immobilized pep- tide immunogen.

Expression and Purification of Recombinant Punctin from Insect Cells—High-Five™ cells (Invitrogen) were routinely cultured on tissue culture plastic and maintained at 27°C in Ultimove™ serum-free media (Invitrogen) as per manufacturer’s directions. The full-length punctin ORF was excised from plcDNA3.1/Myc-His B-TSL1 (see below) with EcoRI and NotI and ligated into the corresponding sites in pIZT/V5-His (Invitrogen). The resulting insect cell expression plasmid pIZT/V5-His-TSL1 generated punctin with a COOH-terminal V5 epitope and 6× His tag. pIZT/V5-His-TSL1 was transfected into High- Five™ cells using Insect-Plus liposomes (Invitrogen) and plated onto 100-mm Petri dishes. After 48 h, antibiotic selection (500 µg/ml Zeocin, Invitrogen) was started and continued for 21 days. Colonies that survived selection were picked manually, expanded, and maintained in medium containing Zeocin (50 µg/ml). Punctin production by isolated cells was tested by Western blot analysis of conditioned medium using anti-His (monoclonal antibody, Invitrogen). For protein production, cells were grown in suspension in either Ultimove™ serum-free insect cell medium or Express-Five serum-free medium containing heparin (5 units/ml, Invitrogen). Production cultures were in spinner flasks, and culture medium was stored at −80°C with 1 mM sodium thiosulfate fluoride until use. For purification, conditioned medium was dialyzed into a binding buffer (20 mM sodium phosphate, 500 mM NaCl, pH 7.8) containing 0.03% Brij-35 (Sigma). Purification was performed using 1-liter batches of dialyzed medium and a 5-ml Ni-Sepharose column (ProBond™, Invitrogen) on an fast protein liquid chromatography instrument (Bio-Rad, Hercules, CA). Following bind- ing, the column was washed with three column volumes of binding buffer. A gradient of 0–42.5 mM imidazole in binding buffer was used to remove nonspecifically bound molecules from the column. Elution was with four column volumes of 250 mM imidazole in binding buffer, pH 7.0, containing 0.03% Brij-35. Elution was monitored by in-line UV and conductivity measurements. 2-ml fractions of eluate were collected and tested by Western blot analysis as described above. Fractions contain- ing punctin were pooled. Protein concentration was determined using the Bradford assay (Bio-Rad) and by phenylthiocarbamyl amino acid analysis using an Applied Biosystems model 420H/130/920 automated analysis system (16).

**Characterization of Recombinant Punctin—**The NH₂-terminal se- quence of recombinant punctin was determined by Edman degradation. Recombinant punctin (5 µg) was electrophoresed on 10% SDS-PAGE, electrotransferred to polyvinyllidene difluoride membrane, and lightly stained with modified Coomassie Blue (Simply Blue Safe Stain, Invitro- gen). Protein bands were excised and subjected to Edman degradation on an Applied Biosystems Procise 492 sequencer in the Molecular Biotechnology Core Facility of the Lerner Research Institute.

To probe for glycosylation, recombinant punctin (4 µg) was electrophoresed on 10% SDS-PAGE and stained for carbohydrate using a periodic acid-Schiff reaction-based method (Pro-Q fuchsin glycoprotein staining kit, Molecular Probes, Eugene, OR). In this reaction, Candy- Cane™ glycoprotein molecular weight standards consisting of alternate bands of glycosylated and unglycosylated proteins were used as controls. Glycoprotein staining was also performed after enzymatic deglycosylation of punctin with peptide N-glycosidase F. Deglycosyla- tion of denatured as well as native punctin was performed with a cocktail of endoglycosidases (Bio-Rad) using bovine tunicin acid. To investigate further whether N-linked carbohydrates were present in punctin, stably transfected insect cells were cultured in the presence or absence of tunicamycin A1 homolog (0.1 µg/ml culture medium, Sigma). Amounts of total protein from culture medium of tunicamycin- treated and untreated cells were assayed by Western blot with antibody 4113 at various time points after the addition of tunicamycin (in Da). Molecular mass of punctin was measured by MALDI-TOF and by LC-ESMS. MALDI-TOF was performed with a PerkinElmer Biosystems Voyager DE Pro-mass spectrometer using sinapinic acid as the matrix and bovine serum albumin as a calibration standard protein (17). MALDI-TOF MS measurements of intact punctin and naturally observed limited proteolysis fragments are reported ≤ 50% peak width (in Da) at half-maximal peak height. LC-ESMS was performed with a PerkinElmer Sciex API 3000 triple quadrupole mass spectrometer (17, 18). Nitrogen was used as the nebulization gas at 40 p.s.i., and curtain gas was supplied from a nitrogen generator (What- man model 75-72). For LC-ESIMS of intact punctin, a scan range of 700–1800 m/z was used with 0.2 atomic mass unit steps, a scan time of 7.5 s, and at an orifice potential of 80 and 5000 V ion spray. Reverse phase-high-pressure liquid chromatography was done at a flow rate of 5 µmin a 5-µm Vydc C18 capillary column (0.3 × 150 mm, LC Packing) using an Applied Biosystems Model 140D high-pressure liquid chromatography system and aqueous acetonitrile/trifluoroacetic acid solvents with 100% of the eluant going to the mass spectrometer. ESMS measurements of intact punctin are reported as the mean ± S.E. (in Da).

For glycopeptide characterization, punctin was excised from a SDS-polyacrylamide gel (~1 µl/lane × 6 lanes), in-gel reduced with 10 µt dithiothreitol, cysteine-alkylated with 20 µt iodoacetamide in 400 µt ammonium bicarbonate, and digested with 0.2 µt of trypsin (Promega) overnight at 37°C in 100 µt ammonium bicarbonate. Peptides from the in-gel tryptic digestes were extracted with 60% acetonitrile containing 0.1% trifluoroacetic acid, dried in a Speed Vac, redissolved in 50 µl
of 0.1% trifluoroacetic acid, and analyzed by LC-ESMS using selective ion monitoring with the PE Sciex API 3000 triple quadrupole mass spectrometer system as described above for intact protein analyses. Glycopeptides were selectively detected based on diagnostic sugar oxoacids. Partial sequence (Hex (m/z) 356) and N-acetylanamideic acid (HexNAc (m/z) 392) (17). Carbohydrate marker ions at m/z 264 (dwell time 200 ms each) were monitored in a positive ion mode at a high orifice potential (180 V), whereas full scans at m/z 300–2300 (0.2 atomic mass unit steps, scan time 3.5 s) were acquired at a lower orifice potential (70 V). This way both intact parent ions and abundant marker ions were observed in the same m/z scan.

**RESULTS**

**Cloning of Punctin cDNA**—We identified a novel EST (GenBank™ accession number AA482392) derived from pooled human melanocyte, fetal heart, and pregnant uterus with homology to ADAMTS proteases. The 1.5-kb insert of the corresponding IMAGE clone 752797 contained a long ORF encoding an amino-terminal TS domain, a cysteine-rich domain, a cysteine-free spacer domain, and three tandem TS modules followed by a short acidic peptide and stop codon (Fig. 1a). The stop codon and 3' untranslated sequence were independently confirmed by 3'-RACE (clone pSHTSL1s3, Fig. 1a) as well as by another EST (GenBank™ accession number W47029). The 3'-untranslated region encoded in IMAGE clone 752797 contained a consensus polyadenylation signal (AATTTAA) followed by a poly(A) tail 14 nucleotides downstream. Completion of the full-length coding sequences by 5'-RACE predicted a putative signal peptide upstream of the central TS domain. The signal peptide was preceded by a methionine codon within a satisfactory Kozak consensus sequence (A at −3, G at +4 relative to ATG) (20) although there was no upstream in-frame stop codon. The 5' sequence obtained by RACE was subsequently validated by independently cloned human and mouse ESTs (GenBank™ accession numbers A1459225 for human skeletal muscle cDNA (see below) as well as by identification of the encoding exons arranged sequentially on human chromosome 9 (Celeria Genomics, Rockville, MD).

**Primary Structure of Punctin Predicts an ADAMTS-like Protein**—The predicted full-length punctin protein contains 525 amino acids and has the typical domain structure of the ancillary noncatalytic regions of an ADAMTS protease (Fig. 1a). The mature secreted form of punctin is 497 amino acids with a molecular mass of 55,240 Da and a calculated pI of 6.2. Like the ADAMTS proteases, each domain in punctin has an even number of cysteine residues. This observation suggests that each domain may have internal disulfide bonds (17 such bonds are predicted in punctin), and that punctin consists of a series of independently folded and disulfide-bonded domains. Punctin shares some of these domains with other domains known from the ADAMTS family, especially in the auxiliary TS domains. The mature protein is predicted to be a membrane-embedded protein, with a single TM (transmembrane) domain.
AMTSL-3 (68% identity, see below). Of the ADAMTS enzymes published to date, punctin is most similar to human ADAMTS-10 (35% identity). The punctin TS domains have a higher degree of similarity to other ADAMTS-like proteins and ADAMTS proteases than to TSP1 and TSP2. The greatest similarities, as indicated by percentage of identity of amino acid sequences identified by BLAST searches of the first TS domain of punctin to TS domains from various molecules, are as follows: human ADAMTS-1, 80%; human ADAMTS-1, ADAMTS-6, and ADAMTS-10, 50%; mouse papilin, 47%; human ADAMTS-8, 44%; human ADAMTS-5, 42%; human TSP2, 40%; human TSP1, 38%. Like most TS domains in the ADAMTS family, punctin TS domains do not contain linear peptide sequences found in TSP1 that have been defined as heparin or CD-36 binding sequences (22). They do not contain degenerate GAG binding sequences such as BBX, where B is the basic amino acid and X is any amino acid (22).

Genomic Location of the Mouse and Human Punctin Genes and Tissue-specific Expression—The mapping of Adamts1 in an interspecific cross resulted in the following most probable gene order (mean ± S.D.): Ptprd-4.4 ± 2.0 centimorgan-Adamts1, Cdkn2a-1.8 ± 1.2 centimorgan-Jun and placed Adamts1 at a consensus position of 42.6 centimorgan on mouse chromosome 4 (Fig. 1c) in the vicinity of the interferon gene cluster. A search of the mouse genome data base (www.informatics.jax.org) did not reveal any pertinent genetic disorders near this locus.

The human-mouse homology maps (www3.ncbi.nlm.nih.gov/Omim/Homology/, accessed September 26, 2001) predict that the ADAMTSL1 locus is on human chromosome 9p21-22. The predicted locus was confirmed by the analysis of the human genome sequence. The punctin ORF is encoded by 13 exons spanning >250 kb of genomic DNA mapping to 9p21.2-22.1. A search of the Online Mendelian Inheritance in Man site (www3.ncbi.nlm.nih.gov/Omim/) revealed three unsolved human disorders in the vicinity of the ADAMTSL1 locus. Diaphyseal medullary stenosis with malignant fibrous histiocytoma (MIM12250) is linked to 9p22-p21, Friedrich’s ataxia type 2 (MIM601992) is linked to 9p23-p11, and neuropathy, distal hereditary motor, Jerash type (MIM605726) are linked to 9p21.1-p12.

ADAMTSL1 is primarily expressed in human and mouse skeletal muscle with a major message size of ~7.0 kb in both species (Fig. 2). A minor messenger RNA species of ~1.0 kb was also seen in some human tissues (Fig. 2, skeletal muscle, heart, colon, kidney, and liver). Expression was not detected in brain, colon, thymus, spleen, placenta, small intestine, lung, testis, ovary, or peripheral blood leukocytes.

Expression and Characterization of Recombinant Punctin—Punctin expressed in High-Five™ cells with tandem COOH-terminal V5 and 6× His epitopes was secreted into the conditioned medium of adherent as well as suspension cultures. Punctin was detected by antibody 4113 and anti-epitope tag antibodies as a ~60-kDa band under reducing conditions. It was substantially purified from the culture medium using Ni chromatography (Fig. 3a). The purification scheme yielded a maximum of 200 µg/liter purified protein as determined by amino acid analysis. Electrophoresis and Western blotting of concentrated punctin preparations frequently demonstrated additional bands of molecular mass (~120 and ~180 kDa, data not shown), suggesting the formation of dimers and trimers at high concentrations.

The conformation of punctin appears to be maintained by disulfide bonds as evidenced by more rapid migration in SDS-PAGE under nonreducing conditions than under reducing conditions (Fig. 3b). Furthermore, on Western blots under nonreducing conditions, the protein was not detectable with antibody 4113 (data not shown), suggesting that the peptide epitope was not accessible without reduction of disulfide bonds. A mass analysis of His-tagged punctin by MALDI-TOF MS yielded a broad peak suggesting that the 60-kDa gel band contained major molecular species of 61,935 ± 595 and 60,873 ± 295 Da, respectively. LC-ESMS analyses of the intact protein defined more precisely the major molecular species to be 61,970 ± 6 and 62,131 ± 5, which are, respectively, 1979 and 2140 Da larger than the calculated mass (59,991) of tagged punctin based on amino acid sequence. NIH-terminal sequencing of the polyvinylidene difluoride-immobilized 60-kDa protein revealed a single sequence, which commenced at Glu29 (i.e., Glu-Glu-Asp-Arg-Asp-Gly and so on). Recombinant Punctin Is Glycosylated—Two closely spaced punctin bands were resolved by Western blot analysis of conditioned medium or purified protein, although Coomassie Blue staining of purified punctin always demonstrated a single band (Fig. 3a). A periodic acid-Schiff-based method of staining carbohydrate chains suggested that recombinant punctin is a gly-
coprotein (Fig. 3c), and mass spectrometry demonstrated multiple molecular species consistent with variable glycosylation. Treatment of recombinant protein with peptide N-glycosidase F did not result in a perceptible decrease in molecular mass, although the intensity of glycoprotein staining was decreased (data not shown). Culture medium from tunicamycin-treated cells exhibited only a single punctin species as demonstrated by Western blotting (Fig. 3d). The difference (161 Da) between the LC-ESMS-observed masses of the major punctin molecular species (61,970 and 62,131 Da) is close to the in-chain chemical average mass of a oligosaccharide residue (Hex, 162). Minor molecular species were also apparent by LC-ESMS analysis, which differed by mass increments that approximated the in-chain chemical average mass of oligosaccharide residues (e.g. Hex, 162; HexNAc, 203; NeuAc, 291). For a further analysis, tryptic digests of the protein were examined by analytical LC-ESMS using stepped collision energy scanning to produce carbohydrate-specific marker ions. Glycopeptides were detected including molecular species with masses of 5881.4 ± 0.4 and 6171.2 ± 0.2 Da. The mass difference (289.8 Da) between these observed glycopeptides appears to correspond to the in-chain chemical average mass of N-acetylneuraminic acid (NeuAc, 291). Taken together, these data indicated that punctin is glycosylated, although specific glycopeptides have yet to be characterized fully. Approximately 65% of the amino acid sequence in punctin was identified by peptide mass mapping including the NH₂-terminal tryptic peptide (Glu²⁹–Arg⁴⁷), verifying that the target protein has been expressed. Based on the difference between the observed and calculated masses of intact punctin, the recombinant protein contains approximately 3–4% carbohydrate by weight.

During purification of punctin in the absence of protease inhibitors, additional components of ~40 and 20 kDa, respectively, were detected on Coomassie Blue-stained gels (data not shown). The 40-kDa band contained two molecular species with measured masses of 38,409 ± 115 and 39,456 ± 156 Da, respectively, as determined by MALDI-TOF MS. The NH₂-terminal sequencing of these bands yielded the same amino terminus as the full-length punctin. The ~20-kDa fragment exhibited an NH₂-terminal sequence ³⁷²–⁴⁰³, indicating that the fragment is from the carboxyl terminus. The addition of 1 mM phenylmethylsulfonyl fluoride to culture medium effectively prevented this proteolysis, suggesting that it was effected by a serine protease.

Visualization of Punctin by Rotary Shadowing—Rotary shadowing of purified recombinant punctin demonstrated a hatchet-shaped or comma-shaped molecule 30–40 μm in length (Fig. 4). Punctin consists of a single globular domain of 10–20 μm in size with a short linear segment at one end. Most of the visualized protein was in monomeric form (Fig. 4). Occasional aggregates with the appearance of dimers and trimers were seen but have not yet been resolved in detail.

Expression and Localization of Punctin in Transfected COS-1 Cells—Transfected cells were stained without fixation or permeabilization and on ice (live staining) to prevent the detection of intracellular punctin or endocytosed antibody, respectively. Under these conditions, punctin was localized underneath the cells (i.e. adjacent to their ventral surface) in the substratum laid down on plastic. The staining pattern was punctate (Fig. 5, a–d) and was preferentially located toward the periphery of the cells (Fig. 5, a, b, and d) and under cellular processes (Fig. 5c). The punctin deposits were of submicron dimension, although fluorescent signals from closely located deposits were frequently merged suggesting larger aggregates. Transfected cells had minimal or no staining on the dorsal cell surface. Punctin was not seen in the substratum in areas not corresponding to the cells. If cells were detached with 10 mM EDTA prior to staining, “footprints” of transfected cells were retained on the substratum with a similar staining pattern as under intact conditions.

**FIG. 2.** Northern analysis of expression of ADAMTS11 (left) and ADAMTS11 (right) in adult human and mouse tissues, respectively. Kilobase markers of RNA are shown at the left of each autoradiogram, and tissue origin is indicated above each lane. Hybridizing transcripts are indicated by arrows.

**FIG. 3.** Analysis of epitope-tagged punctin purified by Ni-chromatography from insect cell culture medium. a, Coomassie Blue (Simply Blue Safe Stain) staining of purified recombinant punctin on reducing SDS-PAGE (left lane) and Western blot analysis with anti-punctin antibody 4113 (right lane). b, Western blot analysis using anti-His tag monoclonal antibody on reducing (left lane) and nonreducing SDS-PAGE (right lane). c, glycprotein staining of recombinant punctin (lane 2 contains 0.6 μg, and lane 3 contains 3 μg) using the periodic acid–Schiff procedure. Glycosylated CandyCane™ markers (1 μg/band) stained similarly are in lane 1. The arrow indicates stained punctin. d, Western analysis of culture medium from insect cell cultures treated without (left lane) or with (right lane) tunicamycin A for 72 h. Each lane contains 2.8 μg of total protein. Double arrowheads are used to indicate two molecular species seen on Western blots.
cells. Staining was seen in some areas not covered with cell processes. In other areas, there were cell processes without underlying punctin (Fig. 5c). We interpret this finding to result from cellular motility (i.e., withdrawal of existing processes and the formation of new ones). Identical results were obtained with anti-FLAG monoclonal antibody or antibody 4113. Fig. 5, a–c, shows staining of FLAG-tagged protein using the FLAG M2 monoclonal antibody, and Fig. 5d shows staining with anti-punctin antiserum 4113. Similar staining patterns were seen whether cells were grown in the presence or absence of serum and using tagged or untagged proteins (data not shown).

Double staining for vinculin (Fig. 5d) or focal adhesion kinase (data not shown), components of focal contacts, indicated that punctin staining did not correspond to sites of focal contacts. No staining was visible in control experiments, i.e., in untransfected COS cells, cells transfected with vector alone, cells stained without a primary antibody, or cells stained with preimmune serum as control.

On Western blots, we found reactive protein bands of the expected size (58–60 kDa for untagged punctin and 62–64 kDa for the His-tagged or FLAG-tagged forms) in the medium, cell layer, and the underlying substratum or ECM of transfected COS-1 cells (Fig. 5e). In contrast, cells transfected with vector alone (Fig. 5e) or untransfected cells (data not shown) did not show a reactive band. As controls, preimmune serum from rabbits in which anti-Punctin antibodies were generated did not produce immunoreactivity on Western blots (data not shown).

**DISCUSSION**

**Punctin/ADAMTSL-1 Is a Novel ADAMTS-like Secreted Protein Belonging to a Distinct ADAMTSL Family of Proteins**—In addition to missing the catalytic domain, the ADAMTS-like proteins (see below) do not possess disintegrin-like domains. This finding suggests that the disintegrin-like domain and catalytic domain may represent a functionally coupled protease domain in ADAMTS enzymes. Further evidence for this comes from the identification of other proteins with a predicted structure similar to punctin. Following the complete cloning of punctin/ADAMTSL-1, we became aware of a second such molecule encoded by the KIAA0605 gene (GenBank accession number AB011177) that we designated as AD-AMTS-like Protein 2 (23). We have cloned a third ADAMTS-like protein, ADAMTSL-3 (GenBank accession number AF237652).

Therefore, punctin belongs to a distinct protein family. ADAMTSL-2 and ADAMTSL-3 differ from punctin in their greater length (951 and 1690 amino acids, respectively) and also have more TS domains (6 and 10, respectively). These molecules will be described in greater detail in subsequent publications. In contrast to ADAMTSL-2 and ADAMTSL-3, which are quite widely expressed, punctin/ADAMTSL-1 is selectively expressed in muscle.

Other secreted ECM molecules such as lacunin and papilin also contain the ancillary domains of the ADAMTS family in the precise order as punctin. However, punctin is more closely related to ADAMTSL-3 and some ADAMTS proteases than it is to mouse papilin (32% identity). Lacunin is a basement membrane glycoprotein in the moth Manduca sexta (24). Lacunin has the structure of ADAMTSL including seven TS modules as

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**FIG. 4.** Rotary shadowing of recombinant punctin. a, overview. b–g, images of individual punctin molecules. Scale bar in panel a indicates molecular dimensions in all panels.

**FIG. 5.** a–d, confocal laser-scanning microscopy of COS-1 cells following transient transfection with ADAMTSL1 expression constructs and immunocytochemistry. Untransfected cells are visible in a and b. Scale bar (10 μm) is shown at lower right of each panel. a and b, punctate staining of FLAG-tagged punctin (red) in nonpermeabilized cells visualized with anti-FLAG M2 antibody. Nuclei are blue 4',6-diamidino-2-phenylindole. c, relationship of punctin staining (red) visualized with anti-FLAG M2 monoclonal antibody to cellular actin as visualized by phalloidin staining (green). The asterisk indicates a cellular protrusion that does not have underlying punctin, and the arrow indicates punctin immunolocalization without an overlying cellular process. d, relationship of punctin staining (red) visualized with anti-punctin antiserum 4113 to vinculin staining (green) as shown by confocal imaging and overlay of single-color images from a double-stained cell. e, Western blot analysis of cell lysates (lane 1), medium (lane 2), and ECM (lane 3) from transfected COS-1 cells using an anti-His tag monoclonal antibody. Cell lysates from untransfected COS-1 cells are shown in lane 4. Molecular mass is indicated on the left.

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3 N. Moore, B. Anand-Apte, and S. Apte, unpublished data.

4 S. Apte, unpublished data.
well as a single COOH-terminal protease and lacunin domain. In addition, it contains 13 repeats of a novel lagrin domain, 11 Kunitz inhibitor domains, 2 antistasin-like domains, 1 serine protease inhibitor domain, and 2 immunoglobulin domains. Lacunin localizes to the basal lamina of the moth wing (24). Papilin from Drosophila melanogaster may be an ortholog of M. sexta lacunin, because the two molecules are similar in their domain content, organization, and primary sequence. Papilin is also a basement membrane protein (25). Although these invertebrate proteins have numerous protease inhibitor domains, mammalian papilin contains substantially fewer such domains (25).

**Characterization of Recombinant Punctin from Insect Cells—**Our experimental data support the likelihood that recombinant punctin is disulfide-bonded. First, its electrophoretic mobility is greater under nonreducing conditions. Second, the punctin epitope is masked under nonreducing conditions. Third, rotary shadowing demonstrated a molecule with a specific and consistent conformation. Limited proteolysis within the linker peptide, connecting TS domains 2 and 3 assigned to the Tyr371-Asp372 peptide bond (Fig. 1b) by a putative serine protease, indicates that there may be a proteolytically susceptible exposed region between the two disulfide-bonded TS domains. It is not yet known whether this is a physiologically relevant processing or whether it is an artifact that is unique to this expression system. The processing event releases the two COOH-terminal TS domains of punctin. Because proteolytically derived fragments of many secreted proteins have distinctive functions, it will be interesting to investigate whether specific functions are associated with the ~40- and ~20-kDa fragments.

A mass measurement of epitope-tagged recombinant punctin by MALDI-TOF MS and LC-ESMS revealed that purified punctin contained multiple species of higher than the predicted mass. Edman degradation indicated that all these species had the same amino terminus. Further MS analysis, glycoprotein staining, and culture in the presence of tunicamycin A confirm that punctin contains N-linked sugars but do not exclude the presence of O-linked sugar. Significant alteration of mobility was not seen after peptide N-glycosidase F treatment, suggesting that the N-linked carbohydrate may be resistant to complete enzymatic removal (26).

Rotary shadowing is useful for demonstrating the physical conformation of a molecule as well as the existence of oligomeric complexes (27–29). The data we have obtained for punctin are relevant to the ADAMTS, lacunin, and papilin. They can be extrapolated to represent the structure of the ancillary domains of an ADAMTS enzyme and the “papilin cassette” (25) and provide the first insight into the conformation of these domain assemblies. Many ECM proteins exist as oligomers. This observation may also be the case with punctin, because rotary shadowing electron microscopy and gel electrophoresis occasionally suggested the presence of dimers and trimers. We anticipate that rotary shadowing will be useful for future studies to investigate punctin oligomerization and interactions of punctin with putative ECM ligands.

**Punctin Is an ECM Glycoprotein That Binds to the Cell Substratum in a Spatially Specific Manner—**Nontransformed cells in culture require a substratum for attachment, spreading, and migration. The substratum present on an unmodified plastic tissue culture surface is derived from the cells themselves as well as from proteins in serum-supplemented culture medium (30–32). Quantitatively significant components of the cell substratum are laminin, fibronectin, vitronectin, collagen, tenasin, PG-M or versican (a chondroitin sulfate proteoglycan), perlecán (a heparan sulfate proteoglycan), hyaluronan, and tissue inhibitor of metalloproteases-3 (30–37). Punctin shares the subcellular distribution of molecules that do not generally co-localize with focal contacts (e.g. versican, hyaluronic, and tenasin) (31, 37). Because punctin is left behind in the ECM after cell detachment with EDTA, we conclude that when expressed in COS-1 cells, punctin binds a component of the ECM. Punctin in culture medium may reflect an excess of more than that which can bind to the substratum or indicate secretion from the free surface of the cell. Punctin does not bind to ECM between the cells, indicating that the punctin ligand is absent from these regions. Because similar staining was seen under serum-supplemented as well as under serum-free culture conditions, it is probable that the ECM binding partner of punctin is a molecule produced by COS-1 cells but not one derived from fetal bovine serum.

**Significance of Punctin and the ADAMTS-like Family—**Molecules comprising ancillary domains of metalloproteases may be generated in biological systems by proteolytic processing or through alternative splicing of protease genes. Brooks et al. (38) found that the proteolytically generated hemopexin domain of MMP-2 circulated in serum and bound to the integrin α2β1. This MMP-2 fragment inhibited angiogenesis by preventing membrane targeting of MMP-2 (38). So far, there are no known examples of ADAMTS-like proteins generated as splice variants of ADAMTS genes. The discovery of punctin demonstrates for the first time the existence of molecules closely resembling the ancillary domains of ADAMTS that are generated as distinct gene products.

The resemblance of ADAMTSL to ADAMTS suggests a functional relationship between these two groups of molecules. From studies on ADAMTS-1 (39) and ADAMTS-2 (40), it is known that the ancillary domains are required to bind and cleave substrates. ADAMTSL may offer a potential mechanism of ADAMTS regulation via one of several possible mechanisms. As a result of noncompetitive inhibition of ADAMTS-2, an inhibitory role has been shown for Drosophila papilin (25). Another possibility is that punctin may compete with ADAMTS for its substrates and protect the substrates from cleavage. The isolated MMP-2 hemopexin domain represents one such example. In a second example, a truncated nonenzymatic version of ADAM-17 was shown to have a dominant negative effect on the activation of tumor necrosis factor-α (41). An intriguing possibility is that the ADAMTS-like proteins may be enhancers of the ADAMTS proteases. For example, the procollagen C-proteinase enhancer protein (42) contains two domains homologous to those found in the C-proteinase that are instrumental in binding to the carboxyl propeptide of procollagen I and enhancing its removal (43). Very little is currently known about the regulation of ADAMTS proteases following their activation, and it is possible that the ADAMTS-like proteins may provide a novel general principle of regulation.

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