UNIVERSITÉ DE SHERBROOKE

IDENTIFICATION DE DÉTERMINANTS MOLÉCULAIRES IMPLIQUÉS DANS LA BIOSYNTHÈSE ET L'ACTIVATION DES ADAMTS (a disintegrin and metalloprotease with thrombospondin type I repeat)

par

JEAN-MICHEL LONGPRÉ

Département de pharmacologie

Thèse présentée à la Faculté de médecine et des sciences de la santé en vue de l'obtention du grade de philosophiae doctor (Ph.D.) en pharmacologie

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RÉSUMÉ

IDENTIFICATION DE DÉTERMINANTS MOLÉCULAIRES IMPLIQUÉS DANS LA BIOSYNTHÈSE ET L'ACTIVATION DES ADAMTS (a disintegrin and metalloprotease with thrombospondin type I repeat)

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Les ADAMTS (a disintegrin and metalloprotease with thrombospondin type I repeat) sont des métallopeptidases sécrétées dans le milieu extracellulaire ayant comme fonction le clivage de différents substrats de la matrice extracellulaire tel le propeptide en N-terminal du collagène, l'aggrécan, ainsi que le von Willebrand factor retrouvé dans le plasma. Une mutation ou un dérèglement d'ADAMTS2, 5 et 13 sont directement responsable du syndrome de Ehlers-Danlos de type VII C, l'arthrose et la purpura thrombotique thrombocytopénique respectivement. De plus, ADAMTS1 et 8 sont reconnues pour avoir des propriétés anti-angiogéniques qui s'avèrent d'un potentiel thérapeutique contre la progression des tumeurs. La biosynthèse et les mécanismes menant à la pleine activité biologique de ces peptidases sont peu connus et ont été étudiés dans cet ouvrage.

Nous avons démontré que les ADAMTS sont initialement synthétisées sous forme de zymogènes qui subissent un clivage protéolytique à la jonction de leur prodomaine et de leur domaine catalytique par différentes sérines peptidases de la famille des pro-protéines convertases de type subtilisine. Des études de marquages métaboliques des différents ADAMTS transfectées dans la lignée cellulaire CHO RPE.40 déficiente en furine ont dévoilé que ADAMTS1, 5, 7 et 9 sont toutes clivées par la furine. D'autres convertases clivent de façon moins efficace que la furine les prodomaines des ADAMTS (PACE4 et PC6B clivent ADAMTS1, PC6B et PC7 clivent ADAMTS7, PC5A clive ADAMTS9 et PC7 clive ADAMTS5). Malgré la présence de plusieurs sites consensus de clivage par la furine dans les prodomaines des ADAMTS, des études de mutagenèse dirigée abolissant les différents sites ont démontré que le site plus près du domaine catalytique est préférentiellement clivé par la furine. Le clivage du prodomaine d'ADAMTS1 et 7 s'effectue au réseau du *trans*-Golgi. Toutefois, des études de marquage à la biotine des protéines de la surface cellulaire démontrent que ADAMTS7 semble aussi être clivée à la surface de la cellule. ADAMTS5 est strictement maturée dans l'espace extracellulaire, soit à la surface cellulaire ou dans le milieu extracellulaire, ce qui révèle un nouveau mécanisme d'activation par la furine pour des substrats endogènes. En outre, ADAMTS9 est clivée à la surface de la cellule par la furine, mais contrairement à la presque totalité des substrats de la furine, celle-ci inactive ADAMTS9. En somme, il existe plusieurs mécanismes d'activation des ADAMTS : activation intracellulaire ou extracellulaire et inactivation extracellulaire par les convertases.

La présence, dans les prodomaines des ADAMTS, d'acides aminés conservés à travers les membres de cette famille d'enzyme nous amène à penser qu'ils pourraient jouer un rôle important dans leur maturation. La mutation des acides aminés conservés des motifs CXYXG et YFIXPL d'ADAMTS1 et 9 ainsi que des sites de N-glycosylation d'ADAMTS9 ont grandement affecté la sécrétion de l'enzyme mature. Cette observation permet de conclure qu'outre le site consensus de clivage par la furine, des motifs conservés ainsi que la glycosylation des prodomaines sont impliqués dans la biosynthèse des ADAMTS. La découverte de nouveaux mécanismes d'activation par la furine a une signification importante dans le domaine d'activation de précurseurs. L'activation extracellulaire d'ADAMTS5, l'enzyme responsable de la dégradation du cartilage, génère une cible thérapeutique potentielle pour un traitement futur de l'arthrose.
Mots clés : précurseur, pro-protéine convertase, furine, ADAMTS et activation

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LONGPRÉ, J.-M., LEDUC, R., (2004) Identification of pro-domain determinants involved in ADAMTS-1 biosynthesis, *J. Biol. Chem.* 279(32):33237-33245

Autres articles supportant cette thèse :

KOO, B. H., **LONGPRÉ, J.-M.**, SOMERVILLE, R. P., ALEXANDER, P., LEDUC, R., APTE, S. S., (2007) Regulation of ADAMST9 secretion and enzymatic activity by its propeptide, *J. Biol. Chem.* 282(22):16145-16154.

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*Dénote une contribution égale des auteurs

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DÉSILETS, A., **LONGPRÉ, J.-M.**, CLOUTIER, W., PARENTA, A., KOMIYAMA, T., FULLER, R. S., LEDUC, R., Matriptase substrate specificity characterization and inhibition by eglin c variants, 4th General Meeting of the International Proteolysis Society, Québec, Canada, 15-19 octobre 2005

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LISTE DES ABBRÉVIATIONS

ABP-280	Protéine de 280 kDa liant l'actine (actine binding protein)
ADAM	A disintegrin and metalloprotease
ADAMTS	A disintegrin and metalloprotease with thrombospondin type I repeat
COMP	« Cartilage oligomeric matrix protein »
EGF	Facteur de croissance épithélial
fur	région en amont de fsp/fes (fsp/fes upstream region
kDa	KiloDalton
MMP	Matrix metalloprotease
PACE	« Paired basic amino acid converting enzyme »
PACS	Protéine de triage du motif acide de la furine phosphorylée (phosphofurin acidic cluster sorting protein)
PC	Pro-protéine convertase de type subtilisine
POMC	Pro-opiomélanocortine
Pro-TGFβ	Précurseur du facteur de croissance transformant β
RE	Réticulum endoplasmique
TGN	Réseau du *trans*-Golgi
TIMP	« Tissu inhibitor of metalloproteinase »
TNF	Facteur de nécrose tumorale
TTP	purpura thrombotique thrombocytopénique
VEGF	Facteur de croissance endothélial vasculaire

INTRODUCTION

1. Biosynthèse de précurseurs protéiques

Les modifications post-traductionnelles telles la formation de ponts disulfures, la glycosylation, la phosphorylation, l'oligomérisation et le clivage protéolytique spécifique permettent de diversifier et réguler l'expression des gènes. Afin d'atteindre leur forme mature et active, la protéolyse peptidique est sans doute le type de modification le plus courant. La protéolyse de précurseurs protéiques permet, entre autres, de réguler plusieurs processus biologiques incluant la coagulation du sang et la fibrinolyse (DAVIE *et al.*, 1991), l'apoptose (THORNBERRY et LAZEBNIK, 1998) et les fonctions neuronales et endocriniennes (CREEMERS *et al.*, 1998; ROUILLE *et al.*, 1995). De plus, la protéolyse est requise lors de nombreux autres processus biosynthétiques de précurseurs.

On appelle les précurseurs, des protéines qui empruntent la voie de sécrétion, qui sont synthétisées sous forme inactive et qui subissent une maturation protéolytique afin d'acquérir leur pleine activité biologique. En effet, la synthèse de protéine sous forme de précurseur s'avère nécessaire lorsque des séquences spécifiques sont requises pour la translocation d'une protéine dans le réticulum endoplasmique (RE), son repliement adéquat, sa localisation intracellulaire et la régulation de son activité. Les séquences devenues caduques doivent être retranchées par des endopeptidases pour libérer des polypeptides biologiquement actifs. La notion d'activation de précurseurs prend ses racines dans les études des enzymes gastriques et pancréatiques. Les auteurs Kunitz et Northrop (1935) ont identifié une hydrolyse peptidique dans la caractérisation des propriétés

biochimiques et biophysiques des molécules détectées pendant la conversion du chymotripsinogène en γ-chymotrypsine. On compte parmi les différents types de protéines synthétisées sous forme de précurseurs des pro-enzymes (ex : pro-ADAM17), des pro-facteurs de croissance (ex : pro-TGF β1), des pro-récepteurs (ex : pro-récepteur de l'insuline), des pro-protéines du sérum (ex : pro-albumine) et des protéines virales et toxines bactériennes (ex : l'antigène de protection de l'anthrax) (MOLLOY *et al.*, 1999).

Chez presque la totalité des précurseurs des cellules eucaryotes, la maturation se déroule dans la voie de sécrétion. Tel que démontré à la figure 1, il existe deux types de voie de sécrétion : la voie de sécrétion constitutive et la voie de sécrétion régulée. La voie de sécrétion constitutive, présente dans tous les types cellulaires, sécrète les protéines de façon continue et non régulée dans le milieu extracellulaire. La voie régulée par contre se retrouve principalement dans les cellules endocrines ou neuroendocrines. La stimulation par une hormone ou un neurotransmetteur, par exemple, permet la sécrétion du contenu des vésicules de sécrétion tels des hormones, des neuropeptides ou encore des enzymes de digestion.

Comme mentionné précédemment, plusieurs peptides ou protéines biologiquement actives proviennent de précurseurs inactifs synthétisés dans le milieu intracellulaire. La majorité des polypeptides sécrétés tels les facteurs de croissance, les neuropeptides, les enzymes protéolytiques et les glycoprotéines virales doivent être clivés pour être activés. Chez les mammifères, la protéolyse des précurseurs protéiques est effectuée en majorité par une famille d'enzymes, les pro-protéines convertases de type subtillisine.

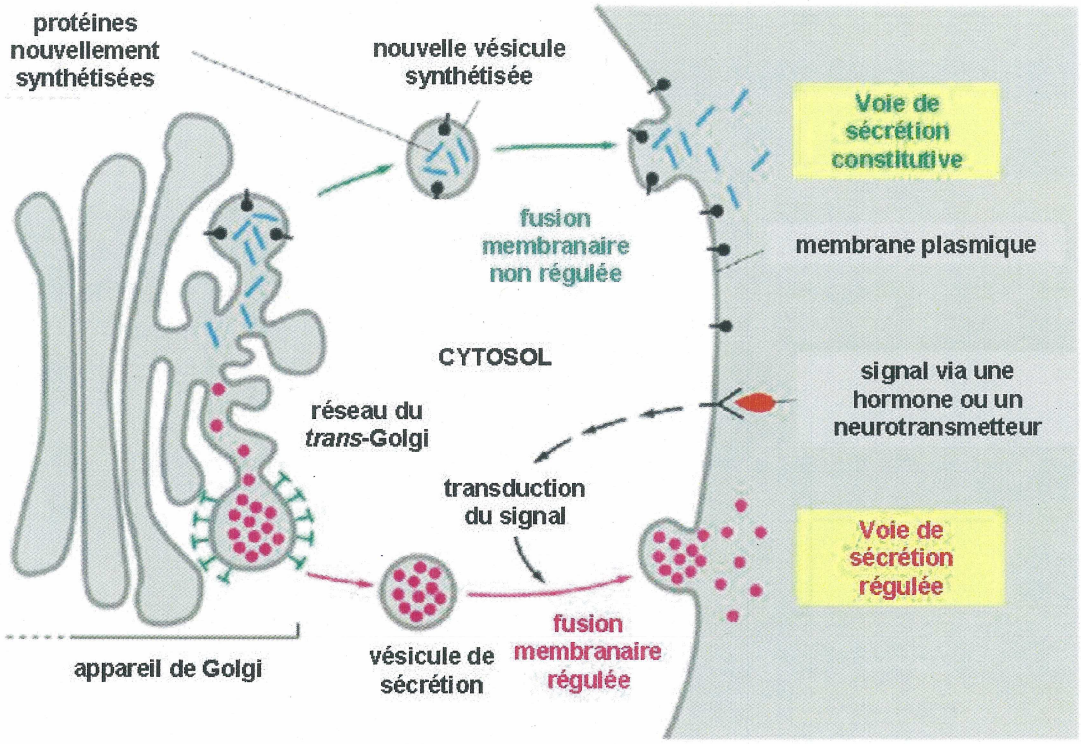

Figure 1. Schéma des voies de sécrétion constitutive et régulée. Les deux voies divergent dans le réseau du *trans*-Golgi. Un grand nombre de protéines solubles sont continuellement sécrétées hors de la cellule par la voie de sécrétion constitutive. Cette voie fournit également des lipides et des protéines transmembranaires à la membrane plasmique. Des cellules sécrétrices spécialisées possèdent également une voie de sécrétion régulée par laquelle des protéines sélectionnées dans le réseau du *trans*-Golgi sont détournées vers des vésicules de sécrétion. Les protéines y sont concentrées et stockées jusqu'à ce qu'un signal extracellulaire stimule leur sécrétion. Figure adaptée de (ALBERTS, 1994)

2. Les pro-protéines convertases de type subtilisine

La quête des pro-protéines convertases remonte à 1967 lorsque Donald Steiner démontra que certaines hormones peptidiques provenaient de l'excision de précurseurs plus larges par clivage à des paires d'acides aminés basiques (STEINER *et al.*, 1967). C'est seulement presque vingt ans plus tard que la kexine fut identifiée chez la levure *Saccharomyces cerevisiae* (JULIUS *et al.*, 1984). La kexine permet la maturation à des paires d'acides aminés basiques du facteur de conjugaison α, (MIZUNO *et al.*, 1988). En raison de la capacité de la kexine à cliver certains précurseurs de mammifères (l'albumine et la pro-opiomélanocortine (POMC)), on pouvait supposer la présence d'orthologues chez les organismes plus complexes (BATHURST *et al.*, 1987; THOMAS *et al.*, 1988). Des recherches d'homologie de séquences à l'intérieur du génome humain permirent d'identifier le gène « *fur* » codant pour la furine/PACE (ROEBROEK *et al.*, 1986b). Une série d'autres enzymes similaires à la furine furent ensuite rapidement découvertes. À ce jour, la famille des convertases de type subtilisine (archétype bactérien des peptidases à sérine active) chez les mammifères compte sept membres : furine (EC 3.4.21.75, (FULLER *et al.*, 1989; ROEBROEK *et al.*, 1986a)), PC2 (EC 3.4.21.94, (SEIDAH *et al.*, 1990; SMEEKENS et STEINER, 1990)), PC1/PC3 (EC 3.4.21.93, (SEIDAH *et al.*, 1990; SMEEKENS et STEINER, 1990)), PACE4 (KIEFER *et al.*, 1991), PC4 (NAKAYAMA *et al.*, 1992; SEIDAH *et al.*, 1992), PC6A et B (LUSSON *et al.*, 1993; NAKAGAWA *et al.*, 1993a; NAKAGAWA *et al.*, 1993b), et PC7 (SEIDAH *et al.*, 1996).

Les convertases sont des enzymes Ca^{2+}-dépendants qui clivent leurs substrats protéiques à des sites très spécifiques. En effet, les convertases ont une spécificité de

clivage en C-terminal de résidus dibasiques. La furine est la plus étudiée des convertases et son motif de reconnaissance minimal est RXXR↓, où X peut être n'importe quel acide aminé. Au niveau du site de clivage, on identifie les acides aminés de la manière suivante : ...P4-P3-P2-P1↓P1'-P2'... (SCHECHTER et BERGER, 1967).

2.1 Structures des pro-protéines convertases (PC)

La spécificité de substrat, l'efficacité catalytique et la localisation intracellulaire des pro-protéines convertases sont conférées par les composantes structurales de chaque enzyme. Les convertases sont des protéines mosaïques, c'est-à-dire qu'elles contiennent des domaines définis remplissant des fonctions particulières (Figure 2). Elles possèdent toutes un peptide signal permettant leur ciblage au réticulum endoplasmique (RE) pour entrer dans la voie de sécrétion. C'est le premier segment des convertases à être enlevé et ce clivage est réalisé par une « signal peptidase » directement après la translocation du polypeptide naissant au travers de la membrane du RE. Le deuxième domaine à être retranché est le prodomaine. Le prodomaine a comme rôle de guider le repliement adéquat de la protéine en agissant comme une chaperone intramoléculaire (EDER *et al.*, 1993; SHINDE et INOUYE, 1993). Le prodomaine sert aussi d'inhibiteur en se repliant sur le site catalytique de l'enzyme. Tout comme leurs nombreux substrats, les convertases sont d'abord synthétisées sous la forme d'un précurseur inactif qui doit être clivé pour s'activer. Cette maturation autoprotéolytique se déroule lors du transit des convertases dans la voie de sécrétion des cellules (LEDUC *et al.*, 1992). Le prodomaine des convertases est donc le deuxième domaine à être clivé afin de s'activer.

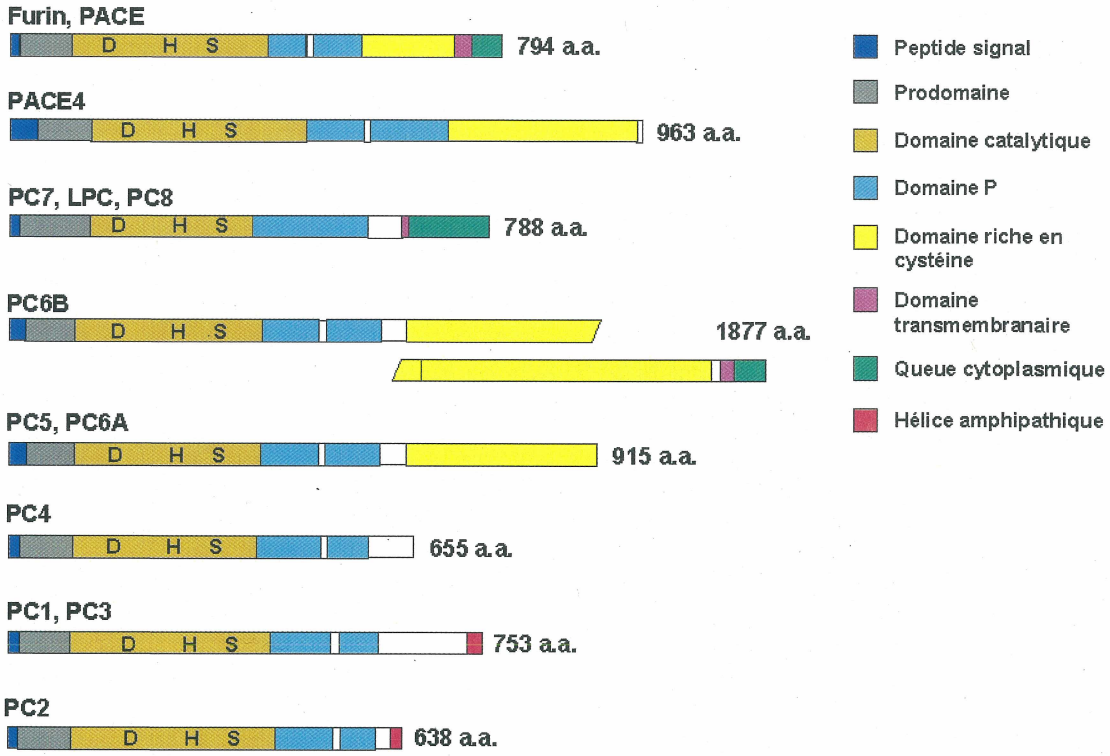

Figure 2. Représentation schématique des pro-protéines convertases de type subtilisine. Chaque domaine des endopeptidases est représenté sous la forme d'une boîte ayant un code de couleur. Les lettres D, H et S représentent la triade d'acides aminés du site actif. Le chiffre à la droite de chaque enzyme indique le nombre d'acides aminés de chaque pro-protéine convertase.

Chez la furine, un premier clivage autocatalytique du prodomaine s'effectue à la séquence Arg-Thr-Lys-Arg↓ et, suite à ce clivage, le prodomaine occupe le site actif de l'enzyme en agissant en tant qu'inhibiteur compétitif (MOLLOY *et al.*, 1999). Lorsque l'enzyme atteint le réseau du *trans*-Golgi (TGN), les conditions de pH permettent à un second site (Arg-Gly-Val-Thr-Lys-Arg↓), qui est situé en amont du site de clivage primaire, d'être auto-clivé par la furine afin d'abolir l'inhibition du prodomaine sur le domaine catalytique (FELICIANGELI *et al.*, 2006). L'interaction du prodomaine avec le site catalytique permet une régulation de l'activité catalytique des convertases afin que celles-ci soit seulement activées lorsqu'elles atteignent leur compartiment cellulaire cible.

Le domaine catalytique contient le site actif de l'enzyme et démontre la plus grande homologie entre les pro-protéines convertases de type subtilisine. Il est caractérisé par la triade catalytique Asp-His-Ser et classe donc les convertases dans le clan SB (peptidase à sérine active, triade catalytique ordonnée Asp-His-Ser), famille S8 (subtilisines) et sous-famille B (type kexine) (RAWLINGS et BARRETT, 1994). En plus de la triade catalytique, les PCs possèdent une cavité oxyanionique permettant la stabilisation de l'intermédiaire tétrahédrique de la réaction d'hydrolyse ainsi que deux sites de liaison au Ca^{2+} (SIEZEN *et al.*, 1994). D'ailleurs, l'analyse du cristal de la furine de souris lié à l'inhibiteur dec-RVKR-cmk révèle les contraintes tridimensionnelles expliquant la spécificité de substrat de la furine pour des arginines en P1 et P4 et des résidus basiques en P3, P5 et P6 (HENRICH *et al.*, 2003).

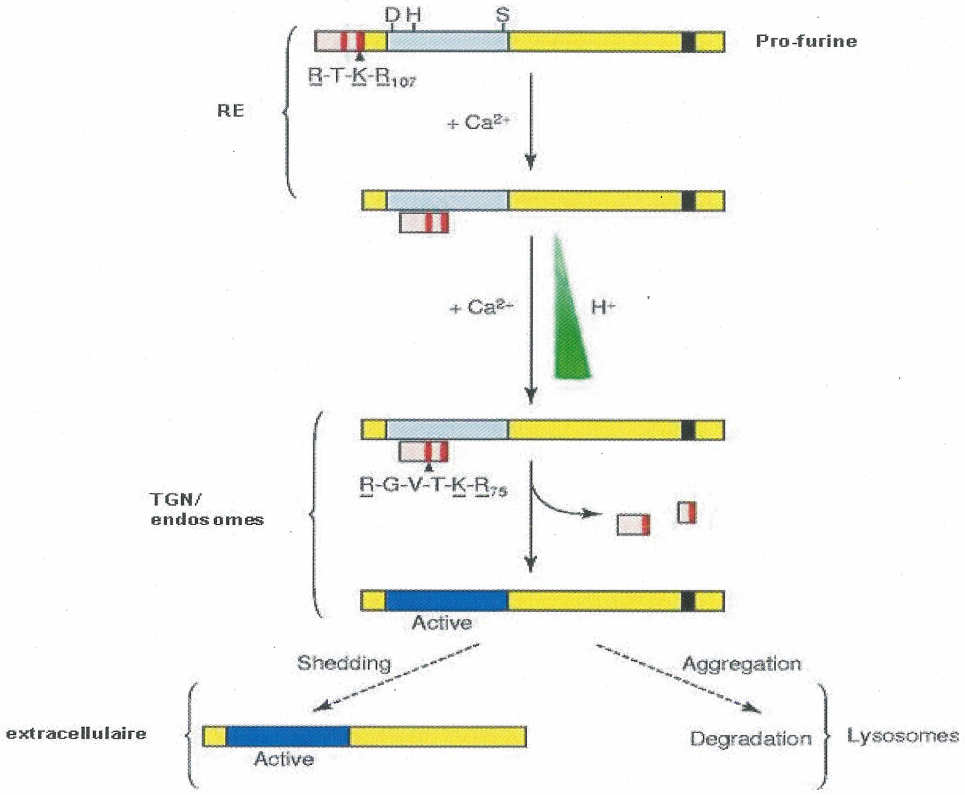

Figure 3. Mécanisme d'autoactivation de la furine. Le prodomaine de la furine est représenté en rouge et le domaine catalytique en bleu. Dans le réticulum endoplasmique, un premier clivage du prodomaine survient au site RTKR107. Lors de la translocation vers les endosomes et au TGN, une baisse du pH entraine le clivage d'un deuxième site de clivage du prodomaine, RGVTKR75, afin de libérer le prodomaine du site catalytique et rendre l'enzyme active.

Le domaine P, est nécessaire pour l'activité catalytique de l'enzyme puisque sa délétion complète ou partielle abolit l'activité catalytique (CREEMERS *et al.*, 1993). Les études de modélisation confirment que le domaine P est associé au domaine catalytique (HENRICH *et al.*, 2003). Ce domaine serait aussi essentiel au bon repliement et à la stabilité de l'enzyme. De plus ce domaine agirait dans la régulation de la dépendance des convertases au Ca^{2+} et au pH (ZHOU, A. *et al.*, 1998).

Les autres domaines en C-terminal du domaine P sont très variables au sein des convertases. PC1 et PC2 possèdent une hélice amphipathique pouvant créer des interactions hydrophobes avec les membranes (SEIDAH *et al.*, 1990). La furine (VAN DE VEN *et al.*, 1990), PACE4 (KIEFER *et al.*, 1991) et PC6A/B (LUSSON *et al.*, 1993) possèdent une région riche en cystéine montrant une homologie avec certains motifs des récepteurs du TNF et de l'EGF (SEIDAH *et al.*, 1994). Dans le cas de la furine, il a été démontré que la région riche en cystéine pourrait être responsable de la bonne conformation de l'enzyme permettant la protéolyse de son ectodomaine (DENAULT, J. *et al.*, 2002). Le domaine riche en cystéine des convertases PC6A et PACE4 ancre ceux-ci à la surface cellulaire (NOUR *et al.*, 2005). La furine, PC6B et PC7 possèdent, quant à eux, un domaine transmembranaire permettant leur ancrage à la membrane plasmique ainsi qu'une queue cytoplasmique. L'implication de ces domaines a été davantage étudiée chez la furine (MOLLOY *et al.*, 1999). La queue cytoplasmique interagit avec certaines protéines cytosoliques permettant, entre autres, à la furine d'entrer dans la voie de recyclage via la membrane plasmique et les endosomes (MOLLOY *et al.*, 1994).

TABLEAU 1. *Profil de distribution tissulaire et intracellulaire des PCs.*

PC	Distribution tissulaire	Localisation intracellulaire
Furine	Ubiquitaire	TGN, endosomes et surface cellulaire
PC1	Cellules neuroendocrines	Granules de sécrétion et TGN
PC2	Cellules neuroendocrines	Granules de sécrétion
PACE4	Ubiquitaire	TGN, surface cellulaire
PC4	Cellules germinales	Non déterminée
PC6A	Ubiquitaire	Granules de sécrétion et TGN
PC6B	Distribution large avec niveaux élevés dans le système digestif et cortex surrénalien	TGN, endosomes et surface cellulaire
PC7	Ubiquitaire	TGN, endosomes et surface cellulaire

Tableau adapté de TAYLOR *et al.* (2003)

2.2 Routage et localisation intracellulaire des PCs

Les convertases se retrouvent soit dans la voie de sécrétion régulée ou dans la voie de sécrétion constitutive. On retrouve dans le système neuroendocrinien les convertases PC1 et PC2 (Tableau 1). Ces enzymes clivent des précurseurs hormonaux ou peptidiques dans les vésicules de la voie de sécrétion régulée des cellules neuroendocrines (ROUILLE *et al.*, 1995). PC4, que l'on retrouve seulement dans les cellules germinales (MBIKAY *et al.*, 1994), ainsi que PC6A (DE BIE *et al.*, 1996), sont eux aussi des enzymes de la voie de sécrétion régulée.

Les autres convertases furine, PACE4, PC6B, PC7 et PC6A, qui se retrouvent dans les deux voies de sécrétion, agissent dans la voie de sécrétion constitutive. La furine, PACE4 et PC7 ont une distribution tissulaire ubiquitaire tandis que PC6B est davantage exprimée dans le système digestif et le cortex surrénalien (BERGERON *et al.*, 2000a). Il est clairement démontré qu'un type cellulaire peut exprimer plus d'une PC et que celles-ci ont toutes la capacité de cliver des substrats à des paires d'acides aminés basiques. Ceci met en évidence la possible redondance d'activités des convertases pour la maturation de précurseurs. Cependant, cela pourrait s'expliquer par leur spécificité enzymatique, la régulation de leur expression ainsi que leur localisation intracellulaire différentielle.

La localisation intracellulaire de la furine peut être qualifiée de dynamique puisqu'elle transige de la voie d'exocytose vers la voie d'endocytose en passant par la membrane plasmique. Une certaine portion de l'enzyme est aussi larguée dans le milieu extracellulaire où elle conserverait son activité catalytique (THIMON *et al.*, 2006; VIDRICAIRE *et al.*, 1993). La furine est principalement localisée au réseau du *trans*-Golgi (TGN) (MOLLOY *et al.*, 1994; SCHAFER *et al.*, 1995). Cette localisation stratégique lui permet d'avoir accès aux précurseurs protéiques allant vers la surface cellulaire via les vésicules de sécrétion constitutive (NAKAYAMA, 1997a). La localisation ainsi que le routage de la furine sont contrôlés par sa queue cytoplasmique. Celle-ci contient deux motifs contenant une tyrosine, YXXϕ (où ϕ = résidu aliphatique), une séquence dileucine et une séquence acide contenant deux sérines phosphorylables par la caséine kinase II. La phosphorylation de ces sérines par la caséine kinase II participe au bourgeonnement de la furine du TGN vers les compartiments endosomaux (MOLLOY *et al.*, 1998), alors que sa

déphosphorylation par la protéine phosphatase 2 entraîne le retour de la furine au TGN de la membrane plasmique via l'endocytose (JONES, B.G. *et al.*, 1995). De plus, la phosphorylation de ces mêmes sérines présentes dans le motif acide génère des sites d'interaction pour la protéine adaptatrice PACS-1 (WAN *et al.*, 1998). L'interaction de PACS-1 avec la furine permet son cheminement entre les endosomes et la surface cellulaire. À la membrane plasmique, la furine interagit avec ABP-280 qui est une protéine de liaison de l'actine du cytosquelette (LIU *et al.*, 1997). Une forme soluble endogène de la furine a été rapportée (LEITLEIN *et al.*, 2001; THIMON *et al.*, 2006). Notre laboratoire ainsi qu'un groupe autrichien ont identifié l'Arg en position 683 comme étant le site de clivage permettant sa relâche de la membrane cellulaire vers le milieu extracellulaire (DENAULT, J. *et al.*, 2002; PLAIMAUER *et al.*, 2001).

Finalement, les convertases PC6B et PC7 contiennent aussi différents motifs YXXϕ, di-leucine et des motifs acides dans leur queue cytoplasmique. Ils seraient d'ailleurs responsables de la localisation post-TGN de PC7 (WOUTERS *et al.*, 1998) et de la localisation endosomale de PC6B (XIANG *et al.*, 2000). Il a été démontré que les convertases PACE4 et PC6A lient les protéoglycans sulfate heparan dans la matrice extracellulaire (TSUJI *et al.*, 2003). Il se pourrait donc que PACE4 et PC6B jouent un rôle dans l'activation de certaines protéines extracellulaires. Il est intéressant de noter que la convertase PC6B est aussi larguée de la membrane plasmique, alors que PC7 ne l'est pas (BISSONNETTE, 2003).

2.3 Fonctions biologiques des convertases

Les fonctions biologiques des PCs vont beaucoup plus loin que la simple maturation de précurseurs protéiques. La présente section décrit l'implication des différentes PCs au niveau biologique en analysant pour chacune d'elles, le phénotype de l'abolition des gènes.

Compte tenu que l'absence de furine chez une souris transgénique cause la mort de l'embryon, la furine est considérée vitale (ROEBROEK *et al.*, 1998). Ce phénomène peut s'expliquer par le vaste éventail de précurseurs sécrétés clivés par la furine incluant le pro-récepteur de l'insuline, le pro-facteur de von Willebrand, le pro-β-nerve growth factor, le pro-transforming growth factor β1 et la pro-endothéline-1 (BERGERON *et al.*, 2000a). Cependant, des cellules déficientes en furine sont viables comme le démontre la lignée cellulaire CHO RPE.40 (SPENCE *et al.*, 1995). Ceci est probablement dû à la présence dans ces cellules d'autres convertases telles PACE4 ou PC7 qui ont une activité compensatoire à la furine et permettent aux cellules de survivre. De plus, cette activité compensatoire des convertases a été notée chez les modèles animaux xenopus et « zebrafish » (NELSEN *et al.*, 2005; WALKER *et al.*, 2006).

L'activité des convertases PC1 et PC2 est restreinte à la voie de sécrétion régulée des cellules neuroendocrines. Elles sont donc responsables de la maturation de pro-hormones contenues dans les granules de sécrétion, telles la POMC (BENJANNET *et al.*, 1991) et la pro-insuline (SMEEKENS *et al.*, 1992). Une absence de PC1 n'est pas toujours létale chez la souris, mais les embryons qui survivent sont atteints de nanisme (dû à une

déficience dans la production d'hormones de croissance), de diarrhées légères et présentent plusieurs défauts dans la maturation d'hormones (ZHU *et al.*, 2002). Chez les humains, une déficience en PC1 conduit à l'obésité, provoque une homéostasie anormale du glucose (maturation de l'insuline défectueuse) et l'aménorrhée, dérègle les fonctions intestinales, provoque un hypogonadisme hypogonadotropique et de l'hypocortisolisme (JACKSON *et al.*, 1997; O'RAHILLY *et al.*, 1995). Une absence de PC2 mène, quant à elle, à une très grande déficience dans la maturation de plusieurs précurseurs protéiques (proglucagon, pro-insuline et pro-somatostatine) et à l'hypoglycémie (FURUTA *et al.*, 1997).

Les autres convertases de la famille des PCs ont été beaucoup moins étudiées que la furine, PC2 et PC1 dont les rôles biologiques sont beaucoup mieux connus. On connaît donc peu les substrats qui sont clivés par ces convertases, mais on connaît tout de même les effets qu'une déficience de ces convertases provoque.

Une déficience en PACE4 donne un phénotype très variable au sein d'une population de souris. L'absence de PACE4 est létale dans 25% des cas, et on observe des défauts cardiaques et cranio-faciaux, de même qu'un mauvais positionnement des organes (CONSTAM et ROBERTSON, 2000). PACE4 aurait donc probablement un rôle important à jouer dans le développement de l'axe antéropostérieur (LOWE *et al.*, 2001).

PC4 est retrouvée seulement dans les cellules germinales testiculaires et ovariennes, ce qui présuppose un rôle de cette convertase dans la fertilité. En effet, une déficience en PC4 réduit la fertilité des souris. Les spermatozoïdes des mâles étaient moins efficaces pour fertiliser des ovules *in vitro* et les ovules fécondées n'étaient pas viables (MBIKAY *et al.*,

1997). Les ovaires des femelles des souris transgéniques déficientes en PC4 ont une folliculogénèse retardée, ce qui pourrait expliquer la fertilité réduite chez les femelles. Un substrat particulier de PC4 dans les gonades, le pro-PACAP (pituitary adenylate cyclase-activating polypeptide), a d'ailleurs été ciblé comme étant en partie responsable des effets négatifs sur la fertilité (LI, M. *et al.*, 2000).

La convertase PC6 subit un épissage alternatif générant les isoformes A et B qui diffèrent par leur extrémité C-terminal (Figure 2). La forme PC6A est soluble et ne possède donc pas de domaine transmembranaire comme PC6B. PC6A et B exercent différentes fonctions de par leur localisation intracellulaire et tissulaire différentes. Il n'existe pas de modèle animal pour illustrer les effets d'une déficience en PC6A, mais il en existe un pour PC6B (TAYLOR *et al.*, 2003). Une absence de PC6B résulte en la mort des embryons, mais peu de détails sont connus à ce sujet.

Il semble que PC7 joue un rôle non essentiel et redondant, puisque le phénotype des souris transgéniques déficientes de cette convertase semble normal. Les souris ont des proportions normales et semblent en parfaite santé. Ceci est relativement surprenant étant donné que PC7 est retrouvée de manière ubiquitaire au niveau de tous les stades du développement et dans plusieurs tissus adultes comme le foie, la rate et le cerveau (CONSTAM *et al.*, 1996). PC7 serait donc impliquée dans la maturation de substrats non essentiels ou bien serait active dans des cellules où la redondance dans la maturation des précurseurs est présente et suffisante.

Les proprotéines convertases sont des enzymes protéolytiques tronquant des précurseurs de leur prodomaine afin les rendre biologiquement actifs. Il s'avère donc pertinent de décrire les différentes fonctions des prodomaines.

3. Fonctions des prodomaines

Le prodomaine des enzymes protéolytiques joue un rôle crucial dans la régulation de l'activité protéolytique. En effet, les peptidases de la voie de sécrétion sont habituellement synthétisées sous la forme de pro-enzymes inactives dont le prodomaine doit être clivé pour générer la forme active. Les prodomaines se lient au domaine catalytique et obstruent la pochette catalytique rendant l'enzyme inactive. Ce mécanisme d'inhibition permet un contrôle de l'activité pour empêcher l'enzyme de dégrader des substrats non voulus et permet aussi une régulation spatio-temporelle de l'activité protéolytique (KHAN et JAMES, 1998). Il est donc logique de retrouver le prodomaine en N-terminal du domaine catalytique, après le peptide signal pour les protéines sécrétées, afin qu'il soit synthétisé en premier lieu afin de prévenir une activité non voulue.

Les prodomaines inhibent l'activité enzymatique de différentes manières selon la classe de peptidases. Des mécanismes d'activation ont été établis grâce à la cristallographie de certaines peptidases types de chacune des classes d'enzymes (KHAN et JAMES, 1998). À l'aide des cristaux étudiés d'enzymes archétypes des sérines peptidases tels la chymotrypsine, il a été établi que la pochette catalytique est immature. Une protéolyse du prodomaine est nécessaire afin d'engendrer des changements conformationnels et la formation de ponts salins pour obtenir une enzyme active. La subtilisine, quant à elle,

possède une pochette catalytique mature, mais le prodomaine bloque celle-ci de façon stérique. Une baisse de pH engendre une autoprotéolyse du prodomaine qui libère la pochette catalytique. Les cystéines peptidases, telles la cathepsine-B, possèdent un prodomaine bloquant la pochette catalytique. Toutefois, l'orientation du prodomaine est inverse de celle d'un substrat peptidique. Cette orientation peptidique est non favorable a un clivage par le site actif de l'enzyme. Il en résulte donc une activation intermoléculaire (ROZMAN *et al.*, 1999). Une diminution de pH est aussi à l'origine de l'activation des aspartates peptidases. Le prodomaine chargé positivement interagit avec la région catalytique chargée négativement via plusieurs ponts salins obstruant l'accessibilité des substrats à la pochette catalytique. Une diminution de pH défait les interactions et le prodomaine est ainsi autoclivé. Le prodomaine des métallopeptidases possède une cystéine interagissant avec l'ion zinc du site actif des métallopeptidases bloquant ainsi la pochette catalytique. Il est à noter que l'émergence de structures cristallographiques contribuent à la découverte de nouveaux mécanismes d'activation. Ces nouvelles structures nous amènent à croire que différents mécanismes d'activation sont possibles à l'intérieur de mêmes familles d'enzymes.

En plus de pouvoir agir comme inhibiteur, une autre fonction courante attribuée au prodomaine est celle de chaperone intramoléculaire (LAZURE, 2002a). Le prodomaine est souvent responsable du bon repliement des enzymes leur empêchant de précipiter ou leur permettant d'obtenir la structure tertiaire nécessaire à l'activité protéolytique. Les chaperones intramoléculaires diffèrent des autres chaperones puisqu'elles sont liées par un lien peptidique à la protéine dont elles facilitent le repliement. Les chaperones intramoléculaires sont essentielles et hautement spécifiques pour les protéines qui les

contiennent (SHINDE et INOUYE, 1996). Leur mode d'action consiste à abaisser les barrières énergétiques entre deux états de la protéine (SHINDE et INOUYE, 1996).

Par ailleurs, le prodomaine peut également jouer un rôle dans la localisation des protéinases. Le prodomaine de la caspasase-2 contient des motifs permettant sa localisation au noyau (BALIGA *et al.*, 2003). Il a aussi été démontré que le « bone morphogenetic protein-7 » (BMP-7) est localisé dans la matrice extracellulaire via l'interaction de son prodomaine avec la fibrilline (GREGORY *et al.*, 2005).

Les connaissances sur les prodomaines nous renseignent sur le rôle probable de ceux-ci lors de découvertes de nouvelles peptidases ayant un prodomaine. Des indices révélateurs tels des sites consensus de clivage par les pro-protéines convertases suggèrent un mécanisme d'activation par les convertases et un possible rôle d'inhibiteur du prodomaine de ces peptidases nouvellement identifiées.

4. ADAMTS

Les ADAMTS (a disintegrin and metalloprotease with thrombospondin type I repeat) forment une nouvelle classe d'enzymes appartenant à la famille des peptidases M12, sous-famille M12B (adamalysin subfamily) (Merops database (http://merops.sanger.ac.uk/)). Les ADAMTS ont une grande similitude avec la famille des ADAM (a disintegrin and metalloprotease) qui n'ont pas de domaine thombospondine, dont la plus connu est ADAM17 qui clive des substrats à la surface de la cellule tels la cytokine tumor necrosis factor (TNFα) et le facteur de croissance épithélial liant l'héparine (HB-

EGF). Par contre, les ADAMTS ne possèdent pas de domaine transmembranaire. Les ADAMTS sont des enzymes sécrétées dans la voie de sécrétion constitutive et pour la plupart associées à la membrane ou à la matrice extracellulaire. Le premier membre, ADAMTS1 de souris, fut identifié en 1997 dans une lignée cellulaire tumorale du colon (KUNO *et al.*, 1997). Présentement, on dénombre 19 ADAMTS chez l'humain : ADAMTS1 (KUNO *et al.*, 1997; VAZQUEZ *et al.*, 1999a), ADAMTS2 (EC 3.4.24.14 (COLIGE *et al.*, 1997; COLIGE *et al.*, 1999a)), ADAMTS3 (NAGASE *et al.*, 1997), ADAMTS4 (EC 3.4.24.82 (TORTORELLA *et al.*, 1999)), ADAMTS5 (ABBASZADE *et al.*, 1999; HURSKAINEN *et al.*, 1999), ADAMTS6 (HURSKAINEN *et al.*, 1999), ADAMTS7 (HURSKAINEN *et al.*, 1999; SOMERVILLE *et al.*, 2004c), ADAMTS8 (VAZQUEZ *et al.*, 1999a), ADAMTS9 (CLARK *et al.*, 2000; SOMERVILLE *et al.*, 2003a), ADAMTS10 (SOMERVILLE *et al.*, 2004a), ADAMTS12 (CAL *et al.*, 2001), ADAMTS13 (FUJIKAWA *et al.*, 2001), ADAMTS14 (BOLZ *et al.*, 2001; COLIGE *et al.*, 2002), ADAMTS15 (CAL *et al.*, 2002), ADAMTS16 (CAL *et al.*, 2002), ADAMTS17 (CAL *et al.*, 2002), ADAMTS18 (CAL *et al.*, 2002), ADAMTS19 (CAL *et al.*, 2002) et ADAMTS20 (SOMERVILLE *et al.*, 2003a).

4.1 Structure moléculaire

Depuis l'émergence de cette nouvelle famille, la structure mosaïque composant les ADAMTS est conservée. En effet, les ADAMTS sont constituées de plusieurs domaines dans un ordre précis du N- au C-terminal comme illustré à la figure 4 : un peptide signal, un prodomaine, un domaine catalytique, un domaine disintégrine, un motif thrombospondine de type 1 central, un domaine riche en cystéine, un domaine séparateur et un nombre

variable de motifs thrombospondine de type 1. Le peptide signal, le prodomaine et le domaine catalytique forment un domaine appelé « peptidase » et les autres domaines en C-terminal forment le domaine auxiliaire.

Les ADAMTS sont synthétisées sous forme pré-pro-enzyme et le peptide signal dirige ces enzymes dans le voie de sécrétion. Une fois le peptide signal clivé, la pro-enzyme subit un autre clivage du prodomaine. Le prodomaine des ADAMTS contient un ou plusieurs sites de clivage par les convertases tel qu'illustré à la figure 4. Il existe aussi plusieurs sites constitués de paires d'acides aminés basiques pouvant être clivés par différentes convertases. Il est à noter qu'ADAMTS10 est la seule ADAMTS ne possédant pas de site consensus de clivage par la furine à son extrémité C-terminal du prodomaine. Par contre, on détecte la présence d'une paire d'acides aminnés basiques KR^{233}. Comme pour certaines MMP (matrix metalloprotease) et ADAM, il est prédit que le prodomaine des ADAMTS garde l'enzyme inactive et qu'il est important pour le repliement et la sécrétion (CAO, J. *et al.*, 2000a; MILLA *et al.*, 1999). Le prodomaine des ADAMTS1, 6, 7, 10, 12 et 15 contient un résidu cystéine faisant partie du motif XXCGVXD. Ce motif est reconnu chez les MMP pour être responsable de l'inhibition enzymatique grâce à l'interaction de la cystéine et l'atome de zinc du site catalytique. Ce mécanisme est appelé le « cysteine switch » (VAN WART et BIRKEDAL-HANSEN, 1990a). Aucune évidence ne permet jusqu'à présent d'identifier ce mécanisme d'activation chez les ADAMTS. Une fois le prodomaine clivé, l'enzyme mature devrait obtenir une activité catalytique due au domaine catalytique de type métallopeptidase contenant la séquence HEXXHXXGXXH liant un ion divalent de zinc.

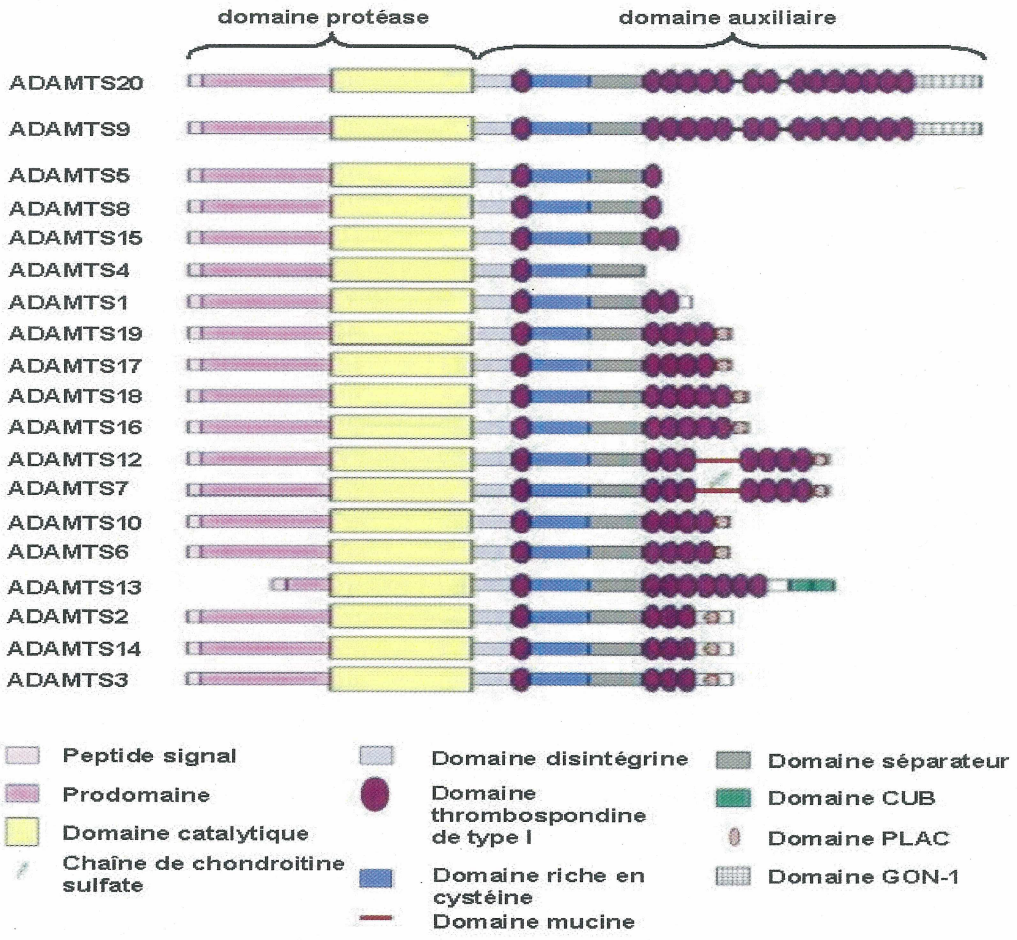

Figure 4. Représentation schématique des ADAMTS. Les différents domaines constituant les ADAMTS sont identifiés par un code de couleur. Le peptide signal, le prodomaine et le domaine catalytique composent le domaine peptidase tandis que les autres domaines en C-terminal composent le domaine auxiliaire. À noter qu'il peut y avoir un nombre variant de 0 (ADAMTS4) à 14 (ADAMTS9 et 20) domaines thrombospondines en C-terminal. Certaines ADAMTS possèdent aussi des domaines PLAC ou mucine dans leur domaine auxiliaire. Tiré de (JONES, G.C. et RILEY, 2005).

Protéinases	Sites potentiels de clivage par la furine dans les prodomaines
ADAMTS1	$RRNR_{174}$, $RKKR_{235}$
ADAMTS2	$RARR_{88}$, $RRRARR_{259}$
ADAMTS3	$RRRR_{249}$
ADAMTS4	$RPRR_{209}AKR_{212}$
ADAMTS5	$RRRR_{260}R_{261}$
ADAMTS6	$RRRR_{71}$, $RQKR_{244}$
ADAMTS7	$RALR_{68}$, $RELR_{92}$, $RRER_{218}$, $RRPR_{229}LRR_{232}$
ADAMTS8	$RGLR_{99}$, $RLQR_{153}$, $RQER_{180}$, $RTKR_{214}$
ADAMTS9	$RKDR_{33}$, $RTRR_{74}$, $RTHR_{283}RTKR_{287}$
ADAMTS10	$RRQRR_{40}$, $RSSR_{70}$, $RAAR_{83}$
ADAMTS12	$RRKR_{82}$, $RSLSRR_{240}$
ADAMTS13	$RHPR_{7}$, $RQRR_{74}$, $RQVR_{193}$
ADAMTS14	$RVAR_{87}$, $RVGR_{103}$, $RPNR_{125}$, $RKRR_{252}$
ADAMTS15	$RSRR_{205}$, $RAKR_{212}$, $RLYR_{254}$
ADAMTS16	$RGWR_{9}$, $RRRR_{87}$, $RSHR_{155}$, $RHKR_{279}$, $RSHR_{286}$
ADAMTS17	$RRRRRPR_{66}$, $RRDLR_{11}$, $RRRGR_{120}RPSR_{217}DWR_{220}ERR_{223}$
ADAMTS18	$RKKR_{88}$, $RPRR_{284}$
ADAMTS19	$REVR_{84}$, $RRDGR_{185}$, $RVYR_{274}QKR_{277}$
ADAMTS20	$RQKR_{66}$, $RRHSR_{250}KKR_{253}$

Figure 5. Schéma des sites potentiels de clivage par la furine des prodomaines des ADAMTS. Les différents sites de clivage consensus par la furine RxxR↓ où x représente n'importe quel acide aminé sont illustrés pour chacune des ADAMTS humaines. La furine peut cliver en C-terminal des arginines (R) numérotées. Il est à noter qu'il existe d'autres paires d'acides aminés basiques pouvant être clivés par les différentes convertases dans les prodomaines des ADAMTS. ADAMTS10 est la seule ADAMTS ne possédant pas de site consensus de clivage par la furine à son extrémité C-terminal du prodomaine.

Bien qu'il est prédit que le domaine disintégrine lie les intégrines, famille de protéines réceptrices transmembranaires ayant comme fonction l'attachement de la cellule à la matrice extracellulaire et la transduction de signaux de la matrice extracellulaire vers la cellule, aucune évidence n'a encore démontré que les ADAMTS se lient aux intégrines. Les domaines thrombospondines de type 1 sont très homologues à ceux retrouvés dans la molécule thrombospondine 1. Cette molécule a une fonction anti-angiogénique (SHEIBANI et FRAZIER, 1995). Les ADAMTS possèdent de 1 (ADAMTS4) à 15 (ADAMTS9 et 20) domaines thrombospondines de type 1. Il a été démontré que ADAMTS1 et 8 possèdent une activité anti-angiogénique grâce aux domaines thrombospondine de type 1 (VAZQUEZ *et al.*, 1999a). De plus, les domaines thrombospondines de type 1 sont impliqués dans la liaison de ADAMTS1, 4 et 9 à la matrice extracellulaire (KASHIWAGI *et al.*, 2004; KUNO et MATSUSHIMA, 1998; SOMERVILLE *et al.*, 2003a).

Quatres autres motifs sont retrouvés dans le domaine auxiliaire de certaines ADAMTS. ADAMTS7 et 12 possèdent un domaine mucine entre deux groupes de domaines thrombospondines de type 1. Leur domaine mucine est hautement glycosylé, ce qui fait des ADAMTS7 et 12 des protéoglycans, et forme par conséquent une sous-famille (figure 6). ADAMTS9 et 20 possèdent un domaine GON-1 retrouvé dans la protéine gon-1 chez le nématode *C. elegans*. La protéine gon-1 est impliquée dans le développement des gonades chez *C. elegans* (BLELLOCH et KIMBLE, 1999a). Les ADAMTS2, 3, 6, 7, 10, 12, 14, 16, 17, 18 et 19 possèdent un domaine PLAC (peptidase et lacunine) de fonction inconnue. Finalement, le domaine CUB (complement C1r/C1s, Uegf (EGF-related sea urchin protein) and BMP-1 (bone morphogenic protein-1)) est présent en C-terminal de

ADAMTS13. Le domaine CUB est retrouvé dans une variété de protéines extracellulaires et serait impliqué dans le processus développemental dont notamment au niveau de l'embryogenèse et l'organogenèse (BORK et BECKMANN, 1993).

4.2 Fonctionnalité et pathologies associées aux ADAMTS

Un alignement de séquences d'acides aminés ainsi qu'une analyse phylogénétique des ADAMTS démontrent l'existence de sous-familles distinctes ayant des fonctions communes ou similaires (Figure 6).

4.2.1 N-propeptide collagénases

Les ADAMTS2, 3 et 14 forment une sous-famille de N-propeptide collagénases, clivant le propeptide en N-terminal du pro-collagène (COLIGE *et al.*, 1997; COLIGE *et al.*, 2002; FERNANDES *et al.*, 2001). ADAMTS2 clive le pro-collagène I, II et III tandis que ADAMTS3 et ADAMTS14 clivent seulement le pro-collagène II et I respectivement. Une déficience dans l'activité protéolytique de ADAMTS2 mène à des désordres du tissu conjonctif appelés dermatosparaxis chez les animaux et le syndrome de Ehlers-Danlos de type VII C chez l'humain (COLIGE *et al.*, 1999a). Cette pathologie provient d'une mutation récessive et est caractérisée par une fragilité de la peau, une hyperextensibilité des articulations et une petite stature. Les souris transgéniques ayant une délétion d'ADAMTS2

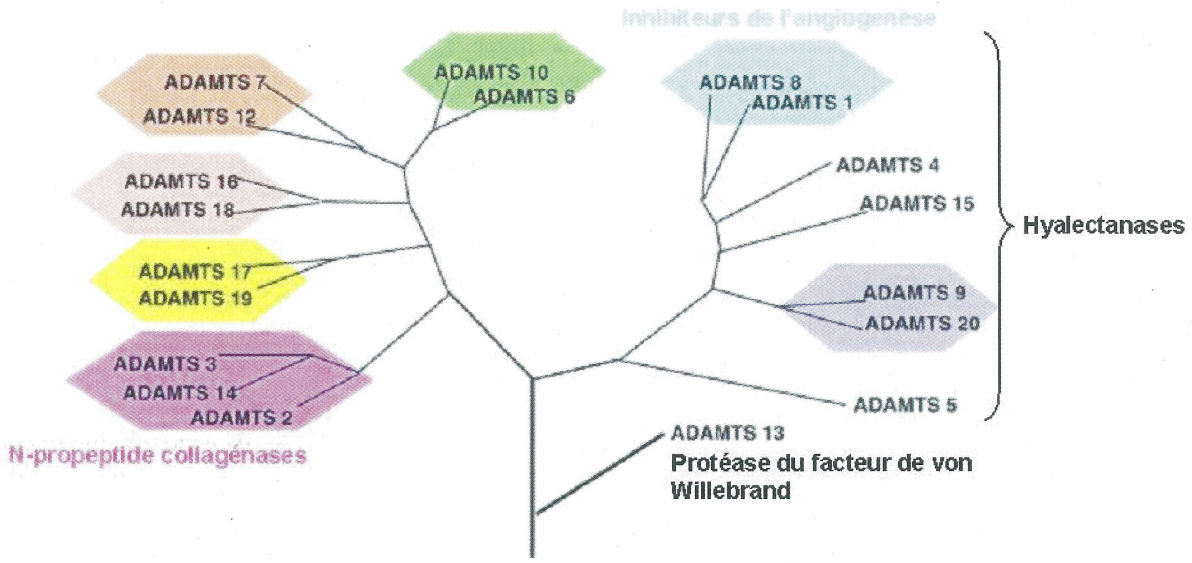

Figure 6. Arbre phylogénétique de la famille des ADAMTS humaines. La séquence entière en acides aminés de chacune des ADAMTS fut utilisée afin de déterminer l'arbre phylogénétique par le Megalign program (LaserGene Software Suite, Madison, WI). ADAMTS11 n'est pas présente dans l'arbre puisqu'elle a été assignée a une enzyme déjà nommée ADAMTS5. Tiré de (APTE, 2004a).

semblent normales à la naissance mais développent une fragilité de la peau après 1 à 2 mois. De plus, les mâles sont infertiles ce qui indique que ADAMTS2 aurait aussi un rôle dans la spermatogenèse (LI, S.W. *et al.*, 2001).

4.2.2 Propriétés anti-angiogéniques

Comme mentionné précédemment, toutes les ADAMTS possèdent un ou plusieurs domaines thrombospondiques de type I. Ce domaine est responsable du pouvoir anti-angiogénique dans la protéine thrombospondine I. Jusqu'à présent, il est surprenant de constater que seulement des études sur ADAMTS1 et 8 ont été publiées afin d'évaluer le rôle potentiel des ADAMTS dans l'inhibition de la formation des vaisseaux sanguins (VAZQUEZ *et al.*, 1999a). Le groupe du Dre. Luisa Iruela-Arispe a démontré que les domaines thrombospondiques de type 1 d'ADAMTS1 étaient responsables de la liaison avec le « vascular endothelial growth factor-165 » (VEGF-165) (LUQUE *et al.*, 2003). Le VEGF a pour fonction d'entraîner la formation de nouveaux vaisseaux sanguins (LEUNG *et al.*, 1989). ADAMTS1 et plus faiblement ADAMTS8 ont la capacité de lier le VEGF-165, ce qui empêche ce dernier de se lier à son récepteur (VEGFR2) et signaler une croissance vasculaire. Donc, ADAMTS1 lie le VEGF-165 mais ne le clive pas, l'action anti-angiogénique proviendrait de la séquestration du VEGF-165.

En outre, les souris transgéniques ne possédant pas d'ADAMTS1 ont un retard de croissance, des malformations du tissu adipeux, des reins, de l'utérus et des ovaires, ce qui suggère son implication dans le développement (SHINDO *et al.*, 2000).

4.2.3 Peptidase du von Willebrand factor

ADAMTS13 été identifiée comme l'enzyme clivant les multimères du facteur de von Willebrand (FUJIKAWA *et al.*, 2001). Le facteur de von Willbrand est une protéine plasmatique impliquée dans l'aggrégation des plaquettes à la paroi vasculaire lésée. Les larges multimères du facteur de von Willebrand doivent être clivés en plus petits fragments pour ne pas adhérer aux plaquettes dans le plasma. La défaillance enzymatique d'ADAMTS13 cause une maladie : la purpura thrombotique thrombocytopénique (TTP) (LEVY *et al.*, 2001a). La maladie TTP est caractérisée par la formation de thromboses due à l'association plasmatique des larges multimères du facteur de von Willebrand non clivés par ADAMTS13 et des plaquettes dans le plasma. Il s'en suit une anémie, une défaillance rénale et une dysfonction neurologique. L'absence d'ADAMTS13 active peut être causée soit par une mutation génétique ou la formation d'anticorps auto-immuns contre ADAMTS13 rendant celle-ci inactive (FURLAN *et al.*, 1998). ADAMTS13 est la plus atypique des ADAMTS et ne requiert pas le clivage du prodomaine pour son activation (MAJERUS *et al.*, 2003a). On remarque par contre que son prodomaine est beaucoup plus court que chez les autres membres des ADAMTS (Figure 4).

4.2.4 Hyalectanases

D'autre part, les ADAMTS1, 4, 5, 8, 9 et 15 ont une activité hyalectanase (PORTER *et al.*, 2005) signifiant qu'elles dégradent avec une efficacité variable soit l'aggrécan, le versican ou le brévican qui sont trois protéines de structure de la matrice extracellulaire. ADAMTS4 et 5 clivent le protéoglycan brevican, retrouvé au cerveau (MATTHEWS *et al.*,

2000; NAKADA *et al.*, 2005). La dégradation du brevican est impliquée dans la progression des glioblastomes. Notre groupe de recherche, en collaboration avec le Dr. Suneel Apte du Cleveland Clinic, a démontré qu'ADAMTS9 dégrade le versican (SOMERVILLE *et al.*, 2003a). Le versican est un protéoglycan de la matrice extracellulaire du cerveau et joue un rôle dans la migration des cellules neuronales (DUTT *et al.*, 2006). Il est aussi présent dans la paroi vasculaire où il semble être impliqué dans l'artériosclérose (KENAGY *et al.*, 2006).

L'aggrécan est le protéoglycan majeur retrouvé dans le cartilage. Il est responsable du pouvoir d'élasticité et de résistance à la compression du cartilage (SANDY *et al.*, 1992). En effet, les glycosaminoglycans (chondroïtine-sulfate et kératane-sulfate) de l'aggrécan sont sulfatés et riches en radicaux acides très hydrophiles qui permettent d'emmagasiner une haute teneur en eau dans le cartilage ce qui le rend élastique et compressible. L'aggrécan protège aussi la dégradation du collagène dans le cartilage. Une dégradation excessive de l'aggrécan entraîne l'effritement du cartilage caractéristique de l'arthrose (ARNER, 2002). Il a été identifié dans des explants de cartilage de patients atteints d'arthrose que l'aggrécan est clivé dans son domaine interglobulaire faisant en sorte que les glycosaminoglycans de l'aggrécan sont relâchés de la matrice extracellulaire (SANDY *et al.*, 1991). Les ADAMTS1, 4, 5, 8, 9 et 15 clivent l'aggrécan à différents endroits, mais seulement ADAMTS4, 5 et faiblement ADAMTS1 clivent l'aggrécan dans le domaine interglobulaire (ABBASZADE *et al.*, 1999; TORTORELLA *et al.*, 1999). De plus, des études à l'aide de souris transgéniques dont le site de clivage du domaine interglobulaire de l'aggrécan a été aboli démontrent bel et bien qu'en bloquant ce clivage, il est impossible d'induire la dégradation du cartilage (LITTLE *et al.*, 2007). Jusqu'à récemment,

ADAMTS4 semblait être l'enzyme la plus susceptible de dégrader l'aggrécan, mais deux études ont été publiées simultanément dans la revue *Nature* afin d'investiguer la contribution relative d'ADAMTS4 et 5 dans la dégradation de l'aggrécan du cartilage retrouvé dans l'arthrose (GLASSON *et al.*, 2005; STANTON *et al.*, 2005a). Les études ont été menées à l'aide de souris transgéniques ayant l'ADN du domaine catalytique soit de ADAMTS4 ou de ADAMTS5 supprimé rendant ces enzymes inactives. Les deux souris transgéniques, ADAMTS4 et 5, ont un phénotype normal. Cependant, lorsque les auteurs induisent l'arthrose dans une articulation des souris transgéniques soit par l'injection d'interleukine-1 ou par chirurgies, seulement l'abolition d'ADAMTS5 active n'entraîne pas la dégradation du cartilage. Ceci indique qu'ADAMTS5 est l'unique enzyme responsable de la dégradation du cartilage chez la souris.

L'inhibition des ADAMTS devient une cible thérapeutique intéressante d'autant plus qu'aucun traitement existe présentement afin de ralentir la progression de l'arthrose. Les « tissue inhibitor of metalloproteinase » (TIMP) inhibent les MMP et les ADAM, deux classes d'enzymes très semblables aux ADAMTS (BAKER *et al.*, 2002). Il semble que TIMP-3, et non TIMP-1, -2 et –4, ait une spécificité encore plus grande envers les ADAMTS4 et 5 avec un K_i dans les nanomolaires (KASHIWAGI *et al.*, 2001). L'activité aggrécanase des ADAMTS 1, 4 et 5 est aussi inhibée par les esters gallate catéchine du thé vert (VANKEMMELBEKE *et al.*, 2003). Un groupe de la compagnie pharmaceutique Pfizer, John Tortorella et collègues, ont publié trois articles relatant les propriétés inhibitrices de différents composés synthétiques contre les ADAMTS4 et 5 (CHERNEY *et al.*, 2003; WITTWER *et al.*, 2007; YAO *et al.*, 2001). Mais le défi d'obtenir des inhibiteurs efficaces contre le site actif semble difficile. Malgré ces quelques publications, peu

d'information sur de potentiels inhibiteurs contre les ADAMTS est disponible jusqu'à présent. La recherche afin d'enrayer l'activité aggrécanase des ADAMTS, plus particulièrement ADAMTS5, s'avère très pertinente pour contrer l'arthrose.

4.2.5 Autres fonctions

Une autre molécule de la matrice extracellulaire du cartilage « cartilage oligomeric matrix protein » (COMP) est dégradée et détectée dans les articulations arthritiques. Il a été démontré que ADAMTS7 et 12 lient et clivent la protéine COMP suggérant qu'elles sont impliquées dans l'initiation et la progression de l'arthrite (LIU, C.J. *et al.*, 2006a; LIU, C.J. *et al.*, 2006b).

En outre, des mutations dans le gène d'ADAMTS10, prédisant une forme tronquée dans le domaine catalytique, ont été attribuées au syndrome de Weill-Marchesani (DAGONEAU *et al.*, 2004a). Ce syndrome est caractérisé par une petite stature, des anomalies aux yeux et au cœur ainsi qu'une raideur articulaire. Cela suggère qu'ADAMTS10 est impliquée dans la croissance, le développement des yeux et du cœur chez l'humain.

En somme, les fonctions déterminées jusqu'à présent ont une importance significative dans la régulation de la modulation de la matrice extracellulaire puisqu'un dérèglement de leur activité est grandement susceptible d'entraîner une pathologie.

5. HYPOTHÈSE DE RECHERCHE ET OBJECTIFS DE L'ÉTUDE

Plusieurs peptides ou protéines biologiquement actives proviennent de précurseurs inactifs synthétisés dans le milieu intracellulaire. La majorité des polypeptides sécrétés doivent être clivés pour être activés par des enzymes de la famille des pro-protéines convertases de type subtilisine de mammifères. Cette famille de convertases comprend sept sérines protéinases dépendantes du calcium (BERGERON *et al.*, 2000a) reconnaissant plusieurs motifs contenant des permutations de résidus basiques en position P1, P2, P4 et P6. Les prodomaines de certains précurseurs possèdent ces motifs pouvant être clivé par les convertases activant ainsi la protéine. Le prodomaine des précurseurs possède donc des déterminants essentiels à l'activation et/ou à la fonctionnalité de protéines.

L'expertise de notre laboratoire dans le domaine des convertases et l'activation de précurseurs par le clivage de prodomaines a mené à étudier l'activation d'une nouvelle famille de protéinases, les ADAMTS. Cette nouvelle famille de métallopeptidases possède des sites consensus de clivage par les convertases dans leur prodomaine ce qui suggère une activation via les pro-protéines convertases.

Les ADAMTS forment cette nouvelle famille peu caractérisée de protéinases extracellulaires jouant un rôle, entre autres, dans la régulation de la dégradation de la matrice extracellulaire. En effet, certaines ADAMTS possèdent des activités enzymatiques contre des substrats de la matrice extracellulaire tel l'aggrécan. Les différents processus physiologiques requièrent une activation des ADAMTS pour obtenir une peptidase active. Le dérèglement de l'activité protéolytique entraîne des pathologies telles l'arthrose et le

cancer. Nouvellement identifiées, la biosynthèse et l'activation des ADAMTS demeurent encore peu documentées.

À cet égard, ce projet de recherche propose de vérifier l'hypothèse suivante : *les prodomaines des ADAMTS possèdent en leur structure primaire des motifs ou des résidus régulant leur biosynthèse et leur activation.*

L'objectif principal de cette thèse est d'identifier les déterminants moléculaires du prodomaine des ADAMTS impliqués dans la biosynthèse et l'activation de ces protéinases. Plusieurs sous-questions émergent de l'hypothèse principale et les cinq articles présentés dans cet ouvrage décrivent les différents motifs impliqués dans la biosynthèse du prodomaine des ADAMTS ainsi que les différents mécanismes d'activation par les pro-protéines convertases.

1ère hypothèse : Les zymogènes pro-ADAMTS sont des substrats des pro-protéines convertases. La séquence d'acides aminés des ADAMTS recensées indique que toutes les ADAMTS possèdent des sites consensus de clivage par les convertases dans leur pro-domaine. Objectifs : Déterminer le profil biosynthétique des ADAMTS : est-ce que les zymogènes pro-ADAMTS sont clivés lors de leur biosynthèse? Identifier quelles pro-protéines convertases clivent les ADAMTS. Déterminer quel(s) site(s) consensus de clivage par les convertases est clivé(s) dans les prodomaines des ADAMTS. Évaluer où le clivage du prodomaine se produit : dans le TGN ou dans le milieu extracellulaire?

La présente thèse fait état de la maturation par les PCs des ADAMTS1, 5, 7 et 9.

2ième hypothèse : Les prodomaines des ADAMTS agissent en tant que chaperones intramoléculaires ainsi qu'inhibiteurs des ADAMTS. Outre les sites consensus de clivage part les PCs, le prodomaine des ADAMTS contient également des motifs ou résidus conservés. Objectif : Déterminer si le prodomaine est nécessaire à la biosynthèse des ADAMTS. Évaluer si la N-glycosylation des prodomaines des ADAMTS est impliquée dans leur biosynthèse. Analyser le rôle des résidus conservés dans les prodomaines des ADAMTS. Déterminer si les prodomaines des ADAMTS ont une fonction inhibitrice contre l'activité de ces peptidases.

Le rôle de chaperones intramoléculaires est analysé en mutant des résidus conservés du prodomaine de ADAMTS1. De plus, le rôle dans la biosynthèse de la N-glycosylation du prodomaine d'ADAMTS9 est évalué. La fonction inhibitrice des prodomaines de ADAMTS5 et 9 est déterminée à l'aide du substrat versican de ces deux enzymes.

RÉSULTATS

ARTICLE 1 – AVANT-PROPOS

Identification of pro-domain determinants involved in ADAMTS-1 biosynthesis

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Contribution: J'ai effectué la totalité des expériences de cette étude. J'ai rédigé la première version du manuscrit.

Résumé de l'article 1

ADAMTS1 (a disintegrin and metalloprotease with thrombospondin type I repeat), est une métalloprotéinase qui est initialement synthétisée sous forme de zymogène, pro-ADAMTS1. Ce zymogène est activé par un clivage protéolytique à la jonction du prodomaine et du domaine catalytique par une serine peptidase de la famille des pro-protéines convertases. Les objectifs de cette étude étaient d'identifier les acides aminés impliqués dans la maturation de ADAMTS1 et de déterminer quelles pro-protéines convertases activent le précurseur pro-ADAMTS1. Afin d'élucider les rôles potentiels des différents acides aminés du prodomaine dans la biosynthèse de ADAMTS1, des études de marquage métabolique dans la lignée cellulaire HEK293 exprimant ADAMTS1 et des mutants du prodomaine de pro-ADAMTS1 furent entreprises. Les cellules exprimant ADAMTS1 de type sauvage produisent initialement un zymogène de 110 kDa qui est converti en une forme de 87 kDa qui est aussi détectée dans le milieu extracellulaire. Malgré que les convertases PACE4 et PC6B clivent pro-ADAMTS1, la furine est l'enzyme qui produit la forme de 87 kDa le plus efficacement. La mutagenèse dirigée des deux sites consensus de clivage par la furine retrouvés dans le prodomaine de ADAMTS1 (RRNR173 et RKKR235) a révélé que l'Arg235 est l'unique site de clivage possible. De plus, en utilisant les composés affectant la sécrétion des protéines, Brefeldine A et monensine, nous avons démontré que le clivage de pro-ADAMTS1 est effectué dans l'appareil de Golgi. La présence, dans les prodomaines des ADAMTS, d'acides aminés conservés dans cette famille d'enzymes, nous amène à penser qu'ils pourraient jouer un rôle important dans leur maturation. La mutation de ces acides aminés conservés Cys106, Tyr108, Gly110, Cys125, et Cys181 ainsi que le motif comprenant les acides aminés 137 à 144 ont grandement

affecté le profil biosynthétique de ADAMTS1. Cette observation permet de conclure que des acides aminés autres que ceux du site consensus de clivage par la furine sont impliqués dans la biosynthèse d'ADAMTS1.

Identification of pro-domain determinants involved in ADAMTS-1 biosynthesis

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Running title: Pro-domain residues involved in ADAMTS-1 biosynthesis

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SUMMARY

The metalloprotease ADAMTS-1, like other members of the ADAMTS family, is initially synthesized as a zymogen, proADAMTS-1, that undergoes proteolytic processing at the pro-domain/catalytic domain junction by serine proteinases of the furin-like family of proprotein convertases. The goals of the present study were to identify residues of the pro-domain that play an essential role in ADAMTS-1 processing and to determine the identity of the convertase which is required for zymogen processing. To gain insight into the putative roles of specific pro-domain residues in ADAMTS-1 biosynthesis, we performed biosynthetic labeling experiments in transiently transfected HEK-293 cells expressing wild-type (wt) and pro-domain mutants of proADAMTS-1. Cells expressing wtADAMTS-1 initially produced a 110 kDa zymogen form that was later converted to an 87 kDa form which was also detected in the media. Although convertases such as PACE4 and PC6B processed proADAMTS-1, we found that furin was the most efficient enzyme at producing the mature ADAMTS-1 87 kDa moiety. Site-directed mutagenesis of the two putative furin recognition sequences found within the ADAMTS-1 pro-domain ($RRNR^{173}$ and $RKKR^{235}$) revealed that Arg^{235} was the sole processing site. Use of the Golgi disturbing agent BFA and monensin suggest that the cleavage of proADAMTS-1 takes place in the Golgi apparatus prior to its secretion. Conserved residues within the pro-domain of other ADAMTS members hinted that they might act as maturation determinants. Replacement with Alanine of selected residues Cys^{106}, Tyr^{108}, Gly^{110}, Cys^{125}, Cys^{181} and residues encompassing the 137-144 sequence significantly affected the biosynthetic profile of the enzyme. Our results suggest that conserved residues other than the furin cleavage site in the pro-domain of ADAMTS-1 are involved in its biosynthesis.

INTRODUCTION

Proteolysis of extracellular substrates by the ADAMTS (A disintegrin and metalloprotease with thrombospondin type I motif) family, which consists of at least 19 members, is an important mechanism regulating events such as cartilage biosynthesis, angiogenesis and cell motility and growth (1). The first member, ADAMTS-1 (peptidase M12.222, Merops database) identified as a cachexia-associated gene expressed in colon tumor cells (2), along with ADAMTS-4, ADAMTS-5 (also called aggrecanases) degrade to different extents the cartilage proteoglycan aggrecan and lectican or aggrecan-like proteins such as brevican and versican. This suggested a significant participation of these enzymes in conditions such as arthritis (3-6). ADAMTS-1 is also anti-angiogenic, a property possibly explained by the recent finding that it sequesters VEGF[1]1 (7). The phenotype of *ADAMTS-1–/–* mice revealed marked reduction in size, with body weights about 70% of their wild-type or heterozygous littermates and fertilization was impaired in females (8). ADAMTS-2, along with ADAMTS-14 and ADAMTS-3, are procollagen N-proteinases that proteolytically remove amino peptides in the processing of type I and type II procollagens to collagens (9-11). Deficiency of ADAMTS-2 lead to an inherited connective tissue disorder called dermatosparaxis in animals and the Ehlers-Danlos syndrome (dermatosparactic type) in humans. Reports have recently demonstrated that

[1] Abbreviations used: VEGF: vascular endothelial growth factor, FBS: fetal bovine serum, FCS: fetal calf serum, ECM: extracellular matrix, TSP: thrombospondin type I repeat, PC: proprotein convertase, ER: endoplasmic reticulum, MMP: matrix metalloproteinase, ADAM: a desintegrin and metalloproteinase, TGN: *trans*-Golgi network, BFA: Brefeldin A.

mutations in the *ADAMTS-13* gene cause thrombotic thrombocytopenic purpura, a coagulation disorder, and that ADAMTS-13 is required for processing of large von-Willebrand factor multimers (12). The functions of most other ADAMTS members remains to be better clarified.

The structure of all ADAMTS members includes a signal peptide for access to the secretory pathway, a pro-domain, a catalytic metalloprotease domain, a disintegrin-like domain and a carboxyl terminal ancillary domain having a conserved modular structure but containing a variable number of TSP-like domains (2). Unlike the ADAM family the ADAMTS members do not contain a transmembrane domain, yet they may be located in the cell's vicinity via binding to cell surface molecules or pericellular matrix (13). Thus ADAMTS are secreted proteins anchored to the cell surface or to the extracellular matrix (14).

ADAMTS proteases, like ADAM and MMPs are synthesized as zymogens, which require activation via proteolytic removal of a pro-domain. The size of the pro-domain varies, since it is in part determined by the location of the C-terminal most furin processing site, but in all ADAMTS except ADAMTS-13, which has an overall atypical primary structure, it is around 200 residues. At least one, but usually multiple furin recognition sequences occur in the pro-domain of most members of the ADAMTS family. ADAMs, MT-MMPs, MMPs and ADAMTS-1, -2, -4, -5, -9, and –12 (3,4,13,15-17) have been shown to be processed by furin-like proprotein convertases. The convertase family is comprised of seven calcium-dependent serine proteases (18) that recognize various sequence motifs containing permutations of basic residues in the P1, P2, P4 and P6

positions (19). One of the most studied convertases, furin, is concentrated in the *trans*-Golgi network (TGN) and cycles between this compartment and the cell surface through the exocytic/endocytic pathway (20). The autoactivation and intracellular trafficking of furin are well-characterized events (21).

Since zymogen activation is a critical post-translational regulatory step, we have addressed, in the present study, the critical determinant affecting this process. Although convertases like PACE4 and PC6B may process proADAMTS-1, we demonstrate that furin is the most efficient convertases at cleaving the proADAMTS-1 precursor intracellularly at Arg^{235} of the furin recognition sequence $RKKR^{235}$. Moreover, we also identify different conserved residues within the pro-domains of ADAMTS family members that could be involved in the maturation of proADAMTS-1. These observations could be of broad significance for understanding regulation of the ADAMTS proteases.

EXPERIMENTAL PROCEDURES

Cell culture and transfection

QBI-293A cells (293A, Quantum Biotechnologies, Montréal, QC, Canada), derived from the hEK-293 cell line, were grown in complete DMEM containing 10% heat-inactivated FBS, 2mM L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin. CHO RPE.40 cells were cultured in RPMI 1640 containing 10% heat-inactivated FBS, 2mM L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin. Sub-confluent cells were transfected with 2 µl of Fugene6 reagent (Roche Diagnostics, Laval, QC, Canada) per µg of DNA 24 h prior to each experiment.

Site-directed mutagenesis

Human ADAMTS-1 cDNA in pcDNA3.1MycHis has been previously described (RODRIGUEZ-MANZANEQUE *et al.*, 2000a). Construction of ADAMTS-1 pro-domain mutants was performed using the QuickChange site-directed mutagenesis kit (Stratagene, San Diego, Ca, USA) with the following oligonucleotides: R173A (5'-CACCTCCTGCGGCGGAATGCTCAGGGCGACGTAGGCGGC, mutated codon is underlined), R235A (5'-ATAAGAAAGAAGGCTTTTGTGTCCAGTC), C106A (5'-GACCTGGCGCACGCTTTCTACTCCGGC), Y108A (5'-GCGCACTGCTTCGCTTCCGGCACCGTG), G110A (5'-TGCTTCTACTCCGCTACCGTGAATGGC), T111A (5'-CTTCTACTCCGGCGCTGTGAATGGCGATC), V112A (5'-CTACTCCGGCACCGCTAATGGCGATCCC), P116A (5'-

GTGAATGGCGATGCTAGCTCGGCTGCC), C125A (5'-GCCCTCAGCCTCGCTGAGGGCGTGCGC), C181A (5'-GTAGGCGGCACGGCTGGGGTCGTGGAC), 8A (amino acids 137-144 mutated to alanine) (5'-GCCTTCTACCTGCTGGGGGCTGCTGCTGCTGCTGCTGCTCCCGCCGCCAGC GAGCGC). The different convertases were expressed using the following constructions. The human furin cDNA in pCI-Neo was described elsewhere (23). The cDNA encoding mouse PC6B was subcloned in NotI of pRcCMV(a gift from K. Nakayama, Tsukuba, Japan). The human PC7 (a gift from R. Day, Université de Sherbrooke, Sherbrooke, Canada) and PACE4 (a gift from R. E. Mains, The University of Connecticut Health Center, Farmington, Connecticut, USA) cDNA were inserted in EcoRI/XbaI-linearized pcDNA3. All constructs were confirmed by DNA sequencing.

Production of anti-hADAMTS-1 and anti-PC7 antibodies

The hADAMTS-1 polyclonal antibodies were obtained from rabbits injected with a peptide (Ile-His-Asp-Glu-Gln-Lys-Gly-Pro-Glu-Val-Thr-Ser-Cys) coupled to KLH (Pierce). The peptide encompasses residues 295-306 of the human ADAMTS-1 catalytic domain. The hPC7 polyclonal antibodies were obtained from rabbits injected with a peptide (Asp-Gly-Pro-His-Gln-Leu-Gly-Lys-Ala-Ala-Leu-Gln-His-Cys) coupled to KLH. The peptide encompasses residues 298-310 of the human PC7 catalytic domain. Rabbits were injected with antigen emulsified in an equal volume of TiterMax adjuvant (Sigma-Aldrich).

Metabolic labeling and immunoprecipitation

Metabolic labeling experiments were performed 24h post-transfection. Cells were washed with warm PBS and incubated in Met/Cys-free medium (MEM Select-Amine kit, Life Technologies) supplemented with 10% dialyzed FCS, 1 mM L-glutamine and 50 μCi of Expre^{35}S^{35}S (NEN Life Science Products, Boston, MA, USA) for the indicated period of time (pulse). Chase was done in complete medium. After recovering the medium, the cell layer was washed with PBS and cells were lyzed with 1 ml of radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% deoxycolic acid, 4 mM EDTA and 0.1% SDS) containing protease inhibitors (1 μM aprotinin, 10 μM pepstatin, 10 μM leupeptin and 1mM PMSF). Samples were centrifuged to remove insoluble material. hADAMTS-1 antiserum, anti-furin (24), anti-PACE4 (Alexis biochemicals) or anti-PC6B (Alexis biochemicals) or anti-PC7 was added and samples were incubated overnight at 4°C. Protein A/G Plus-agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA) beads were added and incubated for 1 h at 4°C. Beads were washed three times with 1 ml of RIPA buffer and labeled proteins were resolved by SDS-PAGE. Gels were treated with ENHANCE reagent (Perkin Elmer Life Science), dried and exposed for fluorography. BFA at 36 μM (Sigma) or monensin at 3.6 μM or 36 μM (Sigma) were added to the labeling mixture when indicated.

Western Blot

Transfected CHO RPE.40 cells were lyzed in 1 ml of RIPA buffer. 30 μl of each samples were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblot analysis was performed with monoclonal mouse anti-actin antibody (Chemicon). The antibodies were visualized with the horseradish peroxidase coupled sheep anti-mouse

immunoglobulin (Amersham LIFE SCIENCE) using the Western Lightning chemilunescence reagent plus according to the manufacturer's instructions (PerkinElmer Life Sciences, Inc., Boston, MA, USA).

Deglycosylation

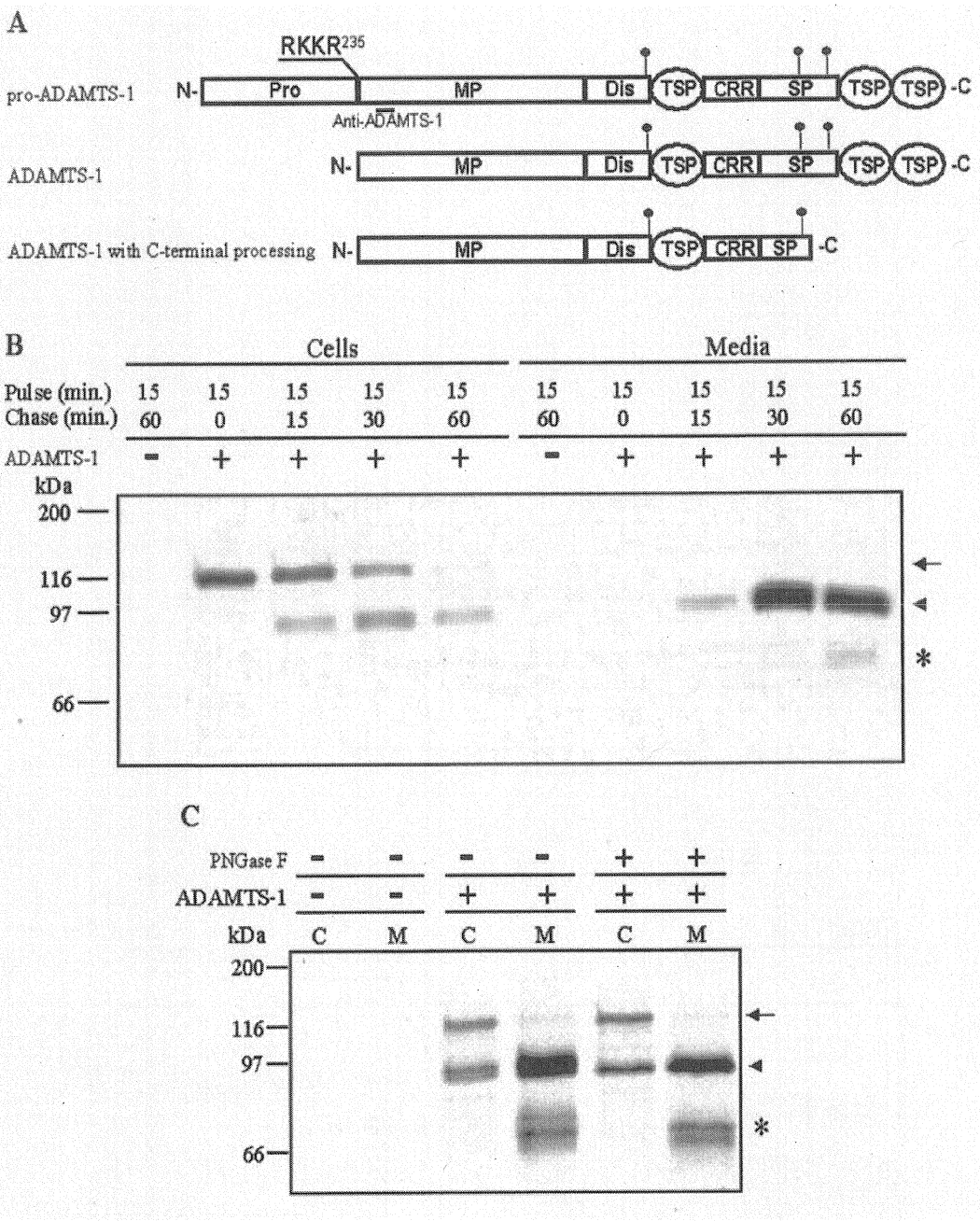
Deglycosylation of immunoprecipitated proteins from cell lysate and conditioned medium of ADAMTS-1 transfected cells was performed following immunoprecipitation using 10 U N-glycosidase F (PNGase F) (Roche, Indianapolis, IN, USA) for 3 h at 37°C in 150 mM sodium phosphate, pH 7.4, 50 mM EDTA, 0.1% SDS, 1% 2-mercapthoethanol, 0.5% Triton X-100, followed by SDS-PAGE and fluorography.

RESULTS

Biosynthesis of ADAMTS-1 – Previous work described how processing of proADAMTS-1 is required for its activation (22). To better characterize how ADAMTS-1's pro-region is involved in the enzyme's biosynthesis we transfected human kidney 293 cells with an expression vector containing the complete human ADAMTS-1 cDNA. Pulse-chase experiments were initially carried out to analyze the onset of ADAMTS-1 synthesis. ADAMTS-1 proteins were immunoprecipitated with a polyclonal anti-ADAMTS-1 recognizing the IHDEQKGPEVTS sequence present in the metalloproteinase domain of ADAMTS-1. As seen in figure 1B, we detected in cell lysates, after a 15 min. pulse, a 110 kDa form which corresponds well to the theoretical molecular weight (100 kDa, 916 residues) of unprocessed proADAMTS-1 (Fig. 1A). Following a 15 min. chase period, a 87 kDa form corresponding to the proteolytically active ADAMTS-1 appears in the cells with a complete processing of proADAMTS-1 after 60 min. of chase. The mature 87 kDa ADAMTS-1 is also detected in the media after 30 minutes. A 2-hour chase period enabled us to detect a doublet at 65 kDa in the media (Fig. 1C), a result of C-terminal processing by a metalloproteinase (22).

Deglycosylation of ADAMTS-1 - Longer pulse labeling and chase times enabled us to detect a doublet at 87 kDa and 65 kDa. Since ADAMTS-1 possesses three N-glycosylation sites we investigated the glycosylated state of the doublets. Deglycosylation with PNGase F after metabolic labeling reveals that the upper band of the doublet at 87 kDa represents a N-glycosylated form as can be seen by the significant shift in electrophoretic mobility to an apparent molecular weight of 82 kDa more in line with the calculated molecular weight of

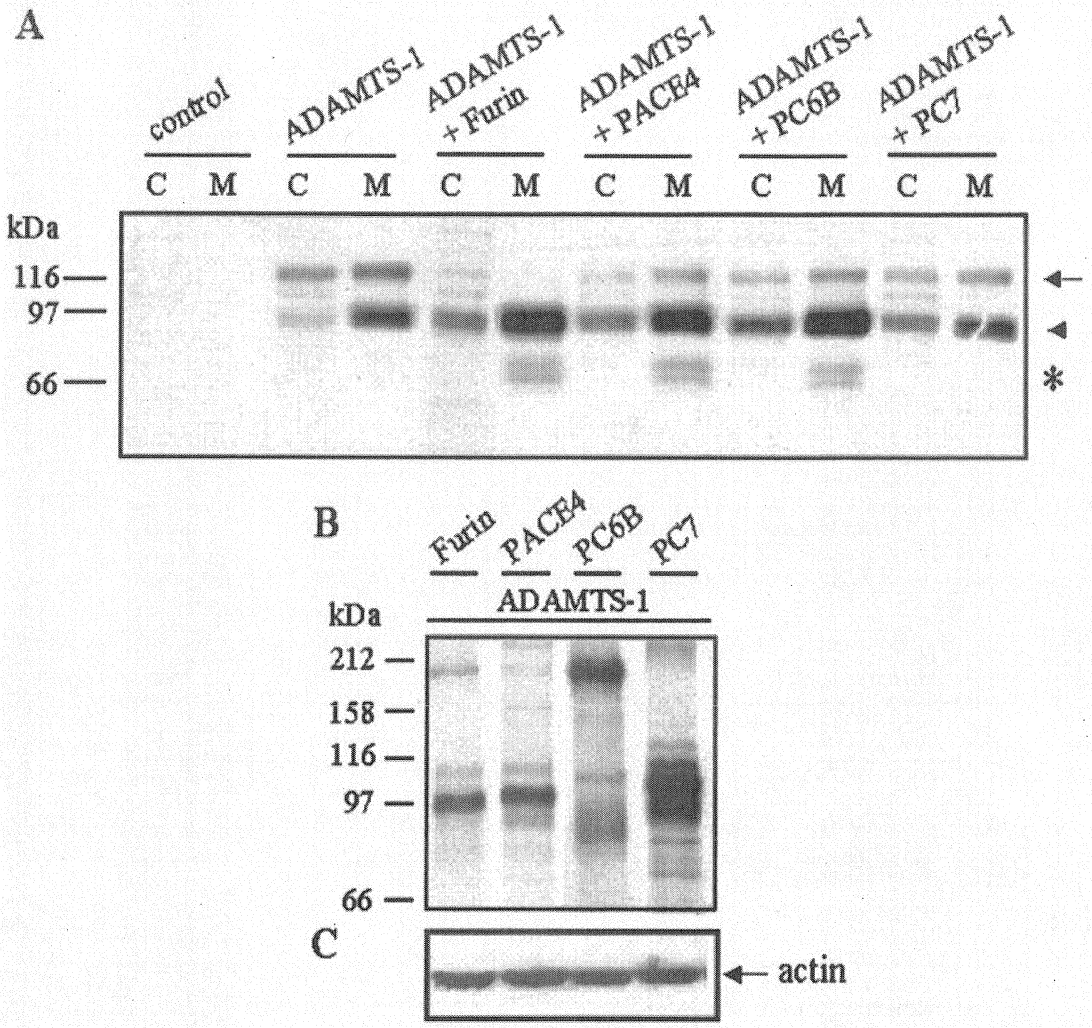
Figure 1. Biosynthesis of ADAMTS-1 in QBI 293A cells. (A) Schematic representation of ADAMTS-1 forms. ADAMTS-1 is a mosaic protein containing different domains labelled *Pro* (prodomain), *MP* (metalloproteinase domain), *Dis* (disintegrin-like domain), *TSP* (thrombospondin type I repeat), *CRR* (Cysteine-rich region) and *SP* (spacer domain). Putative *N*-linked glycosylation motifs are shown (Y). A peptide corresponding to residues 295-306 (IHDEQKGPEVTS) was used to generate polyclonal anti-ADAMTS-1 antibodies. (B) Time course of ADAMTS-1 biosynthesis in QBI 293a cells. Cells transfected with pcDNA3.1*Myc*his/ADAMTS-1 or empty expression vector pcDNA3.1*Myc*his were pulse-labeled with [35S]Met/Cys for 15 min. and chased in complete medium for the indicated periods of time. Labeled proteins from cell extracts prepared in RIPA buffer or cultured medium were immunoprecipitated with anti-ADAMTS-1 (1/1000) polyclonal antibody and detected by fluorography. Arrow indicates the intracellular zymogen form. Arrowheads indicate the mature form and the asterisk indicate the C-terminally processed forms in the medium. (C) *N*-deglycosylation of ADAMTS-1. Pulse-chase was performed as in B except pulse was for 30 min. and chase for 2 hours. Cells extracts (C) and media (M) were treated with 10 U of PNGas F for 3 hours following immunoprecipitation.


A
RKKR235
pro-ADAMTS-1
N-
Pro
MP
Dis
TSP
CRR
SP
TSP
TSP
-C
Anti-ADAMTS-1
ADAMTS-1
ADAMTS-1 with C-terminal processing
B
Cells
Media
Pulse (min.) 15 15 15 15 15 15 15 15 15 15
Chase (min.) 60 0 15 30 60 60 0 15 30 60
ADAMTS-1 - + + + + - + + + +
kDa
200
116
97
66
C
PNGase F - - - - + +
ADAMTS-1 - - + + + +
kDa C M C M C M
200
116
97
66

79 kDa which would correspond to mature ADAMTS-1 (residues 236-950, Fig. 1C). Moreover, the two forms at 65 kDa also appear to be N-glycosylated since both form are shifted to lower molecular weights following PNGase F treatment.

Processing of proADAMTS-1 by convertases – Although furin is the better-characterized proprotein convertase, PACE4, PC6B and PC7 also cleave precursor proteins within the constitutive secretory pathway (25) although their precise function is still unclear. To investigate if these convertases process proADAMTS-1, we co-transfected ADAMTS-1 cDNA with the cDNA of these different convertases in the furin-deficient CHO RPE.40 cells (26). As can be seen in Fig. 2A, proADAMTS-1 is processed to 87 kDa in CHO RPE.40 cells by endogenous proteases but less effectively than in 293 cells as demonstrated by the presence of the 110 kDa zymogen form in the medium (compare Fig. 2A with Fig. 1 where the 110 kDa form is detected in the medium). Co-transfection of furin with ADAMTS-1 led to the complete processing of the 110 kDa form into the 87 kDa band and the 65 kDa doublet. PACE4 and PC6B also cleaved proADAMTS-1 as demonstrated by the reduced intensity of the 110 kDa zymogen form in both cells and media but not as efficiently as furin. Interestingly, PC7-dependent processing of proADAMTS-1 was very weak. Expression levels of the different convertases were verified by immunoprecipitating each convertases when co-transfected with ADAMTS-1 (Fig. 2B). Figure 2B shows that mature furin (apparent molecular weight of 100 kDa, ref.(27)), PACE4 (103 kDa, ref.(28) PC6B (theorical molecular weight of 195 kDa) and PC7 (92 kDa, ref.(29)) are all expressed at similar levels. Sample loading was verified by western blot using anti-actin (Fig.2C). These results suggest that proADAMTS-1 is efficiently converted to the active 87 kDa and

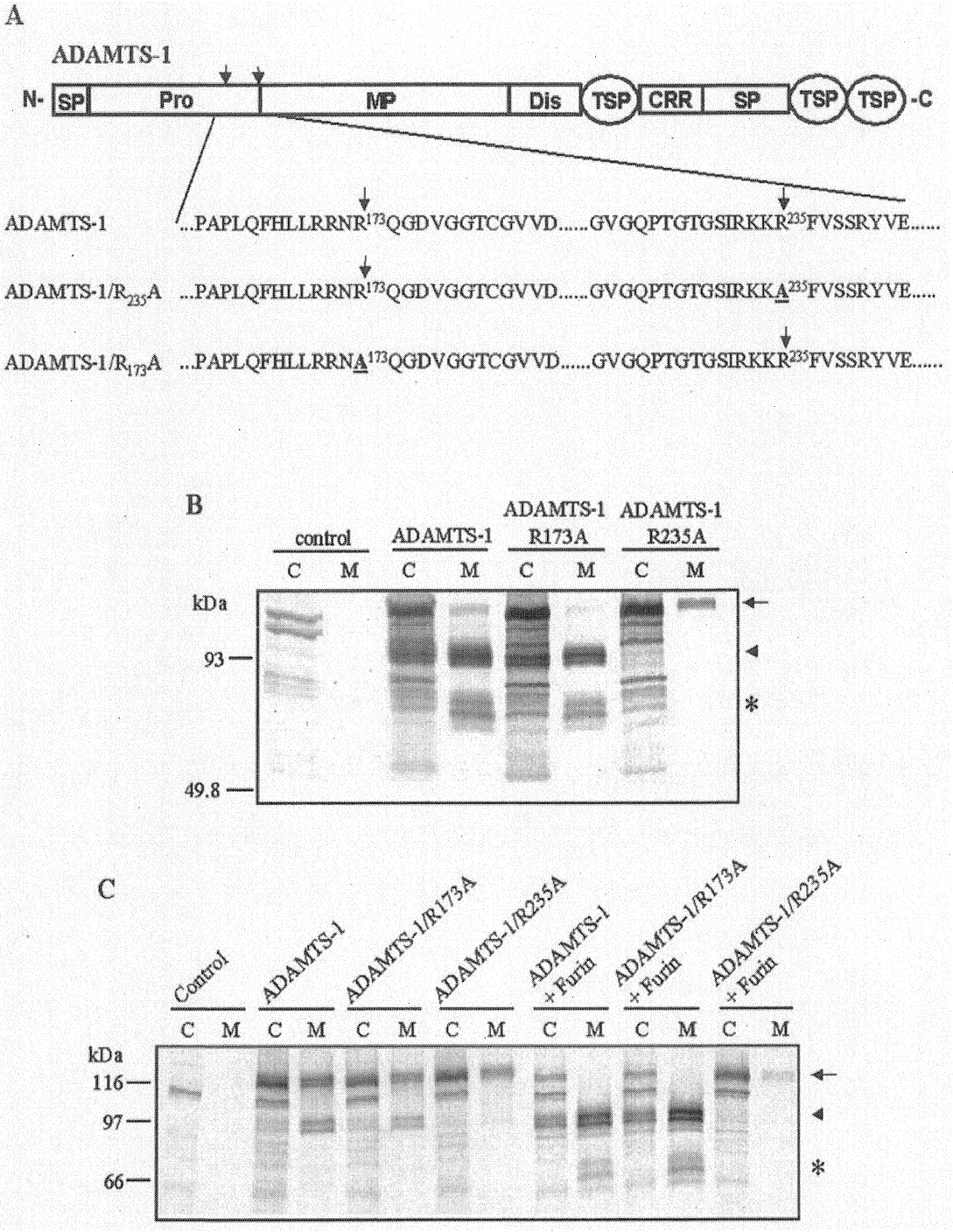
Figure 2. Processing of proADAMTS-1 by the mammalian convertases in CHO RPE.40 cells. (A) CHO RPE.40 cells were transfected with empty vector (control), pcDNA3.1Mychis/ADAMTS-1 (3 μg) and co-transfected separately with different plasmids encoding cDNAs for furin, PACE4, PC6B and PC7 (0.5 μg). Cells were labeled with [35S]Met/Cys for 30 min. and then incubated in non-radioactive medium for 2 hours. Cell extracts (C) and media (M) from pulse-chase incubations were immunoprecipitated with anti-ADAMTS-1 antibody and analyzed by SDS-PAGE followed by fluorography. (B) CHO RPE.40 cells were transfected and labelled as in (A). Cell extracts were immunoprecipitated with anti-furin antibody (1/200, lane 1), anti-PACE4 antibody (1/200, lane 2), anti-PC6B antibody (1/200, lane 3) and anti-PC7 antibody (1/200, lane 4) and analyzed by SDS-PAGE followed by fluorography. (C) Western blot using anti-actin antibody on cell extracts of CHO RPE.40 cells transfected and labelled as in (A).


A
control
ADAMTS-1
ADAMTS-1 + Furin
ADAMTS-1 + PACE4
ADAMTS-1 + PC6B
ADAMTS-1 + PC7
C M C M C M C M C M C M
kDa
116
97
66
B
Furin
PACE4
PC6B
PC7
ADAMTS-1
kDa
212
158
116
97
66
C
actin

65 kDa forms by furin and to a lesser extent by PACE4, PC6B and PC7. Since furin in the most ubiquitously expressed of all convertases, it is therefore very likely to be the bona fide physiological zymogen convertase of the ADAMTS family. However, it is conceivable that other proteases of this family may also play a role in zymogen activation under certain conditions.

Arg235 is required for proADAMTS-1 maturation - Many ADAMTS family members possess more than one putative furin recognition sequence, RXXR, within their pro-domain. There are two putative cleavage sites ($RRNR^{173}$ and $RKKR^{235}$) within the ADAMTS-1 pro-domain. To assess the effect of abolishing either one of these sites on ADAMTS-1 biosynthesis, we performed site-directed mutagenesis on the P1 residue of each cleavage sequence by replacing Arg with Ala (ADAMTS-1/R_{173}A and ADAMTS-1/R_{235}A, Fig. 3A). Figure 3B shows the result of metabolic labeling of 293A cells previously transfected either with ADAMTS-1/R_{173}A or ADAMTS-1/R_{235}A cDNA followed by immunoprecipitation with anti-ADAMTS-1 antiserum. We observed that the biosynthetic profile of the ADAMTS-1/R_{173}A mutant did not differ from that associated with wild-type ADAMTS-1. However, cells transfected with the ADAMTS-1/R_{235}A cDNA did not process the 110 kDa form, which is also found intact in the media, indicating the necessity of Arg at position 235 for cleavage. No other bands were detected suggesting that the alternative furin recognition site at position 173 was not used to produce other maturation fragments. Moreover, no additional processing was observed, such as the C-terminal processing of the 87 kDa form into the C-terminally cleaved forms, indicating that these latter entities require the preliminary cleavage of the zymogen form. Thus, it is possible that the C-terminally processed forms observed at 65

Figure 3. Processing of proADAMTS-1 furin recognition sequence mutants. (A) Schematic representation of the domain organization of ADAMTS-1 and the furin cleavage site mutants. Putative furin cleavage sites are represented by arrows. Bold and underlined characters are mutated amino acids to alanine that abolishes the minimum furin recognition sequence (RXXR). (B) Pulse (30 min) and chase (2 h) were performed on QBI 293A cells transfected with pcDNA3.1*Myc*his/ADAMTS-1 or ADAMTS-1 furin cleavage sites mutants pcDNA3.1*Myc*his/ADAMTS-1/R173A and pcDNA3.1*Myc*his/ADAMTS-1/R_{235}A. Control was transfected with empty expression vector. (C) Pulse-chase experiment performed as in B but in the furin-deficient CHO RPE.40 cells. Wild-type and mutant ADAMTS-1 were co-transfected with pCI-Neo/Furin.


A
ADAMTS-1
N-
SP
Pro
MP
Dis
TSP
CRR
SP
TSP
TSP
-C
ADAMTS-1 ...PAPLQFHLLRRNR173QGDVGGTCGVVD.....GVGQPTGTGSIRKKR235FVSSRYVE.....
ADAMTS-1/R235A ...PAPLQFHLLRRNR173QGDVGGTCGVVD.....GVGQPTGTGSIRKKA235FVSSRYVE.....
ADAMTS-1/R173A ...PAPLQFHLLRRNA173QGDVGGTCGVVD.....GVGQPTGTGSIRKKR235FVSSRYVE.....
B
control
ADAMTS-1
ADAMTS-1 R173A
ADAMTS-1 R235A
C M C M C M C M
kDa
93
49.8
C
Control
ADAMTS-1
ADAMTS-1/R173A
ADAMTS-1/R235A
ADAMTS-1 +Furin
ADAMTS-1/R173A +Furin
ADAMTS-1/R235A +Furin
C M C M C M C M C M C M C M
kDa
116
97
66

kDa arise from the autocatalytic action of the proteolytically active forms of ADAMTS-1.

Because we had observed some processing of proADAMTS-1 in CHO RPE.40 cells, we investigated whether cleavage in these cells was also dependent on the furin recognition sites. Figure 3c shows that proteolysis also required Arg^{235} for proper processing, because only the zymogen form (110 kDa) of ADAMTS-1/$R_{235}A$ was present in the cell extracts and in the media. Supplementing these cells with furin did not promote processing into the mature forms suggesting the absolute requirement of Arg^{235} in the maturation process.

Intracellular localisation of ADAMTS-1 pro-domain cleavage – To further define the intracellular compartment where proADAMTS-1 activation occurs, we treated cells expressing ADAMTS-1 with the Golgi-disturbing agents Brefeldin A (BFA) and monensin (Fig.4). Pulse-labeling analysis revealed that BFA, which inhibits protein transport between the ER and the Golgi apparatus, abolished processing of the 110 kDa proADAMTS-1 form into the 87 kDa and 65 kDa mature forms. The cells were also treated with monensin, a known inhibitor of post-Golgi transport (30). At 3.6 μM, monensin interfered with but did not completely abolish production of the mature 87 kDa form that was detected in the media although this secretion was blocked at higher doses (36 μM). Taken together, these results identify the Golgi network as the major site of proADAMTS-1 processing.

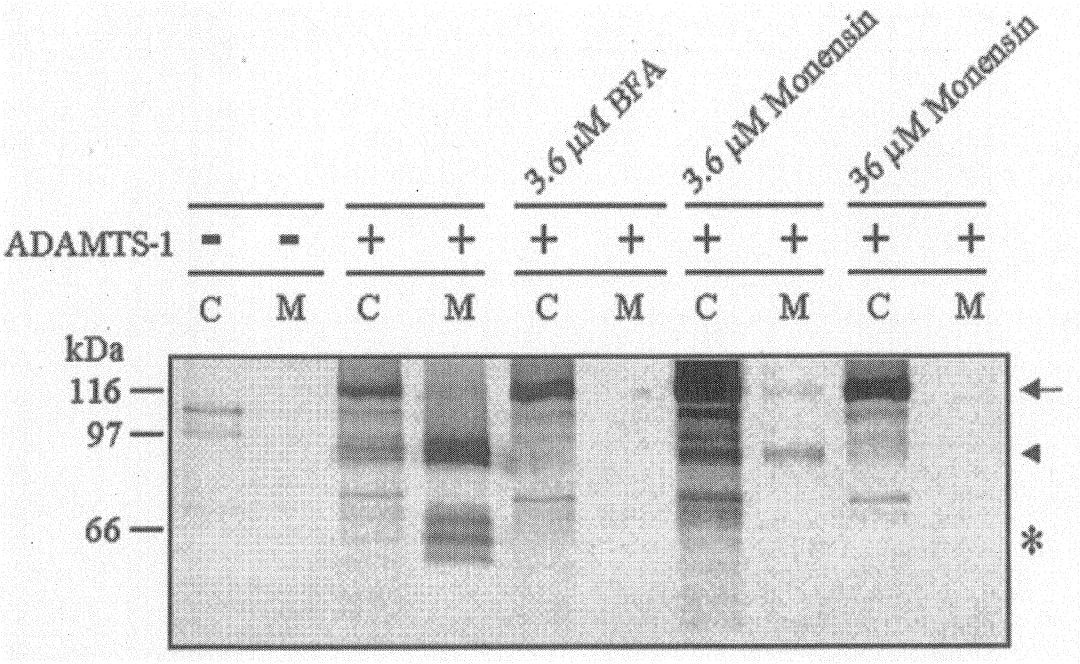


Figure 4. ProADAMTS-1 is processed in the secretory pathway

Transfected QBI 293A cells with pcDNA3.1*Myc*his/ADAMTS-1 or empty vector were pulsed for 3 hours in presence of 3.6 μM Brefeldin A (lane 5-6), 3.6 μM Monensin (lane 7-8) and 36 μM Monensin (lane 9-10).

Mutagenesis of conserved ADAMTS-1 pro-domain residues – To determine whether residues other than the furin recognition sequences are important in the maturation and conserved residues could potentially be required or implicated in these processes. Figure 5 shows the alignment of the 19 human ADAMTS pro-domains using hierarchical clustering. Only that portion of the pro-domains with the highest sequence homology is shown and identical amino acids found in more than 12 ADAMTS pro-domains are termed "consensus" residues. This alignment identifies 15 conserved residues and motifs in the pro-domain of ADAMTS besides the furin recognition sequences found at the c-terminal end of the pro-domains.

Site-directed mutagenesis was performed to investigate the effect of replacing these conserved amino acids in the pro-domain of ADAMTS-1. Figure 6a illustrates the different Ala mutants of ADAMTS-1 used in the study. Pulse-chase analysis was performed in HEK293 cells transfected with nine different ADAMTS-1 pro-domain mutants (Fig. 6B). First, we found that the zymogen of every mutant expressed was detected in cell extracts. The T111A, V112A and P116A mutants essentially behaved similarly to the wild-type ADAMTS-1 with regards to their processing pattern. Replacing residues 137-144 by Ala (ADAMTS-1/8A) completely abolished processing of the 110 kDa zymogen form into the 87 kDa active moiety but this zymogen form failed to be detected in the media. For the C106A, and C125A mutants processing was greatly reduced and, similar to ADAMTS-1/8A mutants, no detectable forms were observed in the media. Conversely, significantly lower amounts of processed forms of Y108A, G110A, and C181A were found in the media. Intriguingly, although zymogen forms of these mutants were detected intracellularly they were not observed as secreted products as where the furin recognition sequence mutant

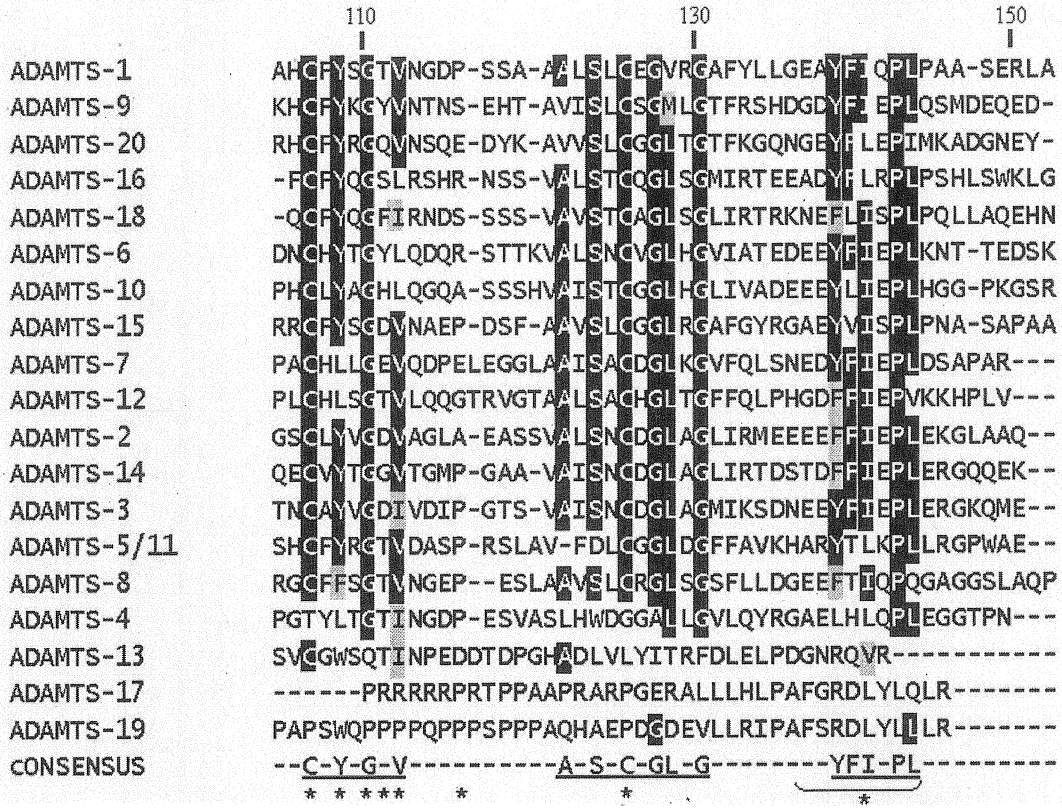


Figure 5. Alignment of ADAMTS pro-domains

ADAMTS (1-20) pro-domains were aligned using hierarchical clustering (43). Highest concensus motifs within proregion of ADAMTS family members are represented. Amino acids numbering correspond to ADAMTS-1 residues 104-152. Identical amino acids are shadowed and residues of similar properties are in gray. Concensus residues are indicated at the bottom line. Underlined amino acids represent the three conserved motifs. Asterisks indicate those residues mutated to Alanine.

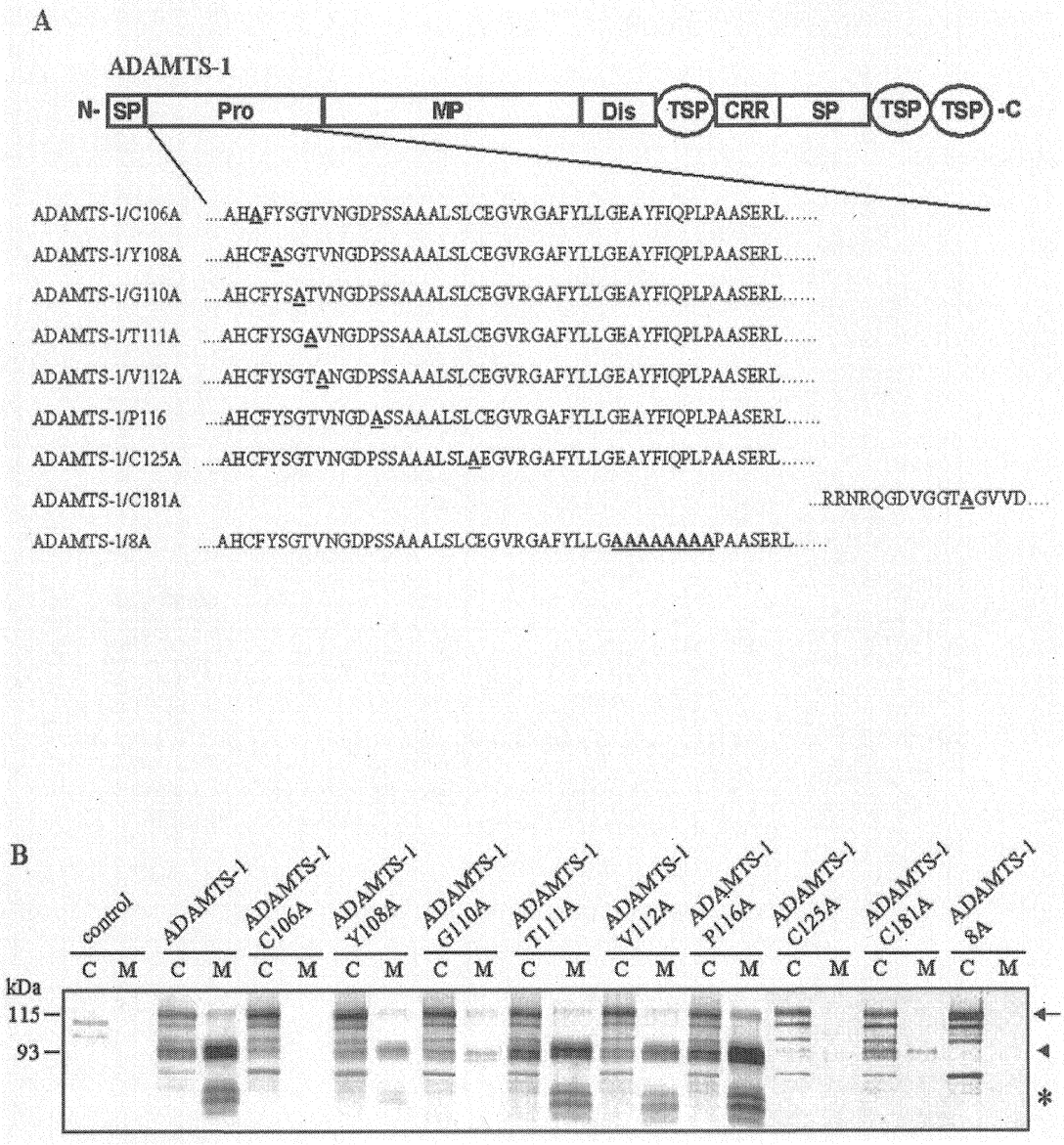


Figure 6. Biosynthesis of ADAMTS –1 pro-domain mutants

(A) Sequence of ADAMTS-1 pro-domain mutants. Underlined and bold amino acids were replaced by Ala. (B) QBI 293A cells were transfected with the ADAMTS-1 pro-domain mutants and were treated as in Fig. 3B.

R235A (Fig. 3B) indicating that zymogen activation is not a pre-requisite for secretion. Moreover, it can be seen that an overall reduction of immunoreactive material can be observed for these mutants suggesting that a percentage of these proteins may undergo inefficient folding leading to degradation. Nevertheless, those immunoreactive moieties that resided intracellularly did not seem to act as good substrates for processing or for transiting through the secretory pathway. Finally, we examined the importance of Cys^{181}, possibly involved in the cysteine switch process (see discussion), an essential component in the biosynthesis of other metalloproteinases (31). The results indicate that replacing Cys^{181} with Ala led to the reduced processing of proADAMTS-1 and reduced levels of processed products in the media suggesting that this particular cysteine may not be directly responsible for latency and activation as demonstrated for most of the MMPs.

DISCUSSION

Proteolysis of the extracellular matrix and cell surface proteins mediated by metalloproteases, including MMPs, ADAMs and ADAMTSs, is of vital importance for tissue-remodeling processes during normal and pathological conditions, such as tissue morphogenesis, wound healing, inflammation, and tumor cell invasion and metastasis (1,32). Metalloproteases are synthesized as inactive proenzymes or zymogens. In most cases, to display any proteolytic activities, the prodomain located N-terminal to the catalytic domain must be removed from the zymogen. For proADAMTS precursors, members of the proprotein convertase family would be responsible for the proteolytic cleavage of the pro-domain at the junction of the catalytic domain.

Here we report that the 110 kDa proADAMTS-1 precursor is rapidly processed intracellularly to a shorter form (87 kDa) that eventually gets secreted as a soluble entity. Moreover, although furin is the most efficient convertase at cleaving proADAMTS-1 within the constitutive secretory pathway, PACE4, PC6B also process the zymogen. These convertases are, like furin, localized intracellularly within the TGN/endosomal compartments (21) but it has been reported that PACE4 is secreted and binds to heparin in the ECM (33). Therefore, it is possible that PACE4 and PC6B may recognize and cleave proADAMTS-1 in different tissues or under various conditions of expression. The mechanism responsible for processing proADAMTS-1 in the furin-deficient CHO RPE.40 cells may well be achieved by compensatory functions of enzymes such as PACE4 or PC6B. However, these proteases are dependent, as is furin, on the presence of a furin-like recognition sequence and Arg^{235} for proper cleavage of proADAMTS-1. The fact that PC7

process very weakly ADAMTS-1 is intriguing since PC7 is the subtilisin-like PC with substrate specificity most similar to that of furin, with which it is highly homologous (34).

Furin or furin-like PCs mediate prometalloprotease activation by cleaving at the C-terminal end of the conserved motif RXXR found between pro- and catalytic domains. Rodriguez-Manzaneque and coworkers have shown that a P4 Arg to Ala proADAMTS-1 mutant (A^{232}KKR) did not exhibit aggrecanase activity. Our data showing that the RKKA^{235} mutant, which also abolishes the furin recognition sequence, was not processed by furin and only the zymogen form (110 kDa) was secreted; this may explain the lack of activity of these mutants. Moreover, it is interesting to note that in metalloproteases such as ADAM19 and MT1-MMP, alternative furin cleavage sites can be used when the primary site is abolished (35,36). However, this is not the case for ADAMTS-1 since replacing the P1 Arg for Ala in the RKKA^{235} mutant did not yield to alternative processing at the RRNR^{173} site. A possible explanation for this is that the RRNR^{173} site is not in an area of the pro-domain that is exposed to the aqueous environment and hence is not accessible to furin. In ADAMTS-9 multiple furin-like recognition sequences are found within the pro-domain but only one is preferentially used (13).

Our results also show that maturation of proADAMTS-1 is an intracellular event. To further define the intracellular compartment where proADAMTS-1 activation occurs, we treated the cells with Brefeldin A, which blocks the trafficking between the ER to Golgi apparatus (37) but permits autoproteolysis of the furin zymogen (38). BFA inhibited the ADAMTS-1 activation and secretion, which suggests that the maturation of proADAMTS-1 by furin requires the specific microenvironment of the *trans*-Golgi network. Monensin, a

known inhibitor of post-Golgi transport (30), interfered with secretion of ADAMTS-1 but not its maturation. Taken together, these results demonstrate that proADAMTS-1 processing takes place in the Golgi apparatus.

Alignment of the 19 ADAMTS family members revealed three conserved regions other then the furin recognition sequence at the C-terminal end of the pro-region. ADAMTS- 13, ADAMTS-17 and ADAMTS-19 pro-regions showed poor homology with other members of the family. In one of these, ADAMTS-13, it has recently been demonstrated that removal of its prodomain is not necessary for proteolytic activity toward the von Willebrand factor precursor (39). The short and poorly conserved ADAMTS-13 pro-region thus seems to be dispensable for latency and activation.

Conserved residues within another metalloprotease pro-region, MT1-MMP's, have been implicated in revealing crucial conformational constraints important for folding and function of the enzyme (40). In a comparable fashion, we employed mutational analysis on proADAMTS-1's pro-region to assess the consequence of replacing conserved residues on biosynthesis. We did not notice significant changes in the biosynthetic patterns of the less conserved residues T111A (found in 6 of 19 ADAMTS sequences), V112A (10 of 19 but all are hydrophobic in this position) and P116A (9 of 19) mutants suggesting that these amino acids are not major determinants in ADAMTS-1 biosynthesis. Substitution of the more conserved residues C^{106} (found in 16 of 19 ADAMTS sequences), Tyr^{108} (12 of 19), Gly^{110} (16 of 19), Cys^{125} (15 of 19), C^{181} and residues 137-144 had a more profound effect on the biosynthesis of the enzyme with processing and secretion being affected. It is important to mention however that it is likely that substitution of these key residues resulted

in a certain degree of folding defects as demonstrated by the lower overall levels of detected mutant proteins. This would imply that highly conserved residues in ADAMTS members are important for proper folding and/or are implicated as recognition motifs required for their maturation and secretion.

The cysteine switch model predicts that metalloproteinases are kept latent by the interaction of a conserved Cys residue of the prodomain and a zinc atom in the catalytic domain that blocks the active site. Disruption of this interaction leads to removal of the prodomain and activation of the enzyme (31). Most ADAMTS, like ADAM and MMP possess a conserved asymmetric (or unpaired) cysteine and the catalytic zinc atom potentially implicated in a cysteine switch mechanism. The cysteine residue within the pro-region sequence G-T-C^{181}-G-V-V-D is potentially the residue participating in the cysteine switch mechanism because of the similarity of the amino acids surrounding Cys^{181} to other cysteine switches found in MMPs and ADAMs (41). Impaired maturation and activation of the Cys^{181}/Ala mutant demonstrated that the Cys^{181} is not crucial for latency. Moreover, the furin Arg^{235} mutant to Ala showed no maturation so the $RKKR^{235}$ motif is thus a clearly identifiable determinant for activation.

Importantly, abrogation of C-terminal post-secretory processing in the activation deficient mutants also supports the likelihood that this could be autocatalytic, occurring either in *cis* or *trans*. Such a C-terminal processing event has been shown to have a regulatory role, and has now been noted with several ADAMTS (-1, -4, -5) (22,42). In conclusion, these studies shed light on critical regulatory mechanisms of broad significance to the ADAMTS proteases. Many important features of the ancillary domain and catalytic

site have been identified by engineered or naturally occurring mutations, yet there has not yet been a systematic analysis of the pro-domain. Although 3-dimensional structure is not yet available for ADAMTS zymogens, we predict that observations we have provided here will be crucial in understanding the structure of the zymogen and the role of the pro-domain.

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ARTICLE 2 – AVANT-PROPOS

Extracellular activation of proADAMTS5 by proprotein convertases

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Contribution: J'ai effectué toutes les manipulations à l'exception des figures 1E, 4 et 6 qui ont été effectué dans le laboratoire de notre collaborateur Dr. Suneel Apte du Cleveland Clinic. Par contre, les plasmides encodant différents mutants de ADAMTS5 utilisés dans les figures 1E, 4 et 6 ont été construits par moi-même. J'ai rédigé la première version du manuscrit.

Résumé de l'article 2

Des études récentes mettent en évidence qu'ADAMTS5 est l'enzyme responsable de la dégradation du cartilage retrouvé dans l'arthrose. Cette enzyme est initialement synthétisée sous forme de zymogène, pro-ADAMTS5, dont sa maturation protéolytique est présumément requise pour son activité. Afin d'en savoir davantage sur la biosynthèse et l'activation de cette métallopeptidase, des études de marquages métaboliques ainsi que d'immunoprécipitations des différentes formes d'ADAMTS5 provenant des cellules transfectées et du milieu extracellulaire furent entreprises. Nous avons fait la découverte surprenante que le clivage du précurseur pro-ADAMTS5 de 120 kDa n'est pas intracellulaire compte tenu de la présence du prodomaine ainsi que de la forme mature de 85 kDa exclusivement dans le milieu extracellulaire. Aucune forme d'ADAMTS5 n'a pu être détectée en biotinylant les protéines de surface ce qui suggère que cette enzyme n'est pas associée à la membrane cellulaire. Nous avons démontré à l'aide de l'inhibiteur des pro-protéines convertases dec-RVKR-cmk, des cellules déficientes en furine ainsi que le sauvetage de la maturation dans ces cellules par la furine et PC7 que pro-ADAMTS5 est clivée par un mécanisme dépendant des convertases. La mutagenèse dirigée des trois site consensus de clivage par les convertases présents dans le prodomaine d'ADAMTS5 (RRR^{46}, RRR^{69} et $RRRRR^{261}$) indique que le clivage se fait préférentiellement à l'Arg^{261}. Finalement, la maturation de pro-ADAMTS5 par le clivage des convertases est essentielle à l'activité protéolytique de l'enzyme puisque seulement la forme mature a une activité versicanase. La découverte du clivage extracellulaire menant à l'activation d'ADAMTS5 a une signification considérable dans le développement de stratégies afin de prévenir cette activation impliquée dans l'arthrose.

Extracellular activation of proADAMTS5 by proprotein convertases

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Running title: Extracellular activation of proADAMTS5

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ABSTRACT

Recent evidence strongly implicates ADAMTS5 as a key enzyme mediating cartilage destruction in arthritis. This metalloprotease is synthesized as a zymogen, proADAMTS5, whose proteolytic processing is presumably a prerequisite for its activity. To gain insight into the biosynthesis and activation mechanism of this enzyme, we performed pulse-chase studies together with analysis of immunoprecipitated ADAMTS5 molecular forms originating from transfected cells or endogenously expressing HeLa cells and their conditioned medium. Interestingly, we found that the 120-kDa proADAMTS5 is not processed intracellularly, since the cleaved propeptide and the mature 85-kDa ADAMTS5 were found exclusively in the conditioned medium. Biotinylation of cell-surface proteins failed to detect proADAMTS5 or mature ADAMTS5 suggesting that this enzyme is not anchored to cell membranes. Using the proprotein convertase inhibitor dec-RVKR-cmk, furin-deficient cells and rescue by furin or PC7, we demonstrate that proADAMTS5 processing is convertase-dependent. Site-directed mutagenesis of three multibasic sequences found within the ADAMTS5 propeptide (RRR^{46}, RRR^{69} and $RRRRR^{261}$) suggests that processing occurs preferentially at Arg^{261}. Finally, processing of proADAMTS5 was deemed essential for activity since only the processed form was able to cleave versican, a new substrate for this protease. The finding that proADAMTS5 is activated by extracellular processing has considerable significance in the context of osteoarthritis.

INTRODUCTION

Proteolysis of extracellular substrates by the ADAMTS (A disintegrin-like and metalloprotease with thrombospondin type 1 repeats) family, which consists of 19 members, is an important mechanism regulating diverse physiological processes such as extracellular matrix biosynthesis, angiogenesis, cell migration and hemostasis (1,2). These roles have been unequivocally established by several natural human and animal mutations as well as by analysis of genetically engineered mice (3-7). There has been considerable interest in the proteolytic activity of ADAMTS proteases in osteoarthritis (8,9). Proteolytic loss of the cartilage proteoglycan aggrecan in osteoarthritis is considered to be a major contributor to joint cartilage damage, since aggrecan is responsible for the mechanical stability of cartilage in response to compressive loads (10,11). Furthermore, the loss of aggrecan exposes collagen fibrils of cartilage, as well as other components to eventual degradation. Although ADAMTS4, ADAMTS5 and (less efficiently) ADAMTS1, ADAMTS9 and ADAMTS8 (8,9,12-15) can degrade aggrecan and are referred to as aggrecanases, experiments with a genetically modified mouse in which the ADAMTS5 gene was inactivated, clearly indicate that this protease is primarily responsible for aggrecan degradation (16,17). Although similarly unequivocal evidence is lacking in humans, ADAMTS5 is believed to be the primary mediator of aggrecan loss in human arthritis as well, since it is present in arthritic cartilage and synovium and is a considerably more efficient aggrecanase than ADAMTS4. Thus ADAMTS5 is currently considered a major drug target in osteoarthritis (16-18). ADAMTS5 also degrades brevican, a component of neural extracellular matrix (19). Brevican proteolysis is thought to promote glioma cell invasion (19,20). As shown here, ADAMTS5 proteolytically processes the core

protein of versican, a major regulator of neural crest cell migration during embryonic development, and adult blood vessels (21-23). Thus, understanding the biosynthesis of ADAMTS5 and its mechanism of activation has considerable biomedical significance.

All ADAMTS members possess a signal peptide for access to the secretory pathway, a propeptide, the catalytic metalloprotease domain, a disintegrin-like domain, and a carboxyl terminal region containing a variable number of thrombospondin type 1 repeats (TSRs). Two such TSRs are present in ADAMTS5 (8,24). Following secretion, ADAMTS proteases may become anchored to the cell surface (e.g. ADAMTS7, ADAMTS9, ADAMTS10 (14,25,26) or to the extracellular matrix (e.g. ADAMTS1, ADAMTS5) (14,27). Like most zinc metalloproteases, ADAMTS proteases, are synthesized as inactive zymogens. Members of the mammalian subtilisin-like proprotein convertase (PC) family (28-31) have a definitive role in processing various zymogens by removal of their propeptides. This mechanism, used by some proMMPs (32,33), proADAMs (34-36) and proADAMTS proteases (37,38), typically occurs intracellularly, consistent with the trans-Golgi network (TGN) location of the processing enzyme, furin.

Furin is the best studied of the PCs implicated in proprotein processing within the constitutive secretory pathway (28). Most furin resides in the TGN, but it has also been detected at the plasma membrane (39-41) and as a shed, soluble form (42). Previous studies on ADAMTS1, ADAMTS4, and ADAMTS7 suggested that their zymogens were processed in the TGN by PCs, although some cell-surface processing of ADAMTS7 was also observed (25,37,38,43,44). Recently, we found that proADAMTS9 processing by furin occurred at the cell-surface (45). We found that proADAMTS9 processing diminished its

proteolytic activity against versican (46). Previously, analysis of ADAMTS13 had shown no role for its propeptide in enzyme latency (47). Thus, ADAMTS proteases appear to be highly idiosyncratic in their processing mechanisms and in the consequences of propeptide processing. Because zymogen activation is a critical post-translational regulatory step, we have investigated how it occurs in ADAMTS5 and asked whether it influences the catalytic activity of this protease. We show that proADAMTS5 processing occurs extracellularly and that this event leads to the enzyme's activation.

EXPERIMENTAL PROCEDURES

Cell culture and transfection–QBI-293A cells (Quantum Biotechnologies, Montréal, QC), derived from the HEK293 cell line, and HeLa cells were grown in complete Dulbecco's Minimum Essential Medium (DMEM) containing 10% heat-inactivated FBS, 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. CHO RPE.40 cells were cultured in RPMI 1640 containing 10% heat-inactivated FBS, 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin. Sub-confluent cells were transfected with 2-6 μl of Fugene 6 reagent (Roche Diagnostics, Laval, QC) per μg of DNA.

Site-directed mutagenesis–Human ADAMTS5 cDNA was purchased from Origene (Rockville, MD). ADAMTS5 cDNA was cloned in-frame into pcDNA3.1/myc-his plasmid (Invitrogen, Burlington, ON) with the myc-his epitope in frame. The oligonucleotide primers 5'-CTTGGTACCATGCTGCTCGGGTGGGCGTCC-3' (*KpnI* site underlined) and 5'-GTCCTCGAGACATTTCTTCAACAAAGCATTG-3' (*XhoI* site underlined) were used for PCR with the ADAMTS5 cDNA as template. The product was inserted into the pcDNA3.1/myc-his vector after digesting the vector and PCR product with *KpnI* and *XhoI*. The same approach was used to introduce the signal peptide, propeptide, and catalytic domain (ADAMTS5 Pro-Cat) in frame with the myc-his epitope of pcDNA3.1/myc-his with the following primers 5′-CTTGGTACCATGCTGCTCGGGTGGGCGTCC-3′ (*KpnI* site underlined) and 5′-CTGTCTAGATGGTAGGTCCAGCAAACAGTT -3′ (*XbaI* site underlined). ADAMTS5 constructs with mutations in the putative furin processing sites and in the active site were generated using the QuikChange site-directed mutagenesis kit

(Stratagene, San Diego, CA). The different proprotein convertase expression plasmids were described elsewhere (43). The plasmids used to express ADAMTS9 Pro-Cat (14) and ADAMTS1 (43) were described previously. All constructs were confirmed by DNA sequencing.

Metabolic labeling and immunoprecipitation– Metabolic labeling experiments were performed 24 h post-transfection. Cells were washed with PBS and incubated in DMEM Met/Cys-free medium (Invitrogen, Burlington, ON) supplemented with 10% dialyzed FCS, 1 mM L-glutamine and 50 μCi of Expre^{35}S^{35}S (NEN Life Science Products, Boston, MA) for the 30-minute pulse period. Chase was done in complete medium. After recovering the medium, the cell layer was washed with PBS and cells were lysed with 1 ml of radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 4 mM EDTA and 0.1% SDS) containing protease inhibitors (1 μM aprotinin, 10 μM pepstatin, 10 μM leupeptin and 1 mM PMSF). Samples were centrifuged to remove insoluble material. The appropriate antibodies were added (anti-myc (Roche Diagnostics, Indianapolis, IN), anti-ADAMTS5 propeptide (Chemicon International, Temecula, CA), anti-ADAMTS5 C-terminal (Abcam, Cambridge, MA), anti-furin (MON-152 from Alexis Biochemicals, San Diego, CA) or anti-ADAMTS1 (as previously described (43))) and incubated overnight at 4°C. Protein A/G Plus-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) beads were added and incubated for 1 h at 4°C. Beads were washed three times with 1 ml of RIPA buffer and labeled proteins were resolved by SDS-PAGE. Gels were treated with ENTENSIFY reagent (NEN Life Science, Boston, MA), dried and exposed for fluorography. Proprotein convertases inhibitor dec-

RVKR-cmk (Bachem Bioscience Inc., Prussia, PA) was added to the labeling mixture when indicated. Each panel corresponds to the same gel or gels from the same experiment run and exposed for the same duration before developing.

Immunoblotting–HEK293 cells were transiently transfected as above, and the medium was replaced by 293 serum free medium (Invitrogen, Burlington ON) 24h later for another 24 h. Conditioned medium was collected and centrifuged (~3000 rpm / 5 min). The cell debris-free medium was collected and concentrated by acetone precipitation or stored at -80°C until further processing. The cell lysates were washed with warm PBS and harvested in 200 µl ice-cold protein lysis buffer (1 % Triton X-100 in TBS) or RIPA buffer containing 1 X complete protease cocktail inhibitor (Roche, Laval, QC). 30 µl of each sample was subjected to reducing SDS-PAGE and transferred to a nitrocellulose membrane (Amersham Biosciences, Baie d'Urfé, QC) or PVDF (Millipore, Bedford, MA). Immunoblot analysis was performed with either anti-myc (Roche Diagnostics, Indianapolis, IN), anti-ADAMTS5 propeptide (Chemicon International, Temecula, CA), anti-ADAMTS5 RP1 (Triplepoint Biologics, Portland, OR), anti-furin (MON-152 from Alexis Biochemicals, San Diego, CA), anti-ADAMTS5 C-terminal (Abcam, Cambridge, MA) or versican V0/V1 neo-epitope antibody (anti-DPEAAE from Affinity BioReagents, Golden, CO). Antibodies were visualized with the appropriate horseradish peroxidase-coupled secondary antibodies, sheep anti-mouse immunoglobulin or donkey anti-rabbit immunoglobulin (Amersham Biosciences, UK), using enhanced chemiluminescence (ECL, Amersham-GE Biosciences Healthcare, Piscataway, NJ) according to manufacturer's instructions.

Detection of cell-surface processing-Biotinylation of cells was done on ice to prevent labeling of intracellular proteins, essentially as previously described (45). As a control to eliminate cell-surface proteins, cells were treated with 0.05% trypsin/0.53 mM EDTA for 20 min on ice, and trypsin was subsequently inactivated with 10% fetal bovine serum-supplemented medium. Biotinylated proteins were captured from cells lysates as described above using streptavidin-agarose (Sigma-Aldrich) and eluted by boiling in Laemmli sample buffer followed by SDS-PAGE and Western blotting with appropriate antibodies.

Versican Processing Assays-Conditioned medium of skin fibroblasts from a patient with Marfan syndrome (provided by Dr. Alana Majors, Lerner Research Institute, without identifiers) was used as a source of versican. For analysis of versican processing, HEK293F cells were transfected with full-length ADAMTS5 and catalytically inactive ADAMTS5 (i.e. E411A mutant) vectors and incubated for 48 h. The medium was changed to 293 serum free medium (Invitrogen, Burlington ON) and incubated in the presence or absence of 100 μM dec-RVKR-cmk (Calbiochem, San Diego, CA) for further 24 h. The conditioned medium was mixed with the versican-rich medium from skin fibroblasts at a 1:1 ratio and incubated at 37°C for 24 h. The conditioned medium was analyzed by Western blotting using Versican V0/V1 neo-epitope antibody (anti-DPEAAE) (Affinity BioReagents, Golden, CO) as previously described (46).

RESULTS

ADAMTS5 biosynthesis in HEK293 cells-To investigate the activation mechanism of ADAMTS5, we transiently transfected HEK293 cells with expression vectors containing the cDNA encoding either full-length or a C-terminally truncated form (ADAMTS5 Pro-Cat) of human ADAMTS5. Both constructs had the myc-his epitope tag at their C-termini (Fig. 1A). Expression of ADAMTS5 was examined in two ways. First, a metabolic labelling approach (pulse-chase analysis) was carried out to analyze initial events during ADAMTS5 biosynthesis. Second, Western blotting with N- and C-terminal specific ADAMTS5 antibodies was carried out to detect unprocessed and processed zymogen in the cell lysates and the conditioned medium using a variety of experimental approaches.

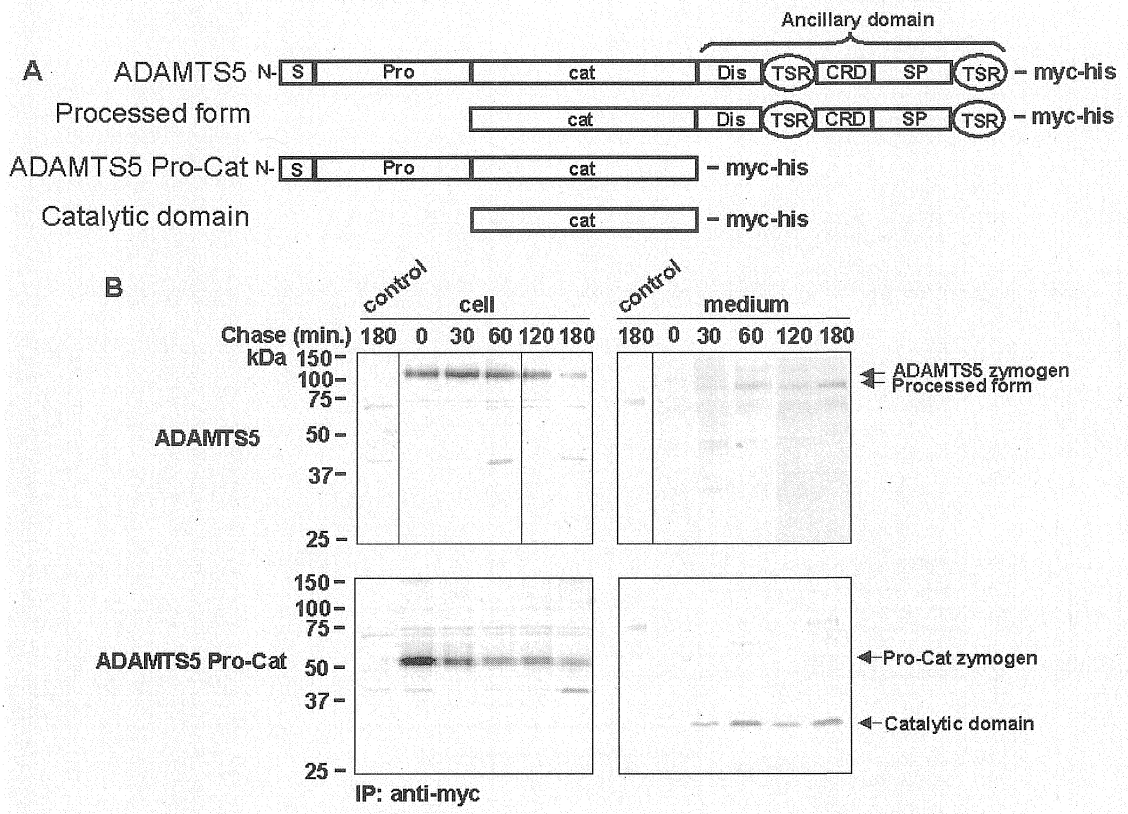
After a 30-minute pulse and no chase period, a 120-kDa band was detected in cell lysates (Fig. 1B) which corresponds to the predicted molecular mass (100 kDa) plus N-glycosylation of unprocessed proADAMTS5. Following a 30-min. chase, an 85-kDa form was detected in the medium, which corresponds to the processed form of ADAMTS5. In these samples and using this approach, the 120-kDa zymogen was not detected in the medium, which may be indicative of rapid processing. The relatively weak immunoreactivity of the 85-kDa processed form in the medium, possibly owing to C-terminal proteolysis and loss of the myc epitope tag (48) or because of binding to the ECM, prompted us to use a shorter construct, ADAMTS5 Pro-Cat (Fig. 1A), lacking the C-terminal ancillary domain known to be involved in anchoring ADAMTS enzymes to the cell (14) and ECM (27). The same approach had previously been used to characterize the propeptide cleavage of ADAMTS7 and ADAMTS9 (14,25). Moreover, a recent

characterization of C-terminally truncated ADAMTS5 forms showed that these forms are more readily secreted (49). HEK293 cells were transfected with ADAMTS5 Pro-Cat and pulsed with radioactive amino acids for 30 min. A 55-kDa band corresponding to the unprocessed proADAMTS5 Pro-Cat zymogen was detected in cell lysates. Following a 30 min chase period, we detected a 30-kDa band in the medium corresponding to the catalytic domain of ADAMTS5 Pro-Cat. This demonstrates that both the wild-type ADAMTS5 and the truncated ADAMTS5 Pro-Cat undergo rapid secretion and proteolytic processing.

Figure 1C shows that Western blot analysis using an anti-myc antibody on transfected cell lysates led to the detection of only the zymogen forms of either full-length ADAMTS5 (120 kDa) or ADAMTS5 Pro-Cat (55 kDa). However, both the zymogen and processed forms were detected in the medium. Similar results were obtained when immunoblotting with an anti-ADAMTS5 propeptide antibody (Fig. 1D) which demonstrated that the cleaved propeptide was detected only in the medium. To complement this, immunoblotting with the anti-ADAMTS5 C-terminal antibody anti-RP1 in cells transfected with the full-length ADAMTS5 revealed only the zymogen form in the cell lysate (120 kDa) while the processed form (85 kDa) was found only in the medium (Fig 1E).

Processed ADAMTS5 is present in the conditioned medium but not in the cell lysate-To enhance the sensitivity for detection of a possible intracellular processed form of ADAMTS5 we transfected HEK293 cells with ADAMTS5 Pro-Cat and performed immunoprecipitation of cell lysate with anti-myc followed by immunoblotting with the same antibody. Thus anti-myc antibody was used to concentrate all ADAMTS5 molecular

Figure 1. Biosynthesis of ADAMTS5 in HEK293 cells. A. Schematic representation of ADAMTS5 constructs used in the present studies. ADAMTS5 modules are indicated thus: S, signal peptide; Pro, propeptide; Cat, catalytic domain; Dis, disintegrin-like module; TSR, thrombospondin type 1 repeat; CRD, cysteine-rich domain; SP, spacer domain. **B.** Time course of ADAMTS5 biosynthesis in HEK293 cells. Cells transfected with pcDNA3.1/myc-his/ADAMTS5, pcDNA3.1/myc-his/ADAMTS5 Pro-Cat or empty expression vector pcDNA3.1/myc-his (control) were pulse-labeled with [^{35}S]Met/Cys for 30 min and chased in complete medium for the indicated periods of time. Labeled proteins from cell extracts (indicated as C) prepared in RIPA buffer or cultured medium (indicated as M) were immunoprecipitated with anti-myc antibody and detected by fluorography. **C-E.** ADAMTS5 and ADAMTS5 Pro-Cat were expressed in HEK293 cells. Immunoblotting using anti-myc (**C**), anti-ADAMTS5 propeptide (**D**) or anti-RP1 antibodies (**E**) was done on cell lysate and acetone-precipitated medium obtained 48 h after transfection. A nonspecific band marked with an *asterisk* (*) was detected with the anti-RP1 antibody in cell lysates.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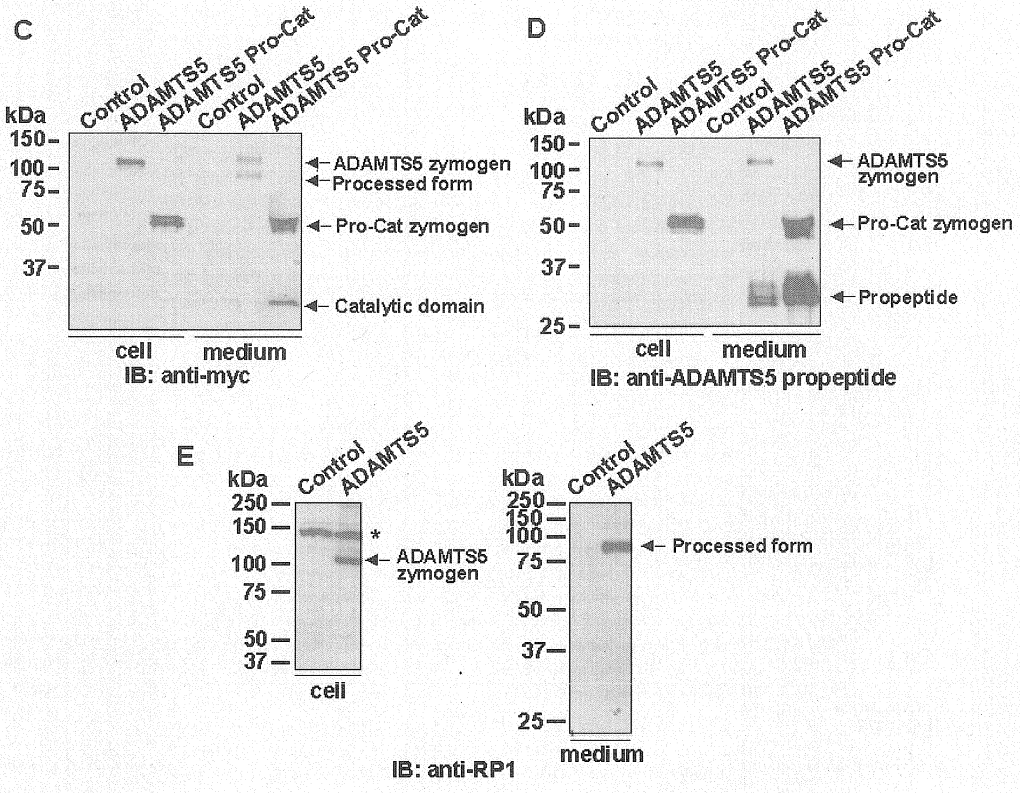
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IB: anti-ADAMTS5 propeptide
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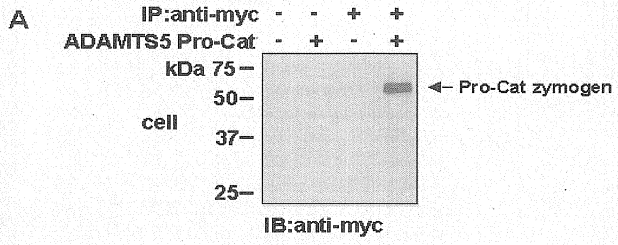
IB: anti-RP1

forms that may reside intracellularly. Figure 2A shows that only the ADAMTS5 Pro-Cat zymogen (55 kDa) but not the processed catalytic domain (30 kDa) was detected in the cell lysate using anti-myc antibodies. To force the accumulation of all forms of ADAMTS5 within the trans-Golgi network where many proprotein precursors are processed, we blocked post-Golgi transport with monensin (50). The cDNA encoding the proprotein convertase furin was co-transfected with ADAMTS5 Pro-Cat cDNA to provide an excess of the convertase and to favour intracellular cleavage. Figure 2B shows that although furin clearly augmented cleavage of proADAMTS5 Pro-Cat, as shown by the complete processing of the zymogen in the medium, no processed forms were detected intracellularly. Incubation with 100 μM monensin greatly diminished the secretion of processed ADAMTS5 Pro-Cat (catalytic domain) (Fig. 2B lower panel) yet no processed forms were detected in the cell lysate (Fig. 2B, upper panel).

The presence of only the intact proADAMTS5 Pro-Cat zymogen in cells and detection of the processed form exclusively in the conditioned medium therefore suggested that processing occurred extracellularly, *i.e.* either at the cell-surface or in the medium/extracellular matrix of transfected cells.

To verify that lack of intracellular processing of proADAMTS5 was not the result of consequence of an intrinsic cell defect affecting the cell's processing machinery, we compared the biosynthetic profiles of ADAMTS5 with ADAMTS1, which is known to undergo intracellular processing (43). Figure 2C (right-hand panel) shows that the ADAMTS1 zymogen form (110 kDa) and processed form (87 kDa) are both clearly present in the cell lysates even without co-transfecting with furin; co-expressing with furin

Figure 2. The ADAMTS5 propeptide is cleaved outside the cell. A. Cell lysates of HEK293 cells transfected with ADAMTS5 Pro-Cat were immunoprecitated with anti-myc followed by Western blotting with anti-myc and a special secondary antibody (TrueBLOT, eBioscience, San Diego, CA) to eliminate interference of IgGs (note absence of the anti-myc immunoprecipitating heavy and light chains). **B.** CHO RPE.40 cells were co-transfected with pcDNA3.1/myc-his/ADAMTS5 Pro-Cat and pCiNeo/furin. Cells were labeled with [^{35}S]Met/Cys for 3 h. Cell extracts and conditioned medium following metabolic labeling were immunoprecipitated with anti-myc antibody and analyzed by SDS-PAGE followed by fluorography. Monensin (100μM) was added for the 3 h pulse time. A nonspecific band marked with an *asterisk* (*) was detected with the anti-myc antibody in cell lysates. **C.** HEK293 cells were co-transfected with pcDNA3.1/myc-his/ADAMTS5 or pcDNA3.1/ myc-his/ADAMTS1 and pCINeo/furin. Metabolic labeling was carried as in (B) and immunoprecipitated with anti-ADAMTS1 or anti-ADAMTS5 C-terminal antibodies. **D.** Cell lysates and conditioned medium of HeLa cells were immunoprecitated followed by Western blotting with anti-ADAMTS5 C-terminal antibody.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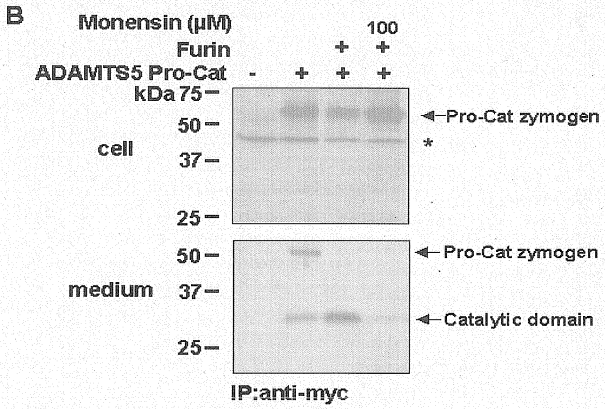
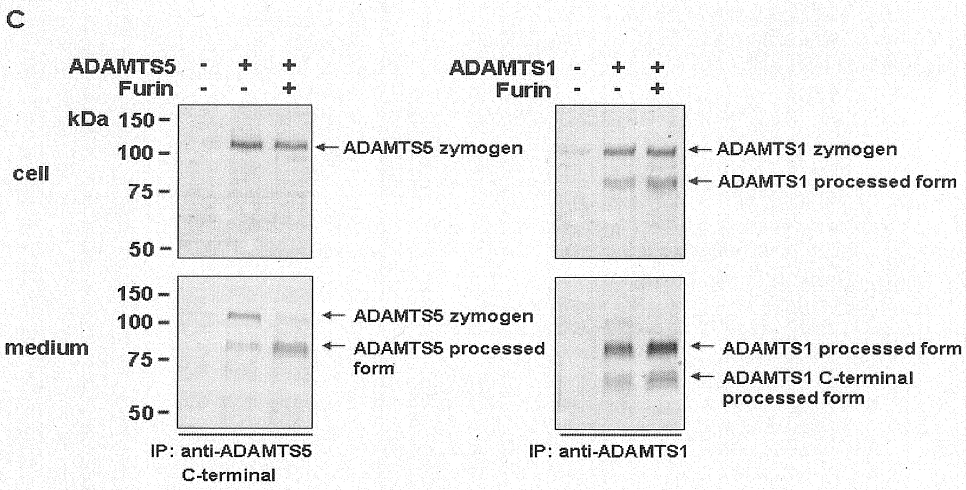
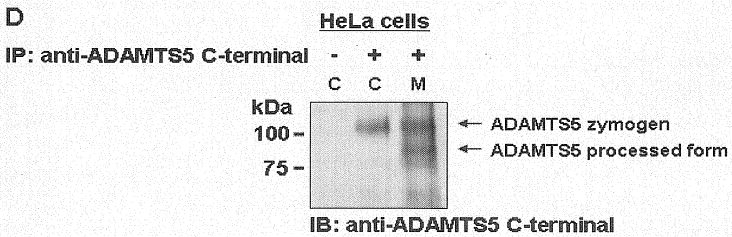
ADAMTS5 Pro-Cat - + - +
kDa 75
50
cell
37
25
Pro-Cat zymogen
IB:anti-myc


B
Monensin (μM) 100
Furin + +
ADAMTS5 Pro-Cat - + + +
kDa 75
50
cell
37
25
Pro-Cat zymogen
*
50
medium
37
25
Pro-Cat zymogen
Catalytic domain
IP:anti-myc


C
ADAMTS5 - + +
Furin - - +
kDa 150
100
cell
75
50
ADAMTS5 zymogen
150
100
medium
75
50
ADAMTS5 zymogen
ADAMTS5 processed form
IP: anti-ADAMTS5 C-terminal
ADAMTS1 - + +
Furin - - +
ADAMTS1 zymogen
ADAMTS1 processed form
ADAMTS1 processed form
ADAMTS1 C-terminal processed form
IP: anti-ADAMTS1


D
HeLa cells
IP: anti-ADAMTS5 C-terminal - + +
C C M
kDa
100
75
ADAMTS5 zymogen
ADAMTS5 processed form
IB: anti-ADAMTS5 C-terminal

enhances processing in the cell. Conversely, we demonstrate that, using an ADAMTS5 C-terminal antibody, no processed form of ADAMTS5 is detected in the HEK293 cell lysates even when these cells overexpress furin (upper left-hand panel). However, the ADAMTS5 zymogen form (120 kDa) and processed form (85 kDa) are present in the conditioned medium with an increase in the processed form when cells are supplemented with furin (Fig. 2C lower left-hand panel). This establishes that two ADAMTS proteins possess different biosynthetic profiles and strongly supports extracellular processing of proADAMTS5 as an intrinsic characteristic of this protease.

To determine whether this profile is observed in cells endogenously expressing ADAMTS5, we performed immunoprecipitation followed by immunoblotting of HeLa cell lysates and conditioned medium using an anti-ADAMTS5 antibody recognizing a C-terminal domain (residues 600-700). Figure 2 D shows that only a 120 kDa band was detected in the cell lysates indicating no intracellular processing while the zymogen (120 kDa) and the processed form (85 kDa) were detected in the conditioned medium. Taken together, these observations demonstrate that ADAMTS5 is not processed intracellularly.

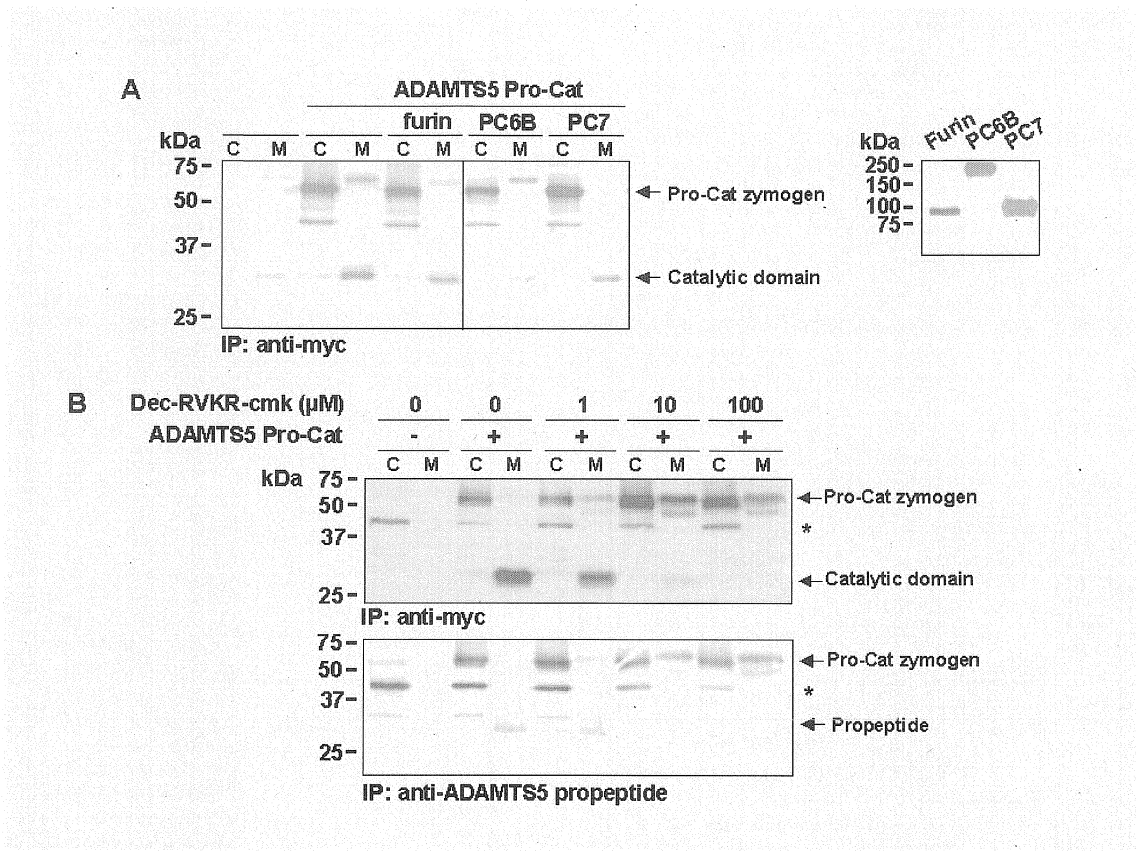
ProADAMTS5 is cleaved by furin and PC7- Proprotein convertases such as furin, PC5/6B and PC7 have been found to process other ADAMTS zymogens (25,43,45,51). To investigate whether furin, PC6B or PC7 process proADAMTS5, we co-transfected ADAMTS5 Pro-Cat cDNA with the cDNAs encoding these convertases in furin-deficient CHO RPE.40 cells (52). Figure 3A shows proADAMTS5 is present as an unprocessed zymogen in CHO RPE.40 cells and partially processed to a 30-kDa form by endogenous proteases (i.e. other than furin). Co-transfection of furin with ADAMTS5 Pro-Cat led to the

complete processing of the zymogen form to the processed 30-kDa catalytic domain. PC7 also efficiently cleaved proADAMTS5 Pro-Cat as demonstrated by the absence of the 55-kDa zymogen in the medium, but processing by PC6B was inefficient (Fig. 3A left-hand panel) as shown by the presence of the zymogen in the medium. Expression levels of the different convertases were verified by immunoprecipitating each convertase after co-transfection with ADAMTS5 Pro-Cat (Fig. 3A right-hand panel). Figure 3A (right-hand panel) shows that mature furin (apparent molecular mass of 100 kDa) (53), PC6B (theoretical molecular mass of 195 kDa), and PC7 (apparent molecular mass of 92 kDa) (54) are all robustly expressed following transfection. These results suggest that proADAMTS5 Pro-Cat is efficiently converted to the processed 30-kDa form by furin and PC7.

To provide further evidence for convertase processing, we incubated HEK293 cells with the dec-RVKR-cmk, a well-known inhibitor of these enzymes. At concentrations of 10 μM only the zymogen form was detected in the conditioned medium indicating that processing had been abolished by the inhibitor (Fig 3B).

Extracellular processing of the ADAMTS5 propeptide-We had previously inquired whether proADAMTS9 processing by convertases occurred at the cell surface by labelling cell-surface proteins with biotin and demonstrating that the proADAMTS9 could be thus labelled (45). To detect ADAMTS5 forms that may be associated with the cell-surface, we cells. In parallel experiments, these cells were treated with trypsin to remove all cell-expressed ADAMTS9 Pro-Cat (as a positive control) or full-length ADAMTS5 in HEK293

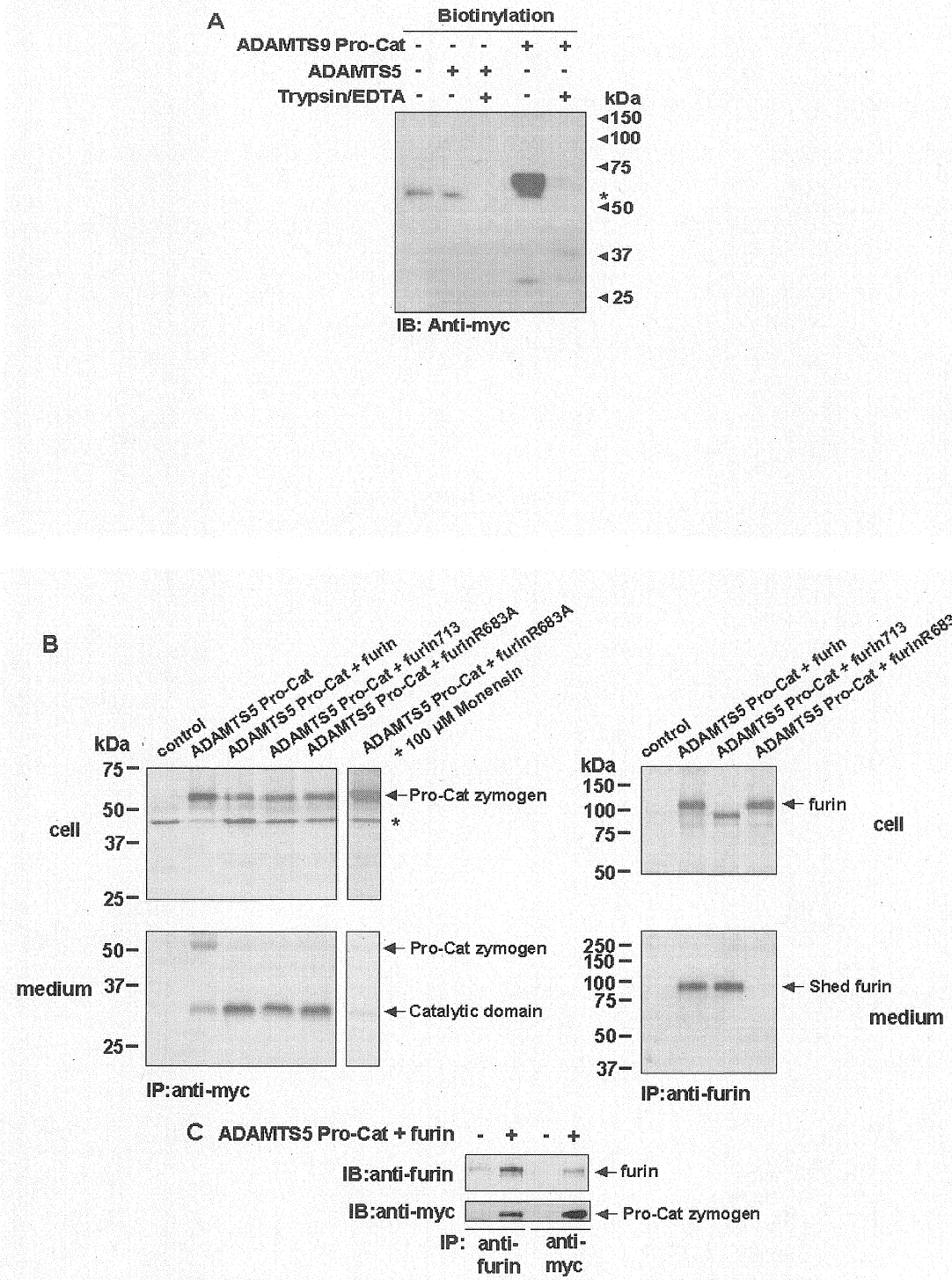
Figure 3. Processing of ADAMTS5 propeptide by proprotein convertases. A. CHO RPE.40 cells were transfected with empty vector or pcDNA3.1/myc-his/ADAMTS5 Pro-Cat (2.5 μg) and co-transfected with plasmids encoding furin, PC6B or PC7 (0.5 μg each). Cells were labeled with [^{35}S]-Met/Cys for 3 h. Cell extracts (C) and medium (M) following metabolic labeling were immunoprecipitated with anti-myc (left hand panel) antibody and analyzed by SDS-PAGE followed by fluorography. To verify expression of the different convertases, they were immunoprecipitated from cell lysates with anti-furin, anti-PC6B or anti-PC7 antibodies (right-hand panel) and analysed by reducing SDS-PAGE and fluorography. **B.** HEK293 cells transfected with pcDNA3.1/myc-his/ADAMTS5 Pro-Cat or empty vector were pulsed for 3 h in presence of increasing amount of the proprotein convertase inhibitor dec-RVKR-cmk as indicated. Labeled proteins from cell lysates and conditioned medium were immunoprecipitated with anti-myc (upper panel) or anti-ADAMTS5 propeptide antibody (lower panel). A nonspecific band marked with an *asterisk* (*) was detected with the anti-myc and anti-ADAMTS5 propeptide antibodies in cell lysates.


A
ADAMTS5 Pro-Cat
furin
PC6B
PC7
kDa
C M C M C M C M C M
75-
50-
37-
25-
Pro-Cat zymogen
Catalytic domain
IP: anti-myc
kDa
Furin
PC6B
PC7
250-
150-
100-
75-
B
Dec-RVKR-cmk (μM)
0 0 1 10 100
ADAMTS5 Pro-Cat
- + + + +
C M C M C M C M C M
kDa
75-
50-
37-
25-
Pro-Cat zymogen
*
Catalytic domain
IP: anti-myc
75-
50-
37-
25-
Pro-Cat zymogen
*
Propeptide
IP: anti-ADAMTS5 propeptide

cells. In parallel experiments, these cells were treated with trypsin to remove all cell-surface proteins. Cell surface proteins were then biotinylated, streptavidin-agarose captured and analyzed by immunoblotting with anti-myc. Figure 4A shows that ADAMTS9 Pro-Cat zymogen was detected at the cell-surface whereas no ADAMTS9 immunoreactivity was found in the trypsin-treated cells. However, under identical conditions, ADAMTS5 was not biotinylated, which suggests that ADAMTS5 forms are not anchored to the cell surface, consistent with previous studies showing that this enzyme is preferentially located in the extracellular matrix (14,49) and the conditioned medium.

We utilized another approach in order to discriminate between processing at the cell-surface or in conditioned medium. Two furin mutants, furin714 and furinR683A were co-expressed with ADAMTS5 Pro-Cat in the furin-deficient CHO RPE.40 cells. Furin714 is a truncated form (Fig. 4B) which does not possess the transmembrane domain and is not retained in membranes but released in the medium (55). FurinR683A is a mutant which is not shed from the cell surface and therefore is not present in the medium as a soluble form (Fig. 4B lower right-hand panel) (56). As demonstrated in figure 4B left hand panel, and consistent with other data, neither furin mutants effected any processing of proADAMTS5 in the cell. However, a processed form of ADAMTS5 was released in the medium of cells transfected with both furin mutants. Monensin treatment of cells expressing the unsheddable furinR683A did not permit detection of an intracellular processed form of ADAMTS5.

Figure 4. ProADAMTS5 does not bind the cell-surface and is processed by secretable and unsheddable furin constructs. A. Proteins from HEK293 cells transfected with ADAMTS5 Pro-Cat or ADAMTS9 Pro-Cat were cell-surface biotinylated with or without treatment with trypsin–EDTA as described in "Experimental procedures". Biotinylated proteins were affinity-purified using streptavidin-agarose and analyzed by immunoblotting (IB) with anti-myc. **B.** CHO RPE.40 cells were co-transfected with ADAMTS5 Pro-Cat and furin, furinR683A (which is not shed from the cell-surface) or furin714 (which does not possess the transmembrane domain). Metabolic labeling was carried out as in Figure 2B. Monensin (100 μM) was added as indicated for the entire duration of the 3-h pulse. Cell lysates and media were immunoprecipitated with anti-myc (left-hand panel) or anti-furin (right-hand panel). **C.** ADAMTS5 Pro-Cat and furin were co-immunoprecipitated 48 h post transfection in HEK293 cells. Endogenous furin is detected when IP and IB are performed. A nonspecific band marked with an *asterisk* (*) was detected with the anti-myc antibody in cell lysates.


A
Biotinylation
ADAMTS9 Pro-Cat - - - + +
ADAMTS5 - + + - -
Trypsin/EDTA - - + - +
kDa
150
100
75
*
50
37
25
IB: Anti-myc
B
control
ADAMTS5 Pro-Cat
ADAMTS5 Pro-Cat + furin
ADAMTS5 Pro-Cat + furin713
ADAMTS5 Pro-Cat + furinR683A
ADAMTS5 Pro-Cat + furinR683A + 100 μM Monensin
kDa
75
50
37
25
cell
Pro-Cat zymogen
*
medium
Pro-Cat zymogen
Catalytic domain
IP:anti-myc
control
ADAMTS5 Pro-Cat + furin
ADAMTS5 Pro-Cat + furin713
ADAMTS5 Pro-Cat + furinR683A
kDa
150
100
75
50
furin
cell
250
150
100
75
50
37
Shed furin
medium
IP:anti-furin
C ADAMTS5 Pro-Cat + furin - + - +
IB:anti-furin
furin
IB:anti-myc
Pro-Cat zymogen
IP: anti-furin anti-myc

Interestingly, immunoprecipitation coupled with immunoblotting using anti-furin and anti-myc antibodies (Fig. 4C) shows that furin and ADAMTS5 co-immunoprecipitate in the cell lysate, suggesting their association throughout the secretory process. The reason for which proADAMTS5 is not processed intracellularly despite being associated with furin is currently unknown. Taken together, these results identify the cell surface and/or the extracellular matrix, as likely to be the major sites of proADAMTS5 processing.

Processing of proADAMTS5 by convertases at Arg^{261}-Many ADAMTS family members possess multiple putative convertase recognition sequences, Arg/Lys-Arg, within their propeptides. The ADAMTS5 propeptide has three putative cleavage sites (RRR^{46}, RRR^{69} and RRRRR^{261} – Fig. 5A). To determine which of these sites is used for ADAMTS5 processing, we performed site-directed mutagenesis of these cleavage sequences by replacing Arg with Ala (ADAMTS5 Pro-Cat R44-46A, ADAMTS5 Pro-Cat R67-69A and ADAMTS5 Pro-Cat R257-261A). Figure 5B shows the result of metabolic labelling of HEK293 cells transfected either with ADAMTS5 Pro-Cat R44-46A, ADAMTS5 Pro-Cat R67-69A, ADAMTS5 Pro-Cat R257-261A or ADAMTS5 Pro-Cat R257-260A cDNAs followed by immunoprecipitation with anti-myc. We observed that the biosynthetic profile of the ADAMTS5 Pro-Cat R44-46A and ADAMTS5 Pro-Cat R67-69A mutants did not differ from that found for ADAMTS5 Pro-Cat i.e. the 55-kDa form was converted to the 30-kDa form. However, the 55-kDa zymogen forms of ADAMTS5 Pro-Cat R257-261A and ADAMTS5 Pro-Cat R257-260A were not processed to the expected 30-kDa form indicating that RRRRR^{261} is the sole motif cleaved by convertases, as previously suggested

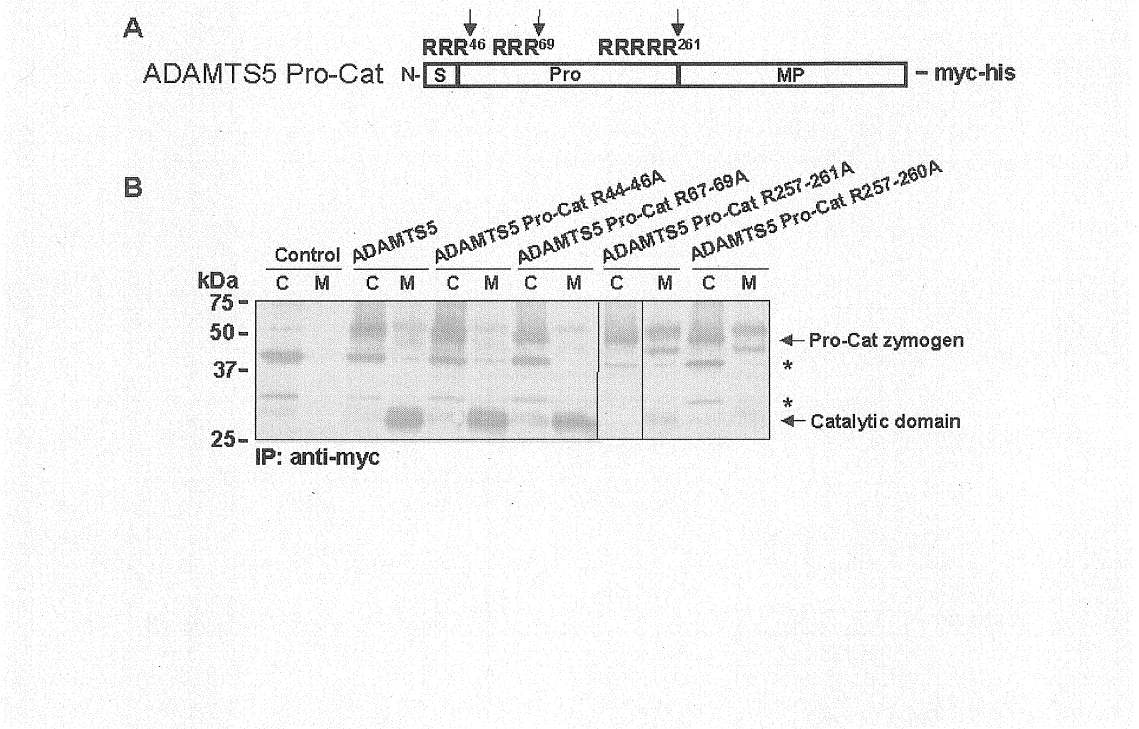


Figure 5. ADAMTS5 propeptide is cleaved by proprotein convertases at the RRRRR[261] motif. A. Schematic representation of the domain organization of ADAMTS5 Pro-Cat and the proprotein convertase cleavage sites (cleavage sites are indicated by arrows). **B**. A 3 h pulse was performed in HEK293 cells transfected with pcDNA3.1/myc-his/ADAMTS5 Pro-Cat or ADAMTS5 Pro-Cat PC cleavage sites mutants pcDNA3.1/myc-his/ADAMTS5 Pro-Cat/R44-46A, pcDNA3.1/myc-his/ADAMTS5 Pro-Cat/R67-69A, pcDNA3.1/myc-his /ADAMTS5 Pro-Cat/R2547-261A and pcDNA3.1/myc-his/ADAMTS5 Pro-Cat/R257-260A. Immunoprecipitation was done with anti-myc antibody and analyzed by SDS-PAGE followed by fluorography. Nonspecific bands marked with *asterisks* (*) were detected with the anti-myc antibody in cell lysates and medium.

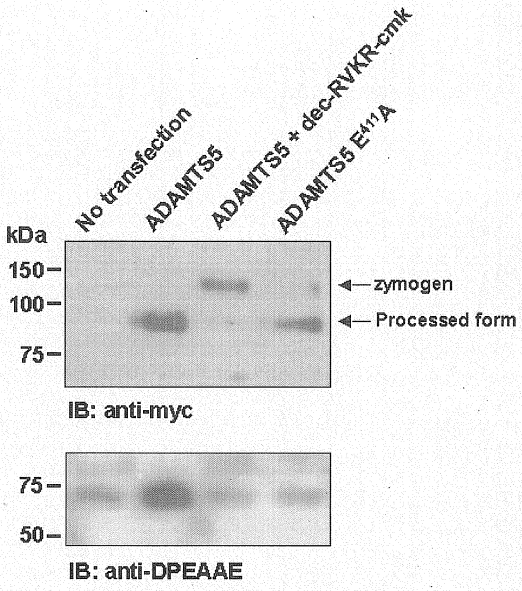


Figure 6. ProADAMTS5 processing is essential for versicanase activity. The conditioned media of HEK293F cells expressing full-length ADAMTS5 with or without 100μM dec-RVKR-cmk or with ADAMTS5 bearing an inactivating mutation in the catalytic site (ADAMTS5E411A) were collected and incubated with medium enriched in versican. Immunoblotting with anti-myc demonstrates that comparable levels of ADAMTS5 were present in the medium (upper panel). The versican neoepitope antibody (anti-DPEAAE) was used to identify proteolytic cleavage on immunoblots as indicated (bottom panel).

by N-terminal sequencing of mature ADAMTS5 (48). The detection, in the media of a 47-kDa form of ADAMTS5 in cells transfected with the ADAMTS5 Pro-Cat R257-261A and ADAMTS5 Pro-Cat R257-260A mutants, suggests that the zymogen may be susceptible to cleavage by other proteases, albeit inefficiently. This alternative form was also present when incubating with 100 μM dec-RVKR-cmk (Fig. 3B) and is, in fact, also seen in medium of cells transfected with the other furin-cleavage site mutants (Fig. 5). Thus, this processing likely precedes or is independent of furin processing. Interestingly, when furin-processing is prevented by dec-RVKR-cmk, the 47 kDa fragment accumulates in medium, but based on absence of versican processing (see below), this partial cleavage of the propeptide does not permit proteolytic activity. Together, these data indicate that the ADAMTS5 zymogen is processed at the junction of the propeptide and catalytic domain following Arg261 and that other sites within the propeptide containing multiple basic residues are not processed.

Processing of proADAMTS5 is essential for versicanase activity-We have recently demonstrated that the cell-surface processing of proADAMTS9 led to diminished versicanase activity (46). Thus, the ADAMTS9 zymogen was more proteolytically active than furin-processed enzyme. Similarly, the propeptide of ADAMTS13 does not perform the function of maintaining the enzyme in an inactive form since proADAMTS13 cleaves its substrate, pro-von Willebrand factor (47). To determine whether the zymogen or processed form of ADAMTS5 is active, versican was used as a substrate in a cellular assay. The anti-DPEAAE neoepitope antibody which recognizes only the cleaved, V1 form (70 kDa) of versican as previously shown (23) was used to detect proteolysis of the versican core protein. Under these conditions versican was proteolytically cleaved in the medium of

cells transfected with full-length ADAMTS5 (Fig. 6 lower panel). Indeed, ADAMTS5-transfected cells produced a significantly stronger immunoreactive band when compared to non-transfected cells, to cells treated with the convertase inhibitor dec-RVKR-cmk and producing only the zymogen form, or to cells expressing the catalytically inactive ADAMTS5 E411A mutant (Fig. 6 upper panel). As shown, the ADAMTS5 E411A mutant undergoes cleavage to yield the 85-kDa processed form in the media. Thus, we demonstrate that the proADAMTS5 zymogen is not active against a proteoglycan substrate until its propeptide is processed by extracellular proprotein convertases.

DISCUSSION

Aggrecan depletion in arthritic cartilage may be considered as the principal pathological feature of osteoarthritis. In a murine model of osteoarthritis, the metalloprotease ADAMTS5 has been identified as the major proteolytic enzyme responsible for aggrecan degradation (16,17). Recent data comparing ADAMTS4 and ADAMTS5 proteolytic activities have revealed that ADAMTS5 possesses far greater activity at cleaving aggrecan than ADAMTS4 (49) supporting its central role in cartilage pathology.

Metalloproteases, like many other proteolytic enzymes, are synthesized as inactive proenzymes or zymogens. Typically, the propeptide located N-terminal to the catalytic domain must be excised from the zymogen in order for the enzyme to exhibit any proteolytic activity. We and others had previously examined the biosynthesis and activation of other ADAMTS proteases and found that their processing profiles differed. For instance, proADAMTS1 (43) and proADAMTS4 (44), are cleaved intracellularly, whereas proADAMTS9, which associates with the plasma membrane, is processed extracellularly (45). ProADAMTS7 showed a combination of both intracellular and cell-surface processing (25). ADAMTS10 is inefficiently processed by proprotein convertases, but whatever little processing occurs appears to take place at the cell surface or in ECM (26). Here, we investigated the biosynthesis of ADAMTS5 and the mechanism leading to its activation.

Examining the biosynthesis of proADAMTS5 in HEK293 cells using various approaches provided evidence that convertase cleavage occurs extracellularly but, in contrast to proADAMTS9 and proADAMTS9 Pro-Cat, proADAMTS5 does not associate with the plasma membrane. Pulse-chase experiments demonstrated that the 120-kDa proADAMTS5 precursor was processed to a shorter 85-kDa form found exclusively in the conditioned media. These experiments were supported by Western blotting using a variety of antibodies (anti-myc, anti-ADAMTS5 propeptide and anti-ADAMTS5 C-terminal), which revealed the presence of processed forms solely in the conditioned medium. Concentrating ADAMTS5 in cell lysates using immunoprecipitation and detection by immunoblotting failed to reveal any processed forms supporting the concept of extracellular cleavage of proADAMTS5. These results were also obtained in HeLa cells which endogenously express ADAMTS5.

Identification of the proteases involved in the maturation process revealed that processing of proADAMTS5 was carried out by members of the proprotein convertase family. Typically, maturation of precursor proteins, such as ADAMTS5, that follow the constitutive secretory pathway, occurs in the trans-Golgi network and is performed by furin, the prototypal proprotein convertase (28). Indeed, furin has been shown to be a *bona fide* ADAMTS convertase but other convertases also have demonstrated ADAMTS processing abilities (25,43-45). In this work, furin and PC7 were shown to process proADAMTS5 while PC6B did not. Apart from the fact that PC7 is a mammalian convertase with substrate specificities most similar to that of furin (57) it is, like furin and PC6B (58), translocated to the cell surface and relatively widely expressed. However, unlike furin, PC7 does not undergo cell surface shedding (54). It is therefore conceivable

that PC7 or other convertases recognize and cleave proADAMTS5 depending on the cell type and/or under different conditions of expression such as we have shown here in the furin-deficient CHO RPE.40 cells.

Furin cleavage of a number of metalloproteases occurs at the C-terminal end of conserved recognition sequences (R*XX*R) found between the propeptide and the catalytic domain. However, potential recognition sequences consisting of basic residues within the propeptide of ADAMTS have been cleaved by convertases. For ADAMTS5, furin and PC7 are dependent on the presence of a furin-like recognition sequence $RRRRR^{261}$ for proper cleavage. Although other potential sites (RRR^{46} and RRR^{69}) are present in the propeptide, only the site nearer the catalytic domain was cleaved. This may be explained by the fact that the RRR^{46} and RRR^{69} cleavage sites are not *bona fide* furin recognition sequences. It has been demonstrated that under certain conditions the ADAMTS4 propeptide could be cleaved at a site near the junction of the propeptide and the catalytic domain by MMP-9 (51). The 47-kDa band detected in the media of cells expressing the ADAMTS5 Pro-Cat R257-261A and ADAMTS5 Pro-Cat R257-260A mutants (Fig. 5) or in cells incubated with the convertase inhibitor dec-RVKR-cmk (Fig. 3) could be generated by such metalloproteases.

To further define the extracellular space as the proADAMTS5 activation site, we made use of two furin mutants, one (furin714) that is secreted and not retained in membranes and the other (furinR683A) which is not shed in the conditioned medium. Processing of proADAMTS5 by furin714 was observed in the medium indicating that proADAMTS5 processing can occur in the conditioned medium by soluble furin. The

unshedable furinR683A also cleaved proADAMTS5. Since no intracellular processing was observed with the furinR683A mutant even in the presence of monensin, a post-Golgi transport inhibitor (Fig. 3B) (50), this suggested that processing of proADAMTS5 occurred at the cell-surface. The ability of PC7, which does not undergo cell-surface shedding (54) to cleave proADAMTS5 would lend support to a mechanism of cell-surface proteolysis of the zymogen. Finally, although biotinylation experiments demonstrated the capacity of another ADAMTS protease (ADAMTS9) to bind the plasma membrane, no ADAMTS5 was detected here using this approach, suggesting the possibility of highly efficient processing at the cell-surface. It is noteworthy in this context, that ADAMTS9 Pro-Cat is not dependent on cell-surface furin for its cell-surface localization (45) and appears to bind through another, as yet undefined mechanism. Thus, the inability to detect proADAMTS5 at the cell-surface and its processing by an unsheddable furin, would be explained by efficient cleavage at the cell-surface without a further continuing association with the cell membrane.

Interestingly, pull-down experiments revealed that the ADAMTS5 zymogen and furin interact in the cell lysates, providing the interesting conundrum of why the zymogen is resistant to intracellular cleavage. It is possible that the processing sites within the propeptide may be conformationally inaccessible to intracellular proteolysis or masked by molecular chaperones. Once outside the cell, the zymogen may undergo a conformational change or detach from chaperones which would enable their processing. Because of the lack of detectable membrane association by ADAMTS5, the processed forms would reside in the extracellular matrix (ECM) of tissues where recognition and cleavage of ADAMTS5 substrate would take place. The presence of ADAMTS5 in the ECM of transfected COS-1

cells (14) and HTB 94 cells has been previously demonstrated (49). Our findings do not however necessarily preclude processing of the zymogen within the medium itself.

In addressing whether the zymogen or convertase-processed ADAMTS5 had proteolytic activity, we also identified a new substrate of ADAMTS5, namely versican, a large chondroitin sulphate proteoglycan of the extracellular matrix. This is the first demonstration that like ADAMTS1, ADAMTS4 and ADAMTS9, ADAMTS5 can also process versican. Versican is a key component of developing extracellular matrices, where it influences neural crest cell migration (21), as well as of the vascular wall, where it is believed to have a role in the pathology of atherosclerosis (59). In future studies it will be important to evaluate the role of ADAMTS5 in these processes.

Only the propeptide excised, processed form of ADAMTS5 was active against versican. Although removal of the propeptide is also necessary for the activities of ADAMTS1 and ADAMTS4, our model of the activation mechanism for ADAMTS5 would suggest that, unlike other "aggrecanases", the zymogen is secreted as an inactive form and is processed at the cell surface or in the extracellular matrix by a member of the convertase family. This mechanism closely resembles that recently demonstrated for ADAMTS9 (46) with the difference that, unlike proADAMTS9, there is no binding of proADAMTS5 on the cell surface and the zymogen is not active. Furthermore, the convertase-activated ADAMTS5 is also not cell-surface associated and presumably binds to and cleaves its substrates in the ECM. Moreover, since our studies were not conducted in cells endogenously expressing ADAMTS5, binding of the enzyme to the plasma membrane of other cell types such as chondrocytes is not ruled out. Nevertheless, the major conclusion of

this study, which is that proADAMTS5 is secreted unprocessed from cells, cleaved/activated by proprotein convertases and that only the processed form is able to cleave versican (and quite probably other proteoglycans) offers potential opportunities for therapeutic intervention in osteoarthritis.

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ARTICLE 3 – AVANT-PROPOS

ADAMTS7B, the full-length product of the ADAMTS7 gene, is a chondroitin sulphate-proteoglycan containing a mucin domain

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Contribution: Cet article est le deuxième d'une collaboration entre mon directeur de recherche Richard Leduc et Suneel S. Apte du Cleveland Clinic. Notre expertise pour la biosynthèse des protéines et l'activation de précurseurs par les pro-protéines convertases fut jumelée à l'expertise du laboratoire de Suneel S. Apte. J'ai planifié et réalisé les expériences ayant trait à la maturation de ADAMTS7 par les pro-protéines convertases ainsi que la caractérisation du site de clivage du prodomaine. Mes travaux ont mené à la réalisation de la figure 8A, B, C et D. Mes commentaires et suggestions ont été incorporés à l'écriture du manuscrit jusqu'à la version publiée.

Résumé de l'article 3

Nous avons caractérisé ADAMTS7B qui est l'authentique protéine complète produite du gène *ADAMTS7* de souris. La structure des domaines de ADAMTS7B est similaire à celle de ADAMTS12 qui compte huit domaines thrombospondines. Sept de ceux-ci sont organisés en deux groupes distincts séparés par un domaine mucine. ADAMTS7B est le seul protéoglycan de la famille des ADAMTS car son domaine mucine est modifié par l'attachement de glycosaminoglycans chondroitine sulfate. L'addition de glycosaminoglycans joue un rôle potentiellement important dans la localisation cellulaire et la reconnaissance de substrats de ADAMTS7B. Même si ADAMTS7B n'est pas une protéine transmembranaire, l'expression transitoire de celle-ci dans les cellules HEK 293 démontre que ADAMTS7B est retenue près de la surface cellulaire via des interactions des domaines auxiliaires et du prodomaine. Un mécanisme furine dépendant est responsable du clivage du prodomaine de ADAMTS7B. Le clivage du lien peptidique Arg220-Ser221 (séquence de la souris) se produit partiellement à la surface cellulaire. ADAMTS7B est une métalloprotéinase active de par sa capacité de cliver l'alpha2-macroglobuline. Cependant, ADAMTS7B ne clive pas les liens peptidiques spécifiques du versican et de l'aggrécan clivés par d'autres ADAMTS. ADAMTS7B ainsi que ADAMTS12, dont la structure primaire révèle aussi un domaine mucine, forment un groupe unique de la famille des ADAMTS.

ADAMTS7B, the Full-length Product of the ADAMTS7 Gene, Is a Chondroitin Sulfate Proteoglycan Containing a Mucin Domain

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ABSTRACT

We have characterized ADAMTS7B, the authentic full-length protein product of the *ADAMTS7* gene. ADAMTS7B has a domain organization similar to that of ADAMTS12, with a total of eight thrombospondin type 1 repeats in its ancillary domain. Of these, seven are arranged in two distinct clusters that are separated by a mucin domain. Unique to the ADAMTS family, ADAMTS7B is modified by attachment of the glycosaminoglycan chondroitin sulfate within the mucin domain, thus rendering it a proteoglycan. Glycosaminoglycan addition has potentially important implications for ADAMTS7B cellular localization and for substrate recognition. Although not an integral membrane protein, ADAMTS7B is retained near the cell surface of HEK293F cells via interactions involving both the ancillary domain and the prodomain. ADAMTS7B undergoes removal of the prodomain by a multistep furin-dependent mechanism. At least part of the final processing event, i.e. cleavage following Arg^{220} (mouse sequence annotation), occurs at the cell surface. ADAMTS7B is an active metalloproteinase as shown by its ability to cleave α_2-macroglobulin, but it does not cleave specific peptide bonds in versican and aggrecan attacked by ADAMTS proteases. Together with ADAMTS12, whose primary structure also predicts a mucin domain, ADAMTS7B constitutes a unique subgroup of the ADAMTS family.

INTRODUCTION

The extracellular matrix (ECM) is an information-rich assembly influencing cell proliferation, apoptosis, and cell migration. Proteases have an essential role in modulating the environmental cues that ECM provides for tissue morphogenesis, homeostasis, and disease progression. Metalloproteases, especially matrix metalloproteases, have a conspicuous role in ECM degradation as well as in proteolysis of cell-surface and soluble proteins (1, 2). Another metalloprotease family, ADAM (a disintegrin and metalloprotease domain), contains transmembrane enzymes with a major role in ectodomain shedding of cell-surface molecules, but a negligible function in ECM proteolysis (3). The active site of ADAM proteases, unlike that of the matrix metalloproteases, is of the reprolysin (snake venom zinc metalloprotease) type (3). The recent discovery of the ADAMTS (a disintegrin-like and metalloprotease domain with thrombospondin type 1 motif) family brought to light metalloproteases that contain a reprolysin-type catalytic site, but, unlike the ADAM proteases, are secreted enzymes with a prominent role in ECM proteolysis.

ADAMTS proteases have a characteristic modular structure whose hallmark is the presence of one or more thrombospondin type 1 repeats (TSRs) (4). In the short period of time since the discovery of ADAMTS1 in 1997 (4), important functions have been attributed to a number of family members, and mutations of two of these enzymes have been shown to cause human genetic disorders. Inactivating mutations of ADAMTS13 cause inherited thrombocytopenic purpura due to a failure to process von Willebrand factor (5). Mutations of ADAMTS2, a procollagen aminopropeptidase, cause skin fragility in a variety of mammals, including humans, through failure of incompletely processed collagen to form

proper fibrils (6). ADAMTS2, ADAMTS3, and ADAMTS14 form a subfamily of procollagen aminopropeptidases (7, 8). Other ADAMTS enzymes (e.g. ADAMTS1, ADAMTS4, ADAMTS5, and ADAMTS9) may mediate aggrecan degradation in arthritis and the turnover of the related proteoglycans versican and brevican in blood vessels and the nervous system, respectively (9–14). *Adamts1* null mice have abnormal adipogenesis, defective angiogenesis in the adrenal gland, and altered ureteric ECM turnover (15). ADAMTS1 inhibits angiogenesis by its ability to sequester the vascular endothelial growth factor (16). A mutation in *Adamts20* gave rise to the mouse mutant Belted, which has defects in skin pigmentation owing to presumed effects on melanoblast migration (17). Although progress in this field has been rapid, the properties and functions of the majority of the 19 ADAMTS proteases remain unknown, and we refer to these as orphan enzymes (i.e. without a cognate substrate).

ADAMTS7 was originally identified as an enzyme containing two TSRs and having a similar domain structure to ADAMTS5, ADAMTS6, and ADAMTS8 (18). In this work, we describe a longer form of ADAMTS7 (designated ADAMTS7B) that we believe is the authentic full-length version of this enzyme and that suggests that our previous characterization of the C terminus of ADAMTS7 may have been incomplete. The primary structure of *ADAMTS7B* presented here places this enzyme in a distinct phylogenetic clade with *ADAMTS12* (19). In addition, we have undertaken a detailed characterization of ADAMTS7B with emphasis on its unusual post-translational modification. ADAMTS7B is shown to contain a mucin domain within which is attached one or more chondroitin sulfate chains. This suggests that ADAMTS7B may exist in some cells and tissues as a proteoglycan, with potentially important functional consequences. In addition, we have

characterized the maturation of the ADAMTS7B zymogen, demonstrated that the zymogen and mature enzyme may be cell-associated through different mechanisms, and shown that the mature enzyme and possibly the zymogen are active proteases.

EXPERIMENTAL PROCEDURES

Materials—Reagents and chemicals were purchased from Sigma unless otherwise stated.

cDNA Cloning of Mouse ADAMTS7B—A partial cDNA encoding the 290 C-terminal amino acids of ADAMTS7B along with ~200 bp of 3'-untranslated sequence was isolated from an embryonic day 17 mouse cDNA library (Clontech). This fragment was used as a starting point to clone cDNAs encoding the entire 1641-amino acid open reading frame (ORF) using the original library plus 5'-rapid amplification of cDNA ends of RNA from 13- and 17-day-old mouse embryos and Sol8 mouse myotubes. All sequences were verified by isolation of independent duplicate clones. To confirm the continuity of the clones and to generate an expression plasmid, primers from the 5'- and 3'-ends were used to amplify the entire ORF using cDNA obtained from C2C12 mouse myotubes.

Northern Blot Analysis—Northern blots containing poly(A)$^+$ RNA (1 μg/lane) from mouse embryos and adult human tissues (BD Biosciences) were hybridized to a [α-^{32}P]dCTP-labeled *Adamts7b* probe, followed by autoradiographic exposure for 7 days.

Human and Mouse ADAMTS7 Expression Plasmids—For PCR amplification of full-length mouse ADAMTS7B, we used the above mouse plasmid as template and oligonucleotide 5'-**gaattc**caccacattggtgtgcacc-3' (with the EcoRI site underlined) as the forward primer and oligonucleotide 5'-**gcggccgc**ccgtctggctaccccgctga-3' (with the NotI site underlined) as the reverse primer. The fidelity of PCR amplification was confirmed by nucleotide sequencing. For expression of mouse ADAMTS7$_{\text{PRO-CAT-Myc/His}}$ (encoding the signal peptide, prodomain,

and catalytic domain, constituting residues 1–444 in mouse ADAMTS7B), PCR amplification was performed utilizing the same forward primer and the reverse primer 5'-**gtcgac**ttgagtgcaatgacatccttgg-3' (with the SalI site underlined). A critical P1 Arg residue within each of the two major furin cleavage sites in $ADAMTS7_{PRO\text{-}CAT\text{-}Myc/His}$ was substituted with Ala (Arg^{60} →Ala and Arg^{220} →Ala) by site-directed mutagenesis (QuikChange site-directed mutagenesis kit, Stratagene). These cDNAs were all cloned into pcDNA3.1(+)-Myc/HisB for expression in-frame with tandem Myc and His_6 tags. The corresponding human catalytic domain construct (encoding residues 1–459) was made by excision from an IMAGE clone (expressed sequence tag N48032) using EcoRI and HindIII and cloning into the matching sites of pcDNA3.1(+)-Myc/HisB. cDNA encoding the mucin domain (mouse Pro^{980}–Arg^{1366}, named $ADAMTS7B_{FLAG\text{-}MUC}$) was amplified using mouse ADAMTS7B cDNA as a template with primers 5'-a**aagctt**cccatgtacatagtggaca-3' (with the HindIII site underlined) and 5'-a**ggatcc**tcacctggcaggcagtggat-3' (with the BamHI site underlined) and cloned into pCMV9–3XFLAG for expression with a preprotrypsin leader sequence and three tandem N-terminal FLAG tags.

Transfection and Selection of Stable Cell lines—DNA for transfection was prepared using the Wizard™ purification system (Promega Corp.). 80% confluent HEK293F cells (Invitrogen) in 6-well plates were transfected with 100 ng of ADAMTS7B, $ADAMTS7B_{FLAG\text{-}MUC}$, or human or mouse $ADAMTS7_{PRO\text{-}CAT\text{-}Myc/His}$ plasmid using FuGENE 6 (Roche Diagnostics) following the manufacturer's instructions. After 24 h, the medium was changed and supplemented with 1 mg/ml active G418 (Mediatech, Herndon, VA). Discrete clones were isolated using cloning discs (PGC Scientifics, Frederick, MD) and expanded in 24-well plates. Western blotting with anti-c-Myc monoclonal antibody

9E10 or anti-FLAG monoclonal antibody M2 was used to determine the level of protein expression in the media of these clones as appropriate for the plasmid used. The highest expressing clones were expanded to purify human or mouse ADAMTS7$_{PRO\text{-}CAT\text{-}Myc/His}$, full-length mouse ADAMTS7B protein, or mouse ADAMTS7B$_{FLAG\text{-}MUC}$.

Protein Purification and N-terminal Sequence Analysis—HEK293F cells expressing human or mouse ADAMTS7$_{PRO\text{-}CAT\text{-}Myc/His}$ were cultured in three-tier flasks (Nunc, Rochester, NY) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. When cultures were 80% confluent, their medium was replaced with serum-free medium (293 SFM medium, Invitrogen) with subsequent culture at 37 °C in the presence of 8% CO_2 for 5 days. The medium was collected, centrifuged to remove cellular debris, and supplemented with NaCl to a final concentration of 0.5 M. ProBond resin (Invitrogen) was prepared by washing with 1 bed volume of binding buffer (0.5 M NaCl and 20 mM sodium phosphate, pH 7.8). The medium and resin were mixed overnight at 4 °C (100 μl of resin/100 ml of medium). The resin was pelleted by centrifugation at 1500 rpm and then washed five times with 10 volumes of binding buffer. Bound protein was eluted by sequential washes with binding buffer containing 50, 100, 150, 200, and 250 mM imidazole. The washes and eluted protein fractions were assayed for the presence and purity of protein by Coomassie Blue staining. Gels were electroblotted onto polyvinylidene difluoride membrane (Millipore Corp.) for Western blotting. The recombinant protein was detected using anti-c-Myc epitope monoclonal antibody 9E10 or anti-pentahistidine antibody (QIAGEN Inc., Valencia, CA) in conjunction with horseradish peroxidase-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Antibody binding was detected by enhanced chemiluminescence (Amersham Biosciences).

Purification and analysis of full-length mouse ADAMTS7B were essentially similar, except that the cell layer was first washed with 0.5 M NaCl to release the protein from the cell surface; this was pooled with the medium from these cultures and purified and analyzed as described above. Purification of full-length ADAMTS7B was challenging and provided extremely small amounts of substantially purified enzyme such that quantitation could not be done. Since most of the studies with this enzyme were qualitative in nature for determination of its intrinsic characteristics, most assays used sufficient enzyme to permit visualization on Western blots. ADAMTS7B$_{FLAG\text{-}MUC}$ was isolated from the conditioned medium of stably transfected cells by affinity purification with anti-FLAG antibody M2-Sepharose beads, following which the beads were extracted in Laemmli sample buffer prior to electrophoresis.

For N-terminal sequencing of human and mouse ADAMTS7$_{PRO\text{-}CAT\text{-}Myc/His}$, purified protein was electroblotted onto polyvinylidene difluoride membrane and lightly stained with Simply Blue Safe Stain (Invitrogen). The 50- and 29-kDa major bands were excised for amino acid sequencing by Edman degradation on an Applied Biosystems Procise 492 sequencer in the Molecular Biotechnology Core Facility of the Lerner Research Institute. In all the subsequent studies, we used full-length mouse ADAMTS7B or mouse ADAMTS7$_{PRO\text{-}CAT\text{-}Myc/His}$ as indicated. No further studies with human ADAMTS7$_{PRO\text{-}CAT\text{-}Myc/His}$ beyond the N-terminal sequencing described above were conducted.

Analysis of N- and O-Glycosylation of ADAMTS7B—For enzymatic removal of *N*-linked carbohydrate with peptide N-glycosidase F (PNGase F), HEK293 cell lysate expressing ADAMTS7B or purified ADAMTS7$_{PRO\text{-}CAT\text{-}Myc/His}$ was denatured in 0.5% SDS and 0.1 M

β-mercaptoethanol at 100 °C for 10 min and cooled to room temperature, and Nonidet P-40 was added to a concentration of 1% (v/v) to neutralize the PNGase F inhibitory effects of SDS. The reduced samples were incubated with 100 units of PNGase F (New England Biolabs Inc., Beverly, MA) in 50 mM sodium phosphate, pH 7.5, for 2 h at 37 °C. SDS-PAGE and Western blotting (with antibody 9E10) were used to compare in-gel migration with an untreated sample.

For ligand blot analysis, ADAMTS7B$_{FLAG\text{-}MUC}$ was electrophoresed on denaturing 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride. Horseradish peroxidase-conjugated peanut agglutinin (from *Arachis hypogaea*) was incubated with the membrane, followed by washes with Tris-buffered saline containing 0.1% Tween 20 and detection by ECL (20, 21). Enzymatic removal of O-linked carbohydrate was done using a mixture of 10 milliunits of sialidase and 0.5 milliunits of O-glycosidase (Roche Diagnostics) in 50 mM sodium phosphate, pH 7.5, and 0.5% Triton X-100, followed by immunoblotting with anti-FLAG antibody M2. Undigested ADAMTS7B$_{FLAG\text{-}MUC}$ was used as a control.

Glycosaminoglycan Analysis—ADAMTS7B and ADAMTS7B$_{FLAG\text{-}MUC}$ were treated with 0.5 units of protease-free chondroitinase ABC (Seikagaku America, East Falmouth, MA) in 100 mM Tris and 50 mM sodium acetate, pH 6.5, for 2 h at 37 °C. HEK293F, COS-1, and CHO-K1 cell lysates obtained from transiently transfected cells expressing ADAMTS7B were treated with 2.0 units of chondroitinase ABC, 5 units each of keratanase I and II, or 2.5 units each of heparinase I and III in serum-free Dulbecco's modified Eagle's medium for

4 h at 37 °C. The proteins from the digests were precipitated with acetone and electrophoresed on reducing 7% SDS-polyacrylamide gel. ADAMTS7B was detected by Western blotting using monoclonal antibodies 9E10 (for ADAMTS7B) and M2 (for $ADAMTS7B_{FLAG\text{-}MUC}$). Samples untreated with the respective enzymes were used as negative controls. The blots were subsequently stripped by treatment with 62.5 mM Tris-HCl, pH 6.8, 2% SDS, and 100 mM 2-mercaptoethanol at 55 °C for 30 min. The membrane was reprobed using mouse anti-chondroitin 6-sulfate monoclonal antibody 2035 (Chemicon International, Inc., Temecula, CA), which specifically recognizes stubs with an unsaturated glucuronic acid terminus derived from chondroitin 6-sulfate in chondroitin sulfate proteoglycans following chondroitinase digestion. These stubs are not present in the native undigested chondroitin sulfate proteoglycans. To further verify the attachment of glycosaminoglycan (GAG) to the ADAMTS7B core protein, we transfected the ADAMTS7B expression plasmid into CHO-K1 cells with a null mutation in β-D-xylosyltransferase (CHO-K1 pgsA-745; American Type Culture Collection, CRL-2242) (22) and analyzed the extracts of these cells by Western blotting using monoclonal antibody 9E10. Transfected parental CHO-K1 cells (CCL-61) were analyzed in parallel as a control. The conditioned medium from HEK293F clones expressing full-length ADAMTS7B was batch-incubated overnight with heparin-Sepharose (Bio-Rad) at 4 °C with end-over-end rotation. The resin was washed five times with phosphate-buffered saline (PBS) and eluted with increasing concentrations of NaCl (0.2–0.5 M). Uneluted protein was removed by boiling the beads in Laemmli sample buffer. Unbound protein was precipitated with cold acetone. Aliquots of the medium before binding, unbound proteins, and proteins bound to the beads were analyzed by Western blotting with antibody 9E10.

Biosynthesis of ADAMTS7B—QBI 293A cells (Quantum Biotechnologies, Montreal, Canada) were maintained, transiently transfected with mouse $ADAMTS7_{PRO\text{-}CAT\text{-}Myc/His}$ using FuGENE 6, and metabolically labeled with a [^{35}S]methionine/cysteine mixture (Expre^{35}S^{35}S, PerkinElmer Life Sciences) essentially as described previously (13). A 15-min label (pulse) was followed by incubation in complete nonradioactive medium (chase) for various times. Immunoprecipitation with anti-pentahistidine monoclonal antibody was done as described previously (13). For inhibition of furin, decanoyl-RVKR chloromethyl ketone, a lipid-permeable inhibitor (furin-1 inhibitor; Calbiochem) (23), was added to HEK293F cells stably expressing $ADAMTS7_{PRO\text{-}CAT\text{-}Myc/His}$ at concentrations ranging from 1 to 100 μM for 24 h, and the culture medium was analyzed by Western blotting using monoclonal antibody 9E10.

Cotransfection of ADAMTS7B and Proprotein Convertases—CHO RPE.40 cells lacking furin were maintained as described previously (24). They were transfected with mouse $ADAMTS7_{PRO\text{-}CAT\text{-}Myc/His}$ alone (as a control) or in combination with plasmids encoding the proprotein convertases furin, PACE4, PC6B, and PC7. QBI 293A cells were transiently transfected with $ADAMTS7_{PRO\text{-}CAT\text{-}Myc/His}$, also as a control. Cells were metabolically labeled for 3 h, and immunoprecipitation and fluorography of the cell lysate and medium were performed as described (13).

Cell-surface Biotinylation—Stably transfected HEK293F cells expressing $ADAMTS7_{PRO\text{-}CAT\text{-}Myc/His}$ were scraped from plates; washed six times with cold PBS, pH 8.0; and diluted to 2.5×10^7 cells/ml. As a control to eliminate all cell-surface proteins, an equal number of cells were treated with trypsin/EDTA for 15 min and washed once with medium containing

serum to inactivate trypsin and then six times with cold PBS. 1 ml of each cell suspension was mixed with 0.5 mg of sulfosuccinimidyl 6-(biotinamido)hexanoate (Pierce Biotechnology, Inc.) and incubated for 30 min at room temperature. The cells were pelleted and washed once with 50 mM Tris-Cl, pH 8.0, and three times with PBS, pH 8.0. The cell pellet was lysed in radioimmune precipitation assay buffer (1x PBS, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, and 0.1% (w/v) SDS) at 4 °C for 30 min and centrifuged. The soluble portion of the lysate was transferred to a fresh tube and incubated overnight with 50 μl of streptavidin-agarose at 4 °C with rotation. The streptavidin-agarose was pelleted by centrifugation at 3000 rpm in a microcentrifuge and washed three times in radioimmune precipitation assay buffer and PBS. The supernatant was discarded, and the streptavidin-agarose was resuspended in 50 μl of Laemmli buffer and boiled for 5 min. The agarose was pelleted, and the supernatant was loaded onto a 10% SDS-polyacrylamide gel for Western blotting with anti-c-Myc antibody 9E10.

Pulse-Chase Analysis of Cell-surface Biotinylated Proteins— HEK293F monolayers expressing mouse ADAMTS7$_{\text{PRO-CAT-Myc/His}}$ were biotinylated as described above for cell suspensions. The monolayers were washed three times with Tris-buffered saline to inactivate the biotinylation reagent. The monolayers were returned to 10 ml of 293 SFM medium and further cultured for 3 h. 1 ml of medium was taken for analysis at sequential time points, and biotinylated protein was purified from the medium using streptavidin-agarose, followed by Western blotting as described above. Cell lysates were taken at fixed time points to investigate the fate of surface-biotinylated proteins by Western blotting as described above.

α_2-Macroglobulin, Versican, and Aggrecan Processing Assays—To determine whether purified ADAMTS7$_{PRO\text{-}CAT\text{-}Myc/His}$ and ADAMTS7B were catalytically active, 5 μl of purified full-length ADAMTS7B or ADAMTS7$_{PRO\text{-}CAT\text{-}Myc/His}$ was incubated with 5 μg of α_2-macroglobulin (Calbiochem) in 50 mM Tris-Cl, pH 7.8, 100 mM NaCl, and 5 mM $CaCl_2$ for 2 h at 37 °C. Laemmli sample buffer was added, and the sample was electrophoresed without heating on 10% SDS-polyacrylamide gel, followed by Western blotting with antibody 9E10 to visualize ADAMTS7B. In similar digests, Coomassie Blue staining was used after reducing SDS-PAGE to visualize α_2-macroglobulin. Processing of specific ADAMTS-susceptible peptide bonds in versican and aggrecan was analyzed using transfected HEK293F cells essentially as described previously (13) as well as in solution using the same volume of enzyme used in α_2-macroglobulin digests. Briefly, ADAMTS7B-transfected and ADAMTS4-transfected (as a positive control) cells were incubated in suspension culture with versican and aggrecan. Subsequently, aggrecan and versican were precipitated from the medium, deglycosylated, and analyzed by Western blotting using anti-neoepitope antibodies specific for the cleaved Glu^{1771}–Ala^{1772} and Glu^{441}–Ala^{442} peptide bonds of aggrecan and versican, respectively (13). Cell lysates were analyzed by Western blotting with antibodies 9E10 and M2 to determine the expression levels of ADAMTS7B and ADAMTS4, respectively.

RESULTS

Primary Structure of ADAMTS7B—Shortly after the acceptance of our original publication on ADAMTS7 (18), a human cDNA (GenBank™/EBI accession number AL110226, submitted August 13, 1999) that overlapped with our DNA sequence (accession number AF140675) at its C terminus was deposited by the German Cancer Research Center. Assembly of AF140675 and AL110226 resulted in a transcript that is 5338 bp in length and contains a polyadenylation signal (AUAAA) near the 3'-end. The mouse ADAMTS7B ORF was determined independently by cDNA cloning from a mouse muscle cDNA library. The human and mouse ADAMTS7B proteins are 1686 and 1641 amino acids long, respectively, with predicted masses of 181 and 178 kDa, respectively (excluding the signal peptide). The difference in length arises primarily within the prodomain and mucin domain. Fig. 1 demonstrates the conserved domain organization and noteworthy structural features of human and mouse ADAMTS7B. The previously described ADAMTS7 protein (now designated ADAMTS7A) terminates in TSR2 (Fig. 1). In ADAMTS7B, TSR2–8 are arranged in two arrays, with TSR4 and TSR5 separated by a polypeptide (420 amino acids long in human and 392 amino acids long in mouse) with sequence attributes typical of mucins, i.e. enrichment in Ser, Thr, and Pro residues, predicting heavy O-glycosylation. We refer to this as the mucin domain.

Human and mouse ADAMTS7B have high amino acid sequence homology (67% identity and 74% similarity, including conserved substitutions) and several potentially significant conserved sequence motifs (Fig. 2). Three putative proprotein convertase (*e.g.*

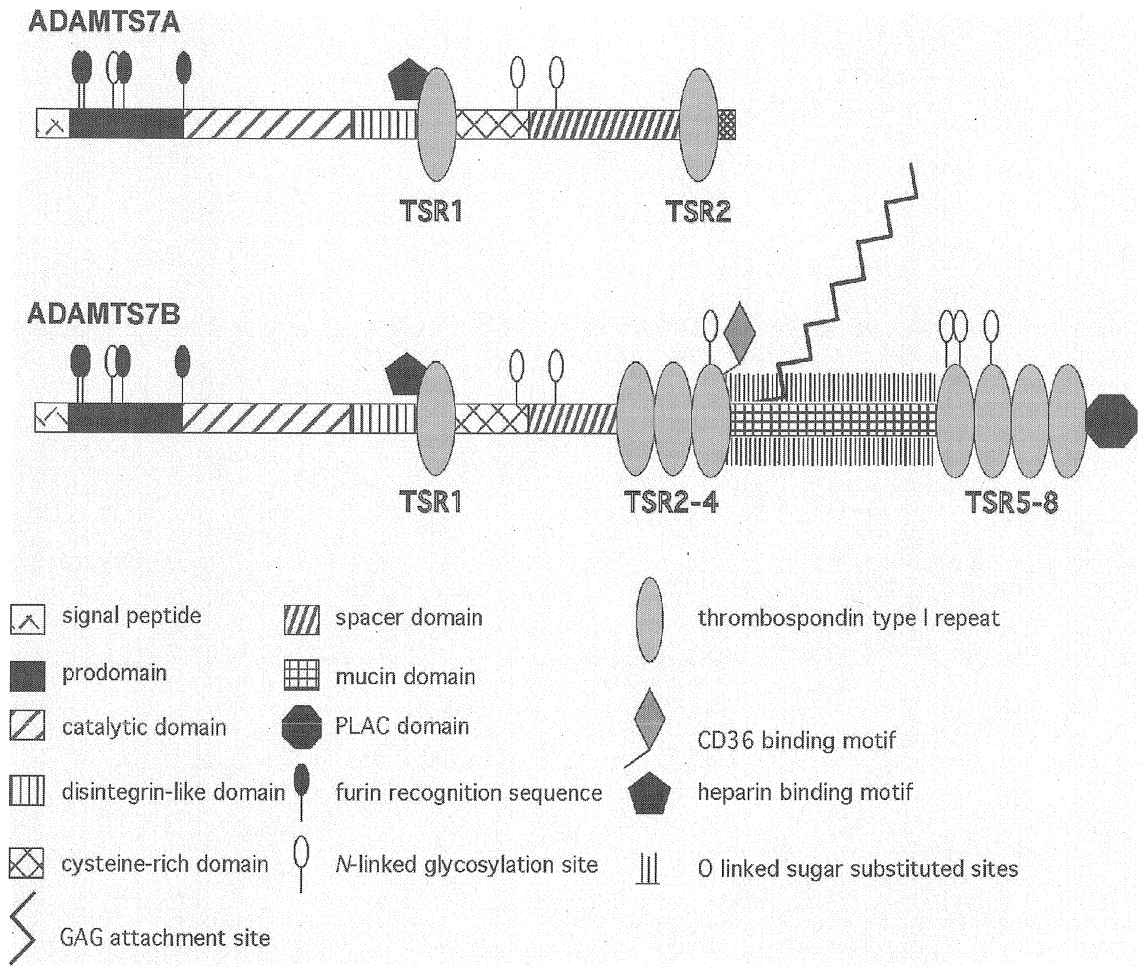
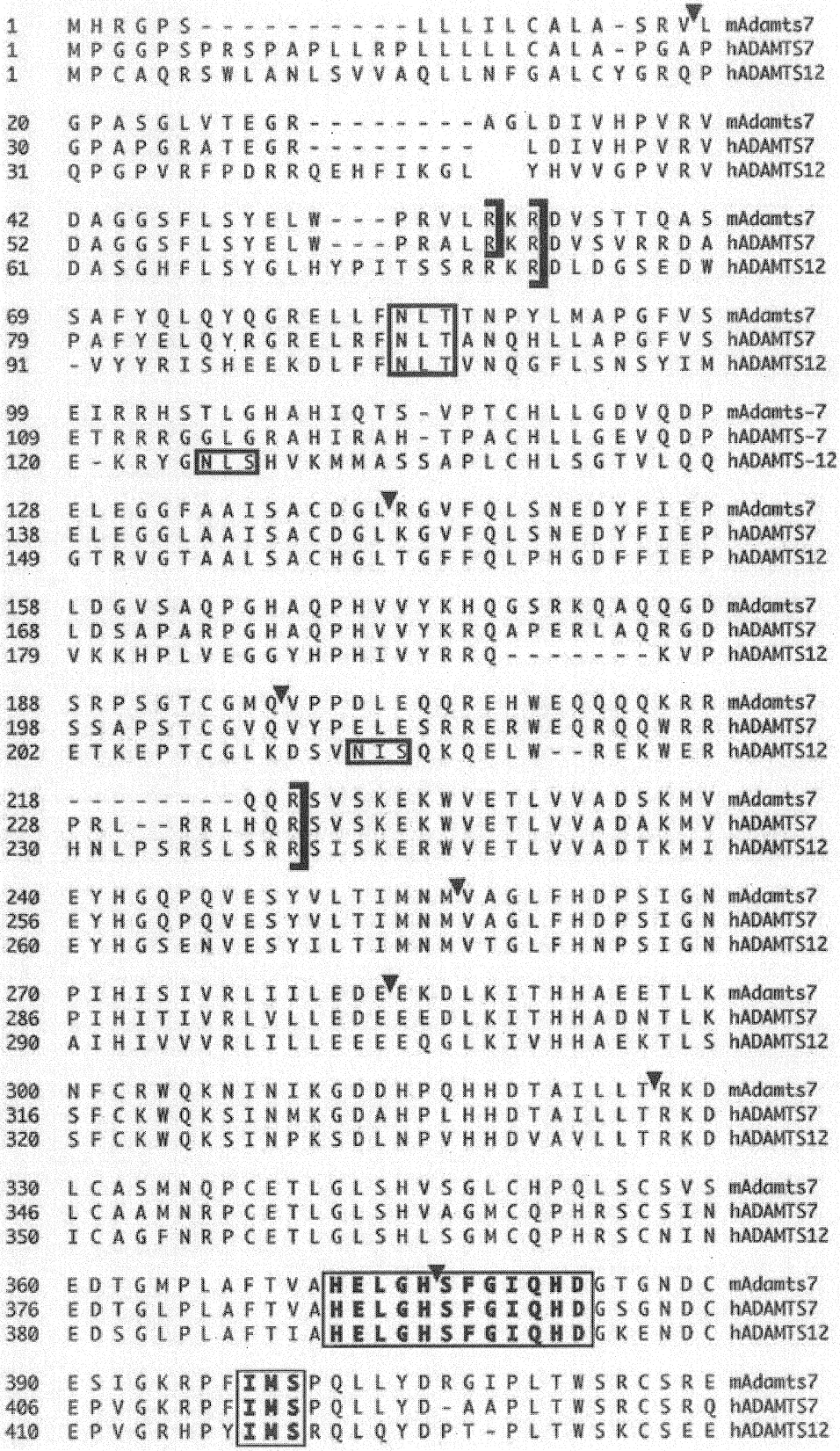


Figure 1. Domain organization of human ADAMTS7A and ADAMTS7B. The precise locations of sequence motifs are indicated in Fig. 2.

furin) recognition sites (RVLR^{58}, RVLRKR^{60}, and RQQR^{220}, mouse sequence annotation) in human and mouse ADAMTS7B are at relatively conserved locations within the prodomain, implying similar activation sites and mechanisms. However, human ADAMTS7B has additional furin sites not present in mouse ADAMTS7B: RGRELR^{92}, RRER^{218}, RRPR^{229}, and RLRR^{232}. The zinc-binding active-site sequence is identical in both species (Fig. 2). TSR1 contains tandem Trp-Ser-Xaa-Trp motifs that have been implicated in heparin and sulfatide binding of thrombospondin (25). A Cys-Ser-Val-Thr-Cys-Gly motif that is suggested to mediate thrombospondin 1 binding to the CD36 receptor (26) is present at an identical location within TSR4 of human and mouse ADAMTS7B. Following prodomain processing at the most C-terminal furin-processing site, mature human and mouse ADAMTS7B are predicted to be 157.9 and 156.4 kDa, respectively. These calculations assume no further post-translational modification. However, both the human and mouse sequences predict considerable post-translational modification, including conserved potential GAG attachment sites (^{989}SGSG in mouse and ^{1006}SGSG in human) (27) within the mucin domain (Fig. 2). Gly-Ser dipeptides (Fig. 2) are also present within the mucin domain, some in appropriate sequence context for GAG attachment (27), but they are not at identical locations in mouse and human ADAMTS7B. Of 10 N-linked glycosylation sites in human ADAMTS7B and nine in mouse ADAMTS7B, nine are at identical locations. Like many ADAMTS proteases (e.g. ADAMTS10, ADAMTS12, and ADAMTS16–19), ADAMTS7B has a C-terminal PLAC (protease and lacunin) domain containing six cysteines in the expected arrangement (Fig. 2) (28).

ADAMTS7B and ADAMTS12 Are Highly Homologous and Constitute a Distinct ADAMTS Phylogenetic Clade—ADAMTS7B has a domain organization identical to that of human

Figure 2. Alignment of the primary amino acid sequences of mouse and human ADAMTS7B with human ADAMTS12 (GenBank™/EBI accession numbers AY551090, NM014272, and NM030955, respectively). Exon junctions are indicated by inverted arrowheads. The zinc-binding active site and "Met-turn" (IMS) within the catalytic domain are indicated in boldface and are boxed. All TSRs are underlined and numbered sequentially. Potential proprotein convertase-processing sites conserved in human (h) and mouse (m) ADAMTS7B are indicated by the thick bracket. N-Linked glycosylation sites (Asn-Xaa-(Ser/Thr), where Xaa is any amino acid except Pro) are boxed. The disintegrin-like domain is highlighted in a gray box with black lettering and extends to TSR1. The cysteine-rich domain is highlighted in a gray box with white lettering. The spacer domain is highlighted in a dashed box and extends to TSR2. The PLAC domain is shown in a rounded box, with each of the cysteine residues highlighted. Potential CD36-binding motifs (CSVTCG) are highlighted in blacked circles. The mucin domain lies between TSR4 and TSR5. Potential GAG attachment motifs in the mucin domain are shown by white letters in black boxes.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1 MPCAQRSWLANLSVVAQLLNFGALCYGRQP hADAMTS12
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52 DAGGSFLSYELW---PRALRKRDVSVRRDA hADAMTS7
61 DASGHFLSYGLHYPITSSRRKRDLDGSEDW hADAMTS12
69 SAFYQLQYQGRELLFNLTTNPYLMAPGFVS mAdamts7
79 PAFYELQYRGRELRFNLTANQHLLAPGFVS hADAMTS7
91 -VYYRISHEEKDLFFNLTVNQGFLSNSYIM hADAMTS12
99 EIRRHSTLGHAHIQTS-VPTCHLLGDVQDP mAdamts-7
109 ETRRRGGLGRAHIRAH-TPACHLLGEVQDP hADAMTS-7
120 E-KRYGNLSHVKMMASSAPLCHLSGTVLQQ hADAMTS-12
128 ELEGGFAAISACDGLRGVFQLSNEDYFIEP mAdamts7
138 ELEGGLAAISACDGLKGVFQLSNEDYFIEP hADAMTS7
149 GTRVGTAALSACHGLTGFFQLPHGDFFIEP hADAMTS12
158 LDGVSAQPGHAQPHVVYKHQGSRKQAQQGD mAdamts7
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228 PRL--RRLHQRSVSKEKWVETLVVADAKMV hADAMTS7
230 HNLPSRSLSRRSISKERWVETLVVADTKMI hADAMTS12
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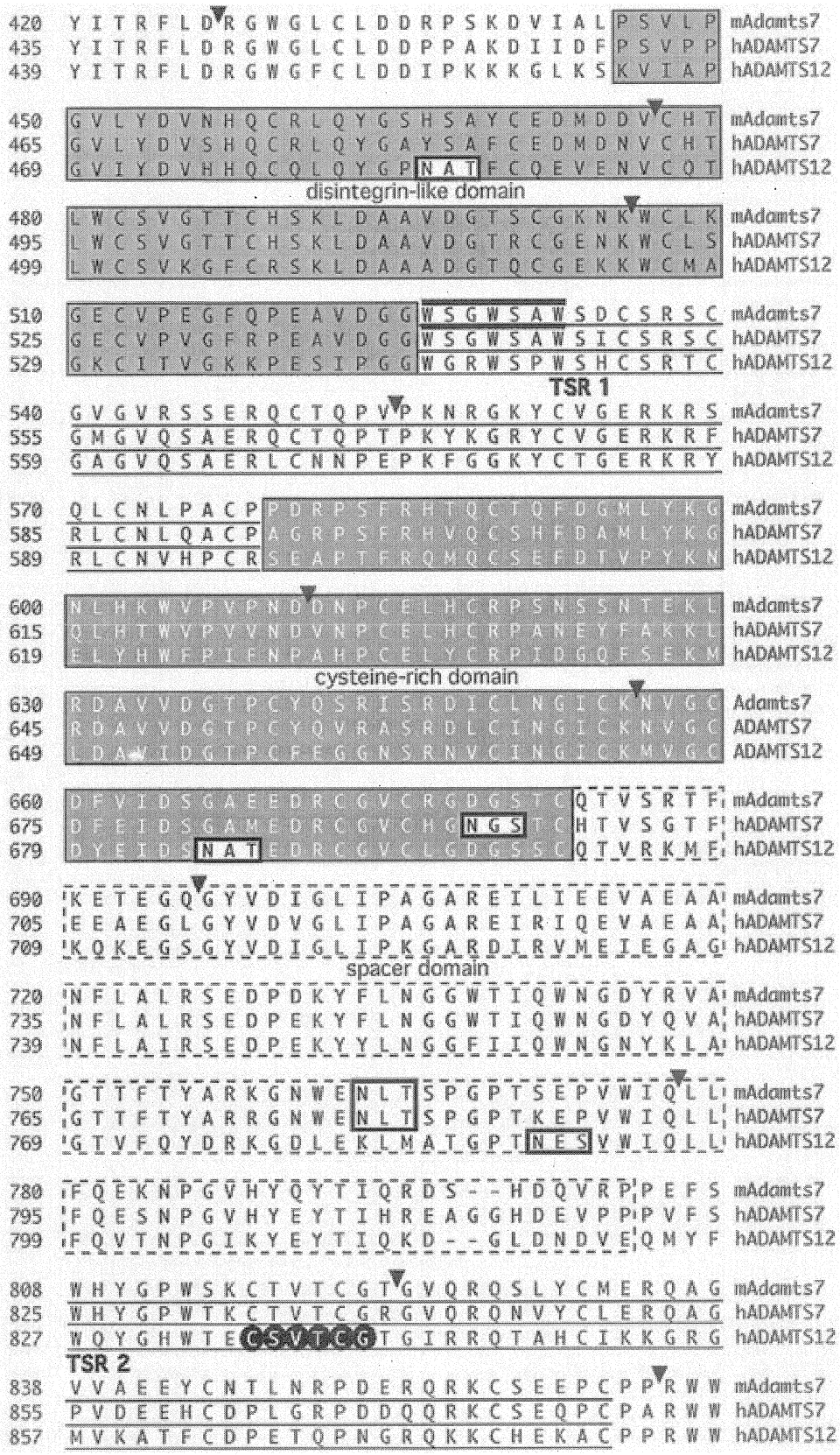
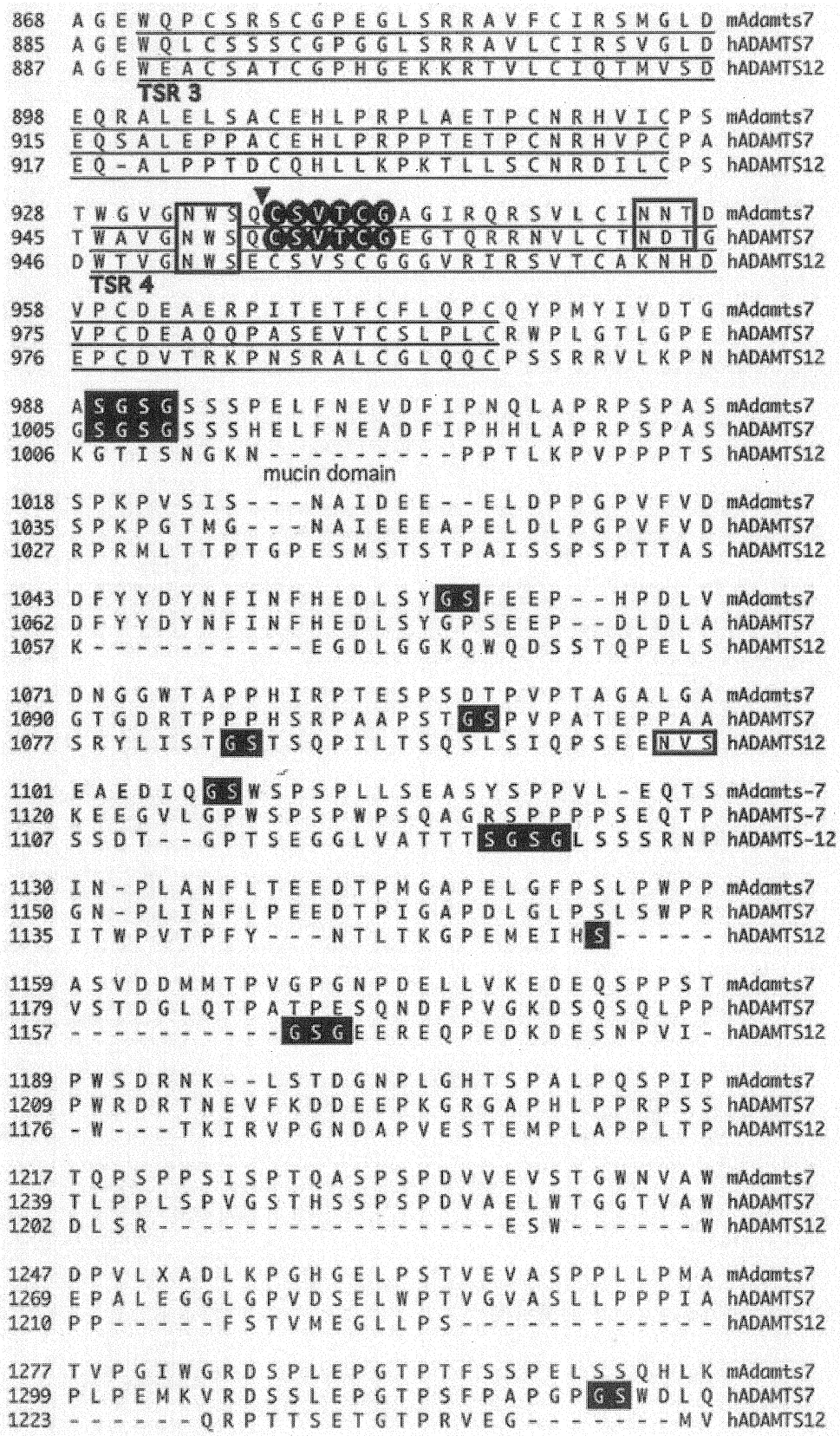
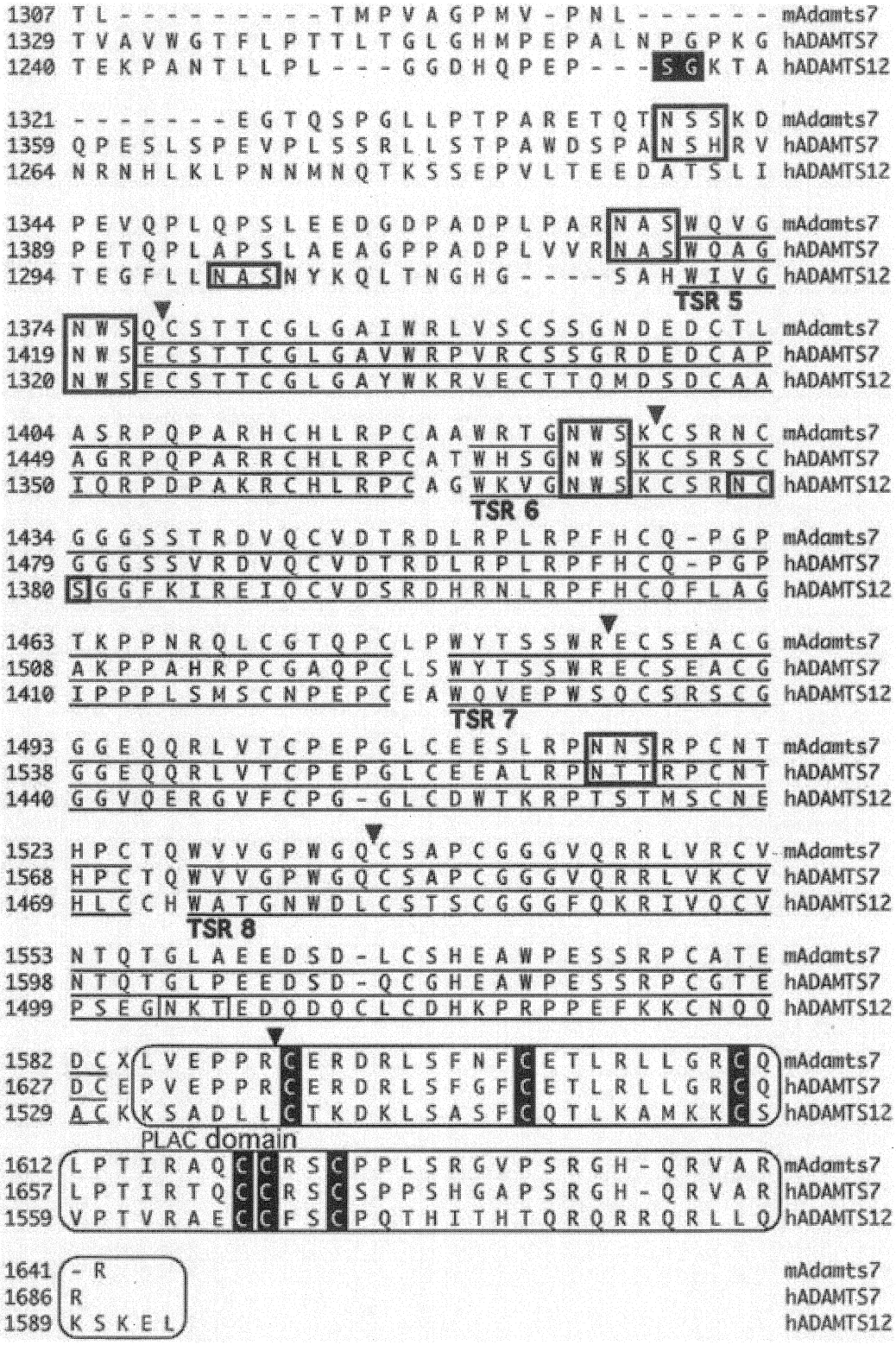

disintegrin-like domain
TSR 1
cysteine-rich domain
spacer domain
TSR 2


TSR 3
TSR 4
mucin domain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1321 -------EGTQSPGLLPTPARETQTNSSKD mAdamts7
1359 QPESLSPEVPLSSRLLSTPAWDSPANSHRV hADAMTS7
1264 NRNHLKLPNNMNQTKSSEPVLTEEDATSLI hADAMTS12
1344 PEVQPLQPSLEEDGDPADPLPARNASWQVG mAdamts7
1389 PETQPLAPSLAEAGPPADPLVVRNASWQAG hADAMTS7
1294 TEGFLLNASNYKQLTNGHG----SAHWIVG hADAMTS12
TSR 5
1374 NWSQCSTTCGLGAIWRLVSCSSGNDEDCTL mAdamts7
1419 NWSECSTTCGLGAVWRPVRCSSGRDEDCAP hADAMTS7
1320 NWSECSTTCGLGAYWKRVECTTQMDSDCAA hADAMTS12
1404 ASRPQPARHCHLRPCAAWRTGNWSKCSRNC mAdamts7
1449 AGRPQPARRCHLRPCATWHSGNWSKCSRSC hADAMTS7
1350 IQRPDPAKRCHLRPCAGWKVGNWSKCSRNC hADAMTS12
TSR 6
1434 GGGSSTRDVQCVDTRDLRPLRPFHCQ-PGP mAdamts7
1479 GGGSSVRDVQCVDTRDLRPLRPFHCQ-PGP hADAMTS7
1380 SGGFKIREIQCVDSRDHRNLRPFHCQFLAG hADAMTS12
1463 TKPPNRQLCGTQPCLPWYTSSWRECSEACG mAdamts7
1508 AKPPAHRPCGAQPCLSWYTSSWRECSEACG hADAMTS7
1410 IPPPLSMSCNPEPCEAWQVEPWSQCSRSCG hADAMTS12
TSR 7
1493 GGEQQRLVTCPEPGLCEESLRPNNSRPCNT mAdamts7
1538 GGEQQRLVTCPEPGLCEEALRPNTTRPCNT hADAMTS7
1440 GGVQERGVFCPG-GLCDWTKRPTSTMSCNE hADAMTS12
1523 HPCTQWVVGPWGQCSAPCGGGVQRRLVRCV- mAdamts7
1568 HPCTQWVVGPWGQCSAPCGGGVQRRLVKCV hADAMTS7
1469 HLCCHWATGNWDLCSTSCGGGFQKRIVQCV hADAMTS12
TSR 8
1553 NTQTGLAEEDSD-LCSHEAWPESSRPCATE mAdamts7
1598 NTQTGLPEEDSD-QCGHEAWPESSRPCGTE hADAMTS7
1499 PSEGNKTEDQDQCLCDHKPRPPEFKKCNQQ hADAMTS12
1582 DCXLVEPPRCERDRLSFNFCETLRLLGRCQ mAdamts7
1627 DCEPVEPPRCERDRLSFGFCETLRLLGRCQ hADAMTS7
1529 ACKKSADLLCTKDKLSASFCQTLKAMKKCS hADAMTS12
PLAC domain
1612 LPTIRAQCCRSCPPLSRGVPSRGH-QRVAR mAdamts7
1657 LPTIRTQCCRSCSPPSHGAPSRGH-QRVAR hADAMTS7
1559 VPTVRAECCFSCPQTHITHTQRQRRQRLLQ hADAMTS12
1641 -R mAdamts7
1686 R hADAMTS7
1589 KSKEL hADAMTS12

ADAMTS12 and has an amino acid sequence identity of 42.6% to it. Two proprotein convertase-processing sites ($RRKR^{82}$ and $RSLSRR^{240}$), the zinc-binding active-site sequence, a putative heparin/sulfatide-binding motif in TSR1, and five N-linked glycosylation sites are conserved in these two proteases (Fig. 2). ADAMTS12 has a putative CD36-binding motif, but it is located in TSR2 instead of TSR4 (ADAMTS7B) (Fig. 2). ADAMTS12 has an additional putative proprotein convertase-processing site not found in ADAMTS7 ($RFPDRR^{41}$).

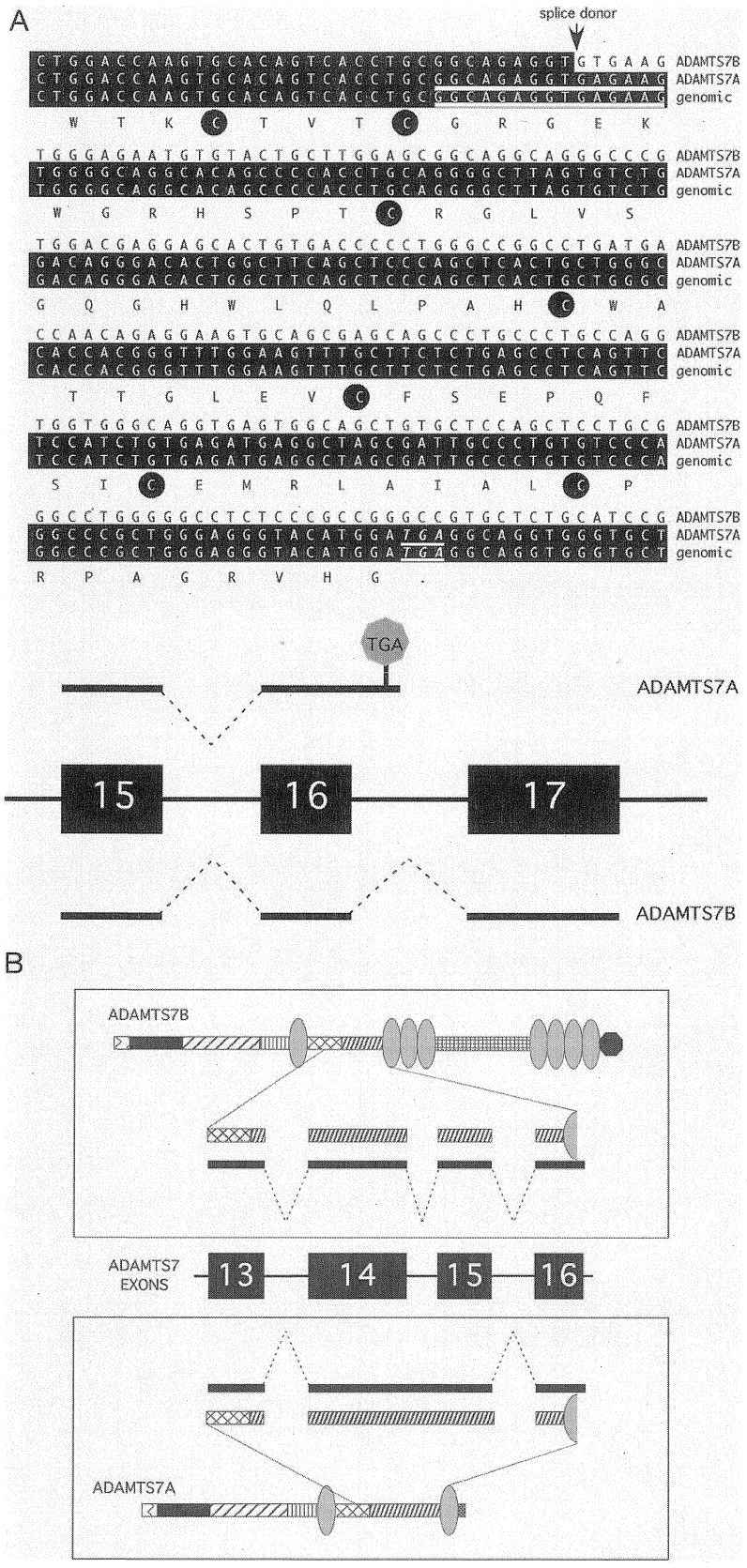
Like ADAMTS7B, the primary structure of ADAMTS12 predicts a mucin domain separating TSR4 from TSR5, although this was not noted in a previous report (19). According to our analysis, the ADAMTS12 mucin domain is considerably shorter (320 amino acids to 420 amino acids in human ADAMTS7B), accounting for much of the length discrepancy between ADAMTS7B and ADAMTS12. The mucin domains in ADAMTS7B and ADAMTS12 are rich in serines (46 residues each) and threonines (25 and 29 residues, respectively). ADAMTS7B has a higher percentage of prolines (18% in human and 17% in mouse) than ADAMTS12 (12%). Analysis of the ADAMTS7B and ADAMTS12 mucin domains at the NetO-Glyc 2.0 Server (www.cbs.dtu.dk/services/NetOGlyc/) (29) predicts that 32 and 40 Ser + Thr residues in the respective domains are likely to be O-glycosylated. The mucin domain in each enzyme contains a high number of acidic residues (12% and 11% Asp + Glu residues in human ADAMTS7B and ADAMTS12, respectively) and has a net negative charge (human ADAMTS7B, -31.5; mouse ADAMTS7B, -43; and human ADAMTS12, -7.2 at pH 7.0). We have also detected two possible GAG attachment sites (^{1119}SGSG and ^{1156}SGSG) in the ADAMTS12 mucin domain, but they are located farther down-stream of the corresponding sequences in ADAMTS7B (Fig. 2). In addition, three

Gly-Ser dipeptides and one Ser-Gly dipeptide are present in the ADAMTS12 mucin domain, but they are not conserved in human or mouse ADAMTS7B (Fig. 2).

ADAMTS7B and *ADAMTS12* have identical gene structures, each having 24 protein-coding exons with identical splice boundaries (Fig. 2). *ADAMTS7* maps to human chromosome 15q24 and spans 52.3 kb of genomic sequence, whereas *ADAMTS12* maps to chromosome 5q35 (19) and spans 372 kb of genomic sequence. The catalytic site of each enzyme is interrupted by an intron, as in other *ADAMTS* genes such as *ADAMTS9* (13), with the exception of *ADAMTS1* (30). Each TSR is encoded by a single exon (except TSR1 and TSR2, which are encoded by two exons each), with the intron/exon boundary lying just upstream of the first cysteine residue of each repeat, a positioning similar to that in other ADAMTS family members (13). The final exon of *ADAMTS7* and *ADAMTS12* encodes the PLAC domain. The largest exon of *ADAMTS7* and *ADAMTS12* encodes the entire mucin domain.

Origin of the ADAMTS7A Transcript Variant—ADAMTS7B diverges from ADAMTS7A (18) at a point in intron 16 that resembles a consensus splice donor site (Fig. 3A). At this point, the ADAMTS7A ORF is contiguous with the genomic sequence and reaches a stop codon 190 bp downstream of the putative splice donor site. In contrast, at the point of divergence, the ADAMTS7B sequence is not continuous with the genomic sequence and is spliced into exon 17 (Fig. 3A). As previously reported (18), ADAMTS7A has a longer spacer domain than human or mouse ADAMTS7B or ADAMTS12. The extraneous region in the ADAMTS7A spacer domain is encoded by an intron (intron 14) that is included in the ADAMTS7A transcript and maintains the ORF from exons 14 to 15 (Fig. 3B). In

Figure 3. A, alignment of ADAMTS7B and ADAMTS7A cDNA sequences at their point of divergence with the ADAMTS7 genomic sequence. The splice donor site is shown by the thick underlines. The ADAMTS7A stop codon is underlined and italicized. Sequences that align are shown by white letters in black boxes. The ADAMTS7A ORF is shown in the lowest line, and Cys residues are highlighted. The lower half of the figure illustrates the failure to splice intron 16 in ADAMTS7A. B, unspliced intron 14 in human ADAMTS7A maintains the ORF and generates a longer spacer domain than ADAMTS7B.


A
splice donor
ADAMTS7B
ADAMTS7A
genomic
TGA
ADAMTS7A
15
16
17
ADAMTS7B
B
ADAMTS7B
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EXONS
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14
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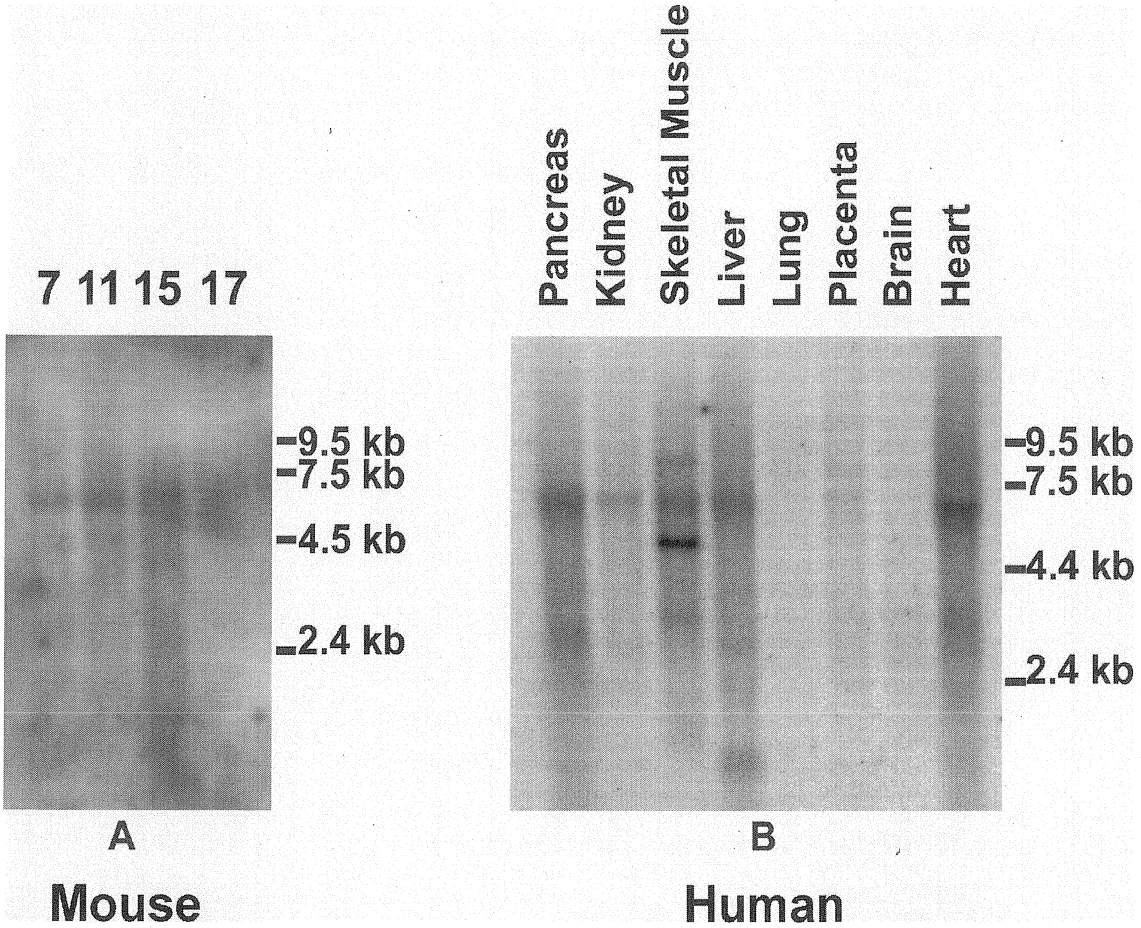


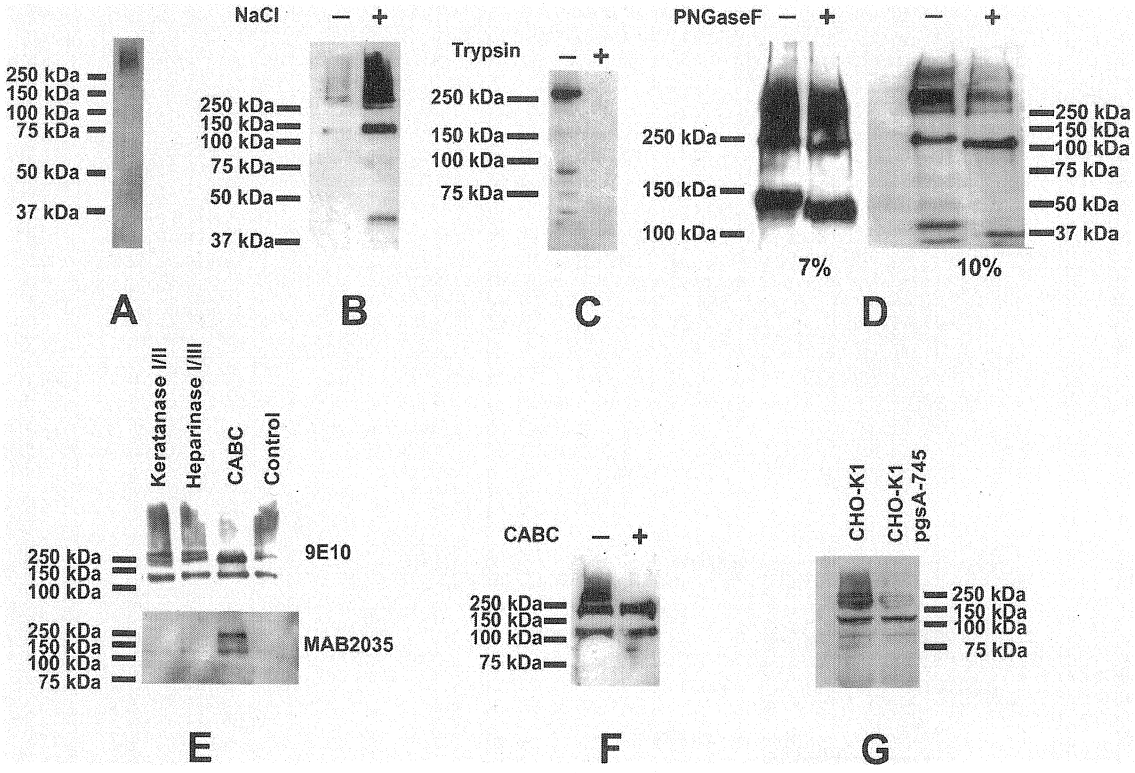
Figure 4. Northern blot analysis of ADAMTS7B gene expression. A, Northern blot of mRNA from mouse embryos. Embryo age in days is indicated above each lane. B, Northern blot of mRNA from adult human organs. Tissue origin is indicated above each lane. RNA size markers (in kb) are shown to the right of the autoradiograms.

mouse, inclusion of this intron does not maintain the ORF (data not shown). The corresponding insertion is not present in ADAMTS12 (19).

ADAMTS7 Is Widely Expressed—Adamts7 expression was detected in mouse embryos as a single transcript of ~5.5 kb at gestational days 7, 11, 15, and 17 (Fig. 4A). In adult human samples, *ADAMTS7* expression was detected as a 5-kb transcript, with heart, pancreas, kidney, skeletal muscle, and liver having the highest level of expression (Fig. 4B). In addition to the 5.5-kb transcript, a transcript of 8 kb and a prominent transcript of 4.5 kb were detected in skeletal muscle. Northern blotting using this probe produced results similar to those previously published using a probe common to both ADAMTS7A and ADAMTS7B (18), although we had not previously detected the 4.5-kb transcript in muscle.

ADAMTS7B Is Cell-associated—Western blotting of Myc-tagged mouse ADAMTS7B from serum-supplemented medium demonstrated that it migrated as two major bands, a broad and smeared band substantially greater than 250 kDa in size (referred to as the >250-kDa smear) and a sharper band just smaller than 250 kDa (referred to as the ~250-kDa band) (Fig. 5, A and B). Each band was larger than the conceptual translation product (181 kDa). Treatment with 0.5 M NaCl released additional ADAMTS7B from cells (Fig. 5B). This result indicates that ADAMTS7B is secreted, but is partially, transiently, or loosely retained in the pericellular region by interaction with one or more molecules at the cell surface or in the pericellular matrix. To confirm that the ADAMTS7B released by treatment with 0.5 M NaCl originated from the cell surface and not from an intracellular pool, we undertook biotinylation of cell-surface proteins. Cells in suspension were briefly treated with trypsin (to eliminate all surface proteins as a negative control) or with serum-free medium 15 min

Figure 5. Characterization of full-length His-tagged ADAMTS7B by Western blotting with anti-c-Myc antibody 9E10. A, ADAMTS7B purified from serum-supplemented medium electrophoresed on reducing 8% SDS-polyacrylamide gel. B, ADAMTS7B expressed in HEK239F cells grown in serum-free medium and eluted from cells with (+) or without (–) 0.5 M NaCl prior to electrophoresis. C, biotinylation of a trypsin-sensitive ADAMTS7B band indicates cell-surface localization. Trypsin (+) abolished salt-extractable ADAMTS7B protein from the cell surface. D, enzymatic removal of N-linked carbohydrate is illustrated by enhanced migration following PNGase F treatment (+). The results from both 7% SDS-PAGE (for high molecular mass bands) and 10% SDS-PAGE (for lower molecular mass bands) are shown. E, enzymatic removal of GAG chains. Only chondroitinase ABC (CABC) eliminated the high molecular mass smear (>250 kDa) with a concomitant increase in the ~250-kDa band. Note the appearance of a faint new band at ~200 kDa following keratanase digestion. The lower panel illustrates that only chondroitinase ABC digests revealed the epitope of monoclonal antibody 2035 (MAB2035). F, chondroitinase ABC digests of the medium from transiently transfected COS-1 cells. G, expression of ADAMTS7B in xylosyltransferase-deficient CHO-K1 pgsA-745 cells. The high molecular mass smear (>250 kDa) visible in the control CHO-K1 medium (left lane) was absent in the mutant cell medium (right lane) and cell lysate (not shown).


NaCl
– +
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
A
B
Trypsin
– +
C
PNGaseF
– +
– +
7%
10%
D
Keratanase I/II
Heparinase I/III
CABC
Control
9E10
MAB2035
E
CABC
– +
F
CHO-K1
CHO-K1
pgsA-745
G

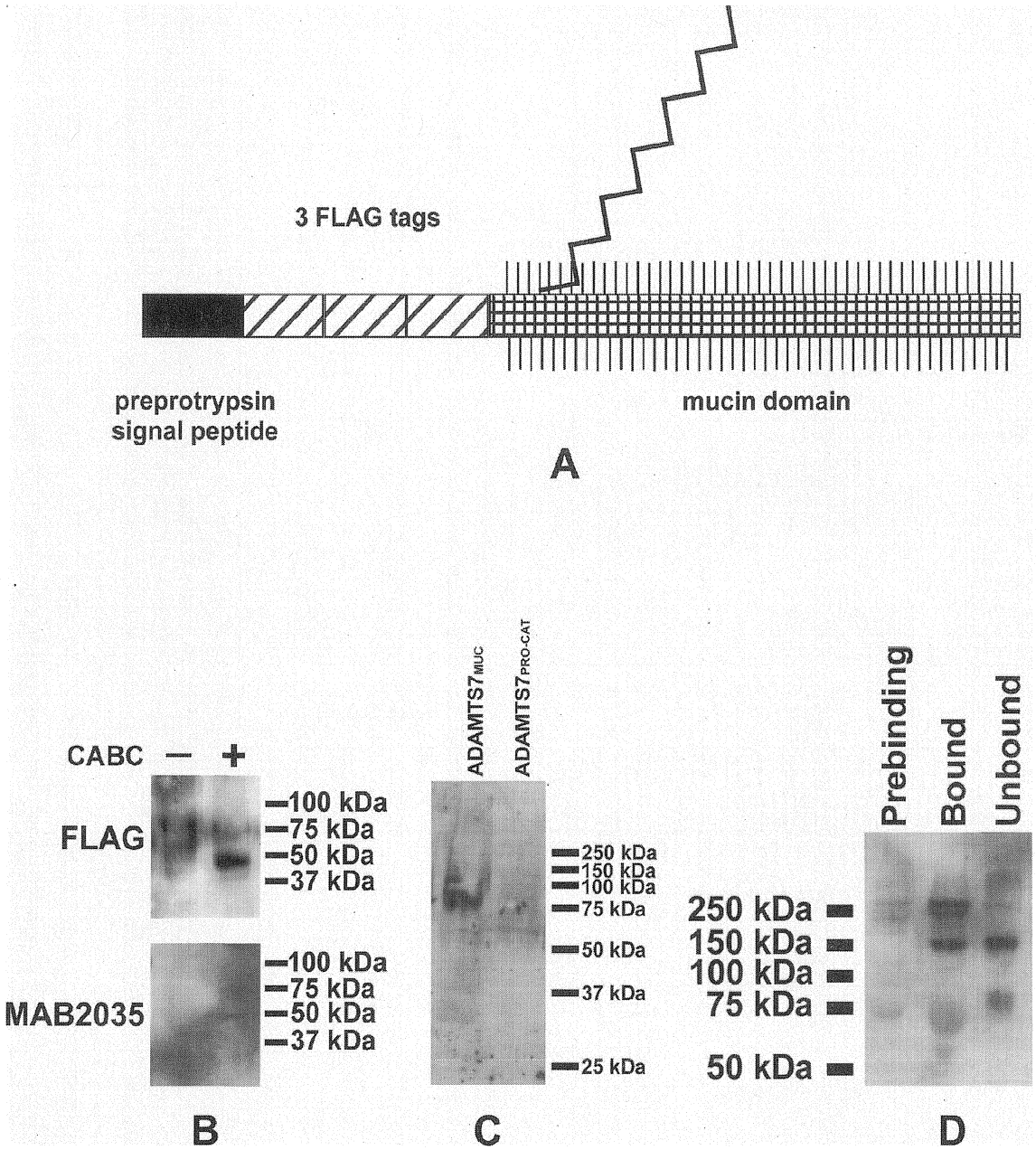
prior to biotinylation. After biotinylation, proteins were salt-eluted and captured with streptavidin-agarose. Untrypsinized cells had a biotin-labeled ~250-kDa anti-c-Myc antibody-immunoreactive band corresponding to ADAMTS7B (Fig. 5C), but not the >250-kDa smeared species. In contrast, samples from the trypsinized cells contained no biotinylated anti-c-Myc antibody-immunoreactive bands (Fig. 5C), but lysates of these cells were positive for ADAMTS7B by Western blotting (data not shown). This indicates that ADAMTS7B is external to the cell membrane, and since HEK293F cells do not make an appreciable amount of ECM, it is likely to be in close proximity to the cell membrane.

Proteolysis of ADAMTS7B in Serum-free Medium—There was no detectable proteolysis of ADAMTS7B when analyzed in cells grown in serum-supplemented medium (Fig. 5A). However, analysis of the ADAMTS7B from serum-free medium demonstrated fragmentation of the protein, presumably proteolytic, with additional anti-c-Myc antibody-immunoreactive bands being generated (Fig. 5, B, D, F, and G). The major anti-c-Myc antibody-reactive fragments observed were ~120 and 40 kDa in size. Both bands showed more rapid in-gel migration after PNGase F treatment (Fig. 5D), indicating that they contained N-linked carbohydrate. The ~120-kDa fragment, but not the ~40-kDa band, was more prominent following chondroitinase digestion and was recognized by antibody 2035 (Fig. 5E), suggesting that the 120-kDa fragment bears the chondroitin sulfate (CS) chain.

Secreted ADAMTS7B Is Post-translationally Modified and Has an Attached Chondroitin Sulfate Chain—Since the mouse ADAMTS7B sequence predicts eight potential N-linked glycosylation sites, we treated it with PNGase F to remove N-linked sugars. The ~250-kDa ADAMTS7B band thus treated migrated faster on Western blots, indicating the presence of

N-linked sugars, but the migration of the >250-kDa smear was largely unaffected by PNGase F (Fig. 5D). Upon treatment with chondroitinase ABC, but not with keratanases or heparinases, the >250-kDa smear largely disappeared; and instead, the amount of enzyme migrating at ~250 kDa increased substantially, indicating conversion of a CS-substituted protein to the core protein form (Fig. 5E). However, the presence of a faint new immunoreactive protein band following keratanase digestion (Fig. 5E) might be indicative of some keratan sulfate substitution as well. Furthermore, the chondroitinase ABC-digested core protein, but not the native protein from these cells, was immunoreactive with a monoclonal antibody (2035) recognizing CS stubs (Fig. 5E). Chondroitin sulfate modification was seen following transient transfection into COS-1 (Fig. 5F) and CHO-K1 (Fig. 5G) cells as well. A CHO cell mutant (CHO-K1 pgsA-745) with diminished xylosyltransferase produced primarily the ~250-kDa form and lacked the >250-kDa smear seen in the parental CHO-K1 cell line that represented the CS-modified form (Fig. 5G). To verify that the mucin domain was indeed heavily O-glycosylated and modified by CS chain attachment, we expressed the mouse ADAMTS7B mucin domain alone with an N-terminal FLAG tag as a secreted protein using the preprotrypsin leader sequence (Fig. 6A). Instead of the expected molecular mass of 37 kDa, the expressed protein migrated as broad bands of 50 and 75- and a smear of >100 kDa (Fig. 6B). When treated with chondroitinase ABC, the smear disappeared, and the 50-kDa band was accentuated (Fig. 6B, upper panel), suggesting conversion of CS-modified protein. The resulting chondroitinase-digested 50-kDa band was reactive with monoclonal antibody 2035, confirming the presence of a CS chain in the mucin domain (Fig. 6B, lower panel). Since the expressed mucin domain contains only one N-linked glycosylation site, we considered the increase in mass of ~13–40 kDa to be a consequence of O-glycosylation. Accordingly, horseradish peroxidase-

Figure 6. Characterization of the ADAMTS7B mucin domain ($ADAMTS7B_{FLAG\text{-}MUC}$) and heparin binding of full-length ADAMTS7B. Unless specified, Western blotting was performed with anti-FLAG monoclonal antibody M2. A, schematic of $ADAMTS7B_{FLAG\text{-}MUC}$ using the symbols shown in Fig. 1. B, chondroitinase ABC (CABC) digest of $ADAMTS7B_{FLAG\text{-}MUC}$ (+) showing attenuation of the broad ~100-kDa smear present in the undigested (–) control (upper panel). Chondroitinase-digested $ADAMTS7B_{FLAG\text{-}MUC}$ subsequently reacted with monoclonal antibody 2035 (MAB2035; lower panel). C, peanut agglutinin ligand blot. Horseradish peroxidase-conjugated peanut agglutinin bound to 75-kDa $ADAMTS7B_{FLAG\text{-}MUC}$, but not to $ADAMTS7_{PRO\text{-}CAT\text{-}Myc/His}$. D, binding of ADAMTS7B to heparin-Sepharose. From a mixture of CS-modified and -unmodified ADAMTS7B present in unconcentrated conditioned medium (Prebinding), the CS-unmodified form (~250 kDa) was enriched by incubation with heparin-Sepharose (Bound). The CS-modified ADAMTS7B smear (>250 kDa) did not bind to the heparin-Sepharose beads (Unbound; acetone-precipitated medium after heparin-Sepharose binding). Western blotting was performed with antibody 9E10.


3 FLAG tags
preprotrypsin
signal peptide
mucin domain
A
CABC
—
+
FLAG
100 kDa
75 kDa
50 kDa
37 kDa
MAB2035
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50 kDa
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B
ADAMTS7MUC
ADAMTS7PRO-CAT
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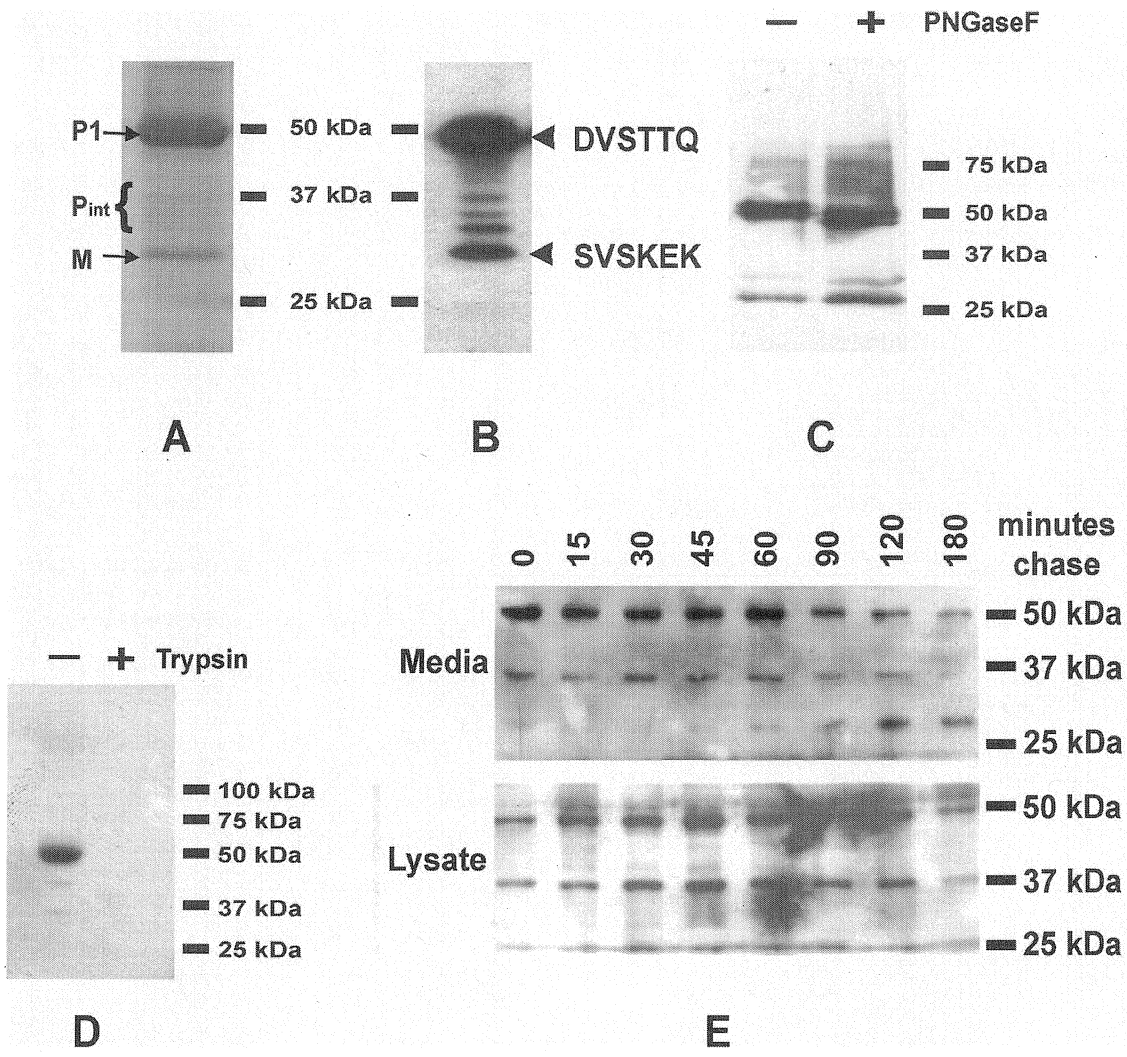
75 kDa
50 kDa
37 kDa
25 kDa
C
Prebinding
Bound
Unbound
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
D

conjugated peanut agglutinin bound to the 75-kDa band in ligand blot experiments (Fig. 6C), but not to ADAMTS7$_{PRO\text{-}CAT\text{-}Myc/His}$ (as a negative control). The 75-kDa band was only moderately altered by digestion with sialidase and O-glycosidase (data not shown), suggesting that the majority of the O-linked sugars were not capped with terminal sialic acid residues.

CS-modified ADAMTS7B Does Not Bind Heparin—To investigate whether ADAMTS7B binds heparin and whether binding is influenced by the GAG chain, the medium from HEK293 cells expressing full-length mouse ADAMTS7B was batch-incubated with heparin-Sepharose. Following elution of beads with 0.5 M NaCl (data not shown), the majority of ADAMTS7B remaining bound to heparin-Sepharose was ~250 kDa in size, with relatively little of the CS form being evident (Fig. 6D). On the other hand, acetone-concentrated protein from the unbound fraction of the medium showed the CS-substituted species as the majority, with very little of the ~250-kDa form (Fig. 6D), indicating that the CS form had relatively poor affinity for heparin-Sepharose. The 150- and 75-kDa bands found in the unbound fraction may have been proteolytically derived.

ADAMTS7$_{PRO\text{-}CAT\text{-}Myc/His}$ Is Sequentially Processed by Proprotein Convertases—SDS-PAGE of His-tagged proteins from human or mouse ADAMTS7$_{PRO\text{-}CAT\text{-}Myc/His}$-expressing HEK293 cells demonstrated two major protein bands of ~50 and 29 kDa on Coomassie Blue-stained gels, with several quantitatively minor intermediate bands (Fig. 7A). Western blotting using anti-c-Myc antibody demonstrated that these bands correspond to major forms of ADAMTS7$_{PRO\text{-}CAT\text{-}Myc/His}$ (Fig. 7B). The amino acid sequences of the 50- and 29-kDa major bands obtained by Edman degradation are ^{61}DVSTTQ and ^{221}SVSKEK, respectively,

Figure 7. Characterization of ADAMTS7$_{PRO-CAT-Myc/His}$ from HEK293 cell medium. A, Coomassie Blue-stained 10% SDS-polyacrylamide gel of purified recombinant ADAMTS7$_{PRO-CAT-Myc/His}$. The P1 band (50-kDa species processed following Arg^{60}), the intermediate processing form(s) of unknown origin (P_{int}), and the M band (mature 29-kDa catalytic domain) are indicated. B, Western blot of the same sample as in A. The N-terminal sequences of the indicated bands are shown on the right C, enzymatic deglycosylation using PNGase F. Note the enhanced migration of the 50-kDa band, but not of the 29-kDa band. D, cell-surface biotinylation. Only the 50-kDa species was present and biotinylated on the surface of cells not treated with trypsin. Trypsin-treated cells were used as a control. E, biotin pulse-chase analysis of the medium (upper panel) and cell lysate (lower panel). Time points (chase duration) following completion of biotinylation are indicated above each lane.

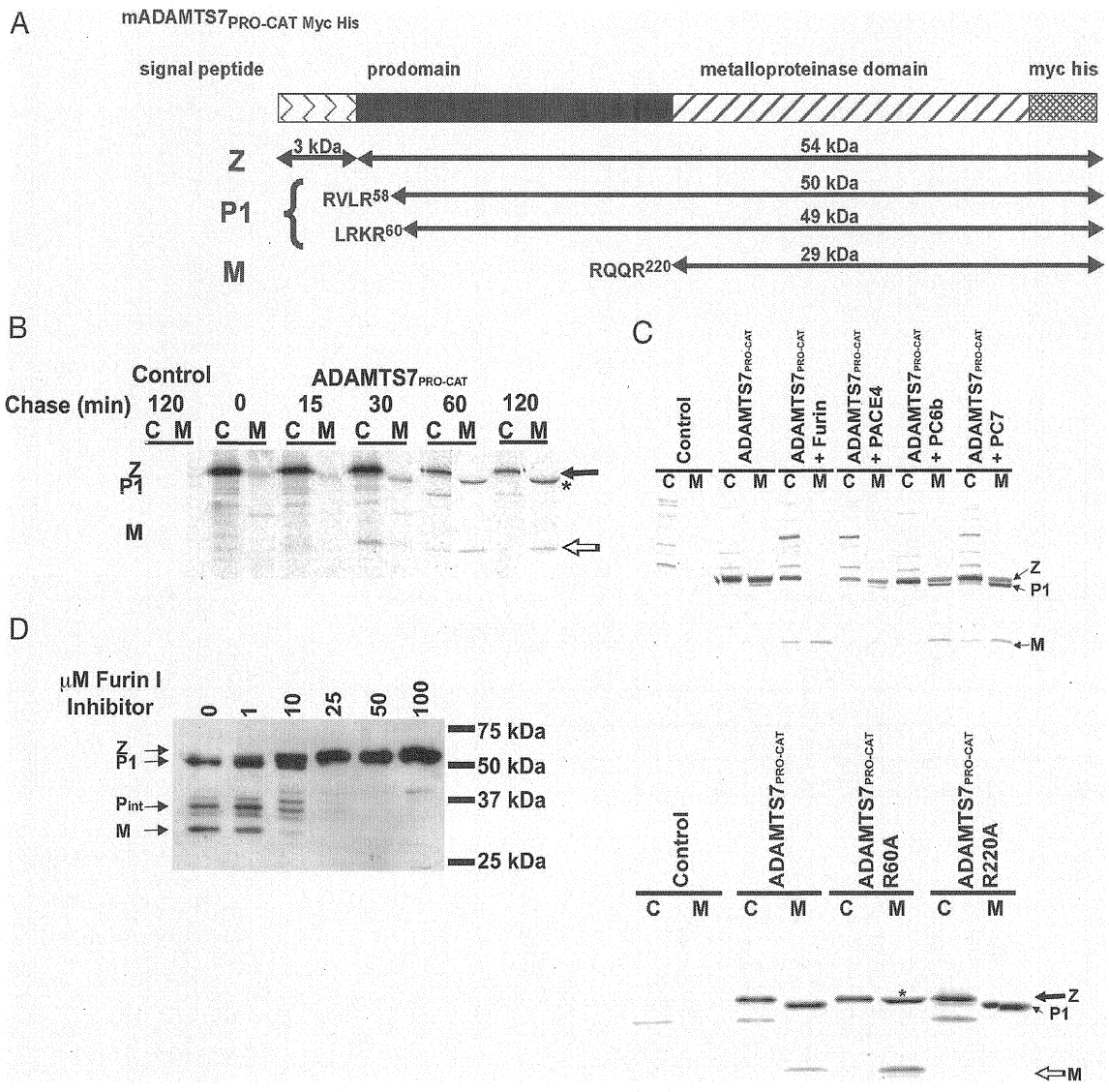

P1
Pint
M
50 kDa
37 kDa
25 kDa
DVSTTQ
SVSKEK
— + PNGaseF
75 kDa
50 kDa
37 kDa
25 kDa
A
B
C
— + Trypsin
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
D
0 15 30 45 60 90 120 180
minutes chase
Media
Lysate
50 kDa
37 kDa
25 kDa
50 kDa
37 kDa
25 kDa
E

indicating processing following Arg^{60} (designated the initial processed or P1 form) and Arg^{220} (designated the final processed, mature, or M form) (mouse sequence annotation), respectively (Fig. 7B). Because the human and mouse sequences around the most C-terminal furin-processing site do not align perfectly, we obtained the N-terminal sequence of the 29-kDa human $ADAMTS7_{PRO\text{-}CAT\text{-}Myc/His}$ band as well. It was ^{237}SVSKEKW, similar to that of mouse, indicating processing following Arg^{236}. PNGase F digestion enhanced migration of the 50-kDa P1 band, but not of the 29-kDa M band, in keeping with the absence of consensus N-linked sites in the catalytic domain and the presence of a single N-glycosylation site in the prodomain (Fig. 7C). In the cell lysate, but not in the medium, a doublet of ~50 kDa was noted (Fig. 7C), presumably corresponding to either the signal peptide processed (zymogen or Z form) and Arg^{60} processed forms or the Arg^{58} and Arg^{60} processed forms. Cell-surface biotinylation was done with HEK293F cells transfected with mouse $ADAMTS7_{PRO\text{-}CAT\text{-}Myc/His}$. Although biotinylated 50- and 29-kDa bands were found in the medium of these cells, the biotinylated 50-kDa P1 form was the major species detected on the surface of cells (Fig. 7D). To clarify this apparent discrepancy, we first biotinylated cell-surface proteins and then tracked biotinylated mouse $ADAMTS7_{PRO\text{-}CAT\text{-}Myc/His}$ in the medium for up to 3 h. In control experiments, cells were treated with trypsin prior to biotinylation to demonstrate that the biotinylated proteins were of cell-surface and not intracellular origin (Fig. 7D). The results show that the 50-kDa P1 form was progressively converted to a soluble 29-kDa M form, with an intermediate 35-kDa form (Fig. 7E), suggesting that processing from the 50-kDa P1 form to the 29-kDa M form occurs at the cell surface, with subsequent release of the 29-kDa M species. However, no 29-kDa M form was found in lysates of biotinylated cells (Fig. 7E), suggesting that its release from the cell surface occurs immediately after the final processing event. The origin

of the 35-kDa species (intermediate processing form) is uncertain since there is not a consensus furin-processing site between Arg^{60} and Arg^{220} that accounts for it, although the size of the intermediate and its inhibition by decanoyl-RVKR chloromethyl ketone (Fig. 8D) suggest processing at an as yet unknown processing site such as $ETRR^{102}$. In addition, since the 50-kDa species could be biotinylated on the cell surface, this suggests an interaction of the prodomain with some unknown moiety on the cell membrane, possible furin itself (31).

Three putative proprotein convertase recognition sequences are located in mouse ADAMTS7B and seven in human ADAMTS7B. The mouse $ADAMTS7_{PRO\text{-}CAT\text{-}Myc/His}$ molecular species derived from processing are illustrated in Fig. 8A. N-terminal sequence analysis suggested that two of these sites are preferentially processed in human and mouse ADAMTS7 (Fig. 7B), but did not indicate the order in which this might occur or the time course of activation. Accordingly, transient transfection of mouse $ADAMTS7_{PRO\text{-}CAT\text{-}Myc/His}$ in QBI 293A cells was followed by metabolic labeling with [^{35}S]methionine/cysteine using a pulse of 15 min, followed by a chase of the radiolabeled protein in cells and medium for up to 120 min. Radiolabeled ADAMTS7 protein was first detected in the culture medium after a chase of 30 min, and the amount of the labeled protein in cells decreased after 60 min (Fig. 8B). The 29-kDa M form first appeared in the medium after a 60-min chase. The 50-kDa P1 form accumulated in the medium from 30 to 120 min, but the 29-kDa M form did not, possibly as a consequence of rapid turnover by autocatalysis. CHO RPE.40 cells, which are furin-deficient, were unable to proteolytically process $ADAMTS7_{PRO\text{-}CAT\text{-}Myc/His}$ (Fig. 8C, upper panel). To determine which proprotein convertases could process ADAMTS7B, we cotransfected CHO RPE.40 cells with mouse $ADAMTS7_{PRO\text{-}CAT\text{-}Myc/His}$

Figure 8. Analysis of zymogen processing. A: schematic representation of the mouse (m) ADAMTS7$_{PRO\text{-}CAT\text{-}Myc/His}$ construct. Below this are the protein species predicted following signal peptide cleavage or processing at three proprotein convertase recognition sequences. B: pulse-chase analysis of ADAMTS7$_{PRO\text{-}CAT\text{-}Myc/His}$-transfected QBI 293A cells. Cells were labeled with radiolabeled amino acids for 15 min and chased for the times indicated. Control cells were transfected with the empty vector. Cell extracts (C) and media (M) were immunoprecipitated with anti-pentahistidine antibody and detected by fluorography. The black arrow indicates the zymogen (Z form), and the white arrow indicates the fully processed form (M form). The Arg^{60} processed form (P1 form) is indicated by the asterisk. C: upper panel, CHO RPE.40 cells were transfected with ADAMTS7$_{PRO\text{-}CAT\text{-}Myc/His}$ or cotransfected with ADAMTS7$_{PRO\text{-}CAT\text{-}Myc/His}$ and the indicated proprotein convertases. Cells were labeled with [^{35}S]Met/Cys for 3 h. Cell extracts and media were collected, extracted, and immunoprecipitated with anti-pentahistidine monoclonal antibody and analyzed by SDS-PAGE, followed by fluorography. Lower panel, furin cleavage site mutants were transfected into QBI 293A cells and labeled with a 3-h pulse, followed immediately by analysis of the cell lysate and conditioned medium. The black arrow indicates the zymogen form, and the white arrow indicates the fully processed form. The protein presumably generated by processing following Arg^{58} is indicated by the asterisk. D: inhibition of furin processing by decanoyl-RVKR chloromethyl ketone. HEK293 cells expressing ADAMTS7$_{PRO\text{-}CAT\text{-}Myc/His}$ were treated with inhibitor at the indicated concentrations, and samples were analyzed by Western blotting with antibody 9E10.


A
mADAMTS7PRO-CAT Myc His
signal peptide
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metalloproteinase domain
myc his
Z
3 kDa
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LRKR60
49 kDa
M
RQQR220
29 kDa
B
Control
ADAMTS7PRO-CAT
Chase (min) 120 0 15 30 60 120
C M C M C M C M C M C M
Z
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Control
ADAMTS7PRO-CAT
ADAMTS7PRO-CAT + Furin
ADAMTS7PRO-CAT + PACE4
ADAMTS7PRO-CAT + PC6b
ADAMTS7PRO-CAT + PC7
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Control
ADAMTS7PRO-CAT
ADAMTS7PRO-CAT R60A
ADAMTS7PRO-CAT R220A
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Z
P1
M

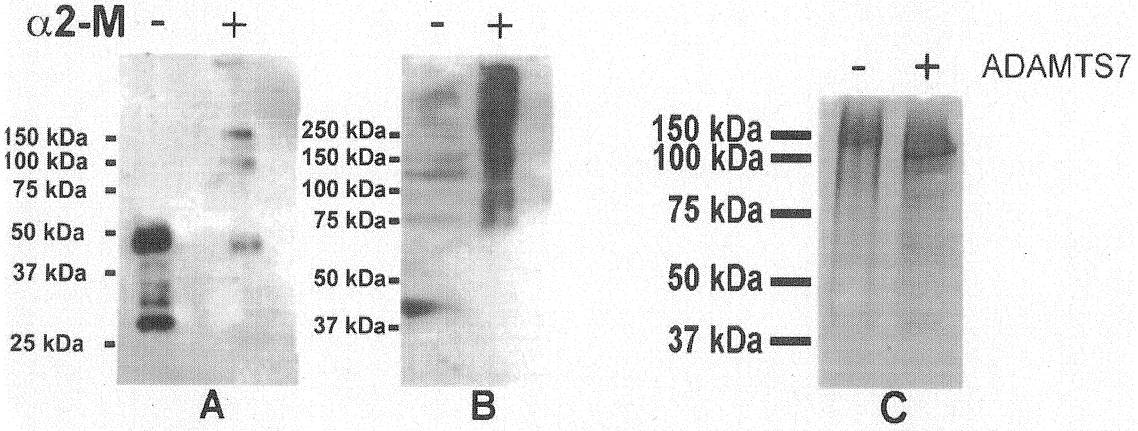
and furin, PACE4, PC6B, or PC7. Analysis of the media of these transfected cells revealed that furin was able to process ADAMTS7$_{PRO\text{-}CAT\text{-}Myc/His}$ completely to the mature form (Fig. 8C, upper panel). Interestingly, the cell lysate showed both the 50-kDa P1 form and the 29-kDa M form, whereas the medium contained only the 29-kDa M form. PC6B and PC7 processed ADAMTS7$_{PRO\text{-}CAT\text{-}Myc/His}$, but the medium contained the 54-kDa Z form as well as the P1 and M forms, indicating that they inefficiently processed ADAMTS7 to its mature form. Interestingly, PACE4 showed predominantly the 54-kDa Z band in the medium, indicating a failure of this enzyme to utilize any of the furin sites within the prodomain of ADAMTS7. To ascertain which of the Arg^{60} and Arg^{220} furin sites was indispensable for zymogen maturation, we obtained site-directed mutants in which the P1 Arg residue was mutated to Ala. Expression of these forms in QBI 293A cells demonstrated that when Arg^{60} was mutated, processing occurred instead at an adjacent alternative site ($RVLR^{58}$) since a slightly decreased migration of the 50-kDa P1 band was noted (Fig. 8C, lower panel, asterisk), and complete processing to the 29-kDa M form was also noted. On the other hand, when Arg^{220} was replaced by Ala, only the 50-kDa P1 band was noted, and the 29-kDa M form was absent in the medium. Although N-terminal sequence analysis suggests that Arg^{60} is preferred, these experiments suggest that when this site is unavailable, Arg^{58} can be used.

Taken together, these data suggest a scenario in which furin is the preferred pro-ADAMTS7B convertase and that it may operate at two locations in the cell to sequentially produce the P1 and M forms. The data suggest that the processing event that produces the M form occurs at the cell surface through a 35-kDa intermediate. To further investigate this, we treated HEK293 cells expressing ADAMTS7$_{PRO\text{-}CAT\text{-}Myc/His}$ with increasing concentrations of the synthetic furin inhibitor decanoyl-RVKR chloromethyl ketone. At low

concentrations (10 μM), it inhibited processing to the 35-kDa intermediate and the M form, but only partially inhibited processing to the 50-kDa form (Fig. 8D). Since this lipophilic inhibitor will permeate cells, higher concentrations (>25 μM) completely inhibited processing to the P1 and smaller forms, and only the zymogen was apparent (Fig. 8D).

ADAMTS7B and ADAMTS7$_{PRO-CAT-Myc/His}$ Are Functional Proteinases—ADAMTS7B was demonstrated to be catalytically active using the broad-spectrum protease substrate and inhibitor α_2-macroglobulin (32). α_2-Macroglobulin, a serum protein, is a circulating endoproteinase inhibitor that is commonly used as a nonspecific protease substrate. Cleavage by many proteases within a proteolytically susceptible "bait" region results in engulfment of the protease and is frequently followed by formation of an irreversible complex. Thus, anomalous migration of a protease upon incubation with α_2-macroglobulin is considered evidence of proteolytic activity. ADAMTS7$_{PRO-CAT-Myc/His}$ and ADAMTS7B were both able to cleave α_2-macroglobulin as shown by altered migration of the protease on SDS-polyacrylamide gel (Fig. 9, A and B) and proteolysis of α α_2-macroglobulin by SDS-PAGE (Fig. 9C). However, using anti-neoepitope antibodies to detect specific ADAMTS cleavage products of aggrecan and versican (12, 33), ADAMTS7B digestion did not reveal the target epitopes (data not shown), suggesting that it does not attack these sites. In these experiments, equivalent amounts of ADAMTS7B that cleaved α_2-macroglobulin were used. ADAMTS7B-transfected cells did not process aggrecan either, whereas ADAMTS4-transfected cells did, despite the ADAMTS7 expression level being considerably higher compared with the ADAMTS4 expression level (Fig. 9D). In combination, these studies demonstrate that ADAMTS7B could not process aggrecan in cells even at much higher

Figure 9. ADAMTS7 is an active protease. A and B, shown is the α_2-macroglobulin effect on ADAMTS7 gel mobility. Purified $ADAMTS7_{PRO\text{-}CAT\text{-}Myc/His}$ (A) and ADAMTS7B (B) were incubated with (+) or without (–) α_2-macroglobulin (α2-M). Analysis of the digest was performed by Western blotting of nonreducing SDS-polyacrylamide gel using anti-c-Myc antibody 9E10. In A, altered migration of both the 50- and 29 kDa forms is seen relative to $ADAMTS7_{PRO\text{-}CAT\text{-}Myc/His}$ not incubated with α_2-macroglobulin (–), indicating that these forms have been shifted by entrapment in α_2-macroglobulin subsequent to its cleavage. In B, incubation of recombinant ADAMTS7B alone (–) for the duration of the digest resulted in extensive proteolysis and a major 40-kDa band, whereas migration was quite different in the presence of α_2-macroglobulin (+). C, α_2-macroglobulin was incubated with (+) or without (–) $ADAMTS7_{PRO\text{-}CAT\text{-}Myc/His}$ for 3 h prior to reducing SDS-PAGE and Coomassie Blue staining, showing reduction in mass following cleavage by $ADAMTS7_{PRO\text{-}CAT\text{-}Myc/His}$. D, ADAMTS7B-transfected cells did not uncover neoepitopes in versican and aggrecan that were revealed in ADAMTS4-transfected cells. The upper and middle panels show immunoblotting with anti-AGEG antibody (anti-aggrecan neoepitope antibody) and anti-DPEAAE antibody (anti-versican neoepitope antibody), respectively. The lower panel indicates the levels of expression of ADAMTS7 and ADAMTS4 in cell lysates by Western blotting with anti-c-Myc and anti-FLAG antibodies, respectively. In cell lysates, ADAMTS7B was easily evident, but expression of ADAMTS4 was low. (Specific bands are indicated by the brace.) Control cells were transfected with the plasmid vector alone.


α2-M - +
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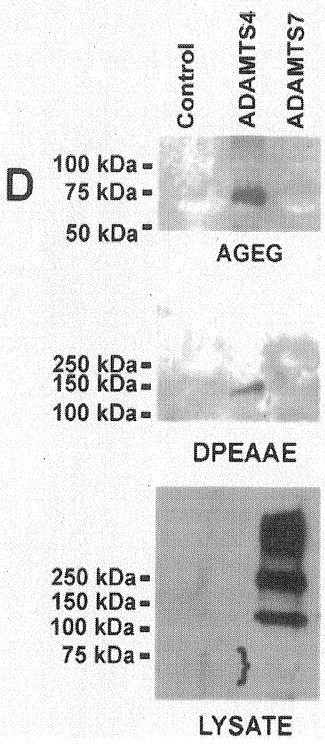

Control
ADAMTS4
ADAMTS7
D
100 kDa
75 kDa
50 kDa
AGEG
250 kDa
150 kDa
100 kDa
DPEAAE
250 kDa
150 kDa
100 kDa
75 kDa
LYSATE

levels compared with ADAMTS4 and in solution at levels at which it could cleave α_2-macroglobulin.

DISCUSSION

The existence of putative ADAMTS7B has been readily apparent by data base analysis since 1999, and its domain organization has been previously noted in the literature (34, 35) and in online resources (www.lerner.ccf.org/bme/apte/adamts/). We have now confirmed by cDNA cloning in the mouse that ADAMTS7B mRNA does exist in tissues. Several considerations suggest that ADAMTS7B encodes the full-length product of the *ADAMTS7* gene and that ADAMTS7A represents an incompletely processed mRNA with retention of two introns. Analysis of expressed sequence tags in the GenBank™/EBI Data Bank validated the sequence of ADAMTS7B, but no expressed sequence tag with the 3'-sequence of ADAMTS7A was found. Incidentally, intron retention confounded the initial characterization of ADAMTS6, ADAMTS9, ADAMTS16, and ADAMTS18 (18, 36, 37), and longer ORFs have subsequently been identified (13). The ADAMTS7A transcript is unlikely to be an alternatively spliced product. It lacks a polyadenylation signal, and the ORF shift in TSR2 has at best a superficial resemblance to other TSR sequences. All other TSRs in ADAMTS proteases have six cysteines, and their spacing and intervening amino acids are quite different from those of TSR2 in ADAMTS7A, which has seven cysteines. Similarly, we do not believe that the longer ADAMTS7 spacer sequence we originally described is authentic since the corresponding intron inclusion in mouse ADAMTS7B truncates the reading frame.

On the basis of identical domain organization and highly similar primary sequence and intron/exon structure, ADAMTS7B and ADAMTS12 clearly form a new phylogenetic clade in the ADAMTS family. It is probable that these genes arose from a gene duplication

event. The active-site motifs in ADAMTS7B and ADAMTS12 are identical to each other, but not to other ADAMTS proteases. This is highly suggestive of a conserved biochemical function distinct from that of other ADAMTS proteases. Consistent with this, ADAMTS7B could not process aggrecan or versican at specific peptide bonds that are attacked by ADAMTS1, ADAMTS4, ADAMTS5, and ADAMTS9, which are on different evolutionary branches of the ADAMTS family tree (34). Nevertheless, ADAMTS7B is an active protease since both the full-length enzyme and $ADAMTS7_{PRO\text{-}CAT\text{-}Myc/His}$ cleaved α α_2-macroglobulin.

Unlike the ADAM proteases and membrane-type matrix metalloproteases, ADAMTS proteases do not contain transmembrane segments or glycophosphoinositol signal anchor sequences. However, cell-surface biotinylation demonstrated that ADAMTS7B localized to the cell surface and was released at elevated salt concentrations. Localization at the cell surface has been previously shown for ADAMTS1 (24), ADAMTS4 (13, 38, 39), and ADAMTS9 (13) and likely occurs via an ionic interaction with a cell-surface or pericellular matrix molecule. Cell-surface interaction of these proteases is mediated by their ancillary domain (13, 38). Since fully activated $ADAMTS7_{PRO\text{-}CAT\text{-}Myc/His}$ was not present at the cell surface, we conclude that the ADAMTS7B ancillary domain mediates its binding to the cell surface. The putative CD36- and heparin/sulfatide-binding motifs in the ancillary domain are possible motifs by which binding of the full-length active enzyme may occur. In support of the latter possibility, ADAMTS7B bound heparin-Sepharose. Since localization of the 50-kDa form of $ADAMTS7_{PRO\text{-}CAT\text{-}Myc/His}$ at the cell surface was followed by furin activation, we surmise that it may be retained at the cell

surface through interaction of the prodomain with furin. Thus, the ADAMTS7B zymogen is retained at the cell surface through interactions involving both the prodomain and ancillary domain, whereas the mature enzyme is retained through interactions involving the ancillary domain alone. A unique feature of ADAMTS7B that sets it apart from all other ADAMTS proteases, with the likely exception of ADAMTS12, is the presence of a putative mucin domain. Interestingly, like the mucin domain of syndecan-3 (40), it is entirely encoded by one exon, implying its introduction by exon shuffling during evolution. Although it is smaller than that in ADAMTS7B, the putative ADAMTS12 mucin domain contains comparatively more predicted O-glycosylation sites and a conserved N-glycosylation site. What are the functional implications of this substantial post-translational modification? The mucin domain is expected to have an extended conformation and to function as a stalk or rigid spacer between the two TSR arrays in ADAMTS7B. It may also have a role in protecting this region from proteolysis (41). In platelet glycoprotein-1Bα, the mucin-like region is required for cell adhesion to immobilized von Willebrand factor (42); and in neutral ceramidase, the mucin-like region facilitates cell membrane localization (43). It could also affect proteolytic specificity since recent studies have shown that glycosylation of MMP14 affects its substrate profile (44).

The mucin domain is further modified by addition of one or more CS chains. The poorly resolved in-gel migration of the >250-kDa smear prompted us to ask whether there was GAG attachment to ADAMTS7B. In three unrelated cell types, ADAMTS7B bears one or more CS chains, suggesting that when modified, it may be an obligatory chondroitin sulfate proteoglycan. However, we cannot exclude the possibility that other cell types may

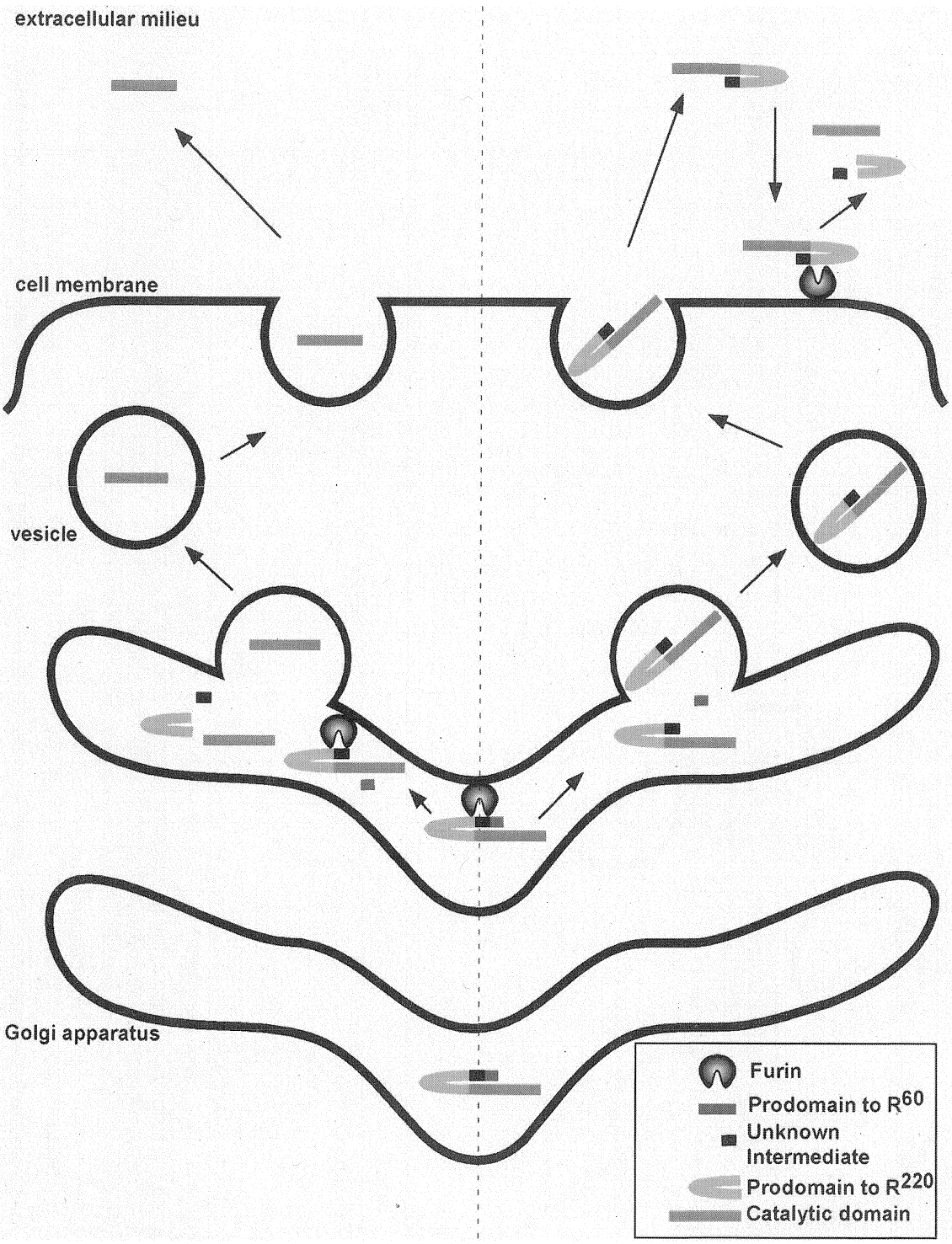
attach a heparan sulfate chain to the acceptor site since the attachment of both GAGs occurs through the carbohydrate primer region Xyl-Gal-Gal-GlcA and at broadly similar acceptor sequences on the core protein (45). Some proteoglycans such as perlecan and syndecan-1 have a hybrid GAG substitution containing both CS and heparan sulfate chains (45, 46). ADAMTS7B could be a part-time chondroitin sulfate proteoglycan, existing in both GAG-substituted and GAG-free forms. Our studies in COS-1 and HEK293 cells support this, although it is possible that high expression levels in transfected cells may allow partial escape from GAG substitution. Although we demonstrated CS chain attachment within the mucin domain, the precise site(s) of GAG attachment and the number of GAG chains are presently undetermined. Comparison of a number of GAG acceptor sequences has shown that the acceptor site is often Ser-Gly-Xaa-Gly (with the Ser residue accepting the initiating xylosyl) and is frequently in the immediate vicinity of acidic residues (27). Both requirements are met at the proposed ADAMTS7B GAG attachment motifs. The Gly-Ser^{1061} (mouse), Gly-Ser^{1108} (mouse), and Gly-Ser^{1324} (human) dipeptides present in the mucin domain also have one or more acidic residues in the immediate vicinity and could support GAG attachment. Of two putative Ser-Gly-Ser-Gly sequence motifs in the putative human ADAMTS12 mucin domain, one (Ser^{1158}) lies within a very acidic amino acid context that may favor substitution with heparan sulfate (45). GAG attachment of ADAMTS12 is presently unresolved since the data in a previous study of ADAMTS12 (19) did not include the upper reaches of the gel. We are addressing the possible attachment of a GAG chain to ADAMTS12 in ongoing studies. GAG attachment is rare in proteolytic enzymes. It has potential regulatory significance since it could influence affinity for ligands and substrates and affect compartmentalization of ADAMTS7B. Indeed, when affinity-purified by heparin-Sepharose chromatography, the dominant ADAMTS7B form captured

on the matrix was the GAG-unsubstituted form, and biotinylation experiments indicated that the major species present near the cell surface lacked the CS chain. Important functions have been shown for GAG chains in other proteoglycans. In Nudel, a *Drosophila* protease, the GAG chain is essential for its proteolytic activity and for dorsoventral patterning (47). The B16F10 cell receptor for a metastasis-promoting laminin-1 peptide is a hybrid CS/heparan sulfate proteoglycan that requires the GAG chain for the metastasis-promoting activity of the peptide (48). In future studies, we will study the fine structure and regulatory role of the GAG chain(s) in more detail.

Proteolysis within the ancillary domain is another potentially significant post-translational event. Western blotting detected two major smaller anti-c-Myc antibody-immunoreactive bands that we estimate to have been produced by proteolysis within the disintegrin-like domain and the C terminus of the mucin domain, respectively. Interestingly, these fragments were not seen in serum-supplemented medium, possibly due to endogenous inhibitors in the serum such as α_2-macroglobulin, shown here to be an ADAMTS7B substrate. The proteolytic fragmentation of several ADAMTS enzymes, including ADAMTS1 (16), ADAMTS4 (39, 49), ADAMTS9 (13), and ADAMTS12 (19), has been reported. C-terminal processing of ADAMTS4 appears to be important in regulating its substrate profile (39, 49). In addition to affecting protease activity, it is tempting to speculate that the released 120- and 40-kDa fragments might have independent biological activities or might competitively inhibit proteolysis by ADAMTS7B. These possibilities can be tested once ADAMTS7B substrates are identified.

Consistent with their forming a distinct clade, ADAMTS7B and ADAMTS12 proprotein convertase sites are situated at similar locations. Proprotein convertases are subtilisin-like, calcium-dependent serine proteases present in the Golgi apparatus or at the cell surface that attack well defined recognition sequences containing dibasic or tetrabasic residues (50, 51). In this work, we have shown that conversion to the mature ADAMTS7 enzyme did not occur in the absence of furin. PC6B and PC7 processed pro-ADAMTS7B less efficiently than furin, whereas PACE4 could not process pro-ADAMTS7B. The emphasis on furin, PACE4, PC6, and PC7 in this study is reflective of widespread distribution observed for ADAMTS7 in tissues. Indeed, all aforementioned convertases have a ubiquitous (furin) or widespread expression in contrast to convertases such as PC1, PC2, and PC4, which are specifically localized only in endocrine/neuroendocrine cells or testicular germ cells (48). In view of the ubiquitous expression and efficient processing of ADAMTS7B, furin is probably its physiological convertase in most cells and organs (50, 51). N-terminal sequencing demonstrated that only two of three putative cleavage sites are preferred in mouse ADAMTS7, but site-directed mutagenesis suggested that Arg^{58} can be used as an alternative to Arg^{60}. The N terminus of mature ADAMTS7B corresponds closely to the position of the N-termini of mature ADAMTS1, ADAMTS2, ADAMTS4, ADAMTS9, and ADAMTS13 (9, 13, 14, 52, 53). Many ADAMTS proteases such as ADAMTS4, ADAMTS9, and ADAMTS20 have multiple furin-processing sites in their prodomains (13, 54), yet final processing appears to occur at the most C-terminal one, as noted here for ADAMTS7B.

Figure 10. Multistep mechanism of ADAMTS7B zymogen processing. Two possible scenarios are separated by the vertical dashed line. Removal of the prodomain by intracellular furin (following Arg^{60}, mouse sequence annotation) occurs within the Golgi apparatus. Some or all of the partially processed zymogen is exported to the cell surface (pathway indicated to the right of the dashed line), where it binds to cell membrane furin through the remainder of the prodomain (Asp^{61}–Arg^{220}). Furin processing following Arg^{220} occurs after an intermediate processing step that is presently uncharacterized. The fully processed 29-kDa species is liberated from the cell surface. Alternatively, as shown to the left of the dashed line, all the processing could be intracellular. The data support the right-hand scenario, but do not exclude the left-hand scenario.


extracellular milieu
cell membrane
vesicle
Golgi apparatus
Furin
Prodomain to R60
Unknown Intermediate
Prodomain to R220
Catalytic domain

The α_2-macroglobulin assay suggested that Arg^{60} processed mouse $ADAMTS7_{PRO\text{-}CAT\text{-}Myc/His}$, which retains most of the prodomain, is catalytically active since the mobility of this form was altered in the presence of α_2-macroglobulin. It was recently shown that the ADAMTS13 zymogen is catalytically competent (55). ADAMTS13 has an unusually short prodomain and is very different from other ADAMTS proteases (55). The proteolytic activity present in the zymogen of ADAMTS7B, an enzyme with a typical prodomain, suggests that it may have an accessible active site and that the cysteine-switch mechanism of matrix metalloprotease zymogen activation (56), in which an unpaired cysteine in the prodomain ligands the active-site zinc atom, may not be operative in this enzyme and ADAMTS13. The reason for the apparent loss of $ADAMTS7_{PRO\text{-}CAT\text{-}Myc/His}$ in the presence of α_2-macroglobulin is unclear, but may be related to inefficient migration or electroblotting of the enzyme-α_2-macroglobulin complex.

The experimental observations from radioactive precursor and biotin pulse-chase experiments, furin inhibition, and protein sequencing together support a multistep model of ADAMTS7B zymogen processing (Fig. 10). In this model, newly synthesized ADAMTS7B is first efficiently clipped intracellularly by furin following Arg^{60} or Arg^{58}. Subsequently, this form is processed by furin following Arg^{220}. Furin inhibition studies indicated that the processing event at Arg^{220} was sensitive to lower concentrations of furin inhibitor compared with the processing event at Arg^{60}, suggesting that it is more easily accessible to the inhibitor, i.e. it may occur at the cell surface or late in the secretory pathway. In contrast, the Arg^{60} processing event occurred early in the secretory pathway and required higher concentrations for inhibition. Since the 50-kDa P1 form was retained at the cell surface, the

Arg^{220}–Ser bond may be attacked less efficiently than the Arg^{60}–Ser bond, and this processing event may be rate-limiting for the release of fully active enzyme. This could be a consequence of there being considerably more intracellular than cell-surface furin (57). Accordingly, when furin was overexpressed in cells along with ADAMTS7, there was complete processing to the 29-kDa M form. However, when ADAMTS7 was expressed without commensurately high expression of furin (as in most of our processing experiments), the processing mechanism at the cell surface was overwhelmed, and there was release of both fully processed (i.e. at Arg^{220}) and incompletely processed (at Arg^{60}) ADAMTS7. Even when there was excess ADAMTS7, the initial processing at Arg^{60} was complete, and the signal peptide processed ADAMTS7 zymogen was not released from the secretory pathway. A two-step activation mechanism has also been proposed for ADAMTS2 through similar approaches (58), although it is unclear if the final processing occurs at the cell surface.

Within the ADAMTS family, there are a number of distinct clades in which structurally identical proteases such as ADAMTS7B and ADAMTS12 appear to have evolved by gene duplication (13). An intriguing possibility is that such homologs have similar biochemical and catalytic properties, but they are differently regulated, and that it is differential gene regulation that may underlie distinctive biological roles of homologs rather than variations in intrinsic properties. In this context, it will be important to investigate whether ADAMTS12 is also a CS- or heparan sulfate-modified mucin domain-containing enzyme and to contrast the gene regulation and substrate profile of these two enzymes.

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The abbreviations used are: ECM, extracellular matrix; TSR, thrombospondin type 1 repeat; ORF, open reading frame; PNGase F, peptide *N*-glycosidase F; CHO, Chinese hamster ovary; GAG, glycosaminoglycan; PBS, phosphate-buffered saline; CS, chondroitin sulfate

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ARTICLE 4 – AVANT-PROPOS

Cell-surface processing of pro-ADAMTS9 by furin

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Contribution: Cet article est le fruit d'une étroite collaboration entre mon directeur de recherche, Dr. Richard Leduc et le Dr. Suneel Apte. J'ai réalisé les expériences menant à la figure 4. J'ai purifié l'inhibiteur des pro-protéines convertases, AT-PDX, utilisé à la figure 6B. Compte tenu de la caractérisation d'un phénomène nouveau allant à l'encontre de la littérature sur la furine, soit le clivage d'un substrat par la furine à la surface cellulaire et non dans l'appareil de Golgi, beaucoup d'efforts ont été entrepris afin de mettre notre hypothèse en faute. De nombreuses expériences ont été exécutées afin de détecter une forme clivée d'ADAMTS9 dans la cellule. Ces dernières n'ont pas été publiées. Les résultats ont par contre été pris en considération dans la rédaction de cet article. J'ai d'ailleurs reproduit les figures 5A, 6A, B et C afin de valider les expériences produites par le groupe du Dr. Apte. Mes commentaires et suggestions ont été incorporés à l'écriture du manuscrit jusqu'à la version publiée.

Résumé de l'article 4

La maturation de précurseurs polypeptidiques par les pro-protéines convertases (PCs) telles la furine se fait typiquement dans le réseau du *trans*-Golgi. Cette étude démontre que le clivage du prodomaine d'ADAMTS9 n'est pas intracellulaire dans différents types cellulaires. Des analyses par marquage métabolique dans les cellules HEK 293 démontrent que le zymogène est sécrété à la surface cellulaire et ensuite clivé à cet endroit avant d'être relâché dans le milieu extracellulaire. L'utilisation d'inhibiteurs des PCs et l'absence de clivage dans des cellules déficientes en furine démontrent que la maturation d'ADAMTS9 est effectuée par un mécanisme furine dépendant. De plus, des ARN d'interférence de la furine réduisent la maturation d'ADAMTS9 dans les cellules HEK 293. La convertase PC5A clive aussi le zymogène pro-ADAMTS9 mais comme pour la furine, ce clivage est extracellulaire. Le clivage à la surface cellulaire via un mécanisme furine dépendant crée un précédent pour la maturation extracellulaire de précurseurs endogènes sécrétés. Ces observations indiquent également l'existence d'une variété de mécanismes possibles pour la maturation des protéinases ADAMTS.

Cell-surface Processing of Pro-ADAMTS9 by Furin

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ABSTRACT

Processing of polypeptide precursors by proprotein convertases (PCs) such as furin typically occurs within the trans-Golgi network. Here, we show in a variety of cell types that the propeptide of ADAMTS9 is not excised intracellularly. Pulse-chase analysis in HEK293F cells indicated that the intact zymogen was secreted to the cell surface and was subsequently processed there before release into the medium. The processing occurred via a furin-dependent mechanism as shown using PC inhibitors, lack of processing in furindeficient cells, and rescue by furin in these cells. Moreover, down-regulation of furin by small interference RNA reduced ADAMTS9 processing in HEK293F cells.PC5Acould also process pro-ADAMTS9, but similarly to furin, processed forms were absent intracellularly. Cellsurface, furin-dependent processing of pro-ADAMTS9 creates a precedent for extracellular maturation of endogenously produced secreted proproteins. It also indicates the existence of a variety of mechanisms for processing of ADAMTS proteases.

INTRODUCTION

Zinc metalloendopeptidases, like most proteases, are synthesized as zymogens, and the N-terminal propeptide is usually excised. Propeptide excision usually leads to enzymatic activation, an important regulatory event, and it can occur intracellularly, at the cell surface, or extracellularly through a variety of proteolytic mechanisms. In one such mechanism, the propeptide is proteolytically excised by serine proteases of the mammalian subtilisin-like proprotein convertase (PC) family (1-5). This mechanism is used by some MMPs (6, 7), many ADAMs (8-10), and all ADAMTS proteases studied thus far (11, 12). In these proteases, removal of the propeptide appears to be mediated by the most widely distributed PC, furin, and occurs within the constitutive secretory pathway, specifically in the trans-Golgi network (TGN) (7-9, 13, 14).

Furin is the best studied of the seven PCs implicated in proprotein processing within the constitutive secretory pathway, and it is present in virtually all cells (1, 15). It is a type I transmembrane protein that is itself synthesized as a zymogen that undergoes autocatalytic intramolecular activation (16). Furin cleaves on the carboxyl side of a consensus recognition site that is rich in basic residues (e.g. Arg-Xaa-Arg/Lys-Arg↓) (2, 4, 5, 17). Most furin resides in the TGN, but some is present at the plasma membrane and shuttles between the cell surface and the TGN (18-20). Furin is also shed from cells and may be functional in the extracellular space (21). Microbial toxins such as the anthrax protective antigen and diphtheria toxin are processed by cell-surface furin, with important implications for their toxicity (20, 22). However, the physiological role of cell-surface or secreted furin in processing endogenous cellular products has remained elusive.

The ADAMTS proteases are a family of 19 secreted enzymes, of which some have critical physiological functions and have been implicated in inherited human disorders, namely Ehlers-Danlos syndrome type VIIC (ADAMTS2), Weill-Marchesani syndrome (ADAMTS10), and inherited thrombocytopenic purpura (ADAMTS13) (23-26). Overexpression of ADAMTS5 and ADAMTS4 is implicated in proteolytic loss of aggrecan, a major cartilage component, in arthritis (27). ADAMTS proteases (except ADAMTS13, the von Willebrand factor-processing protease, whose propeptide contains 41 amino acid residues) are synthesized with propeptides of ~220-240 amino acids, which are larger than the MMP and ADAM propeptides. Almost all ADAMTS propeptides have consensus sites for the attachment of N-linked oligosaccharide (Asn-Xaa-Thr/Ser, where Xaa is any amino acid except Pro). Consensus recognition sequences for PCs are present at the junction of the propeptide and catalytic domain, and ADAMTS proteases may have additional, more N-terminal PC-processing sites within their propeptides. Previous studies of ADAMTS1, ADAMTS4, and ADAMTS7 suggested that, like ADAMs and the furin-processed MMPs, ADAMTS proteases were processed in the TGN by PCs, although some cell-surface processing of ADAMTS7 was also observed (11-14, 28).

ADAMTS9 is one of only two ADAMTS proteases that is highly conserved during evolution (29-31). Its mRNA is widely expressed (29, 30), especially during embryonic development (32). ADAMTS9 is up-regulated by inflammatory cytokines in chondrocytes (33). It may have a role in atherosclerosis and arthritis, because it can proteolytically process the proteoglycans versican and aggrecan (30), which are important constituents of the vessel wall, and cartilage, respectively. This potential biological significance of ADAMTS9 prompted an in-depth analysis of the processing of its propeptide. In the initial

characterization of ADAMTS9, we demonstrated that it could be processed at more than one site (30). In continuing these studies, we have made the unexpected observation that ADAMTS9 processing is exclusively extracellular and occurs at the cell surface in cells that express high levels of furin. These results establish a precedent for the cell-surface activation of precursor endogenous proteins by furin and are possibly of broad biological relevance.

EXPERIMENTAL PROCEDURES

Expression Plasmids and Site-directed Mutagenesis—Plasmids for expression of full-length ADAMTS9, or a truncated form containing the signal peptide, propeptide, and catalytic domain (Pro-Cat) with C-terminal Myc and His tags were described previously (30). Due to low expression levels of full-length ADAMTS9, we excised its open reading frame with the Myc-His tags as a NotI/PmeI fragment and then recloned this into pCEP4 (Invitrogen) digested with KpnI and blunt-ended followed by NotI digestion. To insert a FLAG tag between Thr^{276} and Arg^{277} in the propeptide, site-directed mutagenesis (QuikChange kit, Stratagene) was performed using the forward primer 5'-AATAAGACGGACAACACAGACTACAAGACGATGACGACAAGAGAGAAAAGAG GACCCAC-3' (with FLAG encoding site underlined) and the reverse primer 5'-GTGGGTCCTCTTTTCTCTCTTGTCGTCATCGTCTTTGTAGTCTGTGTTGTCCGTCT TATT-3' (FLAG encoding site underlined). Plasmids for expression of full-length ADAMTS1 or the propeptide and catalytic domain of ADAMTS7 (ADAMTS7-Pro-Cat) were previously described (11, 28).

Antibodies and Immunoblotting—The peptides RP1 and RP4 representing different regions of the ADAMTS9 propeptide were synthesized using Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry and conjugated to KLH. New Zealand White male rabbits (7-8 pounds) were immunized with the conjugates 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 immunosorbent assay using free peptides. Affinity-purified antibodies were prepared using the respective

immobilized antigens. Anti-penta-His monoclonal antibody, anti-Myc monoclonal antibody 9E10, and anti-FLAG M2 monoclonal antibody were obtained from commercial sources (Invitrogen and Sigma-Aldrich). Anti-furin monoclonal antibody was purchased from Alexis Biochemicals (San Diego, CA). Immunoblotting was done using denaturing SDS-PAGE and electroblotting to polyvinylidene fluoride membrane followed by detection of the bound antibody using enhanced chemiluminescence (Amersham Biosciences).

Cell Culture, Transfection, and Cell Treatments—HEK293F cells stably transfected with ADAMTS9 or Pro-Cat, COS-1 cells, LoVo cells (ATCC no. CCL-299), CHO-K1 cells, CHO RPE.40 (35), and rat chondrosarcoma RCS-LTC cells (36) were maintained as described previously (30) or as per vendors' instructions. Transient transfections were done using FuGENE6 (Roche Diagnostics, Indianapolis, IN) as per the manufacturer's recommendations. For inhibition of PCs, cells were treated with the PC inhibitory peptide dec-Arg-Val-Lys-Arg-chloromethyl ketone (dec-RVKR-cmk, Calbiochem) at different concentrations as indicated or with the polypeptide α1-PDX (Portland variant of α1-antitrypsin) (37).

Detection of Cell-surface Processing—Biotinylation of cells was done on ice to prevent labeling of intracellular proteins, essentially as previously described (28). As a control to eliminate cell-surface proteins, cells were treated with 0.05% trypsin/0.53 mM EDTA for 20 min on ice, and trypsin was subsequently inactivated with 10% fetal bovine serum-supplemented medium.

To examine the fate of cell-surface biotinylated protein, biotinylated cells were transferred to serum-free medium, and cells and media were collected at sequential time points. Biotinylated proteins were captured from samples using streptavidin-agarose (Sigma-Aldrich) and eluted by boiling in Laemmli sample buffer followed by SDS-PAGE and Western blotting with appropriate antibodies. Pro-Cat was purified from lysates of stably transfected cells using Pro-Bond resin (Invitrogen). For determination of processing of exogenously added Pro-Cat by cells, untransfected HEK293F cells were cultured to confluence on 6-well plates and then in 293 SFM-II medium (Invitrogen). Purified Pro-Cat (50 μg/well) was added to untransfected HEK293F cells, and the cells were cultured further over an 8-h period. The medium was taken for analysis at successive time points. In parallel experiments purified Pro-Cat was incubated with cell-free conditioned medium from HEK293F cells to investigate whether the processing was cell-mediated or not. The samples were analyzed by Western blotting with anti-Myc.

Enzymatic Deglycosylation—Removal of N-linked carbohydrate with peptide N-glycosidase F (PNGase F, New England Biolabs, Beverly, MA) was performed as previously described (30).

Co-transfection of Pro-Cat and Proprotein Convertases—Furin-deficient CHO RPE.40 cells (35) and LoVo cells were transfected with Pro-Cat alone or together with plasmids encoding the proprotein convertases furin, PACE4, and PC5A (kindly provided by Dr. Nabil Seidah). 48 h after transfection, cells were incubated in 293 SFM-II medium for 24 h, and Western blotting of the cell lysate and medium was performed with anti-Myc.

Cell-surface Cross-linking and Immunoprecipitation—48 h after transfection with the Pro-FLAG-Cat plasmid, HEK293F cells were washed four times with phosphate-buffered saline and treated with the thiolcleavable, membrane-nonpermeable cross-linker 3,3'-dithiobis(sulfosuccinimidylpropionate) (Pierce Endogen, Rockford, IL) to cross-link molecules at the cell surface. The cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, protease inhibitor mixture, Roche Diagnostics) for 1 h at 4 °C and centrifuged. The soluble portion of the lysate was transferred to a fresh tube and incubated overnight with anti-FLAG-agarose (Sigma-Aldrich) at 4 °C with rotation. Anti-FLAG-agarose was pelleted by centrifugation at 1000 rpm in a microcentrifuge and washed six times in lysis buffer. The supernatant was discarded, and the bound protein was eluted with 0.1 M glycine/HCl (pH 3.5) from the resin. The eluted samples were analyzed by Western blotting with anti-furin monoclonal antibody (Alexis Biochemicals) or RP4 antibody.

Metabolic Labeling and Pulse-Chase Analysis—HEK-293F cells stably expressing Pro-Cat were grown to confluence and incubated in Dulbecco's modified Eagle's medium without cysteine and methionine (Sigma-Aldrich). After 24 h, the medium was replaced with cysteinemethionine-free medium containing radioactive cysteine/methionine (50 μCi/well in 6-well plates, Pulse medium) for 15 min using EXPRE^{35}S^{35}S (PerkinElmer Life Sciences). For pulse-chase analysis, the pulse medium was removed, and the cells were washed three times on ice with phosphate-buffered saline containing 0.5 mM $MgCl_2$ and 0.5 mM $CaCl_2$, followed by incubation for 0, 15, 30, 60, or 120 min in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Chase medium) at 37 °C. At the end of the chase period, cells were placed on ice, the medium was collected, and the cells were

washed four times with phosphate-buffered saline and extracted with lysis buffer. In parallel, samples were processed for removal of cell-surface proteins in these assays by treatment with trypsin/EDTA for 20 min on ice, and trypsin was subsequently inactivated with 10% fetal bovine serum supplemented medium. The media and cell lysates were processed for immunoprecipitation with anti-Myc followed by fluorography as described previously (30).

RNA Interference—HEK293F cells stably expressing Pro-Cat were transfected with 20 pmol of Furin ShortCut siRNA Mix (New England Biolabs) using Lipofectamine 2000 (Invitrogen). After 48 h of incubation without antibiotics, the medium was changed to 293 SFM-II medium (Invitrogen), and cells were incubated for a further 24 h. The conditioned medium and cell lysate were analyzed by Western blotting with anti-Myc and anti-RP4. Cell-surface biotinylation was done as above.

RESULTS

The Proteolytically Processed ADAMTS9 Propeptide Is Present in the Conditioned Medium but Not in the Cell Lysate—The ADAMTS9 propeptide (Fig. 1, A and B) has five consensus furin-recognition sequences (Arg-Xaa-Xaa-Arg). Arg-Lys-Asp-Arg^{33} and Arg-Thr-Arg-Arg^{74} are near the signal peptidase processing site, whereas the remaining three sites (Arg-Glu-Lys-Arg^{280}, Arg-Thr-His-Arg^{283}, and Arg-Thr-Lys-Arg^{287}) overlap and are clustered near the junction of the propeptide and catalytic domain. We previously showed that mutation of Arg^{74} and Arg^{287} abrogated processing at these sites, whereas mutation of Arg^{280}, which compromised two putative sites, was without effect (30). The propeptide also contains three sequence motifs for attachment of N-linked oligosaccharides (Fig. 1B), whereas the catalytic domain has none.

Two nonoverlapping synthetic peptides from the propeptide (RP1 and RP4; sequences are not provided for proprietary reasons of Triple Point Biologics) within the propeptide were used for the production of polyclonal antisera. In HEK293F cells stably expressing full-length ADAMTS9 (Fig. 2A), anti-RP4 recognized the expected ~180-kDa zymogen in the cell lysate. Smaller polypeptides (37 kDa and a ~20-22-kDa doublet) were recognized in the conditioned medium. These bands were not seen in untransfected HEK293F cells (Fig. 2A). When the medium was deglycosylated using PNGase F, the 37-kDa band and the ~22-kDa doublet increased in mobility and migrated at 28 and 15 kDa, respectively, demonstrating the presence of N-linked carbohydrate (Fig. 2B), characteristic of propeptide fragments. Notably, the ~22-kDa doublet converted to a ~15-kDa doublet,

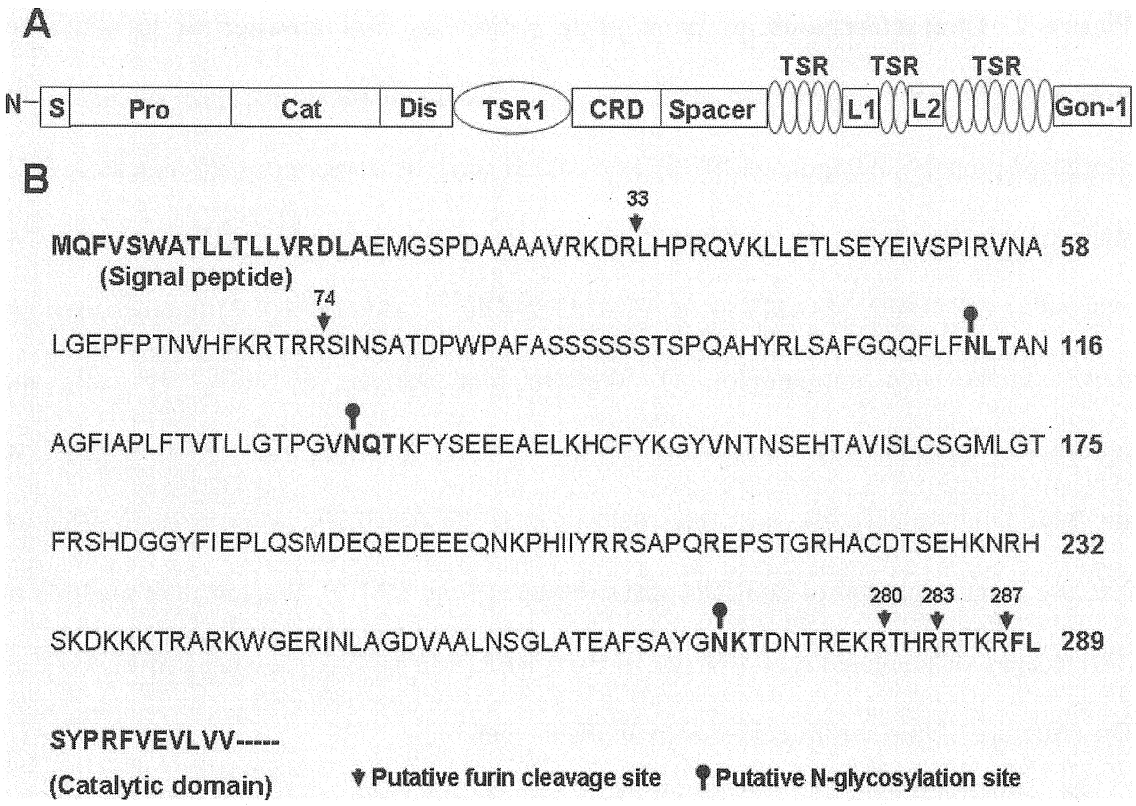
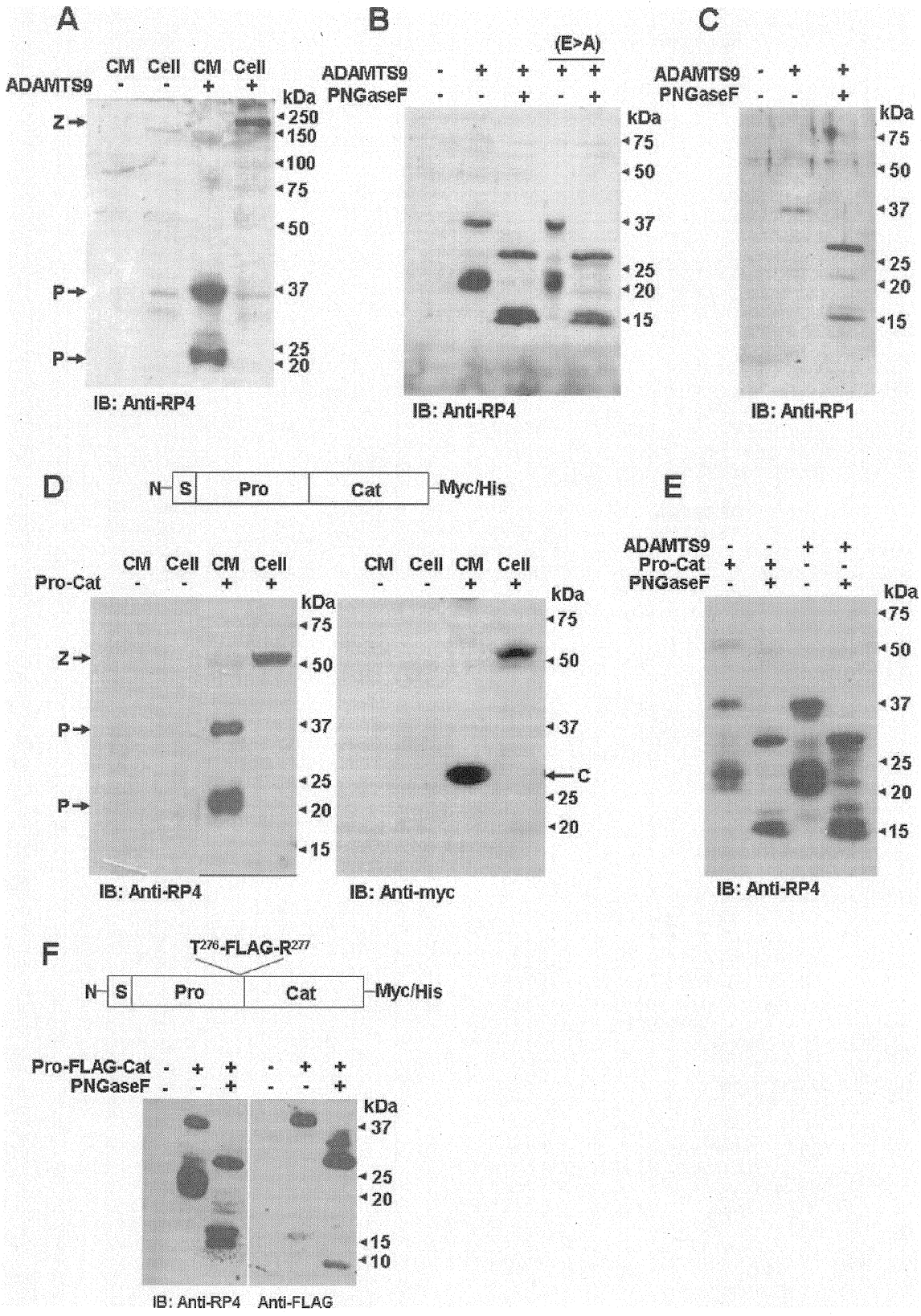


Figure 1. Schematic representation and modular structure of prepro-ADAMTS9. A, the various modules illustrated are: S, signal peptide; Pro, propeptide; Cat, catalytic domain; Dis, disintegrin-like module; TSR, thrombospondin type 1 repeat; CRD, cysteine-rich peptide; Gon-1, the C-terminal module homologous to GON-1. L1 and L2 are linkers between TSR arrays. The full-length enzyme has 1935 amino acids. B, sequence of the signal peptide (bold), propeptide, and start of the catalytic domain (bold). Putative N-linked glycosylation sites and consensus furin-processing sites are shown.

Figure 2. Characterization of propeptide antibodies and absence of intracellular processing in cells transfected with various ADAMTS9 plasmids. The zymogen (Z), processed propeptide fragments (P), and processed catalytic domain (C) are indicated. A-C, Western blot analysis of transfected HEK293F cell and conditioned medium (CM) expressing full-length ADAMTS9 or ADAMTS9-Glu^{381} →Ala (E>A). Antibodies used are shown under each immunoblot. D, Western blot analysis of HEK293F cells and conditioned medium (CM) expressing ADAMTS9 Pro-Cat using anti-RP4 (left panel) or anti-Myc (right panel). The structure of Pro-Cat is illustrated above the gels. E, effect of PNGase F on migration of anti-RP4-reactive bands from CM. F, Western blot analysis of CM, treated, or untreated with PNGase F from HEK293F cells expressing Pro-FLAG-Cat. The structure of this construct is shown above the gel.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indicating two protein species rather than glycoforms of the same fragment. When these experiments were conducted using an ADAMTS9 active site mutant (ADAMTS9 $Glu^{381} \rightarrow$ Ala, a widely used metalloprotease inactivating mutation (12, 38)), no differences were seen (Fig. 2B); thus, the anti-RP4-reative fragments were not autoproteolytic in origin. In Western blotting with anti-RP1 the medium showed a weakly immunoreactive 37-kDa band but not the ~22-kDa doublet (Fig. 2C), whereas PNGase F treatment clearly resolved the 28-kDa band seen with RP4 and a 15-kDa doublet also seen with RP4. The comparable results with RP4 and RP1 as well as the presence of glycosylation identified these fragments in the medium as originating from the propeptide. The RP1 peptide is adjacent to an N-linkage site, and the carbohydrate may mask reactivity of the ~22-kDa fragments without prior PNGase F treatment. Because anti-RP4 provided more robust immunoreactivity, it was used in subsequent experiments.

Because the large size and extensive glycosylation of full-length ADAMTS9 (30) precludes accurate resolution in gels, the above studies were repeated using an expression plasmid encoding the ADAMTS9 signal peptide, propeptide, and catalytic domain (Pro-Cat) with a Myc-His tag at the C terminus (Fig. 2D). The results were identical, i.e. anti-RP4 detected the intact Pro-Cat zymogen (55 kDa) only in the cell lysate, and 37- and 20-22-kDa fragments were found only in the conditioned medium (Fig. 2D). Similarly, anti-Myc detected only the unprocessed 55-kDa Pro-Cat zymogen in the cell lysate, and only the processed catalytic domain (29 kDa) in conditioned medium (Fig. 2D). Incubation of conditioned medium with PNGase F enhanced the mobility of propeptide fragments to the same extent as with full-length ADAMTS9 (Fig. 2E). That these fragments originated from the propeptide was further verified by insertion of a FLAG epitope tag between Thr^{276} and

Arg^{277}, i.e. upstream of the furin processing site at Arg^{287}. Western blotting with anti-FLAG provided similar results to that with anti-RP4, with differences (Fig. 2F) reflecting distinct locations of the FLAG and RP4 epitopes within the propeptide. These results demonstrate that the propeptide is part of the intact zymogen in HEK293F cells, but that it is present as a distinct, proteolytically derived entity in their conditioned medium. We conclude that the 37-kDa band seen with all propeptide antibodies corresponds to the complete propeptide. When deglycosylated, its size (28 kDa), is compatible with processing at the most N- and C-terminal PC processing sites, i.e. Leu^{34}-Arg^{287} (28 kDa). The site at which the processing of the prodomain to the 20- and 22-kDa RP4-reative fragments occurs is not established, but it may represent cleavage at the Arg-Arg^{209}-↓Ser. This site is not a typical furin processing site, but it has certain attributes that favor PC processing, such as His at the P6 position and Ser at the P1' position. We asked whether the conditioned medium of cells endogenously expressing ADAMTS9 or from other transfected cell types had similar processing profiles. RCS-LTC chondrosarcoma cells endogenously expressing ADAMTS9 demonstrated an anti-RP4-reactive 22-kDa band in their conditioned medium, but not in the cell lysate, where only a 180-kDa band corresponding to the intact zymogen was detected (Fig. 3A). When COS-1 and CHO-K1 cell lines were transiently transfected with Pro-Cat, anti-RP4 detected only the unprocessed zymogen in cell lysate, but both the secreted, unprocessed zymogen and proteolytically processed pro-domain in the conditioned medium (Fig. 3, B and C, left panels). Western blotting with anti-Myc detected the intact zymogen in cell lysate and the 29-kDa processed catalytic domain in the conditioned medium (Fig. 3, B and C, right panels). Thus, in all the cell types examined, the ADAMTS9 propeptide was not processed within the cell. Varying amounts of unprocessed Pro-Cat were detected in conditioned medium of transfected COS-1 and CHO-K1 cells (Fig. 3, B and C, left panels)

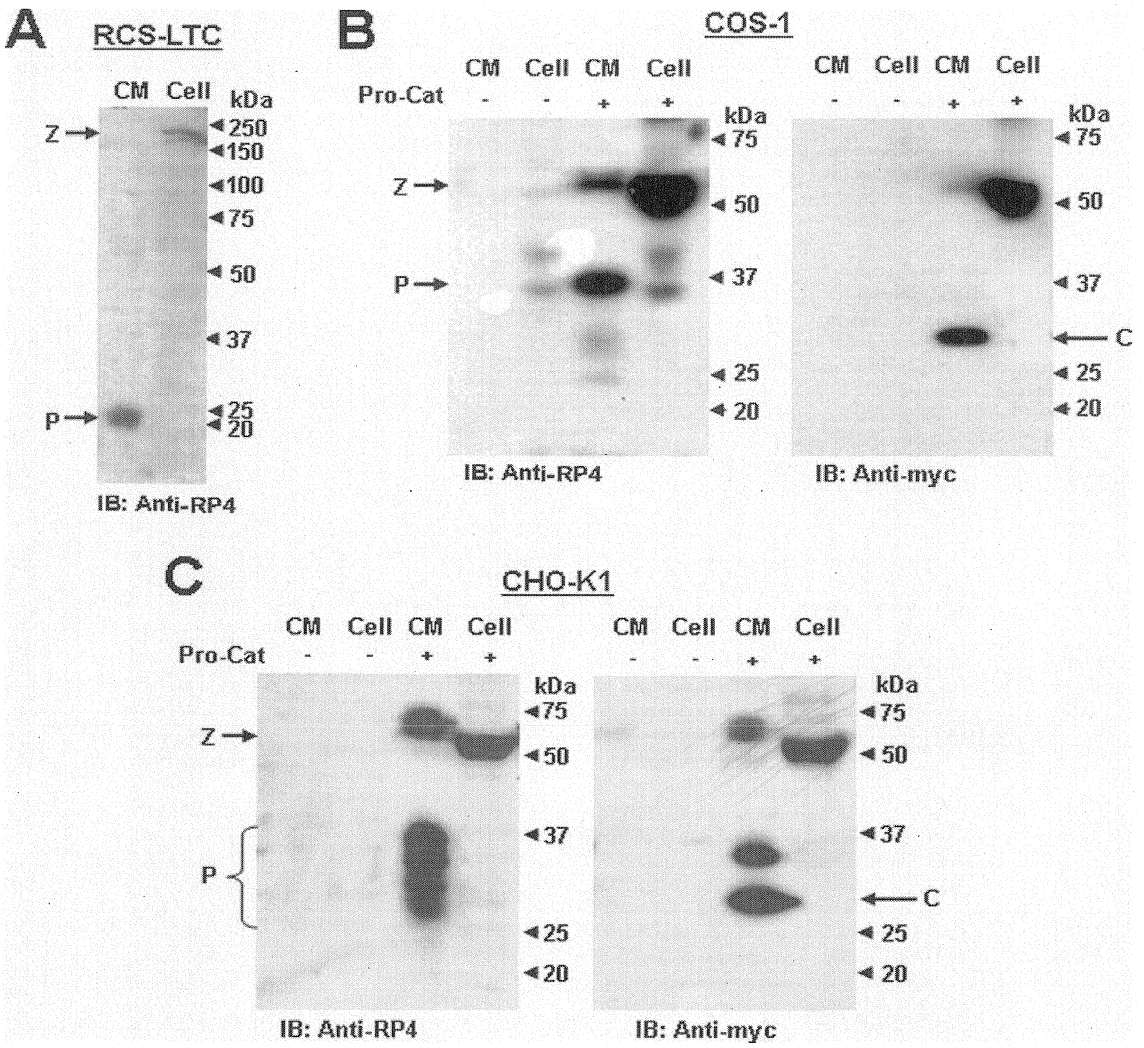


Figure 3. Absence of intracellular processing of ADAMTS9 in cell types other than HEK293F. The zymogen (Z), processed propeptide (P), and processed catalytic domain (C) are indicated. A, Western blotting of untransfected RCS-LTC cells with anti-RP4. A 22-kDa propeptide fragment is seen only in conditioned medium (CM). In cells, only the intact 180-kDa zymogen is detected. B, analysis of Pro-Cat-transfected COS-1 cells and their conditioned medium (CM). Antibodies used are shown below each blot. C, analysis of Pro-Cat-transfected CHO-K1 cells and their conditioned medium (CM). Antibodies used are shown below each blot.

but little in HEK293F cells (Fig. 2). Together, the results suggest that the ADAMTS9 propeptide is intrinsically resistant to removal within the secretory pathway, because it is not processed intracellularly regardless of the cell type.

ADAMTS9 Processing Differs from That of Other ADAMTS Proteases—Previous studies demonstrated intracellular processing of ADAMTS1, ADAMTS4, and ADAMTS7 (11, 13, 14, 28). We directly compared ADAMTS9 processing with ADAMTS1 and ADAMTS7 processing. ADAMTS1 and ADAMTS9 Pro-Cat were transfected into QBI-HEK 293A cells, followed by metabolic labeling with radioactive amino acids and immunoprecipitation with anti-ADAMTS1 and antipenta-His. Fluorography of the cell- and medium-derived proteins demonstrated ADAMTS1 zymogen (110 kDa) and the processed enzyme (87 kDa) in the cell but only the unprocessed 55-kDa ADAMTS9 Pro-Cat in cells (Fig. 4A). In a second set of experiments, ADAMTS9-Pro-Cat was compared with ADAMTS7-Pro-Cat. Cell lysate from ADAMTS7-Pro-Cat-transfected HEK293F cells showed both the intact Pro-Cat and the predicted furin-processed catalytic domain, whereas ADAMTS9-Pro-Cat transfected cells showed only the unprocessed zymogen (Fig. 4B). These results demonstrate that differences in biosynthetic mechanisms exist in the ADAMTS family.

ADAMTS9 Is Processed at the Cell Surface—The presence of only the intact pro-ADAMTS9 in cells and of the processed propeptide exclusively in the conditioned medium, suggested that propeptide processing occurred extracellularly, i.e. at the cell surface or in the medium. We utilized a variety of approaches to examine this possibility further. First, cell-surface proteins were labeled with biotin. Biotinylated proteins were purified from

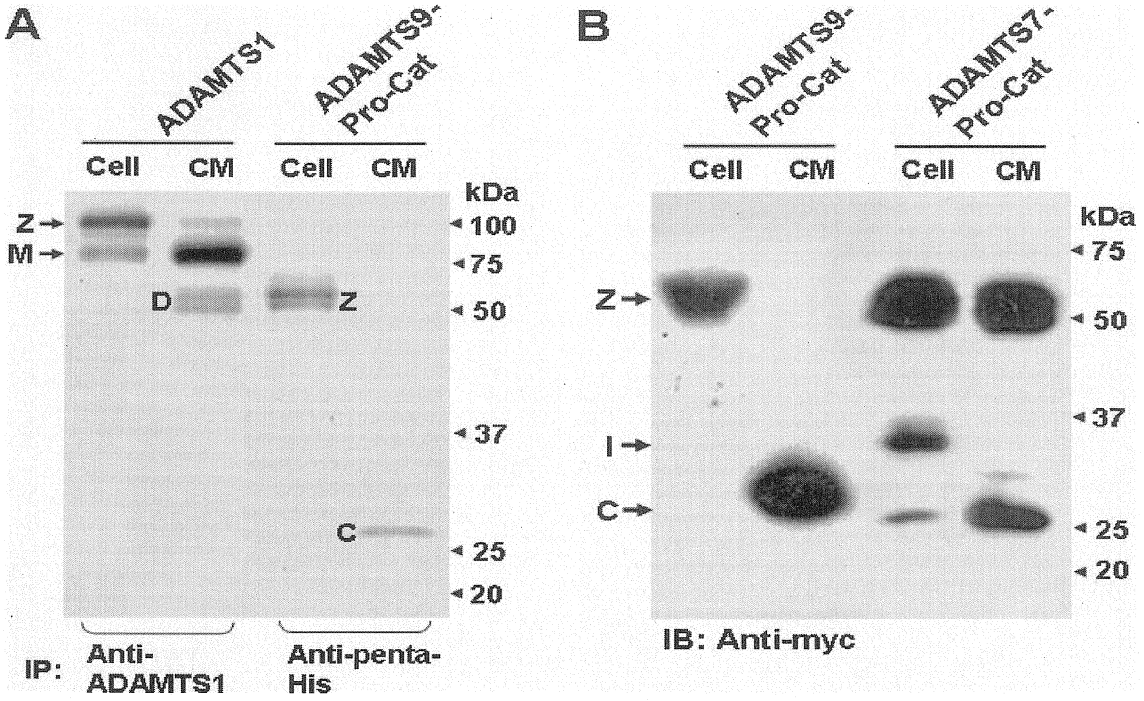
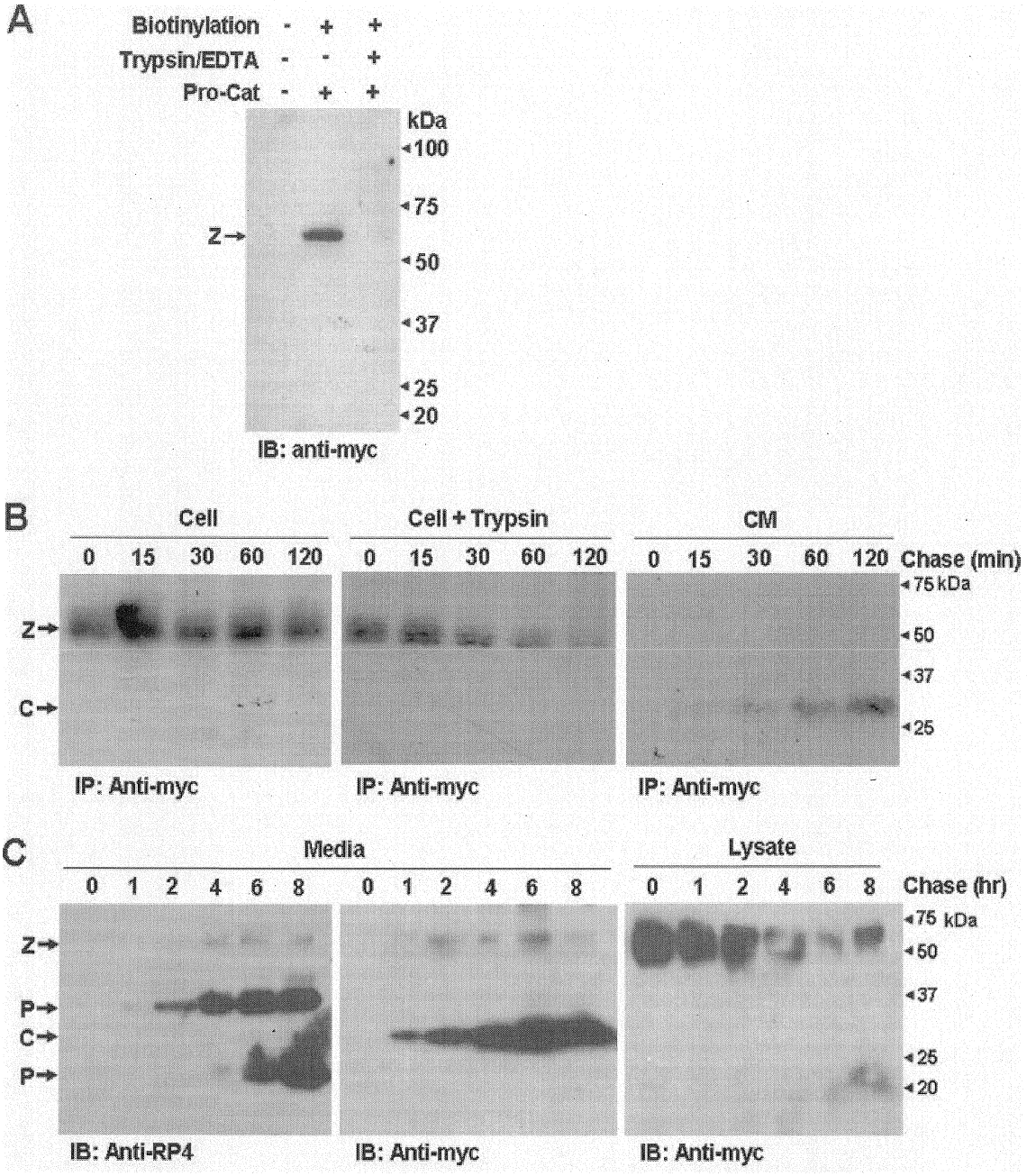


Figure 4. ADAMTS9 is processed differently from ADAMTS1 and ADAMTS7. A, QBI293A cells transiently transfected with ADAMTS1 or ADAMTS9 Pro-Cat were incubated with [^{35}S]Met/Cys for 3 h, followed by immunoprecipitation with anti-ADAMTS1 or anti-penta-His, SDS-PAGE, and fluorography. Note the presence of both the intact zymogen (Z) and mature ADAMTS1 (M) in the cell lysate, whereas only the ADAMTS9 Pro-Cat zymogen (Z) is present in cells and only the catalytic domain (C) in conditioned medium (CM). The 55-kDa ADAMTS1 species (D) represent products of C-terminal proteolysis. B, ADAMTS9 Pro-Cat and ADAMTS7 Pro-Cat were transfected into HEK293F cells followed by immunoblotting of cells and conditioned medium (CM) with anti-Myc. Note that the activated ADAMTS9 catalytic domain (C) is present exclusively in the conditioned medium, whereas the ADAMTS7 catalytic domain (C) is also visible in the cell lysate. An ADAMTS7 processing intermediate (I) is also seen in the cell lysate.

detergent-extracted cells using streptavidin-agarose capture and analyzed by immunoblotting with anti-Myc. As a control, cells were treated with trypsin to remove cell-surface proteins and proteoglycans. Intact Pro-Cat (55 kDa) was present at the surface of HEK293F cells (Fig. 5A). Because biotinylation is very sensitive and may reveal only a small fraction of the total secreted ADAMTS9, we undertook pulse-chase analysis of all newly synthesized protein and followed the labeled protein into the medium. The proportion of cell-surface Pro-Cat at any point during the chase was determined by digesting away cell-surface proteins with trypsin. Over the period of the chase, the total cellular levels of the 55-kDa Pro-Cat zymogen diminished slightly, but the trypsin-sensitive protein accounted for a significant fraction of it 30 min after synthesis and almost all the cellular protein by 2 h (Fig. 5B, left and center panels). This observation was complemented by analysis of the processed catalytic domain in conditioned medium. It was detectable by 30 min after biosynthesis and increased steadily throughout the chase (Fig. 5B, right panel). No zymogen was detected in the conditioned medium. This suggested that the majority of synthesized zymogen was located at the cell surface and that it was processed there prior to release of the catalytic domain into the medium.

To confirm that this was indeed the fate of cell-surface Pro-Cat, biotinylated cells were transferred to fresh medium, and the biotinylated proteins were chased at hourly intervals over an 8-h period in the conditioned medium and cells. As early as 2 h into the chase, detectable levels of processed RP4-reactive propeptide (37 kDa) and Myc-reactive catalytic domain (29 kDa) were detected in the conditioned medium, with a plateau attained at 6 h (Fig. 5C, left and center panels, respectively). Notably, very little intact Pro-Cat was detected in the medium. The levels of cell-surface Pro-Cat gradually declined and were


A
Biotinylation - + +
Trypsin/EDTA - - +
Pro-Cat - + +
kDa
100
75
50
37
25
20
Z
IB: anti-myc
B
Cell
Cell + Trypsin
CM
0 15 30 60 120
Chase (min)
75 kDa
50
37
25
Z
C
IP: Anti-myc
C
Media
Lysate
0 1 2 4 6 8
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P
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IB: Anti-RP4
IB: Anti-myc

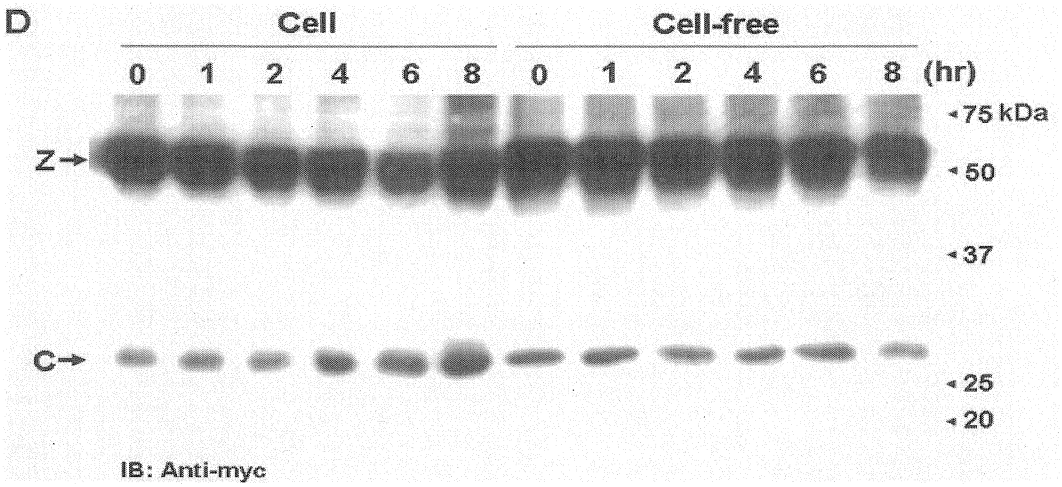


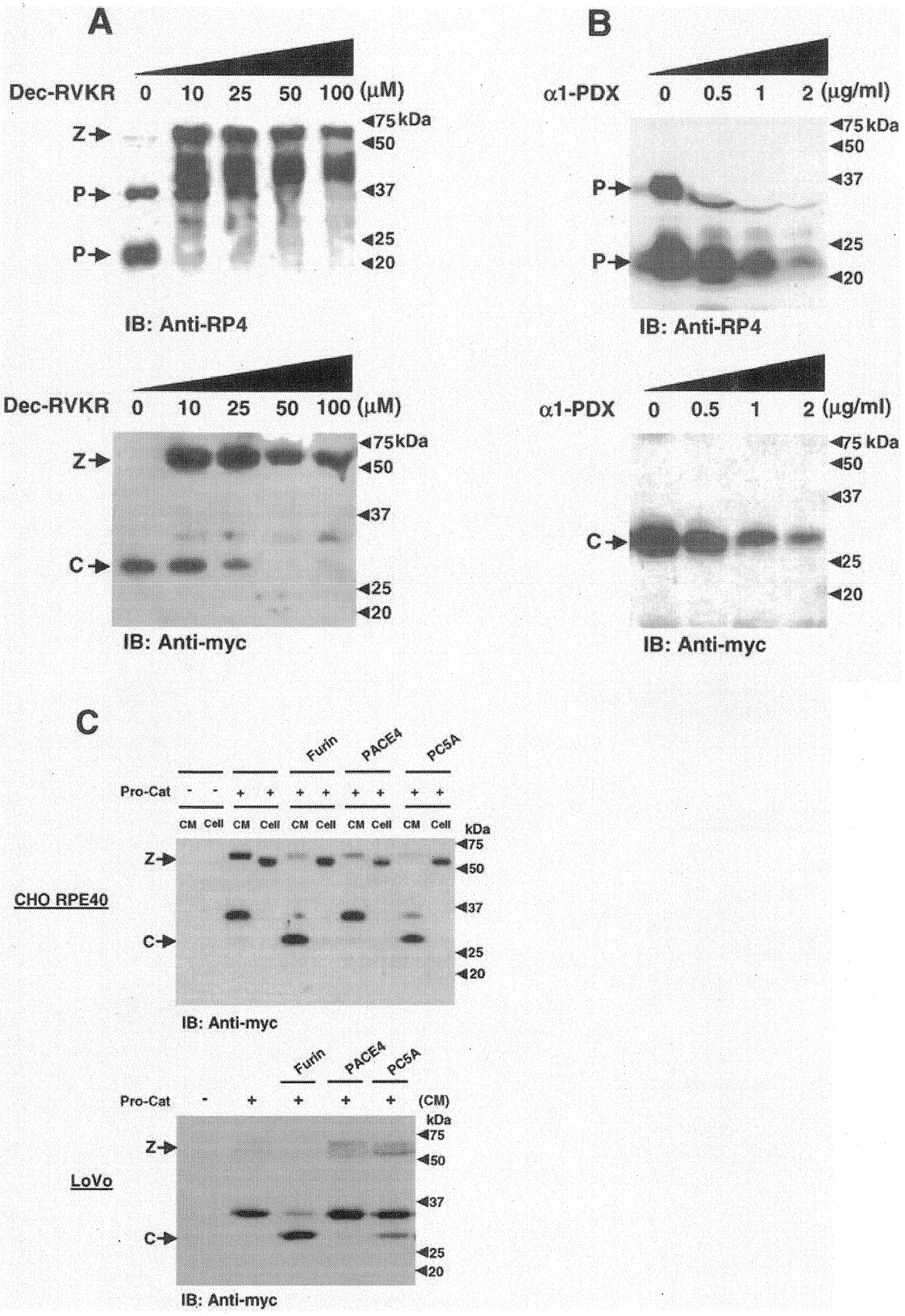
Figure 5. ADAMTS9 Pro-Cat is processed at the cell surface. A, HEK293F cells with stable expression of Pro-Cat were biotinylated as described. Biotinylated proteins were affinity-purified using streptavidin-agarose and analyzed by immunoblotting (IB) with anti-Myc antibodies. The biotinylated protein disappears on trypsin-EDTA treatment of cells, indicating its location at the cell surface. B, Pro-Cat was metabolically labeled and chased over time as described under "Experimental Procedures." Fluorograms of anti-Myc-precipitated protein from total cell lysate (left panel), lysates of cells treated with trypsin (middle panel), and of conditioned medium (right panel) are shown. C, fate of cell-surface biotinylated Pro-Cat in stably transfected HEK293F cells. Cells were biotinylated for 30 min, washed, and returned to fresh medium, and the biotinylated proteins in the medium and cell lysate were captured with streptavidin-agarose. Captured proteins were analyzed by Western blotting at hourly intervals up to 8 h after biotinylation with the antibodies shown below each immunoblot (IB). D, Pro-Cat was purified from cell lysate of transfected HEK293F cells as described under "Experimental Procedures" and incubated with untransfected HEK293F cells or their cell-free medium for varying periods of time as shown followed by Western blotting with anti-Myc.

minimal by 6 h (Fig. 5C, right panel). The 20-22-kDa fragments did not appear until 6 h (Fig. 5C, left panel), suggesting they follow, and may be dependent on, initial PC processing and that the proteolytic activity responsible for these fragments is within the conditioned medium.

To ask whether exogenously added Pro-Cat was processed by the cell-surface proteolytic machinery, Pro-Cat purified from detergent-solubilized cell lysate using nickel-chelating resin was added to untransfected HEK293F cells, and processing was evaluated by Western blotting of the medium. No alteration was seen in conditioned medium incubated in cell-free conditions, but an increase in processing was observed after Pro-Cat was incubated with cells (Fig. 5D).

Proprotein Convertases Mediate Propeptide Processing of ADAMTS9 Zymogen—We previously showed that furin processed ADAMTS9 after Arg^{287} (30). To determine if PCs have an exclusive role in ADAMTS9 zymogen processing, cells were treated with increasing concentrations of the membrane soluble PC inhibitor dec-RVKR-cmk. Processing of Pro-Cat was inhibited at concentrations as low as 10 μM and was essentially abolished by 50 μM (Fig. 6A). In a second approach, HEK293F cells expressing Pro-Cat were treated with increasing amounts of the furin inhibitor, α1-PDX (39), which substantially reduced the processing of Pro-Cat in a 2-h experiment (Fig. 6B). We next investigated which widely expressed PCs associated with the constitutive secretory pathway could mediate processing. When Pro-Cat was expressed in CHO RPE.40 cells, the intact

Figure 6. PC-dependent processing of pro-ADAMTS9. The zymogen (Z), processed propeptide fragments (P), and processed catalytic domain (C) are indicated. A, dose-dependent inhibition of Pro-Cat processing by the PC inhibitor dec-RVKR-cmk. Cells were treated with 0-100 nM inhibitor, and conditioned medium (CM) was analyzed by Western blotting using anti-RP4 or anti-Myc. Note decreasing pro-domain and catalytic domain fragments with increasing concentration of inhibitor. The precise identity of the molecular species migrating between 40 and 50 kDa is unknown, but presumably represents processing intermediates. B, HEK293F cells stably transfected with Pro-Cat were incubated for 2 h with increasing concentrations of α1-PDX as shown. Immunoblotting of conditioned medium showed a dose-dependent reduction of zymogen processing. C, analysis of Pro-Cat processing by furin, PACE4 and PC5A in CHO RPE.40 cells. Pro-Cat was transfected alone or co-transfected with furin or other PCs in furin-deficient CHO RPE.40 cells (top panel) or LoVo cells (bottom panel). Western blotting was done with anti-Myc. Note that, when seen in the CM, the zymogen is consistently 1-3 kDa larger than the main band in cell lysate, indicating the addition of complex terminal carbohydrate structures. Notice significant processing of the zymogen in CM from the furin- and PC5A-transfected cells but not PACE4-transfected cells.


A
Dec-RVKR 0 10 25 50 100 (μM)
Z
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IB: Anti-RP4
Dec-RVKR 0 10 25 50 100 (μM)
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IB: Anti-myc
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α1-PDX 0 0.5 1 2 (μg/ml)
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IB: Anti-myc
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PACE4
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CM Cell CM Cell CM Cell CM Cell CM Cell
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IB: Anti-myc
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IB: Anti-myc

55-kDa zymogen was detectable in the conditioned medium and cell lysate, and no fully processed catalytic domain was detected in conditioned medium (Fig. 6C, upper panel). When furin was co-transfected with Pro-Cat, the 29-kDa processed catalytic domain appeared in the conditioned medium (Fig. 6C, upper panel). Transfection of PC5A (Fig. 6C, upper panel), and PC7 (data not shown) also led to appearance of the 29-kDa His-tagged catalytic domain in the conditioned medium, but not in cell lysate. On the other hand, PACE4 did not produce the fully processed catalytic domain (Fig. 6C, upper panel). When similar experiments were conducted in LoVo cells, the outcomes were comparable to those in CHO RPE.40 cells (Fig. 6C, lower panel). The ability of PACE4 to process Pro-Cat efficiently to a 35-kDa Myc-reactive fragment argues for the presence of a PACE4-susceptible site in the propeptide, possibly, Arg-Arg^{209}-↓Ser, but argues that PACE4 does not process ADAMTS9 following Arg^{287}. Overall, the most efficient conversion was by furin (Fig. 6C).

Furin and ADAMTS9 Are Part of a Complex at the Cell Surface—Because all the above evidence suggested that ADAMTS9 was processed at the cell surface by furin, we asked whether furin was present at the surface of HEK293F cells. Using the same biotinylation approach used to identify ADAMTS9 zymogen at the cell surface, we could also show the presence of furin at the cell surface (Fig. 7A). To ask if furin and Pro-FLAG-Cat existed within a complex, we first cross-linked cell-surface proteins using a reducible cross-linker and immunoprecipitated cellular proteins using anti-FLAG M2 agarose. Furin could be co-immunoprecipitated with FLAG-tagged ADAMTS9 Pro-Cat suggesting its existence as part of a cell-surface complex that also contains ADAMTS9 (Fig. 7B). However, efforts to immunoprecipitate furin and ADAMTS9 Pro-Cat in solution or without cross-linking were

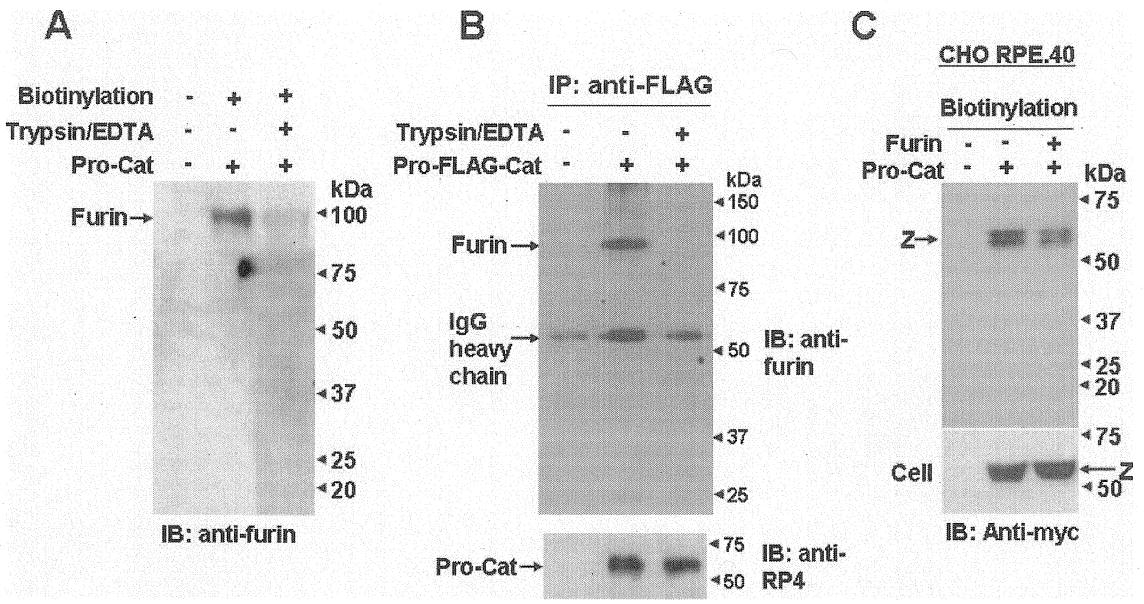

Figure 7. Furin and ADAMTS9 Pro-Cat are part of a complex at the cell surface. A, HEK293F cells with stable expression of Pro-Cat were biotinylated as described under "Experimental Procedure." Biotinylated proteins were affinity-purified using streptavidin-agarose and analyzed by immunoblotting (IB) with anti-furin. The biotinylated furin disappears on trypsin treatment indicating its location at the cell surface. B, furin and Pro-Cat associate at the cell surface of HEK293F cells. Cells transiently expressing Pro-FLAG-Cat were immunoprecipitated with anti-FLAG after treatment with a reducible, cell-impermeable cross-linking agent, and immunoblotting was done using an anti-furin monoclonal antibody. Immunoprecipitated protein was not seen in cells treated with trypsin-EDTA prior to lysis. The bottom panel shows amounts of Pro-Cat in the immunoprecipitated protein of trypsinized or untrypsinized cells. C, conditioned medium (upper panel) and cell lysate of CHO RPE.40 cells expressing Pro-Cat with or without furin were analyzed by cell-surface biotinylation (upper panel) or Western blotting with anti-Myc. Co-transfection with furin leads to decreased cell-surface zymogen. The lower panel shows comparative levels of Pro-Cat.

unsuccessful (data not shown), suggesting that furin was not directly responsible for retention of Pro-Cat at the cell surface. Furthermore, when furin was co-expressed with Pro-Cat in CHO RPE.40 cells, there was not an increase in cell-surface Pro-Cat that would be expected if furin was directly responsible for its sequestration. Instead, the cell-surface ADAMTS9 Pro-Cat was decreased when co-expressed with furin, suggesting that furin contributed directly to processing but not sequestration at the cell surface (Fig. 7C).

Furin Is Essential for ADAMTS9 Propeptide Processing at the Surface of HEK293F Cells—Because the gain-of-function experiments that were done by transfecting furin-deficient cells suggested that furin could most efficiently process the ADAMTS9 propeptide, we asked whether it is responsible for the processing in HEK293F cells in a loss-of-function experiment. To test whether reduction of furin levels in HEK293F cells leads to reduction in cell-surface processing, the cells were transfected with furin siRNA, which significantly reduced total cellular furin (Fig. 8A). In these cells, neither the total amount of ADAMTS9 Pro-Cat nor the total level of cell-surface Pro-Cat were altered (Fig. 8B). However, in contrast to cells that were not siRNA-treated, the conditioned medium contained a significant amount of unprocessed zymogen (Fig. 8C).

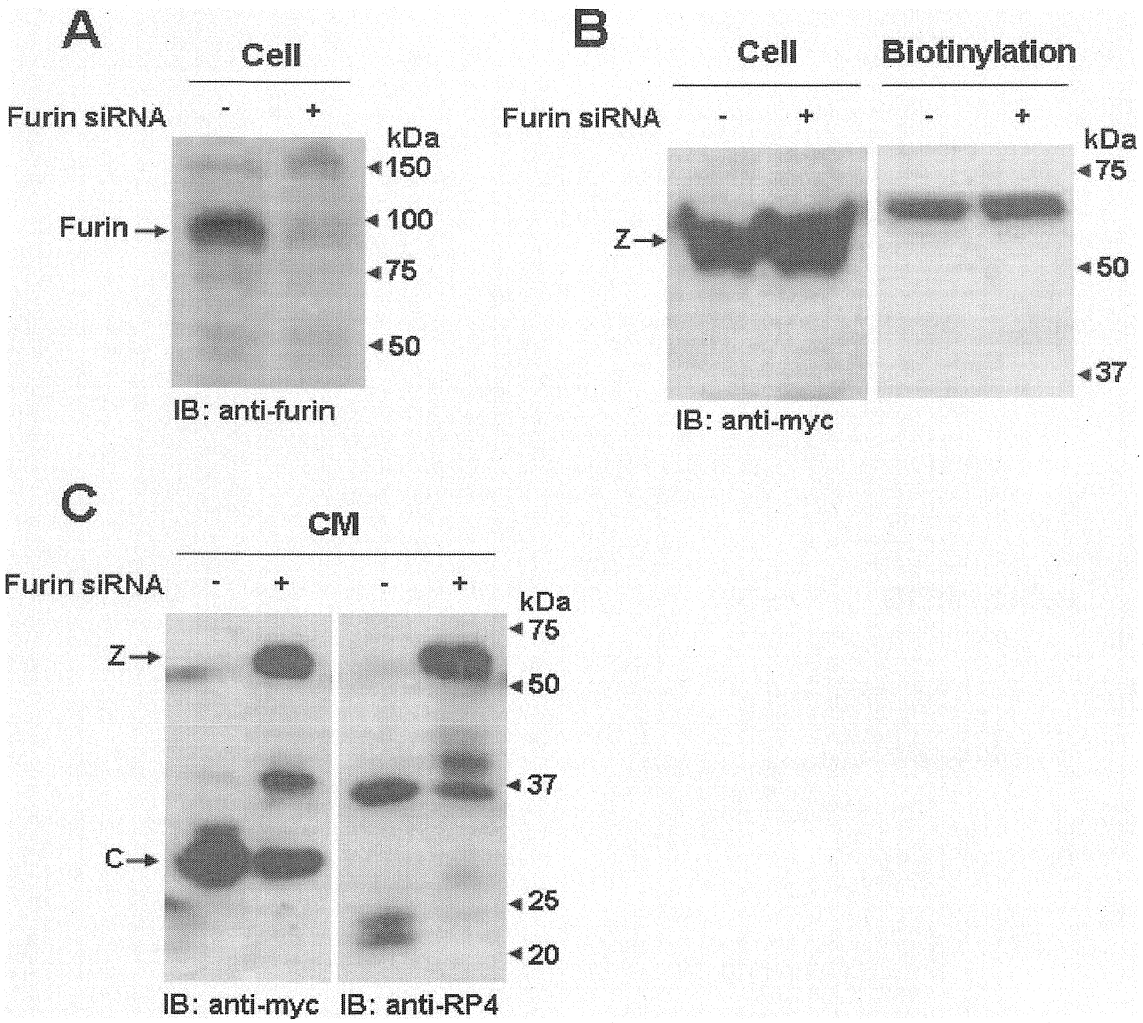

Figure 8. Silencing of HEK293F furin expression suppresses ADAMTS9 Pro-Cat processing. A, furin siRNA leads to suppression of cellular furin. Western blot of cell lysate was with anti-furin monoclonal antibody. B, transfection with furin siRNA does not affect either the total cellular levels of Pro-Cat or the amount located at the cell surface. The panel on the left shows the total cellular Pro-Cat. The panel at right shows cell-surface Pro-Cat labeled by biotinylation. C, expression of furin siRNA suppresses processing of ADAMTS9 Pro-Cat. Note that cells with suppressed furin levels generate mostly the 55-kDa zymogen and little of the processed propeptide and catalytic domain.

DISCUSSION

We previously investigated PC processing of several ADAMTS zymogens (11, 13, 28, 30, 40). The absence of mature ADAMTS9 in cell lysates that was noted in a previous study reporting the complete primary structure of ADAMTS9 (30) led us to carry out the present comprehensive analysis of its maturation. We examined ADAMTS9 propeptide processing using various approaches, none of which provided any evidence for intracellular processing. Both gain-of-function and loss-of-function approaches supported the conclusion that furin processes the ADAMTS9 propeptide. Furthermore, the data suggest that the cell surface is a major site of ADAMTS9 propeptide processing in HEK293F cells or cells that express high levels of cell-surface-processing activity. In contrast, cells expressing no furin activity (CHO RPE.40 or LoVo) or lower levels of furin than HEK293F cells (e.g. COS-1 and CHO-K1) secrete varying amounts of unprocessed pro-ADAMTS9. Whether pro-ADAMTS9 is processed at the cell surface or potentially in the extracellular space, therefore, appears to depend on the relative quantity of furin or other PCs present at these locations. Although CHO RPE.40 and LoVo cells may express other PCs, they processed ADAMTS9 inefficiently. Indeed, the widespread expression of ADAMTS9 (32) is compatible with physiological processing by furin, because this is a ubiquitously distributed convertase. However, there is overlap of ADAMTS9 expression (32) with PC5A during mouse development (17) suggesting that processing by this enzyme may occur *in vivo* as it did in co-transfection experiments.

These data raised the issue of whether or not furin might directly mediate cell-surface binding of ADAMTS9. We argue that it does not. When transfected into CHO RPE

40 cells, furin does not increase the amount of cell-surface zymogen, as one might expect if it contributed significantly to cell-surface binding, but decreases it, presumably because it processes ADAMTS9, and the processed forms are released. In addition, although we could co-precipitate furin and ADAMTS9 after chemical cross-linking of cell-surface molecules, we could not demonstrate an interaction between them without cross-linking. This suggests the existence of a cell-surface complex that contains furin, ADAMTS9, as well as other molecules. However, because unprocessed zymogen is ultimately released into the medium in cells with low levels of furin activity, the binding is likely to be of low affinity. Significantly, neither the cleaved propeptide nor the processed catalytic domain were ever detected at the cell surface. This suggests that once processed, by furin, the cell-surface interactions in which the Pro-Cat zymogen participates are abruptly disrupted. Thus, properties of both the propeptide and the catalytic domain, or the junctional region between them might be crucial in cell-surface binding of this construct.

We propose that cell-surface interactions of ADAMTS9 Pro-Cat could be one way of targeting its proteolytic activity to the pericellular space. Although the function of ADAMTS9 is presently unknown, it is present at the cell surface in cultured cells (30), and its homologs are known to be involved in cell migration (41, 42), which typically requires cell-surface proteolysis (43). Thus, retention of the propeptide may be a mechanism for sequestration of ADAMTS9 at the cell surface, although the ADAMTS9 ancillary domain is also likely to have a role in this context (30).

Traditionally, PCs have been shown to cleave their substrates intracellularly. This is particularly true for furin, the best known member of this protease family (1). Molecular

shedding events that take place at the cell surface are mostly attributable to PC-activated cell-surface proteases such as the ADAMs and membrane-type-MMPs (44, 45). Furin is known to exist at the cell surface (19, 46) and other PCs such as PACE4 and PC6/5A are known to be secreted and anchored in the extracellular matrix (47) and are therefore presumed to have extracellular substrates. Protective antigens of anthrax and diphtheria toxin are cleaved at consensus furin cleavage sites on the exterior of the cell, as well as in intracellular organelles (20, 22). This process nevertheless differs from cell-surface processing of endogenously produced proproteins. Previously, biosynthetic analysis of the extracellular matrix protein fibrillin-1 had suggested that furin processing of profibrillin-1 did not occur intracellularly, although the precise location of processing was not identified (34). ADAMTS9, to our knowledge, is thus the first cellular protein shown to be selectively processed by furin at the cell surface.

Why is the ADAMTS9 propeptide not excised in the TGN? The reasons for this are presently unknown and are the subject of continuing investigation in our laboratory. It is possible that the processing sites in the propeptide may be conformationally inaccessible to PCs intracellularly or masked by molecular chaperones. Once exported from the cell, the zymogen may undergo a conformational change or detach from chaperones and enable furin processing. Although the processing mechanisms of all 19 ADAMTS proteases have yet to be elucidated, the observations reported here and in some other ADAMTS proteases suggest that the constitutive intracellular processing model is not universally applicable to this family. Instead, ADAMTS proteases exhibit a spectrum of activation mechanisms that includes classic intracellular processing (ADAMTS1 and ADAMTS4) (13, 14), cell-surface processing (ADAMTS9), a combination of intracellular and cell-surface processing

(ADAMTS7) (28), and possibly a combination of cell-surface and matrix-associated processing (ADAMTS10) (40). In fact, even in furin-rich HEK293F cells, a significant proportion of ADAMTS10 appears to be secreted as the unprocessed zymogen (40). Beyond ADAMTS proteases, however, the broader significance of these results is that they offer fundamental insights on mechanisms of molecular maturation and suggest that cell-surface processing of proproteins by furin and other PCs may be more widely prevalent than hitherto suspected.

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The abbreviations used are: PC, proprotein convertase; MMP, matrix metalloprotease; TGN, trans-Golgi network; Pro-Cat, signal peptide, propeptide, and catalytic domain; cmk, chloromethyl ketone; PNGase F, peptide *N*-glycosidase F; siRNA, small interference RNA; CHO, Chinese hamster ovary cells; ADAM, a disintegrin and metalloprotease domain; ADAMTS, a disintegrin and metalloprotease domain with thrombospondin type 1 repeats.

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ARTICLE 5 – AVANT-PROPOS

Regulation of ADAMST9 secretion and enzymatic activity by its propeptide

Koo, B. H., Longpré, J.-M., Somerville, R. P., Alexander, P., Leduc, R. & Apte, S. S.

J. Biol. Chem. 282(22):16145-16154. (2007)

Contribution: Notre étroite collaboration avec le groupe du Dr. Suneel Apte a permis la réalisation de cet article. J'ai été impliqué dans l'élaboration de la démarches scientifiques menant aux conclusions. J'ai réalisé les expériences menant à la figure 3. J'ai construit les mutants du prodomaine de ADAMTS9 Pro-cat de la figure 5B. Mes commentaires et suggestions ont été incorporés à l'écriture du manuscrit jusqu'à la version publiée.

Résumé de l'article 5

ADAMTS9 est une métalloprotéinase sécrétée se liant à la surface cellulaire et clivant les protéoglycans versican et aggrécan. Contrairement à la majorité des précurseurs protéiques, le zymogène pro-ADAMTS9 est résistant à la maturation protéolytique intracellulaire. Pro-ADAMTS9 est plutôt clivé par la furine à la surface cellulaire. Dans cette étude, nous avons investigué le rôle du prodomaine dans la régulation de la sécrétion ainsi que l'activité protéolytique d'ADAMTS9. L'expression d'une forme tronquée d'ADAMTS9 sans son prodomaine a abolit sa sécrétion et l'expression du prodomaine *en trans* n'a pu restaurer sa sécrétion. De plus, la substitution des acides aminés Asn pour Ala des trois sites consensus de N-glycosylation dans le prodomaine a diminué la sécrétion d'ADAMTS9. Donc, le prodomaine agit en tant que chaperone intramoléculaire dont la glycosylation est essentielle pour la sécrétion de l'enzyme. En plus des deux sites de clivage par la furine déjà identifiés ($Arg^{74}\downarrow$ et $Arg^{287}\downarrow$), le prodomaine de ADAMTS9 est aussi clivé par la furine à l'Arg^{209}. La substitution pour une Ala des Arg^{74}, Arg^{209} et Arg^{287} simultanément résulte en un zymogène sécrété ne pouvant être clivé par la furine. Les cellules exprimant ce zymogène pro-ADAMTS9 ont une meilleure activité protéolytique envers le versican que la forme de type sauvage. En outre, les cellules traitées avec un inhibiteur des pro-protéines convertases, dec-Arg-Val-Lys-Arg-cmk, ont une plus grande activité versicanase que les cellules non traitées. Suite à la maturation par la furine du précurseur pro-ADAMTS9, le prodomaine clivé maintient une association non-covalente avec le domaine catalytique. En prenant compte de ces observations, le clivage du prodomaine d'ADAMTS9 réduit l'activité catalytique de celle-ci, contrairement aux autres

métalloprotéinases. En conclusion, le prodomaine joue un rôle primordial dans la régulation du mécanisme non conventionnel d'ADAMTS9 qui a dû évoluer afin d'assurer l'activité catalytique de cette protéinase à la surface cellulaire.

Regulation of ADAMTS9 secretion and enzymatic activity by its propeptide

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Running title: ADAMTS9 propeptide

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ABSTRACT

ADAMTS9 is a secreted, cell-surface binding metalloprotease that cleaves the proteoglycans versican and aggrecan. Unlike most precursor proteins, the ADAMTS9 zymogen (proADAMTS9) is resistant to intracellular processing. Instead, proADAMTS9 is processed by furin at the cell-surface. Here, we investigated the role of the ADAMTS9 propeptide in regulating its secretion and proteolytic activity. Removal of the propeptide abrogated secretion of the ADAMTS9 catalytic domain and secretion was inefficiently restored by expression of the propeptide *in trans*. Substitution of Ala for Asn residues within each of three consensus N-linked glycosylation sites in the propeptide abrogated ADAMTS9 secretion. Thus, the propeptide is an intramolecular chaperone whose glycosylation is critical for secretion of the mature enzyme. In addition to two previously identified furin-processing sites ($Arg^{74}\downarrow$ and $Arg^{287}\downarrow$) the ADAMTS9 propeptide was also furin-processed at Arg^{209}. Substitution of Ala for Arg^{74}, Arg^{209} and Arg^{287} resulted in secretion of an unprocessed zymogen. Unexpectedly, versican incubated with cells expressing this proADAMTS9 was processed to a greater extent than when incubated with cells expressing wild-type, furin-processable ADAMTS9. Moreover, cells and medium treated with the proprotein convertase inhibitor dec-Arg-Val-Lys-Arg-cmk had greater versican-cleaving activity than untreated cells. Following furin processing of proADAMTS9, propeptide fragments maintained a non-covalent association with the catalytic domain. Collectively, these observations suggest that unlike other metalloproteases, furin processing of the ADAMTS9 propeptide reduces its catalytic activity. Thus, the propeptide is a key functional domain of ADAMTS9, mediating an

unusual regulatory mechanism that may have evolved to ensure maximal activity of this protease at the cell surface.

INTRODUCTION

ADAMTS proteases have critical roles in many biological processes and in inherited and acquired human disorders (1-5). The 19 enzymes of this family share a conserved organization comprising an N-terminal metalloprotease domain and a C-terminal ancillary domain which contains the thrombospondin type 1 repeats (TSR) that are the hallmark of the family (6). ADAMTS9 is the largest enzyme of the family, containing 15 TSRs, and its mRNA is widely expressed during embryonic development and in adult tissues (7,8). In previously published work, we showed that when expressed in COS-1 or HEK293F cells, ADAMTS9 was located at the cell surface or within pericellular matrix (8), suggesting that despite the lack of a membrane anchor, it could be considered as an operational cell-surface protease. ADAMTS9 can cleave the large aggregating proteoglycans aggrecan and versican (8), suggesting a role in turnover of extracellular matrix. Thus, like its *C.elegans* ortholog, *Gon-1*, which is required for cell-migration during gonadal morphogenesis (9), it is possible that ADAMTS9 participates in extracellular proteolysis of matrix or cell surface molecules.

The ADAMTS9 protease domain contains an N-terminal propeptide, which, as in most zymogens, is believed to maintain enzyme latency and to be proteolytically excised to permit catalytic activity, a process termed "activation". The ADAMTS9 propeptide contains a number of predicted furin-processing sites, and as in many metalloproteases, propeptide excision is mediated by proprotein convertases (PCs) such as furin. We previously made the unexpected observation that proADAMTS9 was resistant to processing by furin within the secretory pathway but was processed by furin at the cell

surface (10). Although a C-terminally truncated zymogen containing only the propeptide and catalytic domain of proADAMTS9 (Pro-Cat) was readily detectable at the cell-surface, the furin-processed propeptide and catalytic domain fragments of the zymogen were never found at the cell surface, but instead, were readily detected in the medium. This suggested that the contiguous propeptide and catalytic domain were essential for cell-surface binding, and that furin-processing either disrupted a cell-binding site or led to a conformational change that resulted in loss of cell-surface binding. Thus, the ADAMTS9 propeptide can potentially contribute both to regulation of ADAMTS9 activity and to its localization. In the present work, we continued our investigation into the role of the propeptide in regulating the secretion and activity of ADAMTS9. The results provide further insights on ADAMTS9 propeptide cleavage by proprotein convertases, and demonstrate an unexpected role for the ADAMTS9 propeptide in regulation of enzyme activity.

EXPERIMENTAL PROCEDURES

ADAMTS9 Expression Plasmids And Site-Directed Mutagenesis— Plasmids for the expression of full-length ADAMTS9, or only the signal peptide, propeptide and catalytic domain (Pro-Cat) with C-terminal myc and His tags were previously described (8,10). New constructs made for the present studies are shown, as appropriate, in Figures 1-3. To make an expression plasmid encoding only the propeptide, site-directed mutagenesis was used to convert the P_1 Arg of the major furin cleavage site to a stop codon (Arg^{287} → Stop) using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA). cDNA encoding the catalytic domain without the prepropeptide (Phe^{288} → Ser^{501}) was amplified using Pro-Cat as a template with primers 5′-TCGATTTTTTATCCTATCCACGGTTTGTAGAAGTCTTG-3′ (with the *Cla* I site underlined) and 5′-GGATCC*TCA*GGATTCAGGTTCGTTAAGCAAAC-3′ (*BamH* I site underlined and introduced stop codon italicized) and cloned into pFLAG-CMV-1™ (Sigma Chemical Co. St. Louis, MO) for expression with a preprotrypsin leader sequence and N-terminal FLAG tag. A plasmid for expression of ADAMTS9 from the N-terminus to the linker 2 peptide (residues 1-1326), named ADAMTS9(N-L2) was generated by PCR and cloned into pcDNA3.1(-)/Myc-his A (Invitrogen, Thousand Oak, CA) for expression with a C-terminal myc and 6X His tag. Two additional constructs were made for expression of mature ADAMTS9 catalytic domain and ADAMTS9 (N-L2) with its endogenous signal peptide, but without the propeptide, by subjecting Pro-Cat and ADAMTS9 (N-L2) cDNAs to consecutive rounds of site-directed mutagenesis. In the first round of mutagenesis, an NheI site was inserted between the propeptide and catalytic domain of each plasmid. In the second mutagenesis step, an NheI site was inserted between the signal peptide and

propeptide of each plasmid. NheI was used to excise the propeptide lying between these two sites and the plasmid ends were ligated to obtain constructs that could express the respective polypeptides lacking the propeptide. N-glycosylation sites within the propeptide of Pro-Cat were substituted with Ala (Asn^{112} → Ala, Asn^{135} → Ala, Asn^{271} → Ala, and $Asn^{112, 135, 271}$ → Ala). A putative PC processing site (Arg^{209}) was converted to Ala by site-directed mutagenesis. Full-length ADAMTS9 that was maturation-deficient (ADAMTS9 $Arg^{74+209+287}$→Ala) and the active site mutant Glu^{435}→Ala were generated by site-directed mutagenesis of the full-length ADAMTS9 expression plasmid as above. All these mutant plasmids were sequence-verified.

Cell Culture—HEK293F cell lines stably transfected with Pro-Cat were maintained as described previously (8,10). Transient transfections with various plasmids were done using FuGENE 6 as per manufacturer's recommendations (Roche Diagnostics, Basel, Switzerland). For protein analysis, transfected HEK293F or QBI293A cells were cultured to confluence in 6-well plates and changed to 293 SFM-II medium (Invitrogen, Carlsbad, CA) for analysis of secreted protein.

Antibodies, Western blot And Immunoprecipitation—The RP4 antibody (Abcam, Cambridge, MA) to the ADAMTS9 propeptide was characterized previously and detects an undisclosed peptide epitope (10). Anti-myc monoclonal antibody 9E10 (Invitrogen) and anti-FLAG M2 monoclonal antibody (Sigma-Aldrich) were obtained from commercial sources. Western blotting was done following reducing or non-reducing SDS-PAGE, using appropriate horseradish peroxidase-conjugated secondary antibodies and enhanced

chemiluminescence (ECL, Amersham-GE Biosciences Healthcare Corp, Piscataway, NJ) for detection. Conditioned medium of stably transfected HEK293F cells was immunoprecipitated with anti-myc agarose (Sigma-Aldrich) or with anti-FLAG agarose (Sigma-Aldrich) as a control followed by reducing SDS-PAGE and immunoblotting with anti-RP4 antibody or anti-myc antibody. For analysis of N-glycosylation mutants, QBI-293A cells were transiently transfected with wild-type Pro-Cat or the various mutants, metabolically labeled with [^{35}S]-Met/Cys mixture for 3 hours, followed by immunoprecipitation with anti-myc, reducing SDS-PAGE and fluorography, all as previously described (10,11).

Cross-Linking And Cell Surface Biotinylation — Conditioned medium was collected from untransfected HEK-293F cells (control) or HEK-293 F cells stably expressing Pro-Cat and dialyzed against phosphate-buffered saline without subsequent concentration. The dialyzed medium was incubated with the thiol-non-cleavable amine-reactive cross-linker BS^3 (Bis(sulfosuccinimidyl)suberate) (Pierce) (final concentration 2mM according to manufacturer's recommendation) for 30 min on ice. The functional groups in this cross-linker are spaced 11.4 °A apart. After blocking unused free functional groups with 50mM Tris-HCl (pH7.5) for 5 min, aliquots of the conditioned medium were analyzed by reducing SDS-PAGE, and western blotting with appropriate antibodies. Cell surface biotinylation of cells was done on ice as previously described (10,12).

Versican Processing Assays —Conditioned medium of skin fibroblasts from a patient with Marfan syndrome (provided by Dr. Alana Majors, Lerner Research Institute, without

identifiers) was used as an enriched source of versican. HEK293F cells were transfected with full-length ADAMTS9, ADAMTS9 Glu^{435}→Ala, or ADAMTS9 $Arg^{74+209+287}$→Ala expression vectors, cultured to confluence in 6-well plates and 1 ml of conditioned medium from the skin fibroblasts was added to the transfected cells. Following 24 hrs incubation, 100 µl of the medium was digested with chondroitinase ABC (Sigma), precipitated with acetone, and analysed by Western blotting using Versican V0/V1 neo-epitope antibody (anti-DPEAAE, also referred to as JSCDPE) (Affinity Bioreagents, Golden, CO) as previously described (8). The same approach was used to analyze versican processing by HEK293F cells transfected with full-length ADAMTS9 but in the presence of 50 µM dec-RVKR-cmk (Calbiochem, San Diego, CA) to prevent proADAMTS9 processing. A variation of this assay using conditioned medium was also developed. HEK293F cells were transfected with full-length ADAMTS9, ADAMTS9 Glu^{435}→Ala, or ADAMTS9 $Arg^{74+209+287}$→Ala expression vectors, and cultured to confluence in 6-well plates. The cells were changed to 293 SFM-II medium (Invitrogen) and incubated in the presence or absence of 50µM deca-RVKR-cmk for a further 24 h. The conditioned medium was collected and incubated with versican-rich medium from Marfan skin fibroblasts at a 1:1 ratio at 37°C for 24 h. The incubated medium was analyzed for versican proteolysis using Western blot analysis as described above. The ADAMTS9 in conditioned medium was evaluated by western blotting using anti-RP4.

RESULTS

ADAMTS9 Catalytic Domain Lacking The Propeptide Is Not Secreted From Cells-We previously showed that both full-length ADAMTS9 and Pro-Cat (Fig. 1A) were efficiently processed by furin at the surface of HEK293F cells. In the case of Pro-Cat, a 29kDa unglycosylated catalytic domain (reactive with anti-myc) and 37kDa, ~22 kDa and ~20 kDa propeptide fragments (reactive with anti-RP4) were readily detectable in the conditioned medium without concentrating it (Fig. 1B). Because of the high furin content of HEK293F cells, we previously showed that very little intact zymogen was secreted into conditioned medium, and moreover, no processed fragments were present at the cell surface, indicating their immediate release upon furin processing (10). Since furin-processed full-length ADAMTS9 is difficult to detect in conditioned medium because of its large size and C-terminal processing which removes the myc-tag, we studied the biosynthetic profile of a shorter form of the precursor, ADAMTS9 (N-L2) (Fig. 1A). This precursor was detected in transfected HEK293F cells as a single 125 kDa species reactive with both anti-myc and anti-RP4 (Fig. 1C), whereas the furin processed Cat-L2 (~100 kDa) and the 37 kDa, 22 kDa and 20 kDa propeptide fragments were seen in conditioned medium using anti-myc and anti-RP4 respectively (Fig. 1C). To examine the role of the propeptide in regulating secretion, a construct for expression of the catalytic domain alone with a preprotrypsin signal peptide and N-terminal FLAG tag, was generated (Fig. 2A). The FLAG-tagged catalytic domain was not secreted into the conditioned medium of HEK293F cells although it was present in the cell lysate (Fig. 2A, left panel). However, the propeptide alone ($ADAMTS9_{1\text{-}286}$) was efficiently secreted into the conditioned medium, and underwent furin processing to generate fragments similar to those previously seen upon Pro-Cat

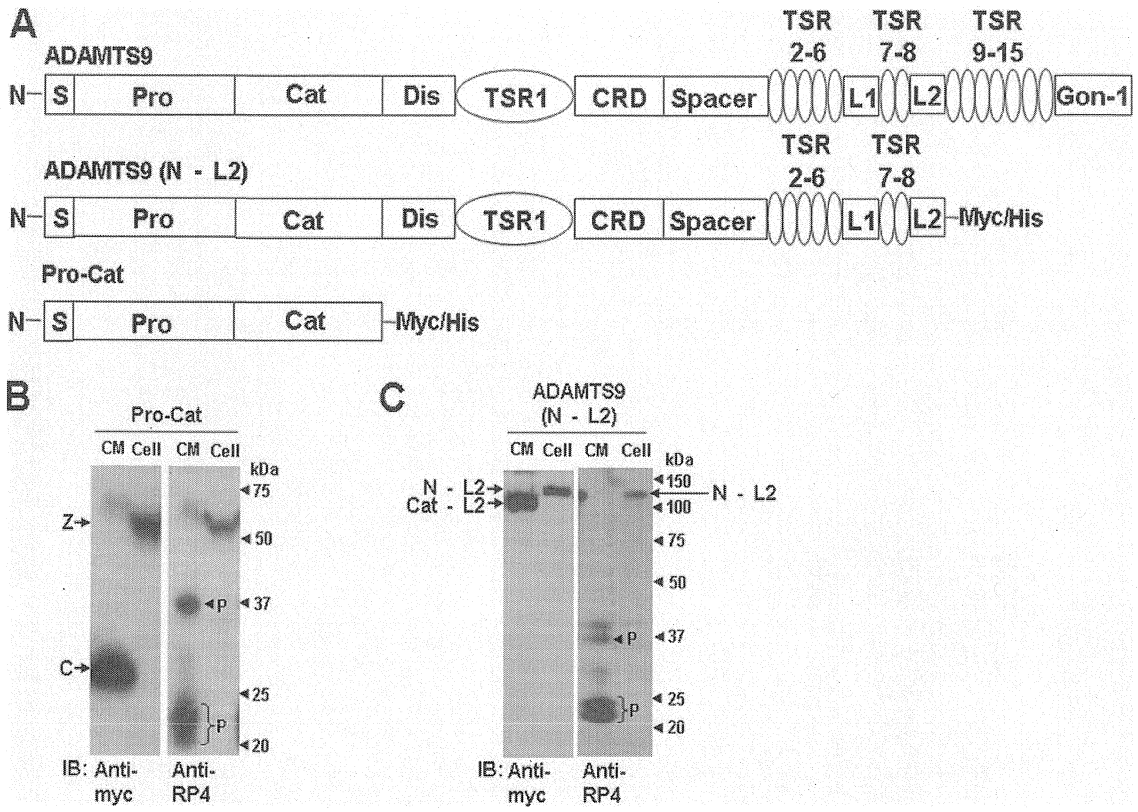
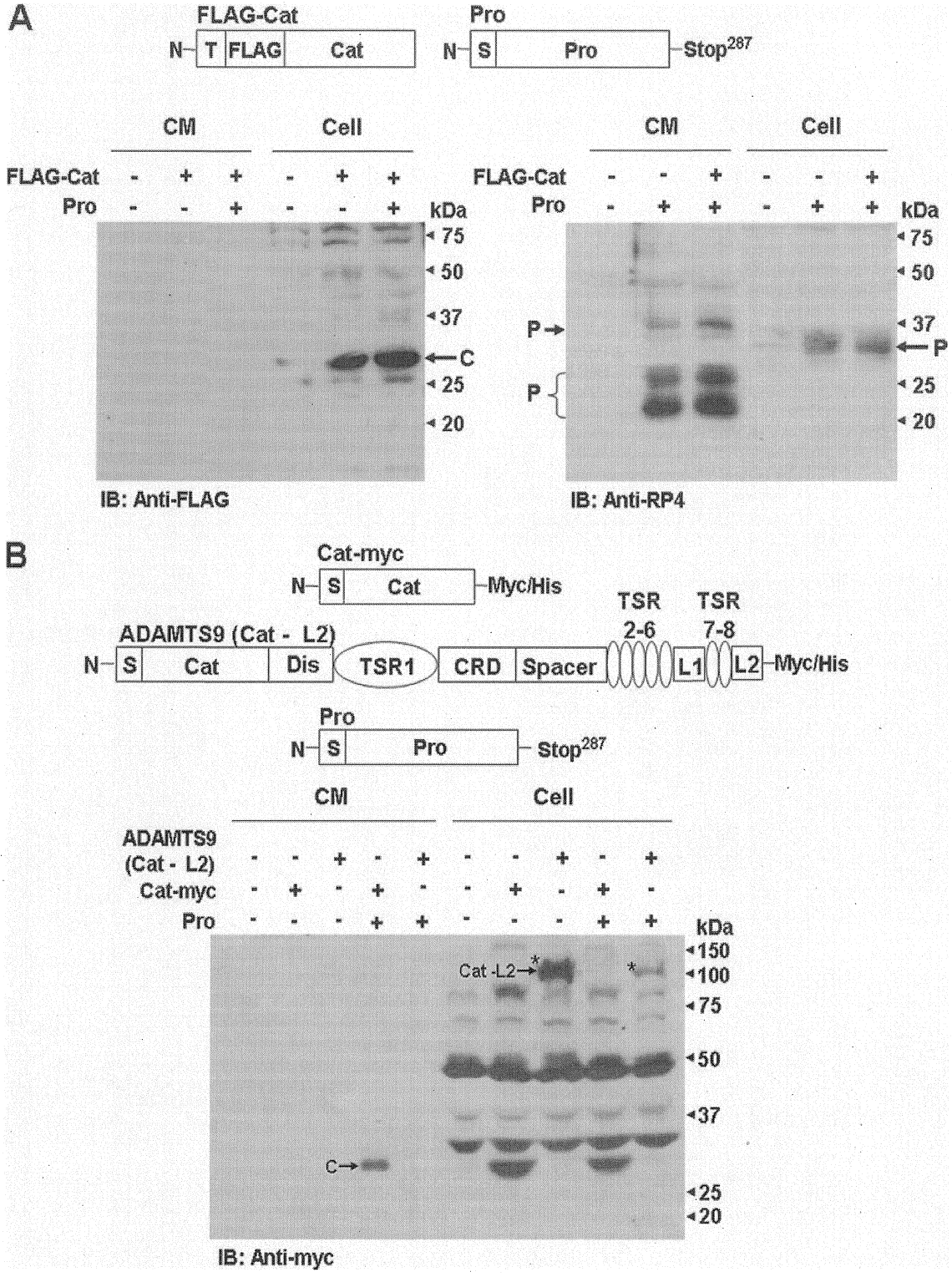


Figure 1. A. Characterization of the major ADAMTS9 constructs used in the present studies. The uppermost drawing shows the domain structure of full-length ADAMTS9. The various modules are represented as S, signal peptide; Pro, propeptide; Cat, catalytic domain; Dis, disintegrin-like module; TSR, thrombospondin type 1 repeat; CRD, cysteine-rich domain; L1 and L2, linker segments 1 and 2; Gon-1, the conserved C-terminal module shared with *C. elegans* Gon-1.**B**,**C**. Characterization of the major molecular entities analyzed in the manuscript. Pro-Cat (B) or ADAMTS9 (N-L2) (C) were expressed in HEK293F cells and immunoblotting of cell lysate or medium was done using anti-myc or anti-RP4 as indicated. The zymogen (Z) and catalytic domain (C) after Pro-Cat expression are indicated. P indicates propeptide fragments. In C, the zymogen (N-L2) and its furin processed derivative (Cat-L2) are indicated.

expression (Fig. 2A, right hand panel). Co-transfection of the propeptide with the FLAG-tagged catalytic domain failed to rescue its secretion (Fig. 2A, left panel). These experiments suggested that the propeptide was essential for secretion of ADAMTS9, but that it was unable to rescue secretion of the mature enzyme when provided in trans. To exclude potential interference of the N-terminal FLAG tag in secretion, as well as to investigate whether a longer form of ADAMTS9 could be secreted without the propeptide, we repeated these experiments using additional constructs that in addition to lacking the N-terminal FLAG-tag, utilized the endogenous signal peptide of ADAMTS9 (Fig. 2B). Both ADAMTS9(Cat) and ADAMTS9 (Cat-L2) were detectable within the cell lysate (Fig. 2B). Co-expression of the isolated propeptide diminished the levels of each construct within the cell lysate (Fig. 2B), but could not restore their secretion to the robust levels seen in conditioned medium upon expression of the Pro-Cat or ADAMTS9(N-L2) constructs (Fig. 1 B,C). For example, the catalytic domain derived from Pro-Cat is robustly detected upon western blot analysis of 20 μl of unconcentrated conditioned medium from transfected cells, requiring only a few seconds of exposure to ECL reagent (e.g. Fig. 1B). However, the myc-tagged isolated catalytic domain co-expressed with propeptide was only detectable following acetone precipitation of 500 μl of medium and long exposure (5 minutes) of the immunoblot to ECL reagent (Fig. 2B). The Cat-L2 construct was undetectable in the medium following co-expression with the propeptide (Fig. 2B). Collectively, these experiments establish i.) that the propeptide is essential for secretion of ADAMTS9 and ii.) The propeptide inefficiently restores secretion of the mature enzyme when provided in trans demonstrating that it can function as a weak chaperone in trans, but that for such function to be optimal, it is required in cis.

Figure 2. The ADAMTS9 propeptide is essential for secretion of the catalytic domain. **A.** A secretable FLAG-tagged catalytic domain or the ADAMTS9 propeptide were expressed individually or together in HEK293F cells using plasmids expressing the constructs shown at the top of the figure. Immunoblotting of cell lysate (Cell) and conditioned medium (CM) was done using anti-FLAG (left hand panel) or anti-RP4 (right panel). Note that the propeptide (P) expressed in isolation is secreted into the CM (right hand panel), but the catalytic domain (C) is not (left hand panel). Secretion of the catalytic domain is not rescued by co-expression of the propeptide. Molecular weight markers are shown on the right of each panel. **B.** Expression of C-terminal myc-tagged Cat (Cat-myc) or ADAMTS9(Cat-L2) and their co-expression with the propeptide in HEK293F cells. Immunoblotting of cell lysate and conditioned medium (CM) was done using anti-myc. The catalytic domain (C) and ADAMTS9(Cat-L2) are indicated and the respective protein species are marked by asterisks. The Cat-L2 construct was used in preference to full-length ADAMTS9, since it is more resistant to C-terminal proteolysis and thus more readily detected in the medium of transfected cells. Note a weakly reactive catalytic domain band in acetone-precipitated conditioned medium (C) following co-expression of propeptide, but no secretion of ADAMTS9 (Cat-L2). The bands of 35 kda and 48 kDa are seen in all lanes and are non-specific.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IB: Anti-RP4
B
Cat-myc
N S Cat Myc/His
ADAMTS9 (Cat - L2)
N S Cat Dis TSR1 CRD Spacer TSR 2-6 TSR 7-8 L1 L2 Myc/His
Pro
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IB: Anti-myc

N-Linked Oligosaccharide In The Propeptide Is Essential For Secretion—Treatment of ADAMTS9 Pro-Cat with PNGaseF previously demonstrated that the propeptide, but not the catalytic domain, was N-glycosylated, as predicted by the primary sequence (10). Since N-linked oligosaccharides are predicted to be attached to Asn^{112}, Asn^{135} and Asn^{271} (these residues are located within the consensus motif Asn-Xaa-Ser/Thr, where Xaa is any residue except proline (13)), and can be involved in regulating correct folding of secreted proteins (14), we mutated these residues individually and in combination by substituting Asn with Ala in Pro-Cat. Pro-Cat is present in transfected QBI-293A cells as a major glycosylated molecular species of 60 kDa, and a 50 kDa unglycosylated species (8) (Fig. 3, upper panel). Protein expressed from each of the single Asn mutant Pro-Cat plasmids was detected in the respective transfected cell lysate, but migrated more rapidly than the fully-glycosylated Pro-Cat, suggestive of a lack of glycosylation at that site (Fig. 3, upper panel). Pro-Cat in which all three putative sites were mutated showed a major band which migrated at the level of the unglycosylated Pro-Cat (Fig. 3, upper panel). Together, these observations demonstrated that the secreted ADAMTS9 propeptide is constitutively glycosylated at Asn^{112}, Asn^{135} and Asn^{271} as suggested by previous analysis (10). Although the 29 kDa furin-processed catalytic domain fragment was present in conditioned medium of wild-type Pro-Cat transfected cells, the catalytic domain derived from N-glycosylation defective proteins was not found in the media of transfected cells (Fig. 3, lower panel). This suggests that N-linked glycosylation at each site within the propeptide is essential for secretion of ADAMTS9 zymogen and as a corollary, that secreted ADAMTS9 normally contains an oligosaccharide at each of these sites.

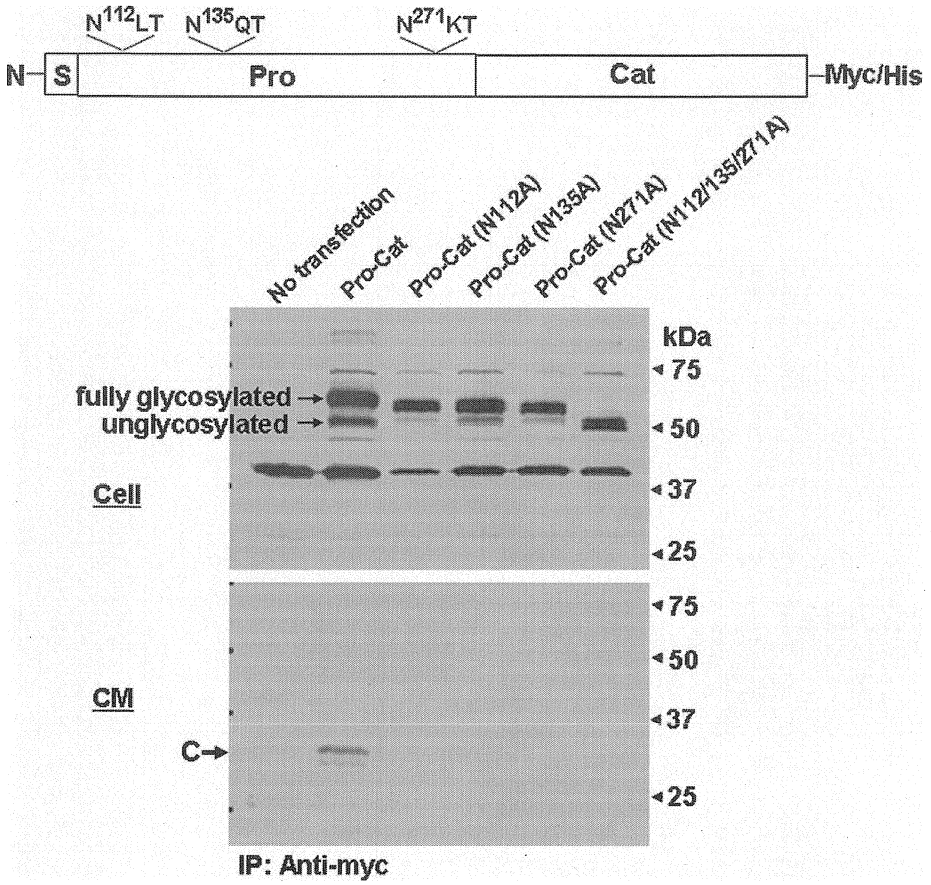
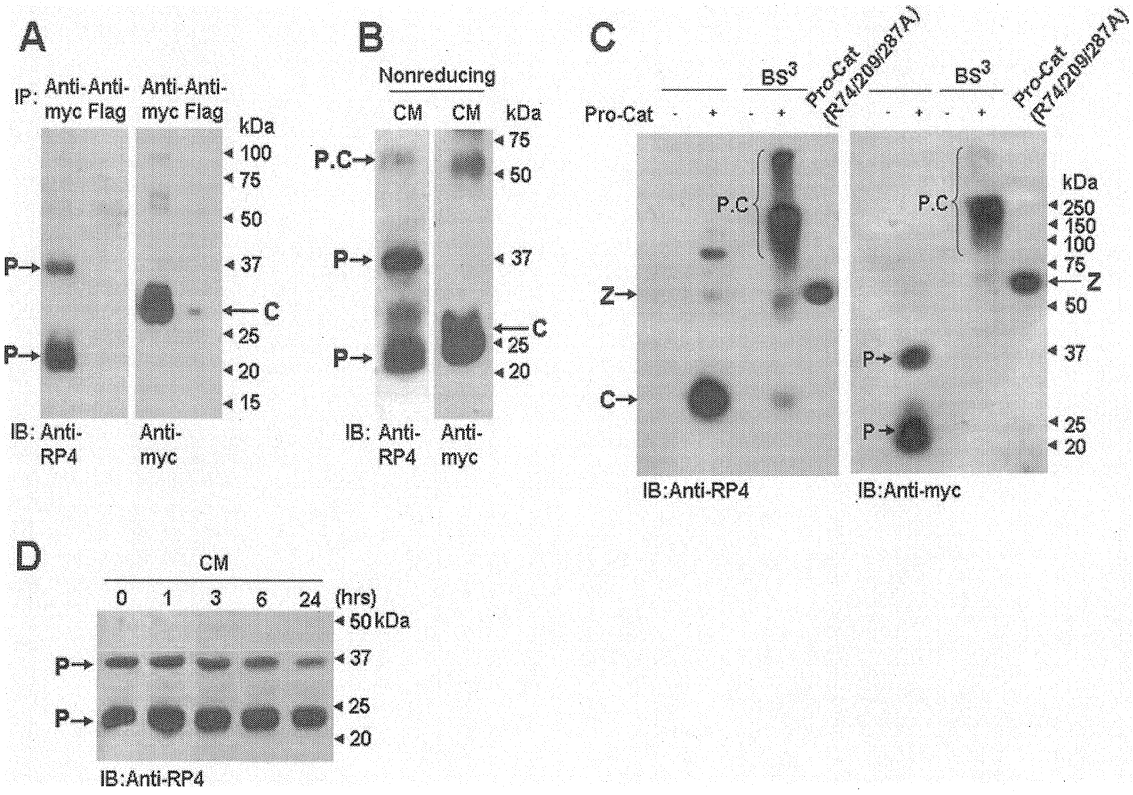


Figure 3. The role of propeptide glycosylation in Pro-Cat secretion. QBI293A cells were transiently transfected with Pro-Cat or the Asn (N) mutants introduced into the N-linked glycosylation sites shown at the top of the figure, followed by incubation with [^{35}S]-Met+Cys amino acids for 3 hours, and immunoprecipitation of labeled proteins with anti-myc, SDS-PAGE and fluorography. Untransfected cells were used as a control. Note the reduction in size of each mutant protein and absence of the corresponding catalytic domain in the conditioned medium (CM). The glycosylated and unglycosylated zymogen and catalytic domain (C) are indicated. Molecular weight markers are shown on the right.

The Propeptide Remains Non-Covalently Associated With The Catalytic Domain After Furin Processing— Previous studies using reducing SDS-PAGE of conditioned medium from ADAMTS9 transfected cells, demonstrated that the propeptide was secreted into conditioned medium following proteolysis of the zymogen at the cell surface (10). To investigate whether the propeptide interacted with the catalytic domain after processing or was secreted as a discrete entity, the conditioned medium of cells stably expressing Pro-Cat was immunoprecipitated with anti-myc agarose and immunoblotted with anti-RP4. Figure 4A shows that the propeptide fragments and catalytic domain could interact with each other. Furthermore, anti-myc immunoprecipitated not just the intact 37kDa propeptide fragment, but also the smaller 20-22 kDa fragments derived from it (Fig. 4A). As a control for these experiments, immunoprecipitation using anti-FLAG agarose, which does not recognize this construct, did not provide any anti-RP4 immunoreactivity (Fig. 4A). We noted that the propeptide was not co-immunoprecipitated with the catalytic domain under conditions where detergents were used for washing the protein-A beads (e.g. Fig. 3). In the immunoprecipitation experiments shown in Fig. 4A, the beads were washed several times with PBS, and no detergents were present. Furthermore, non-reducing SDS-PAGE analysis of the medium showed discrete propeptide and catalytic domain fragments as the major immunoreactive species, although low levels of a propeptide-catalytic domain complex were also detectable (Fig. 4B). This demonstrates that the complex between the propeptide and catalytic domain in the medium is not disulfide-bonded. Taken together, this suggested that the binding of the propeptide to the catalytic domain is not only non-covalent, but is also susceptible to dissociation in the presence of detergents such as SDS. Next, cross-

Figure 4. Association of the cleaved propeptide with the catalytic domain. A. Conditioned medium of HEK293F cells stably transfected with Pro-$Cat_{Myc\text{-}His}$ was immunoprecipitated with anti-myc agarose or anti-FLAG agarose (as a negative control) followed by immunoblotting (IB) with the indicated antibodies. Note that anti-myc immunoprecipitates propeptide fragments (left hand panel). **B.** Non-reducing SDS-PAGE followed by immunoblotting with anti-RP4 or anti-myc shows that the propeptide (P) and catalytic domain (C) did not associate under non-reducing conditions, suggesting a non-covalent, possibly ionic, interaction. A small amount of complexed propeptide and catalytic domain (P-C) is indicated. **C.** Cross-linking of cleaved propeptide and catalytic domain in conditioned medium of Pro-Cat transfected cells by BS3. Conditioned medium was treated with a non-reducible cross-linker. Analysis of conditioned medium by reducing SDS-PAGE and immunoblotting with anti-RP4 and anti-myc shows that the cleaved propeptide (P) and catalytic domain (C) migrate as cross-linked species (P.C) whose major form (asterisk) is larger than the zymogen (Z), for reasons that are presently unclear. **D.** Cell-free conditioned medium from HEK293F cells expressing Pro-Cat was incubated at 37 °C for the indicated durations. Western blot analysis was done using anti-RP4.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linking of the secreted propeptide and catalytic domain was done at low concentrations of the target proteins (unconcentrated medium) using a cross-linker with a short arm (11.4 A°) between the functional groups. Figure 4C shows that the propeptide and catalytic domain were efficiently cross-linked with very little residual isolated catalytic domain or propeptide. The molecular mass of the major complexes (100-250 kDa) reactive with both anti-myc and anti-RP4 is considerably larger than the zymogen (60 kDa) (Fig. 4C). It is possible that other molecules may be included within the complex, but resolution of this issue is beyond the scope of the present work. Cell-free incubation of conditioned medium containing the propeptide and catalytic domain revealed that the propeptide fragments were quite stable over a period of 24 hours (Fig. 4D), although there appears to be progressive conversion of the 37 kDa fragment to the smaller propeptide fragments, as we previously noted (10). Taken together, these data demonstrate that the propeptide and catalytic domain have a close proximity to each other following furin processing and that the resulting complex is quite stable.

The ADAMTS9 Propeptide Is Proteolytically Processed Following Arg^{209}— Next, we investigated the mechanism of conversion of the 37 kDa fragments to smaller fragments that have been consistently noted in this and previous analysis (10), with the goal of generating a zymogen that could not be furin processed. Although Pro-Cat has 5 consensus PC processing sites (8), we previously found that furin processed only two of these, i.e. following Arg^{74} and Arg^{287} (8). The 37kDa propeptide resulting from processing at these two sites was subsequently processed at one or more internal sites, since two smaller fragments of the propeptide (20 and 22 kDa) were consistently observed in the conditioned

medium of cells expressing full-length ADAMTS9, Pro-Cat (10) or propeptide alone (Fig. 2C). These fragments were previously shown to be absent in cells treated with the PC-inhibitor dec-RVKR-cmk, as well as furin siRNA, suggesting that they resulted from the action of furin (10). Their size suggested the possibility that one processing site could be within the sequence Ile-Tyr-Arg-Arg^{209}↓Ser (Fig. 5A). This does not comprise a consensus furin cleavage site since it lacks Arg at the P4, P6 or P8 positions. However, it does have Ser at the P1′ position, which is present in a significant number of furin-processed sites in various precursors (15-17) and a His residue at P6 which may be positively charged in the propeptide. Substitution of Ala for Arg^{209} led to disappearance of the 20 kDa proteolytic fragment of the propeptide in conditioned medium (data not shown) although it did not affect processing at the downstream processing site, Arg^{287} (Fig. 5B). Moreover, substitution of $Arg^{74+209+287}$ with Ala abolished zymogen processing altogether (Fig. 5B). Thus, Ala substitution of Arg^{74}, Arg^{209} and Arg^{287} could be used to produce an uncleavable zymogen.

Furin-Processed ADAMTS9 Zymogen Cleaves Versican Inefficiently— To ask whether the ADAMTS9 propeptide had a role in regulating its latency and enzymatic activity, or if the bound propeptide fragments following furin-processing affected catalytic activity, we compared versican processing by full-length ADAMTS9, full length ADAMTS9 $Arg^{74+209+287}$Ala and full-length ADAMTS9 Glu^{435}Ala. The Glu^{435}Ala mutant replaces the critical catalytic Glu residue in the active site and is predicted to generate a proteolytically inactive enzyme (18,19). Since purification of full-length ADAMTS9 has not been successful despite numerous attempts, and because purified Pro-Cat will not cleave

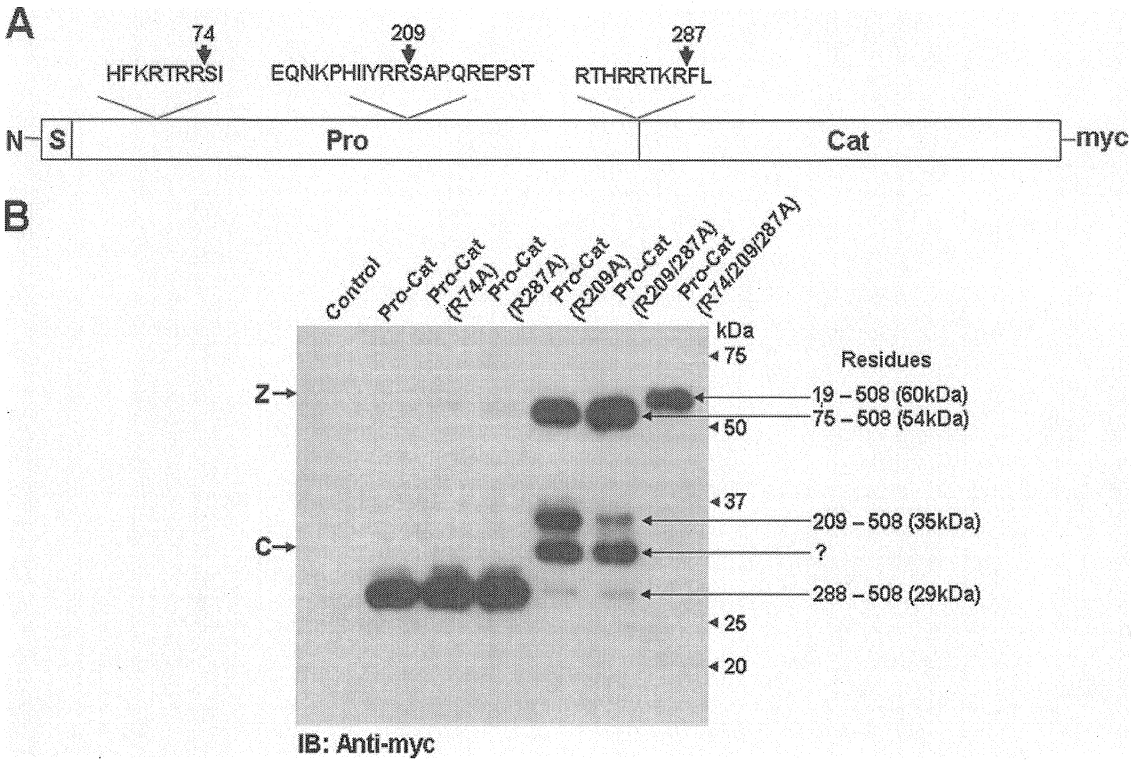
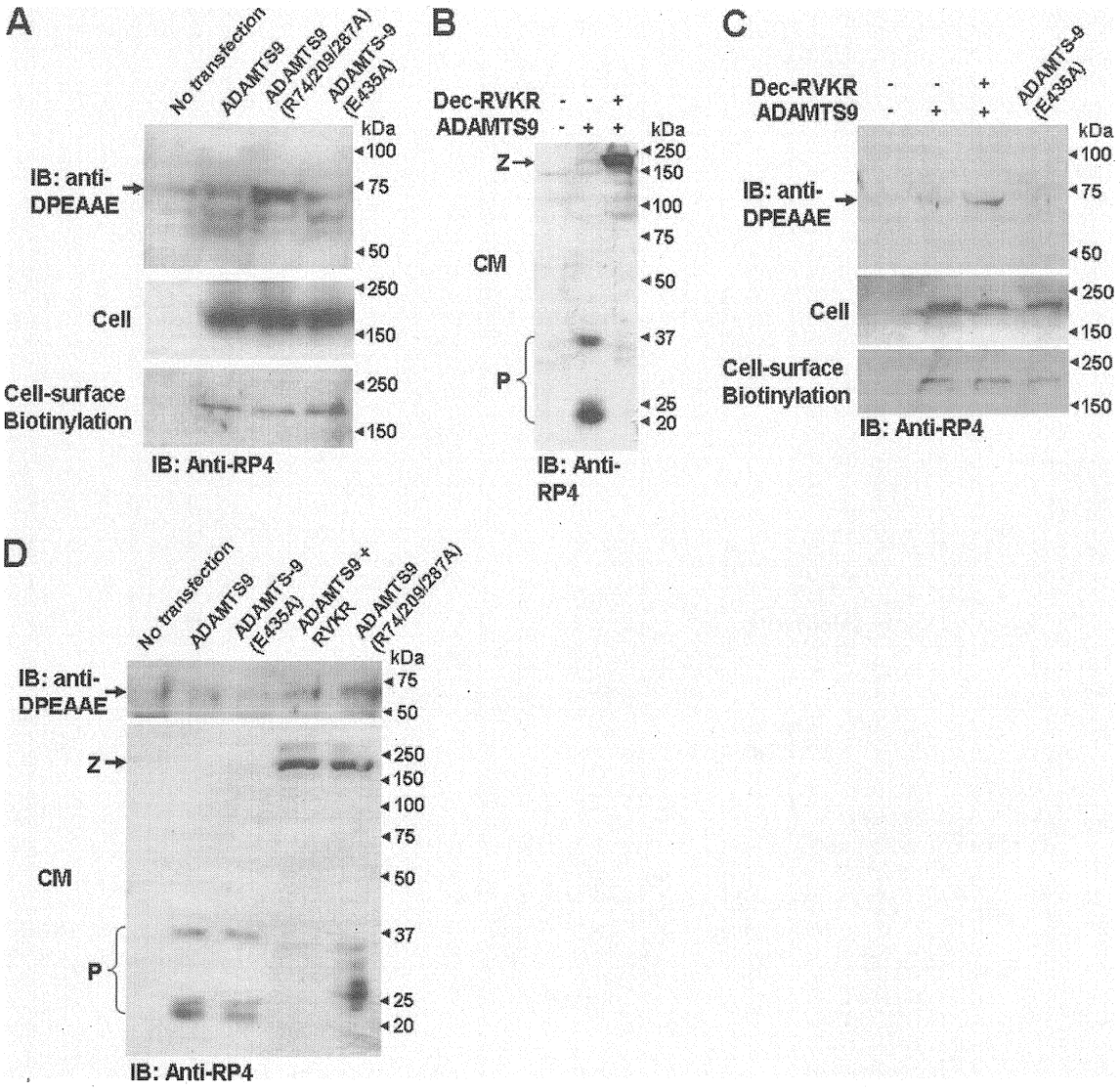


Figure 5. Identification of a novel PC-processing site in the ADAMTS9 propeptide and generation of PC-uncleavable ADAMTS9 Pro-Cat. **A.** The domain organization of ADAMTS9 Pro-Cat is shown with expansion of sequence motifs containing two previously identified processing sites and a putative novel PC processing site ($RR^{209}S$) in the propeptide. **B.** Western blotting (using anti-myc) of conditioned medium from HEK293F cells transfected with wild-type Pro-Cat or various mutants as indicated. Untransfected cells were used as a control. The zymogen (Z) and catalytic domain (C) are indicated. The residues comprising the various fragments are identified on the right of the figure. The 32 kDa fragment is presently unidentified. Note that expression of mutant Pro-$Cat^{R74/209/287A}$ results in unprocessed zymogen.

versican (8), these experiments were first done using a cell-based assay. In this assay, HEK293F cells are transiently transfected with the various constructs described above and incubated with medium obtained from fibroblasts expressing high levels of versican. Untransfected cells were used to ascertain baseline, endogenous levels of versican proteolysis. Since ADAMTS9 binds to the cell-surface (8), a cell-based assay also provides the appropriate context for testing proteolysis, including putative co-factors (20). The V1 isoform of versican, the predominant form made by fibroblasts, is proteolytically processed by some members of the ADAMTS family, including ADAMTS9, within the sequence DPEAAE^{441}-^{442}ARRGQ to generate a 70 kDa fragment reactive with the anti-DPEAAE antibody (8,21). This antibody specifically detects the neo-epitope generated by cleavage at E^{441}-^{442}A site, but it does not react with uncleaved versican core protein (8,21). Untransfected HEK293F cells have low background levels of versicanase activity (Fig. 6A, upper panel). Unexpectedly, cells expressing the ADAMTS9 Arg$^{74+209+287}$ Ala mutant showed more robust processing of versican at the E^{441}-^{442}A site than the wild-type ADAMTS9 (Fig. 6A, upper panel). ADAMTS9 Glu^{435}Ala could not generate an immunoreactive fragment since this mutation renders the protease domain catalytically inactive (Fig. 6A, upper panel). The three constructs were expressed at comparable levels in cells (Fig. 6A, middle panel) and were equally represented at the cell surface as shown by cell-surface biotinylation (Fig. 6A, lower panel). This observation suggests that furin processing of the propeptide could decrease the versicanase activity present in proADAMTS9. Because of concerns about the possible structural consequences of mutating three Arg residues, we undertook parallel experiments in which zymogen processing was prevented by addition of 50μM dec-RVKR-cmk, which effectively

Figure 6. Furin processing of the ADAMTS9 propeptide diminishes versicanase activity. A. HEK293F cells expressing full-length ADAMTS9, uncleavable zymogen ($ADAMTS9^{R74/209/287A}$) or ADAMTS9 with a $Glu^{435}\rightarrow Ala$ mutation in the active site ($ADAMTS9^{E435A}$) were incubated with medium enriched in versican. Neoepitope antibody (anti-DPEAAE) was used to identify proteolytic cleavage on immunoblots as indicated (top panel). Immunoblotting with anti-RP4 demonstrates comparable levels of ADAMTS9 expressed in cells (middle panel) and cell surface biotinylation suggests equivalent levels at the cell surface. The result is representative of four independent experiments. **B.** Inhibition of zymogen processing by 50μM dec-RVKR-cmk. Note that only the zymogen (Z) is seen in the conditioned medium of the treated cells upon western blotting using anti-RP4, and propeptide fragments (P) are only seen without treatment. **C.** Versican processing by ADAMTS9-transfected HEK293F cells treated with dec-RVKR-cmk is greater than untreated cells. Neoepitope antibody (anti-DPEAAE) was used to identify proteolytic cleavage on immunoblots as indicated (top panel). Immunoblotting with anti-RP4 demonstrates comparable levels of ADAMTS9 expressed in cells (middle panel) and cell surface biotinylation suggests equivalent levels at the cell surface. The result is representative of four independent experiments. **D.** Conditioned medium was collected for versican proteolysis assays from untransfected cells, cells transfected with the constructs shown, or cells expressing wild-type ADAMTS9 treated with dec-RVKR-cmk. The upper panel is an immunoblot using the versicanase neo-epitope antibody. The lower panel is an immunoblost with anti-RP4 to show the comparative levels and processing status of ADAMTS9.


A
No transfection
ADAMTS9
ADAMTS9 (R74/209/287A)
ADAMTS-9 (E435A)
kDa
IB: anti-DPEAAE
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Cell
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Cell-surface Biotinylation
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IB: Anti-RP4
B
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ADAMTS9 - + +
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IB: Anti-RP4
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Dec-RVKR - - + ADAMTS-9 (E435A)
ADAMTS9 - + +
kDa
IB: anti-DPEAAE
100
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Cell
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Cell-surface Biotinylation
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IB: Anti-RP4
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No transfection
ADAMTS9
ADAMTS-9 (E435A)
ADAMTS9 + RVKR
ADAMTS9 (R74/209/287A)
kDa
IB: anti-DPEAAE
75
50
Z
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P
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20
IB: Anti-RP4

prevented processing of proADAMTS9 (Fig. 6B). Cells expressing ADAMTS9 in the presence of 50μM dec-RVKR-cmk also showed greater activity towards versican than cells expressing the processable ADAMTS9 (Fig. 6C, upper panel). Dec-RVKR treatment affected neither the cellular levels of ADAMTS9 zymogen nor its localization at the cell-surface (Fig. 6C). In another variation of the cell-based assay, we used conditioned medium from cells expressing the various constructs or following treatment with dec-RVKR-cmk. The results (Fig. 6D), further confirm that intact ADAMTS9 zymogen, but not furin-processed ADAMTS9 has proteolytic activity against versican. Although the data strongly suggest that furin processing of proADAMTS9 reduces its versicanase activity, we cannot presently exclude the formal possibility that this effect of ADAMTS9 in the cell-based assays is an indirect one that results from its effects on another protease.

DISCUSSION

Propeptides of protease zymogens have been shown to have important, if transient, functions in inhibition of catalytic activity (22). Proteolytic processing of propeptides has been studied in detail in the MMP and ADAM family as well as in some ADAMTS proteases. In most secreted, soluble MMPs (e.g. pro-MMP3, pro-MMP7), the propeptide binds to the active site zinc atom through a free sulfydryl group and maintains the enzyme in a latent, inactive state (23). This bond is destabilized by propeptide proteolysis (23) or chemical modification, which occurs extracellularly, and is not proprotein convertase-mediated (24). In these MMPs, removal of the propeptide is an important prerequisite for obtaining enzymatic activity (23). In the case of MMP14 (membrane-type 1 MMP), an MMP that is processed and activated by furin (25), the propeptide is excised in the *trans*-Golgi, and constitutively active MMP14 is transported to the cell surface. The MMP14 propeptide is essential as an intramolecular chaperone, since MMP14 lacking the propeptide is not secreted. However, unlike ADAMTS9, expression of the propeptide *in trans* restores secretion (26). The propeptides of a number of ADAMs (e.g. ADAM9, ADAM12, and ADAM17) (27-29) are constitutively excised in the secretory pathway by furin. The ADAM17 propeptide, like MMP14 and ADAMTS9, is essential for secretion, but unlike ADAMTS9, it can function as a chaperone in *trans* to enable secretion (29,30). Recently, analysis of ADAM12 showed that its propeptide remained associated with the catalytic domain after processing of the zymogen by furin (31).

ADAMTS propeptides are generally quite large, ranging from 200-240 residues (with the exception of ADAMTS13 which is only 41 residues long(32)). Most contain

conserved consensus sites for N-linked glycosylation. Zymogen maturation has been studied in ADAMTS1, ADAMTS4, ADAMTS7, ADAMTS9, ADAMTS10, and ADAMTS13. These studies have indicated that proprotein convertases such as furin mediate the process (8,12,33-36,37 Koo, 2006 #620). The propeptides of ADAMTS1 and ADAMTS4 are excised by furin in the *trans*-Golgi, consistent with the classical mechanism of proprotein convertases (10,11,38,39). However, recent analysis of ADAMTS7, ADAMTS9 and ADAMTS10 showed that furin processing of these metalloproteases occured exclusively (ADAMTS9, ADAMTS10) or substantially (ADAMTS7) in the extracellular space (8,10,12,34). In particular, the cell-surface processing of proADAMTS9 led us to ask what role the propeptide had in secretion and zymogen activation.

The data provided here make several novel observations, some of which may be potentially of broad relevance to the ADAMTS proteases. First, the present studies demonstrate that unlike ADAMTS13 (32), the propeptide is an absolute requirement for ADAMTS9 secretion. Second, the critical role of glycosylation demonstrated here suggests that correct modification of the propeptide is essential for its function as an intramolecular chaperone. Third, in attempting to generate a zymogen that was resistant to furin processing, we characterized a previously unknown, atypical furin processing site. Mutation of this site and of two previously identified sites was successful in generating a furin-resistant zymogen. Finally, and most significantly, we showed that furin processing of ADAMTS9 reduces its proteolytic activity towards versican.

Furin processing of metalloprotease zymogens is generally referred to as "activation", since it typically transforms a latent enzyme activity into a functional proteolytic activity. Consistent with this, the ADAMTS4 propeptide was found to have an inhibitory role in the zymogen, since the ADAMTS4 zymogen lacked proteolytic activity against aggrecan (38). However, retention of the propeptide did not affect the ability of ADAMTS13 to process vWF and moreover, this propeptide was dispensable for folding (32). The ADAMTS13 propeptide is not just unusually small, but unlike the other ADAMTS propeptides, it contains only two cysteine residues (versus three for the others) (32); thus, it is not an appropriate model for the other 18 ADAMTS proteases.

The observation that furin processing of the ADAMTS9 propeptide resulted in loss of proteolytic activity against versican was unexpected, and to our knowledge, is without precedent in the protease field. We are aware of two other examples in the literature in which furin-processing was associated with loss of enzymatic activity (40,41), but in both these instances cleavage occurred at sites unrelated to the propeptide. The mechanism by which furin processing leads to reduced ADAMTS9 catalysis, is presently unclear. A potential mechanism is suggested by the observation that the propeptide and its fragments remain attached to the catalytic domain following processing. The immediate release of furin-processed Pro-Cat from the cell surface (10) suggests that furin-cleavage induces a conformational change in Pro-Cat. We speculate that such a conformational shift may cause the propeptide or its fragments to block the active site and thus hinder proteolysis of versican (Fig. 7). Close contact between the propeptide and catalytic domain is suggested both by effective cross-linking using a short-arm cross-linker in unconcentrated medium, and by the efficient disappearance of individual propeptide and catalytic domain fragments

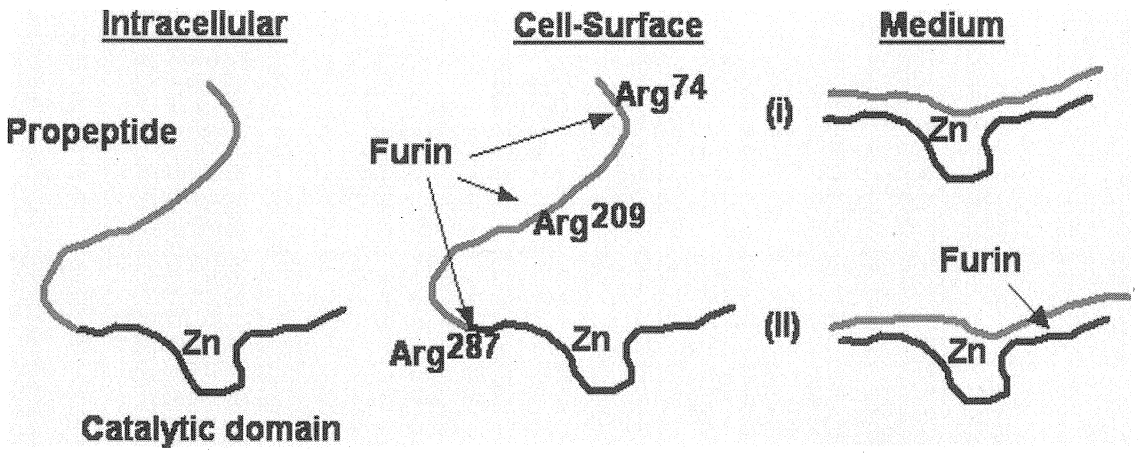

Figure 7. Schematic depicting the proposed consequence of proADAMTS9 by furin.

from reducing SDS-PAGE following cross-linking. The contact between propeptide and catalytic domain likely occurs at several sites, since the derivative fragments of the propeptide are immunoprecipitated with the catalytic domain and cross-linked to it. A hypothetical mechanism for the loss of proteolytic activity upon furin-processing is schematically depicted in Figure 7. The following experimental evidence supports this model: First, that furin processed Pro-Cat shows immediate loss of cell-surface binding, suggestive of a conformational change, second, that all propeptide fragments are closely associated with the catalytic domain after furin processing, and third, that the furin-processed proADAMTS9 has less versicanase activity. Clarification of the structural basis for this model is beyond the scope of the present body of work, and it is also not clear what role a potentially unpaired cysteine in the propeptide plays in this regulatory mechanism. The stable association of the propeptide with the mature ADAMTS9 molecule is of additional potential significance, since it may indicate a possible role for the propeptide in mediating subsequent intermolecular interactions as a distinct domain of ADAMTS9. The stability of the secreted propeptide in association with mature ADAMTS9, also suggests the possibility that it could be useful as a marker for this protease.

Potential Functional Significance Of The Unusual Mechanism Of Propeptide Processing Of ADAMTS9 — Taken together, with previous work (10), it is clear that the ADAMTS9 propeptide constitutes a critical functional domain of this protease, since it has essential roles in secretion, cell-surface binding, as well as maintenance of enzyme activity. Why is ADAMTS9 handled in such an unusual fashion by cells? We propose that these unusual features constitute a mechanism that ensures that ADAMTS9 activity is maximal at the cell

surface. Thus, by a yet-unknown mechanism, proADAMTS9 is protected from furin processing until it reaches the cell surface, ensuring that proteolytic activity is not lost prematurely. Unprocessed proADAMTS9 is retained at the cell surface (10) but once it is processed there, its activity is reduced or lost. Thus maximum catalytic activity, at least against versican, is present at the cell surface. This phenomenon, taken together with the role that the ADAMTS9 homologs *Gon-1* and ADAMTS20 have in cell migration (9,42) and its clearly demonstrated localization at the cell surface (8,10) strongly suggests that ADAMTS9 is operationally a cell-surface protease. The life cycle of a proteolytic activity subject to furin-dependent inactivation, such as of ADAMTS9, is predicted to be short. Furthermore, function of a protease that is regulated in this unusual fashion is likely to be highly dependent on transcriptional regulation of its mRNA. Indeed, developmental analysis has shown ADAMTS9 to be very dynamically regulated at the transcriptional level and to be strongly associated with cell populations actively undergoing migration, such as trophoblast giant cells, endothelial cells and neural crest cells (7).

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DISCUSSION

La protéolyse de la matrice extracellulaire et de protéines membranaires par les métallopeptidases, incluant les MMP, les ADAM et les ADAMTS, joue un rôle important dans la régulation de processus du remodelage tissulaire dans des conditions normales et pathologiques tels la morphogenèse tissulaire et l'invasion de cellules tumorales (CHANG et WERB, 2001). Les métallopeptidases sont généralement synthétisées sous forme de précurseurs pro-enzyme inactifs. Dans la plupart des cas, le prodomaine en N-terminal du domaine catalytique doit être retranché du précurseur afin que l'enzyme ait une activité protéolytique. Chez les précurseurs pro-ADAMTS, les pro-protéines convertases de type subtilisine sont responsables du clivage à la jonction du prodomaine et du domaine catalytique.

Cet ouvrage identifie des déterminants moléculaires du prodomaine impliqués dans la biosynthèse et l'activation par les pro-protéines convertases (PC) des ADAMTS1, 5, 7 et 9. Il appert que les sites de clivage par la furine et que des motifs conservés, CXYXG et YFIXPL, dans les prodomaines de la famille des ADAMTS sont essentiels à la biosynthèse de ces enzymes. De plus, il existe une variété de mécanismes d'activation pour les ADAMTS dont l'activation intracellulaire d'ADAMTS1 par la furine. Un nouveau mécanisme d'activation pour des substrats endogènes par la furine extracellulaire à d'ailleurs été identifié pour ADAMTS5 et 7. Finalement, le mécanisme d'inactivation d'ADAMTS9 par la furine à la surface cellulaire est un mécanisme nouveaux chez les pro-protéines convertases. En plus d'apporter de nouvelles connaissances dans le domaine des

précurseurs protéiques, ces découvertes ont un impact considérable dans le développement de stratégies visant à ralentir la dégradation du cartilage impliquée dans l'arthrose.

1. Clivage du prodomaine des ADAMTS

Grâce à des études de gain de fonction dans les cellules CHO RPE.40 déficientes en furine, nous avons démontré que la furine est la convertase clivant les précurseurs pro-ADAMTS1, 5, 7 et 9 le plus d'efficacement. Il a aussi été démontré que la furine clive les précurseurs pro-ADAMTS2, 4, 12 et 13 (CAL *et al.*, 2001; MAJERUS *et al.*, 2003a; WANG, P. *et al.*, 2004a; WANG, W.M. *et al.*, 2003a). Étant donné la distribution tissulaire ubiquitaire de la furine et le fait que parmi les PCs, la furine clive plus efficacement le prodomaine des ADAMTS, il semble donc que cette dernière représente l'enzyme la plus apte à cliver les précurseurs ADAMTS *in vivo*. Par contre, nos études ont fait état de différentes convertases de la voie de sécrétion constitutive pouvant cliver des précurseurs pro-ADAMTS (PACE4 et PC6B clivent ADAMTS1, PC6B et PC7 clivent ADAMTS7, PC5A clive ADAMTS9 et PC7 clive ADAMTS5). ADAMTS4 peut aussi être activée via PACE4, PC5A et PC6B (TORTORELLA *et al.*, 2005a). Il est donc possible que les autres convertases de la voie de sécrétion constitutive puissent cliver les précurseurs ADAMTS dans différents tissus ou différentes conditions d'expression des enzymes. Par exemple, ADAMTS9 et PC5A sont toutes deux fortement exprimées dans les reins à la fin de l'embryogenèse de la souris (JUNGERS *et al.*, 2005a; ZHENG *et al.*, 1997), ce qui suggère que le clivage de pro-ADAMTS9 par PC5A est possible *in vivo*.

La furine mature plusieurs précurseurs de pro-métallopeptidases en clivant en C-terminal au motif consensus RXXR retrouvé entre le prodomaine et le domaine catalytique. Toutes les ADAMTS possèdent un ou plusieurs sites potentiels de clivage par la furine dans leur prodomaine. À l'aide de mutagenèses dirigées des différents sites potentiels de clivage, nous avons démontré que la furine clive seulement le site le plus près du domaine catalytique pour ADAMTS1 ($RKKR^{235}$) et ADAMTS5 ($RRRR^{261}$). Cependant, lorsque l'on mute le site le plus près du domaine catalytique d'ADAMTS9 ($RTKR^{287}$) d'autres sites en amont sont alors utilisés par la furine ($RTRR^{74}$ et RR^{209}). En plus de cliver mADAMTS7 au site consensus de clivage par la furine à proximité du domaine catalytique ($RQQR^{220}$), mADAMTS7 est aussi clivée au site $RVLR^{58}$. La structure tridimensionnelle des prodomaines fournirait des informations expliquant le fait que les sites potentiels de clivage autres que celui le plus en C-terminal sont peu ou non clivés par les PCs. En effet, ces sites ne sont sûrement pas exposés à l'environnement extérieur de la protéine rendant l'accessibilité à la furine difficile ou impossible.

Bien que nos collègues du groupe de Suneel Apte aient démontré qu'ADAMTS10 subit un clivage pouvant être inhibé par un inhibiteur des PCs (SOMERVILLE *et al.*, 2004a), l'efficacité des PCs à maturer ADAMTS10 reste à prouver. En effet, nous n'avons pas été en mesure de détecter une maturation d'ADAMTS10 par marquage métabolique dans les cellules HEK293 (résultats non publiés). Comme mentionné dans l'introduction, ADAMTS10 ne possède pas de site de clivage potentiel par la furine (RXXR) près de son domaine catalytique. ADAMTS10 possède le site KR^{233} qui n'a pas d'arginine en position P4 ou P6 qui contribuerait à augmenter l'efficacité de la furine (HOSAKA *et al.*, 1991). De plus, la concentration de l'inhibiteur des PCs dec-RVKR-cmk utilisée pour empêcher

totalement la maturation d'ADAMTS10 (>100μM) est plus grande que celle pour empêcher la maturation des ADAMTS7 et 9 (<25μM). Ceci démontre que dec-RVKR-cmk inhibe moins efficacement l'enzyme responsable du clivage d'ADAMTS7 et 9 qu'ADAMTS10 dans les cellules HEK293. Toutefois, il se peut qu'une autre peptidase clivant après une arginine et pouvant être inhibée par dec-RVKR-cmk soit responsable du clivage observé du prodomaine d'ADAMTS10. Il existe plusieurs sérines peptidases ayant la capacité de cliver à des résidus basiques telles les sérines peptidases à domaine transmembranaires de type II (NETZEL-ARNETT *et al.*, 2003).

Le prodomaine d'ADAMTS4 est aussi clivé par des métallopeptidases. Il a été démontré que la MMP-9 clive *in vitro* pro-ADAMTS4 près de la jonction du prodomaine et du domaine catalytique, (TORTORELLA *et al.*, 2005a). Le clivage par les PCs d'ADAMTS4 se fait au site $RAKR^{212}\downarrow$FASL et un anticorps reconnaissant seulement les acides aminés FASL libres n'a pu détecter la forme clivée par MMP-9. D'ailleurs, cette forme clivée par MMP-9 n'a pas d'activité protéolytique envers l'aggrécan, alors que la forme clivée par les PCs est active. Par conséquent, MMP-9 ne clive pas au même site dans le prodomaine que les PCs. Nous avons aussi remarqué un clivage du prodomaine d'ADAMTS5 lorsque celle-ci est exprimée dans les cellules HEK 293 et que le clivage par les PCs est inhibé. Ce clivage n'est donc pas dépendant des PCs et pourrait être effectué par une métallopeptidase. L'importance et le rôle du clivage par MMP-9 est présentement inconnu.

2. Mécanismes d'activation des ADAMTS

La furine est localisée principalement au TGN, mais elle a aussi été détectée à la surface cellulaire (KLIMPEL *et al.*, 1992; MAYER *et al.*, 2003; MAYER *et al.*, 2004) et dans le milieu extracellulaire (THIMON *et al.*, 2006; VIDRICAIRE *et al.*, 1993). La majorité des substrats de la furine sont maturés au TGN. La furine à la surface cellulaire est responsable du clivage de protéines exogènes tels l'antigène protecteur de l'anthrax ou l'α-toxine de *Clostridium* (GORDON *et al.*, 1997; KLIMPEL *et al.*, 1992). Étant donné que les ADAMTS sont localisées à la surface cellulaire ou dans le milieu extracellulaire, il s'avère pertinent d'identifier où l'activation des ADAMTS par la furine s'effectue.

2.1 Clivage intracellulaire par la furine

Les études de marquage métabolique des cellules HEK293 exprimant ADAMTS1 ont démontré la présence, dans les extraits cellulaires, du zymogène d'ADAMTS1 (110 kDa) qui est ensuite clivé par la furine pour générer une forme mature (87 kDa). Cette forme mature est par la suite sécrétée dans le milieu extracellulaire. La maturation d'ADAMTS1 est donc intracellulaire. Seulement la forme dont le prodomaine est clivée par les PCs possède une activité aggrécanase (RODRIGUEZ-MANZANEQUE *et al.*, 2002). Afin d'identifier le compartiment intracellulaire où l'activation d'ADAMTS1 se produit, nous avons traité les cellules avec la Brefeldine A qui bloque le transport des protéines du RE à l'appareil de Golgi (FUJIWARA *et al.*, 1988), et qui permet quant même l'activation de la furine (VEY *et al.*, 1994). Ce traitement a empêché l'activation et la sécrétion

d'ADAMTS1 suggérant que l'activation d'ADAMTS1 nécessite le transport de celle-ci au TGN. La maturation, bloquant le transport vésiculaire du TGN à la surface cellulaire (DINTER et BERGER, 1998), a empêché la sécrétion d'ADAMTS1, mais pas son activation. Ces résultats démontrent donc l'activation d'ADAMTS1 dans l'appareil de Golgi. Ce mécanisme d'activation pour ADAMTS1, clivage au Golgi entraînant l'activation de l'enzyme, est le mécanisme dit « classique » d'activation par la furine (Figure 7). La maturation du précurseur pro-ADAMTS4 par la furine est aussi fait au TGN et seulement la forme mature ayant le prodomaine retranché est active (TORTORELLA *et al.*, 2005a). Il semble aussi que la maturation de pro-ADAMTS2 et 12 soit intracellulaire de par la détection de leur forme mature dans les extraits cellulaires (CAL *et al.*, 2001; WANG, W.M. *et al.*, 2003a). Il est donc évident que certaines pro-ADAMTS sont maturées selon le mécanisme « classique » par la furine.

2.2 Clivage extracellulaire par la furine

Le marquage métabolique des cellules HEK293 exprimant ADAMTS7 Pro-Cat révèle la présence de formes zymogènes (50 kDa) et matures (29 kDa) d'ADAMTS7 Pro-Cat dans les extraits cellulaires. « Pro-Cat » signifie une forme tronquée d'ADAMTS contenant le peptide signal, le prodomaine et le domaine catalytique de l'anzyme. Le précurseur pro-ADAMTS7 est donc clivé à l'intérieur de la cellule. Par ailleurs, des études de biotinylation des protéines de la surface cellulaire ont démontré la présence de pro-ADAMTS7 Pro-Cat (50 kDa) associé à la membrane plasmique. Contrairement aux MMP et aux ADAM, les ADAMTS ne possèdent pas de domaine transmembranaire ou de séquence d'ancrage GPI (glycophosphoinositol) à la surface cellulaire. Les domaines

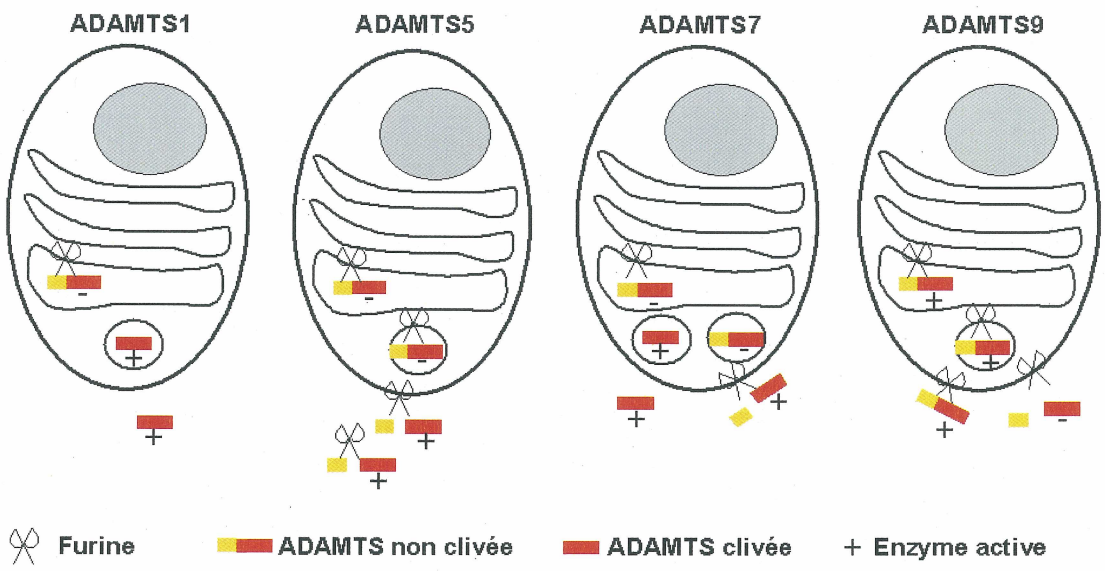

Figure 7. Schéma des différents mécanismes d'activation des ADAMTS1, 5, 7 et 9. Schéma représentant les différents scénarios possibles pour l'activation des précurseurs pro-ADAMTS1, 5, 7 et 9. Le précurseur pro-ADAMTS1 est clivé par les PCs au Golgi et ce clivage entraîne une activation de l'enzyme. Le précurseur pro-ADAMTS5 est, quant à lui, clivé soit à la surface cellulaire ou dans le milieu extracellulaire par les PCs et ce clivage entraîne l'activation d'ADAMTS5. Pro-ADAMTS7 peut être clivé par les PCs au Golgi ou à la surface cellulaire. Pro-ADAMTS9 est clivé à la surface cellulaire par la furine et ce clivage diminue l'activité protéolytique d'ADAMTS9.

auxiliaires des ADAMTS1 et 4 sont responsables de l'interaction de ces peptidases à la surface cellulaire (KASHIWAGI *et al.*, 2004; RODRIGUEZ-MANZANEQUE *et al.*, 2000a). Nous avons aussi observé un fait inusité : le précurseur pro-ADAMTS7 à la surface cellulaire est clivé et relâché dans le milieu extracellulaire en fonction du temps. Pro-ADAMTS7 est donc aussi clivée à la surface cellulaire. Cette observation démontre que le précurseur pro-ADAMTS7 peut être clivé soit à l'intérieur de la cellule ou soit à la surface de la cellule.

Nous avons caractérisé davantage le clivage à la surface cellulaire des ADAMTS en étudiant la biosynthèse d'ADAMTS9. Bien qu'ADAMTS9 Pro-Cat mature (29 kDa) ainsi que le prodomaine (37 kDa) soit présent dans le milieu extracellulaire , nous n'avons pas été en mesure, malgré tous nos efforts, de détecter une forme mature d'ADAMTS9 ou le prodomaine clivé à l'intérieur de plusieurs lignées cellulaires. Seulement la forme zymogène (55 kDa) est détectée dans les extraits cellulaires. Des études de biotinylation des protéines de la surface cellulaire démontrent clairement un clivage dépendant de la furine à la membrane plasmique du précurseur pro-ADAMTS9. En effet, la furine et pro-ADAMTS9 font partie d'un complexe à la surface cellulaire. L'activité protéolytique d'ADAMTS9 a été vérifiée en incubant un milieu de cellules fibroblastes riche en versican non clivé avec le milieu de culture de cellules contenant soit le zymogène pro-ADAMTS9 ou la forme clivée sans prodomaine. Curieusement, le zymogène possède une plus grande activité versicanase que la forme dépourvue de son prodomaine. Ce mécanisme de désactivation par la furine d'ADAMTS9 est inattendu et nouveau dans le domaine des peptidases. Le clivage du prodomaine d'ADAMTS13 n'est pas requis pour l'activité protéolytique de cette enzyme, contre le facteur de von Willebrand d'ADAMTS13

(MAJERUS *et al.*, 2003a). Par contre, le zymogène n'a pas davantage d'activité que la forme clivée par la furine. Les seuls exemples jusqu'à présent dans la littérature où un clivage par la furine inactive un enzyme sont ceux de la lipase endothéliale et pro-MMP-2 (CAO, J. *et al.*, 2005a; GAUSTER *et al.*, 2005a). Le clivage par la furine de la lipase endothéliale ne se fait pas dans un prodomaine de l'enzyme, mais plutôt à l'intérieur du deuxième domaine de liaison à l'héparine de la protéine. Par contre, l'inactivation de la pro-MMP-2 par la furine se fait par un clivage dans le prodomaine. Contrairement à ADAMTS9, le clivage de pro-MMP-2 se fait au TGN et le zymogène pro-MMP-2 n'est pas actif. Pro-MMP-2 doit se faire cliver par la MT1-MMP (membrane type MMP) à la surface cellulaire pour devenir active. Donc, la perte d'activité d'ADAMTS9 par un clivage dépendant de la furine à la surface cellulaire est sans précédent. Le mécanisme moléculaire menant à l'inactivation est présentement non défini. Nous avons observé que le prodomaine d'ADAMTS9 reste associé au domaine catalytique après le clivage par la furine. De plus, suite au clivage par la furine, une forme tronquée, ADAMTS9 Pro-Cat, ne possédant pas le domaine auxiliaire d'ADAMTS9 n'est plus associée à la membrane plasmique. Ces observations concordent avec l'explication suivante : suite au clivage par la furine, ADAMTS9 subit un changement conformationnel qui permet au prodomaine de mieux bloquer le site actif d'ADAMTS9, la rendant ainsi inactive contre le versican. N'étant pas clivée par la furine au TGN, ce mécanisme permettrait de garder une activité catalytique d'ADAMTS9 à la surface cellulaire.

Comme pour ADAMTS9, il fut impossible de détecter une forme mature d'ADAMTS5 à l'intérieur de la cellule. Des études de marquage métabolique ont démontré que le précurseur pro-ADAMTS5 (120 kDa) est clivé en une forme plus petite de 85 kDa

retrouvée seulement dans le milieu extracellulaire. Les résultats de ces expériences sont supportés par des immunobuvardages à l'aide de différents anticorps (anti-myc, antiADAMTS5 prodomaine et anti-ADAMTS C-terminal) révélant seulement la présence de forme clivée dans le milieu extracellullaire. Même la concentration d'ADAMTS5 des extraits cellulaires par immunoprécipitation et détection par immunobuvardage n'a pu révéler de forme clivée dans les extraits cellulaires. Toutes ces expériences supportent le concept de clivage extracellulaire du prodomaine d'ADAMTS5. La biotinylation des protéines de surface des cellules HEK293 surexprimant ADAMTS5 n'a engendré la détection d'aucune forme d'ADAMTS5 à la membrane plasmique. Afin de mieux évaluer si le clivage du prodomaine d'ADAMTS5 s'effectue à la surface cellulaire ou dans le milieu extracellulaire, nous avons utilisé deux mutants de la furine : furine714, ne pouvant s'ancrer à la surface cellulaire (DENAULT, J.B. *et al.*, 2000) et furineR683A, ne pouvant être relâchée de la surface cellulaire dans le milieu extracellulaire (DENAULT, J. *et al.*, 2002). L'analyse de la biosynthèse d'ADAMTS5 a été étudiée en co-transfectant ADAMTS5, la furine714 et la furineR683A séparément. Le clivage de pro-ADAMTS5 par la furine714 et la furine 683A a été observé dans le milieu extracellulaire indiquant que pro-ADAMTS5 peut être clivé à la surface cellulaire et dans le milieu extracellulaire. Le fait que pro-ADAMTS5 est clivé à la surface cellulaire malgré qu'il ne soit pas ancré à la membrane plasmique est supporté par le fait que PC7, qui se retouve à la surface cellulaire mais qui n'est pas relâché dans le milieu extracellulaire (VAN DE LOO *et al.*, 1997), est en mesure de cliver pro-ADAMTS5. D'ailleurs, il se pourrait qu'il soit très difficile de détecter l'association de la furine et pro-ADAMTS5 à la surface cellulaire compte tenu que la réaction enzymatique de la furine est un processus rapide.

Dans le but d'identifier si le zymogène ou la forme clivée d'ADAMTS5 est active, nous avons découvert un nouveau substrat d'ADAMTS5, le versican : protéoglycan de la matrice extracellulaire jouant un rôle dans la migration des cellules neuronales (DUTT *et al.*, 2006) ainsi que dans l'artériosclérose (KENAGY *et al.*, 2006). Seulement la forme clivée sans prodomaine d'ADAMTS5 est actice envers le versican. L'activation d'ADAMTS5 requiert donc un clivage de son prodomaine dans l'espace extracellulaire par les PCs. Ce mode d'activation s'avère très prometteur pour inhiber l'activation d'ADAMTS5 : l'enzyme susceptible d'être directement responsable de la dégradation de l'aggrécan causant l'arthrose (ARNER *et al.*, 1999). Il existe présentement aucun traitement pour ralentir la progression de l'arthrose. Jusqu'à ce jour, ADAMTS5 semble être le seul substrat de la furine à la surface cellulaire. Tous les autres substrats de la furine se font cliver au Golgi hormis des toxines tels l'antigène protecteur de l'anthrax ou l'α-toxine de *Clostridium* (GORDON *et al.*, 1997; KLIMPEL *et al.*, 1992) qui se font cliver à la surface cellulaire. Le design d'inhibiteur spécifique de la furine non perméable à la membrane plasmique serait donc, malgré la multitude de substrats de la furine, spécifique à ADAMTS5. Le groupe de Gary Thomas a identifié des inhibiteurs synthétiques de la furine inhibant le clivage à la surface celllaire de l'antigène protecteur de l'anthrax (JIAO *et al.*, 2006). Ces petites molécules qui inhibent la furine de façon spécifique et compétitive sont d'un potentiel thérapeutique contre l'activation de protéines exogènes telles des toxines virales et bactériennes ainsi que ADAMTS5.

À la lumière des mécanismes d'activation démontrés, une question très intéressante se pose : pourquoi le prodomaine d'ADAMTS5 et 9 n'est-il pas clivé au TGN? Une

explication serait que les sites consensus de clivage par la furine soient conformationnellement inaccesibles à l'intérieur de la cellule ou encore que des chaperones masquent les sites de clivage. Une fois à la surface cellulaire, le zymogène peut subir des changements conformationnels ou les chaperones peuvent se détacher du zymogène et rendre possible la protéolyse par la furine. De plus, il a été démontré qu'ADAMTS4 interagit avec la furine intracellulaire (WANG, P. *et al.*, 2004a). Nous avons aussi démontré par des essais de « pull-down » à l'aide d'anticorps contre la furine et ADAMTS5 que ces deux protéines interagissent dans les extraits cellulaires. Cependant, l'abolition du site consensus de clivage de la furine du prodomaine d'ADAMTS4 n'a pas empêché l'interaction intracellulaire de la furine (WANG, P. *et al.*, 2004a). Ceci démontre que la furine et ADAMTS4 et possiblement ADAMTS5 et 9 interagissent via d'autres motifs que les sites de reconnaissance de la furine. ADAMTS5 et 9 pourraient donc interagir avec la furine intracellulaire même si leurs sites consensus de clivage ne sont pas accessibles à la furine.

Le modèle d'activation des métallopeptidases par le « cysteine switch » prédit que l'enzyme est gardée inactive par l'interaction d'un résidu conservé cystéine dans le prodomaine avec l'ion Zn^{2+} dans la pochette catalytique (RAWLINGS et BARRETT, 1995). La plupart des ADAMTS, comme les MMP et les ADAM, possèdent un motif contenant une cystéine (XXCGVXD) pouvant potentiellement être impliqué dans un mécanisme « cysteine switch ». Jusqu'à présent, aucune ADAMTS ne semble être activée par ce mode d'activation. Les ADAMTS diffèrent donc des MMP et ADAM quant à leur mode d'activation malgré que plusieurs MMP et ADAM soient aussi activées par d'autres modes que le « cysteine switch ». Même la plus connue des ADAM, ADAM17, possède

un prodomaine inhibant son activité catalytique de façon indépendante du « cysteine switch » (BUCKLEY *et al.*, 2005). ADAM17 est aussi activée par le clivage d'un prodomaine par la furine (SROUR *et al.*, 2003).

La cristallographie est une technique qui a contribué de façon significative à l'élucidation des différents mécanismes d'activation de zymogènes (KHAN et JAMES, 1998). Cette technique permet d'évaluer l'importance des différents résidus dans la pochette catalytique et d'établir l'interaction de cette pochette avec des inhibiteurs. La contribution de cette approche technique est essentielle à la découverte des interactions entre le prodomaine et la peptidase. Récemment, des études de cristallographie ont été réalisées pour ADAMTS1 et ADAMTS5 (GERHARDT *et al.*, 2007; SHIEH *et al.*, 2007). Malheureusement, la technique de cristallographie ne permet pas présentement de produire et d'analyser des cristaux de plus de 1000 acides aminés tels que retrouvés chez les ADAMTS. Donc, seul le domaine catalytique de ces peptidases a pu être purifié et cristallisé pour fin d'analyse. Les informations indiquent une sélectivité très large de ADAMTS1 envers ces substrats comparativement à ADAM33 (GERHARDT *et al.*, 2007). Le domaine catalytique d'ADAMTS5 est très similaire a celui d'ADAMTS1 (SHIEH *et al.*, 2007). Ces analyses viennent confirmer le fait que les ADAMTS1 et 5 peuvent cliver l'aggrecan, le brevican et le versican à plusieurs endroits, indiquant ainsi une large sélectivité de substrats.

Dans le but de caractériser davantage le mécanisme d'activation des ADAMTS, il serait intéressant de cristalliser le prodomaine et le domaine catalytique d'une ADAMTS. Cette analyse permettrait entre autres d'identifier les surfaces du prodomaine accessibles à

la furine. Il serait ainsi possible de déterminer si les sites de clivage potentiels sont tous accessibles ou si seulement le site le plus près du domaine catalytique est accessible à la furine. D'ailleurs, une cristallisation du prodomaine et du domaine catalytique est nécessaire pour déterminer les résidus interagissant avec la pochette catalytique. Il serait aussi intéressant de déterminer où sont situés les résidus conservés des prodomaines des ADAMTS (CXYXG et YFIXPL) dans la structure tridimensionnelle. Ainsi, le mécanisme impliquant ces résidus conservés dans la sécrétion des ADAMTS1 et 9 pourrait être dévoilé. Par exemple, ces résidus pourraient notamment interagir avec la pochette catalytique ou être exposés à la surface du prodomaine pouvant ainsi interagir avec d'autres molécules telles des chaperones.

Une cristallisation d'ADAMTS9 et de son prodomaine permettrait d'évaluer si le prodomaine cache ou non la pochette catalytique. Étant donné que le clivage du prodomaine d'ADAMTS9 inactive l'enzyme, il serait pertinent de déterminer où se situe le prodomaine. Cache-t-il la pochette catalytique ou contribue-t-il à exposer la pochette catalytique? Ces expériences de cristallographie du prodomaine et du domaine catalytique nous en apprendraient davantage sur les mécanismes d'activation des ADAMTS. Par ailleurs, il ne faudrait pas oublier de prendre en considération les domaines auxiliaires qui jouent aussi un rôle dans la structure tridimensionnelle des ADAMTS et sont aussi régulés par des clivages protéolytiques.

3. Protéolyse dans le domaine auxiliaire des ADAMTS

Lors de la biosynthèse des ADAMTS, suite au clivage du prodomaine en N-terminal de la protéine, un ou plusieurs clivages en C-terminal de la protéine surviennent dans le domaine auxiliaire. Nous avons démontré qu'ADAMTS5 subit un clivage autocatalytique à la Glu^{753} du domaine séparateur (voir figure 4, représentation schématique des ADAMTS) faisant partie du domaine auxiliaire (résultats non publiés). De tels clivages ont été rapportés pour ADAMTS4, qui tout comme ADAMTS5, clive l'aggrécan. Le clivage en C-terminal joue un rôle important dans la spécificité d'ADAMTS4 contre l'aggrécan (GAO *et al.*, 2002). En effet, la forme longue d'ADAMTS4 possède une meilleure activité pour cliver l'aggrécan dans le domaine globulaire au site Glu^{1480}-Gly^{1481}. Ce site de clivage n'est pas défini comme étant le site aggrécanase entraînant la dégradation du cartilage. On retrouve dans le synovium de patients atteints d'arthrose l'aggrécan clivé au site (Glu^{373}-Ala^{374}) du domaine interglobulaire de l'aggrécan (STRUGLICS *et al.*, 2006). C'est seulement la forme clivée en C-terminal d'ADAMTS4, dont le domaine séparateur est relâché, qui possède l'activité contre le site du domaine interglobulaire de l'aggrécan (Glu^{373}-Ala^{374}) entraînant la dégradation du cartilage (GENDRON *et al.*, 2007). ADAMTS5, quant à elle, possède une plus grande activité catalytique contre le site Glu^{373}-Ala^{374} de l'aggrécan lorsqu'elle n'est pas clivée en C-terminal à la Glu^{753} (GENDRON *et al.*, 2007). ADAMTS5 possède aussi une meilleure efficacité (1000X) *in vitro* contre l'aggrécan qu'ADAMTS4 (GENDRON *et al.*, 2007). Par contre, il est important de noter que ADAMTS5 tout comme ADAMTS4 n'ont presque aucune activité catalytique contre l'aggrécan lorsqu'on tronque le domaine auxiliaire en entier (GENDRON *et al.*, 2007; KASHIWAGI *et al.*, 2004). Nous avons aussi confirmé ces résultats pour ADAMTS5 (résultats non publiés) et pour ADAMTS9 (SOMERVILLE *et al.*, 2003a). En effet, ADAMTS5 Pro-Cat et ADAMTS9 Pro-Cat n'ont aucune activité aggrécanase. L'absence

d'activité du domaine catalytique lorsqu'il est exprimé seul, sans les autres domaines de la peptidase, est plutôt rare chez les peptidases. Habituellement, le domaine catalytique seul possède une activité catalytique et les autres domaines régulent l'activité. Cette observation vient appuyer le fait qu'il semble très difficile d'obtenir des inhibiteurs sélectifs des domaines catalytiques des MMPs (OVERALL et KLEIFELD, 2006; RAO, B.G., 2005) et des ADAMTS (WITTWER *et al.*, 2007). Les différents domaines retrouvés en C-terminal du domaine catalytique seraient importants pour la reconnaissance des substrats comme l'aggrécan qui est une protéine hautement glycosylée (ZENG *et al.*, 2006). Les domaines thrombospondines de type I lient les glycosaminoglycans et sont nécessaires mais non suffisants pour la liaison des ADAMTS4 et 5 à l'aggrécan. De plus, outre une certaine spécificité de clivage au résidu Glu↓, ADAMTS4 reconnaît ses substrats par leur conformation tridimensionnelle. ADAMTS4 possède une meilleure affinité contre un substrat ayant une structure tridimensionnelle triple hélice (LAUER-FIELDS *et al.*, 2007). Étant donné l'implication des domaines auxiliaires dans l'activité aggrécanase des ADAMTS4 et 5, ces domaines sont des cibles thérapeutiques potentielles afin d'inhiber l'activité aggrécanase retrouvée dans l'arthrose.

Tout comme ADAMTS5, le clivage dans le domaine séparateur d'ADAMTS4 est autocatalytique mais il semble que des MMPs, dont notamment MT4-MMP, clivent dans le domaine séparateur d'ADAMTS4 (TORTORELLA *et al.*, 2005a). ADAMTS1 subit d'ailleurs un clivage par des MMPs telles MT2-MMP dans son domaine séparateur pour générer la forme de 65 kDa (RODRIGUEZ-MANZANEQUE *et al.*, 2000a). Le clivage en C-terminal d'ADAMTS12 n'est pas autocatalytique mais est inhibé par un inhibiteur des

métallopeptidases BB94, ce qui indique qu'ADAMTS12 est elle aussi clivée par des MMPs (CAL *et al.*, 2001). Il reste donc beaucoup à découvrir sur l'importance des domaines auxiliaires des ADAMTS car ceux-ci semblent jouer des rôles essentiels dans la régulation des ADAMTS.

4. Autres fonctions des prodomaines des ADAMTS

Le prodomaine des peptidases possède habituellement deux fonctions distinctes : inhiber l'activité enzymatique, ce qui a été discuté précédemment pour les ADAMTS, et une fonction de chaperone intramoléculaire. Les chaperones intramoléculaires agissent directement sur le repliement d'une protéine afin de diminuer les barrières énergétiques d'un état instable à un état stable (LAZURE, 2002a). L'absence de la chaperone intramoléculaire empêche la protéine d'intérêt de se replier par elle-même mais n'empêche pas la fonction de la protéine une fois repliée adéquatement (INOUYE, 1991). Lorsque la protéine est repliée, la chaperone intramoléculaire est clivée et relâchée soit par une action autacatalytique ou par une autre endopeptidase. Les chaperones sont attachées à leur protéine d'intérêt par un lien peptidique et sont spécifiques à cette protéine d'intérêt (INOUYE, 1991). Nous avons démontré que la forme tronquée d'ADAMTS9, contenant le peptide signal et le domaine catalytique, ne peut être sécrétée dans les cellules HEK293. Cette observation indique que le prodomaine d'ADAMTS9 est absolument nécessaire pour la sécrétion d'ADAMTS9. Le prodomaine agirait donc en tant que chaperone intramoléculaire afin de stabiliser ADAMTS9 pour lui permettre d'être sécrétée. Par contre, l'expression en *trans* du prodomaine n'a pu restaurer la sécrétion d'ADAMTS9 comme

c'est le cas pour MT1-MMP (CAO, J. *et al.*, 2000a) et ADAM17 (LEONARD *et al.*, 2005a).

L'investigation du rôle de la N-glycosylation du prodomaine d'ADAMTS9 par des analyses mutationnelles des trois sites potentiels de N-glycosylation démontre que la N-glycosylation de chacun des trois sites est un pré-requis pour la sécrétion d'ADAMTS9. L'addition de glycans au prodomaine d'ADAMTS9 est un facteur essentiel dans la sécrétion d'ADAMTS9. L'ajout de sucres s'est avéré essentiel à la sécrétion d'autres peptidases telles PAP21 (protease-associated domain-containing protein, 21 kDa) (ZHOU, Y.B. *et al.*, 2004) et même de protéines, ADAMTS-like-1/punctin-1, ayant la même structure mosaïque que les ADAMTS mais sans domaine catalytique (WANG, L.W. *et al.*, 2007).

Un alignement de séquences des prodomaines des 19 ADAMTS dévoile certains résidus et motifs conservés dans les prodomaines en plus des sites de N-glycosylation. De tels résidus conservés dans le prodomaine des MMP ont été identifiés dans le prodomaine de MT1-MMP. Il s'est avéré que les résidus conservés étaient impliqués dans des contraintes conformationelles importantes pour le repliement et la fonction de cette enzyme (PAVLAKI *et al.*, 2002). Nous avons identifié deux motifs hautement conservés dans les prodomaines des ADAMTSs, CXYXG et YFIXPL. La mutation en alanine des différents résidus du motif CXYXG ainsi que la mutation en alanine de tous les résidus du motif YFIXPL démontrent que ces résidus conservés sont importants pour la sécrétion d'ADAMTS1. Ces différents mutants non sécrétés semblent aussi ne pas être clivés efficacement par les PCs. Il est important de noter que la substitution de ces résidus

conservés affecte à un certain degré le repliement car l'expression de ces mutants est moindre qu'ADAMTS1 de type sauvage. Afin de s'assurer que nos observations sur les résidus conservés ne s'appliquent pas seulement pour ADAMTS1, nous avons muté ces même résidus conservés dans le prodomaine d'ADAMTS9 (LONGPRÉ, 2005). Effectivement, la mutation des motifs CXYXG et YFIXPL du prodomaine d'ADAMTS9 abolit aussi la sécrétion d'ADAMTS9. Les résidus conservés des prodomaines des ADAMTS sont nécessaires au bon repliement de la protéine et/ou sont impliqués dans des motifs de reconnaissance afin qu'elle soit maturée et sécrétée.

Une étude à démontré que deux enzymes de la même famille, la subtilisine E et l'aqualysine I, peuvent s'interchanger leur prodomaine et que celui-ci peut être en mesure, jusqu'à un certain point, de chaperoner le repliement intramoléculaire de la peptidase (TAKAGI *et al.*, 2001). Nous nous sommes donc demandé si ces résidus conservés pouvaient être suffisants pour permettre la biosynthèse des ADAMTS. Nous avons donc procédé à la construction d'une chimère où le prodomaine d'ADAMTS9 remplace le prodomaine d'ADAMTS1. Un zymogène intracellulaire a été détecté mais aucune forme clivée par les PCs et aucune forme sécrétée n'a été détectée (LONGPRÉ, 2005). Il semble donc que les prodomaines des ADAMTSs, malgré qu'ils possèdent certaines homologies, soient spécifiques à chacune des ADAMTS. Cette observation est d'ailleurs en accord avec le fait qu'il existe une panoplie de mécanismes d'activation des ADAMTS. Donc, chacun des prodomaines doit contenir des déterminants moléculaires impliqués dans leur propre mécanisme d'activation.

Les ADAMTS sont de plus en plus étudiées de par leurs nombreuses implications dans des pathologies telles la purpura thrombotique thrombocytopénique (TTP), l'arthrose et le cancer. Toutefois, jusqu'à présent, peu d'études ont été menées dans des modèles *in vivo*. Une récente étude démontre l'implication du prodomaine de MIG-17, une ADAMTS du nématode *Caenorhabditis elegans,* dans sa localisation tissulaire (IHARA et NISHIWAKI, 2007). Selon cette étude, le prodomaine de MIG-17 est nécessaire au recrutement de cette protéine à la membrane basale des cellules des gonades. En plus de leur fonction inhibitrice et de leur rôle de chaperone intramoléculaire, les prodomaines des ADAMTS jouent un rôle dans la localisation des ADAMTS. Il s'avère donc évident que les prodomaines des ADAMTS sont primordiaux à leur biosynthèse et à leur fonctionnalité.

CONCLUSION

Dans cette étude, nous avons démontré que les ADAMTS sont d'abord synthétisées sous forme de précurseurs pro-ADAMTS. Il s'en suit un clivage dépendant des PCs. La furine semble être la convertase la plus susceptible de cliver le prodomaine des ADAMTS *in vivo* car elle est la plus efficace et est distribuée de façon ubiquitaire. Par contre, il n'est pas exclu que d'autres PCs soient responsables du clivage de certaines ADAMTS (PACE4 et PC6B clivent ADAMTS1, PC6B et PC7 clivent ADAMTS7, PC5A clive ADAMTS9 et PC7 clive ADAMTS5).

Le prodomaine des ADAMTS contient un ou plusieurs sites potentiels de clivage par les PCs. Cependant, le clivage du site le plus près du domaine catalytique ($RKKR_{235}$ pour ADAMTS1, $RRRRR_{261}$ pour ADAMTS5, $RQQR_{220}$ pour mADAMTS7 et $RTKR_{287}$ pour ADAMTS9) produit la forme mature des ADAMTS.

Il existe une variété de mécanismes d'activation pour les ADAMTS. Pro-ADAMTS1 est clivée par la furine au Golgi et ce clivage induit l'activation de l'enzyme. Ce mode d'activation est celui retrouvé chez la plupart des substrats de la furine. L'activation de pro-ADAMTS7 est aussi effectuée par le clivage du prodomaine par la furine intracellulaire. La pro-ADAMTS7 peut aussi se rendre à la surface cellulaire où elle est clivée par les PCs. Pro-ADAMTS5 est, quant à elle, seulement clivée par la furine extracellulaire. Soit la furine à la surface cellulaire ou la furine larguée dans le milieu extracellulaire peuvent induire l'activité hyalectanase d'ADAMTS5. Le retranchement du prodomaine des ADAMTS1, 5 et 7 est nécessaire à leur activité catalytique démontrant la

fonction d'inhibiteur des prodomaines des ADAMTS. Pro-ADAMTS9 est aussi clivée par la furine à la surface cellulaire. Par contre, le clivage du prodomaine par la furine entraîne une diminution de l'activité hyalectanase d'ADAMTS9.

Les prodomaines des ADAMTS contiennent des motifs et des résidus conservés dans la famille des ADAMTS. Certaines ADAMTS possèdent des sites de N-glycosylation dans leur prodomaine. Les trois sites de N-glycosylation du prodomaine d'ADAMTS9 sont glycosylés. La N-glycosylation de chacun des trois sites est essentielle à la sécrétion d'ADAMTS9. Outre les motifs des prodomaines pouvant être glycosylés, les prodomaines des ADAMTS possèdent deux motifs hautement conservés, CXYXG et YFIXPL. La mutation en alanine de ces résidus chez ADAMTS1 empêche la maturation. De plus, la sécrétion d'ADAMTS1 et ADAMTS9 est grandement affectée lorsque ces résidus sont mutés en alanine. Le prodomaine des ADAMTS contient des résidus essentiels à la sécrétion de ces enzymes.

PERSPECTIVES

Il a clairement été démontré que les prodomaines des ADAMTS sont grandement impliqués dans la biosynthèse des ADAMTS. Les prodomaines sont premièrement essentiels pour le repliement adéquat de la protéine afin de poursuivre son cheminement dans la voie de sécrétion constitutive et deuxièmement, ils sont impliqués dans les différents mécanismes d'activation des ADAMTS. Ces connaissances donnent lieu à de nouvelles questions. Il serait intéressant de découvrir par quel mécanisme moléculaire les résidus conservés du prodomaine des ADAMTS sont impliqués dans la biosynthèse de ces enzymes. Est-ce que ces résidus apportent des contraintes conformationnelles? Interagissent-ils plutôt avec d'autres protéines telles des chaperones afin d'induire le repliement adéquat des ADAMTS? Dans un autre ordre d'idées, la furine interagit avec ADAMTS5 dans la cellule mais ne clive pas ce substrat. Quels sont les déterminants moléculaires responsables de ce phénomène intriguant et nouveau? Mais plus encore, pourquoi certaines ADAMTS sont clivées à l'intérieur de la cellule et d'autres à la surface cellulaire? Est-ce que les prodomaines détiennent les déterminants nécessaires pour entraîner un clivage par la furine intracellulaire ou par la furine à la membrane plasmique?

D'ailleurs, des questions d'ordre pharmacologique sont encore plus intéressantes. ADAMTS5 semble être l'enzyme responsable de la dégradation du cartilage chez les patients atteints d'arthrose. L'arthrose est une maladie très répandue (1 canadien sur 10 est atteint selon la Société d'arthrite (2007)) et présentement aucun traitement n'est disponible afin d'arrêter la progression de la maladie. À l'aide des connaissances apportées dans cette thèse, deux approches peuvent être envisageables afin d'inhiber l'activité protéolytique

d'ADAMTS5 : inhiber l'activité d'ADAMTS5 ou inhiber l'activation d'ADAMTS5. En ce qui a trait à l'inhibition d'ADAMTS5, nous avons démontré dans cet ouvrage que le prodomaine attaché à ADAMTS5 inhibait l'activité enzymatique de celle-ci. Il serait donc pertinent de savoir si l'addition exogène du prodomaine d'ADAMTS5 peut inhiber l'activité aggrécanase d'ADAMTS5 et ainsi empêcher la dégradation du cartilage. Sachant qu'ADAMTS5 est activée à la surface cellulaire et qu'elle est le seul substrat endogène connu jusqu'à présent de la furine à la membrane plasmique, nous pourrions tirer avantage de ce mécanisme identifié au cours des présentes études. En effet, l'inhibition de l'activité de la furine pourrait empêcher l'activation d'ADAMTS5 et par le fait même, l'activité aggrécanase d'ADAMTS5. L'idée repose sur le fait qu'il est impératif d'inhiber seulement la furine extracellulaire et non intracellulaire compte tenu de la panoplie de processus physiologiques impliquant la furine intracellulaire. Donc, il serait intéressant d'utiliser des inhibiteurs sélectifs de la furine et non perméables à la membrane plasmique afin de diminuer la progression de l'arthrose.

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