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Function of the Stroma-Derived Metalloproteinase, Stromelysin-3, in Invasive Breast Carcinomas

PRINCIPAL INVESTIGATOR:
Dr. Stephen J. Weiss

CONTRACTING ORGANIZATION:
Regents of the University of Michigan
Ann Arbor, Michigan 48109-1274

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Stromelysin-3, a new member of the matrix metalloproteinase gene family, is specifically expressed in stromal cells surrounding invasive breast carcinoma cells, but its function has remained largely undefined. We now report that human stromelysin-3 is processed from an inactive zymogen to an active endoprotease following intracellular activation by the proprotein convertase, furin. Active stromelysin-3 is then secreted extracellularly where it is able to cleave and inactivate members of the serine proteinase inhibitor family. Given the fact that breast cancer cells have recently been shown to secrete powerful proteinase (e.g., leukocyte elastase, plasmin) whose activities are normally regulated by serine proteinase inhibitors, our data suggest that stromelysin-3 may regulate breast cancer cell function by controlling the local proteinase-antiproteinase balance.
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I. INTRODUCTION

Current evidence suggests that breast carcinoma cells invade local tissues and metastasize by either expressing, or inducing the expression of, proteolytic enzymes that degrade structural barriers established by the extracellular matrix (ECM).\textsuperscript{1-5} Although the identity of the specific proteinases that lend cancer cells their invasive potential remains the subject of conjecture, attention has recently focused on the matrix-degrading metalloproteinases (MMPs) - a family of at least 11 zinc-dependent proteolytic enzymes whose overlapping substrate specificities include all of the major components of the ECM.\textsuperscript{1-5} Consistent with their presumed role in tumor progression, in situ analyses of a variety of cancerous tissues have confirmed heightened levels of expression of one or more MMPs in tumor and/or surrounding stromal tissues.\textsuperscript{6-8} Furthermore, a range of in vitro as well as in vivo studies have demonstrated that invasion and metastasis can be affected by altering MMP activity.\textsuperscript{9-12} Given these findings, efforts have intensified to identify those MMPs that might be used as diagnostic indicators or potential targets for pharmacologic intervention in breast as well as other cancers.

Until recently, attempts to implicate specific MMPs in breast cancer progression were based on the assumption that all of the major proteinases belonging to this gene family had been identified and characterized.\textsuperscript{2} Unexpectedly however, differential screens of breast cancer cDNA libraries led to the tentative identification of a new member of the MMP family, termed stromelysin-3, on the basis of its apparent homology to stromelysin-1 and -2 (two other members of this gene family that had been previously characterized).\textsuperscript{13} The expressed gene product was predicted to encode a 488-residue protein containing i) a candidate leader sequence, ii) a highly conserved PRCGVPD motif believed to maintain the latency of MMP zymogens, iii) a zinc-binding catalytic motif and iv) a carboxyl-terminal domain with sequence homology to the heme-binding protein, hemopexin and the ECM molecule, vitronectin.\textsuperscript{13} Interestingly, in all invasive breast carcinomas examined thus far, ST-3 was not a product of the neoplastic cells themselves, but rather the surrounding stromal cells.\textsuperscript{6,12,13}

Given the structural similarity between ST-3 and other members of the MMP family, it was postulated that the enzyme would be secreted as a zymogen whose extracellular activation at the tumor-stroma interface would follow the destabilization of the ligand formed between the Cys in the PRCGVPD domain and the Zn\textsuperscript{12} in the catalytic domain.\textsuperscript{6,12,13} In a scheme analogous to that established for the other MMPs, ST-3 could then under autoproteolytic processing to a mature, active form which presumably would catalyze the degradation of critical ECM components localized in either the basement membrane or stroma.\textsuperscript{4,5} However, despite the structural similarities that exist between ST-3 and other MMP members, additional studies indicate that i) the primary sequence of ST-3 is distinct from all other members of the MMP family and ii) the assumed role of ST-3 in ECM remodeling cannot be readily confirmed.\textsuperscript{4,14} First, comparisons of the catalytic domains of the MMPs suggest that ST-3 belongs in a new subgroup relative to all other members of this gene family and that its closest homology lies with the bacterial metalloproteinases.\textsuperscript{4} Consistent with the contention that ST-3 is a structurally distinct entity in the MMP family, the human ST-3 gene has been localized to the long arm of chromosome 22, a position which differs from those of all other MMP genes.\textsuperscript{4,12} Second, although the expression of the human ST-3 protein has not yet been reported, the homologous mouse enzyme (~80% homology identity at the amino acid level) could only be isolated in a truncated form that expressed weak proteolytic activity.\textsuperscript{14} Indeed, these results led Murphy and colleagues to conclude that "the evidence that mature full length stromelysin-3 is a metalloproteinase could not be substantiated and the precise role of this protein in vivo remains to be elucidated."\textsuperscript{14} Taken together, these findings indicate that despite the provocative correlative link established between ST-3 expression and
breast cancer progression, the role of this proteinase in invasive events remains undefined. In this proposal, we seek to use a series of in vitro as well as in vivo biochemical and molecular approaches to i) purify and characterize ST-3 expressed by stably-transfected mammalian cells, ii) determine the mechanism by which the ST-3 zymogen is activated and regulated, iii) determine the ability of stromelysin-3 to regulate the invasive potential of breast carcinoma cells in vitro and iv) characterize the role of ST-3 in regulating invasive potential in vivo in a transgenic mouse model.

II. BODY

In order to characterize the structure/function relationships of ST-3, we i) stably expressed human ST-3 in mammalian cells, isolated the mature full-length form of the proteinase and established its catalytic properties, ii) identified the first set of physiologic targets for the mature enzyme and iii) identified a unique ST-3 activation mechanism that is regulated by a 10 amino acid insert located at the junction between the pro-domain and the mature form of the enzyme. All of the data presented are published results (see Appendix).

ISOLATION AND CHARACTERIZATION OF ST-3

a. Expression of recombinant human ST-3 in E. coli. A 1.5 kb cDNA fragment including the entire open reading frame of human ST-3 was cloned by RT-PCR using mRNA extracted from human breast cancer tissue. The authenticity of the clone was verified following sequencing of three different clones from both strands of the 1.5 kb cDNA. Compared to the published sequence of ST-3, only a single discrepancy was found, changing the nucleotide at position 499 from G to C. This change results in a non-conservative substitution of a His residue for an Asp residue at amino acid position 164 (His is the amino acid found at this position in the aligned sequence of interstitial collagenase, ST-1 and ST-2).4,5

ST-3 cDNA was subcloned into the PGEX-KG vector at the Nco I site resulting in an in-frame fusion between glutathione transferase (GST) and ST-3.15 This fusion protein includes a thrombin cleavage site downstream of the GST sequence so that the expressed product could be purified in a single step involving affinity chromatography over a glutathione-agarose column followed by thrombin cleavage.15 Following transformation of E. coli strain DH5α with control or test plasmids, the cells were grown and induced with isopropyl-1-thio-β-D-galactopyranoside as described.15 Cell pellets were lysed and the releasates analyzed for the presence of ST-3 (before or after thrombin cleavage) with antisera raised against a peptide corresponding to 88G97R in the NH2-terminal domain of ST-3. As expected, the fusion protein migrated with an Mr of ~80 K and 54 K before and after thrombin treatment, respectively. However, neither form of ST-3 expressed any enzymic activity and the purified protein was subsequently used to raise polyclonal antisera (see below).

b. Expression of recombinant human ST-3 in COS cells. In order to express ST-3 in mammalian cells, ST-3 cDNA was cloned into the mammalian expression vector pREP9 (under the control of RSV LTR) which contains a selectable neomycin marker and an EBV origin of replication allowing for extrachromosomal replication in COS cells at relatively high copy number.16 The expression vector (or the control plasmid) was then transfected into COS by the calcium phosphate co-precipitation method.17 Following selection with G418, resistant colonies were isolated, expanded and identified by Northern blot analysis. As shown in the Appendix, COS cells transfected with ST-3 (clone 4-2), but not control transfected cells, secreted a series of products that were specifically recognized by the anti-ST-3 polyclonal antisera.
To first determine whether the largest ST-3-immunoreactive product represented pro-ST-3, the mixture was treated with the organomercurial activator, 4-aminophenylmercuric acetate (APMA; 1 mM) for 1-24 h at 37°C.\(^4\,^5\) Although APMA induces all other members of the MMP family to undergo autoproteolytic processing,\(^4\,^5\) no shift in Mr could be observed for any of the bands identified with the ST-3 antisera. These results raised the possibility that all detected forms of ST-3 (including that with the largest Mr) had already undergone processing and lost their N-terminal pro-domains. However, anti-peptide polyclonal sera directed against the pro-domain recognized the ~65 kD species indicating that this form of ST-3 had not undergone processing. These results were consistent with at least 3 different scenarios: i) pro-ST-3 is the first member of the MMP family that is not sensitive to APMA-induced activation, ii) the ST-3 forms detected are catalytically inactive and thus, cannot respond to APMA treatment or iii) pro-ST-3 resists APMA activation, but the smaller Mr forms have already undergone processing to generate enzymically active forms with unknown activities. In an attempt to determine whether any of the forms of ST-3 detected by Western blotting could express enzymic activity, cell-free supernatants from control and ST-3-transfected COS cells were analyzed by gelatin, β-casein and κ-elastin zymography.\(^1\,^8\) With this technique, latent as well as active forms of all known MPPs can be detected on the basis of their ability to digest one or more of the embedded substrates following SDS-PAGE under non-denaturing conditions.\(^1\,^8\) The position of the MMP is then demarcated after enzyme renaturation and substrate degradation by zones of clearing following Coomassie staining and destaining. Under these conditions, the only band that could be identified by gelatin zymography was the endogenous MMP, 72 kDa gelatinase (termed gelatinase A), which was synthesized by control COS cells.\(^4\,^5\) Proteolytic bands associated with ST-3 could not be identified in the gelatin, casein or κ-elastin zymogram either before or after APMA treatment.

Despite the absence of detectable activity by zymography, these results did not rule out the possibility that i) ST-3 only undergoes activation under a restricted set of conditions distinct from those previously established for all other MPPs (i.e., neither APMA nor zymography can activate latent ST-3) or ii) a portion of the ST-3 has undergone activation, but its substrate specificity is distinct from all other MPPs. To unequivocally determine whether any of the recovered forms of ST-3 represented functional proteases, we took advantage of the fact that the plasma endoprotease inhibitor, α2-macroglobulin (α2M), is able to entrap a wide range of proteinases by displaying an ~30 amino acid "bait" region wherein attacking enzymes hydrolyze peptide bonds that overlap their sequence specificities.\(^1\,^9\) Following cleavage anywhere within this domain, α2M undergoes a major conformational change that locks the attacking proteinase in a molecular cage.\(^1\,^9\) Thus, if any of the forms of ST-3 recovered in the transfected COS cell supernatants were capable of expressing proteolytic activity, then they should be recognized by α2M and trapped. Hence, ST-3-containing cell-free supernatants were incubated with an excess of α2M, the mixtures separated by non-reducing SDS-PAGE and analyzed by Western blotting. Significantly, in the presence of α2M, a major ST-3-reactive band of ~50 kDa as well as minor bands of ~30 kDa disappeared, indicating entrapment in the α2M molecule. The fact that the disappearance of these bands reflects entrapment of the active enzyme was confirmed by the demonstration that the distribution of ST-3-immunoreactive bands was not changed in the presence of the metalloproteinase inhibitor, o-phenanthroline, or the synthetic MMP inhibitor, BB-94 (i.e., if ST-3 activity were blocked, the proteinase could not cleave the α2M bait region to signal entrapment).\(^2\,\,\,^0\) Furthermore, when purified ST-3 (see below) was incubated with α2M and the cleaved inhibitor submitted for N-terminal sequence analysis, a new cleavage site was identified between Phe\(^684\) -Tyr\(^685\). Interestingly, this cleavage site is identical to that previously reported for ST-1. Taken together, these results indicate that ST-3 can be processed, albeit by an unknown mechanism, to enzymically active forms that can be recognized by α2M.
c. Substrate specificity of the active mature form of human ST-3. To begin characterizing the properties of ST-3, initial attempts focused on isolating the 50 kDa species. Following a combination of gelatin affinity, Blue-4-Sepharose, heparin-Sepharose and gel filtration chromatography, a purified ST-3 fraction was obtained. N-terminal sequence analyses have demonstrated that ST-3 was hydrolyzed between Arg\textsuperscript{97}-Phe\textsuperscript{98}, corresponding to the N-terminus predicted from the homologous cleavage sites of other MMP members.\textsuperscript{4,5,21} Antisera raised against the COOH-terminal domain reacted with the 50 kDa species in Western blots, suggesting that this product represents the pro-domain truncated, COOH-terminal intact, mature enzyme. Significantly, the mature enzyme did not cleave type I or type IV collagens, elastin, laminin, fibronectin, thrombospondin, vitronectin, tenascin or gelatin. Because ST-3 gene expression is localized to the stromal fibroblasts immediately surrounding invasive breast cancer cells, we considered the possibility that physiologically relevant substrates might be detected among neoplastic or stromal cell-derived secretory products. Thus, active ST-3 was incubated with [\textsuperscript{35}S]methionine-labeled culture media conditioned by the MCF-7 breast carcinoma cell line or mammary fibroblasts, and the status of the secreted proteins was monitored by SDS-PAGE/fluorography. As shown in Fig. 5A, ST-3 rapidly hydrolyzed a major radiolabeled band in the MCF-7-conditioned medium with an Mr of \(\sim 55-60 \times 10^3\) in the absence, but not the presence, of BB-94. When ST-3 was incubated with conditioned media from mammary fibroblasts for 6 h under identical conditions, significant proteolysis could not be detected. To identify the ST-3 substrate in the MCF-7-conditioned medium, the released product was partially purified by anion-exchange chromatography, the fractions containing the \(\sim 55-60\)-kDa band pooled, separated by SDS-PAGE, electroblotted onto a polyvinylidene difluoride membrane, and submitted for N-terminal sequence analysis. After 15 cycles, a single species was identified with a sequence of EDPQGDAAQKTDTS. A search of the existing protein data base indicated an exact match with the N-terminus of the serine proteinase inhibitor, \(\alpha_1\)PI. As predicted by this result, anti-\(\alpha_1\)PI polyclonal antisera specifically and completely immunodepleted the \(\sim 55-60\)-kDa radiolabeled band from the conditioned medium.

To determine the functional status of MCF-7-derived \(\alpha_1\)PI, conditioned medium was incubated with ST-3, and the ability of the antiproteinase to form an ASDS-stable complex with exogenous porcine pancreatic elastase (PPE) was examined.\textsuperscript{22} An \(\alpha_1\)PI-elastase complex was detected in immunoprecipitates following the addition of PPE to tumor cell-conditioned medium that had either been incubated alone or with ST-3 in the presence of BB-94. However, following a 6-h incubation with active ST-3, the hydrolyzed antiproteinase lost its ability to complex PPE. When purified \(\alpha_1\)PI was used in place of the tumor cell-derived serpin, ST-3 similarly inactivated the antiproteinase via a process that was inhibitable by either BB-94, tTIMP-1, or tTIMP-2. Quantitative analyses demonstrated that at an enzyme:substrate ratio of 1:100, the anti-elastase activity of \(\alpha_1\)PI was almost completely destroyed within 90 min. Although fibroblast collagenase and stromelysin-1 have been previously shown to express serpinolytic activity,\textsuperscript{22,23} ST-3 inactivated \(\alpha_1\)PI at rates approximately 2-fold faster than either of these matrix-degrading metalloproteinases. Following inactivation by ST-3, N-terminal sequence analysis of proteolyzed \(\alpha_1\)PI revealed a single cleavage site distinct from those reported for either fibroblast collagenase or stromelysin-1 between Ala\textsuperscript{260} and Met\textsuperscript{251} in the reactive site loop of the antiproteinase. Together, these data demonstrate that ST-3 preferentially hydrolyzes the MCF-7-derived serpin, \(\alpha_1\)PI by attacking the antiproteinase with a sequence specificity distinct from that of other members of the MMP family. Based on the fact that MMPs frequently express the ability to inactivate multiple serpins,\textsuperscript{18,22,23} we considered the possibility that ST-3 might cleave other proteinase inhibitors. Recent studies indicate that the expression of the serine proteinase, urokinase, is - like ST-3 - increased at the mammary carcinoma-stroma interface.\textsuperscript{6} Because urokinase cleaves plasminogen to generate the powerful proteinase, plasin, we next examined the ability of ST-3 to inactivate
α₂-antiplasmin (the physiologic inhibitor of plasmin). Of note, ST-3 also inactivated α₂-antiplasmin at significant rates, raising the possibility that the mature form of the metalloproteinase participates in regulating the urokinase-plasminogen axis.

**ACTIVATION OF THE ST-3 ZYMOCEN**

MMPs expressed in transfected cell populations do not normally undergo spontaneous processing to active forms. Yet, Western blots of transfected COS cell supernatants consistently demonstrated that ~50% of the extracellular ST-3 had undergone processing to the mature 50 kDa form. Furthermore, when ST-3 was stably transfected into a variety of other cell lines including CHO, HT-1080, MCF7 and 293, similar results were obtained. Processing of the other members of the MMP family to mature forms can, however, be induced following the exposure of the zymogen to proteinases (e.g., urokinase or plasmin), oxidants, or plasma-membrane associated factors. To determine whether COS cells participated in ST-3 activation via a previously described mechanism, transfected cells were incubated under serum-free conditions in the presence of urokinase, plasmin, cysteine proteinase, aspartate proteinase or metalloproteinase inhibitors as well as antioxidants. However, none of these interventions impeded the processing of the ST-3 zymogen to the 50 kDa active form. Interestingly, however, the MMP inhibitors, rTIMP-1, rTIMP-2 and BB-94 each inhibited the formation of the small M₆ forms of ST-3. Thus, the ST-3 zymogen was processed to its mature form by an uncharacterized process, while the smaller M₆ forms of ST-3 were generated by a MMP-dependent process.

Interest in the unusual processing events associated with ST-3 expression was heightened by the realization that ST-3 has an unusual 10 amino acid insert which is located at the junction between the pro-domain and the N-terminus of the mature form of the active enzyme. Because an homologous insert cannot be found in any other member of the MMP family, we considered the possibility that this motif might play an important role in the activation cascade. To this end, a PCR-based strategy was developed to generate a domain swap wherein the 10 amino acid domain was deleted from ST-3 (denoted as ST-3-10) and inserted into interstitial collagenase (denoted as IC+10) at the homologous junction between the pro-domain and the N-terminus of the mature enzyme. (IC was chosen as the 10 amino acid recipient since its sequence shows the lowest degree of homology to ST-3 among the full-length MMPs. Following transfection of the mutant cDNAs into COS cells, radiolabeled ST-3, IC, ST-3-10 and IC+10 were immunoprecipitated and analyzed by SDS-PAGE/fluorography. Significantly, while ST-3 and IC were secreted in their expected forms, ST-10 accumulated as a single band with an M₆ of ~65 K (i.e., consistent with the production of the ST-3 zymogen) while IC+10 was recovered as the fully processed, active form. These data demonstrated that the 10 amino acid insert regulates the ST-3 activation sequence.

While attempting to determine the kinetics with which ST-3 or IC+10 underwent processing, pulse-chase experiments demonstrated that the removal of the pro-domain unexpectedly occurred in an intracellular compartment rather than extracellularly. Because no other member of the MMP family has been reported to undergo pro-domain processing during intracellular transport, we examined the 10 amino acid sequence more carefully in an attempt to discern any additional clues that might be presented. Interestingly, we noted that this insert (i.e., GLSARNRQKR) contains an R(X)R(X)R/KR motif (where R=Arg and K=Lys in the single letter amino acid code) which has recently been reported to function as a recognition sequence for members of a new class of intracellular processing enzymes termed the proteolytic convertases (PCs). As recently reviewed by Steiner et al., intracellular processing enzymes have long been known to play a key role in the posttranslational proteolytic events that are required for the
expression of biological activity.\textsuperscript{26,27} While prohormones of neuroendocrine origin often contain a paired dibasic motif (K/RK) at the cleavage site within the precursors, other secretory proteins have an RXK/RR motif containing an additional R residue 4 residues upstream of the cleavage site.\textsuperscript{26,27} Although human enzymes capable of catalyzing these cleavages have long been sought, human homologues of the yeast propeptide processing enzyme, Kex2, have only recently been identified.\textsuperscript{26,27} Presently, two of the convertases, furin and the furin-like enzyme, PACE4, appear to be localized to the Golgi region in a wide variety of cell types, resulting in the suggestion that these enzymes are involved in the cleavage of proproteins secreted via the constitutive pathway.\textsuperscript{26,27} Based on our observations that ST-3 activation occurs intracellularly via a mechanism linked to the presence of a 10 amino acid insert that contains the PC-like recognition sequence RXK/RR (as well as an R at P6 which has recently been reported to enhance recognition by PACE4),\textsuperscript{28} we postulated that ST-3 might represent the first example of a proteinase zymogen that undergoes PC-dependent intracellular processing to generate an active enzyme destined for constitutive secretion. Indeed, mutations inserted at positions -1, -2, -4 or -6 relative to the scissile bond (that is, P\textsuperscript{1}, P\textsuperscript{2}, P\textsuperscript{4} and P\textsuperscript{6}, respectively) almost completely inhibited processing and the mutants were secreted as unprocessed zymogens.

To assess the relative efficiency of furin versus PACE4 in ST-3 processing, COS-7 cells were co-transfected with ST-3 cDNA and either furin or PACE4 cDNAs. While COS cells transfected with either proprotein convertase express the respective proteases comparably,\textsuperscript{29} only furin increased ST-3 processing (Figure 4A). Furthermore, when ST-3-transfected COS-7 or HT-1080 cells were co-transfected with the Pittsburgh mutant of \( \alpha_1 \)PI (\( \alpha_1 \)PI\textsubscript{Pit}), a reactive site variant that inhibits furin (but not PACE4) activity \textit{in situ},\textsuperscript{29} ST-3 processing was completely blocked. Consistent with these findings, LoVo cells, a carcinoma cell line that does not produce functional furin,\textsuperscript{29} were unable to process the ST-3 zymogen to its active form. However, when LoVo cells were co-transfected with ST-3 and furin (but not PACE4) cDNAs, processing was re-established. Finally, to determine whether furin directly mediates ST-3 processing, a soluble form of the convertase was generated by deleting the transmembrane domain\textsuperscript{29} and the purified mutant incubated with the ST-3 zymogen under cell-free conditions. At neutral pH, the soluble furin mutant efficiently cleaved the ST-3 zymogen at the Arg\textsubscript{97}-Phe\textsubscript{98} junction (as determined by N-terminal sequencing) to generate the active 45 kDa form of the proteinase. Together, these data not only demonstrate that ST-3 is the first member of the MMP gene family to undergo intracellular activation, but also identify the existence of a new proprotein convertase-MMP axis.

\section*{III. Conclusion}

The completed research has allowed us to demonstrate that human ST-3 can be processed via an intracellular route directly to an active form which can be constitutively secreted into the extracellular milieu where it can regulate the proteinase-antiproteinase balance at the tumor-stromal cell interface. Should ST-3 prove to play a critical role in tumor invasion (Aims #3 and #4 of the original proposal) these data would indicate that recently developed proprotein convertase inhibitors could be developed as new anti-cancer therapeutics. Furthermore, our results also indicate that a recently discovered new MMP family members (the so-called membrane-type MMP) may be similarly regulated by proprotein convertases.\textsuperscript{29} Because both ST-3 and MT-MMP are up-regulated in primary as well as metastatic disease in breast cancer, our results indicate that pharmacologic interference with proprotein convertase function could have far reaching implications in controlling tumor invasion.
IV. REFERENCES


V. APPENDIX

1. Publications
   

2. List of All Personnel Receiving Pay
   a. Stephen Weiss, M.D.
   b. Duanqing Pei, Ph.D.
   c. Susan Steven, B.S.
Hydrolitic Inactivation of a Breast Carcinoma Cell-derived Serpin by Human Stromelysin-3*

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Duanqing Pei, Gopa Majumdar, and Stephen J. Weiss††

From the Departments of Internal Medicine and Dermatology, University of Michigan Comprehensive Cancer Center, University of Michigan, Ann Arbor, Michigan 48109

To elucidate structure-function relationships of stromelysin-3, a putative matrix metalloproteinase originally identified at the tumor-stromal cell interface in breast carcinomas, the human cDNA was expressed in mammalian cells, and its products were isolated and characterized. In stably transfected cells, stromelysin-3 was recovered as a complex mixture of species ranging in size from ~20 to 65 kDa. Among these products, a major 45-kDa species with an N terminus of Phe68 and an intact C-terminal domain was identified as a serine endopeptidase on the basis of its ability to cleave the bait region of α2-macroglobulin between Phe684 and Tyr685, a site identical to that recognized by stromelysin-1. However, unlike stromelysin-1 or other members of the matrix metalloproteinase family, the mature form of stromelysin-3 was unable to hydrolyze a range of extracellular matrix molecules associated with either the basement membrane or interstitial. To probe for alternate substrates among tumor cell-derived products, purified stromelysin-3 was incubated with [35S]methionine-labeled medium conditioned by the breast cancer cell line, MCF-7. Under these conditions, a single, tumor cell-derived protein was hydrolyzed as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Following anion-exchange chromatography and preparative gel electrophoresis, the stromelysin-3 substrate was identified by N-terminal sequencing as the serine proteinase inhibitor, α1-proteinase inhibitor. Further studies demonstrated that stromelysin-3 rapidly destroyed the antiproteolytic function of α1-proteinase inhibitor by cleaving the proteinase at a distinct site between Ala689 and Met695 within the reactive-site loop. Together, these data not only demonstrate that human stromelysin-3 acts as a powerful endopeptidase with a restricted substrate specificity distinct from all other matrix metalloproteinases, but also serve to identify serine proteinase inhibitors as potential physiologic targets at sites of extracellular matrix remodeling.

Differential screening of breast cancer cDNA libraries recently led to the tentative identification of a new metallopro-

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† To whom correspondence should be addressed: Division of Hematology/Oncology, 5240, MSRB III, 1150 W. Medical Center Dr., Ann Arbor, MI 48105-0668. Tel: 313-763-4725; Fax: 313-763-4226.

‡‡ To whom correspondence should be addressed: Division of Hematology/Oncology, 5240, MSRB III, 1150 W. Medical Center Dr., Ann Arbor, MI 48105-0668. Tel: 313-763-4725; Fax: 313-763-4226.

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EXPERIMENTAL PROCEDURES

ST3 cDNA Cloning—The ST3 cDNA was cloned by reverse transcription-polymerase chain reaction from a primary basal cell carcinoma RNA sample employing two primers designed according to the published sequences (1). 5'-TGGTCTCTAGGCTCCTGACCTATAGCTGGCCTGCA-3' with the XbaI site underlined, 5'-TACAGATT- TCCATGGCGGCTCGGCGCTCCGCGACGGCG-3' with the EcoRI site underlined. Reverse transcription-polymerase chain reaction was carried out according to the supplier (Perkin-Elmer) and a single pre-

1 The abbreviations used are: ST3, stromelysin-3; α1M, α1-macroglobulin; α1i, α1-proteinase inhibitor; APMA, 4-aminophenylmercuric acetate; ECM, extracellular matrix; PPE, porcine pancreatic elastase; rTMP-1, recombinant human tissue inhibitor of metalloproteinases-1; rTMP-2, recombinant human tissue inhibitor of metalloproteinases-2; MMP, matrix-degrading metalloproteinase; DMEM, Dulbecco’s modified Eagle’s medium; PAGE, polyacrylamide gel electrophoresis.
Isolation and Characterization of Human Stromelysin-3

1.5-kb cDNA product cloned into pBlueScript (II) digested with EcoRI and XhoI. The cDNA was verified against the published sequence upon complete sequencing of both strands from three individuals.

Preparation of Polyclonal Antibodies—Peptides corresponding to Phe^3-Lys^6 in the pro-domain and the 25 C-terminal amino acid residues of ST3 were synthesized, coupled to keyhole limpet hemocyanin, emulsified in Freund’s adjuvant, and injected intradermally in specific pathogen-free New Zealand rabbits (7). To obtain polyclonal antibodies against the full-length proteinase, ST3 was subcloned into the pGEX-KG vector at the NcoI site resulting in an in-frame fusion between glutathione S-transferase and ST3 (8). Following transformation into Escherichia coli strain DH5α, inclusion bodies were purified, solubilized in urea and the fusion protein cleaved with thrombin as described (8). The ST3 moiety was purified by preparative SDS-PAGE, electrodialyzed, and injected intradermally into rabbits as described above.

Establishment of Stable COS-7 Cell Lines Producing Human ST3—An ST3 expression vector was constructed by cloning the ST3 cDNA into the HindIII-XhoI site of pREP9 (Invitrogen Co.) under the control of RSV-LTR. The pREP9-ST3 plasmid was then transfected into COS-7 cells by the calcium phosphate co-precipitation method (9). COS-7 cells were grown in DMEM (Life Technologies, Inc.) containing 10% fetal bovine serum (HyClone), 4 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humified 95% air, 5% CO₂ atmosphere. After 24 h, the cells were selected with G418 (Life Technologies, Inc.; 400 μg/ml), and clonal lines were maintained in selection media. Positive clones were screened by Northern and Western blot analyses using a human ST3 cDNA probe and polyclonal antiserum against the ST3 moiety, respectively. In selected experiments, cells expressing ST3 were cultured under serum-free conditions (see below) in the absence or presence of the reversible MMP inhibitor BB-94 (5 μM; British Biotech Industries Ltd.) (10).

Purification of Human ST3—ST3 clone 4-2 cells were cultured on collagen-coated microcarrier beds (Sole Hill) in 1750 flasks and grown to confluence. The cells were then incubated under serum-free conditions in DMEM supplemented with 0.2% bovine serum albumin and 1 μM BB-94. After 48 h, the conditioned medium (5 liters) was collected and concentrated ~25-fold by ultrafiltration through a YM-10 membrane (Amicon). The retained protein was dialyzed (50 mM Tris-HCl, 5 mM DTT, 1 μM BB-94, pH 7.5; termed buffer A), loaded onto a 2-10 cm blue Sepharose column (Sigma), and eluted in buffer A containing 1 mM NaCl. Eluted proteins were concentrated 10-fold, subjected to gelatin-Sepharose affinity chromatography (1 x 5 cm), the flow-through collected, dialyzed against buffer A, and loaded onto a 2 x 10 cm heparin-Sepharose column (Pharmacia Biotech Inc.). The column was washed with buffer A, and bound ST3 was eluted in a gradient of 0-100 mM NaCl. Following Western blot analysis, ST3-containing fractions were divided into pools I and II depending on the forms of the metalloproteinase detected (see “Results”). Pool II was concentrated and fractionated by gel filtration on an Ultragel AcA44 column (1 x 100 cm) in the absence of BB-94 to regenerate active enzyme.

Zymography, SDS-PAGE, and Western Blot Analysis—COS-7 cell supernatants as well as ST3-transfected COS-7 cells were incubated alone or with 4-anilino-7-diethylamino-phenylmercuric acetate (APMA; 0.5-2.0 mM) for 4-12 h at 37°C and analyzed by SDS-substrate gel electrophoresis (i.e. zymography) under non-denaturing conditions in 8.5% SDS-polyacrylamide gels impregnated with either 1 mM gelatin, α- or β-casein, or κ-casein as described elsewhere (11, 12). Purified protein products and substrate hydrolysis products were analyzed by SDS-PAGE on an 8.5% polyacrylamide resolving gel with heat-denatured samples under reducing or non-reducing conditions. Proteins were visualized by Coomassie Brilliant Blue R250 or silver staining as described previously (11, 12), while immunoblot analyses were performed as detailed in Harlow and Lane (7). Alkaline phosphatase-conjugated, goat anti-rabbit IgG was used as the secondary antibody and developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate p-toluidine as outlined by the supplier (Life Technologies, Inc.).

Identification of Active ST3 by α-M Entrapment—Aliquots of 4-2 conditioned media were incubated with purified human α-M (10 μg; Athens Research and Technology) in the absence or presence of BB-94 (5 μM) or sodium nitroprusside (1 μM) at 12 h at 37°C. Samples were separated by SDS-PAGE on an 8.5% polyacrylamide resolving gel under non-reducing conditions (13), electrophobed, and probed with full-length ST3 antisera as described above. Under non-reducing conditions, proteinase entrapped by α-M remained associated with the inhibitor (13).

Enzyme Assays—The amount of active ST3 used in enzyme assays was estimated by titration with α-M assuming a 1:1 stoichiometry. In brief, a 2-fold molar excess of α-M was incubated with ST3 for 4 h at

37°C and the residual α-M content quantitated with porcine pancreatic elastase as described elsewhere (13). In substrate assays, ST3 (20–500 ng) was incubated with 2–5 μg of fibronectin, laminin, vitronectin, thrombospondin, tenasin (all obtained from Calbiochem), β-casein (8 μg; Sigma), gelatin, type I collagen, EHS type IV collagen (Collaborative Research), α-PI, or α-antipain (Athena Research and Technology) in 50 mM Tris-HCl, 5 mM CaCl₂, 200 mM NaCl, and 0.1% NaN₃ (pH 7.5) at 30°C for 2 h in the absence or presence of full-length ST3, respectively. Tissue inhibitor of metalloproteinases-1 (TIMP-1; 0.1 μg/ml; Synergen Inc.), or recombinant tissue inhibitor of metalloproteinases-2 (rTIMP-2; 0.1 μg/ml; Amgen). Elastinolytic activity was determined with 3H-labeled insoluble elastin as described previously (13). The inhibitory activity of α-PI and α-antipain for pancreatic elastase and chymotrypsin, respectively, was quantitated spectrophotometrically in amidolytic assays (13, 14). In hydrolytic assays performed with human fibroblast collagenase or stromelysin-1 (provided by J. Seltzer, Washington University), the MMPs were activated with APMA (11) and active enzyme quantitated with α-M as described above.

Identification of α-M as an ST3 Substrate in MCF-7 Cell-conditioned Media—MCF-7 cells (purchased from ATCC) or primary cultures of mammary fibroblasts (obtained from reduction mammaplasties) were incubated with 100 μM [3H]methylmethionine (Du Pont-NEN) in methionine-free RPMI 1640 (Life Technologies, Inc.) for 6 h at 37°C. Conditioned medium (from ~10⁶ confluent cells) was recovered and unincorporated [3H]methylmethionine removed with a PD-10 desalting column (Pharmacia). Aliquots of medium containing ~3 x 10⁶ cpm (equivalent to ~10⁶ cells) were incubated with 50 ng of ST3 for 3 or 6 h and analyzed by SDS-PAGE (7.5%) and fluorography. To isolate ST3-sensitive species in MCF-7 cell-conditioned medium, cells were grown to confluency in DMEM supplemented with 10% fetal bovine serum, 4 mM l-glutamine, 100 mM/ml streptomycin. The cells were washed and incubated with DMEM supplemented with antibiotics and estradiol (10⁻⁷ M; Sigma). Forty-eight hours later, medium was collected, centrifuged, concentrated by ultrafiltration, dialyzed against 50 mM Tris-HCl (pH 7.5), and fractionated into 4-mll aliquots on a Q-Sepharose anion exchange column (2 x 10 cm) with a 0-1 x NaCl gradient. The ST3-sensitive species was identified by SDS-PAGE, electrophobed, and sequenced as described below. In selected experiments, α-PI was immunoprecipitated from MCF-7 cell-conditioned media. In brief, conditioned media (from ~10⁷ cells) was incubated with 0.5 μl of anti-α-PI polyclonal antiserum (Calbiochem) for 30 min at 4°C followed by the addition of 10 μl of 10% Panisorbin beads (Calbiochem) for an additional 30 min. The immune complexes were washed in radioimmune precipitation buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) and analyzed by SDS-PAGE and fluorography. Immunoprecipitated samples from each immunoprecipitation were either electrophoresed and electroblotted onto Prolab filters (Applied Biosystems) or destained with a polyvinylidene difluoride filter before analysis (12).

RESULTS

Expression of Human ST3 in Mammalian Cells—COS-7 cells transfected with ST3 cDNA secreted a series of products that were specifically recognized by polyclonal antibodies to the bacterially expressed full-length protein (Fig. 1). The molecular mass of the largest products (~65 kDa) is higher than that predicted on the basis of its primary structure (i.e. 51.4 kDa) and reflects the O-glycosylation of one or more sites in the pro-domain. Because the ~65-kDa species are the only ST3 products that reacted with polyclonal antibodies against the N-terminal (as well as the C-terminal; see below), these products have been tentatively identified as the full-lengthzymogen as well as a mixture of N-terminally processed or differentially glycosylated forms.

Given the fact that all previously characterized members of the MMP family can be detected by zymography (11, 12, 15), serum-free conditioned media from control or ST3-transfected COS-7 cells were electrophoresed in gelatin-, casein-, or κ-casein-impregnated SDS-gels and processed as described under “Experimental Procedures.” As shown in Fig. 1, no MMP activity other than that of an endogenous 72-kDa gelatinase synthesized by control COS-7 cells was detected. As expected, the

D. Pei and S. J. Weiss, unpublished observation.
Fig. 1. Expression of ST3 cDNA in COS-7 cells. A. Western blot analysis. Serum-free conditioned medium from COS-7 cells either stably transfected with a control (COS) or ST3 expression vector (4-2) was electrophoresed, blotted, and probed with either the full-length ST3 antisera or pro-domain antisera as indicated. Lane 1, medium from COS-7 cells; lanes 2 and 4, medium from 4-2; lane 3, medium from 4-2 treated with 1 mM APMA for 6 h at 37 °C. B, gelatin-zymography of conditioned medium from A. In lane 4, conditioned medium from 4-2 cells was gelatinase-depleted by gelatin-affinity chromatography (11, 12) prior to zymography.

72-kDa gelatinase underwent autocatalytic processing following a 6-h incubation with APMA (15), but no other bands of activity could be detected. Although the presence of the endogenous gelatinase could conceivably mask the presence of ST3, samples depleted of gelatinase by gelatin affinity chromatography (11, 12) (ST3 does not bind to gelatin; see below) were devoid of additional activities (Fig. 1).

Identification of a 45-kDa Form of ST3 as an Active Endopeptidase—To determine whether the absence of detectable ST3 activity could be attributed to an unusually restricted substrate specificity, we took advantage of the fact that the general endopeptidase inhibitor, α2M, displays a 30-amino acid baintrerin region wherein proteinases from all four major cathepsin classes cleave at sites consistent with their sequence specificities (16). Hydrolysis anywhere within this region signals α2M to undergo major conformational changes that lock the attacking proteinase in a molecular cage (16). Thus, to identify enzymically active forms of ST3, concentrated supernatants from 4-2 cells were mixed with α2M in either the absence or presence of APMA for 12 h, separated on non-reducing SDS-PAGE, transferred to nitrocellulose, and probed with ST3 antibodies. Surprisingly, despite the fact that no active forms were detected by zymography and no exogenous activating agent had been added, both the ~45-kDa and an intermittently detected (two of six experiments) ~30-kDa species were trapped by α2M (Fig. 2). Enzymatic activity was only with active ST3 since α2M did not interact with either of these species in the presence of the metalloproteinase inhibitors 1,10-phenanthroline or BB-94 (Fig. 2). In the absence of APMA, the ST3 forms detected did not undergo detectable proteolytic processing (Fig. 1), and no additional forms of the enzyme were trapped by α2M. Pulse-chase experiments demonstrated that the processing of the ST3zymogen directly to active forms occurred rapidly (within 40 min after a 5-min pulse) and could not be attributed to the use of COS-7 cells for expression, since similar results were obtained with stably transfected HT-1080, MCF-7, 293, or CHO cells (data not shown).

Isolation and Characterization of the 45-kDa Form of ST3—Although the multiplicity of ST3 products generated by COS-7 cells complicated early attempts to purify the active enzyme, the inclusion of MMP inhibitors during culture in serum-free media prevented the formation of the small molecular mass products without interfering with the generation of the 45-kDa species (data not shown). Following a combination of blue 4-Sepharose, gelatin-Sepharose affinity, heparin-Sepharose affinity, and gel filtration chromatography, the purified 45-kDa form of ST3 was isolated. Antibodies directed against the C-terminal 25 amino acids of the intact enzyme recognized the 45-kDa species, and N-terminal sequencing identified the start site of the enzyme as Phe<sup>60</sup>-ST3 (Fig. 3; herein referred to as the active or mature form of ST3).<sup>3</sup> Thus, COS-7 cells processed ST3 to a form which aligns with the fully active, mature forms of other MMPs (15). Although the 65-kDa form of ST3 was also isolated, the N-terminus of this species was blocked.

To probe the sequence specificity of active ST3, the purified enzyme was incubated with α2M and the scissile bond in the bait region identified at Phe<sup>60</sup>-Phe<sup>61</sup>, a cleavage site identical to that reported for stromelysin-1 (Fig. 4) (15). Like stromelysin-1 (15), active ST3 also readily degraded β-casein<sup>1</sup> (Fig. 4B). However, in contrast to the reported substrate specificity of stromelysin-1 (15), mature ST3 did not exhibit significant proteolytic activity against fibronectin, laminin, type IV collagen, gelatin or elastin. In addition, identical results were obtained when the substrate spectrum was expanded to include type I collagen, vitronectin, thrombospondin or tenasin (data not shown). Thus, despite the fact that ST3 and stromelysin-1 display an overlapping P<sub>1</sub>-P<sub>1</sub> cleavage site in α2M, ST3 failed to hydrolyze a panel of ECM substrates recognized by members of the MMP family.

Identification of an ST3 Substrate in Tumor Cell-conditioned Media—Because ST3 gene expression is localized to the stromal fibroblasts immediately surrounding invasive breast cancer cells (1, 5), we considered the possibility that physiologically relevant substrates might be detected among neoplastic or stromal cell-derived secretory products. Thus, active ST3 was in-

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<sup>3</sup>The predicted molecular mass of Phe<sup>60</sup>-ST3 is 44.2 kDa based on its amino acid sequence.

<sup>4</sup>Casinoactivity could not be visualized by zymography presumably because the small reduction in molecular weight following hydroyzation was not of a degrate sufficent to allow the fragments to diffuse out of the SDS-polyacrylamide gel.
Fig. 3. Purification of active ST3. A, fractionation of ST3 products on heparin-Sepharose affinity column. Following blue-Sepharose and gelatin-Sepharose chromatography, flow-through from 4-2 cells was loaded onto a heparin-Sepharose column and washed and eluted in a NaCl gradient. A representative Western blot from each pool is shown in the inset. B, Western blot and silver stain of purified 45-kDa ST3. Pool II was fractionated by gel filtration, and the 45-kDa fractions were pooled, concentrated, and analyzed by Western blot (full-length ST3 or C-terminal peptide antiserum) or silver staining. The amount loaded for Western blot and silver staining was ~100 and 250 ng, respectively. C, the N terminus of the 45-kDa ST3 product as determined after 10 cycles of sequencing (indicated by arrow). Identical results were obtained when ST3 was purified in the absence of BB-94. The open box represents the ST3 open reading frame while the amino acid sequence of full-length ST3 from Pro\(^{18}\) to Lys\(^{187}\) is shown above that of the first 10 cycles of the N terminus of the 45-kDa form of ST3 (1).

Fig. 4. Proteolytic activities of ST3 against the \(\alpha_M\) bait region and \(\beta\)-casein. A, \(\alpha_M\) bait region cleavage. \(\alpha_M\) (20 ng) was incubated with purified ST3 (1 ng) at 37 °C for 3 h, and the reaction mixture was resolved by SDS-PAGE, transferred to Problot membranes, and submitted for sequencing. Cleavage sites of other proteinases (obtained from literature) (15) are indicated as CC, Clostridium histolyticum collagenase; FIB-CL, fibroblast collagenase; ST-1, stromelysin-1; ST3, stromelysin-3; TL, thermolysin. B, hydrolysis of \(\beta\)-casein. \(\beta\)-casein (8 ng) was incubated alone (lane 1) or with ST3 (100 ng) in the absence or presence of 5 μM of BB-94 (lanes 2 and 3, respectively) for 3 h at 37 °C and analyzed by SDS-PAGE and Coomassie Blue staining.

cubated with \(^{35}\)S-methionine-labeled culture media conditioned by the MCF-7 breast carcinoma cell line or mammary fibroblasts, and the status of the secreted proteins was monitored by SDS-PAGE/fluorography. As shown in Fig. 5A, ST3 rapidly hydrolyzed a major radiolabeled band in the MCF-7-conditioned medium with an M, of ~55–60 x 10\(^3\) in the absence, but not the presence, of BB-94. When ST3 was incubated with conditioned media from mammary fibroblasts for 6 h under identical conditions, significant proteolysis could not be detected (data not shown). To identify the ST3 substrate in the MCF-7-conditioned medium, the released product was partially purified by anion-exchange chromatography, the fractions containing the ~55–60-kDa band pooled, separated by SDS-PAGE, electroblotted onto a polyvinylidene difluoride membrane, and submitted for N-terminal sequence analysis. After 15 cycles, a single species was identified with a sequence of EDIPQG-DAAQKTDTSH. A search of the existing protein data base indicated an exact match with the N terminus of the serine proteinase inhibitor, \(\alpha\)-PI. As predicted by this result, anti-\(\alpha\)-PI polyclonal antisera specifically and completely immunodepleted the ~55–60-kDa radiolabeled band from the conditioned medium (Fig. 5B).

To determine the functional status of MCF-7-derived \(\alpha\)-PI, conditioned medium was incubated with ST3, and the ability of
Fig. 5. Identification of α1PI as a substrate for ST3. A, cleavage of a protein species from MCP-7 conditioned media by ST3. Aliquots of MCP-7 conditioned media (~3 x 10^6 cpm) were incubated alone (lane 1) or with 50 ng of ST3 for 3 h (lane 2) or 6 h (lane 3) at 37°C. In lanes 4 and 5, ST3 was incubated with conditioned media for 3 or 6 h, respectively, in the presence of BB-94 (1 μM final). Mixtures were then analyzed by SDS-PAGE and fluorography. The arrow indicates the protein being cleaved by ST3. B, immunoprecipitation of the ST3-sensitive protein by anti-α1PI antisera and assessment of antiproteinase activity. Aliquots of [35S]methionine-labeled conditioned media were incubated alone (lane 1), or with Pansorbin beads preloaded with either normal rabbit sera (lanes 2 and 4), or rabbit anti-α1PI antisera (lanes 3 and 5). After centrifugation, supernatants (lanes 1-3) and pellets (lanes 4 and 5) were separated and analyzed by SDS-PAGE/fluorography as in A. To assess the ability of MCP-7-derived α1PI to complex PPE, conditioned medium was incubated with or without ST3 (50 ng) in the absence or presence of BB-94 (5 μM) for 6 h at 37°C. An aliquot of each mixture was subsequently incubated with PPE for 15 min at 25°C 15 min, and native, cleaved or complexed α1PI immunoprecipitated with anti-α1PI polyclonal antiserum. Pellets were analyzed by SDS-PAGE/fluorography. Immunoprecipitates recovered from conditioned medium incubated alone, with ST3, or with ST3 in the presence of BB-94 are shown before (lanes 6, 8, and 10, respectively) or after incubation with PPE (lanes 7, 9, and 11, respectively). A small amount of inactive α1PI can also be seen migrating at ~50 kDa (see Refs. 11 and 13). The arrow and arrowhead represent the position of α1PI and the α1PI-elastase complex, respectively. C, cleavage of purified α1PI by ST3. α1PI (2 μg) was incubated alone (lane 1) or with 20 ng of ST3 in the absence (lane 2) or presence of 100 ng of rTIMP-1 or 5 μM BB-94 (lanes 3 and 4, respectively) for 3 h at 37°C. PPE was added to α1PI alone (lane 5), α1PI cleaved by ST3 (lane 6) or α1PI incubated with ST3 in the presence of rTIMP-1 (lane 7) or BB-94 (lane 8) and incubated at 25°C for 5 min. Samples were then analyzed by SDS-PAGE. When rTIMP-2 (100 ng/ml) was used in place of rTIMP-1, identical results were obtained. None of the MMP inhibitors used affected PPE activity. D, time course of α1PI inactivation by ST3. α1PI (20 μg) was incubated alone (●), with 200 ng of ST3 in the absence (○) or presence of 5 μM BB-94 (black square with white circle), 200 ng of stromelysin-1 (■) or 200 ng of fibroblast collagenase (○). The residual α1PI activity was calculated as percent activity of control α1PI. Results are the mean ± 1 S.D. (n = 3).

the antiproteinase to form an SDS-stable complex with exogeneous porcine pancreatic elastase (PPE) was examined (11, 12). As shown in Fig. 5B, an α1PI-elastase complex was detected in immunoprecipitates following the addition of PPE to tumor cell-conditioned medium that had either been incubated alone or with ST3 in the presence of BB-94. However, following a 6-h incubation with active ST3, the hydrolyzed antiproteinase lost its ability to complex PPE (Fig. 5B; lanes 8–11). When purified α1PI was used in place of the tumor cell-derived serpin, ST3 similarly inactivated the antiproteinase via a process that was inhibitable by either BB-94, rTIMP-1, or rTIMP-2 (Fig. 5C). Quantitative analyses demonstrated that at an enzyme:substrate ratio of 1:100, the anti-elastase activity of α1PI was almost completely destroyed within 90 min (Fig. 5D). Although fibroblast collagenase and stromelysin-1 have been previously shown to express serpinolytic activity (11, 17), ST3 inactivated α1PI at rates approximately 2-fold faster than either of these ECM-degrading metalloproteinases (Fig. 5D). Following inactivation by ST3, 3-terminal sequence analysis of proteolized α1PI revealed a single cleavage site distinct from those reported for either fibroblast collagenase or stromelysin-1 (11, 18) between Ala358 and Met359 in the reactive site loop of the antiproteinase. Together, these data demonstrate that ST3 preferentially hydrolyzes the MCP-7-derived serpin, α1PI, by attacking the antiproteinase with a sequence specificity distinct from that of other members of the MMP family.

**DISCUSSION**

On the basis of previously described divergencies in sequence homology (2, 3) and the functional data presented herein, human ST3 appears to belong to a new subgroup of the MMPs. Unlike other members of this gene family (15), none of the forms of ST3 secreted by stably transfected cells underwent APMA-induced autoproteolytic processing and neither latent nor active products could be detected by zymography. Nonetheless, by using the bait region of α1M to "fish" for endoproteinase activity independent of prior information regarding sequence or substrate specificity, the 45-kDa processing product of ST3 was identified as the major active form of the metalloproteinase. Given an N terminus of Phe38 and an intact C terminus, ST3 is assumed to undergo an activation cascade similar to that proposed for most MMP members wherein the ~10-kDa prosequence is lost leaving the mature active species (15). Although this rapid and direct processing of an MMP family member to a fully processed form is unusual, we have recently identified an ubiquitous transmembrane serine proteinase that directly cleaves the ST3 zymogen at the Arg63-Phe88 bond to generate the mature enzyme.2
In addition to N-terminal truncation, all MMPs can undergo additional autolytic processing at variable rates within the proline-rich "hinge" region that separates the catalytic and C-terminal hemopexin domains (15, 19, 20). Consistent with this characteristic, an αM-reactive small molecular mass form (~30 kDa) was intermittently detected, but only after prolonged incubation (i.e., >24 h). Our demonstration that the 45-kDa form of ST3, rather than a C-terminal truncation product, represents the fully processed mature form of the enzyme contrasts with the recent characterization of recombinant mouse ST3 by Murphy and colleagues (4). In their study, the single active species detected was a 28-kDa fragment with truncated N and C termini that displayed only weak metalloproteinase activity (4). However, because C-terminal truncation can significantly modify MMP activity (15, 19, 20) and the N-terminal active form of the enzyme was not detected (4), neither the structure nor function of the mature form of mouse ST3 could be defined. Although differences in processing pathways could be related to structural peculiarities between the orthologues (20), truncated mouse ST3 was purified after a 5-day incubation under serum-free conditions, and zymography was used as the primary means of screening for active species (4). In the case of human ST3, prolonged incubation periods favor autolytic processing to small molecular mass forms of the enzyme and zymography does not allow for the detection of the 45-kDa metalloproteinase. Thus, while additional studies are needed to resolve possible differences between the two enzymes, use of the conditions employed to isolate mouse ST3 would have precluded detection or isolation of the human enzyme.

Prior to predicting physiologic substrates for ST3, we noted that the 45-kDa enzyme (i) cleaved the αM bait region at a site identical to that recognized by stromelysin-1, (ii) hydrolyzed the nonspecific MMP substrate, β-casein, and (iii) was sensitive to inhibition by TIMPs. Despite these similarities with other MMPs (15), ST3 did not degrade any one of a large number of ECM components. Although ST3 may yet display proteolytic activities against less well characterized ECM molecules, it is significant that all other known MMPs can cleave one or more of the substrates tested (15). From this perspective, the mature form of ST3 may not be designed to function as a matrix-degrading metalloproteinase. Indeed, when physiologically relevant substrates were sought among breast carcinoma cell- or mammary fibroblast-derived secretory products, the single major target molecule for ST3 was identified as α1PI. Although the primary site of α1PI synthesis is the liver, recent studies indicate that the serpin is also synthesized by epithelial cells where its function as a soluble or matrix-bound antiprotease remains the subject of conjecture (22, 23). Long thought to function specifically as an inhibitor of leukocyte elastase, a tissue-destructive proteinase stored in neutrophils and monocytes (24), increasing evidence indicates that α1PI can also regulate a variety of carcinoma cell functions. While α1PI has been previously shown to affect hepatocyte, fibroblast or erythroid progenitor cell growth (25–27), Finlay et al. (28) have demonstrated that the purified antiproteinase exerts a potent suppressive effect on the anchorage-independent growth of MCF-7 cells. Furthermore, in an unexpected finding, Yamashita et al. (29) recently reported that a wide range of human breast cancer cell lines (including MCF-7 cells) secrete neutrophil elastase and that elastase-α1PI complexes can be detected in carcinomatous breast tissues. Together, these data raise the possibility that the ST3-dependent inactivation of α1PI function could simultaneously affect the proliferative and invasive activity of neoplastic cells. Although we have noted that at least two other members of the MMP family can cleave α1PI (i.e., interstitial collagenase and stromelysin-1) (11, 18), both of these enzymes appear to display higher affinities for ECM molecules and would not be predicted to attack the serpin until competing ECM substrates had been almost completely hydrolyzed. Because ST3 does not appear to recognize ECM substrates, it secreted in an active rather than latent form and hydrolyzes α1PI at rates at least 2-fold faster than either collagenase or stromelysin-1, mature ST3 may act as the predominant regulator of serpin function at the tumor-stroma interface. Finally, these data should not be interpreted to suggest that the serpinolytic activity of ST3 is limited to α1PI. In additional studies, we have found that while ST3 cannot proteolyze α1-antichymotrypsin, plasminogen activator inhibitor-1, plasminogen activator inhibitor-2, or proteinase nexin (data not shown), it will inactivate the plasmin inhibitor, α1-antiplasmin (at an enzyme:substrate ratio of 1:100, ST3 inactivated 45 ± 7% of the antiproteinase during a 24-h incubation; n = 3). Because N-terminal sequence analysis demonstrated that α1-antiplasmin inactivation occurred as a consequence of cleavages between Ala192-Ala193 and Thr204-Ser205 in its reactive site loop, these data demonstrate that the enzymic activity of ST3 can be expanded beyond those target sequences identified in either αM or α1PI. The physiologic significance of α1-antiplasmin inactivation remains to be determined, but the possibility that ST3 might directly or indirectly regulate the activity of recently identified epithelium-derived serpins (30) or serine proteinases (31) deserves consideration.

The analyses of human ST3 function described in this report have by design focused on the enzymic activity of the N-terminally truncated form of the metalloproteinase as the major active species generated by transfected cells. However, it is important to note that a 50-kDa active form of ST3 was also detected, albeit intermittently, in our studies. Although sufficient amounts of this species could not be recovered for analysis, a preliminary report from our laboratory in which mature human ST3 was purported to express gelatin-, fibronectin-, and vitronectin-degrading activity appears to be due to the presence of small quantities of this truncated enzyme in early preparations (21). If these results can be confirmed, the substrate specificity of ST3 may ultimately be regulated by the degree of N-terminal as well as C-terminal processing. Thus, ST3 may be able to alter its substrate specificity from a highly restricted serpin-cleaving proteinase to a wide spectrum matrix-degrading enzyme as a function of the processing events to which it is subjected. Clearly, the final role that ST3 plays in physiologic or pathologic ECM remodeling in vivo awaits the development of the appropriate transgenic and knock-out animal models and these studies are currently underway in our laboratory. Nonetheless, the unusual structural and functional characteristics described herein, coupled with the recent identification of a Xenopus homologue of human ST3 (GenBank™ accession no. D27093), strongly suggest that this evolutionarily conserved metalloproteinase plays a unique role distinct from that of all previously characterized MMP family members.

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Furin-dependent intracellular activation of the human stromelysin-3 zymogen

Duanqing Pei & Stephen J. Weiss

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Duanqing Pei & Stephen J. Weiss

Division of Hematology/Oncology, Department of Internal Medicine, The University of Michigan Comprehensive Cancer Center, Ann Arbor, Michigan 48109-0640, USA

Human stromelysin-3, a new member of the matrix metalloproteinase family, is expressed in tissues undergoing the active remodelling associated with embryonic development, wound healing and tumour invasion. But like all other members of the matrix metalloproteinase gene family, stromelysin-3 is synthesized as an inactive precursor that must be processed to its mature form in order to express enzymic activity. Here we identify stromelysin-3 as the first matrix metalloproteinase to be discovered that can be processed directly to its enzymically active form by an obligate intracellular proteolytic event that occurs within the constitutive secretory pathway. Intracellular activation is regulated by an unusual 10-amino-acid insert sandwiched between the pro- and catalytic-domains of stromelysin-3, which is encrypted with an Arg-X-Arg-X-Lys-Arg recognition motif for the Golgi-associated protease, furin, a mammalian homologue of the yeast Kex2 prohormone convertase. A furin-stromelysin-3 processing axis not only differentiates the regulation of this enzyme from all previously characterized matrix metalloproteinases, but also identifies pro-protein convertases as potential targets for therapeutic intervention in matrix-destructive disease states.

Although previously characterized members of the matrix metalloproteinase (MMP) family are secreted as inactive zymogens, COS-7 cells stably transfected with ST3 complementary DNA (COS 4-2) spontaneously expressed ST3 activity and degraded α1-proteinase inhibitor (α1PI; a substrate for the active MMP). Proteolysis was completely inhibited by tissue inhibitor of metalloproteinase-1 or -2 (TIMP-1 or TIMP-2, respectively) or the synthetic metalloproteinase inhibitor, BB-94 (Fig. 1). Immunoprecipitation with ST3 polyclonal antisera revealed the presence of two major bands in the conditioned medium of ST3-transfected COS cells with approximate relative molecular masses of 65K and 45K, respectively (Fig. 1b). The 65K species represents the ST3 zymogen, and the 45K species was previously identified as the processed mature form of the enzyme on the basis of an amino terminus homologous to that of the mature form of known MMPs (that is, Phe 98), an intact carboxy terminus, and its ability to cleave the bait region of α2-macroglobulin. Importantly, ST3 processing to the active 45K form was similarly observed in ST3-transfected HT-1080 and MCF-7 (Fig. 1b) as well as 293, MDCK and CHO cell lines, where the secreted enzyme was recovered only in its mature form (n = 3). In the extracellular media, pulse-chase analysis of ST3 processing demonstrated that both the 65K zymogen and the 45K active forms of the proteinases were detected in tandem fashion by as early as 40 min post-labelling (Fig. 1c). These results are consistent with a rapid extracellular processing event, but the ratio of active ST3 to zymogen did not increase with continued incubation (Fig. 1c). Furthermore, the activation of the ST3 zymogen was unaffected by proteinase inhibitors capable of preventing processing of all other MMPs (that is, aprotinin, benzamidine, E-64, α2-macroglobulin, TIMP-1/2 or BB-94; data not shown). Although the rapid accumulation of active ST3 in the extracellular milieu and the resistance of the processing event to extracellular antiproteinases is consistent with an intracellular activation cascade, MMPs have been assumed only to undergo activation after secretion. However, when pulse-chase analyses of the intracellular pool of ST3 were examined, the 45K processed form of the proteinase could be detected before its secretion into the extracellular medium (Fig. 1c).

The intracellular maturation of the ST3 zymogen suggested that the zymogen displays an encrypted domain that initiates processing. Amino-acid sequence alignments comparing ST3 with other MMPs have identified a non-homologous 10-amino-acid insert that is sandwiched between the pro-domain and the N terminus of the active proteinase (Fig. 2a). To determine whether this decapetide regulates processing, the effect of switching this domain from ST3 to the homologous region in a structurally distinct MMP, human fibroblast collagenase (HFC), was assessed. As expected, although wild-type ST3 was processed to its active form, HFC was secreted as glycosylated and non-glycosylated zymogens (Fig. 2a, c). After domain switching, however, the deletion mutant of ST3 (termed ST3Δ10) was not processed and secreted only as a single, inactive ~65K species (Fig. 2b, c). In contrast, the insertional mutant of HFC (termed HFC⁺10) was secreted as a fully processed and active

* To whom correspondence should be addressed.
Fig. 1. Activation and processing of ST3. a. Detection of ST3 activity. α5PI was incubated alone (lane 1), or with supernatants from cells stably transfected with the control expression vector (lane 2), or the ST3 expression vector in the absence or presence of tIMP-1 (1 μg ml⁻¹), tIMP-2 (1 μg ml⁻¹) or BB-94 (5 μM) (lanes 3–6), respectively) and examined by SDS-PAGE/Coomassie staining. ααααα, protease inhibitor; ααααα, cleaved αααα; ST3 activation by transfected cell lines. COS, MCF-7 or HT-1080 cells transiently transfected with αααα and either the control (lanes 1, 3, 5) or the ST3 expression vector (lanes 2, 4, 6) were analysed by immunoprecipitation with ST3- or αααα-specific polyclonal antisera (upper and lower panels, respectively). The 65K and 45K species are the zymogen and processed forms of ST3, respectively (upper panels). Cleaved αααα is detected when coexpressed with ST3 in all three cell lines (lower panels; lanes 2, 4, 6) but not in control transfected cells (lanes 1, 3, 5). b, Pulse-chase analysis of ST3 processing in situ. COS-7 cells stably transfected with ST3 were pulse-labelled with [⁹⁵ᵐᵐ₃]methionine for 5 min and chased with unlabelled methionine for 0 min (lanes 1 and 6), 20 min (lanes 2 and 7), 40 min (lanes 3 and 8), 60 min (lanes 4 and 9) and 80 min (lanes 5 and 10). Supernatants (lanes 1–5) and lysates (lanes 6–10) were immunoprecipitated with anti-ST3 polyclonal antisera. The arrow indicates the position of the non-glycosylated ST3 precursor.

Methods. COS-7 cells stably transfected with the control or ST3 expression vector were described previously. Serum-free conditioned medium was collected from either cell population and incubated with αααα (10 μg ml⁻¹) in the absence or presence of MMP inhibitors for 6 h at 37 °C and analysed as described by SDS-PAGE (7.5%), COS-7, HT-1080 or MCF-7 cells were transiently transfected with the αααα and ST3 expression vectors by Lipofectamine treatment (BRL-GIBCO) and labelled with [⁹⁵ᵐᵐ₃]methionine (100 μCi ml⁻¹) for 3 h at 37 °C. For pulse-chase analyses, stably transfected COS-7 cells were labelled with [⁹⁵ᵐᵐ₃]methionine (500 μCi ml⁻¹) for 5 min and chased with 10 mM unlabelled methionine. Cells were lysed in RIPA buffer in the presence of PMSF, E-64, BB-94 and pepstatin. ST3 and αααα were immunoprecipitated with monoclonal polyclonal antisera and processed as described.

Fig. 2. A new 10 amino-acid insert in ST3 encodes an intracellular activation signal. a, Schematic representation of domain swaps between ST3 and HFC. The 10 amino-acid insert of ST3 (black box) is located between the pro- (labelled P) and catalytic (labelled ENZ) domains of ST3. ST3-10 is a deletion mutant lacking the insert between residues Asp 87 and Phe 98 whereas HFC-10 contains this motif inserted between residues Gln 99 and Phe 100 (ref. 5). b, Processing of ST3 and HFC mutants. ST3 (lane 1) but not ST3-10 (lane 2) was processed to its mature form. In contrast, HFC was secreted as glycosylated and non-glycosylated proforms (lane 3) whereas HFC-10 was processed completely to the mature enzyme (lane 4). Products were visualised by SDS-PAGE/trypsinogrophy after immunoprecipitation. Brackets indicate the glycosylated and non-glycosylated forms of HFC. c, Enzymic activities of ST3, ST3-10, HFC and HFC-10. Shown are αααα alone expressed alone (lane 1) or coexpressed with ST3 (lane 2) or ST3-10 (lane 3). The cleaved αααα products includes the ~50K fragment (upper arrowhead) and the ~4AK fragment (lower arrowhead). Native type I collagen (lane 4) was incubated with HFC or HFC-10 (lanes 5 and 7, respectively) or aminophenylmercuric acetate-treated HFC or HFC-10 (lanes 6 and 8, respectively). Although the HFC zymogen expressed no collagenolytic activity until activated with the organomercurial, HFC-10 was secreted as the fully active enzyme. The characteristic 3/4- and 1/4-sized fragments of cleaved type I collagens are indicated.

Methods. A sequential PCR strategy was used to generate both ST3-10 and HFC-10 (ref. 23). A 5′ primer containing the ATG codon and a 3′ primer containing the stop codon of ST3 were paired with two complementary internal primers encoding the deletion of the 10 amino-acid insert to generate two partial PCR fragments which were annealed and reamplified to generate ST3-10. HFC-10 was generated in a similar fashion with the following primers: a 5′ and a 3′ primer of HFC, and two complementary internal primers with a precise insertion of the 10 amino-acid insert of ST3 between Q 99 and F 100 of HFC. The ST-10 and HFC-10 PCR fragments were cloned and sequenced in pCloneAmp (BRL-GIBCO), and cloned into pPRE9. DNA transfection and immunoprecipitation was as in Fig. 1a. For N-terminal sequence determination, [³⁵S]-leucine-labelled HFC-10 was immunoprecipitated with specific polyclonal antisera, electrophoresed, immunoblotted and sequenced as described. Collagenolytic activity was analysed after a 12 h incubation with soluble type I collagen at 25 °C as described.

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Arg→Ala mutants at P-4 (R94K and R94A, respectively), Lys→Asp at P-2 (K96D) or Arg→Ala at P-1 (R97A), were all secreted as unprocessedzymogens (Fig. 3). Although an additional basic residue at P-3 has not been identified in other Arg-X-Lys/Arg-containing precursors, mutants containing this extended recognition sequence have been reported to undergo more efficient processing. Indeed, an Arg→Ala substitution at P-3 was processed, but only at a rate ~10% of that

![Diagram](image)

**Fig. 3** Amino-acid sequence requirements for ST3 processing. The basic residue motif in the 10 amino-acid insert (boxed) of ST3 is shown in bold letters. Mutations were introduced in each of these residues as well as the Phe 98 by a sequential PCR-based strategy. ST3 expression constructs harbouring these mutations were transiently transfected into COS-7 cells and analysed after immunoprecipitation and SDS-PAGE/fluorography as described in Fig. 1.

**METHODS.** Mutations were introduced into the desired positions in ST3 essentially as described for the generation of ST3. In Fig. 2, Mutagenic primers used are as follows: R97A, CGGAGGCGAAGGAGGCGTCGTGTTTCGGC: K96D, GCCGCCGAGCAAGAGGATAGTTGCTGCTTCT: R94K, CTGAGTCGCCCAAGCAAAGAGGCTGCGT: R94A, CTGAGTCGCCCAAGCAAAGAGGCTGCGT: R92A, GATTTGGCAGAGGCGCCGACAAGCAAGAGGCTGCTGTTTCGGC: R98Y, AACGGAGCAAGGAGGATAGTTGCTGCTTCT: TGCGCGCG. Bold nucleotides indicate the altered codons. The mutagenic primers were paired with the ST3 primer to generate C-terminal ST3 fragments carrying the desired mutations by PCR from an ST3 cDNA template. A PCR fragment coding for the pro-domain of ST3 (M1–K 96) was generated by amplifying the ST3 cDNA template with the 5' ST3 primer described previously and the primer, CGCCTTCTGCTGTTGGCGGAGCCTGCGGCATCGCCATC, whose complementary strand encodes D 87–K 96. The M1–K 96 fragment was annealed to each of the mutated C-terminal ST3 fragments generated above and PCR amplified with the 5' and 3' ST3 primers to generate full-length ST3 mutants as described in Fig. 2. The resulting PCR fragments were cloned into pCloneAmp, screened for mutations by DNA sequencing, and cloned into pREP9 expression vector.

![Diagram](image)

**Fig. 4** Processing of ST3 by furin and PACE4. a, Efficiency of furin and PACE4 as ST3-processing convertases. ST3 expression vector (1 μg) was cotransfected with 100 ng control vector (lane 1) or expression vectors for human furin (lane 2) or PACE4 (lane 3) into COS-7 cells. ST3 products were analysed after immunoprecipitation as described in Fig. 1. b, Inhibition of ST3 activation by α1Pl in situ. COS-7 cells (lanes 1–3) or HT-1080 cells (lanes 4–6) were transfected with the following expression vectors: ST3 alone (1 μg, lanes 1 and 4), ST3 and α1Pl (1 μg and 100 ng, respectively; lanes 2 and 5), or ST3 and α2Pl (1 μg and 100 ng, respectively; lanes 3 and 6). The ST3 products were analysed following immunoprecipitation as described in Fig. 1. c, ST3 processing in the furin-deficient cell line, LoVo. LoVo cells were transfected with expression vector for ST3 (lane 1), or with expression vectors for ST3 and either furin (1 μg, lane 2) or PACE4 (1 μg, lane 3). Radiolabelled ST3 products were analysed after immunoprecipitation as described in Fig. 1. d, Processing of proST3 by soluble furin under cell-free conditions. ProST3 (50 ng) was then incubated alone (lane 1) or with purified soluble furin (2 units; lane 2) and the mixtures analysed by western blotting with ST3 polyclonal antiserum. To assess ST3 proteolytic activity, α1PI (200 ng) was incubated alone (lane 3) or with proST3 (20 ng, lane 4), soluble furin (2 units; lane 5), furin-activated ST3 (lane 6) or furin-activated ST3 and TIMP-1 (50 ng, lane 7). Products were analysed by western blotting with α1PI polyclonal antiserum.

**METHODS.** ProST3 was purified from transiently transfected LoVo cells as described. Soluble furin expressed in transiently transfected COS-7 cells was purified and assayed according to ref. 26. Furin-ST3 mixtures were incubated for 2 h at 37°C in 10 mM Tris-HCl, 250 mM NaCl, 1 mM CaCl2 and 0.02% Brij 35 (pH 7.0). N-terminal sequence analysis of furin-processed ST3 was determined as described in the absence or presence of Bb-94. α1PI hydrolysis was determined under conditions in Fig. 1 and the antiprotease immunoassay with polyclonal antiserum (Calbiochem) as described.
observed with the wild-type proteinase (Fig. 3). Finally, given that substitutions in the P24 position are more readily tolerated, F98Y was processed comparably to wild-type ST3 (Fig. 3). Under the assumption that insert substitutions did not significantly perturb the conformation of the Arg-97–Phe-98 cleavage site, these results suggest that ST3 undergoes furin- or PACE4-mediated processing.

To assess the relative efficiency of furin versus PACE4 in ST3 processing, COS-7 cells were cotransfected with ST3 cDNA and either furin or PACE4 cDNAs (Fig. 4a). Although COS cells transfected with either pro-proteinase convertase express the respective proteases comparably, only furin increased ST3 processing (Fig. 4a). Furthermore, when ST3-transfected COS-7 or HT-1080 cells were cotransfected with the Pittsburgh mutant of α2PI (α2PI(p)), a reactive site variant that inhibits furin (but not PACE4) activity in situ, ST3 processing was completely blocked (Fig. 4b). Consistent with these findings, LoVo cells, a carcinoma cell line that does not produce functional furin, were unable to process the ST3zymogen to its active form (Fig. 4c).

However, when LoVo cells were cotransfected with ST3 and furin (but not PACE4) cDNAs, processing was reestablished (Fig. 4c). Finally, to determine whether furin directly mediates ST3 processing, a soluble form of the convertase was generated by deleting the transmembrane domain and the purified mutant incubated with the ST3zymogen under cell-free conditions. At neutral pH, the soluble furin mutant efficiently cleaved the ST3zymogen at the Arg-97–Phe-98 junction (as determined by N-terminal sequencing) to generate the active 45K form of the proteinase (Fig. 4d).

We have demonstrated that ST3 is processed directly to its mature active form by the pro-proteinase convertase, furin. A mammalian homologue of the yeast Kex2 phenrome convertase, furin is a transmembrane serine proteinase concentrated in the trans-Golgi network. Furin efficiently processes serum proteins, growth factors, and membrane receptors containing an Arg-X-Lys-Arg sequence, but an additional Arg residue at P25 was necessary for the efficient processing of the ST3zymogen. Apparently, the inclusion of this enhancing signal for human ST3 processing is evolutionarily conserved because mouse and Xenopus ST3 homologues each contain the identical motif. Importantly, a new transmembrane MMP that controls gelatinase A activation also contains a decapetide insert with an Arg-X-Lys-Arg motif upstream of its catalytic domain. Thus, we propose that pro-protein convertases may serve as important regulators of multiple metalloproteinases involved in extracellular matrix turnover. The unexpected identification of furin as an activator of MMPs that are strongly implicated in tumour progression suggests that recently developed pro-protein convertase inhibitors might be developed as anti-cancer therapeutics.