

Identification of Structural Determinants Controlling Human and Mouse Stromelysin-3 Proteolytic Activities*

(Received for publication, May 30, 1995)

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Matrix metalloproteinases (matrixins) constitute a group of extracellular proteinases belonging to the metzincin superfamily. They are involved in both physiological and pathological tissue remodeling processes, including those associated with cancer progression. Stromelysin-3, which is expressed in most invasive human carcinomas, is a matrix metalloproteinase with unusual functional properties. In particular, its mature form does not cleave any of the major extracellular matrix components. To define critical structural determinants involved in controlling stromelysin-3 proteolytic activity, we have used site-directed mutagenesis. We show that the deletion of at least 175 C-terminal amino acids is sufficient to endow mouse stromelysin-3 with activities against casein, laminin, and type IV collagen. In the case of the human enzyme, however, a further and single Ala-235 → Pro substitution is necessary to observe similar activities. Ala-235, which characterizes human stromelysin-3 among matrixins, is located immediately after the C terminus of the "Met-turn," which forms a hydrophobic basis for the catalytic zinc atom in the metzincin family. We conclude that human stromelysin-3 has gained specific functional properties during evolution by amino acid substitution in the catalytic zinc environment, and that it represents an attractive target for specific inhibitors that may be used to prevent cancer progression.

Matrix metalloproteinases (matrixins) constitute a group of extracellular proteinases of related primary structure, including collagenases, gelatinases, stromelysins, and their activators (1–3). Matrix metalloproteinases are believed to be medi-

ators of physiological and pathological remodeling processes occurring during development, involution, repair, and cancer progression (4–7). In particular, these enzymes have been shown through both correlative and direct inhibitor studies to be essential for cancer cell invasion (8–10). However, the demonstration that stromelysin-1 plays a role in morphogenesis during development (11) and the observation that a matrix metalloproteinase-like enzyme is implicated in tumor necrosis factor- α processing (12) suggest that the contribution of matrix metalloproteinases to tumor progression may be not limited to a role in cancer invasion and also involve other aspects of the malignant phenotype. In agreement with this possibility, matrix metalloproteinase expression is not specific to invasive carcinomas and is also observed in a number of precursor lesions (Ref. 13 and references therein).

Stromelysin-3 (ST3)¹ was identified through its overexpression in fibroblastic cells of invasive breast carcinomas (14), and similar observations were thereafter made in most other human carcinomas (13). ST3-expressing fibroblastic cells are specifically detected in the immediate vicinity of cancer cells, suggesting that ST3 may play a role in stromal-epithelial interactions during carcinoma progression, thereby contributing to tumor growth. Indeed, Engel *et al.* (15) have found that recurrent breast carcinoma was more frequent in patients showing tumors with high ST3 RNA levels than in those with low ST3 RNA levels. Although ST3 has a protein domain organization similar to that of other stromelysins (14, 16), it was found to display both unusual structural and functional properties (17, 18). In particular, the putative mature form of human ST3 is unable to hydrolyze any of the major extracellular matrix components of basement membranes or interstitium (19).

The present study was initiated to identify structural determinants controlling ST3 enzymatic activity. By site-directed mutagenesis, we demonstrated that deletion of the 175 C-terminal amino acids is necessary to endow mouse ST3 with proteolytic activities toward casein and some extracellular matrix molecules, while a similar deletion is ineffective with the human enzyme. However, when Ala-235 flanking the "Met-turn" (20) is replaced by a proline, the C-terminally truncated human protein exhibits proteolytic activities similar to those of the mouse enzyme. These findings add further support to the concept that human ST3 is a matrix metalloproteinase with unique functional properties and that it represents a potential target for specific inhibitors that may lead to the development of new anticancer agents (21, 22).

* This work was supported by funds from INSERM, CNRS, the Centre Hospitalier Universitaire Régional, the Mutuelle Générale de l'Education Nationale, the Groupement de Recherches et d'Etudes sur les Génomes (Grant 94/50), the Association pour la Recherche sur le Cancer, the Ligue Nationale Française contre le Cancer, and the Comité du Haut Rhin, the Fondation pour la Recherche Médicale Française, the Fondation de France, and a grant from the Fondation Jeantet (to P. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ A recipient of postdoctoral fellowships from the European Community, the Fondation Léon Frédéricq, the Fondation Rose et Jean Hoguet, the Fondation cancérologique S^t. Michel, and the Fondation Braconier-Lamarque, all from Belgium.

¶ Recipient of a Ph.D. studentship from the Ligue Régionale du Bas-Rhin contre le Cancer.

‡‡ Supported by the Arthritis and Rheumatism Council (United Kingdom).

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¹ The abbreviations used are: ST3, stromelysin-3; DTT, dithiothreitol; TIMP, tissue inhibitor of metalloproteinase; PAGE, polyacrylamide gel electrophoresis; h, human; m, mouse; E Δ H ST3, C-terminally truncated mature stromelysin-3 form lacking part of the hemopexin-like domain.

MATERIALS AND METHODS

In Vitro Mutagenesis—cDNAs containing the full-length coding region for human (14) and mouse (16) ST3 cloned in the pSG5 vector, were mutagenized *in vitro* by oligonucleotide-directed mutagenesis on single-stranded DNA. Primer 1 (5'-GCCCGCAACCGACAGCATATGTTTCGTGCTTTCTGGC-3') and primer 2 (5'-AACCGACAGCATATGTTTCGTCTG-3') were used to incorporate sequences for an unique *NdeI* restriction site (underlined) together with an initiating methionine (ATG) in human and mouse ST3 cDNAs, respectively. DNA fragments corresponding to the entire coding sequences for the putative mature form of human (hE, starting at Phe-98) and mouse (mE, starting at Phe-102) ST3 were then subcloned into the T7 expression pET-3b vector (23).

C-terminally truncated forms for human (h) and mouse (m) ST3 were obtained by introducing a second mutagenesis in the cDNA described above, using the following primers in order to create 3'-stop codons (italic) followed by an unique *XhoI* restriction site for cloning (underlined); the numbers following EAH indicate the number of amino acids that were C-terminally deleted: hEAH216, 5'-ACCCAGCCCTGGGCTGACTCGAGGGGATAGACACCAAT-3'; hEAH158, 5'-TACCCAGCA-TAATGATCTCGAGACTGGCAGG-3'; mEAH216, 5'-GCCCAACTTTGAGCTGATAGCTCGAGACAGATACC-3'; mEAH205, 5'-GAGATTGCT-ATAGTAGCTCGAGGAAACCCCGCCA-3'; mEAH195, 5'-CCAGATGTCTGATAGCTCGAGTTCGACGCG-3'; mEAH183, 5'-TCCACCATCCG-ATGATAGCTCGAGTTCCTCAAGGCA-3'; mEAH175, 5'-TTCAAGGC-ATGATAGCTCGAGAGGCTCGCGC-3'; mEAH167, 5'-CTGCGCAGTTGATGACTCGAGCCCGGTAT-3'; mEAH158, 5'-TATCTGCTTAG-TAACTCGAGGCACTGGCA-3'.

Plasmids were then digested with *NdeI* and *XhoI* restriction enzymes, and the resulting ST3 DNA fragments were ligated into the corresponding sites of the pET-3b vector modified by introduction of a polylinker containing *BstEII*, *KpnI*, *NdeI*, and *XhoI* restriction sites.

The EAH216 ST3 constructs were further modified by site-directed mutagenesis for substitution of Ala-235 by Pro-235 in the human enzyme (primer 5'-GCCCTGATGTCCTCCGTTCTACACCTTT-3' was designed to remove an *AclI* cleavage site) and for substitution of Pro-239 by Ala-239 in the mouse enzyme (primer 5'-GCCCTCATGTCGCGCT-TCTACACTTC-3' was synthesized in order to create a *BstUI* restriction site, underlined). After mutagenesis, plasmids were digested with *NdeI* and *XhoI* restriction enzymes, and the resulting ST3 cDNA fragments were ligated into the corresponding sites of the pET-3b vector. All recombinant plasmids were sequenced with an automated sequencer (Epicentre Technologies, Madison, WI) in order to check that no mutation was introduced during plasmid construction.

Cloning and Sequencing of ST3 cDNA from Human Placenta—A ST3 cDNA fragment corresponding to amino acid residues 450–838 was amplified by polymerase chain reaction from a human placenta cDNA library constructed in the λ Exlox vector (a gift from J. M. Garnier, IGBMC, Strasbourg, France). Polymerase chain reaction products were then cloned into the pBluescript II vector for sequencing.

Expression of Recombinant ST3 Forms—Escherichia coli BL21 (DE3) cells containing a copy of the T7 RNA polymerase gene under the control of the *lac* promoter were transformed with the various plasmids described above. Cells were grown in Lennox Broth medium containing ampicillin (100 μ g/ml) at 37 °C, with shaking. When a cell density corresponding to an A_{600} of 0.6 was reached, ST3 expression was induced by adding 0.4 mM isopropyl-1-thio- β -D-galactopyranoside, and the incubation was continued for 3 h.

Solubilization from Inclusion Bodies and Purification of ST3 Forms—Cell pellets from bacterial cultures were resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.1% Brij) and sonicated for 1 min. The insoluble cell lysate obtained after centrifugation for 30 min at 10,000 \times g was washed 3 times with 50 mM Tris-HCl, pH 7.5, 2 M NaCl, 0.1% Brij and twice with 50 mM Tris-HCl, pH 7.5. Solubilization of the final pellet was achieved by stirring for 2 h at 22 °C in 50 mM Tris-HCl, pH 8.5, 8 M urea, and 100 mM DTT. The mixture was adjusted to a protein concentration of 100–250 μ g/ml and then dialyzed for 4 h, at 4 °C, against 50 mM Tris-HCl, pH 8.5, buffers containing 100 mM NaCl, 10 mM CaCl₂, and 100 μ M ZnCl₂, with urea at progressively decreasing concentrations in order to slowly eliminate urea. Additional dialyses were performed for 20 h against Tris assay buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM CaCl₂, 1 μ M ZnCl₂). Soluble proteins were collected after centrifugation at 100,000 \times g for 2 h.

In some experiments, EAH216 ST3 preparations were extracted from bacterial inclusion bodies as described above, adjusted to 6 M urea, and loaded onto a Q-Sepharose anion-exchange column (3 ml) previously equilibrated with 50 mM Tris-HCl, pH 8.5, containing 100 mM DTT and 6 M urea. Elution from the column was performed with a NaCl gradient

(0–500 mM) in 50 mM Tris-HCl, pH 8.5, containing 6 M urea. The 100–250 mM NaCl fractions were pooled, and purified EAH216 ST3 was renatured by dialysis as described above.

Preparation of Recombinant TIMPs—TIMP-1 and TIMP-3 were prepared as described previously (24, 25). TIMP-2 was produced from transformed Chinese hamster ovary cells, using dihydrofolate reductase-methotrexate selection and amplification system. TIMP-2 was purified from Chinese hamster ovary-conditioned medium on Hitrap Cu²⁺-chelating affinity column and Hitrap Q-column (Pharmacia Biotech Inc.) according to the procedure described by DeClerck *et al.* (26).

Preparation of Recombinant Mouse ST3 from MCF-7 Cells—A cDNA encoding mouse pre-pro-ST3 (16) was subcloned into the expression vector pCMV and transfected by electroporation into MCF7 breast adenocarcinoma cells. Transfected cells were selected with G418 (400 μ g/ml), and 20 resistant clones were screened by Northern and Western blot analyses. Subconfluent MCF7 cells expressing mouse ST3 (clone AN1) and their parental counterpart were incubated in serum-free Dulbecco's modified Eagle's medium. After 48 h of incubation, conditioned media were harvested and cleared by centrifugation for 1 h at 10,000 \times g, and proteins were precipitated at 4 °C with 80% ammonium sulfate. The protein pellets were collected after centrifugation for 2 h at 10,000 \times g, resuspended in Tris assay buffer, and dialyzed against the same buffer at 4 °C for 3 h.

Protein Analyses—Protein concentrations were determined as described by Bradford, using the Bio-Rad kit (Bio-Rad). SDS-polyacrylamide gel electrophoresis (PAGE) was performed under reducing or nonreducing conditions, as described by Laemmli (27). For Western blot analysis, proteins were transferred onto nitrocellulose membranes (28), and visualized with monoclonal antibody 5ST-4C10 raised against the ST3 catalytic domain (18) followed by peroxidase-labeled goat antibody raised against mouse IgG. Bound antibodies were evidenced by Enhanced Chemiluminescence detection (ECL kit, DuPont NEN). N-terminal amino acid sequencing was performed on proteins transferred onto polyvinylidene difluoride membranes according to Towbin *et al.* (28). Membranes were stained with Coomassie Brilliant Blue R250, the appropriate bands were cut-out, and proteins were immobilized on membranes were sequenced with a protein sequencer (477A, Applied Biosystems).

For casein zymography, samples were electrophoresed under nonreducing conditions in SDS-polyacrylamide (12 or 15%) gels containing 2 mg/ml α β -casein (Sigma, C-7891). After electrophoresis at 4 °C, SDS was exchanged by three washes with Triton X-100 (2.5%) in order to regenerate caseinolytic activity. Gels were then incubated at 37 °C in 50 mM Tris-HCl, pH 7.5, containing 5 mM CaCl₂ and 1 μ M ZnCl₂. After 48 h of incubation, gels were stained with Coomassie Brilliant Blue R250 and destained in order to visualize clear bands representing caseinolytic enzymes on a dark background.

β -casein degradation was quantified using ¹⁴C-labeled β -casein (Sigma, C-6034), as described previously by Murphy *et al.* (29) with incubation at 37 °C for 18 h. In some experiments, β -casein (Sigma, C-6905) degradation was semiquantitatively evaluated after incubation with ST3 in Tris assay buffer at 37 °C for 18 h, followed by SDS-PAGE (15%) and silver or Coomassie Blue staining. Similar tests were performed to evaluate ST3 activities toward human type IV collagen (provided by K. Kuhn, Martinsried, Germany), laminin (extracted from EHS tumor according to Timpl *et al.*, (30)), and human plasma α 1-proteinase inhibitor (provided by J.-P. Martin, Rouen, France).

RESULTS

Expression of Recombinant ST3 in E. coli—cDNAs encoding human and mouse ST3 forms lacking the N-terminal prodomain (Fig. 1A) were expressed in BL21 (DE3) cells using the pET-3b vector. All of these ST3 forms, with or without an intact C-terminal hemopexin-like domain, were found to be strongly expressed in the presence of 0.4 mM isopropyl-1-thio- β -D-galactopyranoside (Fig. 1B). The identity of major proteins species observed at 46–48 kDa (E), 32–34 kDa (EAH158), and 21–22 kDa (EAH216) with ST3 was confirmed by Western blot analysis using monoclonal antibody 5ST-4C10 specific to the ST3 catalytic domain (data not shown).

Recombinant ST3 being predominantly found in the insoluble protein fraction of *E. coli*, bacterial inclusion bodies were treated with 8 M urea in the presence of 100 mM DTT. After inclusion body solubilization, the different ST3 forms were refolded by slowly dialyzing out the urea in the presence of

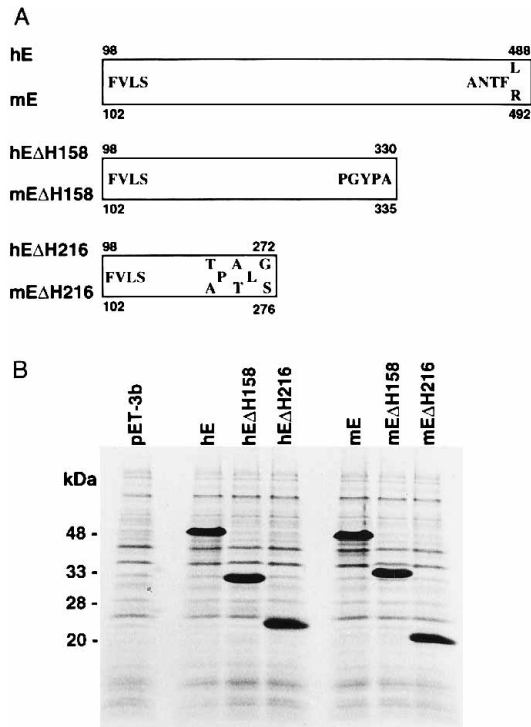


FIG. 1. Electrophoretic analysis of human and mouse ST3 forms expressed in *E. coli*. *A*, schematic representation of human (*h*) and mouse (*m*) putative mature ST3 (*E*) (lacking the prodomain) and of C-terminally ST3 truncated forms (*EΔH*), lacking part (*EΔH158*) or most (*EΔH216*) of the ST3 hemopexin-like domain. $\Delta H158$ and $\Delta H216$ indicate that the 158 and 216 C-terminal amino acids have been deleted, respectively. Numbers above or below frames refer to the first and last amino acids for each form, according to Basset *et al.* (14) and Lefebvre *et al.* (16). C-terminal amino acids for each form are also indicated inside frames by using the single-letter code. *B*, Coomassie Blue staining of crude ST3 preparations after SDS-PAGE. Whole bacterial cells transformed with ST3 cDNAs defined in *panel A* and cloned into the pET-3B vector were collected after 3 h of isopropyl-1-thio- β -D-galactopyranoside induction, lysed, and analyzed by SDS-PAGE (12%) under reducing conditions. pET-3b corresponds to bacteria transformed with the pET-3b vector alone. The mobilities of standard molecular size (kDa) proteins are indicated.

CaCl₂ and ZnCl₂ ions, as described under "Materials and Methods." SDS-PAGE showed that while most bacterial contaminants were eliminated during refolding, and the ST3 preparations obtained after urea removal comprised both protein species at the expected molecular weight and other species at lower molecular weights, the latter being shown to correspond to ST3 degradation products using monoclonal antibody 5ST-4C10 (Fig. 2, *A* and *B* and data not shown). Although the proportions of these low molecular weight ST3 species were found to vary from one preparation to another, they were usually more abundant with mouse than with human ST3.

Structural Determinants Controlling ST3 Caseinolytic Activity—Analysis of proteolytic activities of human and mouse ST3 preparations was first performed using α/β -casein zymography. None of the human ST3 preparations was found to be active, while casein digestion was observed with all three mouse preparations (Fig. 2, *C* and *D*). However, only mouse E Δ H216 ST3 exhibited caseinolytic activity at the expected molecular mass (21 kDa). The activities observed with the preparations of mouse E (46 kDa) and E Δ H158 (32 kDa) ST3 forms were found in both cases around 24 kDa, where a major protein species was observed in both preparations (Fig. 2, *B* and *D*). When zymographic analyses were carried out in the presence of EDTA, no caseinolytic activity was observed for any of the mouse ST3 preparations (data not shown). These observations, showing

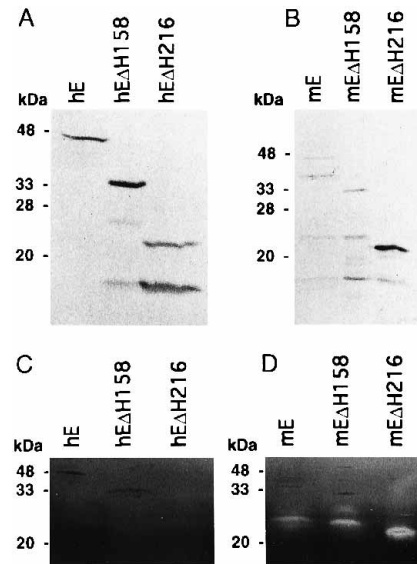


FIG. 2. Electrophoretic analyses of human and mouse recombinant ST3 forms after extraction from bacterial inclusion bodies and refolding. Human (*h*) (*A* and *C*) and mouse (*m*) (*B* and *D*) ST3 forms (2 μ g) solubilized from bacterial inclusion bodies were analyzed after protein refolding by SDS-PAGE (12%) under reducing (*A* and *B*) or nonreducing conditions (*C* and *D*). *A* and *B*, Coomassie Blue staining. *C* and *D*, α/β -casein zymography. *E*, *EΔH158* and *EΔH216* ST3 forms are defined in Fig. 1 and its legend. The mobilities of standard molecular size (kDa) proteins are indicated.

that mouse recombinant ST3 must have lost the majority of its hemopexin-like C-terminal domain to exhibit caseinolytic activity, are in accordance with those previously made by Murphy *et al.* (18) using an eucaryotic expression system. To further demonstrate that the inability of high molecular weight forms of mouse ST3 to display caseinolytic activity was not specific to the prokaryotic expression system used in the present study, we tested the activity of mouse recombinant ST3 from MCF7 cells stably transfected with a full-length mouse ST3 DNA. Although both high and low molecular weight forms of mouse ST3 were detected in conditioned media from these MCF7 cells using Western blot analysis (Fig. 3*A*), only those of lower molecular mass around 28 kDa were found to display caseinolytic activity (Fig. 3*B*).

In order to define the structural determinants present in the hemopexin-like domain and preventing mouse ST3 to digest casein, we performed sequential deletions of the mouse ST3 C-terminal portion (Fig. 4*A*). After extraction from bacterial inclusion bodies and protein refolding, these mouse ST3 preparations were analyzed by SDS-PAGE (Fig. 4*B*) and α/β -casein zymography (Fig. 4*C*). While the mouse E Δ H167 form itself was unable to digest casein, all other C-terminally truncated mouse ST3 forms examined were found to display caseinolytic activity. These findings indicate that the structural determinant preventing mouse ST3 to digest casein corresponds to part or totality of the hemopexin-like domain between Gly-318 and Arg-492 (Figs. 1*A* and 4*A*, and their legends).

While the deletion of the C-terminal part of the hemopexin-like domain endowed mouse ST3 with caseinolytic activity, a similar deletion was ineffective in the case of human ST3 (Fig. 2*C*). This observation was unexpected since the human and mouse ST3 catalytic domains exhibit 94% amino acid identity (16). However, human ST3 is the sole matrixin so far identified having an alanine (residue 235) instead of a proline C-terminal to the "Met-turn" (Table I), which is believed to provide a hydrophobic environment necessary for the catalytic zinc atom (20). Although the presence of Ala-235 in human ST3 was initially deduced from a breast cancer cDNA (14), its presence

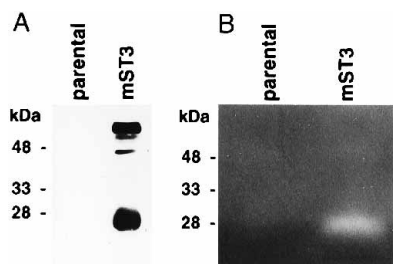


FIG. 3. Electrophoretic analyses of recombinant ST3 expressed in MCF7 cells stably transfected with a mouse ST3 cDNA. Media conditioned by parental MCF7 cells (*parental*) or MCF7 cells transfected with a mouse ST3 cDNA (*mST3*) were collected and concentrated about 50-fold by ammonium sulfate precipitation, and analyzed by SDS-PAGE (12%) under reducing (A) or nonreducing (B) conditions. A, Western blot revealed with monoclonal antibody 5ST-4C10 raised against the ST3 catalytic domain. B, α/β -casein zymography. The high molecular weight species detected by Western blot did not cleave casein, in contrast to a lower molecular weight form. The mobilities of standard molecular size (kDa) proteins are indicated.

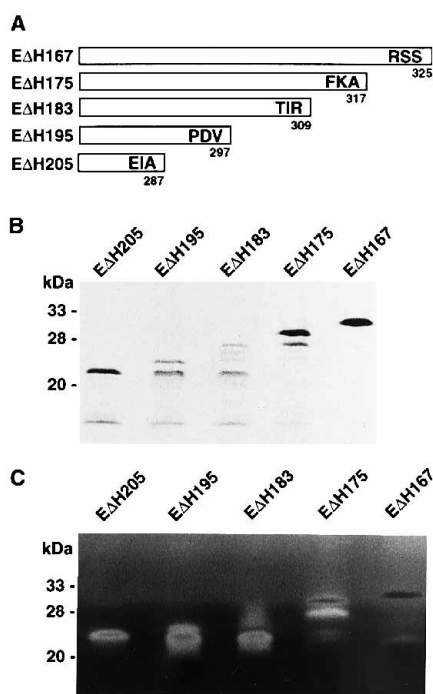


FIG. 4. Electrophoretic analyses of C-terminally truncated forms of mouse recombinant ST3 expressed in *E. coli*. A, schematic representation of putative mature ST3 forms truncated at the C terminus. Numbers following *EΔH* indicate the number of amino acids that were deleted, while those below frames indicate the position of the last C-terminal amino acid in each form, according to Lefebvre *et al.* (16). C-terminal amino acids in each C-terminally truncated ST3 forms are indicated inside frames by using the single-letter code. B, Coomassie Blue staining of refolded recombinant ST3 forms (2 μ g) analyzed by SDS-PAGE (12%) under reducing conditions. C, α/β -casein zymography of the same ST3 forms (2 μ g). The mobilities of standard molecular size (kDa) proteins are indicated.

has been confirmed both by sequencing the ST3 gene² and a cDNA obtained from a human placenta cDNA library (data not shown). Thus, we hypothesized that Ala-235 could be responsible for the inability of human ST3 to digest casein. Indeed, the substitution of Ala-235 by Pro was sufficient to endow human E Δ H216 ST3 with caseinolytic activity by zymographic analysis (Fig. 5). Conversely, when mouse E Δ H216 ST3 was mutated to replace Pro-239 by Ala, its caseinolytic activity was strongly reduced.

² P. Anglard, personal communication.

TABLE I
Amino acid sequence alignment of matrixin catalytic zinc-binding region

Functionally relevant amino acids in the HEXXHXXGXXH zinc-binding region, including the conserved methionine residue of the Met-turn (ALMX) (20), are indicated by boldface letters. The boxed amino acid following the Met-turn is Pro in all matrixins known so far, except in human ST3 (1–3, 14, 16). h, m ST3, human and mouse stromelysin-3, respectively; hST1 and hST2, human stromelysin-1 and -2, respectively; hCOL I, human interstitial collagenase.

hST3	VAAHEFGHVLGLQHHTTAALKALMS	A	FYTFR
mST3	VAAHEFGHVLGLQHHTTAALKALMS	P	FYTFR
hST1	VAAHEIGHSLGLFHSANTEALMY	P	LYHSL
hST2	VAAHELGHSLGLFHSANTEALMY	P	LYNSF
hCOL I	VAAHELGHSLGLSHSTDIGALMY	P	SYTFS

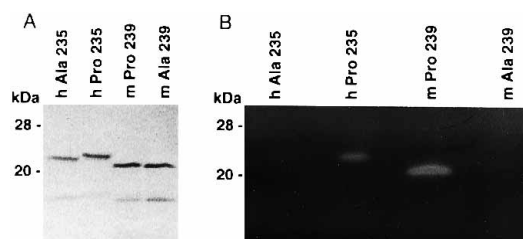


FIG. 5. Electrophoretic analyses of human and mouse E Δ H216 ST3 forms. Wild-type (*h Ala 235*; *m Pro 239*) and mutated (*h Pro 235*; *m Ala 239*) recombinant forms (2 μ g) of human (*h*) and mouse (*m*) E Δ H216 ST3 (see Fig. 1 for definition) extracted from bacterial inclusion bodies were analyzed after protein refolding by SDS-PAGE (15%) under reducing (A) or nonreducing (B) conditions. A, Coomassie Blue staining; B, α/β -casein zymography. The mobilities of standard molecular size (kDa) proteins are indicated.

Enzymatic Properties of ST3 Catalytic Domain—In order to further evaluate the enzymatic properties of the catalytic domains of human and mouse ST3, the corresponding E Δ H216 ST3 forms were purified in order to eliminate bacterial proteolytic contaminants that may interfere with enzymatic analyses performed by using tests distinct from casein zymography. Both E Δ H216 ST3 forms were solubilized from bacterial inclusion bodies with 8 M urea in the presence of 100 mM DTT and applied to a Q-Sepharose column equilibrated with a 50 mM Tris buffer, pH 8.5, containing 6 M urea and 100 mM DTT. Elution was performed using a 0–500 mM NaCl gradient in the same buffer and without DTT. Fractions eluted with 100–250 mM NaCl were pooled and refolded as described under “Materials and Methods.” After protein refolding, both human and mouse ST3 preparations were found by SDS-PAGE analysis to contain two major protein species at 22 and 16 kDa for the human protein and at 21 and 16 kDa for the mouse enzyme (Fig. 6). These ST3 forms were analyzed by N-terminal sequencing using an electroblotting technique, and all of them were found to contain the sequence Met-Phe-Val-Leu-Ser-Gly-Gly-Arg-Trp-Glu, indicating that they corresponded to ST3 forms lacking the prodomain and differing at their C terminus (14, 16). The lack of caseinolytic activity for the 16-kDa mouse protein species (Fig. 5) can thus be explained by a proteolytic cleavage having deleted the C-terminal portion of the ST3 catalytic domain.

In agreement with the observation made using casein zymography, human E Δ H216 ST3 could not degrade ¹⁴C-labeled β -casein (Fig. 7A). When its activity against β -casein was evaluated using a more sensitive assay in which proteolytic products were analyzed by SDS-PAGE (see “Materials and Methods”), only a weak activity could be evidenced (data not shown). However, mouse E Δ H216 ST3 could degrade ¹⁴C-labeled β -casein almost proportionally to the amount of added enzyme between 2 and 8 μ g/ml protein (Fig. 7A). The specific activity of mouse ST3 toward ¹⁴C-labeled β -casein was found to be 20 \pm 5 units/mg

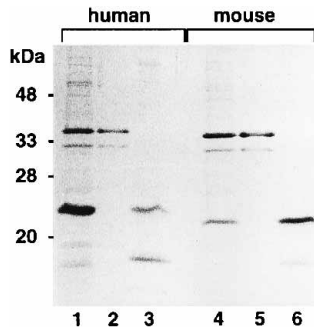


FIG. 6. Electrophoretic analysis of purified human and mouse EΔH216 ST3 forms. Human (lanes 1–3) and mouse (lanes 4–6) EΔH216 ST3 forms (see Fig. 1 for definition) were extracted from bacterial inclusion bodies, and purified by Q-Sepharose chromatography as described under “Materials and Methods.” Samples (2 μg of protein) were analyzed by SDS-PAGE (15%) under reducing conditions and Coomassie Blue staining. Lanes 1 and 4, crude material solubilized in 8 M urea; lanes 2 and 5, unbound materials; lanes 3 and 6, refolded proteins after elution from Q-Sepharose with NaCl (100–250 mM fractions). The mobilities of standard molecular sizes (kDa) proteins are indicated.

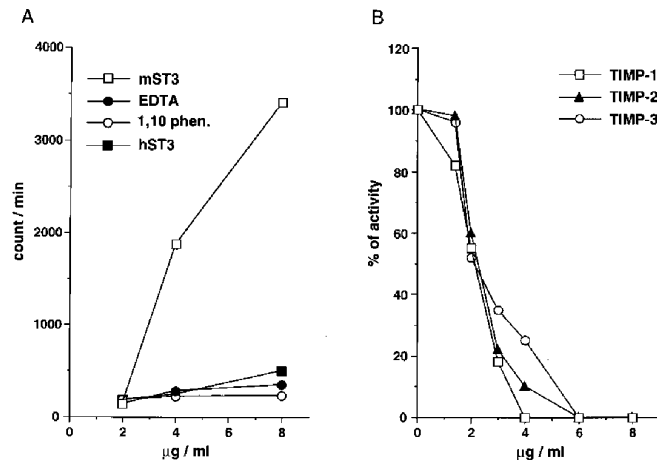


FIG. 7. Quantitation of caseinolytic activities of recombinant human and mouse EΔH216 ST3 forms. Quantitation was performed using ^{14}C -labeled β -casein (400 μg/ml) as substrate and incubation at 37 °C for 18 h. **A**, activities of human (*h*) and mouse (*m*) EΔH216 ST3 (see Fig. 1 for definition) (2–8 μg/ml) in the absence or in the presence of 5 mM EDTA or 2 mM 1,10-phenanthroline (*1,10 phen.*). Results are expressed as the amount of ^{14}C (counts/min) remaining in the supernatant after precipitation with 3% trichloroacetic acid at 0 °C for 30 min. Counts/min observed in the absence of ST3 (average 800) have been subtracted. **B**, inhibition of mouse EΔH216 ST3 caseinolytic activity by tissue inhibitors of metalloproteinases, TIMP-1, TIMP-2, and TIMP-3. Mouse EΔH216 ST3 (8 μg/ml) was incubated with increasing amounts of TIMPs (0–8 μg/ml), at 37 °C for 18 h. Results are expressed as percentage of ST3 activity observed in the absence of TIMP.

versus 350 ± 30 units/mg for trypsin-activated stromelysin-1 (data not shown). Caseinolytic activity was inhibited by 2 mM 1,10-phenanthroline or 1 mM EDTA (Fig. 7A), as well as by the specific matrixin inhibitors TIMP-1, TIMP-2, and TIMP-3 (Fig. 7B). Inhibition (95%) was achieved with a ST3/TIMP-1 molar ratio of 1:1. However, the caseinolytic activity of mouse EΔH216 ST3 was not abolished by 100 μM Pro-Leu-Gly hydroxamate, an inhibitor of human interstitial collagenase (Ref. 31 and data not shown).

Purified mouse and human EΔH216 ST3 forms were then tested against laminin, type IV collagen, and plasma α 1-proteinase inhibitor. Mouse EΔH216 ST3 was able to cleave laminin and type IV collagen at 37 °C (Fig. 8A), thus confirming previous observations obtained with C-terminally truncated mouse ST3 forms produced by using an eucaryotic expression system (18). In the same experimental conditions, human

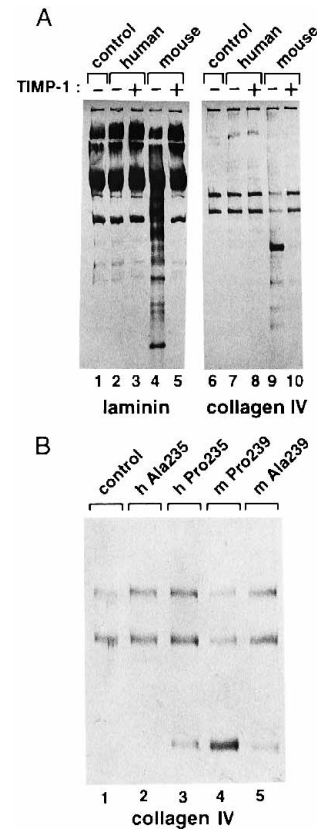


FIG. 8. Degradation of laminin and type IV collagen by human and mouse EΔH216 ST3 forms. Human and mouse EΔH216 ST3 forms (see Fig. 1 for definition), purified by Q-Sepharose chromatography before protein refolding, were incubated with laminin or type IV collagen at 37 °C for 18 h. The products were analyzed by SDS-PAGE (8%) under reducing conditions followed by silver staining. **A**, laminin (2 μg) (lanes 1–5) or type IV collagen (3 μg) (collagen IV) (lanes 6–10) were incubated alone (control, lanes 1 and 6) or with human (2 μg) (lanes 2, 3, 7, and 8) or mouse (2 μg) (lanes 4, 5, 9, and 10) EΔH216 ST3 forms. Where indicated (lanes 3, 5, 8, and 10), incubation was performed in the presence (+) of TIMP-1 (2 μg). **B**, type IV collagen (3 μg) was incubated alone (lane 1, control) or with human (lane 2, *h Ala 235*) and mouse (lane 4, *m Pro 239*) wild-type ST3 forms (2 μg) or with the corresponding mutated human (lane 3, *h Pro 235*) and mouse (lane 5, *m Ala 239*) EΔH216 ST3 forms (2 μg).

EΔH216 ST3 was unable to degrade laminin or type IV collagen (Fig. 8A). However, both mouse and human EΔH216 ST3 showed activity toward plasma α 1-proteinase inhibitor, although the mouse enzyme was more potent than the human enzyme (Fig. 9 and data not shown). Full cleavage of α 1-proteinase inhibitor (2 μg) was achieved with 250 ng of mouse and 2 μg of human EΔH216 ST3, respectively. These activities were completely inhibited by the addition of EDTA, TIMP-1, or TIMP-2 (Figs. 8 and 9, and data not shown).

Importantly, the substitution of Ala-235 by Pro in human EΔH216 ST3 was sufficient to endow the human enzyme with activity against laminin and type IV collagen (Fig. 8B and data not shown). Conversely, the replacement of Pro-239 by Ala in mouse EΔH216 ST3 led to an enzyme that has lost part of its ability to degrade laminin and type IV collagen. Altogether, these observations emphasize the importance of the proline/alanine residues that C-terminally flank the Met-turn in controlling ST3 functional properties.

DISCUSSION

In previous work using an eucaryotic expression system, we had demonstrated that C-terminal truncation of the putative mature form of mouse ST3 was necessary to observe stromelysin-like activity (18). In this respect, mouse ST3 differs from

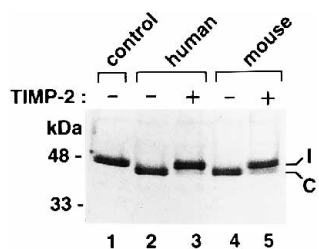


FIG. 9. **Degradation of α 1-proteinase inhibitor by human and mouse EAH216 ST3 forms.** Plasma α 1-proteinase inhibitor (3 μ g) was incubated at 37 °C for 18 h alone (control, lane 1) or with human (lanes 2 and 3) or mouse (lanes 4 and 5) EAH216 ST3 (see Fig. 1 for definition) (2 μ g). Incubation was performed in the absence (–) (lanes 1, 2, and 4) or presence (+) (lanes 3 and 5) of TIMP-2 (2 μ g). After incubation, products were analyzed by SDS-PAGE (8%) followed by Coomassie Blue staining. I and C indicate intact and cleaved α 1-proteinase inhibitor, respectively. The mobilities of standard molecular size (kDa) proteins are indicated.

the other stromelysins whose enzymatic activity is not substantially affected by C-terminal truncation (32, 33) and exhibits partial analogy with interstitial (34, 35) and neutrophil collagenases (36, 37), which acquire stromelysin-like activity after C-terminal truncation. The present study was undertaken to determine 1) whether the previous observations made using an eucaryotic expression system can be reproduced using a pro-caryotic expression system, 2) whether human ST3 behaves similarly to the mouse enzyme, and 3) the nature of the critical structural determinants controlling the unusual functional properties of ST3.

Putative mature forms of ST3 in which the prodomain had been deleted were expressed in *E. coli* using the pET-3b vector and tested for proteolytic activity in casein zymography. Neither the human nor the mouse ST3 forms thus produced exhibited activity by casein zymography, as had been observed for the ST3 forms generated by eucaryotic expression systems (18, 19 and the present study). However, when at least 175 C-terminal amino acids were deleted, we found that mouse ST3 could degrade casein. On the other hand, the deletion of the C-terminal hemopexin-like domain was not sufficient to endow human ST3 with caseinolytic activity. In the case of the human protein, detection of caseinolytic activity required a further mutation in which Ala-235 was replaced by a proline. Ala-235 was thought to represent a critical residue in human ST3 because all other matrix metalloproteinases, including mouse and frog ST3, contain a proline residue at this position (1–3, 38, 39). Ala-235 C-terminally flanks the Met-turn, which is believed to provide a hydrophobic base beneath the catalytic zinc atom (20). Furthermore, by analogy with the structure determined for human neutrophil collagenase (40), Ala-235 should belong to the outer wall of the putative S1' pocket of the active-site cleft. Our finding that the substitution of Ala-235 by Pro is sufficient to endow the catalytic domain of human ST3 with caseinolytic activity, while the corresponding replacement of Pro-239 by Ala in the mouse enzyme decreases proteolytic activity, is the first direct evidence demonstrating the functional importance of this amino acid residue.

The critical role of Ala-235 is further demonstrated by the observation that the human ST3 catalytic domain, in contrast to the mouse one, cannot digest laminin and type IV collagen, while after substitution of Ala-235 by a proline, the human enzyme also degrades laminin and type IV collagen. The inability of human recombinant ST3 produced in *E. coli* to digest laminin and type IV collagen is consistent with the observation recently made by Pei *et al.* (19) using recombinant enzyme produced in COS-7 cells. These data raise the possibility that human ST3 is not a functional proteinase, but a molecule that

has evolved to acquire a new biological function. This has been observed in the case of hepatocyte growth factor (41, 42), haptoglobin (43), and protein Z (44), which are all similar in structure to serine proteinases, although two residues of the catalytic site have been replaced with amino acids that could not support catalysis (45). Alternatively, human ST3 may be an enzyme with a highly restricted substrate specificity. Pei *et al.* (19) have proposed that some serine proteinase inhibitors, including α 1-proteinase inhibitor, are physiologically relevant ST3 substrates. Our finding that α 1-proteinase inhibitor, in contrast to other substrates, is cleaved by both human ST3 forms having a proline or an alanine at position 235, also supports this hypothesis. However, all matrix metalloproteinases can cleave α 1-proteinase inhibitor, and in some cases with an apparently much higher efficiency than ST3 (46, 47). Thus, the most likely hypothesis is that ST3 is a matrix metalloproteinase with functional properties distinct from all other matrix metalloproteinases and whose physiological substrate is presently unknown. In this respect, we note that mouse ST3 with an intact C-terminal domain, in contrast to the corresponding catalytic domain, cannot cleave casein, thereby suggesting that the ST3 hemopexin-like domain is also involved in endowing ST3 with a restricted substrate specificity.

The specific expression of the ST3 gene in fibroblastic cells immediately surrounding cancer cells in invasive human carcinomas (Refs. 13 and 14 and references therein) has raised the possibility that ST3 contributes to tumor progression. This possibility is supported by clinical observations showing that recurrent breast carcinoma is more frequent in patients having tumors with high ST3 RNA levels (15) and by experimental data showing that ST3 expression favors tumor take in nude mice.³ In this context, it appears reasonable to include ST3 among those matrix metalloproteinases that might represent targets for therapeutic intervention using synthetic matrix metalloproteinase inhibitors (21, 22). Such inhibitors have been found in preclinical studies to be efficient and well tolerated (9, 48, 49), and the clinical evaluation of some of them has been recently initiated (21). The demonstration that human ST3 possesses unusual functional properties adds further support to the possibility of obtaining inhibitors with appropriate selectivity to target ST3 for the treatment of human carcinomas.

Acknowledgments—We thank P. Chambon for support and interest in this work. We thank K. Kühn for the gift of type IV collagen and J.-P. Martin for providing the α 1-proteinase inhibitor. We acknowledge Y. Lutz for preparing monoclonal antibodies, J.M. Garnier for the human placenta cDNA library, and S. Vicaire for DNA sequencing.

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³ A. Noël, unpublished data.

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