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The matrix metalloproteinase stromelysin-3 cleaves laminin receptor at two distinct sites between the transmembrane domain and laminin binding sequence within the extracellular domain

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ABSTRACT

The matrix metalloproteinase (MMP) stromelysin-3 (ST3) has long been implicated to play an important role in extracellular matrix (ECM) remodeling and cell fate determination during normal and pathological processes. However, like other MMPs, the molecular basis of ST3 function *in vivo* remains unclear due to the lack of information on its physiological substrates. Furthermore, ST3 has only weak activities toward all tested ECM proteins. Using thyroid hormone-dependent *Xenopus laevis* metamorphosis as a model, we demonstrated previously that ST3 is important for apoptosis and tissue morphogenesis during intestinal remodeling. Here, we used yeast two-hybrid screen with mRNAs from metamorphosing tadpoles to identify potential substrate of ST3 during development. We thus isolated the 37 kd laminin receptor precursor (LR). We showed that LR binds to ST3 *in vitro* and can be cleaved by ST3 at two sites, distinct from where other MMPs cleave. Through peptide sequencing, we determined that the two cleavage sites are in the extracellular domain between the transmembrane domain and laminin binding sequence. Furthermore, we demonstrated that these cleavage sites are conserved in human LR. These results together with high levels of human LR and ST3 expression in carcinomas suggest that LR is a likely *in vivo* substrate of ST3 and that its cleavage by ST3 may alter cell-extracellular matrix interaction, thus, playing a role in mediating the effects of ST3 on cell fate and behavior observed during development and pathogenesis.

Keywords: stromelysin-3, laminin receptor, matrix metalloproteinase, extracellular matrix, *Xenopus laevis*, cell surface substrate.

INTRODUCTION

The extracellular protease stromelysin-3 (ST3) is a member of the matrix metalloproteinase (MMP) family, a large family of zinc-dependent, extracellular or membrane-bound proteolytic enzymes [1-5]. MMPs have different but often overlapping substrate specificities [3, 6, 7]. Collectively, they are capable of cleaving all protein components of the ECM. In addition, MMPs are also capable of degrading non-ECM extracellular or membrane-bound proteins. On the other hand, with a few exceptions, it is unknown whether MMPs cleave these *in vitro* substrates during normal or pathological processes *in vivo*. Thus, it remains to be determined whether and how MMPs affect biological processes *in vivo* by cleaving these or other as yet unknown substrates.

We are using intestinal remodeling during frog metamorphosis [8] as a model to study the *in vivo* role and the underlying mechanisms of ST3 function. Intestinal metamorphosis involves complete degeneration of larval epithelium through apoptosis and concurrent proliferation and differentiation of the adult epithelium, accompanied by the remodeling of basement membrane or basal lamina, the ECM that separates the epithelium from the connective tissue. While drastic, all these changes are controlled by thyroid hormone, T3, just like all other changes during metamorphosis. By isolating genes which are regulated by T3, we and others have previously shown that a number of MMPs are upregulated during metamorphosis [9, 10]. In particular, the expression of ST3 is correlated with cell death

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in different organs during metamorphosis in *Xenopus laevis* [11-15] and ST3 is necessary for ECM remodeling and cell death at least in the animal intestine [16]. These results suggest that ST3 regulates cell fate and behavior via basal lamina remodeling.

Unlike most other MMPs, ST3 has only a weak activity toward ECM proteins *in vitro* but a much stronger activity against the non-ECM proteins α1-proteinase inhibitor (α1PI) and insulin-like growth factor binding protein-1 (IGFBP-1) [17-19]. It is unclear whether α1PI and IGFBP-1 are ST3 substrates during development. Our recent studies indicate that the frog ST3 has little activity against *Xenopus* α1PI compared to human α1PI due to amino acid sequence differences at the cleavage site [20]. Thus, to understand the function of ST3 *in vivo*, it is critical to identify the physiological substrates of ST3. Toward this end, we employed the yeast two-hybrid screen to isolate proteins that interact with ST3 by using mRNAs from metamorphosing intestine and identified the 37 kD precursor of the 67 kD laminin receptor as a potential physiological substrate of *Xenopus* ST3 (the 67 kD laminin receptor is reported to consist of a homodimer of the 37 kD precursor or a heterodimer of this precursor with an, as yet, unknown partner [21, 22]. For simplicity, we will refer to both as LR in this paper).

MATERIALS AND METHODS

ST3 constructs, *in vitro* binding and cleavage assays

The cDNA encoding the catalytic domain or the mature enzyme of ST3 [15] was amplified by PCR with forward and reverse primers which contained *Eco R*I site and a stop codon followed by *Xho* I site, respectively. Enzymatically inactive ST3 was made by introducing a substitution of amino acid E204 to A204 with PCR. The PCR products were cloned into pET28 (N-terminal His- and T7-tag, Novagen), pET30 (N-terminal His- and S-tag, Novagen), and pBD-Gal4 Cam (Stratagene) between *Eco R*I and *Xho* I sites. Both the wild type and mutant ST3 catalytic domains were overexpressed and purified as described [23]. ST3 binding assay was done by mixing S-tagged ST3Cw, ST3Cm, or Trip 7, a transcriptional coactivator [24] as a negative control, with human α1PI (Athens Research and Technology) or *Xenopus* LR. The mixture was incubated overnight at room temperature in the ST3 reaction buffer containing 200 mM NaCl, 10 mM $CaCl_2$, 0.1 mM $ZnCl_2$, 0.1% Triton X-100, and 50 mM Tris-Cl, pH 7.4. The S-tagged proteins were then precipitated with S-protein agarose (Novagen). After several washes with the same buffer, the proteins were eluted with SDS-PAGE sample buffer and subjected to Western blotting. The cleavage assay was done as described [23].

Yeast two-hybrid screen and *in vitro* verification of candidate substrates

A cDNA library in HybriZap 2.1 was made commercially (Stratagene) using intestinal RNA of metamorphosing *Xenopus* tadpoles (between stage 58 and 64). Target cDNA library in pAD-Gal4 2.1 was mass-excised from the HybriZap 2.1 cDNA library using helper phage according to manufacture's manual. The two-hybrid screen was done according to manufacture's protocol (Stratagene). Candidate clones were isolated and their cDNA inserts were ligated into a double strand DNA fragment containing T7 RNA polymerase promoter and used for *in vitro* transcription/translation (Promega). *In vitro* translated ^{35}S labeled-proteins were partially purified using G-50 spin columns (Amersham Biosciences), digested with ST3Cw, and analyzed as above.

Full length cDNA cloning of *Xenopus* LR and purification of LR protein

5'-RACE was done using SMART RACE cDNA Amplification Kit (Clontech) to clone the 5-end of LR. A 1.1 kb full length cDNA sequence was thus determined and deposited in GenBank (AY730625). The full length LR was subcloned into pET30 (N-terminal His- and S-tag, for protein purification) and pCDNA4/HisMax (N-terminal His- and Xpress-tag and additional C-terminal Flag-tag, for Cos7 cell expression, Invitrogen) between *Bam HI* and *Xho* I sites. The protein was overexpressed in *E. coli* BL21 (DE3) and extracted with 8 M urea, 0.5 M NaCl, 0.1% Triton X-100, 20 mM Tris-Cl, pH 7.9. The extract was incubated with Ni-NTA agarose (Qiagen) and the protein was eluted with the same buffer containing 200 mM imidazole. Urea and imidazole were removed by dialysis against PBS containing 4, 2, 1, 0.5, and 0 M urea sequentially. The protein solution was finally concentrated by dialysis against 50% glycerol in PBS. A rabbit polyclonal antibody was generated against purified LR (Covance).

Cloning of human LR

A cDNA for human full length LR was cloned by PCR from human liver total RNA (Ambion). The PCR fragment was subcloned in pET30 and used for *in vitro* transcription/translation.

In vitro binding assay and ST3 cleavage assay of LR

ST3 binding assay was done by mixing 100 ng or 500 ng of T7-tagged ST3Cw or ST3Cm with 500 ng of purified *Xenopus* LR. The mixture was incubated in the ST3 reaction buffer overnight at room temperature and T7-tagged proteins were precipitated with anti-T7 tag antibody agarose (Novagen). After several washes with the same buffer, the proteins were eluted with SDS-PAGE sample buffer and subjected to Western blotting using anti-*Xenopus* LR antibody or ST3 antibody [16]. For *in vitro* cleavage assay of full length and N-terminal truncated (m20141) LR, ^{35}S labeled *in vitro* translated LRs were purified by Ni-NTA agarose followed by G-50 spin columns. LRs were incubated with 40 ng of ST3Cw or ST3Cm in the ST3 reaction buffer overnight at room temperature. The reaction was stopped by adding SDS-PAGE sample buffer, run on a gel, and fluorogramed.

LR cleavage by different MMPs

Purified S-tagged LR (2.6 μg) was incubated in 100 ng of active human MMP-2 or MMP-9, or catalytic domain of human MMP-3 or MT1-MMP (Calbiochem) in 20 μl of the modified ST3 reaction mix (Triton X-100 was substituted by 1% Brij-35) overnight at room temperature. The same amount of S-tagged LR was also incubated with 40 ng of ST3Cw in the ST3 reaction buffer. The N-terminal cleavage products of LR were detected by Western blot analysis with HRP-conjugated S-protein (Pierce).

Amino acid sequencing of degradation products

Purified His- and S-tagged LR (52 μg) was incubated with 1.6 μg of ST3Cw at room temperature overnight. The digested *Xenopus* LR were separated by SDS-PAGE and blotted on PVDF membrane. Membrane was stained with amid black and the regions containing the C-terminal fragments were cut into small pieces. Approximately 0.36 μg of each peptide was subjected to N-terminal sequencing by Edman degradation method (Harvard Microchem).

Western blotting

Anti-*Xenopus* LR rabbit polyclonal antibodies were raised against purified full length LR (Covance). Before protein extraction, isolated small intestines were flushed to remove debris in the lumen. Proteins were extracted from intestine of tadpoles at indicated stages of tadpoles with 9 M urea, 5% 2-mercaptoethanol, 1 mM EDTA, and 50 mM Tris-Cl, pH 6.8, containing proteinase inhibitor cocktail (Calbiochem). Proteins were diluted with SDS-PAGE sample buffer and 1 μg of proteins was electrophoresed on an SDS gel and blotted onto PVDF membrane. The membrane was incubated with 1/10000 dilution of anti-*Xenopus* LR antibody. HRP-conjugated secondary antibody was detected by chemiluminescence.

RESULTS

Identification of laminin receptor (LR) as a putative substrate of ST3 through yeast two-hybrid screen

To isolate ST3 substrates *in vivo*, we used ST3 as bait in the yeast two-hybrid screen. Because this screen is based on protein-protein binding, enzymatically active ST3 as bait may hydrolyze its substrate without forming an interaction stable enough to activate the Gal4 reporter gene. Therefore, we generated inactive mutants of ST3. The inactive ST3 had one amino acid substitution (E204 to A204) in the zinc box in the catalytic domain (Fig.1A) [23]. To test the protease activity and substrate binding of wild type and mutant ST3, purified S-tagged catalytic domains of wild type (ST3Cw) and mutant (ST3Cm) ST3 were incu-

Tab. 1 Summary of yeast two hybrid screening

		Number of positive	
Bait	Clones screened	1st	2nd
ST3Cw	6.8×10^6	13	10
ST3CHw	6.7×10^6	6	3
ST3Cm	7.0×10^6	16	10
ST3CHm	7.8×10^6	4	1

Gene	Number of clones	Bait	Size of insert (kb)	Ga14 reporter	Peptide size (kD)
OL01	5	ST3CHw(1), ST3Cm(4)	1.2-1.5	*	40, 42
OL02	1	ST3Cw	1.3	*	17.5
OL03	1	ST3Cw	1.6	*	41,57 (weak)
OL04	1	ST3Cw	1.1	***	none
OL05	1	ST3Cw	1.3	*	37
OL06	1	ST3CHm	1.6	*	38
OL07	3	ST3Cw(1), ST3Cm(2)	0.9-1.0	**	40
OL08	1	ST3Cm	0.7	**	12
OL09	1	ST3Cw	1.0	*	32 (weak)
OL10	1	ST3Cw	1.3	*	37
OL11	1	ST3Cw	1.5	*	40
OL12	1	ST3Cw	0.9	***	none
OL13	1	ST3Cw	1.0	**	22.5
OL14	1	ST3CHw	0.6	***	none
OL15	1	ST3CHw	1.2	***	none
OL16	1	ST3Cm	1.7	***	none
OL17	1	ST3Cm	1.1	*	n.d.
OL18	1	ST3Cm	1.4	**	n.d.

Peptide size: from *in vitro* translation of the clones; n.d.: not determined; none: no protein detected. *-***: indicate the strength of the signal intensity in the yeast two hybrid screen.

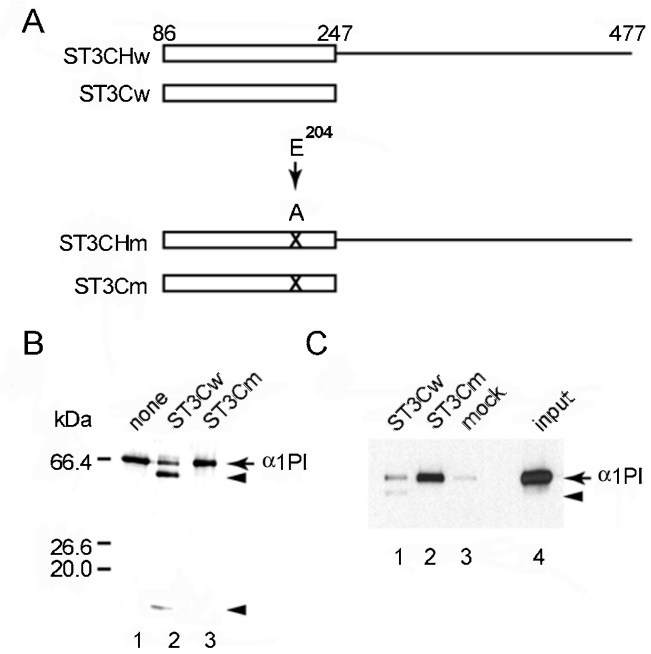

Fig.1 Catalytically inactive mutant ST3 binds α1-protease inhibitor (α1PI) *in vitro*. (**A**) ST3 constructs used as bait for yeast two-hybrid screen. The first construct contains the sequence encoding the wild type mature protein (ST3CHw), which consists of the catalytic domain (box) and the C-terminal hemopexin-like domain (line) but lacks the N-terminal prepropeptide (1-85) of *Xenopus* ST3. The second construct contains only the wild type catalytic domain (ST3Cw). The third (ST3CHm) and fourth (ST3Cm) are the mutant form of ST3CHw and ST3Cw, respectively, and they contain a single amino acid substitution at E204 to A204. (**B**) Mutant ST3 catalytic domain lacks proteolytic activity. Human α1PI (1.5 μg) was incubated with or without equal amounts (40 ng) of purified ST3Cw or its mutant (ST3Cm). The products were run on an SDS-PAGE gel and visualized with silver staining. An arrow and arrow heads point intact and degradation products of α1PI, respectively. The ST3Cw and ST3Cm levels were too low to be detected. (**C**) Mutant ST3 catalytic domain retains the ability to bind α1PI. Equal amount (40 ng) of purified S-tagged ST3Cw or ST3Cm, or S-tagged Trip-7 (mock control) [24] were incubated with 150 ng of α1PI and the reaction mixture was precipitated with S-protein beads. Proteins were eluted by SDS-sample buffer and analyzed by Western blotting with an anti-α1PI antibody. Note that the reduced binding of α1PI by ST3Cw was due in part to the cleavage of α1PI by ST3Cw with the larger cleavage product of α1PI also coimmunoprecipitated (arrow head).

bated with human α1PI, a known *in vitro* substrate [18]. ST3Cw degraded α1PI whereas ST3Cm did not (Fig.1B). On the other hand, co-immunoprecipitation experiments showed that the mutant ST3 was a more efficient binder than ST3Cw to α1PI (Fig. 1C), which was likely due to the hydrolysis of α1PI by the latter and/or likely autodegradation of the enzyme during the incubation. Thus, we made four ST3 bait constructs: ST3Cw and ST3CHw (containing the mature ST3) and their corresponding mutants ST3Cm and ST3CHm.

For the yeast two-hybrid screen, we made a cDNA library from metamorphosing tadpole intestine (stages 58-64), because ST3 is expressed only during this period in the intestine [15] and thus the substrate is likely to be transcribed at this stage. About 7 million clones were screened for each of the four bait constructs ST3Cw, ST3Cm, ST3CHw, and ST3CHm (Tab. 1). Positive clones, i.e., with Gal4 reporter expression, were isolated and purified plasmid DNA was used for a second round of screen. Twenty four clones were thus isolated from the screens, denoting 18 different genes (Tab. 1). Interestingly, most of the clones were isolated using the ST3Cw or ST3Cm and there was little difference between the wild type or mutant ST3 baits (Tab. 1), despite the stronger binding by the mutant form to α1PI *in vitro* (Fig. 1). To examine the ability of these candidate proteins to be cleaved by ST3, the cDNA insert for each was ligated with a double stranded oligonucleotide containing RNA polymerase promoter of phage T7 and used to synthesize the corresponding protein through *in vitro* transcription/translation. Out of 18 genes, eleven clones could be translated into proteins (Tab. 1). Because the *in vitro* translation reaction mixture inhibited ST3 activity (not shown), these *in vitro* translated proteins were partially purified and incubated with purified ST3Cw. Two of them were strongly cleaved by ST3Cw and three others were weakly cleaved by ST3Cw (Tab. 2). All of the 11 translatable candidate genes were sequenced and only three were identified based on sequence homology: the weakly cleaved EF2 (translation elongation factor 2), and the two strongly cleaved candidates, the 37 kD precursor of the 67

Tab. 2 Cleavage of *in vitro* translated protein by ST3Cw

Gene	*In vitro* TXN (kD)	Degradation	Identity
OL01	40, 42	N	unknown
OL02	17.5	N	unknown
OL03	41, 57 (weak)	Y (weak)?	unknown
OL05	37	N	unknown
OL06	38	N	unknownlaminin
OL07	40	Y	receptor
OL08	12	Y (weak)?	unknown
OL09	32 (weak)	n.d.	unknown
OL10	37	N	unknown
OL1I	40	Y	collagen α2 (1)
OL13	22.5	Y (weak)?	EF2

TXN: translation; N: no cleavage; Y: cleaved; Y (weak)?: cleavage was barely detectable; n.d.: not determined.

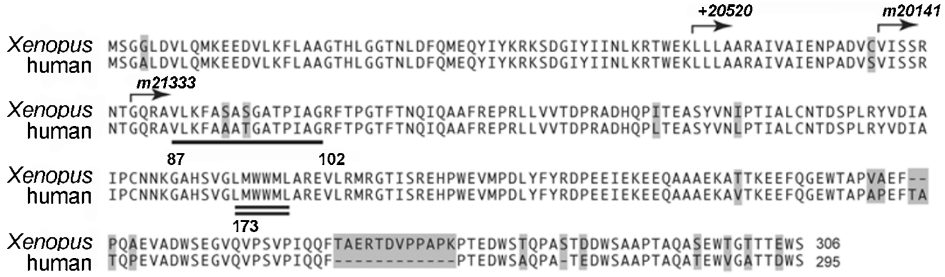

Fig. 2 Laminin receptor (LR) is highly conserved between *Xenopus* and human. The predicted amino acid sequence for *Xenopus* LR was aligned with that of human LR. Shaded boxes indicate amino acids that are different between the orthologs. Dashed lines are gaps. Underline and double underline indicate transmembrane domain and laminin-binding sequence, respectively. Arrows mark the N-terminal ends of three independent clones isolated from yeast two-hybrid screening with clone numbers shown above.

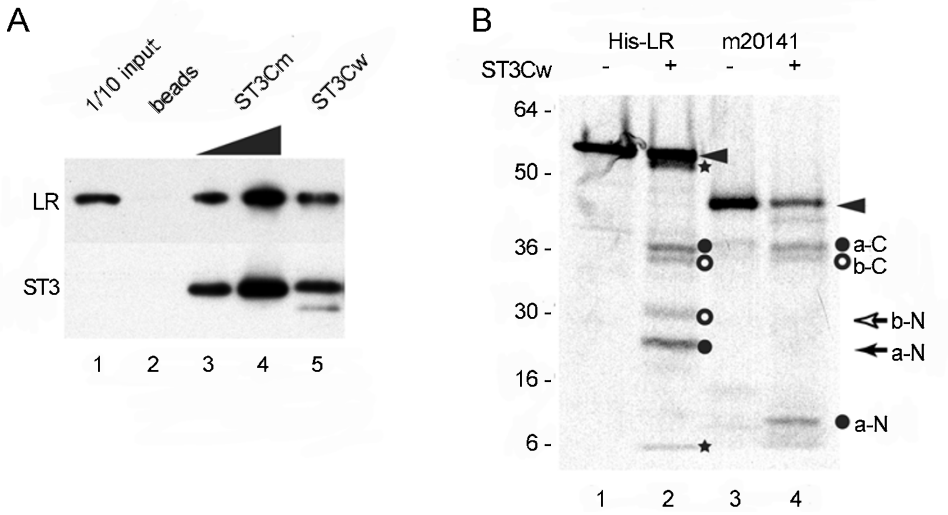

Fig. 3 LR is an ST3 substrate *in vitro*. (**A**) LR coimmunoprecipitates with purified T7-tagged ST3Cm and ST3Cw *in vitro*. Purified LR (500 ng) was incubated alone (lane 2), with 100 ng (lane 3) and 500 ng (lane 4) ST3Cm, and with 500 ng ST3Cw (lane 5), respectively. The complexes were pulled down with anti-T7-tag antibody beads. Proteins were eluted with SDS sample buffer and subjected to Western blotting with anti-*Xenopus* LR antibody (LR) or anti-ST3 antibody (ST3). Lane 1 was input LR before immunoprecipitation. Note that LR binding to ST3Cm was concentration dependent with more LR pulled down in the presence of more ST3Cm (lanes 3 and 4). Much less LR was co-immunoprecipitated with STCw because of reduced amount of intact STCw resulted from autodegradation during incubation. (**B**) ST3Cw cleaves LR at two sites *in vitro*. ^{35}S-labelled full-length LR (His-LR) and N-terminal truncated version of LR (m20141) were synthesized *in vitro* and incubated with purified ST3Cw. The reaction mixture was electrophoresed and detected by autoradiography. His-LR contained histidine-tag and S-tag at N-terminal end of full length LR and migrated at around 53 kDa on an SDS-PAGE gel. N-terminal truncated version of LR migrated at 44 kDa. Closed and open circles indicated two different sets of cleavage products with the C-terminal fragments (a-C and b-C) larger than the N-terminal fragments (a-N and b-N). The N-terminal degradation product (b-N) from m20141 was not detectible due to low intensity of the band, likely a result of further cleavage to produce a-N. Stars in the His-LR lane indicate degradation fragments due to cleavage in the tag region.

kD laminin receptor (LR) and α2 subunit of type I collagen (Tab. 2). Because α2 subunit of type I collagen exists in the collagen fiber form *in vivo*, which is not a good *in vitro* substrate for ST3, we focused our study on LR (we will refer both the 67 kD laminin receptor and the 37 kD precursor simply as LR for the reason given in the Introduction).

We isolated 3 independent clones of LR from the yeast two hybrid screens and sequence analysis showed that they were N-terminally truncated (Fig. 2). We obtained the full

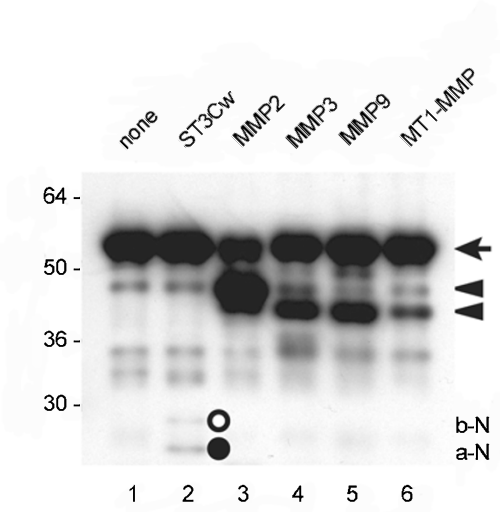

Fig. 4 ST3 but not other MMPs cleaves LR between the transmembrane domain and LR binding site. *In vitro* cleavage of purified N-terminal His and S-tagged LR by various MMPs. The cleavage reactions were carried out with 100 ng of indicated MMPs or 40 ng of ST3 and 2.6 μg of LR in the 20 μl reaction. N-terminal degradation products were detected by HRP-conjugated S-protein. The arrow and arrowheads indicate intact LR and degradation products, respectively. Open and closed circles indicate N-terminal degradation products by ST3. Note that ST3 has distinct cleavage pattern compared to other MMPs. The large sizes of the N-terminal products from the cleavages by other MMPs indicate that they cleave after the laminin binding sequences (LB) (see panel C).

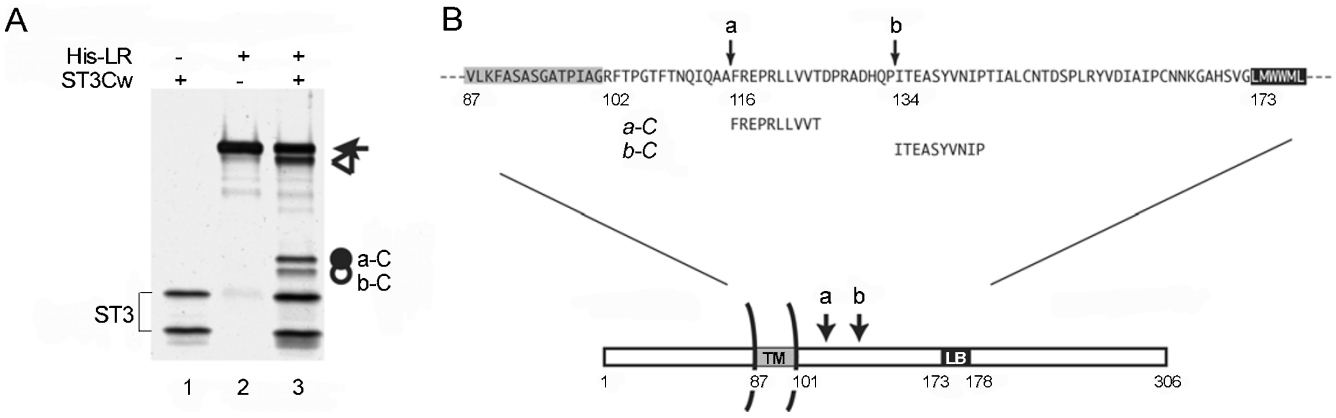

Fig. 5 ST3 cleaves LR in between the transmembrane domain (TM) and laminin binding sequence (LB). (**A**) Isolation of *in vitro* cleavage products to identify the cleavage sites by ST3. One micro gram of purified tagged LR (His-LR) was incubated with or without 40 ng of ST3Cw in 20 μl reaction solution. The reaction mixtures were electrophoresed and the gel was stained by silver. The open arrowhead indicates degradation product within the tag sequence and circles (a-C, b-C) indicate the C-terminal fragments of LR from LR cleavage by ST3. Both a-C and b-C from a larger scale cleavage reaction (see Materials and Methods) were isolated and subjected to N-terminal sequence. Note that there were two ST3 bands detected. During the incubation, ST3 autodegraded to produce a lower molecular weight band that was present both in the presence or absence of LR (lanes 1 and 3). (**B**) ST3 cleavage sites are located between the transmembrane domain (TM) and the laminin binding sequence (LB) of LR. Top: The C-terminal fragments (bands a-C and b-C in the panel A) were sequenced by using the Edman degradation method and the resulting N-terminal sequences of the fragments are shown below with the LR sequence. ST3 hydrolyzed between A115 and F116 (a), and P133 and I134 (b). Bottom: Schematic diagram of LR localization on cell surface. The two cleavage sites by ST3 are indicated by two arrows. LR is either a homodimer of the 37 kD or a heterodimer with another protein [21, 22]. Only the 37 kD precursor protein (referred here and in the text simply as LR) is shown here for simplicity.

length coding region by 5'-RACE. The deduced amino acid sequence of *Xenopus* LR showed very high homology (>90%) to the human homolog (Fig. 2). The six amino acid laminin binding motif (LMWWML) was perfectly conserved among multicellular organisms, suggesting that *Xenopus* LR is capable of laminin binding. All three LR cDNAs obtained from the yeast two hybrid screens contained the coding region from the transmembrane domain to the stop codon and a poly A tail (Fig. 2).

To confirm ST3 binding to LR, recombinant LR was expressed in *E. coli,* and purified LR was incubated with T7-tagged ST3 catalytic domain. ST3 was precipitated with

anti-T7 tag antibody beads, and co-precipitated proteins were detected by Western blotting (Fig. 3A). ST3Cm pulled down LR in a concentration dependent manner (Fig. 3A, lanes 3 and 4). At similar amounts of ST3Cm and ST3Cw, less LR was co-precipitated with ST3Cw (Fig. 3A, lanes 4 and 5). Western blot using anti-ST3 antibody showed that the reduced binding of LR by ST3Cw was mostly due to ST3 autodegradation since under the incubation conditions, only a small fraction of LR was likely degraded by ST3Cw (see Fig. 3B below).

The ability of ST3 to cleave LR was investigated by using partially purified, *in vitro* translated, ^{35}S-labeled LR of full length (His-LR, containing a His tag and an S tag at the N-terminus) and N-terminally truncated LR (m20141, a clone obtained from the yeast two-hybrid screens). ST3 generated 3 sets and 2 sets of degradation products on full length LR and m20141, respectively (Fig. 3B). A pair of bands from full length LR (indicated by stars) was likely the result of cleavage within the N-terminal tag sequence based on the sizes of the products. The second largest (a-C) and third largest bands (b-C) in His-LR were of the same sizes as the largest and second largest bands in m20141, respectively, indicating that these fragments were derived from the C-terminal end since the N-terminal portion of His-LR was longer than m20141. The N-terminal fragments corresponding to these C-terminal fragments were indicated by a-N and b-N, respectively, based on their sizes. The b-N fragment corresponding to b-C of m20141 was faint and not visible in most gels, due to the lower amount of this cleavage product compared to a-N (Fig. 3B).

ST3 cleaves LR at two unique sites

To investigate whether LR can be cleaved by other MMPs, we purchased several different human MMPs and incubated them with purified N-terminal S-tagged *Xenopus* LR. Western blot analysis of the cleavage products using S-protein-HRP (S-protein binds S tag) showed that all four human MMPs cleaved LR with MMP2 (gelatinase A) most efficiently, MT1-MMP weakly, and MMP3 (stromelysin-1) and MMP9 (gelatinase B) in between (Fig. 4). It should be pointed out that it is difficult to accurately compare the cleavage efficiency as the quality of different commercial MMPs likely differs. Interestingly, the cleavage products by these MMPs were distinct from those by ST3. MMP2 generated predominantly a fragment migrating at 47 kD, MMP9 and MT1-MMP generated a 44 kD band, and MMP3 produced both 47 kD and 44 kD N-terminal fragments. In contrast, ST3 generated two N-terminal fragments of 24 kD and 21 kD, respectively.

ST3 is secreted into the extracellular space as an active enzyme and is thought to hydrolyze extracellular proteins. Therefore, LR should be cleaved by ST3 in the extracellular C-terminal domain if it is a physiological substrate of ST3. To determine ST3 cleavage sites, purified *Xenopus* LR was incubated with ST3Cw and the products were separated on a gel and visualized with silver staining. The two C-terminal fragments were easily detectible while the N-terminal fragments were not visible, in part because they migrated in similar locations as the autodegraded products of ST3Cw (Fig. 5A). To map the cleavage sites, the C-terminal cleavage products were isolated and subjected to N-terminal sequencing. Alignment of the sequences with LR sequence revealed that ST3 cleaved between A115 and F116, and between P^{133} and I^{134}, to produce the a- and b-sets of fragments, respectively (Fig. 5B). Interestingly, both cleavage sites were located between the transmembrane domain and laminin-binding sequence (Fig. 5B). Thus, LR cleavage by ST3 would lead to the dissociation of cell bound C-terminal half of the LR from laminin, suggesting a possible role of ST3 cleavage in modulating cell-laminin binding via LR. On the other hand, based on the size of the N-terminal LR fragments, the cleavage by all other MMPs tested above occurs after the laminin-binding sequence and thus is unlikely to affect cell-laminin interactions.

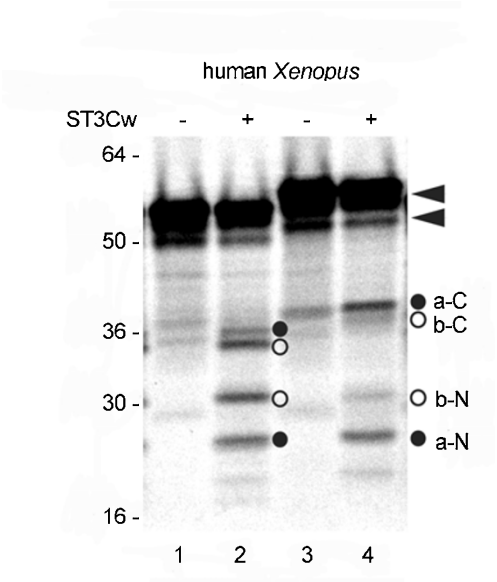

Fig. 6 ST3 cleaves human LR at the same sites as in *Xenopus* LR. ^{35}S labeled full-length human and *Xenopus* LR was synthesized *in vitro* and digested with ST3Cw as described in the panel C. Note that N-terminal cleavage products (a-N and b-N) of human and *Xenopus* are of the same sizes whereas full-length (arrowheads) and C-terminal cleavage products of human LR were smaller than the corresponding ones in *Xenopus* due to amino acid insertions in C-terminal part of *Xenopus* LR (Fig. 2).

ST3 cleaves human LR

Because of the high degree of conservation between *Xenopus* and human LR, we then examined if ST3 could also cleave human LR. ^{35}S labeled full-length human and *Xenopus* LR was synthesized *in vitro* and incubated with ST3. As shown in Fig. 6, human LR was cleaved by ST3 at the same two sites as in *Xenopus* LR, yielding two N-terminal fragments of sizes identical to the corresponding *Xenopus* LR fragments, and two C-terminal fragments slightly smaller than the corresponding *Xenopus* LR fragments due to amino acid insertions in the C-terminus part of *Xenopus* LR. Thus, LR is a conserved substrate for ST3 in vertebrates.

DISCUSSION

The matrix metalloproteinase stromelysin-3 is highly expressed in a number of pathological processes, including atherosclerotic lesions, rheumatoid arthritis, and most, if not all, human carcinomas [25-30], and in many developmental processes, including embryo implantation, organogenesis and tissue remodeling [7, 8, 12, 13, 31-35]. Interestingly, unlike essentially all other MMPs, ST3 does not degrade any ECM proteins efficiently but cleaves the non-ECM proteins α1PI and IGFBP-1 *in vitro* [17-19]. Our recent study suggests that α1PI is not a physiological substrate for ST3 in *Xenopus* due to sequence divergence between human and frog α1PI [20]. Thus, the search for other substrates is indispensable to reveal the function of ST3. Here, we have taken advantages of the yeast two-hybrid screen to identify a likely substrate, laminin receptor, for ST3.

A number of different methods can be used to isolate protein-protein interaction partners, including phage display and biochemical purification. We chose the yeast two-hybrid screen because it allows detection of *in vivo* interactions with high sensitivity. We were further encouraged by the recent success in identifying a substrate of MMP2 (gelatinase A) with such an approach [36]. As an enzyme, ST3 may be expected to interact with its substrate only transiently, followed by cleavage of the substrate, thus requiring a sensitive method or inactive mutant of ST3 to form a stable intermediate with its substrate. Surprisingly, our screens with wild type ST3 and its catalytically inactive mutant forms yielded similar results, suggesting that ST3-substrate interaction is sufficiently long, at least in yeast, to allow the activation of the reporter gene. On the other hand, despite extensive screening, we were able to isolate only two candidate genes of known identities, the laminin receptor and the α2 subunit of type I collagen. Most of the remaining clones were not cleavable by ST3 *in vitro* and they showed no significant homologies with known genes in the data bank. Thus, it is quite possible that this screen failed to isolate substrates rapidly cleaved by ST3, although we cannot rule out the unlikely scenario that there are only a very limited number of ST3 substrates in the tadpole intestine during metamorphosis.

Our *in vitro* analyses confirmed that ST3 is capable of binding and cleaving LR. Amino acid sequencing of the cleavage products of LR by ST3 showed that LR is cleaved by ST3 at two distinct sites between the transmembrane domain and the laminin binding sequence. The sequences of the cleavage sites QAAFRE and HQPITE (p3-p3') are distinct compared to those in other known ST3 substrates, including α2-macroglobulin (VGFYES) [18], α1PI (AGAMFL) [18, 20], and IGFBP-1 (ALHVTN) [19]. While limited information on ST3 cleavage site sequences does not allow a compilation of a reliable consensus sequence to compare with, the LR cleavage site is compatible with ST3 cleavage site preference as obtained from a screen of a random peptide library [37] and with our recent mutational analysis of the αl PI cleavage site [20]. In particular, we showed earlier that an R at the P1 position, a T at P1', or an F at P3' interferes with ST3 cleavage *in vitro* and these residues are absent at the ST3 cleavage sites in LR. Thus, it may not be surprising that like αl PI, the most studied *in vitro* ST3 substrate, LR is a good ST3 substrate *in vitro*.

LR was first identified as a 67 kD protein which binds laminin with high affinity [38] and is expressed in a wide variety of cells [22]. Although LR has no obvious signal peptide and precursor of LR remained in cytoplasm as a component of the translational machinery [39], cell surface association of mature LR has been well documented [40, 41]. It is unclear how the 37 kD precursor is converted to the 67 kD LR, although it has been suggested that it is due to either homo or heterodimerization of the 37 kD LR [21, 22]. Regardless, in the membrane bound state, the C-terminal two-thirds of the LR is located extracellularly and contains a 6 amino acid laminin-binding sequence, whereas the N-terminal third faces the cytoplasm preceded by a short transmembrane domain [40]. ST3 cleaves LR between the transmembrane domain and the laminin-binding sequence. This cleavage pattern is unique to ST3, and the cleavage sites of other MMPs are C-terminal to the laminin-binding sequence, thereby unlikely to affect cell-laminin interactions.

Thus, it is tempting to speculate that the cleavage of cell surface LR by ST3 is likely to affect cell-ECM interaction, thereby playing a role in regulating cell fate and/or cell migration during development and pathogenesis. In fact, like ST3, LR is particularly abundant in cancers [22, 42, 43] and there is a strong correlation between LR overexpression and the metastatic property of tumor cells [44, 45]. Furthermore, our result here shows that human

LR is also a substrate for ST3. The coexistence of ST3, which is expressed in the fibroblasts within the tumors but not actually in tumor cells directly, and LR in tumors may be expected to lead to the cleavage of tumor cell surface LR. This may alter tumor cell-ECM interaction to affect tumor development and cell migration. It is possible that LR expression is needed for tumor cell survival and/or proliferation while its cleavage by ST3 may be necessary for the proliferative cancer cells to migrate.

Clearly, further studies are needed to ascertain the role of LR cleavage by ST3 in developmental and pathological processes and to understand the molecular mechanisms by which the cleavage affects cell fate and behavior. This would require experiments such as mutagenizing the ST3 cleavage sites in LR and determine its effects on development and cancer development *in vivo*. Nonetheless, the data present here support laminin receptor as a potentially important *in vivo* substrate for the interesting MMP ST3.

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REFERENCES

1 Alexander CM, Werb Z. Extracellular matrix degradation. In: Hay ED, ed. Cell Biology of Extracellular Matrix. 2nd Edition. New York: Plenum Press 1991:255-302.
2 Birkedal-Hansen H, Moore WG, Bodden MK, et al. Matrix metalloproteinases: a review. Crit Rev Oral Biol Med 1993; **4:** 197-250.
3 McCawley LJ, Matrisian LM. Matrix metalloproteinases: they're not just for matrix anymore! Curr Opin Cell Biol 2001; **13:** 534-40.
4 Nagase H. Cell surface activation of progelatinase A (proMMP-2) and cell migration. Cell Res 1998; **8:**179-86.
5 Parks W C, Mecham R P. Matrix metalloproteinases. New York: Academic Press, 1998.
6 Overall CM. Molecular determinants of metalloproteinase substrate specificity. Mol Biotechnol 2002; **22:**51-86.
7 Uria JA, Werb Z. Matrix metalloproteinases and their expression in mammary gland. Cell Res 1998; **8:**187-94.
8 Shi YB, Ishizuya-Oka A. Thyroid hormone regulation of apoptotic tissue remodeling: Implications from molecular analysis of amphibian metamorphosis. Prog Nucleic Acid Res Mol Biol 2001; **65:**53-100.
9 Amano T, Fu L, Ishizuya-Oka A, Shi Y-B. Thyroid hormone induced apoptosis during amphibian metamorphosis. In Molecular Mechanisms of Programmed Cell Death. In: Shi Y et al, eds. New York: Kluwer Academic/Plenum 2003:9-19.
10 Shi YB. Amphibian Metamorphosis: From morphology to molecular biology. New York: John Wiley & Sons, Inc., 1999.
11 Damjanovski S, Ishizuya-Oka A, Shi YB. Spatial and temporal regulation of collagenases-3, -4, and stromelysin - 3 implicates distinct functions in apoptosis and tissue remodeling during frog metamorphosis. Cell Res 1999; **9:**91-105.
12 Berry DL, Schwartzman RA, Brown DD. The expression pattern of thyroid hormone response genes in the tadpole tail identifies multiple resorption programs. Dev Biol 1998; **203:**12-23.
13 Berry DL, Rose CS, Remo BF, Brown DD. The expression pattern of thyroid hormone response genes in remodeling tadpole tissues defines distinct growth and resorption gene expression programs. Dev Biol 1998; **203:**24-35.
14 Ishizuya-Oka A, Ueda S, Shi YB. Transient expression of stromelysin-3 mRNA in the amphibian small intestine during metamorphosis. Cell Tissue Res 1996; **283:**325-9.
15 Patterton D, Hayes WP, Shi YB. Transcriptional activation of the matrix metalloproteinase gene stromelysin-3 coincides with thyroid hormone-induced cell death during frog metamorphosis. Dev Biol 1995; **167:**252-62.
16 Ishizuya-Oka A, Li Q, Amano T, et al. Requirement for matrix metalloproteinase stromelysin-3 in cell migration and apoptosis during tissue remodeling in Xenopus laevis. J Cell Biol 2000; **150:**1177-88.
17 Murphy G, Segain JP, O'Shea M, et al. The 28-kDa N-terminal domain of mouse stromelysin-3- has the general properties of a weak metalloproteinase. J Biol Chem 1993; **268:**15435-41.
18 Pei D, Majmudar G, Weiss SJ. Hydrolytic inactivation of a breast carcinoma cell-derived serpin by human stromelysin-3. J Biol Chem 1994; **269:**25849-55.
19 Manes S, Mira E, Barbacid MD, et al. Identification of insulin-like growth factor-binding protein-1 as a potential physiological substrate for human stromelysin-3. J Biol Chem 1997; **272:** 25706-12.
20 Amano T, Fu L, Sahu S, Markey M, Shi YB. Substrate specificity of Xenopus matrix metalloproteinase stromelysin-3. Int J Mol Med 2004; **14:**233-9.
21 Buto S, Tagliabue E, Ardini E, et al. Formation of the 67-kDa laminin receptor by acylation of the precursor. J Cell Biochem 1998; **69:**244-51.
22 Menard S, Castronovo V, Tagliabue E, Sobel ME. New insights into the metastasis-associated 67 kD laminin receptor. J Cell Biochem 1997; **67:**155-5.
23 Damjanovski S, Amano T, Li Q, Pei D, Shi YB. Overexpression of matrix metalloproteinases leads to lethality in transgenic *Xenopus laevis*: implications for tissue-dependent functions of matrix metalloproteinases during late embryonic development. Dev Dyn 2001; **221:**37-47.
24 Amano T, Leu K, Yoshizato K, Shi Y-B. Thyroid hormone regulation of a transcriptional coactivator in *Xenopus laevis*: Implication for a role in postembryonic tissue remodeling. Dev Dyn 2002; **223:**526-35.
25 Ahmad A, Hanby A, Dublin E, et al. Stromelysin 3: an independent prognostic factor for relapse-free survival in node-positive breast cancer and demonstration of novel breast carcinoma cell expression. Am J Pathol 1998; **152:**721-8.
26 Basset P, Bellocq JP, Lefebvre O, et al. Stromelysin-3: a paradigm for stroma-derived factors implicated in carcinoma progression. Crit Rev Oncol Hematol 1997; **26:**43-53.
27 Kossakowska AE, Huchcroft SA, Urbanski SJ, Edwards DR.

Comparative analysis of the expression patterns of metalloproteinases and their inhibitors in breast neoplasia, sporadic colorectal neoplasia, pulmonary carcinomas and malignant non-Hodgkin's lymphomas in humans. Br J Cancer 1996; **73:**1401-8.
28 Lochter A, Bissell MJ. An odyssey from breast to bone: multistep control of mammary metastases and osteolysis by matrix metalloproteinases. APMIS 1999; **107:**128-36.
29 Schonbeck U, Mach F, Sukhova GK, et al. Expression of stromelysin-3 in atherosclerotic lesions: Regulation via CD40-CD40 ligand signaling *in vitro* and *in vivo*. J Exp Med 1999; **189:**843-53.
30 Tetu B, Brisson J, Lapointe H, Bernard P. Prognostic significance of stromelysin 3, gelatinase A, and urokinase expression in breast cancer. Hum Pathol 1998; **29:**979-85.
31 Alexander CM, Hansell EJ, Behrendtsen O, et al. Expression and function of matrix metalloproteinases and their inhibitors at the maternal-embryonic boundary durng mouse embryo implantation. Development 1996; **122:**1723-36.
32 Basset P, Bellocq JP, Wolf C, et al. A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinomas. Nature 1990; **348:**699-704.
33 Chin JR, Werb Z. Matrix metalloproteinases regulate morphogenesis, migration and remodeling of epithelium, tongue skeleta muscle and cartilage in the mandibular arch. Development 1997; **124:**1519-30.
34 Lefebvre O, Regnier C, Chenard MP, et al. Developmental expression of mouse stromelysin-3 mRNA. Development 1995; **121:**947-55.
35 Lefebvre O, Wolf C, Limacher JM, et al. The breast cancer-associated stromelysin-3 gene is expressed during mouse mammary gland apoptosis. J Cell Biol 1992; **119:**997-1002.
36 McQuibban GA, Gong JH, Tam EM, et al. Inflammation dampened by gelatinase A cleavage of monocyte chemoattractant protein-3. Science 2000; **289:**1202-6.
37 Pan W, Arnone M, Kendall M, et al. Identification of peptide substrates for human MMP-11 (stromelysin-3) using phage display. J Biol Chem 2003; **278:**27820-7.
38 Rao NC, Barsky SH, Terranova VP, Liotta LA. Isolation of a tumor cell laminin receptor Biochem. Biophys. Res Commun 1983; **111:**804-8.
39 Auth D, Brawerman G. A 33-kDa Polypeptide with Homology to the Laminin Receptor: Component of Translation Machinery. Proc Natl Acad Sci U S A 1992; **89:**4368-72.
40 Castronovo V, Taraboletti G, Sobel ME. Functional domains of the 67-kDa laminin receptor precursor. J Biol Chem 1991; **266:** 20440-6.
41 Starkey J R, Uthanyakumar S, Berglund DL. Cell surface and substrate distribution of the 67-kDa laminin-binding protein determined by using a ligand photoaffinity probe. Cytometry 1999; **35:**37-47.
42 Sanjuan X, Fernandez PL, Miquel R, et al. Overexpression of the 67-kD laminin receptor correlates with tumor progression in human colorectal carcinoma. J Pathol 1996; **179:**376-80.
43 Sobel ME. Differential expression of the 67 kDa laminin receptor in cancer. Semin Cancer Biol 1993; **4:**311-7.
44 Martignone S, Menard S, Bufalino R, et al. Prognostic significance of the 67-kilodalton laminin receptor expression in human breast carcinomas. J Natl Cancer Inst 1993; **85:**398-402.
45 Castronovo V. Laminin receptors and laminin-binding proteins during tunor invasion and matastasis. Invasion Metastasis 1993; **13:**1-30.