Characterization of structural determinants and molecular mechanisms involved in pro-stromelysin-3 activation by 4-aminophenylmercuric acetate and furin-type convertases

Maria SANTAVICCA*, Agnès NOEL*, Herbert ANGLIKER†, Isabelle STOLL*, Jean-Pierre SEGAIN*, Patrick ANGLARD*, Michel CHRETIEN‡, Nabil SEIDAH§ and Paul BASSET*‖

*Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), Centre National de la Recherche Scientifique/Institut National de la Santé et de la Recherche Médicale U184/Université Louis Pasteur, BP 163, 67404 Illkirch Cédex, C. U. de Strasbourg, France, †Friedrich Miescher-Institut, P. O. Box 2543, CH-4002 Basel, Switzerland, and Laboratories of ‡Molecular and §Biochemical Neuroendocrinology, Clinical Research Institute of Montreal, 110 Pine Avenue West, Montreal, Quebec, H2W 1R7 Canada

Stromelysin-3 (ST3) is a matrix metalloproteinase (MMP) which has been implicated in cancer progression and in a number of conditions involving tissue remodelling. In contrast to other MMPs which are secreted as zymogens requiring extracellular activation, ST3 is found in the extracellular space as a potentially active mature form, suggesting that the activation of the ST3 proform differs from that of other MMPs. We show in the present study that the ST3 proform is not autocatalytically processed in the presence of 4-aminophenylmercuric acetate (APMA). By using ST3/ST2 chimeras, we demonstrate that resistance to APMA is due to properties associated with both the ST3 pro- and catalytic domains. In agreement with the observation made by Pei and Weiss [Pei and Weiss (1995) Nature (London) **375**, 244–247], we find that the requirement for activation of the ST3 proform by the furin convertase is entirely contained within a stretch of 10 amino acids located at the junction between the ST3 pro- and catalytic domains. Furin cleaves human and mouse ST3 equally well. However, PACE-4, a furin-like convertase, is much more efficient on the mouse enzyme, suggesting that ST3 protein determinants other than the conserved Ala-Arg-Asn-Arg-Gln-Lys-Arg sequence preceding the furin cleavage site are implicated in PACE-4 action. Finally, we show that processing of the ST3 proform is inhibited by a furin inhibitor in human MCF7 breast cancer cells stably transfected to constitutively express a full-length human ST3 cDNA. Using brefeldin A, we demonstrate that, in these MCF7 cells, the 56 kDa precursor form of ST3 is post-translationally modified in the *cis*- or *media*-Golgi into a 62 kDa proform. Thereafter, its processing into the 47 kDa mature form occurs in the *trans*-Golgi network and is followed by secretion into the extracellular space.

INTRODUCTION

Stromelysin-3 (ST3) [1] belongs to the matrix metalloproteinase (MMP) family, enzymes which are believed to be mediators of physiological and pathological tissue remodelling processes [2–5]. ST3 is expressed in most invasive human carcinomas ([6] and references therein), in some precursor lesions [6,7], and in a number of other conditions including wound healing [8], mammary gland involution [9], cycling endometrium [10], embryonic development [11] and metamorphosis [12]. As for the other stromelysins, ST3 has four protein domains, these being an N-terminal signal peptide followed by a propeptide, a catalytic domain containing the zinc-binding site, and a C-terminal haemopexin-like domain [1,13]. However, ST3 differs from the other stromelysins in that mature forms of human ST3 cannot cleave any of the major components of the extracellular matrix [14,15].

With the exception of the recently described membrane-type MMPs (MT-MMPs) [16,17], all known MMPs are secreted as soluble zymogens and require activation in the extracellular space [18,19]. *In vitro*, zymogen activation can be achieved without prodomain cleavage by agents such as SDS, or by autocatalytic cleavage of the prodomain in the presence of organomercurial compounds such as 4-aminophenylmercuric acetate (APMA). *In vivo*, zymogen activation is believed to be initiated by serine proteinases such as plasmin, or by cell-surface binding as demonstrated for gelatinase A, both leading to prodomain removal. However, the predominant human ST3 form detected in culture media conditioned by cells expressing either recombinant [14] or the endogenous [20] enzyme corresponds to a mature form which has lost the prodomain.

These observations, suggesting that activation of the ST3 proform differs from that of other MMPs, prompted us to characterize the molecular mechanism implicated in this activation. We found that the ST3 proform is not autocatalytically processed in the presence of APMA. By using ST3/ST2 chimeras, we demonstrated that the resistance to APMA is due to properties associated with both the ST3 pro- and catalytic domains. In agreement with the observation made by Pei and Weiss [21], we found that the requirement for activation of the ST3 proform by the furin convertase was entirely contained within a stretch of 10 amino acids located at the junction between the ST3 pro- and

Abbreviations used: APMA, 4-aminophenylmercuric acetate; ST3, stromelysin-3; h, human; m, mouse; hproST3-hEST2, chimera composed of the hST3 prodomain in front of the hST2 mature form; hproST3(Δ10)-hEST2, chimera composed of the hST3 prodomain minus its 10 C-terminal amino acids in front of the hST2 mature form; (N)ST3-ST2(C), either of these two chimeras; hproST2-hEST3, chimera composed of the hST2 prodomain in front of the hST3 mature form; hproST2-(10)hEST3, chimera composed of the hST2 prodomain in front of the hST3 mature form N-terminally extended with the 10 C-terminal amino acids of the hST3 prodomain; (N)ST2-ST3(C), either of these two chimeras; MAb, monoclonal antibody; MCF7hST3/9, a clone of MCF7 cells stably transfected to express a full-length hST3 cDNA; MMP, matrix metalloproteinase; TGN, *trans*-Golgi network; PMA, phorbol 12-myristate 13-acetate; lmwPE, low-molecular-mass ST3 proform; hmwPE, high-molecular-mass ST3 proform.

‖ To whom correspondence should be addressed.

catalytic domains. Finally, by using human MCF7 breast cancer cells stably transfected to express a full-length human ST3 cDNA, we showed that intracellular processing of the ST3 zymogen is prevented in the presence of a furin inhibitor. These findings add support to the concept that ST3 is an MMP with unique functional properties, and that the intracellular processing of ST3 by furin is biologically relevant.

EXPERIMENTAL

cDNA constructions

Full-length cDNAs for human (h) [1] and mouse (m) [9] ST3, and for hST2 [13] were cloned into the pSG5 expression vector [22]. Human furin [23] and PACE-4 [24] cDNAs were cloned into the pcDNA3 vector (Invitrogen). To generate chimeric proteins in which the hST2 prodomain was swapped with that of hST3 [(N)ST3-ST2(C)], or in which the hST3 prodomain was swapped with that of hST2 [(N)ST2-ST3(C)], site-directed mutagenesis was performed on hST3 and hST2 cDNAs in order to introduce an *Xba*I cleavage site at nucleotide positions 256–261 or 286–291 of hST3 cDNA, and 289–294 of hST2 cDNA (nucleotides numbered from the initiation codon). Since the pSG5 vector contains an unique *Xba*I site 5′ to the cloning site, *Xba*I restriction fragments were generated and exchanged between the two plasmids containing either the hST3 or the hST2 cDNA. Thus, the cDNA fragments encoding the 87 [hproST3Δ(10)] or 97 (hproST3) N-terminal amino acids of hST3 were ligated 5′ to the portion of the cDNA encoding the hST2 mature form (hEST2, amino acids 99–476), to generate the hproST3(Δ10)-hEST2 and hproST3-hEST2 chimeras respectively. Conversely, the cDNA fragment encoding the 98 N-terminal amino acids of hST2 (hproST2) was ligated 5′ to the cDNA portion encoding the hST3 mature form (hEST3, amino acids 98–488) or an N-terminally extended hEST3 form [(10)hEST3] (amino acids 88–488), to generate the hproST2-hEST3 and hproST2-(10)hEST3 chimeras respectively. The introduction of *Xba*I cloning sites in the ST3 and ST2 cDNAs having led to modifications of amino acid sequences, these were corrected by performing a second site-directed mutagenesis to restore the correct sequence. Thus, Ser-Arg residues (TCTAGA corresponding to the *Xba*I restriction site) were replaced by Ser-Asp (residues 86–87 of hST3) or Lys-Arg (residues 96–97 of hST3) and Gly-His (residues 97–98 of hST2).

Conditioned media and cell extracts

All cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) fetal-calf serum. COS-1 cells (A.T.C.C. CRL1650) were transiently transfected using the calcium phosphate precipitation procedure, with 5 μg of each expression vector (completed to 20 μg with pBluescript DNA) per dish (10-cm-diam., Falcon) [25]. Conditioned media were collected after 48 h of incubation in serum-free conditions and centrifuged at 100000 ***g*** for 1 h, in order to eliminate cell debris. Cleared media were concentrated 100-fold by 80 % ammonium sulphate precipitation and then dialysed against 20 mM Tris/HCl, pH 7.4, 100 mM NaCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.005 % $BrijS_{35}$ prior to analyses. MCF7hST3/9 cells corresponding to human MCF7 breast cancer cells stably transfected with a pCMV expression vector containing a full-length hST3 cDNA (A. Noël, unpublished work) were cultured to subconfluence in 1-cm-diam. dishes (Falcon) in the presence of 10 % (v/v) fetal-calf serum. Cells were then cultured in serum-free medium supplemented with the furin inhibitor Dec-Arg-Val-Lys-Arg-CH_2Cl [26] at concentrations varying from 0 to 100 μM. After 24 h, conditioned media were centrifuged at 10000 ***g*** for 30 min to eliminate cell debris and used directly for subsequent analyses. Cells were washed with cold PBS and then lysed in 100 μl of the same buffer supplemented with 1 % Nonidet P40 and 10 mM EDTA. For brefeldin A (Sigma, B-7651) treatment, subconfluent MCF7 cells were preincubated with 0.5 μg/ml brefeldin A for 1 h in serum-free medium, washed with serum-free medium and then incubated in fresh serum-free medium with 0.5 μg/ml brefeldin A for 0 to 24 h, before harvesting cells as described above.

Protein analyses

Proteins in conditioned media and cell extracts were separated by SDS/12 %-PAGE under reducing conditions. After Western blotting, proteins were detected by using three distinct antibodies: monoclonal antibody (MAb) 5ST-4C10 directed against the hST3 catalytic domain [27]; MAb 6ST-1E11 against the ST3 prodomain was obtained by immunizing mice with a synthetic peptide corresponding to amino acids 41–60 of hST3; R654 polyclonal antibody directed against the mature form of hST2 was obtained by immunizing rabbits with recombinant hEST2 expressed in *Escherichia coli* as an N-terminal six-histidine fusion protein and recovered from bacterial inclusion bodies, according to the procedure described by Santavicca et al. [27]. Western blots were revealed by enhanced chemiluminescence (ECL, Amersham) using peroxidase-coupled anti-(mouse IgG) or anti-(rabbit IgG) (Jackson).

RNA analyses

Subconfluent human diploid HFL1 fibroblasts (A.T.C.C. CCL153) were cultured in serum-free medium supplemented or not with 10 ng/ml of phorbol 12-myristate 13-acetate (PMA). After 24 h of culture, RNAs extracted according to the protocol of Chomczynski and Sacchi [28] were separated on formaldehyde-containing 1 % agarose gels prior to transfer on to Hybond N membranes (Amersham). Filter hybridizations were performed using a hST3 cDNA probe [7], and a 420 bp human furin cDNA fragment (nucleotides 170–590) generated by the PCR. Both probes were labelled with [^{32}P]dCTP by random-primed synthesis. Filters were reprobed with the ^{32}P-labelled 36B4 cDNA [29] to check for loading and transfer.

RESULTS

APMA-induced processing of ST2, ST3 and (N)ST3-ST2(C) chimeras

Media conditioned by COS-1 cells transiently transfected with pSG5 plasmids encoding hST3 or hST2 were incubated in the presence of 1 mM APMA. After incubation at 37 °C for 0 to 16 h, conditioned media were analysed by Western blotting using MAb 5ST-4C10 directed against the hST3 catalytic domain, and using polyclonal antibody R654 directed against the mature form of hST2. In the absence of APMA treatment, two major protein species were detected both for hST3 and hST2, at the expected molecular masses for zymogens and mature forms (Figures 1A and 1B, lanes 1). After 16 h of APMA incubation, the hST3 pattern remained unchanged (Figure 1A, lane 2), while the hST2 proform was entirely converted into lower-molecular-mass forms (Figure 1B, lane 3).

Since molecular perturbation of the zymogen has been shown to initiate the activation of MMP proforms by APMA [30], we tested whether the unusual structure of the ST3 prodomain played a role in the inability of the ST3 proform to be processed

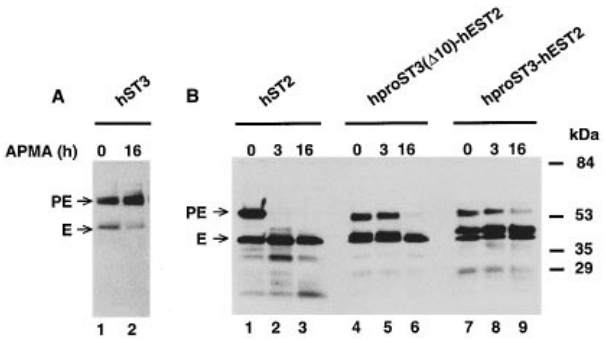

Figure 1 Western blot analysis of ST3 and ST2 proforms, and (N)ST3-ST2(C) chimeras treated with APMA

(**A**) Media conditioned by COS-1 cells transiently transfected with an expression vector encoding the hST3 proform were concentrated by ammonium sulphate precipitation and aliquots containing 20 μg of protein were incubated at 37 °C with 1 mM APMA for the indicated times. After SDS/PAGE and Western blotting, hST3 was detected using MAb 5ST-4C10 directed against the hST3 catalytic domain. (**B**) Media conditioned by COS-1 cells transiently transfected with appropriate expression vectors encoding the hST2 proform or (N)ST3-ST2(C) chimeras were collected as described in (**A**), and incubated at 37 °C with 1 mM APMA for the indicated times, before analysis by Western blotting using polyclonal antibody R654 directed against the hST2 mature form. In the (N)ST3-ST2(C) chimeras, the prodomain of hST2 was swapped with that of hST3 without [hproST3(Δ10)-hEST2] or with (hproST3-hEST2) its 10 C-terminal amino acids (see also the Experimental section). In (**A**) and (**B**), PE and E indicate zymogens and mature forms respectively.

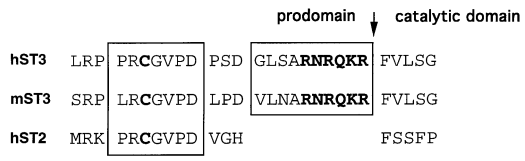

Figure 2 Comparison of ST3 and ST2 amino acid sequences at the junctions of pro- and catalytic domains

Amino acids are represented using the one-letter code. The conserved amino acids surrounding the conserved MMP prodomain cysteine residue, and the 10 extra amino acids characteristic of the ST3 prodomain, are boxed. The conserved cysteine and the residues corresponding to the furin recognition site [32] within these 10 amino acids are in bold letters, and the corresponding cleavage site is marked by an arrow.

in the presence of APMA. Apart from the characteristic 'Pro/Leu-Arg-Cys-Gly-Val-Pro-Asp' sequence, the ST3 prodomain does not exhibit significant amino acid sequence similarities with other MMPs [1], and it is further characterized by the presence of 10 extra amino acids located at its junction with the catalytic domain (Figure 2). (N)ST3-ST2(C) chimeras, having the hST3 prodomain with (hproST3-hEST2) or without [hproST3(Δ10)-hEST2] the 10 extra amino acids characteristic of ST3 in front of the mature hST2 form, were transiently expressed in COS-1 cells and tested for autoactivation in the presence of APMA for 0–16 h. While the hST2 zymogen was completely converted into the corresponding mature form within 3 h of APMA treatment (Figure 1B, lane 2), full processing of the hproST3(Δ10)-hEST2 chimera required 16 h (Figure 1B, lanes 4–6) and processing of the hproST3-hEST2 chimera was incomplete even after 16 h of APMA incubation (Figure 1B, lanes 7–9). Importantly, these (N)ST3-ST2(C) chimeras were found to have retained enzymic activities against casein as tested by zymography (results not shown).

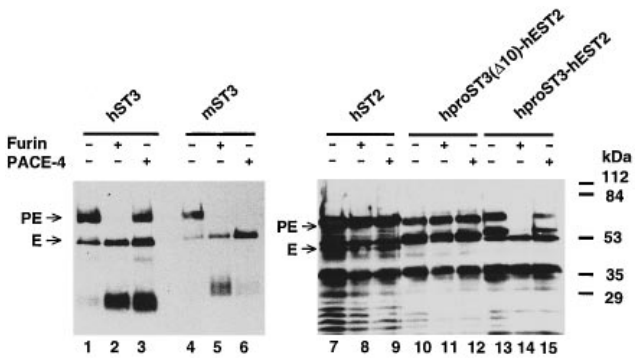

Figure 3 Western blot analysis of ST3 and ST2 proforms and (N)ST3-ST2(C) chimeras processed by furin or PACE-4

COS-1 cells were transiently co-transfected with expression vectors for furin or PACE-4, and vectors for hST3 or mST3 or hST2 or the indicated (N)ST3-ST2(C) chimeras (see the legend of Figure 1 for definition). Conditioned media were collected and analysed as described in the legend of Figure 1. PE and E indicate zymogens and mature forms respectively.

Convertase-dependent processing of ST3, and (N)ST3-ST2(C) and (N)ST2-ST3(C) chimeras

These results indicated that the 10 extra amino acids characteristic of ST3 at the junction between the pro- and catalytic domains (Figure 2) were in part responsible for the inability of the ST3 proform to autoactivate in the presence of APMA. Interestingly, these amino acids have been recently shown to contain a functional furin cleavage site [21]. Consistently, when expression vectors encoding either hST3 or mST3 and human furin were co-transfected into COS-1 cells (containing only low levels of endogenous furin; [31]), the ST3 proform was entirely converted into lower-molecular-mass forms, these corresponding to the putative ST3 mature form and to another species of about 28 kDa (Figure 3, lanes 1, 2, 4 and 5). Furthermore, while the hST2 proform and the hproST3(Δ10)-hEST2 chimera were not processed by furin (Figure 3, lanes 7, 8, 10 and 11), the hproST3-hEST2 chimera was processed by furin (Figure 3, lanes 13 and 14). These results indicated that the sole requirement for furin action was located within the Gly-Leu-Ser-Ala-Arg-Asn-Arg-Gln-Lys-Arg sequence corresponding to the C-terminal portion of the hST3 prodomain. Accordingly, the replacement of the hST3 prodomain by that of hST2 led to an (N)ST2-ST3(C) chimera (hproST2-hEST3) which could not be activated by furin (Figure 4, lanes 1 and 2), while the hproST2-(10)hEST3 chimera, in which the Gly-Leu-Ser-Ala-Arg-Asn-Arg-Gln-Lys-Arg sequence was inserted, was fully processed (Figure 4, lanes 3 and 4). However, it appears that protein determinants other than the Arg-Asn-Arg-Gln-Lys-Arg furin recognition site [32] found in the prodomains of both hST3 and mST3 (Figure 2), play a role in the ability of the furin-like convertase PACE-4 to activate the ST3 proform. Indeed, while PACE-4 was found to process the mST3 proform almost as efficiently as furin (Figure 3, lanes 5 and 6), PACE-4 was much less active on the human enzyme (Figure 3, lane 3) and on the hproST3-hEST2 chimera (Figure 3, lane 15).

Inhibition of ST3 processing by a furin inhibitor

MCF7hST3/9 human breast cancer cells stably transfected to constitutively express a full-length hST3 cDNA (A. Noël, unpublished work) were cultured in the presence or absence of the furin inhibitor Dec-Arg-Val-Lys-Arg-CH_2Cl [26], and ST3 ex-

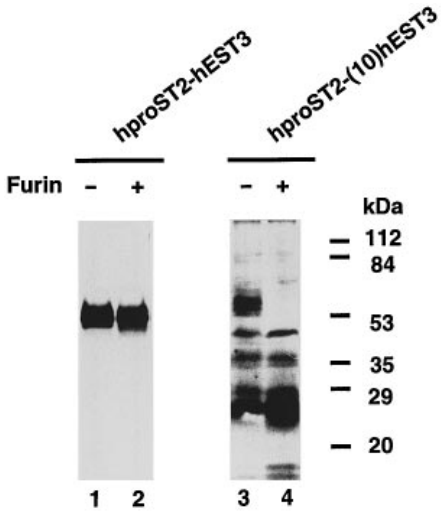

Figure 4 Western blot analysis of (N)ST2-ST3(C) chimeras processed by furin

COS-1 cells were transiently co-transfected with expression vectors for furin and for the indicated (N)ST2-ST3(C) chimeras in which the hST3 prodomain was swapped with the hST2 prodomain (hproST2-hEST3) or with the hST2 prodomain C-terminally extended with the 10 C-terminal amino acids of the hST3 prodomain [hproST2-(10)hEST3] (see also the Experimental section). Conditioned media were collected and analysed using polyclonal antibody R654 directed against the hST2 mature form, as described in the legend of Figure 1.

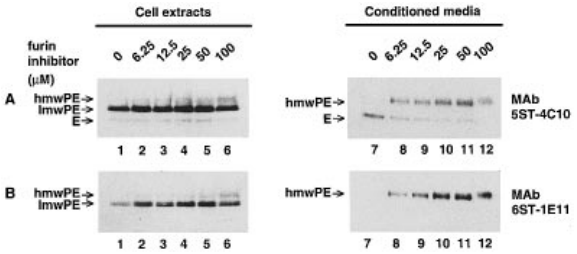

Figure 5 Western blot analysis of ST3 expression in MCF7hST3/9 cells treated with a synthetic furin inhibitor

MCF7hST3/9 cells stably transfected with an expression vector encoding a full-length hST3 cDNA were cultured in serum-free conditions for 24 h in the presence of increasing concentrations (0 to 100 μM) of the synthetic furin inhibitor Dec-Arg-Val-Lys-Arg-CH_2Cl. Cell extracts and conditioned media were analysed by Western blotting using MAb 5ST-4C10 directed against the hST3 catalytic domain (**A**) and MAb 6ST-1E11 directed against the hST3 prodomain (**B**). Abbreviations: hmwPE and lmwPE, high- and low-molecular-mass hST3 proforms respectively; E, hST3 mature form.

pression patterns were evaluated by Western blot analysis. In the absence of the inhibitor, we could detect a major protein species at an average molecular mass of 56 kDa (lmwPE) in MCF7hST3/9 cell extracts. This form corresponded to the ST3 proform since it was recognized by both MAb 5ST-4C10, raised against the ST3 catalytic domain (Figure 5A, lane 1), and MAb 6ST-1E11, directed against the hST3 prodomain (Figure 5B, lane 1). A minor protein species at an average molecular mass of 47 kDa was specifically detected by MAb 5ST-4C10, and it corresponded to the mature ST3 form. However, in media conditioned by MCF7hST3/9 cells, only the 47 kDa mature ST3 form could be detected (Figures 5A and 5B, lanes 7). By incubating MCF7 hST3/9 cells with increasing concentrations of furin inhibitor for 24 h, we observed the appearance in cell

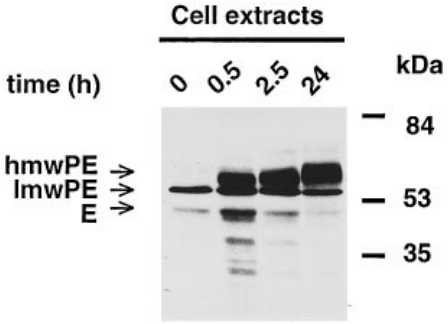

Figure 6 Western blot analysis of ST3 expression in MCF7hST3/9 cells treated with brefeldin A

MCF7hST3/9 cells stably transfected with an expression vector encoding a full-length hST3 cDNA were cultured in serum-free conditions in the presence of 0.5 μg/ml brefeldin A for the indicated times. Cell extracts were analysed by Western blotting using MAb 5ST-4C10 directed against the hST3 catalytic domain. Abbreviations: hmwPE and lmwPE, high- and low-molecular-mass ST3 proforms respectively; E, hST3 mature form.

extracts of a second higher-molecular-mass ST3 proform (hmwPE), at about 62 kDa (Figures 5A and 5B, lanes 2–6). Concomitantly in media conditioned by these cells, we also detected the 62 kDa ST3 proform, while the amount of the 47 kDa mature ST3 form was found to progressively decrease (Figures 5A and 5B, lanes 8–12).

Taken together, these results suggested that the hmwPE protein species was a post-translationally modified ST3 proform derived from the lmwPE form, and that it corresponded to the ST3 proform cleaved by furin in the *trans*-Golgi network (TGN) [33]. To evaluate this possibility further, we disrupted the transport of proteins from the endoplasmic reticulum to the Golgi complex by using brefeldin A, a fungal metabolite which is known to functionally isolate the TGN from the rest of the Golgi complex [33–35]. When MCF7hST3/9 cells were incubated in the presence of 0.5 μg/ml brefeldin A, increasing amounts of the hmwPE form were detected in cell extracts, while the levels of the lmwPE remained stable (Figure 6). This indicates that the ST3 proform (lmwPE) is post-translationally modified in the *cis*- or *media*-Golgi before its processing in the TGN to the mature form.

Comparative expression of furin and ST3 RNAs in human fibroblasts

Since the ST3 gene is specifically expressed in fibroblastic cells of human carcinomas [6], we tested whether the ST3 and furin genes could be co-ordinately expressed in human HFL1 fibroblasts known to express ST3 and other MMPs implicated in cancer progression [1,36]. While the levels of ST3 RNA were found to progressively increase in HFL1 cells treated with PMA (10 ng/ml), furin RNA was found to be constitutively expressed in these cells, and its expression level was not affected by PMA addition (Figure 7).

DISCUSSION

In media conditioned by cells expressing the endogenous ST3 gene [20] or stably transfected ST3 cDNAs constructs [14,15], ST3 is detected in a form which has lost the N-terminal propeptide rather than being detected in a proform. These observations contrast with those made for other MMPs which are secreted as zymogens, requiring activation in the extracellular space [5,18,19]. The present study was undertaken to determine how this unusual

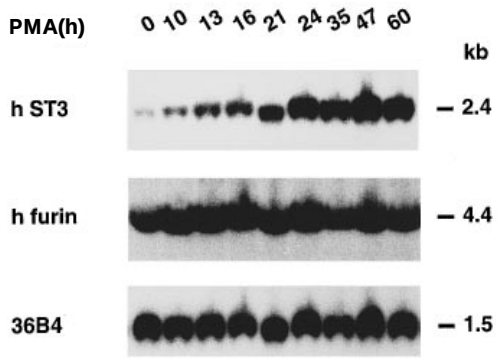

Figure 7 Northern blot analysis of ST3 and furin RNAs in HFL1 fibroblasts

HFL1 cells cultured in serum-free conditions were incubated in the presence of 10 ng/ml PMA for the indicated times. Total RNA (8 μg of RNA per lane) was separated by electrophoresis and transferred to a nylon filter which was successively hybridized with ^{32}P-labelled cDNA probes specific for hST3 and furin. In order to check loading and transfer, the filter was re-rehybridized with a 36B4 cDNA.

expression pattern could be related to an activation mechanism for the ST3 zymogen which differs from those reported for other MMPs.

The latent forms of MMPs can be activated by a variety of means, collectively known as the 'cysteine-switch mechanism' [37]. This involves disruption of the bond existing between the conserved cysteine of the MMP prodomain and the zinc atom of the catalytic site. The 'cysteine switch' is regarded as requiring two steps, in which the initial destabilization of the prodomain is usually followed by its autoproteolytic cleavage or its removal with the help of another MMP ([30,38] and references therein). *In vitro*, the proforms of all secreted MMPs so far characterized have been found to be readily activated by APMA, which is believed to unlock the active centre of the zymogen, and thus permit the subsequent autocatalytic cleavage of the propeptide. In the case of ST3, however, we observed that the ST3 zymogen was not processed to its corresponding mature form in the presence of APMA. We previously proposed that this was due to the unusual structure of the ST3 prodomain [39]. Indeed, the ST3 prodomain does not exhibit significant homologies with the corresponding domains of most other MMPs, apart from the Pro/Leu-Arg-Cys-Gly-Val-Pro-Asp sequence [1,9]. Consistently, in the present study using (N)ST3-ST2(C) chimeras we found that hST3 resistance to APMA activation could be transferred to hST2 by swapping the hST2 prodomain with that of hST3. However, a small amount of the hproST3-hEST2 chimera, but not of the hST3 zymogen, could be converted into lower-molecular-mass species after prolonged activation (16 h) with APMA, suggesting that functional characteristics of the ST3 catalytic domain also contribute to this APMA resistance. In this regard, we note that human, and to a certain extent mouse, ST3 are characterized by both their limited spectra and low levels of activity [14,15,39]. Accordingly, we observed that the (N)ST2-ST3(C) chimera in which the hST3 prodomain was swapped with that of hST2 could not be activated in the presence of APMA (results not shown).

Altogether, these findings indicate that the activation mechanism for the ST3 zymogen differs from those of other MMPs, and that at least part of this difference is due to the particular structure of the ST3 prodomain. In addition to its weak homology with corresponding domains of other MMPs, the ST3 prodomain is characterized by the presence of 10 extra amino acids at its junction with the catalytic domain [1,9]. Comparable stretches of amino acids have been described in the membrane-type MMPs [16,17], and have been proposed to play a role in zymogen activation because they each contain a potential cleavage site for furin or furin-type convertases [16]. In agreement with this hypothesis and with the recent finding of Pei and Weiss [21], we found that the hST3 proform was processed to its mature form by furin when cDNAs encoding both proteinases were transiently co-transfected into COS-1 cells. By using (N)ST3-ST2(C) and (N)ST2-ST3(C) chimeras, we demonstrated that the information necessary to allow processing of the hST3 proform by furin was entirely contained within the 10 extra amino acids characteristic of the ST3 prodomain. The mST3 proform was also found to be processed by furin. However, while the hST3 proform was much less processed by PACE-4, another convertase of the furin-type [31], the mouse enzyme, was processed equally well by PACE-4 and furin. This observation suggests that ST3 protein determinants other than the conserved Ala-Arg-Asn-Arg-Gln-Lys-Arg sequence containing the furin recognition site (Figure 2) may play roles in the capacity of furin-type convertases to cleave the ST3 prodomain.

To evaluate further the possibility that furin or furin-type convertases are the biologically relevant activators of the ST3 proform, we examined ST3 expression in human MCF7 breast cancer cells which had been stably transfected with a full-length hST3 cDNA. In medium conditioned by these cells, the major protein species which was immunodetected corresponded to the 47 kDa mature form of hST3. In cell extracts, however, a 56 kDa proform was mainly found, with only low levels of mature form. In the presence of increasing concentrations of the furin inhibitor Dec-Arg-Val-Lys-Arg-CH_2Cl [26], the 47 kDa form progressively disappeared from conditioned media, while the predominant secreted ST3 form was found to correspond to a 62 kDa proform. These observations suggest that ST3 is first translated into a precursor form of 56 kDa and then post-translationally modified into a 62 kDa proform which is translocated to the cell surface through the TGN, where it is proteolytically processed and finally secreted as a 47 kDa mature form. When MCF7 cells were cultured in the presence of brefeldin A, the 62 kDa proform accumulated intracellularly to high levels. This indicates that the post-translational modification of the ST3 proform occurs early after ST3 protein synthesis, probably in the endoplasmic reticulum and/or the *cis*- and *media*-Golgi compartments, while its processing by furin into a mature form occurs in the TGN [35]. Thus, furin appears to be required for ST3 zymogen activation, although its ubiquitous tissue expression [31] and its constitutive expression in fibroblasts known to express the ST3 gene under various stimuli (the present study), suggest that the expression level of furin itself does not play a critical role in the regulation of the amount of secreted mature ST3 form.

The secretion of ST3 as a potentially active molecule, in contrast to other MMPs which are secreted as proforms requiring activation in the extracellular space, has important implications. This suggests that ST3 activity does not need to be as tightly controlled as other secreted proteinases, which can be explained if ST3 has limited proteolytic activity toward a precise substrate, or if it has functions other than proteolysis. Both possibilities are compatible with the unusual enzymic properties of ST3 previously described [14,15,39]. Alternatively, ST3 might acquire strong and/or broad proteolytic properties only after interaction with another protein whose expression would be tightly controlled. Finally, it cannot be presently excluded that ST3 may exert its proteolytic activity on a substrate present in the TGN, since we could detect the presence of the ST3 mature form in cellular extracts of MCF7 cells stably transfected with the hST3 cDNA.

In this case, the ST3 mature form could be secreted together with its already processed substrate, and immediately inhibited by the tissue inhibitors of MMPs (TIMPs) once in the extracellular space.

We are most grateful to P. Chambon for his support, to G. Murphy for helpful discussions, to J. Byrne for critical reading, to Y. Lutz for MAb preparation, and to G. Duval for polyclonal antibody preparation. This work was supported by funds from the Institut de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique, 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 contre le Cancer and the Comité du Haut-Rhin, the Fondation de France, a grant to P. Chambon from the Fondation Jeantet, and an MRC CANADA programme grant PG11474. M.S. was a recipient of a PhD studentship from the Comité du Bas-Rhin de la Ligue Nationale contre le Cancer. A.N. was a recipient of post-doctoral fellowships from the European Community, and from the Fondation Léon Frédéricq, the Fondation Rose et Jean Hoguet, the Fondation Braconier-Lamarche and the Fondation Cancérologique Saint Michel, all from Belgium.

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Received 13 October 1995/21 December 1995; accepted 22 December 1995