Proprotein Convertase PC1/3-related Peptides Are Potent Slow Tight-binding Inhibitors of Murine PC1/3 and Hfurin*

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The proproteolytic convertase PC1/3 belongs to the subtilisin/kexin-like endoprotease family and is synthesized as a proenzyme. To investigate the function of its propeptide, murine proPC1/3 and preproPC1/3 were isolated from the inclusion bodies of recombinant preproPC1/3 baculovirus-infected insect cells, rendered soluble with 6 M guanidine HCl and 20 mM dithiothreitol, and purified by gel filtration and metal-binding affinity chromatography. Two 

NH2-terminal fragments containing the complete propeptide 1–84 region were obtained after CNBr cleavage, purified, and chemically characterized. Progress curve kinetic analysis with enzymatically active murine 71-kDa PC1/3 or 50-kDa human furin demonstrated that both fragments were potent slow tight-binding inhibitors of either enzyme with $K_i$ in the low nanomolar range. Additional cleavages at Trp residues yielded fragment 9–71, which no longer represents a potent inhibitor. Upon incubation at pH 5.5 in the presence of excess 71-kDa murine PC1/3, NH2-terminal fragment 9–71 is cleaved at two sites, as revealed through Western blotting using NH2-terminal-directed PC1/3 antibodies. Finally, murine PC2 is inhibited by the proPC1/3/31–85 peptide, albeit at a much lesser extent with a micromolar $K_i$ and in a strictly competitive manner. These results suggest that the proregion of PC1/3 is an important feature in regulating its activity.

Many eukaryotic and prokaryotic secretory proteins are synthesized as precursors in the form of preproproteins and include, for example, peptidyl hydrolases, hormones, and growth factors. Whereas the 20–30-amino acid-long presequences usually act as the signal peptide for transport within the endoplasmic reticulum, numerous roles for the following presequences have been proposed. For many proteins, presequences have been shown to serve as an intramolecular chaperone that is essential for the correct folding of the protein (for a review, see Refs. 1–4). Presequences may also facilitate the transport and secretion of hormones (5). Finally, in the case of many peptidyl hydrolases such as α-lytic protease, carboxypeptidases, cathepsins, papain, and subtilases, this presegment was shown to be a very potent inhibitor that is highly specific for its associated protease. This latter observation led to the proposal that the presegment represents an additional strategy to regulate the enzymatic activity of proteases and keep them inactive until they reach their proper destination for action (6–11).

Recently, a new family of subtilisin/kexin-like proteases has emerged (reviewed in Refs. 12 and 13). So far, all seven known members of this family have been shown to be synthesized as preproproteins. Whereas their catalytic regions are all chemically closely related, there appears to be little similarity between the respective proregion sequences (14), hinting that the role, if any, of the proregion might be very specific to its cognate convertase. Because they belong to the subtilisin family, some understanding can be obtained based on the role of the propeptide of subtilisin-BPN’, a prototype model of the eukaryotic convertases. Thus, this proregion was shown to interact intimately and specifically with enzymatically active subtilisin-BPN’ both as a necessary intramolecular chaperone and as a very potent inhibitor (10, 15). Both aspects were recently illustrated through an elegant crystallography study of the subtilisin-BPN’/proregion complex (16). However, the question remains as to whether the conservation of either mode of action has been accomplished through the evolution to the eukaryotic convertases. Thus far, it has been shown that proregion deletion within Kex2, furin, and PC2 leads to a very inefficient activation into enzymatically active enzyme and/or to a failure in transport to the correct compartments (17–19). Moreover, it has been shown that the proregions of furin and PC1/3 are interchangeable, whereas the corresponding region of PC2 is not (20). Finally, while this study was being completed, both the proregion of furin (21) and the proregion of kexin (22) were shown through molecular biology to be potent autoinhibitors and need to be cleaved to release fully active furin and kexin.

Considering that prepeptides may represent an important factor in regulating enzymatic activity and ultimately convertase activation and expression, it is necessary to assess their role and mode of action toward their respective convertases. This report describes the purification of murine preproPC1/3 and proPC1/3 from inclusion bodies arising from the expression of recombinant preproPC1/3 in the baculovirus/insect cells expression system. In addition, it demonstrates their use in generating two NH2-terminal fragments that were used after purification and complete chemical characterization to probe their inhibitory activities toward enzymatically active PC1/3, PC2, and furin. In doing so, it shows for the first time that

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1 The abbreviations used are: PC, protein convertase; mPC, murine PC; DTT, dithiothreitol; IEF, isoelectric focusing; MCA, 4-methylcoumarin-7-amide; PAGE, polyacrylamide gel electrophoresis; PGlu, pyroglutamic acid; RP-HPLC, reverse phase high performance liquid chromatography.
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PC1/3 and furin enzymatic activity can be abolished in a very potent manner by proPC1/3-related peptides but not by mPC2, and that the PC1/3 pror region is cleaved at two sites after incubation with an excess of the enzymatically active 71-kDa form of PC1/3.

MATERIALS AND METHODS

Recombinant mPC1/3 Expression and Purification—Typically, S. frugiperda SF9 insect cells were infected for 1 h at a multiplicity of infection of 5 with the recombinant murine proprePC1/3 baculovirus. After infection, the medium was removed, and the cells were harvested in 400 ml of fresh SF900 II serum-free medium (Life Technologies, Inc.) at a density of 5 x 10⁶ cells/ml in a 1-liter flask. The cells were incubated under mild agitation at 120 rpm at 28 °C for 40 h. After this period, the medium containing the cells was centrifuged at 3,000 x g for 15 min at 4 °C. The pelletted cells were frozen at -20 °C until necessary. After this step, the resulting supernatant was decanted and centrifuged again. The soluble and secreted PC1/3 was purified to homogeneity from the infected insect cell supernatant by polyethylene glycol precipitation followed by immobilized lectin, hydroxylapatite, and gel permeation chromatography as described previously (23). The resulting purified 71-kDa enzyme is homogeneous and is more than 95% pure as judged by SDS-PAGE analysis and had enzymological properties identical to the previously described vac- cinia virus recombinant enzyme (25). Recombinant murine PC2, which was purified from the conditioned medium of Chinese hamster ovary PC2/7B2-expressing cells (26), was a generous gift from Dr. Iris Lindberg (Louisiana State University, New Orleans, LA). The purified mPC2 contains the 71- and 75-kDa proenzyme forms, and to obtain the 66-kDa autoactivated form of this enzyme (26), the mPC2 was preincubated for 2 h at room temperature in the presence of 0.1% (w/v) Brij-35 and 5 mM CaCl₂, pH 5.6, and kept on ice after this activation period.

Purification of Recombinant Zymogen Forms of PC1/3—Typically, 10 g of frozen insect cells harvested at 40 h after infection were added to 4 ml of ice-cold buffer, pH 8.0, containing 100 mM Tris-HCl, 10 mM EDTA, and 500 mM NaCl (Buffer A). The resulting suspension was homogenized using a Polytron homogenizer and centrifuged at 60 x g, supernatant was collected, and the pellet was centrifuged at 30,000 x g for 15 min at 4 °C. The resulting supernatant was filtered through a 0.22-μm filter, and the peptides were separated by gel permeation chromatography as described previously (23).

The recombinant soluble form of human furin corresponds to a highly purified baculovirus-expressed COOH terminus-truncated form of furin obtained after the transfer of the previously described vaccinia construct (24) into the baculovirus vector. The human furin preproform was more than 95% pure as judged by SDS-PAGE analysis and had enzymological properties identical to the previously described vaccinia virus recombinant enzyme (25). Recombinant murine PC2, which was purified from the conditioned medium of Chinese hamster ovary PC2/7B2-expressing cells (26), was a generous gift from Dr. Iris Lindberg (Louisiana State University, New Orleans, LA). The purified mPC2 contains the 71- and 75-kDa proenzyme forms, and to obtain the 66-kDa autoactivated form of this enzyme (26), the mPC2 was preincubated for 2 h at room temperature in the presence of 0.1% (w/v) Brij-35 and 5 mM CaCl₂, pH 5.6, and kept on ice after this activation period.

The NH₂-terminal fragments, each dried fraction was dissolved in 200 μl of water, and 10 μl were used for screening the inhibitory activity or analyzed by SDS-PAGE using Western blotting with the previously described PC1/3 NH-terminal antibody (27). In both cases, the fractions were pooled after purification.

Production and Characterization of the proPC1/3-related 1–98, 26–98, and 9–71 Peptides—The NH₂-terminal proPC1/3-related fragments encompassing residues 1–8 and 26–98 were obtained by CNBr chemical cleavage of the purified zymogen forms described above or directly from the zymogen fraction after solubilization but before gel permeation column chromatography. In the latter case, the 6 x guanidine HCl/20 mM Tris-HCl stable material was first precipitated with 10 volumes of ice-cold 95% EtOH for 30 min. The solution was centrifuged at 27,000 x g at 4 °C for 30 min, and the pellet was washed twice with ice-cold 95% EtOH. At this stage, this fraction and/or the above-described fraction was dissolved in 2–3 ml of 70% (v/v) acetonitrile, and the resulting solution was filtered for 10 min with a stream of nitrogen before the addition of solid CNBr (Sigma) at about 50 mg of pellet wet weight. After an overnight incubation in the dark at room temperature, the reaction mixture was diluted 10-fold with water and lyophilized. The dried material was dissolved in 6 ml of 6 x guanidine HCl, centrifuged, filtered through a 0.22-μm filter, and injected onto a semipreparative Vydac-C₁₈ RP-HPLC column (214TP510; 25 x 0.10 cm; The Separation Group, Hesperia, CA). The adsorbed material was first eluted isocratically for 15 min with a solution containing 5% (v/v) acetonitrile/0.1% (v/v) trifluoroacetic acid and then eluted using a linear gradient of 1%/min from 5 to 60% acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 2.0 ml/min. Fractions of 1 ml were collected and dried under a vacuum on a speed vac evaporator. To identify the NH₂-terminal fragments, each dried fraction was dissolved in 200 μl of water, and 10 μl were used for screening the inhibitory activity or analyzed by SDS-PAGE using Western blotting with the previously described PC1/3 NH-terminal antibody (27). In both cases, the fractions were pooled after purification.

For the production of the NH₂-terminal fragment9–71, the zymogen fraction (5 g of wet weight) was dissolved in 2 ml of 6 x guanidine HCl to which 3 ml of glacial acetic acid together with 40 ml of L-tyrosine and 200 mg of isodosbenzoic acid (Pierce Chemical Co.) were added. The reaction was allowed to proceed at room temperature in the dark for 24 h before centrifuging at 27,000 x g for 1 h. The resulting supernatant was filtered through a 0.22-μm filter, and the peptides were separated by RP-HPLC as described above. After drying under a vacuum, the recovered 100 μl of double-distilled water, and 10 μl were used for screening the inhibitory activity. The most inhibitory fractions were pooled together; analyzed by IEF, gel electrophoresis, amino acid analysis done in duplicate, Edman degradation, and mass spectrometry (matrix-assisted laser-desorption time of flight); and kept at -20 °C before use.

Edman Degradation and Amino Acid and Mass Spectrometry Analysis—Automated Edman degradation of the intracellular PC1/3 as well as the chemical fragments thereof was performed as described previously (23), using an Applied Biosystems gas-phase sequenator (Model 470A updated to Model 475A specifications) directly coupled to an Applied Biosystems phenylthiodyantoin analyzer (Model 120A) and using N-methylpyrroldine as coupling buffer. Amino acid analyses were accomplished in duplicate after an 18–24-h hydrolysis in the presence of 5.7 x HCl in vacuo at 110 °C on a Beckman analyzer (Model 6300) with the post-column ninhydrin detection system generously loaned from the Canadian National Research Council and coupled to a Varian DS 604 integrator/plotter. Matrix-assisted laser-desorption time of flight was done on a TofSpec MicroMass instrument using a-cyano 4-hydroxy-cinnamic acid as the matrix as a pay service by Dr. Gilles Lajoie (Chemistry Department, University of Waterloo, Waterloo, Canada).

SDS-PAGE, Isoelectric Focusing, and Western Blotting—All gel electrophoreses were carried out on a Mini-PROTEAN II gel apparatus (Bio-Rad) according to the procedure described by Laemmli (28). The analysis of the proPC1/3-related fragments was done on 25% acrylamide gels, whereas the analysis of the zymogen forms was done on 10% gels. Analytical IEF was performed using an Xcell II Mini-cell apparatus (Novex, San Diego, CA) and precasted IEF gel cassettes (pH gradien 3 to 10). Western blot analysis was performed after electro transfer to an Immobilon-P membrane (Millipore), and the immune complex was detected using either 3,3-diaminobenzidine staining or the enhanced chemiluminescence kit (Amersham) as described previously (29). In both cases, the primary antibody was used the previously described NH₂-terminal PC1/3 antibody (27), whereas the secondary antibody was a horseradish peroxidase-linked donkey anti-rabbit IgG...
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Expression and Purification of proPC1/3—To obtain large amounts of recombinant enzymatically active PC1/3, we have used the baculovirus/insect cell expression system and found that enzymatically active PC1/3 was secreted into the medium from which it can be purified, yielding 1–2 mg/liter of medium of highly pure 85–71-kDa enzyme (25). Under these expression conditions, enzymatically inactive PC1/3-related proteins also accumulate intracellularly in inclusion bodies as disulfide-linked aggregates at a level of about 10 μg/ml of cell culture (Fig. 1). Considering this sizable amount, intracellular proPC1/3 represents a good material before dissolving it in the guanidine HCl/DTT mixture. The ensuing soluble material was further purified by gel filtration under reducing and denaturing conditions. The proteins eluting from the gel permeation column no longer require reducing conditions to remain soluble and can be purified further by using a copper-chelating column. After this last step, a highly enriched purified fraction containing the PC1/3 zymogen forms is obtained.

Purification and Characterization of the NH2-terminal proPC1/3-related Fragments—Based upon the deduced amino acid sequence of murine proPC1/3 (31), numerous sensitive amino acids can be identified as potential targets for enzymatic and/or chemical cleavage to generate a variety of peptides incorporating elements present in the many predicted conver- tase functional domains such as the proregion, the catalytic region, and the P-domain as well as the COOH-terminal do- main. Among the potential peptides produced, we noticed that cleavage at methionyl residues would generate a fragment encompassing residues 1–98, thus containing the complete previously proposed proregion corresponding to position 1–84. Conveniently, the former also contains the epitope recognized by our NH2-terminal PC1/3-directed antibody (27) that can serve to follow its purification. In addition, cleaving at trypto- phanyl residues could yield a smaller fragment derived from the proregion that corresponds to positions 9–71, as shown in Fig. 2. Both approaches were used and, as shown in Fig. 3, A and B, yielded complex RP-HPLC chromatograms after cleavage by cyanogen bromide and iodosobenzoic acid. Based upon the hypothesis that the prosegment was an inhibitor of PC1/3 enzymatic activity, each separated fraction was individually assayed against the enzymatically active 71-kDa PC1/3 form. In all cases, a unique and single region of the chromatograms was found to contain inhibitory activity. Further analysis of the peptides contained therein, whether by NH2-terminal sequencing, SDS-PAGE, and/or Western analysis, confirmed the presence of peptides exclusively related to the proregion (Fig. 4). From 10 g of cells (wet weight) and further purification by RP-HPLC, the procedure leads to about 0.5–1 mg of the purified NH2-terminal peptide 1–98 and the NH2-terminal elongated peptide 26–98 from the CNBr digest, whereas the purified NH2- terminal peptide 9–70 can be obtained in a similar yield from the iodosobenzoic digest. Edman degradation yielding a single homo- geneous NH2-terminal sequence, amino acid analysis and mass spectrometry yielding a mass of 11,306 (calculated, 11,317.8), 14,260 (calculated, 14,278.2), and 7,151 (calculated, 7,066.7), respectively, as well as SDS-PAGE and IEF analysis (shown in Fig. 4) confirmed their identities and their purity exceeding 95%.

Characterization of the Inhibitory Activity of the proPC1/3 Peptides—After the isolation and characterization of each peptide related to the proPC1/3 and using the highly purified PC1/3 71-kDa form (Fig. 5), we used on-line assays to characterize the type of inhibition by the various peptides. When the
enzyme was incubated with the fluorogenic pGlu-Arg-Thr-Lys-Arg-MCA substrate in the presence of the proPC1/3 segment 1–98 (Fig. 6), representing a COOH-terminal extended form of the complete proPC1 1–84, it was observed that the inhibition followed a typical slow-binding kinetic based upon the progress curves (30). The analysis of the various progress curves as described previously (30) yielded a computed $K_i$ of 6.6 nM. To determine whether this inhibition occurs through a single- or two-step mechanism, the observed $K_{obs}$ values were plotted as a function of the inhibitor concentration; as seen in Fig. 6, a straight line is obtained, which is indicative of a single-step mechanism. Thus, the proPC1/3 1–98 once bound to the enzymatically active mPC1/3 yields a bimolecular complex that does not undergo any further modification in the presence of excess inhibitor. Furthermore, under these conditions, this complex remains stable upon dilution and/or gel permeation, thus confirming the tight-binding properties of the proPC1/3 31–98 peptide (data not shown). However, as discussed later on, this inhibitor-enzyme complex is no longer stable when excess enzymatic activity is present.

Interestingly, when the same peptide is incubated in the presence of highly purified 50-kDa hfurin (Fig. 5), an entirely similar inhibition is observed, yielding progress curves closely following those of PC1/3. It should be noted that this type of inhibition cannot be observed upon using crude vaccinia virus-produced hfurin, because this preparation degrades the peptide rapidly. The amount of peptide necessary to achieve maximal inhibition is slightly higher than that with PC1/3, as evidenced by the computed $K_i$ of 10 ± 1.0 nM. Even though it appears to indicate that the proregion-related peptide is not able to dis-
criminate between those two convertases, one should consider the following aspects. First, it is well established that PC1/3 and furin exhibit similar if not very close affinity for a myriad of substrates, hinting that their substrate binding sites could share a close similarity (25) and hence should be able to interact in a similar fashion with the proPC1/31–98 peptide. Second, it has been shown through elegant studies based on chimeric molecules that the propeptide regions from PC1/3 and furin are completely interchangeable upon expressing the recombinant proteins (20). However, one must remember that furin is mostly active in vivo in the constitutive secretory pathway, whereas PC1/3 is mostly active within secretory granules and hence the regulated secretion pathway. Thus, although it cannot be ruled out, this different localization thereby minimizes the in vivo likelihood that proPC1/3 peptide could interact and/or inhibit furin, because the resulting complex formed should not be dissociated before reaching the secretory granules. Furthermore, in the case of furin, it has been clearly shown that the profurin/furin complex remains intact until it reaches its eventual site of activity, namely, the trans-Golgi network (21).

In the case of the two secretory granule-associated convertases, PC1/3 and PC2, it becomes essential to analyze the effect of the proPC1/31–98 peptide on the enzymatic activity of recombinant PC2. Thus, as illustrated in Fig. 7, a completely different picture emerged. Indeed, it is seen that: (i) a much increased amount of peptide is needed to abolish the enzymatic activity, (ii) the inhibition mechanism is no longer characterized by a slow tight-binding mechanism but, on the contrary, is strictly competitive in nature, and (iii) a stable complex is not formed upon the incubation of mPC2 with proPC1/31–98 because the latter is cleaved (data not shown). The observed $K_i$ determined in the presence of the fluorogenic substrate corresponds to 10 ± 1.0 $\mu$M, a thousandfold higher value than that observed in the case of PC1/3 and/or furin. Hence, it appears that with these two similarly located convertases, the propeptide region is highly specific to its cognate convertase and efficiently discriminates between the two enzymes. This conclusion agrees with the results obtained upon expressing proPC1/3-PC2 or proPC2-PC1/3 chimera, whereupon failure to produce enzymatically active enzymes in the cell culture medium is observed (20). To properly resolve this issue, it is mandatory to obtain the related proPC2 peptide to examine its action upon PC1/3 and its interaction with the 7B2 molecule, because the latter was shown to interact closely with PC2 (32, 33). Such studies are currently in progress.
To further extend our understanding of the mode of action of the proPC1/3\textsubscript{1–98} peptide, we used two other peptides isolated from full-length preproPC1/3. The first one, as described above, was isolated after CNBr treatment of the inclusion bodies sequestered proteins and corresponds to position –26–98. This peptide thus contains the 1–98 region and, as determined by amino acid sequencing, corresponds to an NH\textsubscript{2}-terminal sequence elongated by 26 residues corresponding to the signal peptide devoid of the Met residue occupying position –27 (data not shown). Progress curves obtained through the incubation of PC1/3 as accomplished above indicate a behavior entirely consistent with the one obtained with peptide\textsubscript{1–98}. Both the calculated $K_i$ and the inhibition mechanism are identical to the latter. The second peptide was obtained after cleavage at tryptophanyl residues by iodosobenzoic acid and corresponds to proPC1/3\textsubscript{39–71} (Fig. 4), hence lacking COOH-terminal residues 72–84 that contain the zymogen activation site. Upon incubation with PC1/3, this peptide proved to be a considerably weaker inhibitor of PC1/3, having completely lost the slow tight-binding characteristics of its longer 1–98 counterpart (data not shown). Indeed, to achieve a comparable extent of inhibition, much higher micromolar amounts of peptide must be used. In fact, its behavior toward mPC1/3 is closer to being a competitive substrate, because it is cleaved by PC1. This behavior is therefore in agreement with the proposal whereby the primary site of interaction between the proregion and the enzyme is directly related to the zymogen activation sequence. Interestingly, preliminary results (34) obtained with a 34-residue synthetic peptide related to the proPC1/3\textsubscript{50–84} region indicate that: (i) the presence of the Lys-Arg pair at positions 83 and 84 is essential for inhibition, because carboxypeptidase E treatment of the peptide completely abolishes the inhibition, and (ii) elongating toward the NH\textsubscript{2}-terminal does not improve upon its nanomolar inhibition. These conclusions agree entirely with the results obtained herein with proPC1/3\textsubscript{39–71} and the NH\textsubscript{2}-terminal elongated proPC1/3\textsubscript{26–98} peptide.

Finally, by using very low picomole amounts of proPC1/3\textsubscript{1–98} with an increased amount of PC1/3 enzymatic activity yielding an enzyme:inhibitor ratio much in favor of the former, we demonstrate that, \textit{in vitro}, the proPC1/3\textsubscript{1–98} immunoreactivity, as detected using the antibodies raised against positions 84–99 of mature PC1/3, disappears in the presence of active enzyme, as shown in Fig. 8. The higher band (approximately 13 kDa) corresponds to the position of the intact 1–98 peptide. The lower band (approximately 6 kDa) is likely derived from an internal cleavage but still contains the COOH-terminal-located epitope. As can be seen, both immunoreactive bands disappear with time through similar loss of the epitope. The faster disappearance of the intact proPC1/3\textsubscript{1–98} immunoreactivity is best explained by the fact that this peptide is cleaved at two internal sites; whereas cleavage at the first one leads directly to the loss of epitope, cleavage at the second one leads to the appearance of the lower molecular weight band. This result agrees with the previously observed intracellular cleavage of the profrumin peptide by furin (21) and with the activation pathway of \textit{S. pombe} prokexin (22). In both cases, a primary site and a secondary site of cleavage within the proregion were observed before obtaining full activation of either enzyme. The primary cleavage site is located COOH-terminal to the zymogen activation sequence, namely, the Arg\textsuperscript{50}Ser-Lys-Arg\textsuperscript{84} sequence just preceding the epitope recognized by the antibodies. Given the molecular weight observed for the lower molecular weight band arising from cleavage at the secondary site and considering the results obtained by Anderson \textit{et al.} (21), it can be proposed that this site, by analogy to furin, is located COOH-terminal to the internal Arg\textsuperscript{50}Arg-Ser-Arg-Arg\textsuperscript{84} sequence within the proPC1/3\textsubscript{1–98} peptide. Cleavage at this site leads to the appearance of a fragment, tentatively identified as proPC1/3\textsubscript{55–98}, which undergoes further cleavage into the fragment proPC1/3\textsubscript{55–84}, which no longer contains the epitope recognized by the PC1/3 NH\textsubscript{2}-terminal antibodies. Considering that two cleavage sites exist in proPC1/3\textsubscript{1–98} that serve as recognition sites by PC1/3, it can be proposed that the processing of proPC1/3 likely involves an initial cleavage into the expected 1–84 peptide, yielding the enzymatically active 85-kDa form and, ultimately, the 71-kDa form (23), either of which could be responsible for the subsequent cleavage of the 1–84 peptide into the smaller peptide exhibiting decreased inhibitory properties, a conclusion that leads to a model very close to what has been proposed in the case of furin (21), kexin (22), and the prosubtilisin/subtilisin model (16), whereupon the initial cleavage at the zymogen activation site, followed by a second one within the proregion, yields a fully active enzyme. However, much work clearly remains to be done to fully acquire a complete understanding of the interactions between FC1/3 and its proregion.

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