Evidence for proprotein convertase activity in the endoplasmic reticulum/early Golgi

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Abstract Processing of precursor proteins by the proprotein convertases is thought to occur mainly in the *trans*-Golgi network or post-Golgi compartments. Such cleavage is inhibited by the prosegment of the convertases. During our studies of the use of the inhibitory prosegment of PC1, we noticed that a construct containing the prosegment fused to the C-terminal secretory granule sorting domain was cleaved in the endoplasmic reticulum (ER) at a pair of basic residues, best recognized by furin and PC7. This was further confirmed when this construct was fused at the C-terminus with a KDEL ER-retention signal. This suggests that the convertases could cleave some substrates within the ER, possibly by displacing the inhibitory prosegment associated with them.

Keywords: Proprotein convertase; Furin; PC7; PC1; Prosegment; Endoplasmic reticulum

1. Introduction

Limited proteolysis of inactive polypeptide precursors often generates biologically active proteins and peptides. Such cleavage is performed within the secretory pathway by the mammalian serine proteinases related to bacterial subtilisin, known as the proprotein convertases (PCs). The PC family counts seven basic-amino acid (aa) specific kexin-like convertases: furin, PC1/3, PC2, PC4, PACE4, PC5/6 and PC7/LPC [1]. These enzymes specifically recognize single or paired basic residues within a general motif (K/R)–$(X)_n$–(K/R)↓, where $n = 0$, 2, 4 or 6 and X is any amino acid. PCs contain an N-terminal signal sequence, followed by a prosegment, a catalytic domain and a P-domain, as well as a C-terminal (CT) segment that varies between the different members. PC1 and PC2 are the main convertases of the regulated secretory pathway [1]. Except for PC2, the first zymogen cleavage of the convertases occurs in the endoplasmic reticulum (ER), and the final activation is thought to occur in the *trans*-Golgi network (TGN) or in immature secretory granules. Accordingly, because of this ordered zymogen activation of PCs, the cleavage of precursor proteins by the basic-aa specific PCs is thought to occur mainly in the TGN, cell surface, endosomes (furin, PC7, PC5 and PACE4) [2] or immature secretory granules (PC1 and PC2) [3]. Analysis of the biosynthesis of furin, PC1 and PC2, PACE4, PC5 and PC7 revealed that their N-terminal prosegment acts as an intramolecular chaperone and, except for PC2, as a nanomolar inhibitor of its cognate enzyme. Indeed, expression of the prosegments of furin, PC7, PC5 and PC1 as independent domains confirmed their inhibitory potency and revealed that the C-terminus of the prosegment contains the critical inhibitory elements [4].

We previously showed that the CT segment of PC1 contains an amphiphatic α-helix that seems to be critical for the sorting of this enzyme to secretory granules [5,6]. Accordingly, in this work, we thought to direct the prosegment of PC1 to granules by fusing its inhibitory domain to the CT granule-sorting domain of PC1. Unexpectedly, we observed that this chimera was rapidly processed very early on in the secretory pathway at the CT dibasic site of the prosegment. This led to the hypothesis that convertases may be active on some substrates within the early secretory pathway. This concept supports and extends the scope of an earlier observation that misfolded insulin receptor is processed by furin-like convertases in the ER [7].

2. Materials and methods

2.1. Constructs and antibodies

The prosegment (aa 1–110) and the CT tail (aa 619–753) of mouse PC1 cDNAs [8] were fused by PCR ($RSKR_{110–619}GVEK$) and the cDNA coding for the fusion protein "proCT" was inserted into the pIRES2-EGFP vector (Clontech). The R110A mutant of the primary processing site used to generate the "pro*CT" mutant was obtained generated by PCR. Finally, we also generated a proCT-V5-KDEL construct inserted into the pIRES2-EGFP vector and in which the ER-retention signal KDEL was at the CT end. The cDNAs of human furin, human PACE4, mouse PC5-A/B and rat PC7 [9] cloned into the pIRES2-EGFP vector were as described. The anti-V5 monoclonal antibody (V5-mAb) was from Invitrogen and anti-CT mPC1 [10] antibodies were previously described.

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Abbreviations: aa, Amino acid; PC, proprotein convertase; ER, endoplasmic reticulum; CHO, Chinese hamster ovary; WT, wild type; EGFP, enhanced green fluorescent protein

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2.2. Cell culture, transfection and metabolic labelling

HEK293 and AtT20 cells were grown at 37 °C in DMEM + 10% FBS (heat inactivated for HEK293) + gentamycin 28 μg/ml; while Chinese hamster ovary (CHO)-K1, FD11 cells were grown in DMEM:F12 (1:1) + 5% FBS + gentamycin 28 μg/ml. HEK293 cells were transfected by Effectene (Qiagen), while other cell lines were transfected by Lipofectamine 2000 (Invitrogen). Transfections were done in a 35 mm dishes. Twenty-four hours after transfection, FD11, HEK293 and AtT20 cells were labelled with 0.2 mCi [^{35}S]Met/Cys (Amersham) for various periods in RPMI 1640 + 0.5% dialysed FBS + GTM 28 μg/ml. The different cell lines were either analysed as such after the pulse or chased in their respective growth medium, as described [10]. For experiments using Western blots, 24 h post-transfection of CHO-K1 or FD11 cells, the latter were then washed and further incubated for 6 h at 37 °C in DMEM:F12 (1:1) + GTM 28 μg/ml. Brefeldin A (Calbiochem) was used at concentrations of 2.5 μg/ml.

2.3. Immunoprecipitation, Western blot and protein sequencing

Cells were lysed in RIPA buffer 1X (100 mM Tris–HCl, pH 8, 300 mM NaCl, 0.2% SDS, 2% NP-40 and 1% sodium deoxycholate) for 30 min. Immunoprecipitations in RIPA buffer 0.5× were performed with a V5-mAb (1/500) antibody to which was added 30 μl of protein A/G plus agarose (Santa Cruz biotechnology). We used anti-V5 HRP (Invitrogen) (1/2000) or anti-CT mPC1 (1/2000) antibodies for Western blotting. The anti-CT mPC1 antibody was detected with anti-rabbit HRP antibody (Sigma) (1/10000). Proteins were separated by SDS–PAGE and visualized by autoradiography with X-OMAT or BIOMAX films (Kodak). For microsequencing of proCT and pro*CT products, HEK293 cells were labelled with 1.5 mCi [^{3}H]valine for 4 h and the products immunoprecipitated, separated by SDS–PAGE and microsequenced [10].

3. Results

3.1. Endoplasmic reticulum cleavage by the PCs

The original aim was to engineer a PC1 inhibitor targeted to secretory granules. Accordingly, we fused the signal peptide and the N-terminal inhibitory prosegment (pro) of PC1 [11] to its CT segment reported to contain an amphiphatic α-helix critical for targeting to secretory granules [5], thereby generating a proCT construct (Fig. 1). In order to prevent a PC-dependent cleavage at the C-terminus of the prosegment and to drastically reduce the inhibitory protency of the prosegment [4], we generated an R110A mutant, whereby the P1 Arg_{110} of the primary cleavage site KERSK**R**$_{110}$ of the prosegment is mutated to Ala (called pro*CT; Fig. 1). Both constructs were tagged at the C-terminus with a V5-epitope. We chose the corticotroph AtT20 cell line to perform our analyses, since it is a widely used regulated cell line endowed with secretory granules and endogenously expressing PC1, some PC2 and their substrate proopiomelanocortin [12]. AtT20 cells transiently expressing either proCT or pro*CT were pulse-labelled with [^{35}S]Met/Cys for 5 min (P5) followed by a chase in the absence of label for 15 (P5C15) and 45 (P5C45) min (Fig. 1). To our surprise, we found that with a pulse as short as 5 min, the 33-kDa proCT (form A; likely representing the full-length proCT) was processed to a 21 kDa CT V5-immunoreactive product (form B; likely representing proCT cleaved at the primary cleavage site KERSKR_{110}, thus releasing the CT). This cleavage was not observed with pro*CT that bears an R110A mutation. Only at 45 min chase do we begin to observe an intermediate 26 kDa product (form C) in both proCT and pro*CT, likely representing proCT and pro*CT cleaved at the secondary cleavage site RRSRR_{81}↓ of the prosegment (Fig. 1). These data suggest that proCT is processed very early on during its synthesis, possibly in the ER to produce the B-product. In contrast, the C-form, is obtained later in time along the secretory pathway, likely in the TGN where most convertases were reported to be active [2].

In order to obtain sufficient amounts of A and B products for microsequencing, we selected HEK293 cells that are much better transiently transfected than AtT20 cells. In HEK293 cells, proCT and pro*CT are qualitatively similarly processed as in AtT20 cells (Fig. 1, bottom), with the exception that the proCT processing is much less efficient, both following pulses of 15 min (Fig. 1) and 5 min (not shown). Following transient transfection of HEK293 cells with a proCT recombinant, the cells were pulse labelled for 4 h with [^{3}H]Val, and the A and B products microsequenced. The data revealed a Val at sequence position 5 for the A-form of either proCT or pro*CT, in agreement with it representing the complete proCT or pro*CT following signal peptidase cleavage at …KA_{27}↓KRQF**V** [10]. In contrast, the 21-kDa B-product revealed a Val in positions 2, 6, 8 and 9 demonstrating that it begins following the dibasic cleavage site $RSKR_{110}$↓G**V**EKM**V**N**VV**, and confirms that form B corresponds to the CT alone. Unfortunately, HEK293 cells did not produce enough material for sequencing of the C form, and we can only speculate from its apparent molecular mass that it likely corresponds to proCT and pro*CT cleaved at the secondary processing site of the PC1-prosegment [13]. Notably, treatment of HEK293 cells with the fungal metabolite brefeldin A (BFA) revealed only form B and not C, suggesting that the first cleavage can occur in the ER, but not the second.

Since proCT is cut very early along the secretory pathway, it was no longer useful to direct the inhibitory prosegment to secretory granules and we did not expand on this approach. Rather, we were intrigued by the fact proCT, and not pro*CT, is cleaved very early on in the secretory pathway at a typical basic aa-specific PC-recognition motif $RSKR_{110}$↓ [14]. Since such cleavage occurs in the presence of BFA, which causes the fusion of the ER with most of the Golgi saccules, except the TGN [15], it was plausible that such processing occurs within the ER itself. However, the accepted model of PCs

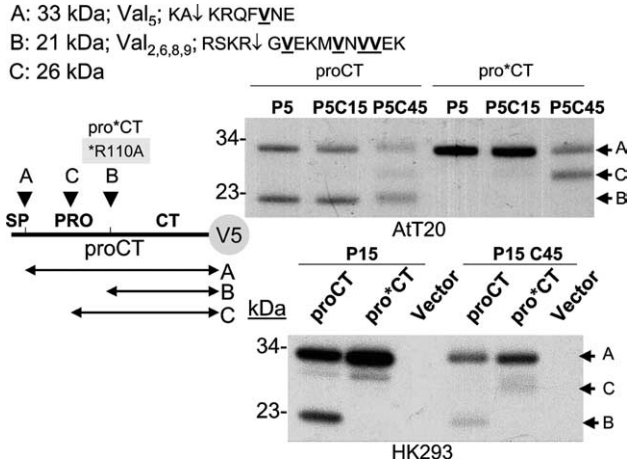

Fig. 1. Processing of proCT and pro*CT occurs early in the secretory pathway of AtT20 and HK293 cells. Transiently transfected cells with either proCT, pro*CT or empty vector were labelled with [^{35}S]Met/Cys for either 5 or 15 min (P5; P15) and chased for 15 or 45 min (C15; C45), and cell lysates analyzed on 14% tricine SDS–PAGE. The [^{3}H]Val sequence of each product is shown at the top left corner. The V5-tagged products were immunoprecipitated with a V5 antibody.