A 36-Residue Peptide Contains All of the Information Required for 7B2-mediated Activation of Prohormone Convertase 2*

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The prohormone convertases (PCs) are serine proteinases responsible for the processing of secretory protein precursors. PC2 is the only member of this family whose activation requires intracellular interaction with a helper protein, the neuroendocrine protein 7B2. In order to gain a better understanding of the mechanism of proPC2 activation, we have characterized the structural determinants of 7B2 required for proPC2 activation. We had already identified a proline-rich binding determinant in the 21-kDa domain, the portion of 7B2 responsible for proPC2 activation. We have now investigated the function of the weakly conserved amino-terminal portion of 21-kDa 7B2 by sequential deletions. Mutant proteins were analyzed in four assays: binding to proPC2, facilitation of proPC2 maturation, and activation of proPC2 in vivo and in vitro. We found that the amino-terminal half of 7B2 is not involved in proPC2 activation, and we identified an active 36-residue peptide that contains the previously characterized proline-rich sequence as well as an α-helix and the only disulfide bond of 7B2. Mutation of the α-helix and of the cysteines demonstrated that these determinants are absolutely required for PC2 activation. Thus, the 186-residue full-length 7B2 rat protein can be functionally reduced to an internal segment of only 36 residues.

Endoproteolytic processing is one of the major post-translational modifications that hormones and neuropeptides precursors must undergo during their biosynthesis. A family of mammalian subtilisin-like endoproteinases responsible for these processing events has been recently identified, the prohormone convertases (PCs) reviewed in Refs. 1 and 2. PC1 and PC2 are the prohormone convertases specific for neuroendocrine cells; both enzymes are active late in the secretory pathway, i.e., the trans-Golgi network (TGN) and the secretory granules.

* This work was supported in part by National Institutes of Health Grant DK49703 (to I. L.) and by the Fundacao de Amparo Pesquisa do Estado de Sao Paulo and Conselho Nacional de Desenvolvimento Cientifico e Tecnologico-PADCT Project 620498/98-6. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡Supported by the Neurosciences Section of Excellence, Louisiana State University Medical Center.
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The abbreviations used are: PC, prohormone convertase; CT-peptide, 7B2155–186; ER, endoplasmic reticulum; TGN, trans-Golgi network; CHO, Chinese hamster ovary; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; Fmoc, N-(9-fluorenyl)methoxycarbonyl.

Precursors such as proinsulin or proopiomelanocortin are processed sequentially, first by PC1, then by PC2 (reviewed in Ref. 3). In agreement with the ordered activity of these enzymes, PC1 and PC2 have different activation pathways (reviewed in Ref. 4). Whereas the propeptide of proPC1 is first processed in the endoplasmic reticulum (ER) (5–7), as are those of furin (8, 9), PC5 (10), and LPC/PC7/PC8 (11), the proPC2 propeptide is processed only in the acidic compartments of the TGN/secretory granules. In addition, proPC2 is the only PC that specifically interacts with a helper protein, the neuroendocrine-specific protein 7B2 (12–14). We have demonstrated that this interaction is absolutely required for proPC2 activation, both in vivo in transfected AtT-20 cells (14) and in 7B2 null mice (16), as well as in vitro (17).

The neuroendocrine protein 7B2 was originally purified from pituitary extracts (18). It is an 185-residue prohormone that is cleaved at a pentabasic site at residue 155 (Fig. 1), most probably by furin (19–21). The two domains generated by this cleavage have distinct functions: the amino-terminal domain of 21 kDa is sufficient for PC2 activation (14), and the COOH-terminal domain of 31 residues is a potent inhibitor of PC2 activity (22, 23). Based on the homology between the 90 amino-terminal residues with members of the chaperonin 60 family, 7B2 was originally proposed to act as a molecular chaperone specific for proPC2 (12). We have already demonstrated that the 90 amino-terminal residues alone cannot bind to proPC2 (14); they could nonetheless be involved in PC2 activation. Thus, the function of the amino-terminal half of 7B2 is not yet known. In order to analyze the role of the amino-terminal half of 7B2 and to better understand the involvement of 7B2 in PC2 activation, we have investigated the effect of amino-terminal deletions. Deleted 7B2s were tested in four functional assays: binding to proPC2; facilitation of its maturation in stably transfected AtT-20 cells; and activation of PC2 both in vivo, by transient transfection in CHO cells, and in vitro, using Golgi subcellular fractions enriched in proPC2. As these experiments demonstrated that the 86 amino-terminal residues of 7B2 are not required for PC2 activation, we further investigated the minimum structural determinants of 7B2. We were able to identify a 36-residue peptide that contains the PPNPSCP motif, as well as an α-helix and a disulfide bridge, which are all absolutely required for binding to proPC2 and for proPC2 activation.

MATERIALS AND METHODS

Cell Culture—AtT-20 cells were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies Inc.) containing 10% Nusenum (Becton Dickinson, Mountain View, CA), 2.5% fetal calf serum (Irvine Scientific, Santa Ana, CA), and the appropriate selection agents, 200 μg/ml G418 (Life Technologies Inc.) and/or 100 μg/ml hygromycin (Sigma). The AtT-20 cells stably expressing PC2 (AtT-20/PC2) were provided by R. E. Mains (Johns Hopkins University School of Medicine, Baltimore, MD) (24). The control AtT-20 cells that co-express PC2 and 7B2 have already
been described (14). CHO cells were maintained in α-minimum essential medium without nucleosides, containing 10% dialyzed fetal calf serum (Irvine Scientific) and 50 µM methotrexate (Sigma). CHO cells stably expressing PC2 (CHO/PC2), which have been amplified by the dithiofradolate reductase method, have already been characterized (25).

Purification of Recombinant 7B2—In a similar way to the purification of the rat 7B2, 7B2 was purified by anion exchange and reverse phase chromatography (59). The ammonium sulfate precipitate was dissolved in a 20 mM sodium acetate, pH 5.7, buffer containing 0.1% Triton X-100, 50 µM EDTA, 0.1% 2-mercaptoethanol, and 5% glycerol. The sample was then run through a Q-Sepharose column (Pharmacia, Uppsala, Sweden) and eluted with a gradient of sodium acetate. The 7B2 protein was collected as described previously (17).

Instability of the disulfide bridge in recombinant 7B2 was determined by transfection experiments with 21-kDa 7B2 (26). The formation of an actual disulfide bridge within recombinant 7B2 was verified by DNA sequencing. Proteins were expressed in the guanidine HCl method as described previously for 21-kDa 7B2 (26).

The formation of a disulfide-containing cysteine was determined by the guanidine HCl method as described previously for 21-kDa 7B2 (26). For formation of the disulfides, the peptides were dissolved in water (at a final concentration of about 1 mM), the pH maintained at 7.5, and air oxidation was allowed to proceed with slow stirring. Aliquots of the solution were removed at different periods of time to monitor the oxidation of the two cysteines by HPLC and by Ellman’s assay.

RESULTS

The 86 Amino-terminal Residues of 7B2 Are Not Involved in PC2 Activation—We analyzed the role of amino-terminal residues of 7B2 (56) in proPC2 activation by serial deletion (Fig. 1A). We have already demonstrated that the capacity of 7B2 to activate proPC2 resides in the 150-residue amino-terminal domain referred to as 21-kDa 7B2 (14), we terminated the common carboxyl-terminal primer was used: 5′-GGCGCGGTTCGAATTCATCAGCTGGCGCGGTTCGAATTC-3′, and the primers containing the mutation were 5′-GAACAATTCGTTATGCAAATTCGTTATGAACTGGCGGTCGTAATC-3′, and 5′-GGCGCGGTTCGAATTCATCAGCTGGCGGTCGTAATC-3′ for the 7B2-21–150 construct; 5′-GGCGCGGTTCGAATTCATCAGCTGGCGGTCGTAATC-3′ for the 7B2-151–210 construct.

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As these three truncated 7B2s were able to bind to proPC2, we tested their capacity to activate proPC2 in vivo. For this purpose, we transiently expressed different 7B2 constructs in CHO/PC2 cells. These cells express very high levels of proPC2, as this gene has been amplified using the dihydrofolate reductase method (25). In addition, CHO/PC2 cells do not express 7B2 and therefore possess no endogenous PC2 activity (25). After transient transfection of mutant 7B2 constructs, PC2 activity was tested in the overnight conditioned medium. In line with their binding to proPC2, the three deleted 7B2s were able to activate PC2 (Fig. 2A); however, 7B2 86–150 was not as efficient as 7B2 30–150 and 7B2 68–150.

The capacity of these deleted 7B2s to activate proPC2 was confirmed using an in vitro activation assay, which is based upon the use of purified Golgi membranes from the same CHO/PC2 cells described above. We have already characterized this cell-free assay and have demonstrated that addition of recombinant 21-kDa 7B2 to these membranes is sufficient to achieve activation of proPC2 (17). The recombinant 7B2 30–150 and 7B2 68–150 proteins were able to activate proPC2 in vitro (Fig. 2B). We were not able to obtain the recombinant protein corresponding to the shortest of the truncated 7B2s since it was not expressed in bacteria, perhaps due to its short size (64 residues).

Taken together, these experiments demonstrate that the 86 amino-terminal residues of 7B2 are not required for 7B2 binding to proPC2 or for activation of proPC2. In addition, the analysis of proPC2 conversion kinetics shows that the 68–87-residue segment of 7B2 is required for the increase of proPC2 conversion rate generated by 7B2.

**A Peptide Corresponding to Residues 86–121 Is Sufficient for ProPC2 Activation**—We have previously demonstrated that carboxyl-terminal deletion of the 21-kDa domain following residue 109 prevents its interaction with proPC2, whereas deletion after residue 121 does not prevent binding to proPC2 (26). We combined these results with the results of the amino-terminal deletions described above, and synthesized a set of peptides corresponding to residues 86–121 or to shorter constructs.

The 86–121 peptide contains the PPNNCP motif that we have already identified as a binding determinant (26), as well as the only disulfide present in mammalian and Xenopus 7B2s, and a COOH-terminal putative α-helix. The various deletions in the 86–121 peptide were thus designed to selectively remove these three determinants (Fig. 3A). The peptides were tested in the in vitro Golgi membrane proPC2 activation assay. The synthetic peptides (5 μM final concentration) were added to CHO/
PC2 cell Golgi membranes, and the pH was lowered to 5 for measurement of PC2 activity. The 86–121 sequence was the only peptide that could activate proPC2 (Fig. 3B). All of the other peptides were not able to effect PC2 activation, including those with deletions within the proline-rich sequence (96–121) and those with deletions within the α-helix (86–112). On a molar basis, the 86–121 peptide was almost as efficient as the entire amino-terminal 7B2 domain in activating proPC2 in vitro (Fig. 3C).

The α-Helix Present in the 86–121 Peptide Is Required for PC2 Activation—We then investigated the involvement of the α-helix in PC2 activation. The recent cloning of 7B2 from Caenorhabditis elegans (27) and Drosophila melanogaster2 showed that this putative α-helix is conserved, even though the overall conservation of 7B2 through evolution is quite poor. In order to test the requirement for the α-helix, we introduced two mutations: the replacement of an arginine by a proline (R116P), which should disrupt the helical structure, and the insertion of a histidine at the same position (H116A). This mutation, unlike R116P, is not predicted to disrupt the helical structure (28–30). These synthetic peptides were used for their ability to activate PC2 in vivo using the Golgi membrane assay (Fig. 4D). The +A115 mutant and the two phenylalanine mutants could only activate PC2 at 20–30% of the activity of the control 86–121 peptide (Fig. 4D). This result suggests that the presence of the two hydrophobic residues on one face of the helix constitutes a determinant directly involved in proPC2 binding or PC2 activation. The H116A mutation had no effect on PC2 activation (Fig. 4D), thus confirming that the effect of the R116P mutation was due to the disruption of the helical structure and not to the role of the arginine residue itself.

The Disulfide Bond Is Required for PC2 Activation—Another feature of the 86–121 peptide is the presence of the sole two cysteine residues in mammalian 7B2a, which are also conserved in all of the invertebrate 7B2s cloned thus far (27, 31).2 These cysteines form a disulfide bond that is apparently not required for correct processing and for secretion of 7B2 (17, 32). However, in vivo reduction of the disulfides does prevent binding of 7B2 to proPC2 (17). In order to determine whether this lack of binding is a direct result of the reduction of the disulfide bond, we mutated cysteine 104 into alanine (C104A). This mutant was unable to bind to proPC2 (Fig. 5A) and was unable to facilitate its maturation in AtT-20 cells (Fig. 5B). The C104A mutant was also not able to activate proPC2 in transiently transfected CHO/PC2 cells (Fig. 5C). Finally, the requirement for the 7B2 disulfide bond in proPC2 activation was confirmed with synthetic peptides in which the cysteines were blocked by methylation. Our data clearly show that, whereas the 86–121 peptide could activate PC2 in vitro, the peptide containing methylated cysteines could not (Fig. 5D). Thus four different assays demonstrate the necessity for the 7B2 disulfide bond for binding to proPC2 as well as for activating proPC2.

DISCUSSION

The production of enzymatically active PC2 requires the interaction of its proenzyme with the neuroendocrine protein 7B2 (14). Whereas we have been able to delineate the cellular mechanism of the interaction between proPC2 and 7B2 (14, 17), the analysis at a molecular level remains puzzling (reviewed in Ref. 4). Interestingly, although intracellular encounter with 7B2 is required for proPC2 activation, the lack of interaction between these two proteins does not prevent the cleavage of the PC2 propeptide. However, the mature enzyme generated under these conditions (lack of intracellular interaction with 7B2) exhibits absolutely no catalytic activity, and this particular maturation process has thus been referred to as
“unproductive” propeptide cleavage (16, 17, 33). In order to gain a better understanding of the molecular mechanism of 7B2-mediated activation of proPC2, we have investigated the structural requirements of 7B2. We were able to identify a 36-residue peptide that contains a proline-rich motif, a disulfide bridge, and an α-helix. These three determinants are necessary and sufficient to generate a proPC2 species capable of actual activation. This 36-residue peptide (position 86–121) is not localized in the amino-terminal half of 7B2, which is homologous to the chaperonin 60 GroEL (12). The lack of importance of this chaperone-like domain for proPC2 activation is in agreement with its weak conservation through evolution. Whereas the sequences of D. melanogaster (34) and mammalian 7B2s among species, the hydrophobic phenylalanines are, which suggests that they could be functionally important. In agreement with this conservation, mutation of one or two phenylalanine residues(s) (F114A and F114A/F118A) demonstrates their essential role in activation of proPC2. We thus propose that these two hydrophobic residues constitute an important site of interaction between the 7B2 α-helix and proPC2.

The third structural element required for binding to proPC2 and for PC2 activation is 7B2’s only disulfide. This disulfide involves the cysteine present in the PPNPCCP motif. We had already shown that exchanging the position of this cysteine with the fourth proline residue of the motif prevented binding to proPC2 and PC2 activation. We had thus proposed that this effect resulted from the disorganization of the proline-rich motif. Further work had then demonstrated that, even though the formation of 7B2 disulfide is not required for 7B2 maturation and secretion (17, 32), the in vitro reduction of disulfide bridges in newly synthesized proteins prevents the interaction between 7B2 and proPC2 (17). We have now demonstrated that the presence of the 7B2 disulfide is required for effective interaction with proPC2. The most probable hypothesis that explains the effect of the cysteine 104 mutation is that the disulfide bridge stabilizes the proline-rich motif in a conformation nec-
Structural Determinants of 7B2 Required for PC2 Activation

There are, however, still no data available concerning the crystal structure of any PC, and the structural study of these enzymes is based on the model of the related enzyme subtilisin (40–42).

ProPC2 exits from the ER as a proenzyme that is autocatalytically processed in the acidic compartments of the TGN and secretory granules (5, 7, 25, 43, 44), whereas the propeptides of the other PCs are initially processed in the ER. A recent study demonstrated that furin-cleaved propeptide remains associated with the mature enzyme (15). These authors also suggested that this interaction could be required for furin transport out of the ER. If such a hypothesis also holds true for proPC2, it must occur without actual cleavage of the propeptide. The binding of 7B2 to several proPC2 determinants could thus provide an alternative for the formation of a transport-competent propeptide/mature enzyme complex. 7B2 binding to the proenzyme could protect proPC2 either from premature unproductive processing, or from inactivation of the proenzyme due to exposure to an acidic pH. Indeed, acidification of proPC2-enriched Golgi fractions prior to addition of recombinant 7B2 completely blocks proPC2 activation (17). 7B2 could fulfill such a passive role, or it could promote proPC2 activation more actively. In both cases, bringing together several domains of proPC2 through binding to different 7B2 determinants could constitute the actual mechanism of 7B2-mediated activation of proPC2. Our new data suggest that a productive interaction between proPC2 and 7B2 requires a specific conformation not only for proPC2, as previously proposed, but also for 7B2. The characterization of a 36-residue peptide that contains this conformation and that is sufficient for effecting proPC2 activation in a cell-free system should prove helpful for the final molecular analysis of the proPC2 activation mechanism.

Acknowledgments—We thank Joelle Finley for expert assistance with cell culture, members of the Lindberg laboratory for helpful comments, and R. E. Mains for AtT-20/PC2 cells.

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