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Involvement of a Polyproline Helix-like Structure in the Interaction of 7B2 with Prohormone Convertase 2*

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The neuroendocrine protein 7B2 is known to be involved in the biosynthesis and activity of prohormone convertase 2 (PC2). Previous studies have demonstrated that while the carboxyl-terminal portion of 7B2 (residues 155–186) regulates the enzymatic activity of PC2, the amino terminus of the molecule (residues 1–151) is required for maturation of proPC2. In this study we employed four different experimental approaches (co-immunoprecipitation with proPC2, facilitation of proPC2 maturation, acquisition of enzymatic activity, and thermal protection assays) to identify structural elements of 7B2 important for bioactivity. Inspection of the sequence of 7B2 indicated potential involvement of a polyproline helix-like (PPII) structure, with similarities to those present within SH3 domain ligands, in the interaction of 7B2 with proPC2. Site-directed point mutagenesis of this proline-rich region confirmed the involvement of this area. Replacement of prolines in positions critical to helix formation (Pro^{90}, Pro^{91}, Pro^{93}, and Pro^{95}) either severely impaired or totally abolished 7B2 bioactivity, as gauged by the four assays described. In addition, constructs longer than residues 1–121 were still functional, whereas those shorter than residues 1–109 were not. Computer-assisted analysis predicts the presence of an α-helix structure between residues 107 and 123. We conclude that both the proline-rich region and the α-helix contribute to 7B2 activity. Polyproline-containing peptides have been shown to be involved in cytoplasmic protein-protein interactions; our results suggest that the polyproline helix motif may also be used to mediate protein-protein interactions within the secretory pathway.

Prohormone convertase 2 (PC2),[1] a member of the subtilisin family of serine proteases, is believed to mediate the proteolytic cleavage of prohormones, such as proinsulin, proopiomelanocortin, and proenkephalin, into bioactive peptide hormones (for review, see Seidah and Chretien, 1992; Rouille *et al.*, 1995). The maturation of PC2 and the regulation of its activity, however, require the neuroendocrine protein 7B2. 7B2 (Hsi *et al.*, 1982) is a molecule with dual functions: its amino-terminal domain (residues 1–151) is capable of facilitating the maturation of proPC2, and its carboxyl-terminal peptide (CT-peptide, residues 156–186) is a potent inhibitor of PC2 (Braks and Martens, 1994; Martens *et al.*, 1994; Zhu and Lindberg, 1995; Lindberg *et al.*, 1995; Van Horssen *et al.*, 1995). The CT-peptide is thought to inhibit PC2 activity during intracellular transport or within secretory granules (Zhu *et al.*, 1996), and the probable mechanism for the termination of this inhibition has been recently described (Zhu *et al.*, 1996). However, structure-function analysis of the 21-kDa amino terminus of 7B2 has been lacking. Since all known 7B2s are extremely well conserved (Waldbieser *et al.*, 1991), amino acid sequence comparison of 7B2s from various vertebrate species yields no information as to which residues in the amino-terminal region are important for the facilitation of maturation of proPC2. It has been proposed that the first 90 amino acid residues of 7B2, which share weak homology with human, wheat, and *Escherichia coli* chaperonin-60, could function as a chaperone in the folding of proPC2 (Braks and Martens, 1994). However, this region alone cannot bind to proPC2 (Zhu and Lindberg, 1995); therefore, amino acid residues downstream of this region must be important. To define the extent of this region, we have constructed serial deletions of this protein to further narrow down the amino acid sequence essential for its binding to proPC2 and for its facilitating function.

An interesting feature of 7B2 is that immediately downstream of the proposed chaperone region there lies a proline-rich stretch of amino acids (Pro^{88}-Asp^{89}-Pro^{90}-Pro^{91}-Asn^{92}-Pro^{93}-Cys^{94}-Pro^{95}). The distribution of these prolines is very similar to that observed in the ligand for SH3 (Src homology 3) domains, although crucial arginine and leucine/valine residues are not present in this region in 7B2. SH3 domains are globular protein modules of about 60 amino acids, present in a large variety of proteins (Koch *et al.*, 1991; Mayer and Baltimore, 1994). The protein ligands for SH3 domains, here referred to as SH3 domain ligands, contain short proline-rich peptides (approximately 10 amino acid residues; Ren *et al.*, 1993) that represent key components in modulating the function of SH3 domain-containing proteins (Feng *et al.*, 1994; Lim *et al.*, 1994). The sequences of both SH3 domains and their ligands are well conserved throughout the evolutionary spectrum, from lower eukaryotic organisms, such as yeast, to mammals. Recent reports indicate that in addition to the regulation of enzymatic activities (Gout *et al.*, 1993; Mayer and Baltimore, 1994; Pleiman *et al.*, 1994), binding of SH3 domain ligands can also mediate the substrate recognition of enzymes (Feller *et al.*, 1994; Ren *et al.*, 1993) and the targeting of proteins to specific subcellular locations (Bar-Sagi *et al.*, 1993). It has been suggested that the proline-rich SH3 domain ligand forms a polyproline II (PPII) helix-like structure (Musacchio *et al.*,

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[1] The abbreviations used are: PC1 and PC2, prohormone convertase 1 and 2; CHO, Chinese hamster ovary; CT-peptide, carboxyl-terminal peptide of 7B2 ($7B2_{155–185}$); PPII, left-handed poly-L-proline II; RIA, radioimmunoassay; SH3, Src homology 3; PCR, polymerase chain reaction.

1994; Yu *et al.*, 1994). In such a PPII helix-like structure three amino acids form a turn, and amino acids that are three positions apart lie in the same plane (Adzhubei and Sternberg, 1993; Lim *et al.*, 1994; Feng *et al.*, 1994). This PPII helix-like scaffold, usually located on the surface of the protein (Adzhubei and Sternberg, 1993), is crucial for the assumption of the correct steric and hydrogen bonding conformation of the ligand (Feng *et al.*, 1994; Lim *et al.*, 1994). To test if the proline-rich region of 7B2 might be involved in its binding to PC2, we mutated the five prolines in this area individually. To further determine whether the PPII-like structure is required for 7B2-PC2 interactions, we disrupted the putative PPII helix-like structure by switching the positions of critical prolines. Our results provide support for the idea that a polyproline helix-like motif exists in 7B2 and that it is vital to the function of 7B2.

MATERIALS AND METHODS

Construction of 7B2 Mutants—The serial deletions and point-directed mutation constructs were created by PCR-mediated procedures, as described earlier (Zhu and Lindberg, 1995). Three deletions were made using the common amino-terminal primer, 5′-GGCGCAAGCTTCACCATGACCTCAAGGATGG-3′; the carboxyl-terminal primers used in each construct are as follows: for 7B2-109, which is truncated at residue 109, 5′-CGGCGGGATCCTTAAGGGGCGTTTTCTAGACA-3′; 7B2-121, truncated at residue 121, 5′-CGGCCGGATCCTTAGTCTAACTGGAATTCTCG-3′; and 7B2-131, the 1–131 truncation, 5′-CGGCCGGATCCTTAGTAGTCATGTTCTGGATC-3′. Seven site-directed mutants were constructed in this research using the two-step PCR method, as described previously (Zhu and Lindberg, 1995). All the mutants employed the same first and fourth primers, specifically, as follows: 5′-GGCGCAAGCTTCACCATGACCTCAAGGATGG-3′, and 5′-CGGCCGGATCCTTATTCTGGCTCCTTCTC-3′. Each desired mutation was incorporated into both the second and third primers, collectively, for 7B2-PR1, 5′-AGGGTCTGCGTAGCCTTGGTCCTCACT-3′, 5′-CAAGGCTACGCAGACCCTCCAAATCCC-3′; for 7B2-PR2, 5′-ATTTGGAGCGTCTGGGTAGCCTTGGTC-3′, 5′-TACCCAGACGCTCCAAATCCCTGTCCT-3′; for 7B2-PR3, 5′-GGGATTTGCAGGGTCTGGGTAGCCTTG-3′, 5′-CCAGACCCTGCAAATCCCTGTCCTCTT-3′; for 7B2-PR4, 5′-AGGACAGGCATTTGGAGGGTCTGGGTA-3′, 5′-CCTCCAAATGCCTGTCCTCTTGGGAAA-3′; for 7B2-PR5, 5′-CCCAAGAGCACAGGGATTTGGAGGGTC-3′, 5′-AATCCCTGTGCTCTTGGGAAAACTGCA-3′; for 7B2-PRS, 5′-AAGAGGGGGACATTTGGAGGGTCTGGGTA-3′, 5′-CCAAATTGTCCCCCTCTTGGGAAAACTGCA-3′; and for 7B2-P23, 5′-GGGATTGACGACGTCTGGGTAGCCTTGGTC-3′, 5′-CCAGACGTCGTCAATCCCTGTCCTCTTGGG-3′.

All of the mutated fragments were cloned into pCEP4 (Invitrogen), and the authenticity of each of the fragments was verified by DNA sequencing.

Cell Culture, Transfection, and Selection—An AtT-20/PC2 cell line stably expressing PC2 (Zhou and Mains, 1994) and CHO/PC2 cells (Shen *et al.*, 1993) were used in this study. Transfection and isolation of 7B2-expressing clones was performed following the procedures described previously (Zhu and Lindberg, 1995). To avoid possible complications caused by clonal variation, at least two and often three clones were analyzed for each transfection.

Metabolic Labeling and Immunoprecipitation—Co-immunoprecipitation experiments were performed with AtT-20/PC2 cells stably transfected with 7B2 and 7B2 mutants following the methods described earlier (Zhu and Lindberg, 1995). Briefly, 5×10^5 cells were labeled with 0.5 mCi of [^{35}S]Pro-mix (Amersham Corp.) for 20 min in all instances. The cells were transfected with the 7B2-109, 7B2-121, and 7B2-131 constructs were treated with iodoacetamide solution (0.25 M iodoacetamide in 200 mM NaCl, 50 mM HEPES, pH 7.5) on ice (all procedures concerning immunoprecipitation were performed at 4 °C) and extracted with 2% cholate buffer (2% cholate, 50 mM HEPES, 200 mM NaCl, pH 7.5; Ou *et al.*, 1993) immediately after labeling. Cell lines that contained site-directed, mutated 7B2s were chased with methionine-containing Dulbecco's modified Eagle's medium (with 2% fetal bovine serum) for 20 min after labeling. They were then treated with iodoacetamide solution and extracted with 2% cholate. These samples were clarified by centrifugation and then subjected to co-immunoprecipitation using PC2 antiserum 18B10 or 7B2 antiserum 13B6, as described previously, except that 0.5% cholate buffer (0.5% cholate, 50 mM HEPES, 200 mM NaCl, pH 7.5) was used in place of AG buffer. Pulse-chase experiments were carried out as described previously (Zhu and Lindberg, 1995). SDS-polyacrylamide gel electrophoresis (8.8% for pulse-chase samples, 15% for co-immunoprecipitated samples) was also performed as described previously (Shen *et al.*, 1993). The gels were treated with Amplify (Amersham Corp.) following the manufacturer's recommendation before fluorography. Quantitation of radioactivity within each band was performed using a PhosphoImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Collection of Conditioned Medium from CHO Cells and Enzyme Assay—CHO/PC2 cells (Shen *et al.*, 1993) were stably transfected with the same mutant 7B2 constructs used for AtT-20/PC2 cell transfection, and three 7B2-expressing clones of each mutant 7B2 were selected; for each cell line, the two highest 7B2 expressors and a low expressor were saved. For examination of the ability of 7B2 molecules to facilitate the acquisition of PC2 activity, 400,000 cells of each of two clones were plated per 35-mm dish. One day after plating, the wells were rinsed with 5 ml of Optimem (Life Technologies, Inc.), and 1 ml of Optimem containing 100 μg/ml aprotinin was placed on the cells for 15 h. The medium was removed, centrifuged briefly to remove floating cells, and stored frozen prior to analysis for PC2 activity, Western blotting, and 7B2 radioimmunoassay (RIA). The PC2 assay was accomplished as described previously (Zhu and Lindberg, 1995), using 25 μl of conditioned medium assayed in triplicate for a 6-h incubation period; 200 μM Pyr-Arg-Thr-Lys-Arg-aminomethylcoumarin (Peptides International, Lexington, KY) was used as a substrate, and an enzyme inhibitor mixture was included to block nonspecific proteases. In order to verify equal expression of proPC2 in all cell lines, Western blotting for proPC2/PC2 was performed using 25 μl of conditioned medium. Expression of 7B2 in each sample of conditioned medium was estimated by RIA (Zhu and Lindberg, 1995).

Preparation of Recombinant 21-kDa Forms of the 7B2 Mutants—The QIAexpress system (QIAGEN Inc., Chatsworth, CA) was used to express 21-kDa 7B2 mutants from the above-mentioned single proline-replaced pCEP4 constructs in *E. coli* following the procedure used to obtain purified recombinant 21-kDa 7B2 (Martens *et al.*, 1994; Lamango *et al*. 1996). In order to clone the fragments from eukaryotic vectors into the pQE30 vector, two primers, 5′-CGGCCGGATCCTATAGTCCACGGACTCCT-3′ and 5′-CCGGCAAGCTTTTACTGTCCTCCCTTCATC-3′, were used in the PCR reaction. The PCR products were digested with *Bam*HI and *Hin*dIII and ligated into the *Bam*HI- and *Hin*dIII-linearized QE30 plasmid. The ligation mixture was then used to transform *E. coli* XL1-Blue (Promega, Madison, WI). The 21-kDa 7B2 mutant-expressing clones were selected following the manufacturer's instructions, and the cloned fragments were verified by DNA sequencing. Large scale production of these proteins was performed using the guanidine-HCl denaturing method essentially according to the manufacturer's instructions for denatured proteins. Single bands of purified mutant proteins were observed on a 15% SDS-polyacrylamide gel.

Effect of 21-kDa 7B2 Mutants on the Thermal Stability of PC2—Purified recombinant PC2 (20 ng, ~8 nM) obtained from size-exclusion chromatography (Lamango *et al.*, 1996) was incubated at 52.5 °C for 1 h in 100 mM sodium acetate buffer containing 6.25 mM $CaCl_2$ and 0.2% Brij in the presence of each of the 21-kDa 7B2 mutants or of wild-type 21-kDa 7B2 (100 nM). The residual enzyme activity after heat denaturation was then assayed in the presence of 200 μM carbobenzoxy-Arg-Ser-Lys-Arg-aminomethylcoumarin for 16 h. Since 7B2 can act to stabilize enzyme activity during the enzyme assay (Lamango *et al.*, 1996), 100 nM wild-type 21-kDa 7B2 was added to all samples during the enzyme assay period.

RESULTS

A 7B2 Mutant Truncated at 1–121 Can Facilitate the Maturation of ProPC2—Our previous study demonstrated that the amino-terminal 151 amino acid residues of 7B2 can confer full facilitation of proPC2 maturation and that the 7B2 1–90 truncation construct does not carry this potential (Zhu and Lindberg, 1995). Having determined that a construct corresponding to 7B2 1–138 still functioned properly,[2] we constructed serial deletions roughly 10 amino acids apart, as illustrated in Fig. 1, to determine the boundary of the region responsible for conferring bioactivity. Three constructs, namely 7B2-109, 7B2-121, and 7B2-131, were made and were stably transfected into AtT-20/PC2 cells. Two clones from each transfection were analyzed on two separate occasions. Cells were pulse-labeled with

[2] X. Zhu, N. S. Lamango, and I. Lindberg, unpublished results.

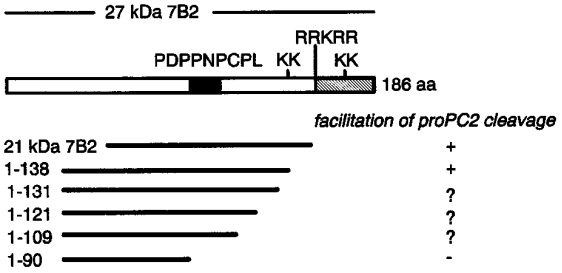

FIG. 1. **Diagram of 7B2 deletion constructs.** The *filled area* depicts the proline-rich region of 7B2; the *hatched area* depicts the CT-peptide. 7B2 deletion constructs used in this study are depicted by *question marks*.

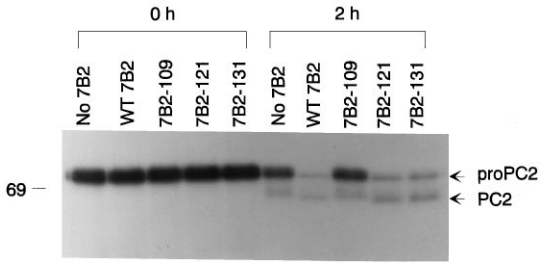

FIG. 3. **7B2-121 and 7B2-131 facilitate the maturation of pro-PC2.** AtT-20/PC2, AtT-20 PC2-7B2, AtT-20/PC2-7B2-109, AtT-20/PC2-7B2-121, and AtT-20/PC2-7B2-131 cells were pulsed for 20 min and then either lysed or chased for 2 h in methionine-containing medium.

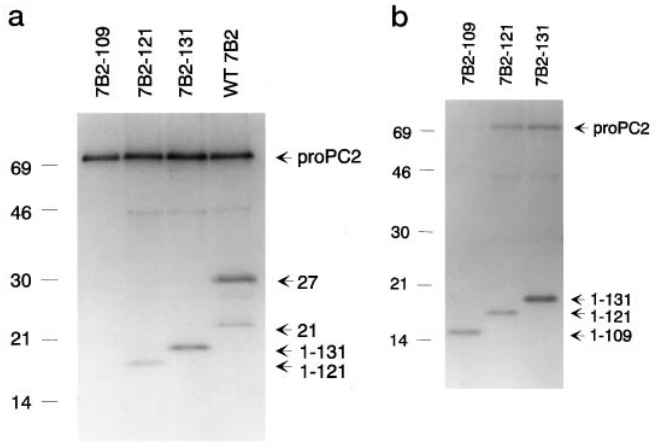

FIG. 2. **A truncated form of 7B2, as short as residues 1–121, is still capable of co-immunoprecipitation with proPC2.** *Panel a*, 7B2-121 and 7B2-131 (residues 1–131 and 1–121) co-immunoprecipitate proPC2. AtT-20/PC2-7B2, AtT-20/PC2-7B2-109, AtT-20/PC2-7B2-121, and AtT-20/PC2-7B2-131 cells were pulsed for 20 min. Cells were extracted by freezing and thawing in 2% cholate buffer. The samples were diluted to 0.5% cholate with HEPES buffer, immunoprecipitated with PC2 antiserum 18B10, and subjected to electrophoresis. *Panel b,* truncated forms of 7B2 are expressed normally. AtT-20/PC2-7B2-109, AtT-20/PC2-7B2-121, and AtT-20/PC2-7B2-131 cells were pulsed for 20 min; samples were prepared as in *A* except that 7B2 antiserum 13B6 was used in place of 18B10.

[^{35}S]methionine and then analyzed for potential co-immunoprecipitation with proPC2 using PC2 antiserum 18B10. The proPC2-7B2 co-immunoprecipitation results indicated that 7B2-131 and 7B2-121 still bound to proPC2; however, 7B2-109 completely lost the ability to bind to proPC2 (Fig. 2*a*). All truncated 7B2s were expressed normally (Fig. 2*b*). Pulse-chase experiments further indicated that proPC2 maturation was hindered in cells containing the 7B2-109 mutant as compared with wild-type 7B2, whereas proPC2 processing of the 7B2-121 and 7B2-131 mutants was comparable with that of wild-type 7B2 (Fig. 3). We conclude that a region between 109 and 131 is critical to the facilitatory function of 7B2.

The Proline-rich Region in 7B2 Is Crucial for the ProPC2-7B2 Interaction—Polyproline helix motifs are known to mediate various types of protein-protein interactions. In SH3 domain ligands, short proline-rich peptides appear to be vital to these interactions (Ren *et al.*, 1993; Feng *et al.*, 1994; Lim *et al.*, 1994). A proline-rich peptide, PDPPNPCP (residues 88–95 in rat 7B2) is present within 7B2. To test whether this region is important to the PC2-7B2 interaction, we used site-directed mutagenesis to replace each individual proline with alanine. These mutated 7B2s were then independently transfected into AtT-20/PC2 cells. Two clones that expressed 7B2, as determined by RIA, were isolated from each transfection. The PC2-binding ability of the various 7B2 mutants was then analyzed by co-immunoprecipitation using PC2 antiserum 18B10 (Fig. 4*a*; the faintness of co-immunoprecipitated 7B2 in this figure is the result of decreased affinity between proPC2 and 21-kDa 7B2, which occurs as a gradual process immediately following protein synthesis and appears in chased samples only. This phenomenon has been previously observed in other experiments.). The results show that substitution of the fourth proline with alanine (7B2-PR4, $Pro^{93} \rightarrow Ala^{93}$) totally abolishes the ability of 7B2 to co-immunoprecipitate with proPC2. In addition, as indicated by phosphoimage analysis (Fig. 4*b*), the apparent binding affinity of proPC2 was decreased in mutants containing alanine at either the second proline (7B2-PR2, $Pro^{90} \rightarrow Ala^{90}$) or the fifth proline (7B2-PR5, $Pro^{95} \rightarrow Ala^{95}$). Similar results were obtained both in a repetition of this experiment with a different cell preparation, as well as with different clones (not shown). We conclude that the fourth proline is crucial to the binding of 7B2 to proPC2 and that the second and fifth prolines are also important for this interaction. In line with the idea that co-immunoprecipitation of 7B2 with proPC2 is correlated with the facilitation of maturation of proPC2, pulse-chase analysis of the 7B2-PR4 cells confirmed that the fourth proline is also vital to proPC2 maturation, *i.e.* a single $Pro^{93} \rightarrow Ala^{93}$ mutation at this position incapacitated 7B2 in terms of its facilitation of the maturation of proPC2 (Fig. 5).

The Arrangement of the Prolines Is Important to the 7B2-PC2 Interaction—The arrangement of two of the prolines in the 7B2 proline-rich region (P*XX*P) is similar to that present within typical SH3 domain ligands. It has been proposed that this proline-rich motif forms a polyproline helix-like structure (PPII) and that this structure is vital to the stabilization and presentation of the protein (Lim *et al.*, 1994; Feng *et al.*, 1994). To test whether this is the case with the 7B2-PC2 interaction, we switched the fourth proline with its neighboring cysteine (7B2-PRS, $Pro^{93} \rightarrow Cys^{93}$; $Cys^{94} \rightarrow Pro^{94}$), which would be expected to move the proline into the opposite plane of the helix (Fig. 6). In addition, we constructed a mutant 7B2 in which both the second and third prolines were replaced with alanines (7B2-PR23, Pro^{90}-$Pro^{91} \rightarrow Ala^{90}$-$Ala^{91}$); this would be predicted to make the PPII structure less favorable (Adzhubei and Sternberg, 1993; Lim *et al.*, 1994; Feng *et al.*, 1994). Both constructs were stably transfected into AtT-20/PC2 cells. Analysis of the transfected cell lines showed that both 7B2-PRS and 7B2-PR23 failed to co-immunoprecipitate with proPC2 (Fig. 7). Pulse-chase results indicated that the facilitation of proPC2 maturation is also diminished in these two mutants (Fig. 8). These results demonstrate that the position of the fourth proline is crucial for its function and that for efficient 7B2-PC2 interaction there must be a proline in either the second or third positions, with proline in the second position being more favorable.

Mutation at Specific Prolines Abolishes the Ability of 7B2 to

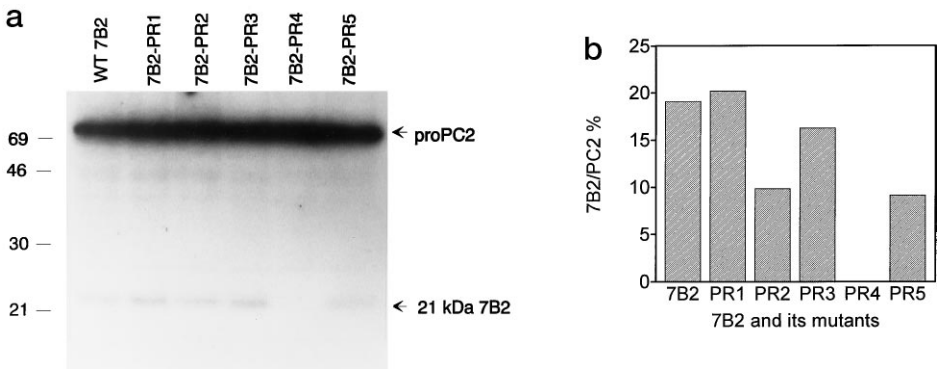

FIG. 4. **Replacement of Pro^{93} with Ala^{93} totally abolishes binding of 7B2 to proPC2.** *Panel a,* co-immunoprecipitation of the individual proline-substituted mutations. AtT-20/PC2-7B2, AtT-20/PC2-7B2-PR1, AtT-20/PC2-7B2-PR2, AtT-20/PC2-7B2-PR3, and AtT-20/PC2-7B2-PR4, and AtT-20/PC2-7B2-PR5 cells were pulsed for 20 min and chased for 30 min (to enable cleavage of the CT-peptide from 27-kDa 7B2). *Panel b,* Pro^{90}, Pro^{91}, and Pro^{95} are also important for 7B2 activity. PhosphoImage analysis of *panel a*. The ratio of the density of the 7B2 band to that of the proPC2 band was multiplied by 100.

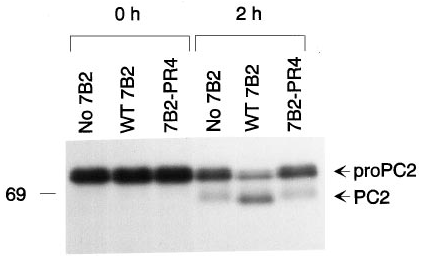

FIG. 5. **7B2-PR4 cannot facilitate the maturation of proPC2.** AtT-20/PC2, AtT-20 PC2-7B2, and AtT-20/PC2-7B2-131 cells were pulsed for 20 min and then either lysed or chased for 2 h in methionine-containing medium.

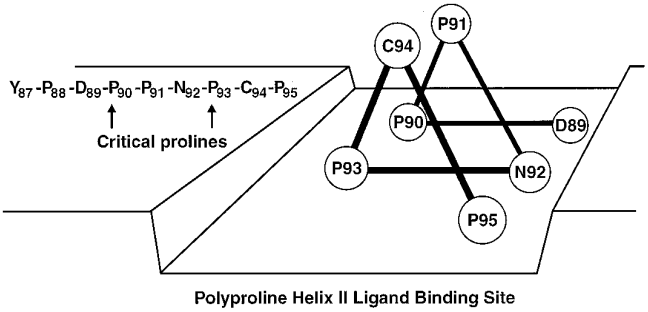

FIG. 6. **Diagram of the proline-rich region in 7B2 (adapted from Feng *et al.*, 1994)**

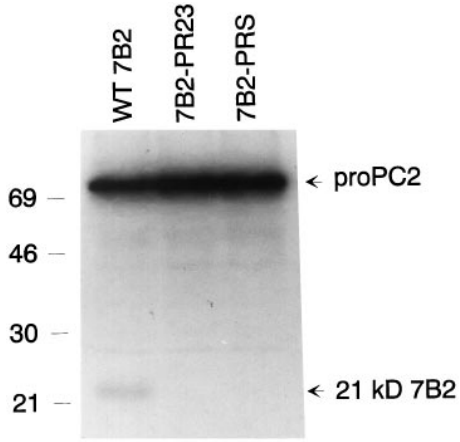

FIG. 7. **A positional switch at Pro^{93} (7B2-PRS) as well as double mutation at Pro^{90}, Pro^{91} (7B2-PR23) abolishes binding of 7B2 to proPC2.** Co-immunoprecipitation of the PRS and PR23 mutants with proPC2 was performed as described under "Materials and Methods." AtT-20/PC2-7B2, AtT-20/PC2-7B2-PR23, and AtT-20/PC2-7B2-PRS cells were pulsed for 20 min, followed by 20 min of chase.

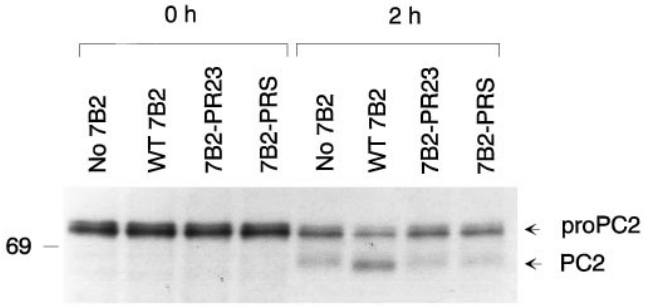

FIG. 8. **7B2-PRS and 7B2-PR23 cannot facilitate the maturation of proPC2.** AtT-20/PC2, AtT-20/PC2-7B2, AtT-20/PC2-7B2-PR23, and AtT-20/PC2-7B2-PRS cells were pulsed for 20 min or pulsed and then chased for 2 h.

Generate Enzymatically Active PC2 in CHO/PC2 Cells—We have previously demonstrated that medium obtained from CHO/proPC2 cells expressing high levels of proPC2 (by virtue of DHFR-mediated amplification of PC2 expression) can be rendered enzymatically active upon supertransfection with 7B2 cDNA (Zhu and Lindberg, 1995). In order to provide a functional assay for the ability of mutated 7B2s to bind to proPC2, we constructed a series of stable CHO/PC2 cell lines containing proline-mutated 7B2s. Medium obtained from two to three independent clones was examined for each mutant cell line. ProPC2/PC2 expression in each was evaluated using Western blotting and shown to be approximately equivalent (not shown). As previously observed, conditioned medium obtained from cells lacking 7B2 did not exhibit any PC2 activity, whereas cells containing wild-type 27-kDa 7B2 did secrete active enzyme (Fig. 9; it should be noted that the background hydrolysis of substrate was about 100 units (*dotted line*), and, for purposes of clarity, had not been subtracted). Only one proline-containing mutant exhibited enzyme activity, the construct containing alanine at the first proline (PR1). All other mutant 7B2-containing cell lines were unable to generate PC2 activity in conditioned medium, despite fairly efficient expression of immunoreactive 7B2 (shown by *triangles*). These data confirm the functional necessity for proline 4 as well as the necessary positioning of prolines (as observed by the lack of activity of the Pro/Cys switched mutant (PRS)).

In Vitro Analysis of 21-kDa 7B2 Supports the Requirement for Prolines 2–5: Mutation of 7B2 at Certain Prolines Affects Its Ability to Stabilize PC2—The above experiments were carried out with constructs containing full-length rat 7B2s. In order to rule out an effect of the carboxyl-terminal inhibitory peptide and to provide an independent means of confirmation of the proposed role of the proline-rich region, bacterial expression was used to generate mutant 21-kDa 7B2s containing the individual proline to alanine mutations. Previous experiments have shown that 21-kDa 7B2 can protect PC2 from thermal denaturation (Lamango *et al.*, 1996); we therefore assayed the ability of the mutant proteins to protect PC2 activity. 7B2-PR1

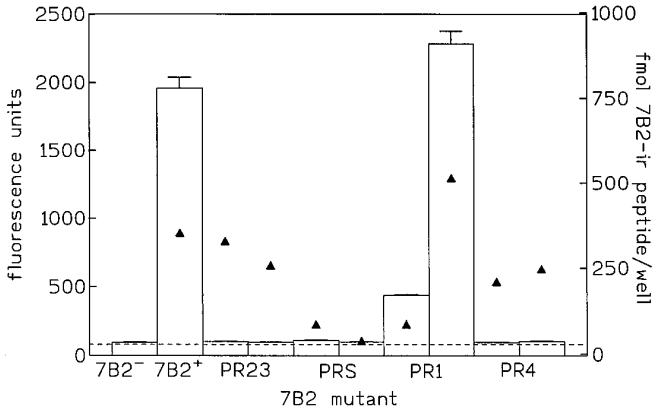

FIG. 9. **Ability of 7B2 constructs bearing proline mutations to generate enzymatically active PC2.** Expression of PC2 enzymatic activity in conditioned medium obtained from two clones from each transfection was assayed; results represent the mean ± S.E. of triplicate determinations. Background hydrolysis of substrate (shown by *dotted line*) was 100 ± 7 units and has not been subtracted. The expression of 7B2 immunoreactivity in this conditioned medium was estimated by radioimmunoassay (*filled triangles*); results represent duplicate determinations that differed by an average of less than 15%.

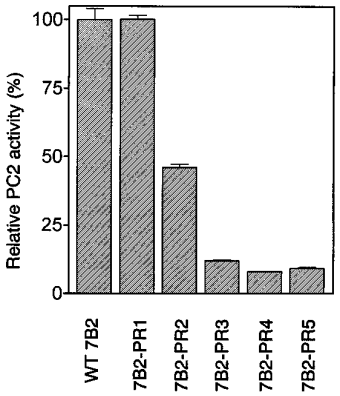

FIG. 10. **Protection from thermal denaturation of PC2 by 21-kDa 7B2s bearing proline to alanine mutations.** Purified recombinant mPC2 (20 ng) was incubated with each type of 7B2 for 1 h at 52.5 °C. The residual PC2 activity was determined as described under "Materials and Methods."

was as effective as wild-type 21-kDa 7B2 in stabilizing PC2 against thermal denaturation. 7B2-PR2 conferred only about 50% stabilization under these conditions, whereas little or no stabilization was observed when 7B2-PR3, 7B2-PR4, and 7B2-PR5 were used (Fig. 10). We conclude that all of the prolines except the first are important for the binding of the amino-terminal portion of 7B2 to PC2.

DISCUSSION

At least seven subtilisin-like proteases are known to be present within eukaryotic organisms (for review, see Rouille *et al.*, 1995). Thus far, PC2 is the only enzyme in the family known to require another specific binding protein, 7B2, for its maturation. It has been previously determined that while the enzyme inhibitory activity of 7B2 lies within the CT-peptide (residues 156–186 in rat, Lindberg *et al.*, 1995; van Horssen *et al.*, 1995), it is residues 1–151 (a natural product) that are involved in PC2 maturation (Zhu and Lindberg, 1995).

Recent results obtained by Braks and Martens (1995) indicate that a 7B2 construct corresponding to residues 1–131 is capable of enhancing proopiomelanocortin conversion by PC2 in *Xenopus* intermediate pituitary lysate, whereas a truncated construct corresponding to residues 1–86 did not show any effect (Braks and Martens, 1995). Our data confirm the observation of successful interaction of 7B2 1–131 and PC2; in our hands, 7B2-131 was found to co-immunoprecipitate with pro-PC2 and was able to facilitate the maturation of proPC2. In addition, our data further show that of the truncated forms of 7B2, a construct corresponding to residues 1–121 is still functional, whereas one corresponding to 1–109 is not. This places the boundary of the sequence required for 7B2 function between residues 109 and 121. Interestingly, computer-assisted secondary structure analysis of 7B2 revealed that this region is likely to form an α-helix. The Gascuel and Golmard basic statistical method predicted the presence of an α-helix for the sequence 113–123, and the method of Garnier predicted an α-helix for the sequence 107–122. We speculate that this putative α-helix is essential for the binding of 7B2 to proPC2.

Our results further indicate that the proline-rich region of 7B2 is vitally important to its function. Four lines of evidence (co-immunoprecipitation with proPC2, facilitation of proPC2 maturation, enzymatic activity assay, and an *in vitro* enzymatic activity thermal protection assay) indicate that the fourth proline (Pro^{93}) is critical and that the second and fifth prolines (Pro^{90} and Pro^{95}, respectively) are also important. A proline to alanine switch in position 93 totally abolished 7B2 activity, and replacement of Pro^{90} or Pro^{95} with alanine decreased 7B2-proPC2 binding by about 50%, as judged from phosphoimage quantitation. Our data indirectly demonstrate that the third proline is also important for 7B2 function, since the double mutation of Pro^{90}-Pro^{91} totally abolished 7B2 activity, although there was no detectable effect when Pro^{91} alone was mutated. The actual position of the fourth proline within the proline-rich region is also crucial. A positional change of this proline from 93 to 94 (Pro^{93}-Cys^{94} → Cys^{93}-Pro^{94}), which moves this proline to the other side of the helix (Fig. 6), totally disabled 7B2 from interacting with proPC2. Based upon these findings, we conclude that Pro^{90} and Pro^{93} and, moreover, their relative positions (three amino acid residues apart) are critical for the proper function of 7B2. These results were confirmed by enzymatic activity assays in 7B2-transfected CHO/PC2 cells. In this system, 7B2 bearing a single Pro^{93}-Ala^{93} mutation was unable to facilitate the generation of enzymatically active PC2 in conditioned medium. It is striking that absolutely no PC2 activity was observed in CHO/PC2 cells expressing the proline-containing mutants (other than 7B2-PR1). These data suggest that this functional assay may represent a more sensitive indicator of intracellular interactions than the co-immunoprecipitation assay, possibly because the latter is performed under noncellular conditions.

PPII helix-like structures such as those present within SH3 domain ligands have been shown to mediate protein-protein interactions in intracellular signaling (reviewed in Mayer and Eck, 1995). These structures are also involved in substrate recognition of enzymes (Feller *et al.*, 1994; Ren *et al.*, 1993) and in cytoskeletal architecture (Cedergren-Zeppezauer *et al.*, 1994). These studies have amply demonstrated that the prolines in these structures are vital to successful interaction of the protein with SH3 domains. The P*XX*P sequence in these proteins has been proposed to adopt a left-handed PPII helix-like structure, and such a structure appears in itself to constitute a stable SH3 domain ligand (Adzhubei and Sternberg, 1993; Feng *et al.*, 1994, Lim *et al.*, 1994). We speculate that a similar helical structure also exists in the proline-rich region of 7B2. In such a helix, Pro^{90} and Pro^{93} lie on the same face and may be critical to the presentation and/or stabilization of the 7B2 binding domain. However, the proline-rich region alone is not sufficient for binding of 7B2 to proPC2, since as mentioned above, a mutant truncated at residues 1–109 is inactive. Fur-

thermore, a synthetic peptide corresponding to this region (GYPDPPNPCPLGKT) failed to block co-immunoprecipitation of 7B2 with proPC2 (data not shown). This may be due either to the fact that the affinity of the peptide is low, *i.e.* the peptide cannot successfully compete with intact 7B2 molecules, or more likely, that this proline-rich peptide alone does not carry sufficient information for binding to proPC2. Therefore, it appears that in addition to the proline-rich domain, the region immediately downstream (residues 95–121), *i.e.* the probable α-helix formed between residues 107–123, is also required for successful binding of 7B2 to PC2. (It should be pointed out that there is no evidence for the involvement of such an α-helix in the interaction of SH3 domain ligands and SH3 domains.)

Since 7B2 contains a putative PPII helix-forming sequence that shares certain similarities with SH3 domain ligands, it might have been predicted that proPC2 would contain an SH3 domain-like structure. However, computer-assisted comparisons of proPC2 with all SH3 domains identified thus far reveal no significant sequence similarity. In view of the fact that the arrangement of prolines is similar between the 7B2 proline-rich region and SH3 domain ligands, while the interlacing nonproline residues (such as the arginine commonly found within this motif) are not conserved between 7B2 and either class of SH3 domain ligands, we speculate that the proPC2-7B2 interaction belongs to a new family of polyproline helix-like structures that mediate protein-protein recognition and potentially provides the first example of proline-mediated protein interactions within the secretory pathway.

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