7B2 Facilitates the Maturation of proPC2 in Neuroendocrine Cells and Is Required for the Expression of Enzymatic Activity

Xiaorong Zhu and Iris Lindberg

Department of Biochemistry and Molecular Biology, Louisiana State University Medical Center, New Orleans, Louisiana 70112

Abstract. The prohormone convertase PC2, which is thought to mediate the proteolytic conversion of many peptide hormones, has recently been shown to interact with the neuroendocrine-specific polypeptide 7B2 in *Xenopus* intermediate lobe (Braks, J. A. M., and G. J. M. Martens. *Cell.* 78:263. 1994). In the present work we have stably transfected neuroendocrine cell lines with rat 7B2 constructs and found that overexpression of 27 kD 7B2 greatly facilitates the kinetics of maturation of proPC2, both in AtT-20/PC2 cells and in Rin5f cells. The half-life of conversion of proPC2 was reduced from 2.7 to 1.7 h in AtT-20/PC2 cells stably transfected with 27 kD 7B2 cDNA. The previously proposed "chaperone" domain was not sufficient for this facilitation event; however, a construct corresponding to the 21-kD 7B2 protein (which represents the naturally occurring maturation product) functioned well. A 7B2 construct in which maturation of 27 kD 7B2 to its 21-kD form was blocked was unable to facilitate maturation of proPC2.

To correlate effects on PC2 maturation with the actual generation of PC2 enzymatic activity, a similar transfection of 21 kD 7B2 was performed using CHO cells previously amplified for the expression of proPC2. Enzymatic activity cleaving the fluorogenic substrate Cbz-Arg-Ser-Lys-Arg-AMC was highly correlated with the expression of immunoreactive 21 kD 7B2 in the conditioned medium; medium obtained from the parent cell line was completely inactive. Enzymatic activity was identified as PC2 on the basis of inhibition by the carboxy-terminal peptide of 7B2, which has previously been shown to represent a potent and specific PC2 inhibitor. Taken together, our in vivo results indicate that the interesting secretory protein 7B2 is a bifunctional molecule with an amino-terminal domain involved in proPC2 transport as well as activation.

BIOLOGICALLY active peptide hormones secreted from neuroendocrine cells are derived through the processing of prohormones through a series of post-translational modifications. This process begins with co-translational secretion of precursor molecules into the lumen of the endoplasmic reticulum. The precursor undergoes proteolytic cleavage, oligosaccharide addition and other required refinements during transport through the secretory pathway before release as bioactive peptide. For most polypeptide hormones, proteolytic cleavage occurs at paired basic residues; this cleavage is mediated by a subset of enzymes in the subtilisin-like enzyme family, known as the prohormone convertases (PCs[1]; for review see Hutton, 1992; Seidah and Chretien, 1992; Steiner et al., 1992). Although much information is available regarding the biochemistry and distribution of the PCs, the biosynthesis and regulatory aspects of these enzymes are not fully understood. The removal of the pro sequence in PC1 appears to occur rapidly and autocatalytically (Benjannet et al., 1993; Lindberg, 1994; Milgram and Mains, 1994; Zhou and Lindberg, 1993; Goodman and Gorman, 1994). The processing of proPC2, on the other hand, is considerably slower than that of proPC1 (Benjannet et al., 1993; Guest et al., 1992; Shen et al., 1993; Zhou and Mains, 1994), and a substantial amount of proPC2 is stored as such in many cell lines (Lindberg et al., 1994; Zhou and Mains, 1994). Since PC2 is thought to cleave intermediates produced by PC1 during peptide processing (Benjannet et al., 1991; Thomas et al., 1991; Breslin et al., 1993; Zhou and Mains, 1994), the regulation of availability of active PC2 could represent an important controlling step in peptide hormone production.

The 7B2 protein, which was first isolated from porcine and human pituitary glands over a decade ago, is selectively distributed in the central nervous system and in endocrine tissues (Hsi et al., 1982; Seidah et al., 1983; Iguchi et al., 1984). The predominant form of this protein stored

Please address all correspondence to I. Lindberg, Department of Biochemistry and Molecular Biology, Louisiana State University Medical Center, 1901 Perdido Street, New Orleans, LA 70112. Tel.: (504) 568-4799. Fax: (504) 568-3370. email: ilindb6lsumc.edu.

1. *Abbreviations used in this paper*: AMC, aminomethylcoumarin; PC, prohormone convertases; POMC, proopiomelanocortin; 7B2 CT peptide, human $7B2_{155\text{-}185}$.

in neuroendocrine tissues is a 21-kD species. Recent research has shown that in *Xenopus* intermediate lobe, newly synthesized 27-kD 7B2 can be coimmunoprecipitated with proPC2 using PC2 antiserum (Braks and Martens, 1994). In line with the idea that 7B2 represents a PC2-binding protein, the amino-terminal region of 7B2 (residues 1-90 of mature 7B2) shares weak amino acid sequence similarities with members of the 60-kD subclass of molecular chaperones, such as human, wheat, and *E. coli* chaperonin-60 (Braks and Martens, 1994). Interestingly, the carboxy-terminal portion of 7B2 is distantly related to a family of subtilisin inhibitors known as the potato inhibitor I family, and our in vitro experiments have shown that intact 27 kD 7B2, but not the processed 21-kD product, represents a potent and specific inhibitor of PC2 (Martens et al., 1994). We have recently demonstrated that the inhibitory activity of 27 kD 7B2 resides entirely within the carboxyl-terminal 31 amino acid peptide removed upon maturation of 7B2 to its 21-kD form (Lindberg et al., 1995; Van Horssen et al., 1995). Taken together, these observations suggest an important role for 7B2 in the maturation of proPC2 as well as in the regulation of PC2 activity. To clarify the nature of the interaction of 7B2 and PC2, we have transfected various rat 7B2 constructs into two neuroendocrine cell lines and examined the kinetics of proPC2 maturation. In addition, we have used PC2-expressing AtT-20 and CHO cells to directly demonstrate a role for 7B2 in the generation of enzymatically active PC2.

Materials and Methods

Construction of 7B2 Vectors

Rat 7B2 cDNA (obtained from J. E. Dixon; Waldbieser et al., 1991) was subcloned directly into the BamHI and HindIII sites of pCEP4 (Invitrogen), immediately downstream of the CMV promoter, using standard methods. Other truncations and mutations of 7B2 were generated by PCR-mediated methods using the rat 7B2 cDNA mentioned above and cloned into the BamHI–HindIII sites of pCEP4. The primers used in each construction were for the 1-90 amino acid truncation: 5′-GGCGCAAGCTTCACCATGACCTCAAGGATGG-3′ and 5′-CGGCGGGATCCTTAATTTGGAGGGTCTGGGTA; for the 21-kD construct, 5′-GGCGCAAGCTTCACCATGACCTCAAGGATGG-3′ and 5′-CCGGCGGGCCCGGATCCTTACTGTCCTCCCTTCATC-3′.

The resulting PCR products were digested with BamHI and HindIII and ligated into pCEP4.

The cleavage blockade mutant of 7B2 was created by a two-step PCR method, as described by Vallette et al. (1989). The following primers were used: 5′-GGCGCAAGCTTCACCATGACCTCAAGGATGG-3′, 5′-GTTGCTGTTCTGGCTCTGTCCTCCCTTCAT-3′, 5′-AGCCAGAACAGCAACAGTGTCAATCCCTATC-3′ and 5′-CGGCCGGATCCTTATTCTGGCTCCTTCTC-3′.

The second and third primers contained the desired mutations (elimination of the pentabasic processing site). The first and second primers were used as a pair in one reaction; and the third and the fourth in a separate reaction in the first round of PCR. In the second round of PCR, the resulting two individual fragments were purified and mixed, and then amplified using the first and the fourth primers. This fragment was then digested with BamHI and HindIII, and ligated into pCEP4. All inserts derived from PCR were verified by DNA sequencing.

Cell Culture, Transfection, and Selection

An AtT-20 cell line stably expressing PC2 (Zhou and Mains, 1994) was kindly provided by Dr. R. E. Mains (Baltimore, MD); this cell line served as the host for transfection of 7B2 cDNAs described below. Data were confirmed in Rin/PE cells (a derivative of the rat insulinoma Rin5f which has been stably transfected with rat proenkephalin cDNA; Lindberg, I., unpublished results). Rin5f cells were obtained from Dr. Gary Thomas (Portland, OR).

All cell culture media were obtained from GIBCO-BRL (Gaithersburg, MD); cells were cultured at 37°C in an atmosphere of 5% CO_2. Culture medium used for AtT-20/PC2 cells consisted of DMEM high glucose medium containing 10% Nuserum, 2.5% FBS and 200 μg/ml G418, while Rin/PE cells were cultured in low glucose DMEM containing 10% FBS and 500 μg/ml G418. CHO/PC2 cells, which represent a CHO cell line amplified for the expression of mouse PC2 using the dihydrofolate reductase–coupled method (Shen et al., 1993), were grown as previously described.

Transfection of each cell line was accomplished with Lipofectin (GIBCO/BRL). Briefly, ~1 × 10^6 cells in a 10-cm plate were used for each transfection. The cells were incubated in 3 ml Optimem (GIBCO/BRL) containing 30 μg vector DNA and 30 μg Lipofectin for 5 h at 37°C followed by the addition of 7 ml growth medium containing 100 μg/ml hygromycin (Sigma Chem. Co., St. Louis, MO). Approximately 2 wk later, 10–30 hygromycin-resistant colonies were picked and screened for 7B2 expression either by Western blotting, by radioimmunoassay or by radiolabeling and immunoprecipitation. Generally, the first experiment employed the two highest-expressing clones, while confirmatory experiments employed only the highest-expressing clone. 7B2-expressing cell lines were found to be quite stable and could be passaged for three months without apparent alteration in expression levels.

Metabolic Labeling and Immunoprecipitation

Half a million cells/well in a six-well plate were labeled with [^{35}S]methionine-labeling mix (Amersham Corp., Arlington Heights, IL) in methionine-deficient DMEM, or with Trans-label Mix (ICN Biomedicals, Costa Mesa, CA) in methionine- and cysteine-deficient DMEM. The cells were pulsed for 10 or 20 min and chased for the indicated times before being subjected to immunoprecipitation. Cells were boiled for 5 min in 0.1 ml boiling buffer (50 mM Na-phosphate, pH 7.4, 1% SDS, 50 mM β mercaptoethanol, and 2 mM EDTA). These samples were then diluted with 0.9 ml AG buffer (0.1 M Na-phosphate, pH 7.4, 1 mM EDTA, 0.1% Triton, 0.5% NP-40, and 0.9% NaCl) for immunoprecipitation. The samples (0.5 ml each) were preincubated with 0.1 ml 20% Protein A–Sepharose CL-4B (Pharmacia, Sweden, previously hydrated and washed three times with AG buffer) for 1 h, and then centrifuged. Antiserum (5 μl) was then added to the supernatant, along with 25 μl of 10 mM PCMS (*p*-chloromercuriphenylsulfonic acid) and 25 μl of 100 mM PMSF, and incubated for either 6 h or overnight at 4°C. Protein A–Sepharose (100 μl of 20%, hydrated and washed three times with AG buffer) was added and the samples rocked at 4°C for 1 h. The samples were then washed two times with AG buffer, once with 0.5 M NaCl in PBS, and once with PBS. Immunoprecipitates were resuspended in Laemmli sample buffer and analyzed using either 8.8% (for PC2) or 15% (for 7B2) SDS-PAGE. The quantitative nature of the immunoprecipitation was verified by immunoprecipitating spent extracts; no further 7B2 nor PC2 forms were recoverable after a second round of immunoprecipitation. A similar procedure was used in the coimmunoprecipitation of 7B2 and proPC2, except that cells were lysed by scraping into AG buffer and were frozen and thawed once at −20°C; samples were not boiled before immunoprecipitation. All labeling experiments/immunoprecipitations were repeated at least once, on separate preparations of cells.

The procedure for SDS-PAGE (8.8% gel for analysis of PC2 immunoprecipitations, 15% for 7B2 immunoprecipitations) has been previously described (Shen et al., 1993). After electrophoresis, gels were treated with Entensify (DuPont, New England Nuclear, Wilmington, DE) following the manufacturer's recommendation before fluorography. Quantitation of radioactivity within each band was carried out using a PhosphoImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

7B2 Radioimmunoassay

Polyclonal antiserum to the sequence $7B2_{23\text{-}39}$ (Iguchi et al., 1983) conjugated to keyhole limpet hemocyanin was raised in rabbits (Hazleton JRH, Denver, PA). $7B2_{23\text{-}39}$ was purchased from Peninsula (Belmont, CA) for use as standard and as radiolabel. The peptide was iodinated using chloramine T and purified on a C-18 Sep-Pak cartridge. The radioimmunoassay was carried out overnight in duplicate using antiserum diluted 1:30,000, 10,000 cpm of iodinated peptide, and samples in a total volume of 300 μl RIA buffer (0.1 M sodium phosphate, pH 7.4, 50 mM NaCl, containing 0.1% BSA and 0.1% sodium azide. 50-μl duplicate samples of conditioned medium were diluted 1:1 with RIA buffer and heated to 100°C

for 2 min to destroy potential proteinase activity before radioimmunoassay. Standards were prepared in Optimem (GIBCO/BRL): RIA buffer in a 1:1 ratio. Free radiolabel was separated from bound using polyethylene glycol precipitation (as described in Mathis and Lindberg, 1992). The range of the standard curve was 1-500 fmol and the IC_{50} was 42 fmol.

Collection of Conditioned Medium from AtT-20/PC2 and CHO Cells and Enzyme Assay

Subclones of AtT-20/PC2 cells expressing either no 7B2, 21 kD 7B2, 27 kD 7B2, the pentabasic blockade mutant, and the amino-terminal domain were subcultured at 500,000 cells per well in a 6-well plate. The following day, the wells were washed twice with 5 ml of Optimem and then incubated with 1 ml of Optimem containing 100 μg/ml aprotinin (Miles) overnight. The conditioned medium was removed from each well, centrifuged to remove any floating cells, and 35–50-μl aliquots then subjected to radioimmunoassay for 7B2 (in duplicate) and enzyme assay for PC2 (in duplicate). Specificity of the enzymatic reaction was monitored in separate reactions containing 100 μM synthetic 7B2 carboxy-terminal peptide (human sequence, residues 155-185), which represents a PC2-specific inhibitor (Martens et al., 1994; Lindberg et al., 1995). The experiment was repeated once with similar results.

For the clonal comparison study, various CHO/PC2-7B2 clones were isolated and subcultured to approximately similar confluence (70–80%) in a 12-well plate. After two 2-ml washes with Optimem, 1 ml of Optimem containing 100 μg/ml aprotinin was placed on each well and the plate was returned to the incubator for 16 h. The following morning, medium was collected from each well, centrifuged at low speed to remove any floating cells, and subjected to radioimmunoassay for 7B2 and enzyme assay.

For the comparison of the two best 7B2-expressing CHO clones with the parent cell line, 35-μl duplicate aliquots of 6 h conditioned medium (80% confluent 35-mm dish with 1 ml Optimem, containing 100 μg/ml aprotinin) were incubated in the reaction mixture described above (with or without 100 μM 7B2 CT peptide) for 16 h. The production of free aminomethylcoumarin (AMC) from clones expressing 7B2 was linear over time. To observe potential activation of proPC2 in the control CHO/PC2 cell line by exogenous 7B2, recombinant rat 7B2 was prepared by bacterial expression (Martens et al., 1994) and included in the medium as described above at a concentration of 100 μg/ml (5.4 μM) during a 16-h incubation at 37°C; a parallel control culture received an equivalent amount of bovine serum albumin. The following day the medium was removed, centrifuged, and tested for enzymatic activity. The experiment was repeated once with similar results.

The assay for PC2 (Lindberg et al., 1992) was carried out using 35 μl of each conditioned medium sample in a total volume of 50 μl, containing 0.1M sodium acetate, pH 5.0, 5 mM calcium, 0.1% Brij 35, 2 μg of bovine serum albumin, an inhibitor mix (1 μM pepstatin A, 100 μM tosyllysyl chloromethylketone; 100 μM tosyl phenylalanylchloromethyl ketone, and 1 μM E-64) and a final concentration of 200 μM Cbz-Arg-Ser-Lys-Arg-aminomethylcoumarin substrate. The liberation of the highly fluorescent product AMC was monitored by fluorescence spectroscopy (380 excitation, 460 emission).

Results

AtT-20/PC2-7B2 Cells Express 7B2 which Coimmunoprecipitates with proPC2

Braks and Martens (1994) have demonstrated coimmunoprecipitation of 27 kD 7B2 with proPC2 in the *Xenopus* intermediate pituitary. To investigate the nature of this association in a cell culture system, which offers more experimental control, we stably transfected PC2-overexpressing AtT-20 cells (Zhou et al., 1993; kindly provided by R. E. Mains) with a hygromycin resistance–conferring construct containing rat 7B2 cDNA. 7B2-expressing clones were selected by Western blotting, which revealed normal cleavage of the transfected 27-kD 7B2 precursor to the 21-kD product (results not shown). Using radioimmunoassay, the expression level of endogenous 7B2 in the parent cell line was estimated to be at or less than 10 fmol/10^6 cells (the limit of detection of the assay), while the best transfected cell line contained 120 fmol 7B2/10^6 cells. Metabolic-labeling experiments with [^{35}S]methionine were carried out on these cells as well as the parent AtT-20/PC2 cell line. In agreement with the results of Braks and Martens (1994), when cell extracts were immunoprecipitated with PC2 antiserum, 27 kD 7B2 was observed to coimmunoprecipitate with proPC2 (Fig. 1). After excision of the bands corresponding to proPC2 and 7B2 and estimation of the radioactivity in each, the molar ratio of proPC2 and 7B2 was found to be approximately 1.1:1.0 when the number of methionines in each molecule was taken into account (Table I).

7B2 Facilitates proPC2 Maturation in AtT-20/PC2 and RinPE Cells

We next performed a kinetic analysis of proPC2 maturation in control and 7B2-transfected AtT-20 cells. To avoid the possibility of conversion of proPC2 to PC2 during the overnight immunoprecipitation, we harvested the cells in the presence of SDS (Milgram and Mains, 1993), boiled, and then diluted this material into nonionic detergent for immunoprecipitation. Fig. 2 *A* shows the results of an experiment in which AtT20/PC2-r7B2 or control AtT-20/PC2 cells were pulsed for 20 min with [^{35}S]methionine, then either terminated (lane *0*) or further incubated for 1, 2, 3, or 4 h in the presence of unlabeled methionine. This figure demonstrates that the presence of 27 kD 7B2 dramatically facilitated the maturation rate of proPC2. Using phosphorimage analysis, we estimate that the half-life of the newly synthesized proPC2 in 7B2-expressing cells was 1.7 h, whereas in parent cells the half-life was 2.7 h. Similar

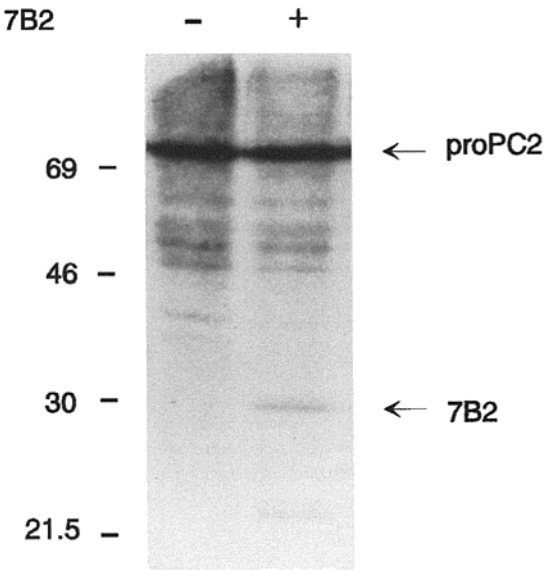


Figure 1. 27 kD 7B2 coimmunoprecipitates with proPC2. AtT20/PC2 and AtT20/PC2-7B2 cells were pulsed with [^{35}S]methionine for 20 min. Cell extracts, prepared in AG buffer, were immunoprecipitated with PC2 antiserum and subjected to electrophoresis. The bands corresponding to PC2 and 7B2 were excised and the radioactivity in each was determined using a scintillation counter. The results are shown in Table I.

Table I. Quantitation of Relative Amounts of Coimmunoprecipitated proPC2 and 7B2

	Clone #1		Clone #2	
	PC2	7B2	PC2	7B2
CPM	4790	1010	5130	1050
No. of Met	16	3	16	3
Molar ratio	1	1.12	1	1.09

results were obtained in one other independently derived clone.

We also found that the rate of secretion of mature PC2 was increased in cells transfected with 7B2 (Fig. 2 *B*). Despite this increased secretion, analysis of steady-state labeling of control and 7B2-expressing cells revealed increased storage of mature PC2 in 7B2-expressing lines (Fig. 2 *C*). However, analysis of the kinetics of processing of newly synthesized proopiomelanocortin (POMC) in AtT-20/PC2-7B2 cells did not reveal any detectable differences from the parent cell line (Mains, R. E., personal communication).

The AtT-20/PC2 cell line has been obtained through artificial engineering of AtT-20 cells with PC2 cDNA (Zhou and Mains, 1993). To extrapolate these results to a cell line which naturally expresses PC2, we performed a similar overexpression of 7B2 in RinPE cells, a derivative of the rat insulinoma cell line Rin5f which has been stably transfected with rat proenkephalin cDNA (Lindberg, I., unpublished results). The parent cell line is known to synthesize large quantities of PC2 (Shen et al., 1993). Fig. 3 shows that the presence of transfected 27 kD 7B2 in these cells was also able to facilitate the maturation of proPC2. No differences in the initial processing of newly synthesized proenkephalin to Peptide B/Met-enk-Arg-Phe could be detected between RinPE cells and RinPE-7B2 cells (Blake, A., and I. Lindberg, unpublished results).

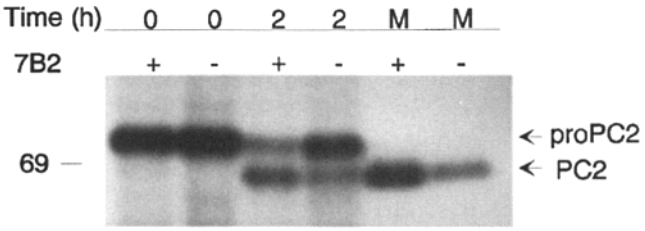
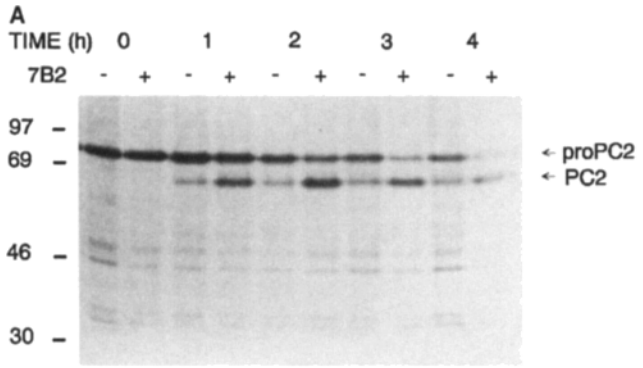
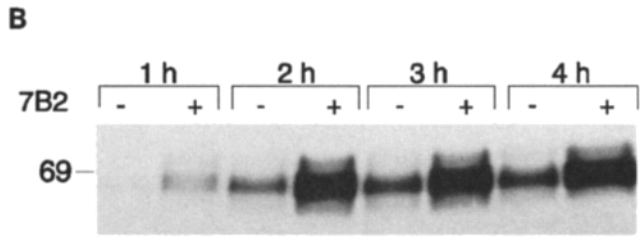


Figure 3. 27 kD 7B2 facilitates the maturation of proPC2 in RinPE cells. RinPE-7B2 or control RinPE cells were pulsed for 20 min with [^{35}S]methionine and then either terminated (lane *0 h*) or chased for 2 h (*2 h*). *M*, 2-h chase medium.

Structure-Function Analysis of 7B2 Indicates a Requirement for the Full 21-kD Protein

Braks and Martens (1994) have previously proposed that the binding of proPC2 to 7B2 is mediated by an amino-terminal domain weakly homologous to a chaperonin-related domain; this domain was proposed to extend to residue 90. To examine whether this region was sufficient to account for the effects on proPC2 transport, we constructed an expression vector containing this region for transfection of AtT-20/PC2 cells. Clones expressing this construct were selected using a radioimmunoassay against an amino-terminal epitope of 7B2. Fig. 4 depicts the maturation of proPC2 in 1–90 7B2 and 27 kD 7B2 cells as well as in the





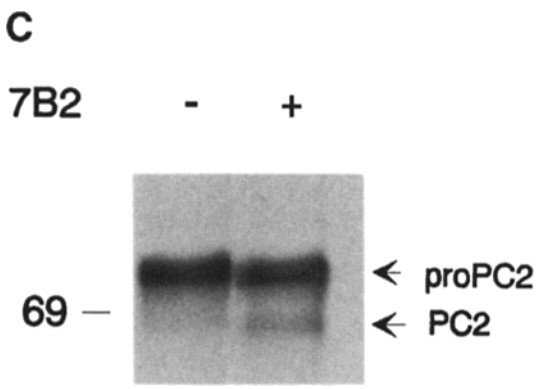


Figure 2. 27 kD 7B2 facilitates the maturation of proPC2in AtT-20/PC2 cells. AtT20/PC2-7B2 or control AtT-20/PC2 cells were pulsed for 20 min with [^{35}S]methionine, then either terminated (lane *0*) or chased for 1, 2, 3, or 4 h. Cell extracts were boiled for 5 min in the presence of 1% SDS and 50 mM-mercaptoethanol. These samples were then diluted 10× with 1% NP-40 for immunoprecipitation with PC2 antiserum. *A* shows that the rate of proPC2 processing was increased in 7B2-transfected cells. *B* depicts the effect of 7B2 expression on the secretion of mature PC2. *C* shows the ratios of intracellular proPC2 to mature PC2 at 6 h in a steady-labeling experiment.

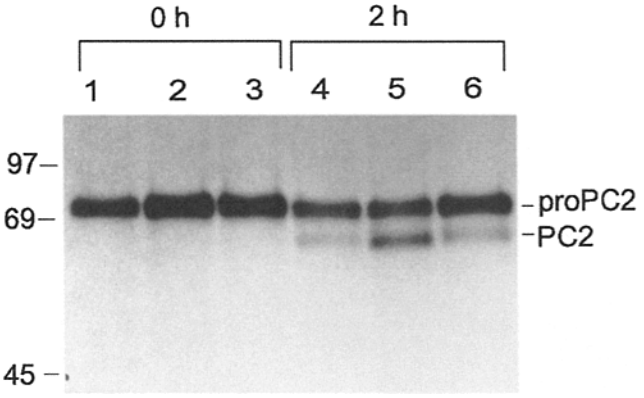


Figure 4. The proposed chaperone domain does not facilitate proPC2 processing. Overexpression of the proposed chaperone domain (Braks and Martens, 1994) had no effect on PC2 maturation in AtT-20/PC2 cells. AtT20/PC2 (lanes *1* and *4*), AtT20/PC2-7B2 (lanes *2* and *5*), and AtT20/PC2-NT 7B2 (lanes *3* and *6*) were pulsed for 20 min (lanes *1–3*) or pulsed and then chased for 2 h (lanes *4–6*). The samples were boiled as described before immunoprecipitation. Truncated 7B2 (NT-7B2) could not be coimmunoprecipitated with PC2 (not shown).

parent cell line; no detectable effects of 1–90 7B2 were observed. In independent experiments, the remaining carboxy-terminal region of 7B2 ($7B2_{95\text{-}185}$) was similarly transfected into AtT-20 cells; again, no effect on proPC2 maturation was observed (results not shown). Using PC2 antiserum, no coimmunoprecipitation of either $7B2_{1\text{-}90}$ or $7B2_{95\text{-}185}$ with PC2 was observed (data not shown). Similarly to the result obtained using intact 7B2, no effects on POMC processing were detected in either the $7B2_{1\text{-}90}$ or the $7B2_{95\text{-}185}$-expressing cell lines (Mains, R. E., personal communication).

The mature form of 7B2, 21-kD 7B2, represents the predominant form of this peptide stored within neuroendocrine cells (Iguchi et al., 1984; Hsi et al., 1984; Ayoubi et al., 1990; Paquet et al., 1994). We therefore transfected a construct containing this region into AtT-20/PC2 cells. Fig. 5 depicts the maturation of proPC2 in cells containing this construct. The results of this 2-h pulse-chase experiment show that the 21-kD 7B2 protein is able to efficiently facilitate proPC2 cleavage. Further analysis revealed that in cells transfected with 21 and 27 kD constructs, the kinetics of proPC2 cleavage and the kinetics of secretion of PC2 are identical (results not shown). These results suggest that the functional region of 7B2 which accomplishes the facilitation of proPC2 cleavage is entirely included within the first 156 amino acids.

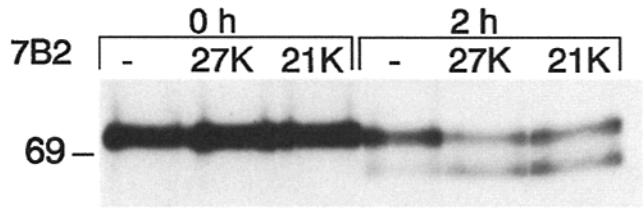


Figure 5. The 21-kD form of 7B2 facilitates the maturation of proPC2. AtT20/PC2, AtT20/PC2-7B2 (27 kD 7B2), and AtT/20/PC2-N560 7B2 (21 kD 7B2) cells were pulsed with [^{35}S] methionine for 20 min or pulsed and then chased for 2 h.

Blockade of Cleavage of 27 kD 7B2 Results in a Molecule which Cannot Facilitate proPC2 Maturation

To investigate whether the cleavage of 27 kD 7B2 to 21 kD 7B2 and the carboxy-terminal peptide is obligatory for the facilitatory function of 7B2, and the role the carboxy-terminal peptide plays in the binding of 7B2 to PC2/proPC2, we mutated the normal cleavage site (residues 151-155) from RRKRR to SQNSN and transfected AtT20/PC2 cells with the construct. Fig. 6, panel *A* (denatured immunoprecipitate using 7B2 antiserum) shows that this 7B2 mutant protein is expressed in transfected cells (lane *0*); it was not processed intracellularly but instead was rapidly secreted. At 2 h no intracellular 7B2 remained (lane *2*; no visible band). Fig. 6, panel *B*, which represents a non-denaturing coimmunoprecipitation of medium obtained at 2 h of chase (7B2 antiserum), shows that the mutant protein was secreted not as the 21-kD form, but as the 27-kD form, supporting the idea that the normal cleavage site was blocked. This figure further shows that this mutant 7B2 protein can efficiently coimmunoprecipitate proPC2. It is interesting that both proPC2 as well as the unprocessed mutant 27-kD 7B2 were secreted intact into the growth medium (panel *B*); the secretion of proPC2 was not observed in experiments with other 7B2-expressing cell lines. It is likely that this proPC2/noncleavable 7B2 complex cannot be further processed by the cellular machinery and is secreted as such. Fig. 6, panel *C* depicts a pulse-chase experiment of PC2 maturation in AtT-20/PC2-blockade mutant cells showing that this unprocessable 7B2 mutant cannot facilitate the maturation of proPC2. We conclude that proteolytic processing of 27 kD 7B2 is required for its facilitatory function.

Kinetics of 7B2 Processing in AtT20/PC2-7B2 Cells

While the maturation of proPC2 in AtT-20/PC2-7B2 cells is rather slow (with a half time of ~1.7 h in 7B2-overexpressing cells), the conversion of 27 kD 7B2 to 21 kD 7B2 occurred much more quickly. After 30 min of chase, virtually all of the newly synthesized 27 kD 7B2 had been cleaved (Fig. 7, panel *A*). Furthermore, the secretion of 21 kD 7B2 was also much faster than that of PC2; even at 20 min, 21 kD 7B2 was detectable in the medium (Fig. 7, panel *B*). At 120 min of chase, however, ~1/3 of the newly synthesized 21 kD still remained inside the cells (Fig. 7, panel *A*), as judged from phosphorimage analysis.

7B2 Is Synthesized and Secreted More Rapidly than PC2

To calculate the relative synthesis rates of 7B2 and PC2, AtT-20/PC2-7B2 cells were labeled with [^{35}S]methionine for 20 min. The cells were then extracted using 1% SDS and 50 mM β-mercaptoethanol and boiled. Half of the extract was immunoprecipitated with 7B2 antiserum, and the other half immunoprecipitated with PC2 antiserum. The resulting samples were then resolved by SDS-PAGE and visualized by fluorography. The 27/21-kD 7B2 bands and the proPC2 band were excised, the radioactivities mea-

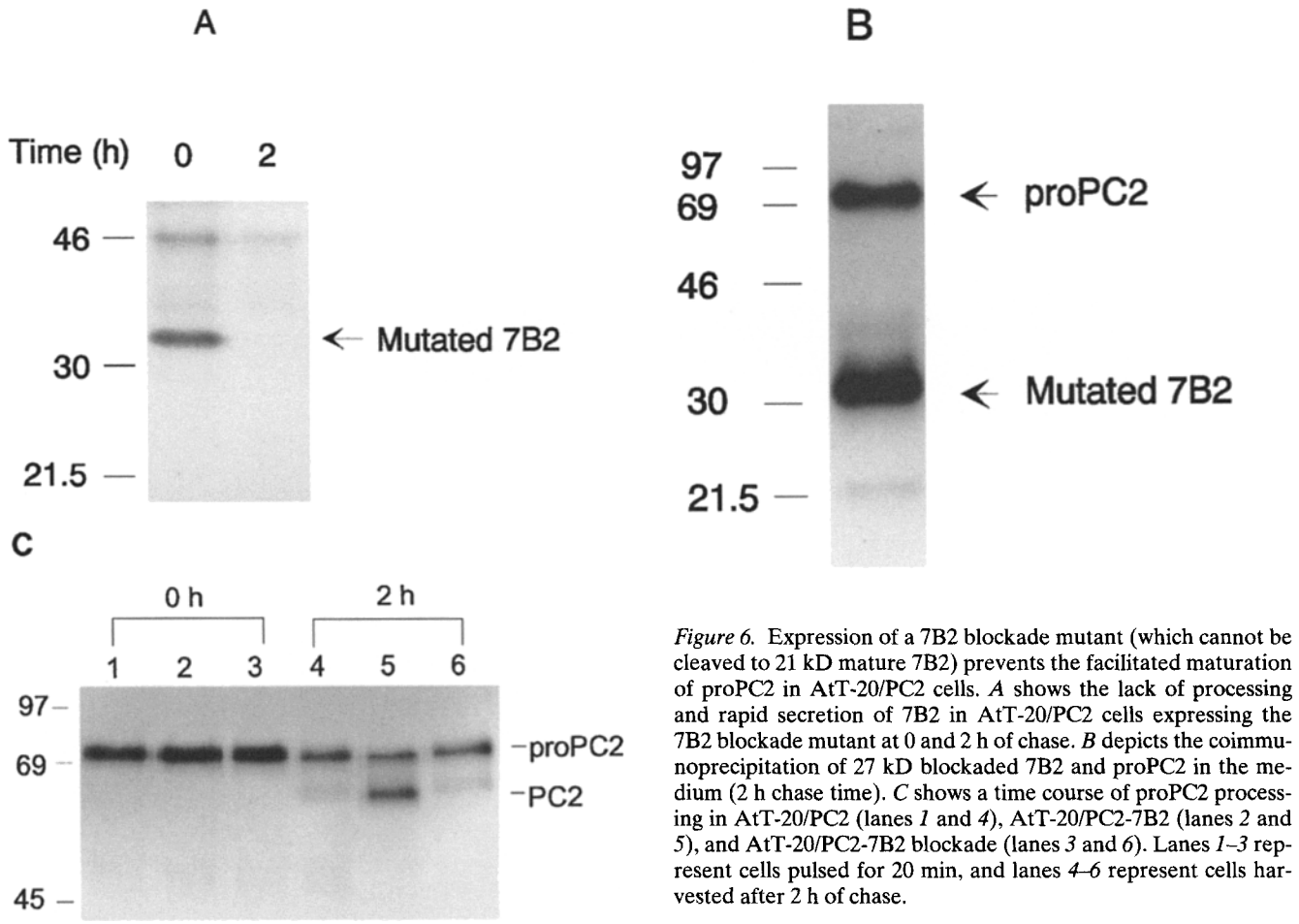


Figure 6. Expression of a 7B2 blockade mutant (which cannot be cleaved to 21 kD mature 7B2) prevents the facilitated maturation of proPC2 in AtT-20/PC2 cells. *A* shows the lack of processing and rapid secretion of 7B2 in AtT-20/PC2 cells expressing the 7B2 blockade mutant at 0 and 2 h of chase. *B* depicts the coimmunoprecipitation of 27 kD blockaded 7B2 and proPC2 in the medium (2 h chase time). *C* shows a time course of proPC2 processing in AtT-20/PC2 (lanes *1* and *4*), AtT-20/PC2-7B2 (lanes *2* and *5*), and AtT-20/PC2-7B2 blockade (lanes *3* and *6*). Lanes *1–3* represent cells pulsed for 20 min, and lanes *4–6* represent cells harvested after 2 h of chase.

sured, and the molar ratio of the two molecules was calculated, taking the number of methionines in each molecule into account (the 21- and 27-kD forms of 7B2 have the same number of methionines). These results revealed that the synthesis rate of 27 kD 7B2 is greater than that of PC2; for every 3.6 ± 0.3 7B2 molecules synthesized, only one proPC2 molecule was produced (mean ± SD of three determinations). However, in a 6-h steady labeling experiment, the intracellular molar ratio of all forms of these two molecules (predominantly 21 kD 7B2 and proPC2) was only 1.4:1.0. Therefore, relative to PC2, 7B2 must be disproportionately rapidly secreted.

Secretion of PC2 Activity from AtT-20/PC2 Cells Occurs only in Cells Transfected with 21 or 27 kD 7B2

The above results demonstrate that 7B2 interacts with proPC2 to facilitate the maturation of this enzyme precursor. However, these results do not reveal whether this proteolytic processing event is associated with the production of active enzyme; indeed, the lack of effect on POMC processing could be interpreted as implying that 7B2 overexpression does not result in the net generation of active PC2. We therefore measured the release of active PC2 into the medium from AtT-20/PC2 cell lines expressing the various forms of 7B2. We were not able to measure significant levels of activity in short-term basal medium or medium from phorbol ester–stimulated cells; however, overnight-conditioned medium yielded measurable levels of PC2 activity (Fig. 8). Only cells transfected with 27 or 21 kD 7B2 were able to secrete active PC2 into the medium. The origin of this enzymatic activity as PC2 was confirmed through its ability to be blocked by the PC2-specific inhibitor, $h7B2_{155-185}$ (Lindberg et al., 1995).

In CHO/PC2 Cells, Expression of 21 kD 7B2 Results in the Generation of Ezymatic Activity

To confirm the above results in a constitutive cell line which potentially offered a greater ability to measure PC2 activity, we tested the effect of 7B2 expression on the production of enzymatically active PC2 from CHO cells amplified for mouse PC2 expression using the dihydrofolate reductase–coupled method (Shen et al., 1993). These cells have previously been shown to produce large quantities of proPC2 which is slowly secreted into the medium and is enzymatically inactive (Shen et al., 1993). CHO/PC2 cells were transfected with the expression vector containing 21 kD 7B2 described above and clones selected on the basis of secretion of immunoreactive 7B2. Table II shows an analysis of cleavage of the fluorogenic substrate Cbz-Arg-Ser-Lys-Arg-aminomethyl coumarin by medium conditioned for only 6 h by control CHO/PC2 cells or by two in-

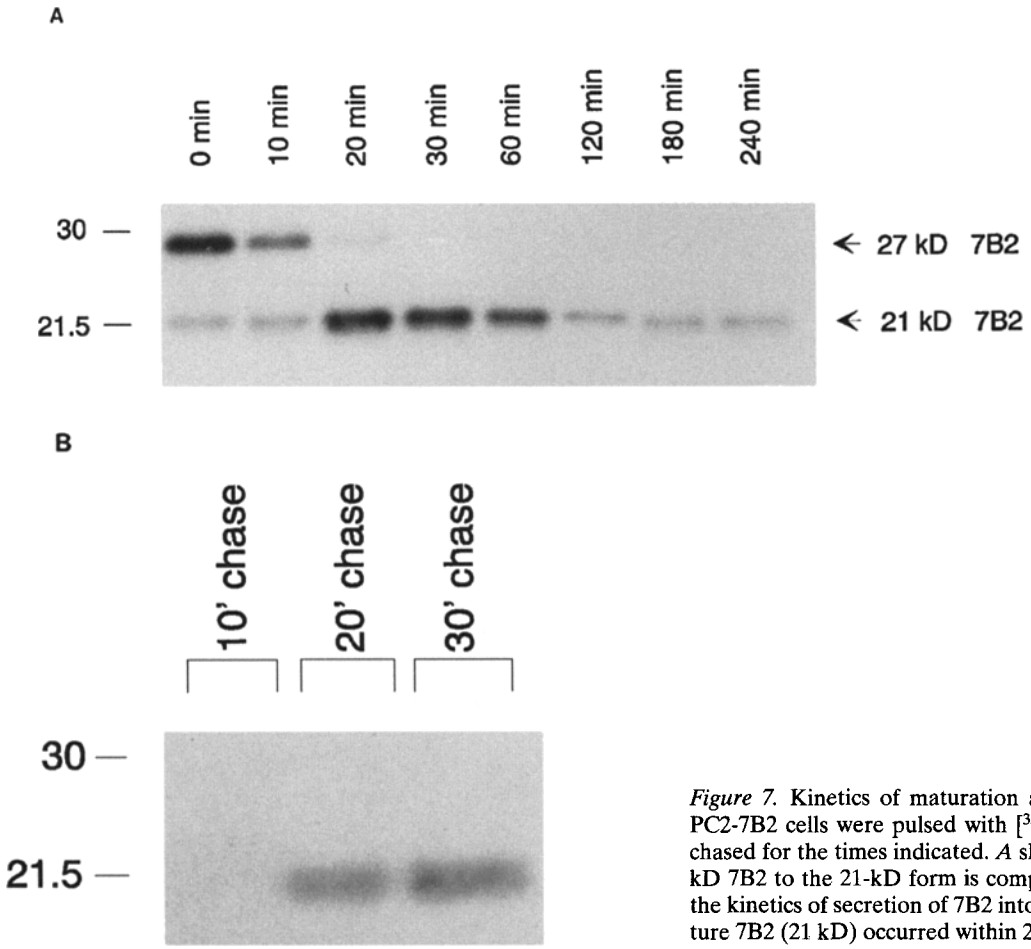


Figure 7. Kinetics of maturation and secretion of 7B2. AtT20/PC2-7B2 cells were pulsed with [^{35}S]methionine for 10 min and chased for the times indicated. *A* shows that the conversion of 27 kD 7B2 to the 21-kD form is completed within 30 min; *B* shows the kinetics of secretion of 7B2 into the medium; secretion of mature 7B2 (21 kD) occurred within 20 min.

dependent clones expressing 21 kD 7B2. As expected from previous results, medium obtained from non–7B2-transfected CHO/PC2 cells was enzymatically inactive; however, medium derived from each of the two 21-kD 7B2-expressing clones exhibited extremely high levels of activity. A high degree of correlation of secreted PC2 activity with 7B2 expression was observed using independent clones (Fig. 9). Incubation of CHO/PC2 cells overnight with 100 μg/ml recombinant rat 21 kD 7B2 did not result in the generation of detectable enzymatic activity in the conditioned medium (not shown).

Discussion

The proteolytic activation of PC2 has been the subject of several previous investigations. Studies using oocyte extracts and site-directed mutants of proPC2 have shown that the conversion of proPC2 (75 kD) to mature PC2 (64 kD) is autocatalytic, and that it is apparently mediated through an intermolecular reaction which is extremely slow in this system (Matthews et al., 1994; Shennan et al., 1995). In previous experiments using a CHO cell overexpression system, we were unable to demonstrate any autocatalysis of proPC2, and proPC2 was largely secreted as an intact, inactive form (Shen et al., 1993). Efforts to activate proPC2 in vitro, for example using recombinant PC1, were not successful (Zhou, Y., and I. Lindberg, unpublished results). These findings strongly implied that PC2 required a separate cofactor, either for activation or activity; or, as suggested by Braks and Martens (1994), that PC2 required the presence of another protein, namely 7B2, for proper folding. The results presented above provide experimental support for the involvement of 7B2 in proPC2 activation. Our data demonstrate that 27 kD 7B2 can be coimmunoprecipitated with proPC2 using anti-PC2 antiserum in extracts of 7B2-overexpressing, PC2-containing cells, confirming the similar association reported by Braks and Martens (1994) in *Xenopus* pituitary. In addition, this association is correlated with a profound acceleration of maturation of proPC2 to mature PC2 in cells expressing 7B2, with a half-life decreasing from ~2.7 h–1.7 h. The observation of stoichiometric binding of proPC2 to 27 kD 7B2 further supports the idea that 7B2 represents a PC2-binding protein involved in the maturation of proPC2 to PC2.

We found that proPC2 required 2–3 h for half-maximal conversion to mature PC2 in AtT-20/PC2 cells; these kinetics are in good agreement with those obtained by Zhou and Mains (1994) for the same cell line. Similarly slow processing of proPC2 was observed in other neuroendocrine cell lines and in rat pancreatic islets (Guest et al., 1992; Shen et al., 1993; Benjannet et al., 1993; Shennan et al.,

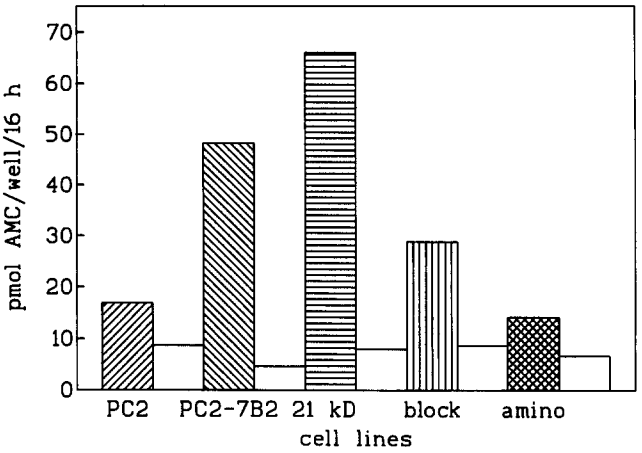


Figure 8. Secretion of PC2 activity in AtT-20/PC2 cell lines is correlated with the expression of certain forms of 7B2. (*Hatched bars*) Cell lines expressing (*from left to right*) no 7B2 (*control*), 27 kD 7B2, 21 kD 7B2, the blockade mutant (*block*), and the amino-terminal domain (*amino*). Clear bars to the right of each hatched bar indicate activity measured in the presence of the PC2 inhibitor $h7B2_{155\text{-}185}$ (Lindberg et al., 1995). Cells were plated at 500,000 cells/well in a six-well plate; 2 d later, serum-free medium was placed on the cells and assayed in duplicate the following day for the expression of enzymatic activity. The total amount of immunoreactive 7B2 in the conditioned medium was 440 fmol (*control*); 790 fmol, 27 kD 7B2; 770 fmol, 21 kD 7B2; 452 fmol, blockade mutant (*block*); and 530 fmol, amino-terminal domain (*amino*). Variance between duplicates was always less than 5%.

1995). (An exception may exist for *Xenopus* intermediate lobe, in which proPC2 appears to be cleaved much more rapidly than in neuroendocrine cell lines and in pancreatic islets; Braks and Martens, 1994). Previous studies from several laboratories have shown that the subcellular site of conversion of proPC2 to mature PC2 is likely to be a late secretory granule compartment such as the *trans*-Golgi network or immature secretory granules (Guest et al., 1992; Shen et al., 1993; Zhou and Mains, 1994; Braks and Martens, 1994; Shennan et al., 1995). Similarly, 27 kD 7B2 is converted to the 21-kD form in a brefeldin-sensitive, late secretory pathway cellular compartment, most probably by furin (Paquet et al., 1994). The fate of the remaining carboxy-terminal 31 amino acids of 7B2—which represents the inhibitory peptide—is uncertain. This peptide could be recovered intact from AtT-20 cells (Paquet et al., 1991); however, it may also be proteolyzed in certain tissues since Sigafoos et al. (1993) have described the isolation of a carboxy-terminal fragment of this peptide from bovine adrenal medullary granules.

Confirming previous results obtained by Braks and Martens (1994) and Paquet et al. (1994), we observed rapid maturation of newly synthesized 27 kD 7B2 ($t_{1/2}$ = 15 min). In AtT-20/PC2 cells, secretion of newly synthesized 7B2 into the medium was also unexpectedly rapid, in contrast to results obtained using vaccinia virus vectors (Paquet et al., 1994) and *Xenopus* intermediate lobe (Braks and Martens, 1994). We found that following only 20 min of chase, newly synthesized 21 kD 7B2 could already be detected in the medium, and by 120 min, only a third of the labeled 7B2 protein remained inside the cells;

*Table II. Only CHO/PC2 Cells Expressing 21 kD 7B2 Exhibit PC2 Activity**

	pmol AMC generated/16h/35 μl 6 h conditioned medium		
	Parent CHO/PC2	21 kD 7B2#7	21 kD 7B2#20
Control	58	700	667
+PC2 inhibitor	62	58	48

* 70-80% confluent 35 mm wells of two 21-kD 7B2-transfected cell lines and the parent, nontransfected cell line were incubated with Optimem for 6 h. The conditioned medium was then removed, centrifuged, and assayed in duplicate for PC2 activity as described in the text. The specific PC2 inhibitor used was $h7B2_{155\text{-}185}$ (Lindberg et al., 1995). Control reactions containing only Optimem (not conditioned by cells) contained 58 pmol AMC. Variance between duplicates was always less than 5%.

this remaining 7B2 may represent the pool which binds to proPC2. Expression of 7B2 also increased the rate of secretion of mature PC2 from the cells; however, not all of the additional mature PC2 produced by 7B2 expression was secreted since the steady state intracellular content of mature PC2 was slightly, but consistently, increased in 7B2-expressing cells.

Based upon homology with chaperonins, Braks and Martens (1994) suggested that the first 1-90 amino acids of 7B2 would represent the putative "chaperone domain." Our data showing that expression of this protein is not sufficient to facilitate proPC2 cleavage indicates that further structural information must be required. We found that expression of the first 151 amino acids, which encode the 21-kD natural processing product of 7B2, were sufficient for the facilitation of proPC2 processing. These data indicate an affinity of this truncated protein for proPC2, and support in vitro experiments which show that recombinant 21 kD 7B2 can bind to proPC2 immunoprecipitates (Braks and Martens, 1994). Our data also show that expression of 21 kD 7B2 is sufficient for the actual activation of PC2, as demonstrated by PC2 activity experiments using AtT-20/PC2 and CHO/PC2 cells in which only cells which have been cotransfected and express 7B2 secrete active PC2. Which enzyme actually performs the activation of proPC2, or whether proPC2 is autocatalytically activated, is as yet

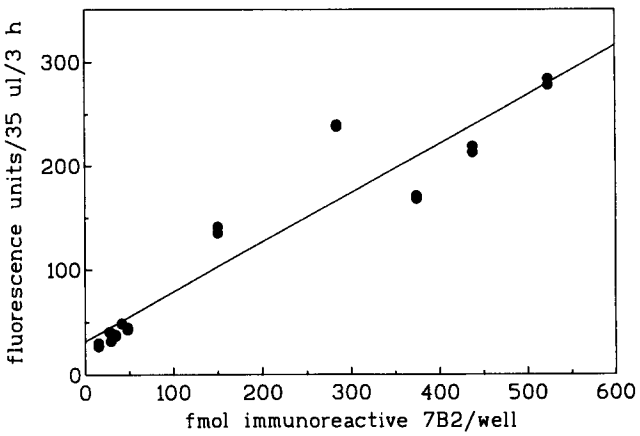


Figure 9. Secretion of PC2 activity is correlated with the expression of 21 kD 7B2 in CHO/PC2 cells. Overnight-conditioned medium samples obtained from 11 independent clones of CHO/PC2 cells stably transfected with 21 kD 7B2 cDNA were assayed for both 7B2 immunoreactivity and for PC2 activity. The correlation coefficient r^2 was 0.90.

unclear. Since postsynthesis activation of CHO cell-secreted proPC2 with recombinant 21 kD rat 7B2 was not successful, it appears that 7B2 must be present intracellularly, either during synthesis of proPC2 or shortly thereafter (however, it should be noted that postsynthesis activation was attempted only at neutral pH). These CHO/PC2-7B2 cells express considerably more proPC2 (expressed via the DHFR-coupled amplification) than 7B2 (expressed through simple transfection and selection). However, enzyme activity produced by CHO/PC2-7B2 cells has proven to be sufficient for the purification and characterization of active recombinant PC2, and such studies are now in progress (Lamango, N., and I. Lindberg, unpublished results).

The carboxy-terminal peptide of 7B2 has been shown to be inhibitory to PC2 activity as well as to proPC2 activation (Martens et al., 1994; Lindberg et al., 1995). The fact that both the 21-kD and the 27-kD 7B2 proteins exhibit a similar facilitatory function on proPC2 processing leads to the question of the normal role of the carboxy-terminal peptide in the processing of proPC2. When processing of 7B2 to the 21-kD protein and the carboxy-terminal peptide was blocked by mutation of the normal pentabasic processing site, the mutated protein bound to proPC2, but did not facilitate proPC2 processing. Moreover, expression of the blocked 7B2 protein prevented the cleavage of the PC2 proregion since in these cell lines proPC2 was secreted intact into the medium (a process which was never otherwise observed). It appears that the removal or cleavage of the carboxy terminal peptide is required for the autocatalytic cleavage of proPC2, most probably because the carboxy-terminal peptide occupies the active site of proPC2 (thus preventing autoactivation). We observed that the binding of cleavage-site blocked 7B2 to proPC2 is tighter than that of 21 kD 7B2 (unpublished results), indicating that the presence of the inhibitory carboxy-terminal peptide (which has been shown to have a strong affinity for proPC2; Lindberg et al., 1995) increases the affinity of 7B2 for proPC2. The mechanism for removal of the 7B2 carboxy-terminal peptide is unclear at present.

Based on the results discussed above, we propose the following model for the interaction of PC2 with 7B2 in AtT20/PC2-7B2 cells (see Fig. 10):

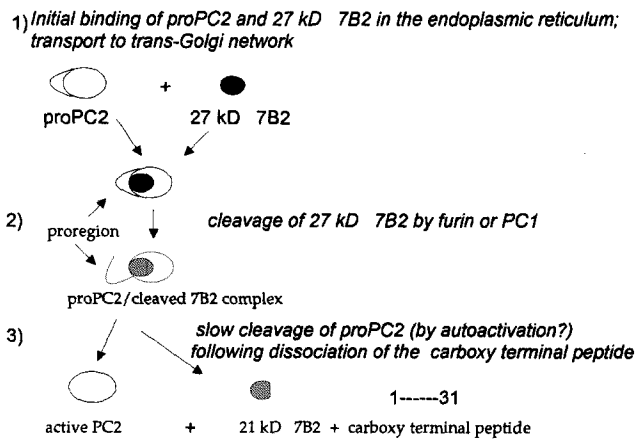


Figure 10. Proposed mechanism of interaction of PC2 and 7B2.

(*1*) Soon after synthesis, a portion of the available 27 kD 7B2 and proPC2 bind each other in the endoplasmic reticulum; this binding results in the translocation of the complex to the TGN/secretory granules. The presence of the carboxyl-terminal inhibitory peptide of 27 kD 7B2 prevents the premature activation of proPC2 before arrival of the complex in the Golgi apparatus.The remainder of the 27 kD 7B2 (i.e., the portion not bound to proPC2) is quickly transported through the secretory pathway, with a half-time of conversion of less than 15 min, and is secreted.

(*2*) In the TGN/immature secretory granule compartment(s), the 7B2 within the proPC2/27-kD 7B2 complex is rapidly cleaved to 21 kD 7B2 and the carboxy terminal inhibitory peptide. The resulting complex, with the various molecules weakly associated, is now competent for cleavage to the active form of PC2, possibly by autoactivation (Matthews et al., 1994; Shennan et al., 1995). The rate of activation of each molecule of proPC2 may depend on the rate of dissociation/cleavage of its associated 7B2 carboxy-terminal inhibitory peptide.

(*3*) Mature PC2 resulting from the above process is then free to cleave prohormone substrates/intermediates.

No effects on POMC processing were observed in cell lines expressing either intact 27 kD 7B2, 21 kD 7B2, or the carboxy-terminal domain alone (Mains, R. E., unpublished data). The lack of effect on peptide processing of the 21- and 27-kD 7B2 constructs, both of which increased the production of mature PC2, may be explained by positing that POMC cleavage is already maximal in the parent cell line, and that it cannot be increased beyond this maximum by the introduction of further active enzyme. This interpretation is supported by the finding of almost complete cleavage of ACTH to α-MSH-sized peptides in AtT-20/PC2 cells (Zhou et al., 1993). The expression of endogenous 7B2 in these cells is apparently sufficient to support this high rate of cleavage. The lack of effect on POMC processing of cell lines overexpressing a carboxy-terminal inhibitory domain (residues 95-185) was expected since expression of this construct had no effect on the kinetics of maturation of proPC2.

In conclusion, it is likely that complex regulatory mechanisms limit the rate of peptide production, involving control of the availability of active PC2 enzyme molecules in the correct subcellular compartment and regulation of PC2 enzyme activity though the association/dissociation of inhibitor molecules from activated and zymogen forms of PC2. Several questions regarding the interaction of PC2 and 7B2 remain to be answered, for example the site and mechanism of dissociation of the inhibitory 7B2 carboxy-terminal peptide from proPC2. Future pulse-chase studies employing antisera to this portion of the 7B2 molecule will provide a better understanding of these cellular events and may one day provide a practical basis for the manipulation of peptide hormone levels.

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Note Added in Proof. Benjannet et al. (1995) have recently reported intracellular binding of proPC2/PC2 to 7B2 forms (Benjannet, S., D. Savaria, M. Chretien, and N. G. Seidah. 1995. *J. Neurochem.* 64:2303–2311). 7B2 is a specific intracellular binding protein of the prohormone convertase PC2.

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