

The neuroendocrine chaperone 7B2 can enhance in vitro POMC cleavage by prohormone convertase PC2

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Abstract We previously showed that the neuroendocrine polypeptide 7B2 transiently interacts with prohormone convertase PC2 in the secretory pathway of neuroendocrine cells. Here we demonstrate that the processed, but not the intact, form of 7B2 can enhance the in vitro cleavage of newly synthesized prohormone proopiomelanocortin (POMC) in lysates of *Xenopus* intermediate pituitary cells. PC2 is presumably the cleavage enzyme involved since intact 7B2 abolishes the enhancing effect of processed 7B2 and is known to act as a specific inhibitor of PC2. Furthermore, processed 7B2 stimulates in vitro POMC cleavage by immunopurified *Xenopus* PC2. Our results indicate that 7B2 can display chaperone activity towards PC2.

Key words: 7B2; PC2; Chaperone; POMC cleavage; *Xenopus* intermediate pituitary

1. Introduction

The prohormone convertases PC1 (also referred to as PC3) and PC2 are responsible for proteolytic cleavage of prohormones and neuropeptide precursors at pairs of basic amino acids [1–2]. The activity of these subtilisin-like enzymes in the secretory pathway of neuroendocrine cells is regulated at multiple levels. PC1 and PC2 exhibit an acidic pH optimum and Ca^{2+} dependence which limits their activity mainly to the *trans*-Golgi network (TGN) and the secretory granules [3–6]. Furthermore, PC1 and PC2 are synthesized as proenzymes and their activation involves proteolytic removal of the N-terminal proregion, a process that is thought to be autocatalytic [7–9]. The conversion of proPC1 to PC1 has been demonstrated to occur in the endoplasmic reticulum (ER) [10–12] whereas maturation of proPC2 was shown to take place only in the later compartments of the secretory pathway [13–14]. The delayed maturation of proPC2 is consistent with the fact that PC2 is involved in the later steps of prohormone cleavage [15–16].

Recently we proposed that activation of proPC2 is regulated by the neuroendocrine polypeptide 7B2 [17]. Like PC2, the 7B2 protein is present in the secretory pathway of a wide variety of neural and endocrine cells [18–21]. 7B2 is synthesized as a 25–29-kDa protein that is C-terminally processed in the TGN, resulting in an 18–21-kDa secretory product [22–23]. We have

found that the intact form of 7B2 interacts with proPC2 in the ER and Golgi region of *Xenopus* and mouse intermediate pituitary cells ([17], J.A.M. Braks and G.J.M. Martens, unpublished results). Based on its functional characteristics and the finding that the N-terminal half of 7B2 is distantly related to members of the Hsp60 family of chaperones, 7B2 has been referred to as a neuroendocrine chaperone [17]. In vitro, the recombinant intact form of 7B2 (and not its processed form) inhibits PC2 convertase activity and blocks autocatalytic conversion of proPC2 into its mature form [24,25]. These observations indicate that intact 7B2 regulates PC2 activity in neuroendocrine cells by preventing premature proPC2 conversion. Here we report that the N-terminal processed form of 7B2 enhances in vitro cleavage of newly synthesized proopiomelanocortin (POMC) by *Xenopus* PC2.

2. Materials and methods

2.1. Recombinant 7B2 proteins

The prokaryotic expression constructs encoding normal and mutated forms of 7B2 were generated with synthetic primers and PCR [25]. Production and purification of the recombinant proteins was performed as described previously [24]. Recombinant intact 7B2 (amino acid residues 1–185; numbering refers to [26]), processed 7B2 (residues 1–151), the C-terminally truncated 7B2 mutants pNK171 (1–171), pNF177 (1–177), pNG162 (1–162), pNP131 (1–131), and pNG86 (1–86), and the N-terminally truncated 7B2 mutants pCP93 (93–185) and pCD125 (125–185) were used in the present study.

2.2. Radiolabeling of *Xenopus* intermediate pituitaries and preparation of tissue lysates

Intermediate pituitaries were dissected from black adapted *Xenopus laevis* and radiolabeled with $\text{Tran}^{[35\text{S}]}$ label (ICN Radiochemicals) as described previously [17]. Pulse labeling was performed for 2 h in the presence of brefeldin A (2.5 $\mu\text{g}/\text{ml}$, Sigma) and the pituitaries were homogenized in lysis buffer (10 mM Hepes, pH 7.2; 140 mM NaCl; 0.1% Triton X-100; 0.1% sodium deoxycholate; 1% Tween-20; 1 mM EDTA; 1 mM phenylmethylsulfonylfluoride; 0.1 mg/ml soybean trypsin inhibitor). The lysates were cleared by centrifugation ($10,000 \times g$, 7 min).

2.3. Immunopurification of *Xenopus* PC2

Intermediate pituitaries of black-adapted *Xenopus* were dissected, homogenized in lysis buffer containing 2 mM EDTA and cleared by centrifugation. Polyclonal anti-PC2 antiserum (4BF [13], kindly provided by Dr. Iris Lindberg, New Orleans, LA, USA) was added and incubation was performed for 2 h at 4°C. Immune complexes were collected with protein A-Sepharose and the precipitates were rinsed three times in lysis buffer with 2 mM EDTA and once in lysis buffer without EDTA. Immunopurified PC2 was used when still attached to protein A-Sepharose.

2.4. POMC cleavage experiments

For POMC cleavage experiments, radiolabeled *Xenopus* intermediate pituitary lysate was diluted 15 times in assay buffer (50 mM MES, pH 6.4; 140 mM NaCl; 3 mM Ca^{2+} ; 0.02% bovine serum albumin). Samples (30 μl , equivalents of ~0.05 intermediate pituitary) were incubated for the indicated periods of time (usually 4 h) at 24°C in the presence or

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Abbreviations: PC1 and PC2, prohormone convertase 1 and 2; ER, endoplasmic reticulum; TGN, *trans*-Golgi network; POMC, proopiomelanocortin; MSH, melanophore stimulating hormone; ACTH, adrenocorticotrophic hormone; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; MES, 4-morpholineethanesulphonic acid.

absence of 0.5 μg recombinant 7B2 ($\sim 1 \mu\text{M}$ final concentration, unless otherwise indicated). In some experiments, ACTH^{1–39} (2.5 μg) or EDTA (5 mM final concentration) was present during the incubations. Samples were analyzed by SDS-PAGE [27]. When newly synthesized intermediate pituitary proteins were used as a source of radiolabeled POMC, the lysates were heated for 5 min at 90°C. The sample was diluted in assay buffer as described for the non-heated lysate and incubations were performed at 24°C in the presence or absence of recombinant 7B2 for 4 h. In some experiments, non-radiolabeled tissue lysate (equivalents of ~ 0.15 *Xenopus* or mouse intermediate or anterior pituitary), prepared by homogenization in lysis buffer, or immunopurified PC2 (purified from an equivalent of ~ 0.4 intermediate pituitary) was present during the incubation.

3. Results

3.1. Effect of recombinant 7B2 on POMC conversion in *Xenopus* intermediate pituitary lysate

After pulse labeling of *Xenopus* intermediate pituitaries with Tran[³⁵S] label, the main radiolabeled protein in the tissue lysate is the prohormone POMC. When a fresh radiolabeled lysate was incubated at 24°C under conditions corresponding to the TGN (pH 6.4/3 mM Ca²⁺), only a small amount of 37-kDa newly synthesized POMC was converted to lower molecular

weight products of 30 kDa and 19–20 kDa (Fig. 1A, lane 1). However, when the recombinant processed form of 7B2 (residues 1–151, $\sim 1 \mu\text{M}$) was present during the in vitro incubation, most of 37-kDa POMC was converted into the 19–20 kDa protein. In addition, the 30-kDa protein and a series of proteins with sizes between 13 and 18 kDa were generated (Fig. 1A, lane 2). Other radiolabeled proteins were not affected during the incubations. In the presence of 10 times less processed 7B2 (~ 100 nM) POMC cleavage was still enhanced, although to a lesser extent ($\sim 30\%$) (Fig. 1A, lane 3). No stimulation of POMC conversion was observed using ~ 10 nM of processed 7B2 (Fig. 1A, lane 4). Incubation of the lysate in the presence of intact 7B2 (residues 1–185, $\sim 1 \mu\text{M}$) inhibited rather than enhanced POMC conversion, since virtually no 30-kDa and 19-kDa products were formed (Fig. 1A, lane 5). A 10-fold lower concentration of intact 7B2 (~ 100 nM) still prevented POMC cleavage (Fig. 1A, lane 6). Intact 7B2 has been shown to act as a specific inhibitor of prohormone convertase PC2 in an in vitro enzyme assay [24]. Incubation of the lysate in the presence of both processed and intact 7B2 (both at $\sim 1 \mu\text{M}$) also led to a complete block of POMC conversion, indicating that the intact form of 7B2 can abolish the stimulating effect of the processed form

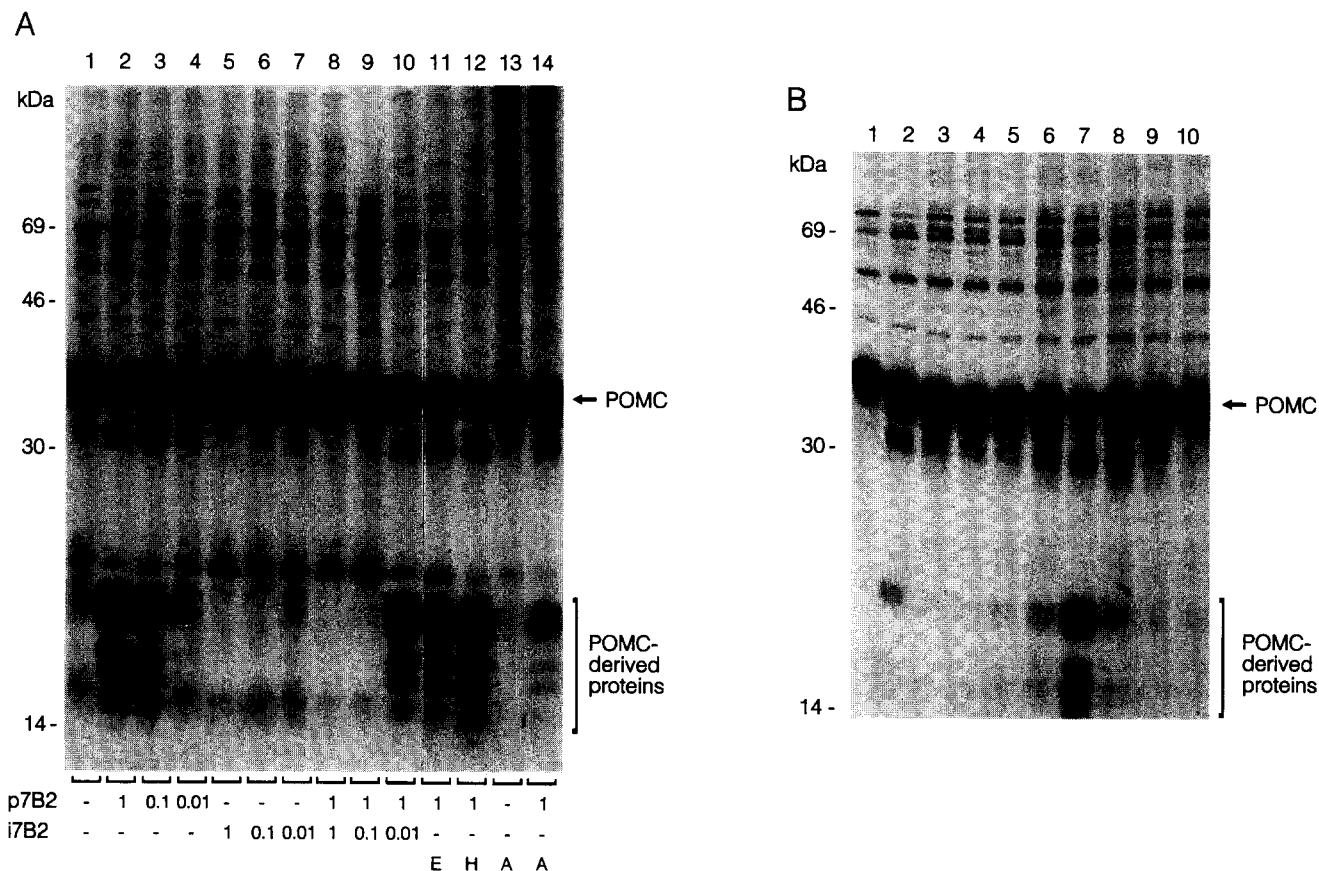


Fig. 1. Effects of recombinant 7B2 proteins on POMC cleavage in *Xenopus* intermediate pituitary lysates. Newly synthesized proteins produced by *Xenopus* intermediate pituitaries were incubated for 4 h and analyzed by SDS-PAGE. (A) Incubations were performed in the absence of 7B2 (lane 1) or in the presence of 1 μM (lane 2), 0.1 μM (lane 3), 0.01 μM (lane 4) recombinant processed 7B2 (p7B2, residues 1–151), in the presence of 1 μM (lane 5), 0.1 μM (lane 6), 0.01 μM (lane 7) recombinant intact 7B2 (i7B2, residues 1–185), or in the presence of both 1 μM p7B2 and 1 μM (lane 8), 0.1 μM (lane 9), 0.01 μM (lane 10) i7B2. In addition, incubations were performed in the presence of 1 μM p7B2 and 5 mM EDTA (lane 11), 1 μM heat-treated p7B2, 0.1 $\mu\text{g}/\mu\text{l}$ ACTH in the absence (lane 13) or presence (lane 14) of 1 μM p7B2. (B) Lysates were not incubated (lane 1), incubated in the absence of 7B2 (lane 2), in the presence of i7B2 (lane 3), or in the presence of recombinant truncated forms of 7B2, pNF177 (residues 1–177; lane 4), pNK171 (residues 1–171; lane 5), pNG162 (lane 6), p7B2 (lane 7), pNG86 (residues 1–86; lane 8), pCP93 (residues 93–185; lane 9), or pCD125 (residues 125–185; lane 10).

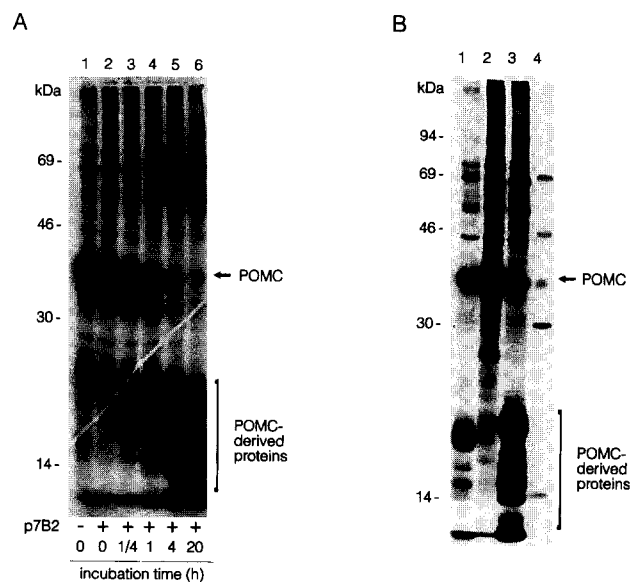


Fig. 2. Analysis of the effect of processed 7B2 on POMC conversion in *Xenopus* intermediate pituitary lysates. (A) Time course of POMC conversion. Newly synthesized proteins produced by *Xenopus* intermediate pituitaries were extracted (lane 1) and incubated for 0 min (lane 2), 15 min (lane 3), 1 h (lane 4), 4 h (lane 5), or 20 h (lane 6) in the presence of 1 μM processed 7B2 (p7B2) and analyzed by SDS-PAGE. (B) POMC cleavage products generated in vivo and in vitro. Newly synthesized proteins produced by *Xenopus* intermediate pituitaries after a 2-h pulse were extracted and analyzed by SDS-PAGE (lane 1). Newly synthesized proteins were extracted and directly analyzed (lane 2) or incubated for 4 h in the presence of 1 μM p7B2 (lane 3). Lane 4, molecular weight marker proteins.

(Fig. 1A, lane 8). Even when processed 7B2 was present in a 10-fold excess to intact 7B2, the conversion was completely blocked (Fig. 1A, lane 9).

Cleavage of POMC in the presence of the processed form of 7B2 was partially blocked by EDTA (Fig. 1A, lane 11), an agent known to inhibit many proteolytic enzymes, including PC2 [3]. Boiling of processed 7B2 did not affect its potency to enhance POMC cleavage (Fig. 1A, lane 12), indicating that 7B2 is heat-resistant. The fact that the dibasic-site containing peptide ACTH (adrenocorticotrophic hormone) served as a competing substrate for POMC and inhibited 7B2-enhanced POMC conversion (Fig. 1A, lanes 13–14) suggests that POMC is cleaved at pairs of basic amino acids.

Subsequently, we examined the effects of a number of mutant forms of recombinant 7B2 on POMC conversion. The C-terminally truncated 7B2 mutant pNF177 (residues 1–177) and the N-terminally truncated 7B2 mutants pCP93 (residues 93–185) and pCD125 (residues 125–185) inhibited POMC cleavage to a degree similar to that of intact 7B2 (Fig. 1B, lanes 3–4, 9–10). On the contrary, mutants pNK171 (residues 1–171), pNG162 (residues 1–162), and the short mutant pNG86 (residues 1–86) hardly affected POMC conversion (Fig. 1B, lanes 5, 6 and 8). 7B2 mutant pNP131 (residues 1–131) was the only mutant having an enhancing effect on POMC cleavage similar to that of processed 7B2 (Fig. 1B, lane 7 and data not shown). The experiments with the mutants show that the C-terminal region of 7B2 is able to inhibit POMC conversion, whereas an N-terminal fragment lacking this region can stimulate POMC cleavage activity in the lysate.

3.2. Analysis of POMC conversion

A time course of 7B2-mediated POMC conversion in the *Xenopus* intermediate pituitary lysate shows a gradual decrease in the amount of 37-kDa POMC and the appearance of a set of smaller proteins ranging in size from 10 to 30 kDa (Fig. 2A). The larger cleavage products gradually disappeared after longer incubations and a set of proteins ranging in size from 10 to 16 kDa were generated. In the absence of processed 7B2, hardly any POMC conversion occurred during a 4-h incubation (see Fig. 1A, lane 2). When the lysate was incubated at 24°C for 1 h before the addition of processed 7B2, POMC was cleaved to the same extent as without the preincubation step (data not shown). This indicates that under the assay conditions the potency of 7B2 to stimulate POMC cleavage is not affected for at least 1 h. The POMC-derived proteins produced in vivo are slightly smaller (~1–2 kDa) than the in vitro generated cleavage products (Fig. 2B, lanes 1 and 3), presumably as a result of additional in vivo processing through carboxypeptidases.

3.3. Effect of recombinant 7B2 on POMC conversion by different tissue lysates and by immunopurified *Xenopus* PC2

POMC converting activity in the *Xenopus* intermediate pitu-

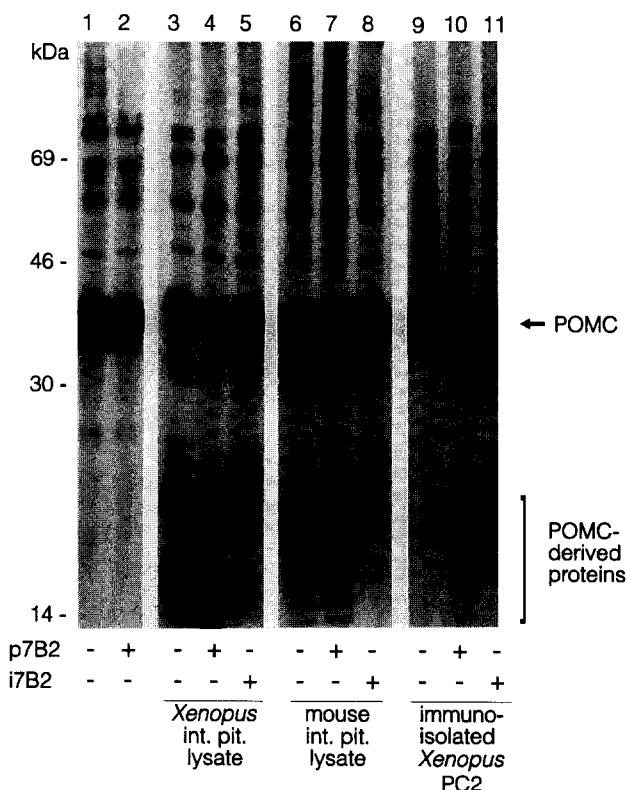


Fig. 3. Effect of processed and intact 7B2 on POMC conversion by different lysates and immunopurified *Xenopus* PC2. Newly synthesized proteins produced by *Xenopus* intermediate pituitaries were heat-treated and incubated for 4 h in the absence (lane 1) or presence (lane 2) of processed 7B2 (p7B2), with fresh non-labeled lysate of *Xenopus* intermediate pituitaries in the absence (lane 3) or presence of 1 μM p7B2 (lane 4) or 1 μM intact 7B2 (i7B2, lane 5), with lysates of mouse intermediate pituitaries in the absence (lane 6) or presence of 1 μM p7B2 (lane 7) or 1 μM i7B2 (lane 8), and with immunopurified *Xenopus* PC2 in the absence (lane 9) or presence of 1 μM p7B2 (lane 10) or 1 μM i7B2 (lane 11). Proteins were analyzed by SDS-PAGE.

itary lysate was irreversibly blocked by heating the lysate for 5 min at 90°C (Fig. 3, lane 1). Addition of only processed 7B2 to the heat-treated lysate did not result in prohormone conversion (Fig. 3, lane 2), showing that 7B2 itself is not able to cleave POMC. We therefore could use the heat-treated lysate to test POMC cleavage activity in other tissue lysates. Addition of fresh, unlabeled *Xenopus* intermediate pituitary lysate to the heat-treated radiolabeled proteins, led to some conversion of POMC (Fig. 3, lane 3). This conversion was greatly enhanced by processed 7B2 (Fig. 3, lane 4) and completely blocked by intact 7B2 (Fig. 3, lane 5). Similar results were obtained when *Xenopus* anterior pituitary lysate was used instead of intermediate pituitary lysate (data not shown). Incubations of lysates of *Xenopus* brain or heart with the heat-treated radiolabeled lysate did not lead to any POMC conversion (data not shown). Together, these results indicate that the enzyme responsible for POMC cleavage is enriched in *Xenopus* pituitary.

To examine whether neuroendocrine lysates from other species also possess 7B2-inducible POMC cleavage activity, mouse intermediate pituitary lysate was added to the heat-treated radiolabeled *Xenopus* lysate. In the presence as well as in the absence of processed 7B2, considerable conversion of POMC occurred (Fig. 3, lanes 6 and 7). This conversion could be blocked by intact 7B2 (Fig. 3, lane 8). When diluted mouse lysate was used, the extent of POMC cleavage was less, but also under these conditions no enhancing effect by processed 7B2 was observed (data not shown). Thus, the effect of processed 7B2 on prohormone cleavage appeared to be species specific.

To show that PC2 represents the POMC-cleaving enzyme in these experiments, PC2 was immunopurified from *Xenopus* intermediate pituitaries and the immunisolated PC2 was incubated with heat-treated radiolabeled lysate. Addition of purified PC2 alone did not result in POMC conversion (Fig. 3, lane 9) but when processed 7B2 was included, POMC was partially cleaved into smaller products with the same sizes as those produced with the intermediate pituitary lysate (Fig. 3, lane 10).

4. Discussion

In the present study, we investigated the effect of recombinant 7B2 on the in vitro processing of POMC. The intact form and several mutants of 7B2 (i.e. pNF177, pCP93 and pCD125) blocked POMC conversion; these 7B2 forms have previously been demonstrated to act as specific inhibitors of PC2 activity in in vitro enzyme assays [24–25]. Surprisingly, the processed form of 7B2 had a strong enhancing effect on POMC cleavage and caused conversion of newly synthesized 37-kDa POMC into a number of 10–30 kDa proteins in a dose-dependent manner. Incubations with truncated forms of 7B2 revealed that a region between residues 86 and 131 is critical for the enhancing effect and that the presence of the 7B2 region between residues 151 and 162 abolishes the ability of 7B2 to enhance POMC processing.

The experiments with heat-treated lysate indicate that not 7B2 itself, but a component of the intermediate pituitary lysate is responsible for POMC cleavage. Several observations suggest that the responsible component in the lysate is the prohormone convertase PC2. First, the PC2-blocking agent EDTA and the PC2 substrate ACTH had an inhibitory effect on the enhanced POMC conversion. Second, only lysates of tissues that are known to produce considerable amounts of PC2, like *Xenopus*

intermediate and anterior pituitaries, showed 7B2-inducible POMC processing. Third, the enhanced conversion of POMC by processed 7B2 was completely abolished when intact 7B2, a specific PC2-inhibitor, was present during the incubation. Fourth, in the presence of processed 7B2, immunopurified *Xenopus* PC2 was able to cleave newly synthesized POMC.

Since 7B2 is known to interact with PC2 and not with POMC [17], the enhancing effect of processed 7B2 on POMC conversion presumably results from activation of PC2 rather than an effect on POMC. Processed 7B2 was found to enhance POMC cleavage in *Xenopus*, but not in mouse pituitary lysate, indicating that the characteristics of PC2 differ between the two species. Possibly, in the mouse lysate, PC2 is already present in an active form, whereas in the *Xenopus* lysate the enzyme is virtually inactive and apparently needs processed 7B2 to become active. Also in our in vitro enzyme assays, processed 7B2 did not have any effect on mouse or rat PC2 activity [25].

Concerning the mechanism underlying the effect of processed 7B2 on POMC processing by *Xenopus* PC2, several possibilities should be considered. Since the N-terminal half of 7B2 (amino acids 1–95) is distantly related to a subclass of molecular chaperones [17], this region of 7B2 might be involved in protein-protein interactions and as such may affect the conformation of its substrate PC2. In the *Xenopus* lysate, PC2 may be present in a misfolded state or as a folding intermediate, and processed 7B2 could then assist in its proper folding or prevent its aggregation. The enhancing effect of processed 7B2 on PC2 activity could also arise from an interaction of 7B2 with the proregion of PC2 rather than with the mature enzyme. For the propeptides of subtilisin and α -lytic protease it has been shown that they can function as strong competitive inhibitors of their respective enzymes [28–29]. If the proregion of PC2 has a similar effect, release of the propeptide from the mature enzyme is required for its activation and this process may be mediated by 7B2. Using an in vitro binding assay, we previously showed that processed 7B2 associates with proPC2, but not with mature PC2 [17], indicating that processed 7B2 is indeed able to interact with the proregion of proPC2. Interestingly, Zhu and Lindberg recently demonstrated that the processed form of 7B2 facilitates production of enzymatically active PC2 in transfected cell lines [30]. Collectively, the results indicate that the N-terminal portion of 7B2 might well act as a chaperone for PC2.

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