Enzymatic Characterization of Immunopurified Prohormone Convertase 2: Potent Inhibition by a 7B2 Peptide Fragment†

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ABSTRACT: Prohormone convertases (PCs) are thought to mediate the controlled proteolysis of prohormones and neuropeptide precursors. While recombinant PC1 and furin are currently available, thus far it has not been possible to produce recombinant PC2. We have used conditioned medium obtained from the mouse insulinoma cell line βTC3 to generate a working preparation of enzymatically active PC2 through immunopurification. Immunopurified PC2 cleaved the fluorogenic substrate Cbz-Arg-Ser-Lys-Arg-AMC in a time- and calcium-dependent manner. It was half-maximally stimulated at 75 μM Ca^{2+}, had an optimum pH of 5, and exhibited PCMS and EDTA sensitivity similar to that reported for furin and PC1. The tight-binding inhibitor 27 kDa 7B2 was used to calculate the K_d for this inhibitor and the active enzyme concentration. The K_d was 7.3 ± 1.7 nM, and the turnover rate of PC2 was 5.2 molecules substrate per enzyme molecule per minute. The specific activity was 4.9 nmol/μg/h (assuming a molecular mass for PC2 of 64 kDa). The enzyme preparation was able to cleave recombinant proenkephalin at at least four of the expected paired basic sites in the absence, but not in the presence, of 27 kDa 7B2. Since 21 kDa 7B2 is functionally inactive as a proteinase inhibitor, we examined the inhibitory activity of the carboxy-terminal portion of 27 kDa 7B2 (7B2 CT-peptide). Synthetic peptides were used to demonstrate that the 7B2 CT-peptide (a) represents a potent inhibitor of PC2 (K_i = 57 nM), (b) can block the conversion of proPC2 to PC2, and (c) can block the PC2-mediated conversion of proenkephalin to smaller peptide fragments. This peptide thus may represent a useful tool in the study of prohormone conversion.

Prohormones require the action of specific prohormone convertases (PCs)[1] for maturation into bioactive species [reviewed in Lindberg (1991); Hutton, 1992]. These enzymes, now known to be subtilisin-like serine proteinases, are preferentially expressed in neuroendocrine tissues and have been shown, through transfection and antisense experiments, to participate in the physiological conversion of several important peptide hormone precursors (Benjannet et al., 1991; Thomas et al., 1991; Bloomquist et al., 1991; Zhou et al, 1993; Breslin et al., 1993). While it has been possible to produce enzymatically active PC1 (also known as sPC3; Smeekens et al., 1992) through several eukaryotic expression systems (Jean et al., 1993; Rufaut et al., 1993; Zhou & Lindberg, 1993), to date the production of significant quantities of recombinant, enzymatically active PC2 has not been reported, despite the fact that this enzyme was first identified 5 years ago (Seidah et al., 1990; Smeekens et al., 1990). The type II enzyme partially purified from insulinoma granules obtained from solid tumors has recently been shown to represent PC2 (Bennett et al., 1992); however, the limited availability of tumor tissue and the difficulty of purifying enzymes from subcellular organelles severely limit this approach to obtaining significant quantities of purified PC2. Recombinant PC2 thus far has been characterized only by expression in *Xenopus* oocytes, where it displayed an optimum pH of 5.5 and was activated by Ca^{2+} in the millimolar range ($K_{0.5}$ = 1 mM) (Shennan et al., 1991). Overexpression of PC2 in Chinese hamster ovary cells by a strategy similar to that used successfully for PC1 did not result in the production of active enzyme (Shen et al., 1993).

In order to facilitate the study of this important prohormone convertase, we have developed an immunopurification system to prepare active PC2 from the conditioned medium of a mouse insulinoma cell line. In this report, we present the enzymatic characterization of this enzyme as authentic PC2 and describe a method for the active-site titration of the enzyme preparation.

Many proteolytic conversion systems are regulated through inhibitor systems; we have recently shown that the neuroendocrine peptide 7B2 represents a potent inhibitor of PC2 (Martens et al., 1994). This interesting protein contains two domains homologous to other proteins: an amino-terminal domain with similarity to the heat shock family of chaperonins (Braks & Martens, 1994) and a carboxy-terminal domain weakly homologous to the potato I family of subtilisin inhibitors (Martens et al., 1994). *In vivo,* however, 27 kDa 7B2 (pro7B2) is cleaved within the Golgi apparatus into a 21 kDa protein and a 3 kDa carboxy-terminal peptide (Ayoubi et al., 1990; Paquet et al., 1991, 1994) (molecular masses refer to apparent sizes during gel electrophoresis rather than actual molecular masses). Since 21 kDa 7B2 is

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[1] Abbreviations: AMC, aminomethylcoumarin; POMC, proopiomelanocortin; PC1 and PC2, prohormone convertase 1 and 2; DMEM, Dulbecco's Modified Eagle's medium; NP40, Nonidet P-40; DTT, dithiothreitol; ir, immunoreactive; PMSF, phenylmethanesulfonyl fluoride; *p*-CMS, *p*-(chloromercuri)benzenesulfonic acid; E-64, *trans*-epoxysuccinic acid; TPCK, L-1-tosylamido-, 2-phenylethyl chloromethyl ketone; TLCK, *N*-tosyl-*l*-lysyl chloromethyl ketone; 7B2 CT-peptide, human $7B2_{155-185}$.

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functionally inactive as a proteinase inhibitor (Martens et al., 1994), we have explored the possibility that the 3 kDa carboxy-terminal piece (referred to here as the 7B2 CT-peptide) is responsible for the observed inhibition of 7B2. In addition, we have used this peptide to block the *in vitro* conversion of proPC2 to PC2 as well as the PC2-mediated cleavage of a model prohormone precursor, proenkephalin.

MATERIALS AND METHODS

Materials. Proteinase inhibitors were obtained from Sigma Chemical Company (St. Louis, MO), except for recombinant human 27 and 21 kDa 7B2s (Martens et al., 1994) and peptidyl chloromethyl ketones (a kind gift of Dr. Elliott Shaw). 7B2 proteins were generated in *E. coli* using a tag of six histidines at the amino terminus (Qiagen) and were a generous gift of Dr. Gerard Martens and Martin van Horssen (Nijmegen, The Netherlands). Custom synthesis of the substrate Cbz-Arg-Ser-Lys-Arg-AMC was performed by Enzyme Systems Products (Dublin, CA). Synthetic peptides were obtained from both LSUMC Core Laboratories and Genosys Biotechnologies (Woodland, TX).

Cell Culture. βTC3 cells (obtained from S. Efrat, Albert Einstein College of Medicine) were grown in DMEM (Gibco) containing 1 g/L glucose and 10% fetal bovine serum (FBS). They were maintained at 37 °C and 5% CO_2 and split at a 1:3 ratio twice a week.

Collection of the Conditioned Medium. Sequential collections of 100 mL each of conditioned medium (OptiMEM, GIBCO, containing 100 μg/mL aprotinin (Sigma)) from confluent roller bottles were performed every 24 h for 3−4 days, and the medium was centrifuged immediately at low speed (to remove floating cells) before storage at −20 °C.

Anti-PC2 Antiserum. Polyclonal antiserum was raised in rabbits against the 10 carboxy-terminal amino acids of mouse PC2 conjugated to keyhole limpet hemocyanin (Pierce) as previously described (Shen et al., 1993); the antiserum was pretreated with PMSF (1 mM) and heated for 10 min at 60 °C to destroy complement proteases.

Inhibitor cocktail was prepared at 10× concentration. It contained 10 μM *trans*-epoxysuccinic acid (E-64), 10 μM pepstatin, 2.8 mM tosylphenylalanyl chloromethyl ketone (TPCK) and 1.4 mM tosyllysyl chloromethyl ketone (TLCK).

Immunopurification of PC2. Two hundred milliliters of the conditioned medium was thawed and centrifuged at 10000*g*, and the supernatant was concentrated by 10−15-fold using an Amicon ultrafiltration device fitted with a YM10 membrane. Following concentration, dithiothreitol (DTT) and phenylmethanesulfonyl fluoride (PMSF) were added to final concentrations of 10 μg/mL and 0.5 mM, respectively. PC2 was immunoprecipitated by mixing the concentrated medium with 500 μL of anti-PC2 antiserum (final dilution 1:30 to 1:60) and one-tenth volume of the inhibitor cocktail. After the mixture was incubated for 6−7 h at 4 °C, 1 mL of washed 50% protein A−Sepharose in Dulbecco's PBS was added, followed by shaking for 60 min at 4 °C. The beads were pelleted at 4 °C by low-speed centrifugation and quickly washed twice with 5 mL of cold AG buffer (0.1 M sodium phosphate (pH 7.4), 1 mM EDTA, 0.1% Triton X-100, 0.5% Nonidet P-40, and 150 mM NaCl) and three times with 5 mL of cold Dulbecco's PBS. The enzyme preparation, including the protein A−Sepharose beads, was stored frozen in aliquots in a final volume of 2−3 mL of PBS containing 10% glycerol, in the presence of one-twentieth volume of 10× inhibitor cocktail. Ten microliters of this 10% suspension was used as the enzyme source in the enzyme assay. PC2 enzymatic activity and immunoreactivity (tested by Western blotting) are absent if either preimmune serum or antigen-blocked anti-PC2 serum is used in the immunopurification (Shen et al., 1993). No enzymatic activity was precipitated using PC1 antisera directed to either the amino or carboxy terminus of PC1; amino-terminal PC2 antiserum precipitated only one-fifth as much activity as carboxyl-terminal antiserum (results not shown). It was important to use conditioned medium obtained from earlier passages (numbers 38−52) as prolonged subculturing appeared to result in the loss of secretion of active PC2. Overnight immunoprecipitation could also be performed with good recovery of activity; however, this resulted in the loss of the upper immunoreactive band during Western blotting.

Enzyme Assay. Duplicate or triplicate reactions were performed in a 50 μL volume containing 0.1 M sodium acetate (pH 5.0−5.5), 0.2 mM RSKR-AMC (Lindberg et al., 1992), 5 mM calcium chloride, the above-mentioned inhibitor cocktail, and 10 μL of the enzyme suspension. All experiments were repeated at least twice with different enzyme preparations. Reactions to test the inhibitors were preincubated for 30 min at room temperature with inhibitors before adding substrate. Released AMC was measured every hour by fluorometry using a Cambridge Technologies microtiter plate fluorometer (excitation 380 nm, emission 460 nm). Values were compared to a standard curve of free AMC.

All peptides were synthesized through standard solid-phase chemistry using Fmoc/*tert*-butyl protection; successive elongations of the peptide chain were performed by coupling with TBTU in dimethylformamide (Genosys Biotechnologies). Purity was verified using mass spectroscopy and reverse-phase high-pressure liquid chromatography; all peptides were at least 85% pure. Stock solutions of peptides were prepared at 10 mM in water, and further dilutions were made into 0.1% Brij and 0.01 mg/mL BSA (Calbiochem, crystalline grade). Preincubation with enzyme was carried out for 30 min at room temperature before the addition of substrate and incubation at 37 °C as described earlier; the reactions also contained 2 μg of BSA and 0.1% Brij to avoid losses of peptide by nonspecific adsorption. From five to seven concentrations of each peptide were tested in duplicate. Results were plotted as percent inhibition *vs* peptide concentration, and the K_is were calculated through nonlinear regression. Three independent determinations were performed using three enzyme preparations; the results are presented as the mean ± the standard error of these three determinations.

Titration Curve. The K_d of the 7B2−PC2 complex and the concentration of PC2 reacting with 7B2 were determined by fitting the predicted inhibition curve to the data. The model assumed that 7B2 and PC2 form a 1:1 complex and that the enzyme in the complex was completely inhibited. The equation describing this phenomenon is

$$v = \frac{v_o(E_t - [EI])}{E_t}$$

where

$$[EI] = \left\{K_d + E_t + I_t - \sqrt{(K_d + E_t + I_t)^2 - 4E_tI_t}\right\}/2$$

The Marquardt−Levenberg algorithm (Bevington & Robinson, 1992) was used to determine the best least-squares fit to the data; fitted parameters were K_d, total enzyme concentration (E_t), and uninhibited velocity (v_o). Uncertainty in the fitted parameters was determined in two ways: from the curvature matrix of the Marquardt algorithm and by Monte Carlo simulation as described by Bevington and Robinson (1992). The range in each parameter is that in which the increase in $X^2 \leq$ 2-fold. Both methods gave equivalent results.

Proenkephalin Cleavage by PC2. Twenty micrograms of recombinant rat proenkephalin (Lindberg et al., 1991) was incubated with 20 μL of immunopurified PC2 in a 100 μL reaction volume containing 5 mM calcium, 100 mM sodium acetate (pH 5.0), 0.1% Brij-35, and the inhibitor cocktail described earlier (final concentration of proenkephalin = 7.1 μM). A parallel sample also contained 1 μg of 27 kDa 7B2 (0.45 μM final concentration). Following 7 h of incubation at 37 °C with rocking, samples were stored frozen prior to the analysis of cleavage using high-pressure gel permeation chromatography (Mathis & Lindberg, 1992). Radioimmunoassay for Met-enk-Arg-Phe-immunoreactive (ir) peptides was carried out on duplicate aliquots of column fractions dried in the presence of 10 μg of bovine serum albumin; this assay detects proenkephalin, peptide B, and Met-enk-Arg-Phe (Breslin et al., 1993). Peptide standards were obtained from Peninsula (Belmont, CA) or prepared from acid extracts of bovine adrenal medullary chromaffin granules through reverse-phase HPLC [Lindberg (1983) and unpublished results]. Radioimmunoassay for Met-enk-Arg-Gly-Leu-ir peptides was carried out as described in Lindberg and White (1986); aliquots of column fractions were treated for 20 min with 3.6×10^{-6} unit of carboxypeptidase B (CPB, Worthington, PMSF-treated) in 0.1 M Tris Cl and 0.01% BSA (pH 7.5) for 20 min in order to remove carboxy-terminal basic amino acids (to reveal immunoreactivity generated by PC2). The CPB reaction was terminated by boiling, and the samples were subjected to radioimunoassay. It should be noted that the cross-reaction of the 18 and 5.3 kDa peptides (previously identified in bovine adrenal medulla; Udenfriend & Kilpatrick, 1983) in this assay is not known, and the results shown are likely to represent underestimates of their actual values. Three chromatographic experiments were carried out using independent enzyme preparations; similar product profiles were observed each time.

For the detection of cleavage of proenkephalin over time in the presence and absence of 7B2 CT-peptide, it was necessary to employ an assay that could discriminate between proenkephalin and the cleavage product. Since the assay for Met-enk-Arg-Phe cross-reacts with proenkephalin and peptide B, we monitored the production of Met-enk-Arg-Gly-Leu-ir peptides (which arise from an interior portion of proenkephalin). For these experiments, 20 μg of proenkephalin was incubated with PC2 using the reaction conditions described earlier in the presence or absence of 1 μM 7B2 CT-peptide for the times indicated (50 μL total volume; final proenkephalin concentration, 14 μM). The samples were stored frozen at −20 °C until assay for the liberation of Met-enk-Arg-Gly-Leu-ir. Radioimmunoassay for peptides terminating in this sequence was carried out on duplicate diluted aliquots of the original reaction mixtures, which were treated with carboxypeptidase B as described earlier.

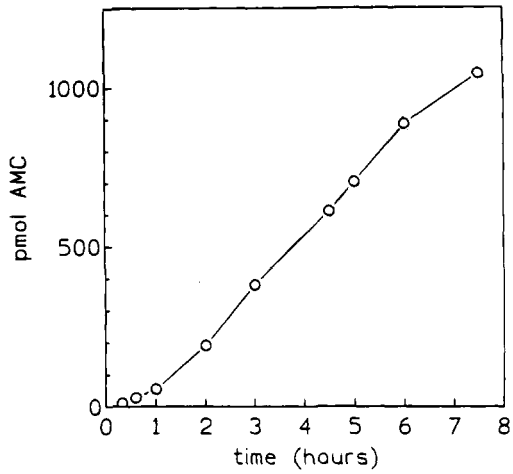

FIGURE 1: Time dependence of enzymatic activity. Enzyme activity was measured every hour. All values are averages of at least two independent observations, which typically differed from the mean by less than 15%.

Western Blotting. In order to determine whether cleavage of proPC2 could be prevented by 7B2 peptides, we incubated immunopurified PC2 for varying lengths of time in the presence or absence of 1 μM 7B2 CT-peptide. The reactions were stopped by the addition of one-tenth volume of 10× Laemmli sample buffer, boiled, centrifuged, and subjected to electrophoresis on 8.8% gels. Following blotting to nitrocellulose, samples were probed with an antiserum to PC2 as previously described (Shen et al., 1993).

RESULTS

PC2 immunopurified from conditioned medium of the insulinoma cell line βTC3 was used to produce and characterize an RSKR-AMC-cleaving activity, which was obtained as a solid-phase enzyme linked to PC2 antibody, protein A, and Sepharose. The proteolytic activity of the immunopurified enzyme was dependent on time (Figure 1). After a variable lag period (0−60 min), the time course became linear. Immunopurified PC2 was activated by Ca^{2+} in the micromolar range ($K_{0.5}$ = 75 μM; Figure 2A). Optimum activity was found at pH 5.0 (Figure 2B), and no activity was present at pHs below 3.5 or above 7.5.

The profile of inhibition of various agents is given in Table 1. EDTA, *p*-CMS, and the two active-site-directed chloromethyl ketones (D-Tyr-Ala-Lys-Arg-CH_2Cl and Pro-Gly-Lys-Arg-CH_2Cl) represented potent inhibitors. The other two chloromethyl ketones tested (TLCK and TPCK), as well as soybean trypsin inhibitor and E-64, did not decrease the activity substantially. PMSF, pepstatin, aprotinin, 1,10-phenanthroline, and recombinant human 21 kDa 7B2 did not inhibit at all. In the presence of DTT, enzyme activity was slightly, but consistently, increased. As expected, recombinant human 27 kDa inhibited PC2 activity very efficiently (Martens et al, 1994). The addition of the detergent Brij to 0.1% (as previously described for PC1; Zhou & Lindberg, 1993) was not stimulatory.

The operational molarity of the PC2 enzyme preparation was calculated by titration with the tight-binding inhibitor

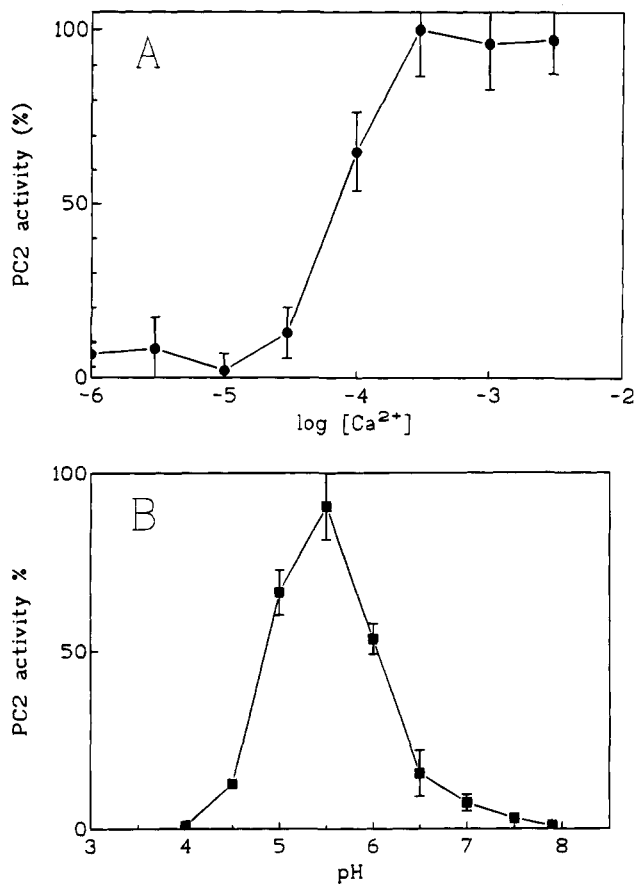

FIGURE 2: (A) Calcium dependence of enzymatic activity. Before the assays were started, the PC2 beads were washed three times with 10 vol of PBS without calcium (22 mM Na_2HPO_4, 8.3 mM NaH_2PO_4, and 150 mM NaCl (pH 7.4)). EDTA (2 μM) was added to all samples. Fluorescence was measured after 6 h. The effect of the different calcium concentrations is expressed as a percentage of maximum activity. Each value is the mean ± SE of three independent observations. (B) pH optimum of enzymatic activity. Before the assays (as described in Materials and Methods) were started, the immunopurified enzyme was washed in buffers of different pHs (100 mM sodium acetate and 100 mM bis-tris). Fluorescence was measured after 6 h of incubation at 37 °C. Activity at the different pHs is expressed as a percentage of the maximum activity. Each value is the mean ± SE of three independent observations.

27 kDa 7B2 (Figure 3). Assuming 1:1 stoichiometry, the amount of active PC2 during the enzyme assay was calculated to be 6.8 ± 3.1 nM, and the turnover rate of PC2 was estimated at 5.2 molecules substrate per enzyme molecule per minute (range within error limits 3.4−10). The equilibrium constant K_d for the dissociation of 27 kDa 7B2 was 7.3 ± 1.7 nM, and the specific activity was 4.9 nmol/μg/h (assuming a molecular mass of 64 000 Da).

Immunopurified PC2 cleaved micromolar quantities of recombinant rat proenkephalin to several of the expected lower molecular weight enkephalin-immunoreactive products, namely, peptide B and Met-enk-Arg-Phe (Figure 4A), peptides produced by paired basic cleavages. Approximately one-half of the substrate remained intact following the 7 h incubation. PC2 also generated the 18 and 5.3 kDa Met-enk-Arg-Gly-Leu-immunoreactive peptides from proenkephalin, but was unable to generate free Met-enk-Arg-Gly-Leu, which by comparison to standard peptide would have eluted in fraction 48 (Figure 4B). The PC2-specific inhibitor 27 kDa 7B2 was able to block the cleavage of proenkephalin by immunopurified PC2 (Figure 4). The sites of cleavage

Table 1: Effect of Protein Inhibitors on PC2 Activity[a]

inhibitors	final concentration	% inhibition
27 kDa 7B2	1 μM	100
	4.4 nM	50
EDTA	5 mM	98
	0.5 mM	82.8
p-CMS	1 mM	94.8
D-Tyr-Ala-Lys-Arg-CH_2Cl	0.1 mM	93
Pro-Gly-Lys-Arg-CH_2Cl	0.1 mM	83.8
TPCK	0.28 mM	29.6
soybean trypsin inhibitor	0.15 mg/mL	19.7
TLCK	0.14 mM	14.6
trans-epoxysuccinic acid	0.1 mM	12.4
PMSF	1 mM	0
pepstatin	0.1 mM	0
aprotinin	0.1 mM	0
1,10-phenanthroline	1 mM	0
21 kDa 7B2	0.1 mM	0
dithiothreitol	1 mM	−14

[a] Before the substrate was added, the enzyme and the inhibitor were preincubated for 30 min at room temperature. After 6 h of reaction, the fluorescence was measured. The effect of the different inhibitors is expressed as a percentage of the control (without proteinase inhibitors). Values represent averages of at least two independent observations.

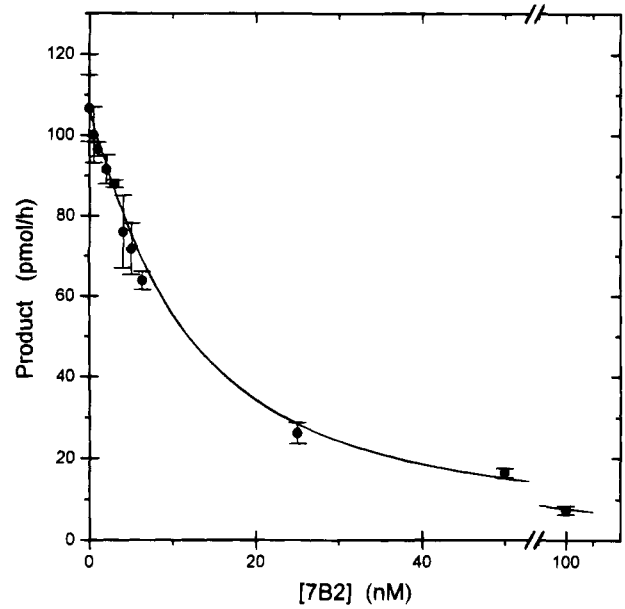

FIGURE 3: Titration curve. Varying concentrations of recombinant 27 kDa 7B2 were incubated with PC2 for 30 min at room temperature prior to substrate addition. Fluorescence was measured following 6 h of incubation at 37 °C. The concentration of inhibitor chosen is given on the *x* axis (in nM); the *y* axis represents the fractional amount of enzyme activity as a percentage of the control without inhibitor. Each value is the mean ± SE of three independent observations.

of proenkephalin by immunopurified PC2 involved in these cleavages are indicated in Figure 5. In three independent experiments, the production of free Met-enk-Arg-Gly-Leu by PC2 was not observed.

In order to test the possibility that the carboxyl-terminal region of 7B2 was responsible for the observed inhibition of PC2, we incubated synthetic peptides corresponding to human $7B2_{155-185}$ (termed 7B2 CT-peptide, a natural cleavage product; Paquet et al., 1991; Sigafoos et al., 1993) and $7B2_{155-178}$ with the enzyme preparation (Figure 6). The experiments revealed that these peptides represent potent inhibitors of PC2, with K_is of 57 and 180 nM, respectively.

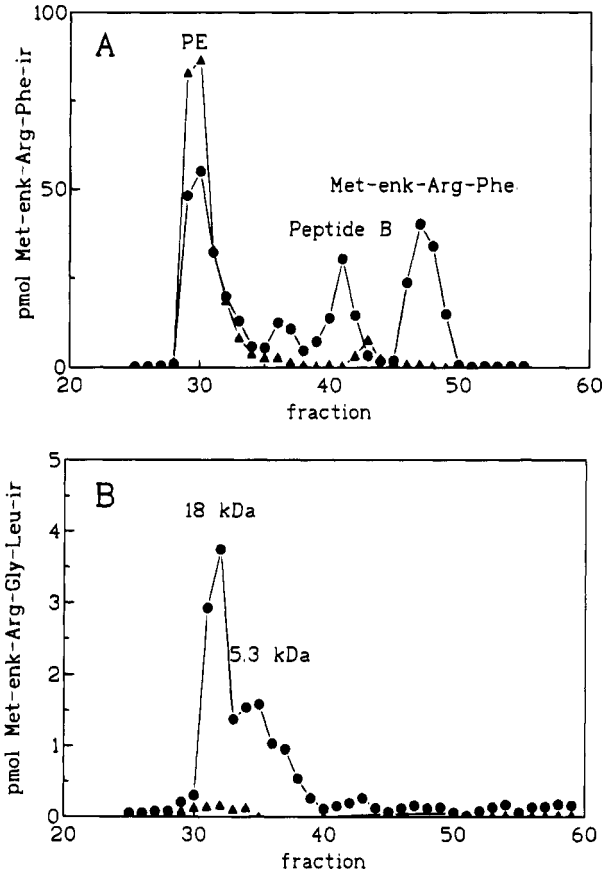

FIGURE 4: Cleavage of recombinant proenkephalin by immunopurified PC2. Recombinant rat proenkephalin was incubated with PC2, and the products were separated by high-pressure gel permeation chromatography. Peptides in each fraction were quantitated by radioimmunoassay. Panel A: Met-enk-Arg-Phe-ir peptides. Panel B: Met-enk-Arg-Gly-Leu-ir peptides. (●) PC2 alone; (▲) PC2 plus 1 μg of 7B2. PE: rat proenkephalin.

When a series of truncated and substituted peptides was incubated with PC2, the results indicated that peptides smaller than 16 amino acids were inactive and that the presence of an intact Lys-Lys site was critical for the potent inhibition of PC2 (Table 2).

The ability of the 7B2 CT-peptide ($7B2_{155-185}$) to block the generation of PC2 from proPC2 was demonstrated by the incubation of this peptide (at 1 μM concentration) with freshly immunopurified PC2 (which contains a small amount of proPC2) for varying lengths of time (Figure 7). These results show that, *in vitro*, the conversion of proPC2 to PC2 is relatively rapid, mainly occurring within the first hour of incubation. The 7B2 CT-peptide was as efficient as EDTA in preventing this cleavage; these results are in agreement with previous data, which show that 27 kDa 7B2 can block this conversion (Martens et al., 1994).

The 7B2 CT-peptide also proved to be very useful in the blockade of proenkephalin conversion to smaller fragments (Figure 8). This figure shows that PC2 mediated the time-dependent conversion of proenkephalin to Met-Enk-Arg-Gly-Leu-ir peptides in the absence, but not in the presence, of the 7B2 CT-peptide. The maximal rate of production of Met-enk-Arg-Gly-Leu-ir peptides (mainly the 18 kDa peptide; Figure 4) was about 8 pmol/h (from a starting amount of 707 pmol of proenkephalin or 14 μM); this figure most likely represents an underestimate since the cross-reaction of the 18 kDa peptide in this assay is unlikely to be quantitative.

DISCUSSION

The prohormone convertase PC2 is thought to mediate the conversion of proopiomelanocortin (Zhou et al., 1993; Thomas et al., 1991; Benjannet et al., 1991), proenkephalin (Breslin et al., 1993), proinsulin (Bennett et al., 1993; Smeekens et al., 1992), and prosomatostatin (Mackin & Noe, 1991; Galanopoulou et al., 1993) to smaller peptide products. However, biochemical studies of PC2 have been limited to date since it has not been possible to obtain purified recombinant PC2 through standard eukaryotic overexpression methods.

In this report, we present the enzymatic characterization of PC2 immunopurified from medium conditioned by the mouse insulinoma cell line βTC3. The time course of activity of PC2 indicated a linear relationship between time and enzyme activity, except for a brief lag during the initial portion of the enzyme assay, which varied considerably in duration depending on the enzyme preparation. The lag period is most likely related to the restoration of correct conformation/calcium binding following purification at high pH and/or in EDTA-containing solutions. The lag period is not related to the *in vitro* conversion of proPC2 to PC2 since enzyme that has been isolated by overnight immunoprecipitation no longer contains visible quantities of proPC2, but still often exhibits a lag phase.

The inhibitor profile of immunopurified PC2 is generally similar to that of kexin, furin, and PC1, i.e., sensitivity to sulfhydryl-directed reagents and EDTA (Brenner & Fuller, 1992; Molloy et al., 1992; Hatsuzawa et al., 1992; Jean et al., 1993; Rufaut et al., 1993; Zhou & Lindberg, 1993), with the exception of inhibition by 27 kDa 7B2, which does not inhibit PC1 (Martens et al., 1994). Our data indicate that the enzyme preparation is slightly contaminated with traces of thiol protease activity, which is well inhibited by a combination of E-64, TLCK, and TPCK. It is therefore necessary to use these inhibitors during the enzyme assays, and all assays were carried out in the presence of the inhibitor cocktail.

The optimum activity of immunopurified PC2 is at about pH 5, while kexin (Fuller et al., 1989; Brenner & Fuller, 1992) and furin (Molloy et al., 1992; Hatsuzawa et al., 1992) have their optima at higher pHs. Furin and kexin are thought to be restricted to the Golgi region (Bresnahan et al., 1990; Wilcox & Fuller, 1991) where the pH is close to neutral (pH 6.4−7.2). PC1 and PC2 are expressed in neuroendocrine cells with a regulated secretory pathway and are localized both to the Golgi and to secretory granules (Christie et al., 1991; Hornby et al., 1993; Egger et al., 1993; Lindberg et al., 1994), in which the pH is more acidic (5.5−6.4).

Like kexin (Brenner & Fuller, 1992), furin (Molloy et al., 1992; Hatsuzawa et al., 1992), recombinant PC1 (Jean et al., 1993; Rufaut et al., 1993; Zhou & Lindberg, 1993), and oocyte-expressed PC2 (Shennan et al., 1991), immunopurified PC2 is a Ca^{2+}-dependent enzyme. Half-maximal stimulation occurred at about 75 μM calcium. This resembles the Ca^{2+} dependency of the insulin secretory granule type II endopeptidase, which is half-maximally activated at 100 μM (Hutton, 1992). Surprisingly, however, oocyte-synthesized PC2 exhibited half-maximal activity at millimolar calcium concentrations (Shennan et al., 1991); the reason for this discrepancy is not clear at present. Unlike

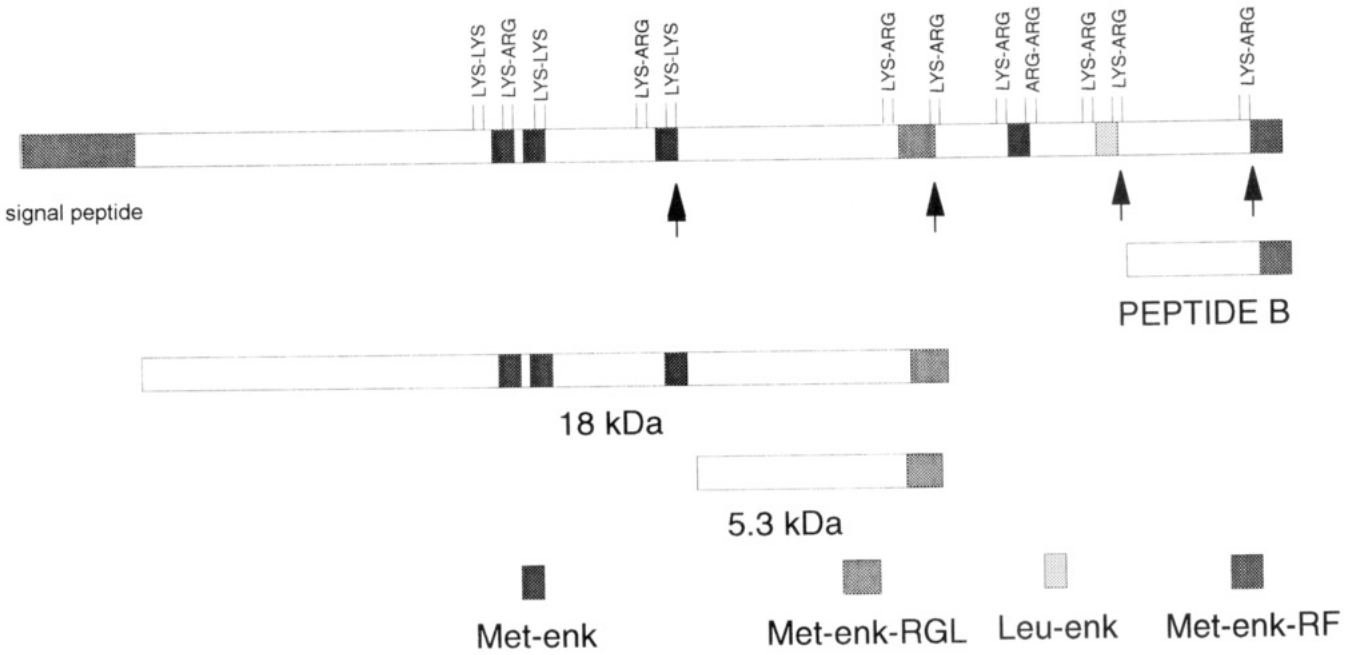

FIGURE 5: Sites of PC2-mediated cleavage of recombinant rat proenkephalin. Only sites for which data are available are shown; other sites may also be cleaved. However, free Met-enk-Arg-Gly-Leu was not produced.

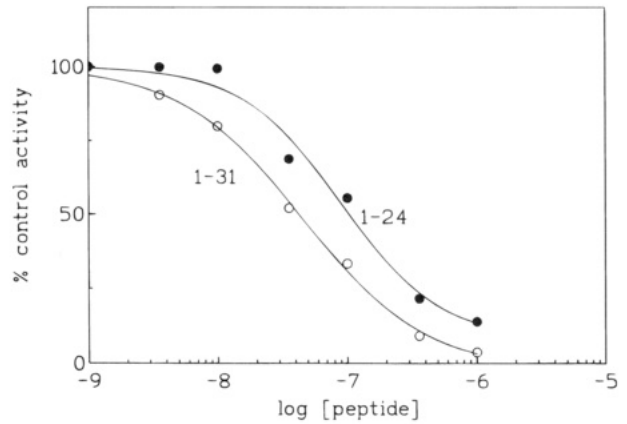

FIGURE 6: Inhibition of PC2 activity by peptides derived from the carboxy terminus of human 7B2. Immunopurified PC2 was incubated with fluorogenic substrate for 5 h in the presence of varying concentrations of $7B2_{155\text{-}185}$ (7B2 CT-peptide) (○); or $7B2_{155\text{-}178}$ (●).

Table 2: Inhibition of PC2 by 7B2 CT-Peptide Derivatives[a]

peptide	K_i
1−31 (7B2 CT-peptide)	57 ± 10 nM
1−24	180 ± 31 nM
1−24 K22→ S22	131 ± 35 μM
1−16	430 ± 42 μM
8−16	>1 mM
11−23	>1 mM
17−31	>1 mM

[a] Results indicate the mean ± the SE of K_is determined from three separate enzyme preparations. Human 7B2 CT-peptide sequence (Martens, 1988): Ser-Val-Asn-Pro-Tyr-Leu-Gln-Gly-Gln-Arg-Leu-Asp-Asn-Val-Val-Ala-Lys-Lys-Ser-Val-Pro-His-Phe-Ser-Asp-Glu-Asp-Lys-Asp-Pro-Glu.

the experiments reported here, Bennett et al. (1992) were unable to effectively immunoprecipitate PC2 activity from insulinoma granule lysates; this may be due to their use of antiserum directed against PC2 produced as a fusion protein (which possibly blocks the catalytic portion of PC2) rather than against the carboxy terminus, as used in our work.

The tight-binding inhibitor 27 kDa 7B2 was used to estimate the K_d for this inhibitor and the concentration of active enzyme. This protein, which represents a naturally occurring, potent inhibitor for PC2, inhibits only PC2 and not PC1 (Martens et al., 1994). By assuming a stoichiometric relationship between this inhibitor and PC2, the active enzyme concentration in the preparation was calculated to be 6.8 nM, yielding a specific activity of immunopurified PC2 of 4.9 nmol/μg/h. Despite the fact that this enzyme preparation contains enzyme tethered by its carboxy-terminal end to a solid support, this figure is comparable to that of purified recombinant PC1 and furin, which possess specific activities of about 3.6 and 1.2−10.7 nmol/μg/h, respectively (Zhou & Lindberg, 1993; Molloy et al., 1992; Hatsusawa et al., 1992). The turnover rate of active PC2 was estimated at 5.2 molecules of substrate per enzyme molecule per minute. It should be noted that our figures represent lower estimates for specific activity since the possibility of the binding of 7B2 to inactive enzyme forms cannot be excluded at this point.

Immunopurified PC2 also demonstrated the appropriate specificity against a recombinant prohormone substrate, rat proenkephalin, which it cleaved at certain Lys-Arg and Lys-Lys sites to yield peptide B and Met-enk-Arg-Phe. It is interesting that PC1 produces primarily peptide B under similar incubation conditions (I. Lindberg and Y. Zhou, unpublished results), confirming the previously reported wider specificity of PC2 as compared to PC1. When the production of immunoreactive peptides terminating in Met-enk-Arg-Gly-Leu was examined, the 18 and 5.3 kDa peptides, but not free Met-enk-Arg-Gly-Leu, were observed. These results are similar to data obtained using vaccinia virus infection of cell lines with PC2 and proenkephalin (Breslin et al., 1993) and leave open the question of how free Met-enk-Arg-Gly-Leu—an abundant proenkephalin-derived opioid peptide in certain tissues, such as brain—is generated from proenkephalin *in vivo*. It is interesting to note that both intact human 7B2 and its carboxy-terminal 31 amino acid peptide were able to completely block proenkephalin cleavage *in*

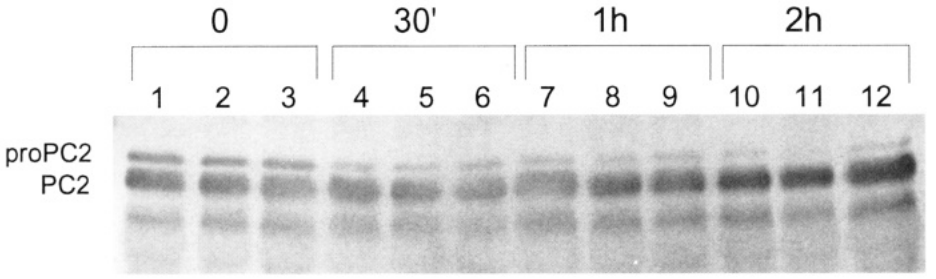

FIGURE 7: Inhibition of proPC2 conversion by 7B2 CT-peptide. Immunopurified PC2 was incubated at 37 °C for varying lengths of time in the presence or absence of 1 μM 7B2 CT-peptide; the reaction was terminated by the addition of sample buffer, and the conversion of proPC2 to PC2 was estimated by Western blotting. Lanes 1, 4, 7, and 10 represent controls containing 5 mM EDTA; lanes 2, 5, 8, and 11 contain 5 mM calcium; and lanes 3, 6, 9, and 12 contain 5 mM calcium and 1 μM 7B2 CT-peptide.

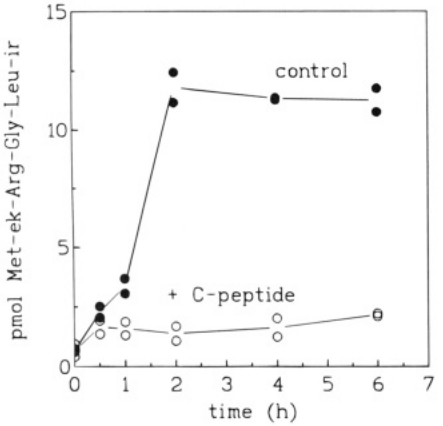

FIGURE 8: Inhibition of Met-enk-Arg-Gly-Leu-ir peptide production from proenkephalin by 7B2 CT-peptide. Recombinant proenkephalin (17 μM) was incubated with PC2 for varying lengths of time in the presence (○) or absence (●) of 1 μM 7B2 CT-peptide. The liberation of internal fragment(s) terminating in Met-enk-Arg-Gly-Leu was determined by radioimmunoassay.

vitro; recent data indicate that the recombinant rat 7B2 protein is also able to inhibit proenkephalin cleavage (X. Zhu and I. Lindberg, unpublished results).

The neuroendocrine protein 7B2 is initially synthesized as a proprotein of 27 kDa, which is cleaved to a 21 kDa mature form (Ayoubi et al., 1990; Paquet et al., 1994) through the action of furin in the trans-Golgi network (Paquet et al., 1994). The 7B2 proprotein has been shown to associate with proPC2 early in biosynthesis (Braks & Martens, 1994) and represents a potent inhibitor of PC2 activity (Martens et al., 1994). However, the 21 kDa mature form is completely inactive against PC2 [the apparent inhibition at the highest concentration in Martens et al. (1994) is due to the presence of high concentrations of imidazole in the recombinant protein preparation]. These results suggested that the inhibitory portion of 7B2 might reside in the carboxy-terminal segment removed during maturation of 27 kDa 7B2. Our present results obtained using synthetic peptides support this view. The high potency of the 7B2 CT-peptide, 57 nM, as well as its truncated derivative lacking the acidic 7 amino acid terminus, implies that it may be possible to use these peptides to experimentally manipulate PC2 activity, for example, within a cell culture system or within an *in vitro* system containing both PC1 and PC2. The identification of Lys-Lys$_{171\text{-}172}$ as the basic residues required for active-site binding to PC2 supports structure−activity results obtained through extensive 27 kDa 7B2 mutagenesis experiments (van Horssen et al., in press). Interestingly, the combination of two peptides that completely cover the 7B2 CT-peptide (residues 155−170 and 171−185) did not result in the additional inhibition of PC2 (not shown). These results imply that the binding of these peptides to PC2 is abolished by the presence of free amino and carboxyl termini near the PC2 binding site.

It is interesting that PC2 is capable of cleaving precursors at Lys-Lys sites (Breslin et al, 1993; this work), yet the 7B2 CT-peptide, which contains such a site, represents a potent inhibitor rather than a substrate for PC2. Comparison of the sites within proenkephalin and proglucagon (Rothenberg et al., in press) which are cleaved by immunopurified PC2 with the sequence of the 7B2 CT-peptide reveals that PC2 processing sites frequently contain an acidic residue in the immediate vicinity of the cleavage site; however, the region immediately surrounding the KK site within the 7B2 CT-peptide contains relatively few charged residues. Site-directed mutagenesis of this region has revealed an important role for the flanking valine residues (van Horssen et al., submitted for publication). It is likely that in the 7B2 CT-peptide the Lys-Lys site and its flanking regions form a discrete structure that is able to bind tightly to the active site of PC2.

In summary, our findings suggest that immunopurified PC2 is a calcium-dependent enzyme with an optimum pH of 5.0; it has a specific activity comparable to that of recombinant furin and PC1. Immunopurified PC2 is active against recombinant proenkephalin, which it cleaves at at least four of the expected dibasic sites. The immunopurification procedure using carboxy-terminal antiserum thus appears to be an effective working method to provide active PC2 for use in prohormone cleavage studies. In addition, we found that the recombinant protein 7B2 was very helpful in determining the operational molarity of PC2 preparations. The fact that the 31 amino acid carboxy-terminal peptide of 7B2 also proved to be a potent inhibitor of PC2 implies that it will represent a useful tool to distinguish the activity of PC2 from that of PC1 in mixed enzyme preparations.

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REFERENCES

Ayoubi, T. A. Y., Duijnhoven H. L. P., van de Ven, W. J. M., Jenks, B. G., Roubos, E. W., & Martens, G. J. M. (1990) *J. Biol. Chem. 265*, 15644−15647.

Bailyes, E. M., Shennan, K. I. J., Seal., A. J., Smeekens, S. P., Steiner, D. F., Hutton, J. C., & Docherty, K. (1992) *Biochem. J. 285*, 391−394.

Benjannet, S., Rondeau, N., Day, R., Chretien, M., & Seidah, N. G. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 3564−3568.

Bennett, D. L., Bailyes, E. M., Nielsen, E., Guest, P. C., Rutherford, N. G., Arden, S. D., & Hutton, J. C. (1992) *J. Biol. Chem. 267*, 15227−15236.

Bevington, P. R., & Robinson, D. K. (1992) in *Data Reduction and Error Analysis for the Physical Sciences*, McGraw-Hill, New York.

Bloomquist, B. T., Eipper, B. A., & Mains, R. E. (1991) *Mol. Endocrinol. 5*, 2014−2023.

Braks, J. A. M., & Martens, G. J. M. (1994) *Cell 78*, 263−273.

Brenner, C., & Fuller, R. S. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 922−926.

Breslin, M. B., Lindberg, I., Benjannet, S., Mathis, J. P., Lazure, C., & Seidah, N. G. (1993) *J. Biol. Chem. 268*, 27084−27093.

Bresnahan, P. A., Leduc, R., Thomas, L., Thorner, J., Gibson, H. L., Brake, A. J., Barr, P. J., & Thomas, G. (1990) *J. Cell Biol. 111*, 2851−2859.

Christie, D., Batchelor, D. C., & Palmer D. J. (1991) *J. Biol. Chem. 66*, 15679−15683.

Egger, C., Kirchmair, R., Hogue-Angeletti, R., Fischer-Colbrie, R., & Winkler, H. (1993) *Neurosci. Lett. 159*, 199−201.

Galanopoulou, A. S., Kent, G., Rabbani, S. N., Seidah, N. G., & Patel, Y. C. (1993) *J. Biol. Chem. 268*, 6041−6049.

Hatsuzawa, K., Nagahama, T., Takahashi, S., Takada, K., Murakami, K., & Nakayama, K. (1992) *J. Biol. Chem. 267*, 16094−16099.

Hornby, P. J., Rosenthal, S. D., Mathis, J. P., Vindrola, O., & Lindberg, I. (1993) *Neuroendocrinology 58*, 555−563.

Hutton, J. C. (1992) *Curr. Opin. Cell Biol. 2*, 1131−1142.

Jean, F., Basak, A., Rondeau, N., Benjannet, S., Hendy, G. N., Seidah, N. G., Chretien, M., & Lazure, C. (1993) *Biochem. J. 292*, 891−900.

Lindberg, I. (1991) *Mol. Endocrinol. 5*, 1361−1365.

Lindberg, I., & White, L. (1986) *Arch. Biochem. Biophys. 245*, 1−7.

Lindberg, I., Yang, H.-Y. T., & Costa, E. (1983) *Life Sci. 33* (Suppl. I), 5−8.

Lindberg, I., Shaw, E., Finley, J., Leone, D., & Deininger, P. (1991) *Endocrinology 128*, 1849−1856.

Lindberg, I., Lincoln, B., & Rhodes, C. J. (1992) *Biochem. Biophys. Res. Commun. 183*, 1−7.

Lindberg, I., Ahn, S. A., & Breslin, M. B. (1994) *Mol. Cell. Neurosci. 5*, 614−622.

Mackin, R. B., & Noe, B. D. (1991) *Endocrinology 129*, 2263−2265.

Martens, G. J. M. (1988) *FEBS Lett. 234*, 160−164.

Martens, G. J. M., Braks, J. A. M., Eib, D. W., Zhou, Y., & Lindberg, I. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 5784−5785.

Mathis, J. P., & Lindberg, I. (1992) *Endocrinology 131*, 2287−2296.

Molloy, S., Bresnahan, P. A., Leppla, S. H., Klimpel, L. R., & Thomas, G. (1992) *J. Biol. Chem. 267*, 16396−164210.

Paquet, L., Rondeau, N., Seidah, N. G., Lazure, C., Chretien, M., & Mbikay, M. (1991) *FEBS Lett. 294*, 23−26.

Paquet, L., Bergeron, F., Boudreault, A., Seidah, N. G., Chretien, M., Mbikay, M., & Lazure, C. (1994) *J. Biol. Chem. 269*, 19279−19285.

Rothenberg, M. E., Eilertson, C. D., Klein, K., Zhou, Y., Lindberg, I., McDonald, J. K., & Noe, B. D. (1995) *J. Biol. Chem.* (in press).

Rufaut, N. W., Brennan, S. O., Hakes, D. J., Dixon, J. E., & Birch, N. P. (1993) *J. Biol. Chem. 268*, 2091−2098.

Seidah, N. G., Gasper, L., Mion, P., Marcinkiewicz, M., Mbikay, M., & Chretien, M. (1990) *DNA Cell Biol. 9*, 145−424.

Seidah, N. G., Marcinkiewicz, M., Benjannet, S., Gaspar, L., Beaubien, G., Mattei, M. G., Lazure, C., Mbikay, M., & Chretien, M. (1991) *Mol. Endocrinol. 5*, 111−122.

Shen, F. S., Seidah, N. G., & Lindberg I. (1993) *J. Biol. Chem. 268*, 24910−24915.

Shennan, K. I. J., Smeekens, S. P., Steiner, D. F., & Docherty K. (1991) *FEBS Lett. 284*, 277−280.

Sigafoos, J., Chestnut, W. G., Merril, B. M., Taylor, L. C. E., Diliberto, E. J., & Viveros, O. H. (1993) *J. Anat. 183*, 253−264.

Smeekens, S. P., & Steiner, D. F. (1990) *J. Biol. Chem. 265*, 2997−3000.

Smeekens, S. P., Avruch, A. S., LaMendola, J., Chan, S. J., & Steiner, D. F. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 340−344.

Smeekens, S. P., Montag, A. G., Thomas, G., Albiges-Rizo, C., Carroll, R., Benig, M., Phillips, L. A., Martin, S., Ohagi, S., Gardner, P., Swift, H. H., & Steiner, D. F. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 8822−8826.

Thomas, L., Leduc, R., Thorne, B. A., Smeekens, S. P., Steiner, D. F., & Thomas, G. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 5297−5301.

Udenfriend, S., & Kilpatrick D. L. (1983) *Arch. Biochem. Biophys. 221*, 309−323.

Van Horssen, A. M., Van den Hurk, W., Bailyes, E. M., Hutton, J. C., Martens, G. J. M., & Lindberg, I. (1995) *J. Biol. Chem.* (in press).

Wilcox, C. A., & Fuller, R. S. (1991) *J. Cell Biol. 115*, 297−307.

Zhou, Y., & Lindberg, I. (1993) *J. Biol. Chem. 268*, 5615−5623.

Zhou, A., Bloomquist, B., & Mains, R. E. (1993) *J. Biol. Chem. 268*, 763−1769.

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