

RESEARCH COMMUNICATION**A member of the eukaryotic subtilisin family (PC3) has the enzymic properties of the type 1 proinsulin-converting endopeptidase**

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PC3, a mammalian homologue of the yeast subtilisin-like proteinase Kex2, was expressed in *Xenopus* oocytes and its activity was characterized. PC3 cleaved human proinsulin at one of the two dibasic sites (KTRR³² but not LQKR⁶⁵). The specificity, inhibitor profile, pH optimum (5.5) and Ca²⁺-dependence ($K_{0.5} = 2.5\text{--}3\text{ mM}$) paralleled those of the insulin-granule type 1 endopeptidase activity, suggesting a role for PC3 in the conversion of prohormones.

INTRODUCTION

The processing of most prohormones and propeptides involves endoproteolytic cleavage at pairs of basic amino acids (Docherty & Steiner, 1982). Several such activities have been described (see Lindberg & Hutton, 1991), but the only endopeptidase unequivocally shown to be involved in proprotein processing is the yeast KEX2 gene product, a Ca²⁺-dependent serine proteinase with homology to the bacterial subtilisins (Mizuno *et al.*, 1988; Fuller *et al.*, 1989). Kex2 cleaves at paired basic amino acid sites and can process several mammalian proproteins correctly, including proinsulin (Thim *et al.*, 1986) and POMC (Thomas *et al.*, 1988). Several mammalian homologues of Kex2 have been cloned recently, i.e. furin, PC2, PC3 (also known as PC1) and PAC4 (Van de Ven *et al.*, 1990; Smeekens & Steiner, 1990; Seidah *et al.*, 1990, 1991; Smeekens *et al.*, 1991; Kiefer *et al.*, 1991). PC2 and PC3, unlike furin and PACE4, are found principally in neuroendocrine tissues and may therefore be prohormone-processing endopeptidases [see Hutton (1990), Barr (1991) and Steiner (1991)]. Co-transfection of these Kex2 homologues with proproteins has shown that they encode endopeptidases which cleave at paired basic amino acid sites. Furin processes pro-von Willebrand factor (Wise *et al.*, 1990) and pro- β -nerve growth factor (Bresnahan *et al.*, 1990) correctly, and co-transfection of PC2 and/or PC3 with POMC generates patterns of cleavage similar to those obtained *in vivo* in the anterior and intermediate lobes of the pituitary (Thomas *et al.*, 1991).

PC2 has been expressed in *Xenopus* oocytes and its activity characterized with small fluorogenic peptide substrates (Shennan *et al.*, 1991a). PC2 had a pH optimum of 5.5 and required millimolar Ca²⁺ for activity, and its inhibitor profile corresponded to that of Kex2 in that it was unaffected by inhibitors of serine (PMSF), thiol (E-64) and aspartyl (pepstatin A) proteinases and

inhibited by an organomercurial compound. Recent immunological studies indicate that PC2 corresponds to the type 2 proinsulin endopeptidase (Bennett *et al.*, 1992). This Ca²⁺-dependent acidic proteinase cleaves human proinsulin predominantly at the Lys⁶⁴-Arg⁶⁵ sequence at the CA-junction. It acts in concert with a second Ca²⁺-dependent acidic enzyme (the type 1 endopeptidase) which cleaves on the C-terminal side of Arg³¹-Arg³² at the BC-junction (Davidson *et al.*, 1988).

The presence of PC3 mRNA in insulinoma tissue raises the possibility that PC3 is related to the type 1 enzyme. This hypothesis was therefore investigated by comparing the enzymic activity of PC3 expressed in *Xenopus* oocytes with those present in extracts of insulin-secretory granules. These studies were facilitated by the recent development of rapid and sensitive assays for the type 1 and type 2 endopeptidase activities based on monoclonal antibodies which recognize precursor and intermediate forms of proinsulin conversion (Bailyes & Hutton, 1992; Bailyes *et al.*, 1992).

EXPERIMENTAL**Materials**

¹²⁵I-labelled proinsulin, des-31,32-proinsulin and des-64,65-proinsulin (monoiodinated at Tyr¹⁴ of the A-chain) were gifts from Lilly Research Laboratories, Indianapolis, IN, U.S.A. ATP[S] was obtained from Boehringer Mannheim, Lewes, Sussex, U.K.

Methods

Preparation of expressed PC3. Mouse PC3 (Smeekens *et al.*, 1991) was expressed in *Xenopus* oocytes essentially as described previously for PC2 (Shennan *et al.*, 1991b). Medium containing expressed PC3 and medium from the water-injected control oocytes were collected 24 and 48 h after injection and stored

Abbreviations used: Tos-Phe-CH₂Cl, tosylphenylalanylchloromethane; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; PMSF, phenylmethanesulphonyl fluoride; BC-junction, B-chain/C-peptide junction; CA-junction, C-peptide/A-chain junction; ATP[S], adenosine 5'-[γ -thio]triphosphate; POMC, pro-opiomelanocortin.

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at -70°C . [^{35}S]Methionine-labelled secreted proteins were analysed by SDS/PAGE and fluorography.

Preparation of insulin-granule endopeptidases. The proinsulin endopeptidases were solubilized from insulin-secretory granules, which were prepared from a transplantable rat insulinoma as described by Bailyes & Hutton (1992).

Assay of enzyme activity. Samples ($7.5\text{--}15\ \mu\text{l}$ of oocyte medium or $5\text{--}15\ \mu\text{l}$ of solubilized granules) were incubated at 30°C for $3\text{--}21\ \text{h}$ with ^{125}I -labelled substrate (proinsulin, des-64,65-proinsulin or des-31,32-proinsulin) at pH 5.5, in $5\ \text{mM-Ca}^{2+}$, essentially as described by Bailyes & Hutton (1992). The reaction products were separated either by alkaline-urea/PAGE (Davidson *et al.*, 1988) or by site-specific monoclonal-antibody immuno-adsorbents (see below). The values for the activity of PC3-injected oocyte medium are given after subtraction of any activity in the matched water-injected control medium. Three batches of PC3 medium were used in this study: two of the water-injected controls were inactive in the enzyme assay, and one had activity which was 24% of that measured in the matched PC3-injected oocyte medium.

Site-specific monoclonal-antibody immuno-adsorbents. The A6 monoclonal-antibody immuno-adsorbent was used to determine the extent of processing at the BC-junction of proinsulin and des-64,65-proinsulin as described by Bailyes & Hutton (1992). A6 binds proinsulin, 65–66-split proinsulin and des-64,65-proinsulin, but not 32–33-split proinsulin, des-31,32-proinsulin or insulin (Sobey *et al.*, 1989). Processing at the CA-junction of des-31,32-proinsulin was determined by a similar method using a monoclonal antibody (ANT-1) which binds des-31,32-proinsulin but not insulin (Bailyes *et al.*, 1992).

Determination of Ca^{2+} - and pH-sensitivity. Assays were performed as above, except that in the Ca^{2+} studies $2\ \text{mM-EDTA}$ with variable concentrations of CaCl_2 was used to generate the Ca^{2+} concentrations (Davidson *et al.*, 1988). In the pH studies, a buffer system composed of $30\ \text{mM-sodium acetate}$, $30\ \text{mM-Mes}$ and $30\ \text{mM-N-ethylmorpholine}$, adjusted with HCl or NaOH, was used.

RESULTS

Expression of PC2 and PC3

Injection of mRNA encoding human PC2 and mouse PC3 into *Xenopus* oocytes resulted in the secretion of the translated products into the medium. As previously described (Shennan *et al.*, 1991b), PC2 was secreted as a 71 kDa protein which was converted in the extracellular medium into a 68 kDa product (Fig. 1, lane 2). In contrast, PC3 was secreted as a protein of approx. 80 kDa which did not appear to be processed in the extracellular medium (Fig. 1, lane 3).

Characterization of PC3 catalytic activity by using the site-specific immuno-adsorbents

PC3 hydrolysed proinsulin and des-64,65-proinsulin at the BC-junction in a time-dependent manner up to 9 h of incubation (Fig. 2). Beyond this time the enzyme was essentially inactive (results not shown). PC3 did not hydrolyse des-31,32-proinsulin. Even when hydrolysis of proinsulin reached 44%, conversion of des-31,32-proinsulin did not exceed 1.7% (results not shown).

The effect of the thio-nucleotide ATP[S] on the activity of PC3 was determined, because it was found to have a stabilizing effect on the type 1 activity during various purification procedures that have been developed for the enzyme (Bailyes & Hutton, 1992). ATP[S] has little effect on type 1 activity solubilized from granules (1.2-fold stimulation); however, it re-activates the enzyme after ion-exchange chromatography (approx. 3-fold

stimulation) and after gel filtration (up to 20-fold stimulation), thereby restoring the activity to the original level. This stimulation has been attributed to the presence of sulphur-containing amino acid(s) susceptible to reversible oxidation, since micromolar dithiothreitol, adenosine 5'-[β -thio]diphosphate and guanosine 5'-[γ -thio]triphosphate had the same effect (Bailyes & Hutton, 1992). ATP[S] stimulated PC3 by 1.34-fold (± 0.02 , S.E.M., $n = 4$; Fig. 2). With PC3 medium that had been collected 48 h after injection instead of 24 h, the stimulation by ATP[S] increased to 2.5-fold (± 0.2 , S.E.M., $n = 3$). Dithiothreitol (at $50\ \mu\text{M}$) had the same effects as ATP[S] (results not shown). Addition of $250\ \mu\text{M-HgCl}_2$ to the assay cocktail completely inhibited PC3 (results not shown), again suggesting the presence of a reactive thiol group.

Proteinase inhibitors ($10\ \mu\text{M-E-64}$ and -pepstatin A, $0.1\ \text{mM-Tos-Phe-CH}_2\text{Cl}$, $1\ \text{mM-PMSF}$) were normally present in the assay cocktail, but omitting them had little or no effect on the activity measured. Increasing the assay temperature from 30°C to 37°C decreased the net proinsulin conversion by 43% by the end of an 18 h incubation. The type 1 endopeptidase activity is also more active at 30°C than at 37°C (Bailyes & Hutton, 1992).

Analysis of assay reaction products by electrophoresis

The assay reaction products were also separated by alkaline-urea/PAGE. In this system, the two split-proinsulin conversion intermediates (des-31,32-proinsulin and des-64,65-proinsulin) comigrate, but can be distinguished from proinsulin and insulin. Incubation of proinsulin with PC3 generated only one reaction product. No insulin was produced even at substrate conversion rates approaching 80% (Fig. 3, lanes 1–3). Similarly, incubation of the type 1 endopeptidase with proinsulin generates only one reaction product, which has been shown to correspond to des-31,32-proinsulin (Davidson *et al.*, 1988). In contrast, incubation of proinsulin with solubilized granule extracts which contain both the type 1 and type 2 activities generated split-proinsulin and insulin (Fig. 3, lanes 4–6). PC3 produced insulin from des-64,65-proinsulin, but did not cleave des-31,32-proinsulin (result not shown). Linear-regression analysis showed a clear correlation

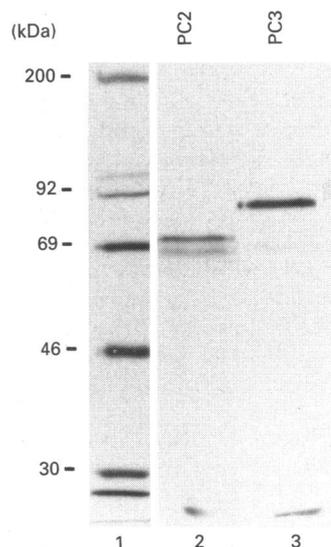


Fig. 1. Expression of PC2 and PC3 in micro-injected *Xenopus* oocytes

Xenopus oocytes were micro-injected with $50\ \text{nl}$ ($25\ \text{ng}$) of mRNA encoding PC2 (lane 2) or PC3 (lane 3) and incubated for 24 h at 20°C in $30\ \mu\text{l}$ of medium containing $120\ \mu\text{Ci}$ of [^{35}S]methionine. Medium was collected, and $10\ \mu\text{l}$ samples were analysed by SDS/PAGE and fluorography. ^{14}C -labelled protein molecular-mass markers are shown in lane 1.

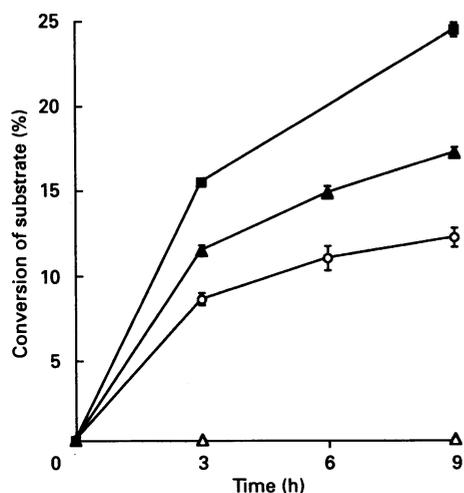


Fig. 2. Substrate specificity of PC3

PC3-injected oocyte medium samples (7.5 μ l) were assayed for enzyme activity at the indicated times by using the following substrates and conditions: \circ , 125 I-proinsulin; \blacktriangle , 125 I-proinsulin plus 250 μ M-ATP[S]; \blacksquare , 125 I-des-64,65-proinsulin plus 250 μ M-ATP[S]; \triangle , 125 I-des-31,32-proinsulin plus 250 μ M-ATP[S]. The values shown are means \pm s.e.m. ($n = 3$). The substrate conversion was determined by using either the A6 (proinsulin and des-64,65-proinsulin) or the ANT-1 (des-31,32-proinsulin) monoclonal-antibody immunoabsorbent.

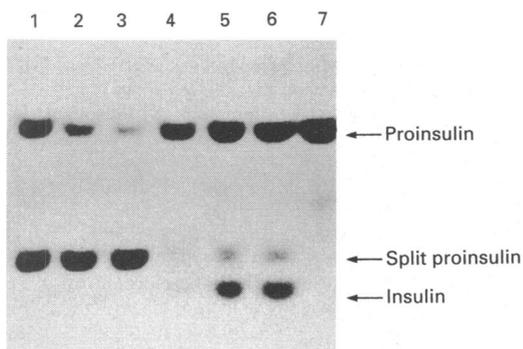


Fig. 3. Electrophoretic separation of proinsulin-conversion products produced by PC3 and insulin-granule endopeptidase activities

Lanes 1–3: PC3 assayed with (1) 3 mM- Ca^{2+} , (2) 5 mM- Ca^{2+} , (3) 8 mM- Ca^{2+} . Lanes 4–6: solubilized endopeptidases assayed with (4) 2 mM- Ca^{2+} , (5) 5 mM- Ca^{2+} , (6) 8 mM- Ca^{2+} . Lane 7: proinsulin incubated in the absence of enzyme. The positions of proinsulin, split proinsulin (des-31,32-proinsulin and/or des-64,65-proinsulin) and insulin are indicated.

($r = 0.985$, slope = 1.16) between the values obtained in the BC-junction-specific immunoabsorbent assay of PC3 and those obtained by alkaline-urea/PAGE analysis of the same samples (results not shown).

pH-dependence of enzyme activity

The pH optimum of both PC3 and the type 1 endopeptidase activity was 5.5 (Fig. 4a). As found previously for the type 1 endopeptidase, performing the assay in the presence of ATP[S] gave a slightly broader profile, owing to its stabilizing effect on enzyme exposed to higher pH values (E. M. Bailyes & J. C. Hutton, unpublished work).

Ca^{2+} -dependence of enzyme activity

Both PC3 and the type 1 endopeptidase showed an absolute

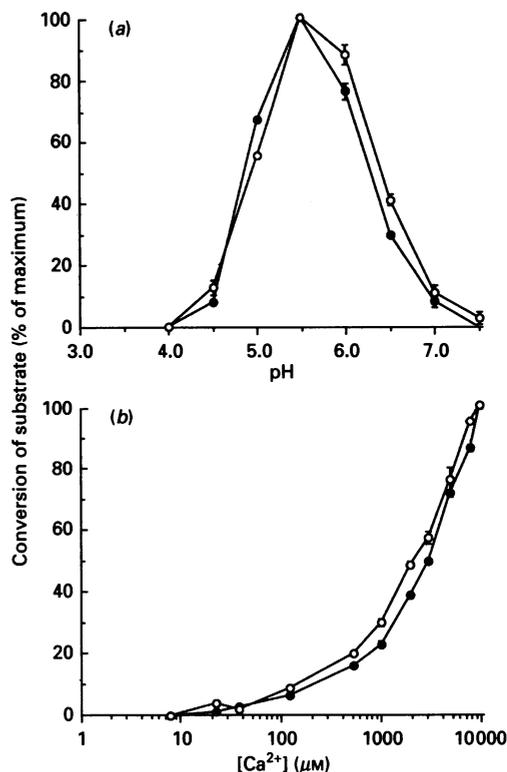


Fig. 4. pH- and Ca^{2+} -dependence of the activity of PC3 and the insulin-granule type 1 endopeptidase activity

The pH-dependence (a) and Ca^{2+} -sensitivity (b) of PC3 (\bullet) and the insulin-granule type 1 endopeptidase activity (\circ) were determined in the presence of 250 μ M-ATP[S] as described under 'Methods'. The results are means \pm s.e.m. ($n = 3$).

dependence on Ca^{2+} for activity, and were activated half-maximally in the concentration range 2.5–3 mM- Ca^{2+} (Fig. 4b). The profiles correspond closely to that reported previously for the type 1 endopeptidase (Davidson *et al.*, 1988; Bailyes & Hutton, 1992).

DISCUSSION

The conversion of proinsulin into insulin in the pancreatic β -cell occurs, for the major part, after the proprotein has been sorted into storage granules. Morphological studies show that the loss of proinsulin immunoreactivity correlates with acidification and accumulation of Ca^{2+} in the nascent granule, indicating that modification of the intragranular ionic environment plays an important role in determining the onset and possibly the extent of proteolytic conversion (Orci *et al.*, 1985, 1986; Steiner *et al.*, 1987). The insulin-granule type 1 and type 2 endopeptidase activities show marked Ca^{2+} - and pH-dependence, which suggests that the kinetic properties of the endopeptidases are a key regulatory factor in the conversion process (Davidson *et al.*, 1988).

The present study shows that the mammalian Kex2 homologue PC3 cleaves at the proinsulin BC-junction, but not at the other processing site in the molecule, the CA-junction. The apparent specificity of PC3 towards the Arg-Arg cleavage site in human proinsulin parallels that of the type 1 endopeptidase. Indeed, the activity of PC3 expressed in *Xenopus* oocytes was indistinguishable from the type 1 activity with respect to Ca^{2+} -sensitivity, pH-dependence, inhibitor profile and sensitivity to thiol agents. Furthermore, recent studies have shown that PC3 immuno-reactive protein co-migrates with a peak of type 1 endopeptidase

activity on ion-exchange chromatography of solubilized insulin-secretory granules, whereas PC2 immunoreactive protein coincides with type 2 endopeptidase activity (Baillyes *et al.*, 1992). These findings, together with the data that equate PC2 with the type 2 endopeptidase activity (Bennett *et al.*, 1992), suggest that the proteins encoded by PC2 and PC3 mRNAs are sufficient to account, at least qualitatively, for the post-translational conversion of proinsulin in islets.

Although in the present study PC3 showed a clear preference for the Arg³¹-Arg³² cleavage site over the Lys⁶⁴-Arg⁶⁵ cleavage site in human proinsulin, transfection studies of PC3 with other proproteins have shown that the enzyme is also capable of processing at Lys-Arg cleavage sites (Hosaka *et al.*, 1991; Korner *et al.*, 1991; Thomas *et al.*, 1991). This indicates that the sequence of amino acids at the -2, -1 positions relative to the cleavage site is not the only factor involved in determining substrate preferences (Docherty *et al.*, 1989). Approximately one-third of paired basic processing sites also have a basic residue at the -4 position (Hutton, 1990), and there is evidence that furin recognizes primarily a motif consisting of RXKR or RXRR (Hosaka *et al.*, 1991). Similarly, the rat proalbumin convertase, which has catalytic properties resembling those of the type 1 proinsulin endopeptidase (Rhodes *et al.*, 1989), processes chick proalbumin at a -4, -1 Arg cleavage site (RFAR) faster than it cleaves human proalbumin at its -2, -1 Arg cleavage site (VFRR) (Brennan & Peach, 1991). The type 1 endopeptidase activity also cleaves chick proalbumin (S. O. Brennan, E. M. Baillyes & J. C. Hutton, unpublished work). Whether a basic residue at position -4 affects the activity of PC2 or PC3 remains to be determined, but there is some evidence to suggest that it does. At the BC-junction of proinsulin, the -4 position is usually Lys, a notable exception being mouse and rat proinsulin II (B29 = Met). This substitution may explain the slower processing of rat proinsulin II observed *in vivo* (Sizonenko & Halban, 1991). At the CA-junction, there is no consensus at the -4 position, but in the rat I and II and mouse I sequence it is occupied by Arg, and this may be correlated with the finding that a rat CA-junction peptide was a very efficient inhibitor of the processing of human ¹²⁵I-proinsulin at the BC-junction (Baillyes & Hutton, 1992). Thus the possibility that PC3 can cleave at both the BC- and CA-junctions of rat and mouse proinsulin warrants further investigation. However, if PC3 is involved in the conversion of human proinsulin, and its substrate specificity *in vivo* corresponds to that determined *in vitro*, then a separate endopeptidase is required for cleavage at the CA-junction. Taken together, the available evidence strongly favours PC3 and PC2 as the enzymes involved in human proinsulin conversion.

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REFERENCES

Baillyes, E. M. & Hutton, J. C. (1992) *Biochem. J.*, in the press

- Baillyes, E. M., Bennett, D. L. & Hutton, J. C. (1992) Enzyme, in the press
- Barr, P. J. (1991) *Cell* **66**, 1-3
- Bennett, D. L., Baillyes, E. M., Nielson, E., Guest, P. C., Rutherford, N. G., Arden, S. D. & Hutton, J. C. (1992) *J. Biol. Chem.*, in the press
- Brennan, S. O. & Peach, R. J. (1991) *J. Biol. Chem.* **266**, 21504-21508
- 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
- Davidson, H. W., Rhodes, C. J. & Hutton, J. C. (1988) *Nature (London)* **333**, 93-96
- Docherty, K. & Steiner, D. F. (1982) *Annu. Rev. Physiol.* **44**, 625-638
- Docherty, K., Rhodes, C. J., Taylor, N. A., Shennan, K. I. J. & Hutton, J. C. (1989) *J. Biol. Chem.* **264**, 18335-18339
- Fuller, R. S., Brake, A. & Thorner, J. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 1434-1438
- Hosaka, M., Nagahama, M., Kim, W., Watanabe, T., Hatsuzawa, K., Ikemizus, K., Murakami, K. & Nakayama, K. (1991) *J. Biol. Chem.* **266**, 12127-12130
- Hutton, J. C. (1990) *Curr. Opin. Cell Biol.* **2**, 1131-1142
- Kiefer, M. C., Tucker, J. E., Joh, R., Landsberg, K. E., Saltman, D. & Barr, P. J. (1991) *DNA Cell Biol.* **10**, 757-769
- Korner, J., Chun, J., Harter, D. & Axel, R. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6834-6838
- Lindberg, I. & Hutton, J. C. (1991) in *Peptide Biosynthesis and Processing* (Fricker, L. D., ed.), pp. 141-173, CRC Press, Boston and London
- Mizuno, K., Nakamura, T., Ohshima, T., Tanaka, S. & Matsuo, H. (1988) *Biochem. Biophys. Res. Commun.* **156**, 246-254
- Orci, L., Ravazzola, M., Amherdt, M., Madsen, O., Vassalli, J.-D. & Perrelet, A. (1985) *Cell* **42**, 671-691
- Orci, L., Ravazzola, M., Amherdt, M., Madsen, O., Perrelet, A., Vassalli, J.-D. & Anderson, R. G. W. (1986) *J. Cell Biol.* **103**, 2273-2281
- Rhodes, C. J., Brennan, S. O. & Hutton, J. C. (1989) *J. Biol. Chem.* **264**, 14240-14245
- Seidah, N. G., Gaspar, L., Mion, P., Marcinkiewicz, M., Mbikay, M. & Chretien, M. (1990) *DNA Cell Biol.* **9**, 415-424
- Seidah, N. G., Marcinkiewicz, M., Benjannet, S., Gaspar, L., Beaubien, S., Mattei, M. G., Lazure, C., Mbikay, M. & Chretien, M. (1991) *Mol. Endocrinol.* **5**, 111-122
- Shennan, K. I. J., Smeekens, S. P., Steiner, D. F. & Docherty, K. (1991a) *FEBS Lett.* **284**, 277-280
- Shennan, K. I. J., Seal, A. J., Smeekens, S. P., Steiner, D. F. & Docherty, K. (1991b) *J. Biol. Chem.* **266**, 24011-24017
- Sizonenko, S. & Halban, P. A. (1991) *Diabetologia*, A55
- Smeekens, S. & Steiner, D. F. (1990) *J. Biol. Chem.* **265**, 2997-3000
- Smeekens, S., Avruch, A. S., La Mendola, J., Chan, S. J. & Steiner, D. F. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 340-344
- Sobey, W. J., Beer, S. F., Carrington, C. A., Clark, P. M. S., Frank, B. H., Gray, I. P., Luzio, S. D., Owens, D. R., Schneider, A. E., Siddle, K., Temple, R. C. & Hales, C. N. (1989) *Biochem. J.* **260**, 535-541
- Steiner, D. F. (1991) *Curr. Biol.* **1**, 375-377
- Steiner, D. F., Michael, J., Houghten, R., Mathieu, M., Gardner, P. R., Ravazzola, M. & Orci, L. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6184-6188
- Thim, L., Hansen, M. T., Norris, K., Hoegh, I., Boel, E., Forstrom, J., Ammerer, G. & Fiil, N. P. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 6766-6770
- Thomas, G., Thorne, B. A., Thomas, L., Allen, R. G., Hruby, D. E., Fuller, R. & Thorner, J. (1988) *Science* **241**, 226-241
- 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
- Van de Ven, W. J. M., Voorberg, J., Fontijn, R., Pannekoek, H., Ans, M. W., Van den Ouweland, A. M. V., Duijnhoven, J. L. P., Roebroek, A. J. M. & Seizen, R. J. (1990) *Mol. Biol. Rep.* **14**, 265-275
- Wise, R. J., Barr, P. J., Wong, P. A., Kiefer, M. C., Brake, A. J. & Kaufman, R. J. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 9378-9382

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