Sequence requirements for processing of proinsulin in transfected mouse pituitary AtT20 cells

Neil A. TAYLOR and Kevin DOCHERTY*

Department of Medicine, University of Birmingham, Queen Elizabeth Hospital, Birmingham B15 2TH, U.K.

To investigate the sequence requirements for proteolytic processing of prohormones at pairs of basic amino acids, normal and mutant proinsulins were expressed in the mouse pituitary corticotrophic cell line AtT20. The extent of processing was determined by h.p.l.c. analysis of insulin-like immunoreactivity secreted into the media of transfected cells. In this model system, normal proinsulin was efficiently processed to insulin. The mutant des-38–62-proinsulin, in which all but six amino acids of the C-peptide were deleted, was also processed to insulin but less efficiently than the wild-type. The mutant Lys⁶⁴-Arg⁶⁵ to Thr⁶⁴-Arg⁶⁵ was partially processed to insulin, while the mutant Arg³¹-Arg³² to Arg³¹-Gly³² was not processed at either site. These results indicate: (i) that a six-amino-acid spacer between the two pairs of basic amino acids in proinsulin is sufficient to permit processing at both sites; (ii) that the endoprotease responsible for cleavage at the Lys⁶⁴-Arg⁶⁵ site will also recognize Thr⁶⁴-Arg⁶⁵; (iii) that the endoprotease responsible for cleavage at the Arg³¹-Arg³² site will not recognize Arg³¹-Gly³²; and (iv) that the change Arg³¹-Arg³² to Arg³¹-Gly³² affects processing at the Lys⁶⁴-Arg⁶⁵ site.

INTRODUCTION

Almost all polypeptides destined for secretion from the cell or for insertion into the plasma membrane are synthesized as larger precursors, known as prepolypeptides. The prepeptide is removed by proteolysis within the lumen of the endoplasmic reticulum. Further cleavage occurs at sites usually marked by pairs of basic amino acids (Docherty & Steiner, 1982; Shields, 1991), although in some cases cleavage can occur at single basic residues (Schwartz, 1986).

The structural requirements for processing of propolypeptides are not fully understood. Thus not all paired basic residues within precursors are cleaved, while others are cleaved only in a tissue-dependent manner. Secondary structural features surrounding these sites may be important in the recognition of specific cleavage sites (Geisow & Smyth, 1980; Rholam *et al.*, 1986; Bek & Berry, 1990; Oda *et al.*, 1991).

One approach to understanding the mechanisms involved in the selection of specific propolypeptide cleavage sites involves mutagenesis of sequences within or close to the processing site and studying the effect of such changes on processing following transfection of cDNAs into cells that normally process propolypeptides (Powell *et al.*, 1988; Gross *et al.*, 1989; Gomez *et al.*, 1989; Dickerson *et al.*, 1990; Thorne & Thomas, 1990; Noel *et al.*, 1991; Ferber *et al.*, 1991; Quinn *et al.*, 1991; Nagahama *et al.*, 1991).

In a previous study we were able to characterize *in vitro* the substrate-specificity of partially purified type 1 and type 2 proinsulin-processing endopeptidases (Davidson *et al.*, 1988) using mutant insulin precursors generated following expression in microinjected *Xenopus* oocytes (Docherty *et al.*, 1989). In the present paper we extend these studies by transfecting the cDNAs encoding the mutant insulin precursors into the mouse corticotrophic cell line AtT20. These cells efficiently process the adrenocorticotrophic hormone (ACTH) precursor pro-opiomelanocortin (POMC) at pairs of basic amino acids. The aim was to compare proinsulin processing in these cells with the processing requirements of the type 1 and 2 endopeptidases.

EXPERIMENTAL

Materials

The AtT20 cell line was provided by Dr. R. E. Mains (John Hopkins University, Baltimore, MD, U.S.A.), and the expression vector MtNeo-1 by Dr. K. Peden (NIH, Bethesda, MD, U.S.A.). Proinsulin and split proinsulin standards were obtained from Dr. B. H. Frank (Eli Lilly, Indianapolis, IN, U.S.A.). G418 was purchased from Gibco/BRL, Paisley, Scotland, U.K. All other chemicals, unless indicated, were purchased from Sigma, Poole, Dorset, U.K., or from BDH, Poole, Dorset, U.K.

Cell culture

AtT20 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal calf serum, penicillin (400 units/ml) and streptomycin (400 µg/ml).

Generation of stably transfected cell lines

Mutant human preproinsulin cDNAs were generated using oligonucleotide site-directed mutagenesis as described previously (Docherty *et al.*, 1989). These cDNAs were cloned into the *Eco*R1 site of pMtNeo1 under the control of the mouse metallothionein promoter (Dickerson *et al.*, 1987). The resulting constructs were then transfected into AtT20.D16v cells using the calcium phosphate DNA co-precipitation method (Graham & van der Eb, 1973). Transformed colonies appeared after 10–14 days of selection in culture medium containing 500 µg of G418/ml. Colonies were expanded in 24-well plates and screened by radioimmunoassay of the medium for insulin-like immunoreactivity (ILI) using a previously described assay (Shakur *et al.*, 1989). Two clones from each transfection were selected for further study and cultured in medium containing 250 µg of G418/ml. To obtain maximal expression from the metallothionein promoter under experimental conditions, medium was supplemented with 90 µM-ZnCl₂ and 5 µM-CdCl₂ (Taylor & Docherty, 1991).

Abbreviations used: ACTH, adrenocorticotrophic hormone; POMC, pro-opiomelanocortin; ILI, insulin-like immunoreactivity; IBMX, isobutylmethylxanthine.

* To whom correspondence should be addressed.

Effect of isobutylmethylxanthine (IBMX) on release of ILI

Semi-confluent cultures in 10 cm dishes were incubated in media supplemented with 90 μM -ZnCl₂ and 5 μM -CdCl₂ for 24 h. The culture medium was removed, and the cells were rinsed twice with serum-free medium and incubated for 2 h in 3 ml of serum-free medium supplemented with 90 μM -ZnCl₂ and 5 μM -CdCl₂. The medium was then collected, and the cell monolayer was rinsed twice with serum-free medium and incubated for 2 h in 3 ml of serum-free medium supplemented with 90 μM -ZnCl₂, 5 μM -CdCl₂ and 1 mM-IBMX at 37 °C. The medium was then removed and the ILI was measured by radioimmunoassay.

To measure the cellular content of ILI, the medium was removed and the cells were rinsed twice with phosphate-buffered saline (137 mM-NaCl/2.7 mM-KCl/9.6 mM-phosphate) before treatment with 0.05% (w/v) trypsin. Cells were harvested and resuspended in 2 ml of phosphate-buffered saline. An aliquot was removed for determination of the cell number using a haemocytometer. The cells were then centrifuged at 1500 g for 10 min, and the cell pellet was resuspended in 1 ml of 5 M-acetic acid and freeze-thawed three times to ensure lysis of the cells. The cell lysate was centrifuged in a microcentrifuge for 5 min to pellet the cell debris, and the supernatant was removed and lyophilized. The dried cell extract was resuspended in 50 μl of 10 mM-HCl, neutralized with NaOH and made up to 1 ml with serum-free medium. An aliquot of this material was removed for radioimmunoassay.

H.p.l.c. analysis of released ILI

Immediately following removal from the cells, the medium was made 5 M with respect to acetic acid and loaded on to a Sep-Pak C18 cartridge (Millipore, Watford, Herts., U.K.). The cartridge was washed once with 10 ml of trifluoroacetic acid/water (1:99, v/v) and once with 10 ml of trifluoroacetic acid/acetonitrile/water (1:20:79, by vol.), and the insulin-like material was eluted in 2 ml of trifluoroacetic acid/acetonitrile/water (1:60:39, by vol.). The samples were then lyophilized and resuspended in 110 μl of 10 mM-HCl.

Samples (100 μl) were then loaded on to a Vydak C18 column and eluted in 37% solvent B for 20 min, followed by a linear gradient over 50 min where solvent B increased to 43%. Solvent A was 50 mM-phosphoric acid, 100 mM-sodium perchlorate and 5 mM-heptane sulphonic acid (pH 3), and solvent B was acetonitrile/water (9:1, v/v). Fractions (0.8 ml) were collected, neutralized by addition of 0.08 ml of 0.5 M-borate (pH 9.5) containing 0.5% (w/v) BSA and lyophilized. The fractions were then resuspended in 0.8 ml of phosphate-buffered saline and radioimmunoassayed for ILI.

RESULTS

The mutant insulin precursors used in this study are shown in Fig. 1. Human proinsulin contains a 30-amino-acid insulin B chain connected to the 31-amino-acid C-peptide by way of two basic amino acids, Arg³¹ and Arg³². The C-peptide in turn is joined to the 21-amino-acid insulin A chain by the basic amino acids Lys⁶⁴ and Arg⁶⁵. The mutant des-38–62-proinsulin contains a major deletion in the C-peptide such that the resultant 'mini C-peptide' has the sequence Glu-Ala-Glu-Asp-Leu-Gln. R32G proinsulin, K64T proinsulin and the double mutant R32G/K64T proinsulin have the pairs of basic amino acids Arg-Arg and Lys-Arg changed to single basic amino acids Arg-Gly and Thr-Arg as indicated. These mutant proinsulins were designed to determine the role of the C-peptide and the requirement for pairs of basic amino acids in the processing of proinsulin.

cDNAs encoding normal and mutant insulin precursors were transfected into AtT20 cells and a number of clonal G418

resistant lines were expanded in tissue culture. The five cell lines selected for this study varied in content of ILI from 0.35 to 53.3 ng/10⁶ cells (Table 1). These highest levels of ILI are less than 0.5% of the levels of ILI in pancreatic β cells, and less than 10% of that of the endogenous ACTH-related peptides in AtT20 cells. There was no detectable ILI in cell extracts or medium from untransfected AtT20 cells. These cells released approx. 40% of their cell content of ILI and ACTH-like immunoreactivity (results not shown) per hour. This high basal release resulted in a low (approx. 2-fold) stimulation of release of ILI by secretagogue. This is similar to the stimulation of release of cholecystokinin in transfected AtT20 cells (Lapps *et al.*, 1988), but is lower than that reported by Gross *et al.* (1989) for stimulation of ILI release from transfected AtT20 cells.

The ILI released into the media from the five transfected cell lines was analysed by h.p.l.c. (Fig. 2). The column was calibrated using human proinsulin, des-31,32-split-proinsulin, des-64,65-split-proinsulin and bovine insulin. There were no standards available for the mutant proinsulins, and so these were identified on the basis of their expected elution relative to wild-type proinsulin as previously described (Docherty *et al.*, 1989). It was more difficult to definitively identify the individual split forms of the mutant proinsulins, and so these were tentatively grouped together as split intermediates, i.e. with no distinction between cleavage at the B-chain-C-peptide junction (B/C) or C-

Fig. 1. Schematic representation of the normal and mutant proinsulins used in the study

The insulin B chain, C-peptide and insulin A chain sequences of proinsulin are indicated.

Table 1. Insulin content and secretion from transfected AtT20 cells

Cells were incubated for 4 h in medium containing 90 μM -ZnCl₂ and 5 μM -CdCl₂. The medium was then removed and the ILI in the cell extract and media measured as described in the Experimental section. For IBMX stimulation, cells were incubated in the absence or presence of 1 mM-IBMX for 2 h. Results are the means $\pm$ s.d. of at least four determinations (plates of cells).

Clone	Cell content (ng/10 ⁶ cells)	Basal release (ng/4 h per 10 ⁶ cells)	IBMX stimulation (% of basal release)
AtT20Mtlins-1	0.35 $\pm$ 0.09	0.58 $\pm$ 0.05	178 $\pm$ 31
AtT20Mtlins-3	3.79 $\pm$ 0.29	10.6 $\pm$ 0.01	170 $\pm$ 19
AtT20Mtlins-G32	1.05 $\pm$ 0.23	1.39 $\pm$ 0.09	206 $\pm$ 33
AtT20Mtlins-T64	0.64 $\pm$ 0.01	0.99 $\pm$ 0.07	170 $\pm$ 11
AtT20Mtlins-G32,T64	53.3 $\pm$ 3.13	63.5 $\pm$ 3.8	217 $\pm$ 38

Fig. 2. Processing of normal and mutant proinsulins in transfected AtT20 cell lines

The AtT20 cell lines AtT20MtIns-1 (a), AtT20MtIns-3 (b), AtT20MtIns-G32 (c), AtT20MtIns-T64 (d) and AtT20MtIns-G32,T64 (e) were incubated for 4 h in medium containing IBMX (1 μ M). The medium was then removed and analysed by h.p.l.c. The elution positions of human proinsulin, des-31,32-proinsulin and des-64,65-proinsulin (intermediates), and bovine insulin are indicated. The results are shown as percentages of total ILI recovered from the column. Each chromatogram is representative of at least two, and in most cases three, separate experiments.

peptide-A-chain junction (C/A). The recovery of ILI from the column was in the range 80–90 % for all samples applied.

The normal proinsulin expressed in clone AtT20MtIns-1 was processed efficiently to insulin, with almost 90 % of the ILI co-eluting with insulin (Fig. 2a). Des-38–62-proinsulin (from clone AtT20MtIns-3) was also processed to insulin, although less efficiently than the wild-type proinsulin (Fig. 2b). Of the recovered

ILI, 67 % co-eluted with the insulin marker while the remaining 33 % eluted as a single peak at the expected position of the unprocessed des-38–62-proinsulin. There were no detectable processing intermediates.

The mutant R32G proinsulin was poorly processed in the transfected AtT20 cells; 53 % of the ILI eluted in fraction 36 slightly ahead of the wild-type human proinsulin standard, 30 % eluted as intermediate(s), and 17 % eluted at fraction 11, slightly ahead of bovine insulin standard. The elution of the insulin-like material ahead of the standard may be explained if this material represented insulin extended at the B chain with an arginine and a glycine, i.e. the C-terminal glycine may be a poor substrate for the endogenous AtT20 carboxypeptidase H. This would render the molecule more hydrophilic, with the result that it would elute ahead of insulin.

The K64T proinsulin was processed to insulin, or a molecule closely related to insulin (48 % of the ILI). There was, however, some accumulation of intermediates which eluted as a broad peak (52 % of the ILI). This suggested that the Thr⁶⁴-Arg⁶⁵ site was recognized by a processing activity within the AtT20 cells, but that overall processing of this mutant was less efficient than for the wild-type.

In keeping with the above observations, the double mutant R32G/K64T proinsulin was not efficiently processed; 57 % of the ILI eluted at fraction 38, 24 % as a broad intermediate peak, and 17 % as insulin or insulin-related molecules.

DISCUSSION

In a previous study we were able to characterize the substrate specificities of the type I and type II proinsulin processing endopeptidases *in vitro* using mutant insulin precursors (Docherty *et al.*, 1989). The type I activity normally cleaves at the B/C (Arg³¹-Arg³²) junction, while the type II activity cleaves preferentially at the C/A (Lys⁶⁴-Arg⁶⁵) junction (Davidson *et al.*, 1988). We confirmed that these enzymes displayed a requirement for pairs of basic residues, i.e. the R32G mutant was not cleaved by the type I enzyme and the T64K mutant was not cleaved by the type II enzyme. Secondary structural features were also important, since neither enzyme was active against the des-38–62-mutant, and mutation of the Arg³¹-Arg³² site affected cleavage at Lys⁶⁴-Arg⁶⁵ site.

The major findings of the present study in transfected AtT20 cells were that: (i) des-38–62-proinsulin was processed to insulin, although much less efficiently than the wild-type; (ii) the mutant K64T proinsulin was processed at the Thr⁶⁴-Arg⁶⁵ site, although again less efficiently than the wild-type proinsulin; and (iii) the mutant G32R proinsulin was not a substrate for the processing activity within the AtT20 cells. These results for the most part confirm the finding of the study on the type I and type II enzymes *in vitro*, although there were some noticeable differences. These differences may relate to the way in which the experiments were performed; the *in vitro* study conditions were optimized such that the major products would be processing intermediates, while the *in vivo* study was performed under conditions whereby the wild-type proinsulin was almost completely converted to insulin.

The finding that des-38–62-proinsulin was processed to insulin in the AtT20 cells is in keeping with the processing of a similar construct when expressed in yeast (Thim *et al.*, 1986). In this respect, the sequence requirements for processing of proinsulin in yeast and AtT20 cells are similar. On the other hand, a mutant insulin precursor completely lacking C-peptide sequences, i.e. B chain-Arg-Arg-Lys-Arg-C chain, is not cleaved in yeast or in AtT20 cells (Thim *et al.*, 1986; Powell *et al.*, 1988). The six-amino-acid C-peptide sequence present in des-38–62-proinsulin

(Glu-Ala-Glu-Asp-Leu-Gln) is highly conserved between species, and it is noteworthy that deletion of four amino acids of the equivalent sequence from rat II proinsulin (Glu-Val-Glu-Asp) inhibits the processing of proinsulin in AtT20 cells (Gross *et al.*, 1989). These results emphasize the importance of the potential for these amino acids to interact with conserved sequences in insulin (Snell & Smyth, 1975), possibly resulting in the presentation of the Arg³¹-Arg³² processing site for cleavage.

The mutant K64T proinsulin was processed to insulin in the AtT20 cells. In a related study, the efficiently processed Lys¹⁶³-Arg¹⁶⁴ site separating the ACTH and β -lipotropin sequences of POMC were changed to His-Arg and Met-Arg. When transfected into a pancreatic β cell line, the His,Arg site was not cleaved, but the Met,Arg site was cleaved (Thorne & Thomas, 1990). Thus Met-Arg and Thr-Arg can substitute to some extent for Lys-Arg, but His-Arg cannot.

The mutant R32G proinsulin was a poor substrate for the AtT20 processing enzyme(s), and as observed *in vitro* with the type I and type II enzymes, mutation of Arg³¹-Arg³² to Arg³¹-Gly³² affected processing at the Lys⁶⁴-Arg⁶⁵ site. This can be explained if the structural requirement for processing at the C/A junction was disrupted by changing the Arg³¹-Arg³² sequence at the B/C junction to Arg³¹-Gly³². It is thus relevant that although the C-peptide within proinsulin is predominantly unstructured (Frank *et al.*, 1972), n.m.r. and photochemically induced nuclear polarization studies show that a stable structure is formed at the C/A junction, the 'C/A knuckle' (Weiss *et al.*, 1990). The change of Arg³¹-Arg³² to Arg³¹-Gly³² at the B/C junction may therefore affect the C/A knuckle in such a way that it becomes less accessible to proteolytic cleavage. Alternatively, cleavage at the normal B/C junction may affect the C/A knuckle in such a way that the Lys⁶⁴/Arg⁶⁵ sequence becomes more accessible to proteolytic cleavage. However, this sequential cleavage model is not compatible with the production of C/A split proinsulin from intact proinsulin (Davidson *et al.*, 1988; Docherty *et al.*, 1989).

Recently cDNAs encoding candidate prohormone-processing endopeptidases have been cloned. These proteinases are related to the bacterial subtilisins (Hutton, 1990), and include furin, PC2 and PC3/mPC1 (Smeekens & Steiner, 1990; Seidah *et al.*, 1990, 1991; Smeekens *et al.*, 1991). Our present data on the sequence requirements for the processing of proinsulin in AtT20 cells may help establish the relationship between these subtilisin-like enzymes and the endogenous AtT20 prohormone-processing proteinase(s).

This work was supported by a grant from the Medical Research Council.

REFERENCES

Bek, E. & Berry, R. (1990) *Biochemistry* **29**, 178–183

- Davidson, H. W., Rhodes, C. J. & Hutton, C. J. (1988) *Nature (London)* **333**, 93–96
- Dickerson, I. M., Dixon, J. E. & Mains, R. E. (1987) *J. Biol. Chem.* **262**, 13646–13653
- Dickerson, I. M., Dixon, J. E. & Mains, R. E. (1990) *J. Biol. Chem.* **265**, 2462–2469
- Docherty, K. & Steiner, D. F. (1982) *Annu. Rev. Physiol.* **44**, 625–628
- Docherty, K., Rhodes, C. J., Taylor, N. A., Shennan, K. I. J. & Hutton, J. C. (1989) *J. Biol. Chem.* **264**, 18335–18339
- Ferber, S., Gross, D. J., Villa-Komaroff, L., Daheny, F., Vollenweider, F., Meyer, K., Loeken, M. R., Khan, C. R. & Halban, P. A. (1991) *Mol. Endocrinol.* **5**, 319–326
- Frank, B. H., Pekar, A. H. & Veros, A. J. (1972) *Biochemistry* **11**, 4926–4931
- Geisow, M. J. & Smyth, D. G. (1980) in *The Enzymology of Post-Translational Modification of Proteins* (Freedman, R. B. & Hawkins, H. C., eds.), vol. 1, pp. 259–287, Academic Press, London
- Gomez, S., Boileau, G., Zollinger, L., Nault, C., Rholam, M. & Cohen, P. (1989) *EMBO J.* **8**, 2911–2916
- Graham, F. & van der Eb, A. (1973) *Virology* **52**, 456–467
- Gross, D. J., Villa-Komaroff, L., Kahn, C. R., Weir, G. C. & Halban, P. A. (1989) *J. Biol. Chem.* **264**, 21486–21490
- Hutton, J. C. (1990) *Curr. Opin. Cell Biol.* **2**, 1131–1142
- Lapps, W., Eng, J., Stern, A. S. & Gubler, U. (1988) *J. Biol. Chem.* **263**, 13456–13462
- Nagahama, M., Nakayama, K. & Murakami, K. (1991) *Eur. J. Biochem.* **197**, 135–140
- Noel, G., Keutmann, H. T. & Mains, R. E. (1991) *Mol. Endocrinol.* **5**, 404–412
- Oda, K., Ikeda, M., Tsuji, E., Sohda, M., Takami, N., Misumi, Y. & Ikehara, Y. (1991) *Biochem. Biophys. Res. Commun.* **179**, 1181–1186
- Powell, S. K., Orci, L., Craik, C. S. & Moore, H.-P. (1988) *J. Cell Biol.* **106**, 1843–1851
- Quinn, D., Orci, L., Ravazzola, M. & Moore, H.-P. (1991) *J. Cell Biol.* **113**, 987–996
- Rholam, M., Nicolas, P. & Cohen, P. (1986) *FEBS Lett.* **207**, 1–6
- Schwartz, T. W. (1986) *FEBS Lett.* **200**, 1–5
- 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, G., Mattei, M. G., Mbikay, M. & Chretien, M. (1991) *Mol. Endocrinol.* **5**, 111–122
- Shakur, Y., Shennan, K. I. J., Taylor, N. A. & Docherty, K. (1989) *J. Mol. Endocrinol.* **3**, 155–162
- Shields, D. (1991) in *Peptide Biosynthesis and Processing* (Fricker, L. D., ed.), pp. 37–70, CRC Press, Boca Raton
- 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
- Snell, C. R. & Smyth, D. G. (1975) *J. Biol. Chem.* **250**, 6291–6295
- Taylor, N. A. & Docherty, K. (1991) *Biochem. Soc. Trans.* **19**, 202
- 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
- Thorne, D. A. & Thomas, G. (1990) *J. Biol. Chem.* **265**, 8436–8443
- Weiss, M. A., Frank, B. H., Khait, I., Pekar, A., Heiney, R., Shoelson, S. E. & Neuringer, L. J. (1990) *Biochemistry* **29**, 8389–8401

Received 3 January 1992/18 March 1992; accepted 26 March 1992