Diabetologia (1994) 37 [Suppl 2]: S48–S56

Diabetologia

© Springer-Verlag 1994

John Charles Hutton
Minkowski Award, 1988, Paris

Dr. Hutton was born in Australia in 1948. He obtained a B. Sc. from the University of New South Wales, Australia in 1969 and in 1974 he obtained a Ph. D. From 1973 Dr. Hutton was Research Officer, School of Biochemistry of the University of New South Wales. Between 1974 and 1976 he was visiting Research Scientist, Instituto de Genetica Humana, Universidad Mayor de San Andres, La Paz, Bolivia. From 1976–1979 he was a Postdoctoral Research Fellow at the Laboratoire de Medecine Experimentale, Universite Libre de Bruxelles, Belgium. From 1979–1989 he was Research Fellow and Senior Research Fellow at the Department of Clinical Biochemistry, University of Cambridge, UK. During 1985 Dr. Hutton was a Research Scientist at the Hagedorn Research Laboratories, Gentofte, Denmark and in 1992 was a visiting Professor at the Hormone Research Institute, Department of Biochemistry and Biophysics, University of California, San Francisco, Calif., USA. Since 1989 Dr. Hutton has been a Reader at the University of Cambridge.

Insulin secretory granule biogenesis and the proinsulin-processing endopeptidases

J. C. Hutton

Department of Clinical Biochemistry, Addenbrooke's Hospital, Cambridge, UK

Summary The insulin storage granule of the pancreatic beta cell is assembled within the trans Golgi network from around 50 or so gene products many of which are synthesized coordinately with the major component, proinsulin. An important contribution to our understanding of the regulation of this process has come from studies of the post-translational processing of proinsulin and of other proteins which are stored in the granule, particularly the processing enzymes themselves. The present review focusses on recent insights into the molecular nature of the processing machinery, and the granule Ca^{2+}-dependent subtilisin-related endopeptidases which catalyse the initial rate-limiting step in the enzymic conversion of proinsulin. [Diabetologia (1994) 37 [Suppl 2]: S48–S56]

Key words Proinsulin, convertases, biosynthesis.

Corresponding author: Dr. J. C. Hutton Department of Clinical Biochemistry, University of Cambridge, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QR, UK

Abbreviations: TGN, Trans Golgi network; CPH, carboxypeptidase H; NMR, nuclear magnetic resonance; Lys, lysine; Arg, arginine, Thr, threonine; Leu, leucine; Gln, glycine; Asp, asparginine; His, histidine; Ser, serine; POMC, pro-opiomelanocortin.

The cellular biology of the regulated pathway of secretion

Formation of the insulin secretory granule is conventionally perceived as a singular vesiculation step occurring at the trans Golgi network (TGN) by which a condensed secretory product is enveloped in a simple phospholipid bilayer [1–4] Figure 1. Subsequent maturation is characterized by loss of the partial

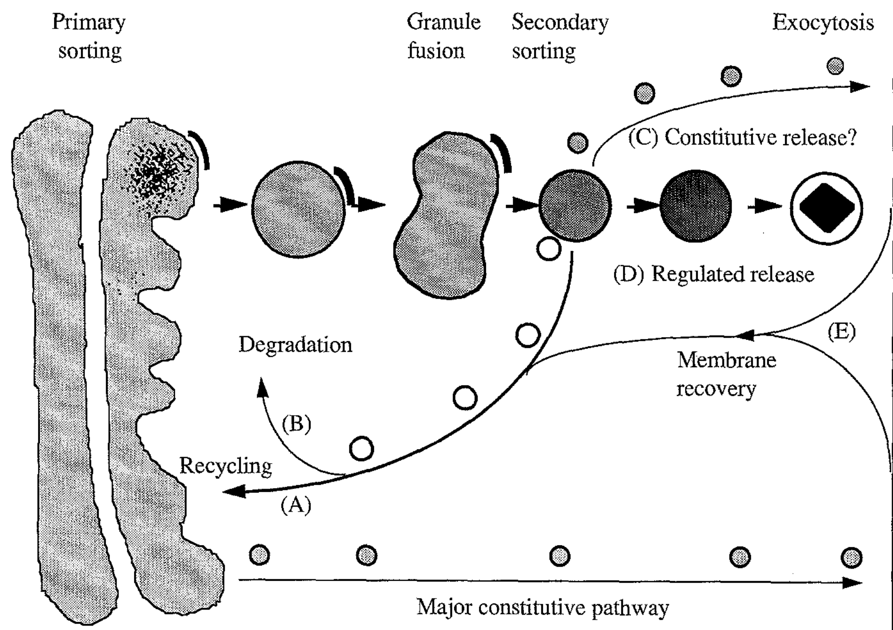

Fig. 1. Secretory granule maturation and pathways of membrane traffic in the beta cell. The formation of dense core secretory granules in the cell (upper pathway) is preceded by condensation of the secretory product in the TGN and is often associated with membranes with a partial clathrin coat. Proteins not sorted at this stage appear to be secreted constitutively in smaller vesicles with electronlucent cores (lower pathway). The initial sorting event may be followed by granule-granule fusion and vesicular budding from the maturing granule. Such vesicles may serve as a mechanism of retrieval (A), disposal (B) of granule membrane components and contribute to a secondary constitutive release process (C) distinct from the major regulated exocytotic pathway (D). Membrane proteins associated with either regulated or constitutive exocytosis are presumably recycled (E) to the TGN or degraded

clathrin coat of the granule membrane and further physical changes in the granule core which lead to the characteristic morphological features of the mature secretory granule [5]. The proteolytic conversion of proinsulin to insulin occurs during the maturation process and is initiated by changes in the ionic composition of the newly-formed "immature" or "nascent" granule [6, 7]. However it is evident that there are other changes occurring in the maturation process particularly at the level of the granule membrane [8, 9]. The existence of these events has been postulated on the basis of biochemical studies which show that the stoichiometry of insulin to C-peptide, which are released constitutively soon after granule formation, deviates from the theoretical 1:1 ratio expected. There is as yet no morphological correlate of this process, probably because the intermediate compartments involved are transitory and not easily distinguishable morphologically from immature granules and other vesicular structures (Fig. 1). The types of maturation processes envisaged include a homotypic fusion of nascent granules, heterotypic fusion between nascent granules and other vesicular elements and retrieval of membrane and possibly granule contents through a clathrin-mediated process. Such processes could function as secondary sorting events, serve the purpose of salvaging granule membrane-associated components for re-use in the Golgi, or provide a means of redirecting granule constituents to lysosomes after they have fulfilled their function in the granule.

The initial sorting of the major granule lumenal proteins into the regulated pathway coincides with the formation of aggregates of secretory products in the TGN [3, 4]. Mutational analysis and transfection experiments indicate that the amino acid sequence of a sorted molecule is an important determinant of its entry into the regulated pathway [10, 11] but there is little clear evidence of a consensus sequence or sorting domain in proinsulin [12–15]. The mechanism of sorting is generally conceived as either an association of the sorted protein with a membrane receptor [16, 17] or spontaneous self-association of the secreted product in response to changes in the TGN environment, in particular pH and Ca^{2+} concentration [18]. Neither model of sorting, however, provides an adequate account of how proteins other than the quantitatively major constituents are packaged in the granule. It is clear that at least six other soluble proteins are co-packaged with insulin in the mature granule [19, 20]. Although most of these proteins are subject to translational control in the same way as proinsulin [21, 22], it seems unlikely that their inclusion into the granule is simply the consequence of their presence in the TGN at the moment when proinsulin condenses. Indeed there is clear evidence that at least two proteins are actively sorted into the regulated pathway [23, 24]. It might be that the granule proteins have common physical properties which permit them to co-aggregate or interact with the TGN membrane. The fact that many have relatively acidic isoelectric points which correspond to the pH of the granule compartment and that they bind Ca ions may be important in this regard. A number of granule proteins also interact with membranes at acidic pH values, a phenomenon which might serve to promote sorting and which could be reversed after sorting by proteolytic cleavage of the molecules [24, 25].

The movements of proteins which are intrinsic to the granule membrane proteins in the cell with a regulated secretory pathway are very poorly understood. Among these there are proteins which are rela-

Table 1. Typical proproteins and the recognition sequence for convertases processing

Protein	Sequence	Cellular site	Consequence
Proinsulin	TPKTRR.EA	Storage granules	Bioactivity
Proalbumin	RGVFRR.DA	Golgi	Chelation activity
Nerve growth factor	THRSKR.SS	Golgi	Bioactivity
von Willebrand factor	SHRSKR.SL	Golgi	Bioactivity
Renin	SQPMKR.ST	Storage granules	Enzyme activity
HIV glycoprotein 160	VQREKR.AV	Golgi	Fusigenicity
Anthrax toxin PA	NSRKKR.ST	Plasma membrane	Internalization

Examples are given of a number of proteins which are processed in post Golgi compartments involved in the secretion or internalisation of proteins. Cleavage occurs in every case on the carboxy-terminal side of an Arg residue which is preceded by another basic amino acid. A basic amino acid at position P4 or P6 appears to be an additional requirement for substrates processed in the constitutive but not the regulated secretory pathway. Precursor forms in every case show low biological activity

Table 2. The principal mammalian subtilisin-related proprotein convertases

Enzyme	Tissue	Location	Specificity	pH	M^{2+}
Furin	Broad	trans Golgi	R X (K/R) R	6–9	Ca
Type 1 (PC1)	Neuroendocrine	Dense core granule	RR (KR)	4–6	Ca
Type 2 (PC2)	Neuroendocrine	Dense core granule	RR (KR)	4–6	Ca
PC4	Testis	?	?	?	?
PACE 4	Broad	? Golgi	RR	?	Ca
PC6/6A	Gastrointestinal	?	?	?	?

The wide tissue distribution, and Golgi localisation and neutral pH optimum of enzymes like furin indicate a ubiquitous function in the processing of membrane receptors and constitutively-secreted proteins in all cells. Other members by contrast show very specific tissue and cellular localisation and unique catalytic properties which are more consistent with specialised function and regulatory properties

tively organelle- and tissue specific, e.g. dopamine hydroxylase in the chromaffin cell [26, 27], those which are specific to secretory tissues but occur in a variety of organelles e.g. SCAMP proteins [28], and those which are widely distributed in post-Golgi compartments of all cells such as the vacuolar proton translocator [29]. Two potential membrane markers have been identified in the insulin granule [30, 31] but at present little is known about their targetting to granule membranes or their movements during the process of granule formation, maturation and exocytosis. Their synthesis, like proinsulin, responds to stimulation of the cell with glucose, a hallmark of proteins of the granule lumen. This finding raises questions concerning the concept that the majority of granule membrane constituents recycle back to the TGN after exocytosis to be incorporated into nascent vesicles.

Post-translational processing at sites marked by basic amino acids

Post-translational proteolysis at sites marked by basic amino acids is ancient in evolutionary terms and appears to serve a similar physiological role in all eukaryotes in the activation of biological function of proteins (Tables 1 and 2). Other molecular, cellular and physiological functions are also affected by processing and roles can be envisaged also in folding, intracellular movement, sorting, and regulation of the solubility of the product.

Polypeptide neurotransmitters and hormones which are secreted via the regulated secretory pathway in neuroendocrine tissues are generally processed at pairs of basic amino acids, typically Lys Arg and Arg Arg and much less frequently at Lys Lys or Arg Lys sites. Plasma membrane receptor molecules, viral glycoproteins and proteins which are secreted constitutively are usually processed at sites marked by a more complex array of basic amino acids, typically a dibasic site with a further basic amino acid in the P4, 5 or 6 position (Table 2). The reaction sequence has been deduced from structural analysis of the products for a number of small peptides and is well illustrated by the pathway of proinsulin to insulin conversion (Fig. 2) which involves the action of two separate endopeptidases in conjunction with a carboxypeptidase H (E) (CPH) which removes the basic amino acids exposed at the C-termini after endoproteolytic cleavage. The endopeptidases of the insulin granule appear directed towards either Arg Arg or Lys Arg in the substrate although it should be pointed out that additional basic amino acids appear in the P4 position in the case of the B-C and C-A junction of a number of species. This arrangement is similar to the processing sites of constitutively secreted proteins and it seems likely that the presence of a basic amino acid in the P4 position is kinetically favourable though not essential in this case.

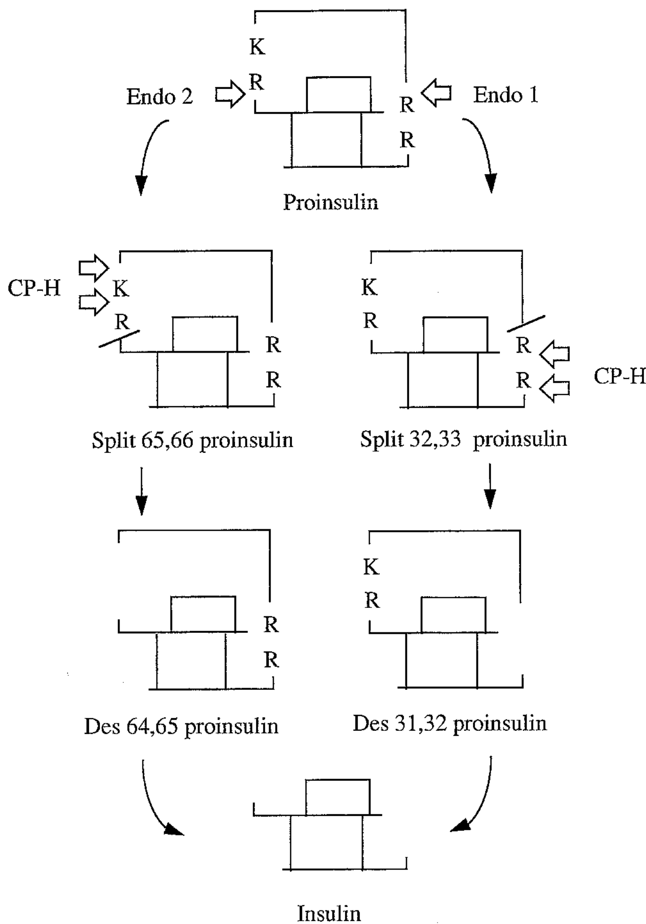

Fig. 2. Reaction pathway for proinsulin conversion. The initial endoproteolytic cleavage of proinsulin at the sites marked by paired basic amino acids is followed by the rapid removal of the basic amino acids exposed at the new carboxy terminil by carboxypeptidase H. A second round of endoproteolytic cleavage and carboxypeptidase H cleavage produces the mature products

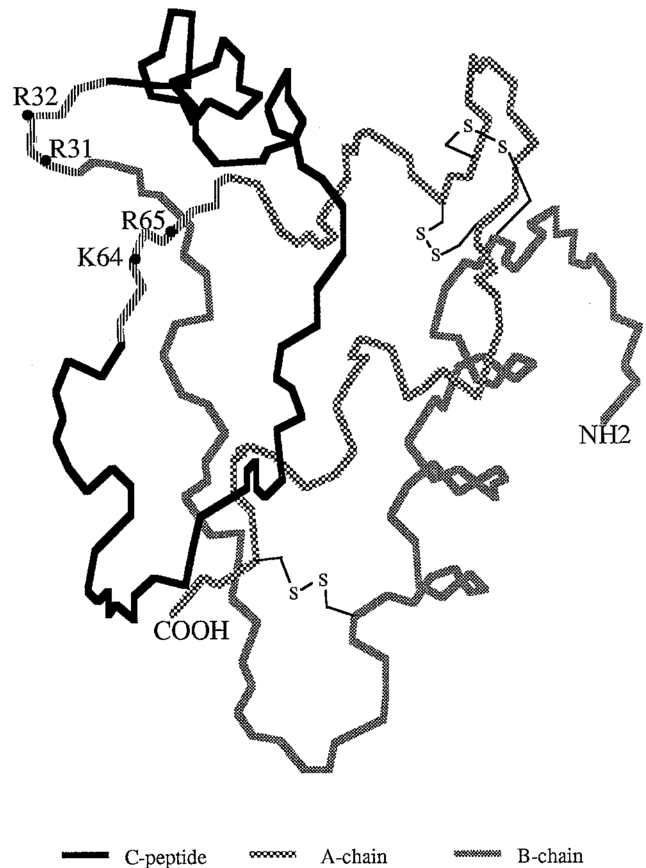

Fig. 3. A model of proinsulin structure (reproduced with permission from Snell and Smythe (1975) J Biol Chem 250 : 6291). The Arg_{31} Arg_{32} and Lys_{64} Arg_{65} processing sites are in close proximity (4–8Å) and on the surface of the molecule. Their location on the face opposing those involved in dimer (left-hand side) and hexamer (right hand side) formation indicates that they would be accessible to the solvent and thus proteases even after hexamerisation of the prohormone

An additional feature of the recognition of proinsulin by the endopeptidases is the secondary structure around the proinsulin processing sites. Proinsulin residues 19–31 and 53–66 are predicted to form β-turns, and residues 46–54 and 69–76 to form omega loops. NMR data point to a close conformational relationship of the A and B chains in proinsulin to that in insulin and although the three dimensional structure of proinsulin is not documented a number of useful predictions can be made based upon molecular modelling (Fig. 3). Within this model it is notable that the two processing sites lie in close proximity and thus cleavage at one site may produce conformational changes around the other.

Enzymological studies of proinsulin endopeptidases

Two soluble, Ca^{2+}-dependent, acidic endopeptidase activities capable of correctly processing human proinsulin *in vitro* to give insulin [32] have been identified. The activity designated type 1 cleaves exclusively on the C-terminal side of the Arg_{31} Arg_{32} sequence of proinsulin whereas the type 2 activity cleaves predominantly on the C-terminal side of the Lys_{64} Arg_{65} sequence (Fig. 2). The two activities differ in their sensitivity to Ca^{2+}, with the type 1 half-maximally activated at around 2.5 mmol/l and the type 2 at 100 µmol/l. The optimum pH for both type 1 and type 2 endopeptidase activity is 5.5 At pH 7.4 both enzymes show little activity and at pH values of 8.0 or above both enzymes are irreversibly inactivated. The pH profile and Ca^{2+} responses of the two enzymes are thus consistent with their being active in the secretory granule and has led to the hypothesis that the ionic environment of the various compartments of the secretory pathway may be a key factor in regulating processing.

Studies with active site-directed tripeptide sulphonium salt inhibitors have shown that the precise sequence at the dibasic site is an important determinant of the specificity of these two endopeptidase ac-

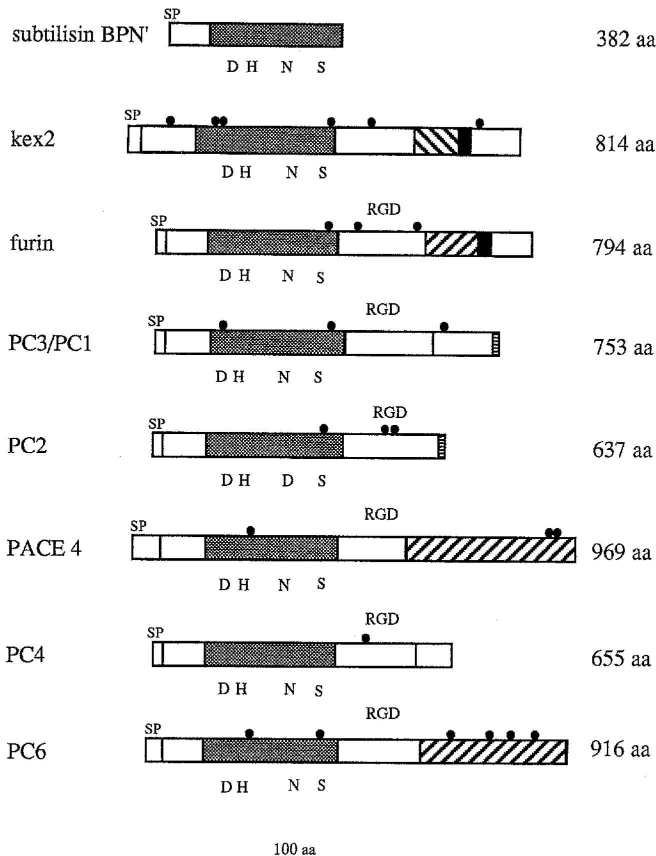

Fig. 4. Structural relationships between the subtilisin-related family of proprotein processing enzymes. Each family member shows the presence of a conserved catalytic domain incorporating the amino acids of the catalytic triad (D, H and S) and oxy-anion hole (N except for PC2). This is preceded by a pro-domain and signal peptide of variant structure and followed by a weakly conserved P-domain that is important for catalytic activity. The P-domain in mammalian members contains a fibronectin-binding concensus sequence (RGD). Other features showing varying degrees on conservation include the presence of Cys-rich domain ▨, Thr/Ser-rich domain ▧, transmembrane anchor ■, and a C-terminal amphipathic helical sequence ▩, and sites of N-linked glycosylation ●

tivities [33]. Studies with mutant proinsulins in vitro and in vivo have confirmed the specificity of the two activities for dibasic sequences and have shown that the structural context of the cleavage site is crucial in determining whether or not it is subject to proteolysis [34]. That the conformation of the peptide backbone around the dibasic sites affects their susceptibility to proteolytic attack is further borne out by the finding that the type 2 activity preferentially cleaves the intermediate des-31, 32-proinsulin over intact proinsulin whereas the type 1 activity recognises the des-64, 65-proinsulin intermediate and proinsulin to a similar extent [35]. It has been argued [35] that this effectively results in sequential processing of proinsulin via the des-31, 32-proinsulin intermediate, however further kinetic data and cell biological studies are needed to support this conclusion.

Molecular cloning of the proprotein endopeptidases

The key to the molecular cloning of the enzymes responsible for the endoproteolytic conversion of proinsulin came from genetic studies in conjugation-deficient yeast strains. These resulted in the cloning of a defective serine protease KEX2 [36] and were followed by the serendipitous discovery of a mammalian homologue, furin [37], and the subsequent systematic search for other family members (Table 2; Fig. 4). The list of subtilisin-related protease family members is expanding and now includes at least nine distinct eukaryote genes together with a number of splice variants. The properties of the currently-documented members is outlined below.

Furin is a Golgi-localised, Ca^{2+}-dependent ($K_{1/2}$ = 200 μmol/l), neutral (pH optimum 6.0–8.5) serine endoprotease of broad tissue distribution [38]. Cell transfection studies have shown that it recognises the consensus motif Arg X X Arg and is capable of processing a variety of proproteins including pro-von Willebrand factor [39], mouse pro-β-nerve growth factor [40], complement pro-C3 and proalbumin [41]. Its processing-site specificity and proprotein substrate specificity indicate a role in the processing of proprotein precursors as they pass through the *trans*-Golgi, rather than in the conversion of prohormones and other peptides secreted by the regulated pathway. Nevertheless it is capable of converting the human proinsulin molecule at the B-chain/C-peptide (Lys Thr Arg Arg) but not the C-peptide/A-chain junction (Leu Gln Lys Arg) in vitro (Bailyes, Thomas and Hutton; personal communication). This raises the question of whether the actual levels of furin activity in the pancreatic beta cell are sufficient to contribute to insulin processing and whether its localisation overlaps with the compartment involved in sorting of the prohormone.

PC1 and PC2 [42, 43], unlike furin, appear to be soluble proteins. There is no apparent transmembrane domain and the C-terminal domain is similar to that of furin and KEX2 only in as much as it is rich in acidic amino acids. However, both sequences contain a potential C-terminal amphipathic helical segment similar to the putative membrane anchor of carboxypeptidase H [44]. The subtilisin-related domains of PC1 and PC2 exhibit 45–65 % amino acid identity with other members of the subtilisin-related family and the Asp-His-Ser catalytic triad is placed in an equivalent position.

PC1 and PC2 mRNAs are associated with neuroendocrine tissues suggesting that they are likely candidates for endopeptidases associated with the regulated pathway of secretion. *In situ* hybridisation studies have demonstrated a distinct localisation of the PC1 and PC2 transcripts in pituitary and brain and it is clear in several endocrine tissues that the relative proportions of the enzymes vary. Such varia-

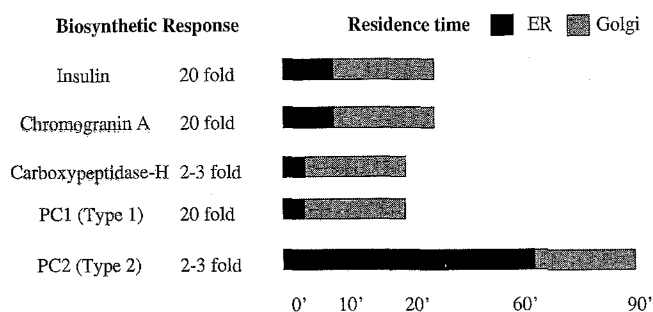

Fig. 5. The dynamics of granule biogenesis deduced from studies of the biosynthesis and post-translational modification of insulin granule proteins. Individual secretory granule proteins show marked differences in the response of their rates of biosynthesis in islets to stimulation by glucose 16.7 mmol/l. Some (PC-1 and CP-H) exit the endoplasmic reticulum more rapidly than insulin and are incorporated into nascent granules within 20 min, whereas others (PC2) do not reach this destination until some 60 min later. A consequence of these variations is that the protein composition of nascent granules is likely to vary according to the intensity and duration of the glycaemic stimulus

tion is thought to account for region-specific differences of post-translational processing of proproteins which contain multiple bioactive sequences within their structure.

Cell transfection studies have shown that PC1 and PC2 process proproteins such as POMC, proinsulin and prorenin at dibasic amino acid sites [45, 46]. The prorenin cleavage site mutant Arg Arg is cleaved but not the cleavage site mutants, Lys Lys, Arg Lys, Gln Arg, Arg Gln or Lys Arg Pro [47]. PC3 is also capable of cleaving mutant mouse prorenins in GH_4C_1 cells at the monobasic Arg X X Arg site. The enzymes expressed in *Xenopus* oocytes show a dependence on millimolar concentrations of Ca^{2+} ions and acidic pH (optimum 5.5) [48, 49].

PC2 immunoreactivity is localised to insulin secretory granules and coincides with type 2 activity on ion-exchange chromatography of insulinoma secretory granule proteins [50]. The identity of PC2 with the type 2 activity is further supported by the ability of PC2 antisera to immunoprecipitate type 2 (but not type 1) activity from extracts of insulinoma secretory granules. Cell transfection studies show that PC2 selectively cleaves rat proinsulin I at the C-peptide/A-chain (Lys Arg) junction while PC1 generates mature insulin but cleaves preferentially at the B-chain/C-peptide (Arg Arg) junction [51]. This is consistent with them being the type 2 and type 1 proinsulin endopeptidases, respectively. The ability of PC1/type 1 to cleave at the C-peptide/A-chain junction in this study may be due to the presence of an Arg residue at the P4 position relative to the cleavage site (Arg Gln Lys Arg) in the rat proinsulin sequence compared to the human cleavage site (Leu Gln Lys Arg) (see article by Halban in this issue).

PACE 4 features an extended signal peptide, a relatively large C-terminal cysteine rich region and no obvious transmembrane domain or C-terminal amphipathic α-helix [52]. The mRNA of PACE 4 has a widespread tissue distribution with the highest levels found in liver.

PC4 is also soluble but restricted to the germ cells of the testis where it is developmentally regulated [53, 54]. The nature of its native substrate or cleavage specificity remains elusive. It is not able to cleave prorenin at its Lys Arg processing site or a prorenin mutant with an Arg X Lys Arg sequence in cell transfection studies.

PC5 demonstrates a ubiquitous pattern of tissue distribution similar to that of furin mRNA [55] with the highest levels being found in the adrenal cortex and gut with lower levels in many other peripheral tissues and tissues of the central nervous system. PC5 is an alternatively spliced form of PC6. PC6 itself is most abundant in the intestine but also present in many other tissues and cell lines. Its cleavage profile is similar to that of PC3 and it is conceivable that it plays a role in the processing of propeptides in enterochromaffin cells with substrates such as enteroglucagon.

Biosynthesis and post-translational processing of the proinsulin convertases

The structures of PC1 and PC2, indeed all the eukaryote subtilisin members, contain sites upstream of the conserved catalytic domain which bear a consensus motif for furin processing and are clearly cleaved at these sites during the process of intracellular maturation in *Xenopus* oocytes [48], bovine adrenal [56], anglerfish islets [57] and rat insulinoma tissue [48].

PC2 is synthesised in isolated rat islets of Langerhans initially as 75kDa precursor which undergoes limited proteolytic processing to a 65kDa form by removal of an 84 amino acid prosequence at a site marked by the sequence Arg Lys Lys Arg^{108} [58, 59]. Other minor forms which are cleaved at an upstream Lys Arg Arg Arg^{81} site and a downstream Arg Gly Tyr Arg^{111} have also been found. The processing of proPC2 in rat islets ($t_{1/2} = 140$ min) (Fig. 5) is significantly slower than that of proinsulin ($t_{1/2} = 40$ min). By contrast PC1 is rapidly converted to an 80kDa protein by removal of the 83 amino acid prosegment presumably at a site marked by the sequence Arg Ser Lys Arg^{110} [43]. PC1 also undergoes C-terminal processing [60] with slower kinetics, possibly at one of several pairs of basic residues that occur in the molecule. The truncated 66kDa protein produced is the major form found in the islets of Langerhans. Both PC1 and PC2 are co-released with insulin in response to glucose and there is little evidence for se-

cretion via the constitutive pathway thus suggesting that they are efficiently sorted into the regulated pathway of secretion. The initial processing of PC2 and PC3 which probably occurs in the endoplasmic reticulum is possibly autocatalytic and perhaps intramolecular (see [61]). The formation of the C-terminally truncated forms may, in contrast, occur after sorting to the secretory granule. At the present time it is not known if the pro-forms, intermediate forms and truncated forms of the enzymes have intrinsic activity, and if so, whether they differ in kinetic properties.

Insulin mRNA translation undergoes rapid and marked changes in the beta cell in response to ambient glucose concentrations (Fig. 5). Chromogranin A and PC1 biosynthesis respond in a similar manner whereas PC2 and carboxypeptidase H usually change no more than a few fold [21, 62]. As a consequence the ratio of PC2 to proinsulin in nascent granules would be expected to vary with stimulus whereas PC1 to insulin would remain constant. The trend to relative depletion of PC2 would be further exacerbated by the fact that delivery of PC2 to the site of granule assembly in the TGN is delayed relative to that of proinsulin and PC1. This might result in a net decrease in the rate of conversion of proinsulin to insulin since PC2 will determine the flux through des 64, 65 proinsulin (Fig. 2), and a relative increase in the concentration of des 31, 32 proinsulin at steady state during the conversion process. This in turn would be reflected in the composition of the insulin peptides secreted, since the newly-formed granules in which most of the processing occurs are competent for exocytosis. Such phenomena might account for the relative rise of proinsulin and des 31, 32 proinsulin with impaired glucose tolerance in humans (see article by Hales in this issue). There is a danger here however of oversimplification since there are many other factors which control the rate of processing and release of intermediates, including the relative size of the mature and nascent granule pool, the total islet tissue mass, the functional islet mass and the proportion of pancreatic beta cell in the population which are biosynthetically active (see article by Pipeleers in this issue).

Acknowledgements. Work from the author's laboratory was supported by the British Diabetic Association, The Wellcome Trust, Medical Research Council and the European Community Bridge Program.

References

1. Burgess TL, Kelly RB (1987) Constitutive and regulated secretion of proteins. Ann Rev Cell Biol 3: 243–294
2. De Camilli P, Jahn R (1990) Pathways to regulated exocytosis in neurons. Ann Rev Physiol 52: 625–645
3. Griffiths G, Simons K (1986) The trans Golgi network: sorting at the exit site of the Golgi complex. Science 234: 438–443
4. Shields D (1991) Signals for intracellular protein sorting: implications for peptide hormone precursor processing and secretion. In: Fricker LD (ed) Peptide biosynthesis and processing CRC Press, Boca Raton, London pp 37–70
5. Orci L, Ravazzola M, Amherdt M, Madsen O, Vassalli JD, Perrelet A (1985) Direct identification of prohormone conversion site in insulin-secreting cells. Cell 42: 671–681
6. Orci L, Ravazzola M, Amherdt M, Madsen O, Perrelet A, Vassalli JD, Anderson RG (1986) Conversion of proinsulin to insulin occurs co-ordinately with acidification of maturing secretory granules. J Cell Biol 103: 2273–2281
7. Hutton JC (1982) The internal pH and membrane potential of the insulin secretory granule. Biochem J 20: 171–178
8. Arvan P, Castle D (1992) Protein sorting and secretion granule formation in regulated secretory cells. Trends Cell Biol 2: 327–331
9. von Zastrow M, Castle JD (1987) Protein sorting among two distinct export pathways occurs from the content of maturing exocrine storage granules. J Cell Biol 105: 2675–2684
10. Stoller TJ, Shields D (1989) Retrovirus-mediated expression of preprosomatostatin: posttranslational processing, intracellular storage and secretion in GH3 cells. J Cell Biol 107: 2087–2095
11. Wagner DD, Saffaripour S, Bonfanti R, Sadler JE, Cramer EM, Chapman B, Mayadas TN (1991) Induction of specific storage organelles by von Willebrand factor propolypeptide. Cell 64: 403–413
12. Powell SK, Orci L, Craik CS, Moore H-PH (1988) Efficient targeting to storage granules of human proinsulins with altered propeptide domain. J Cell Biol 106: 1843–1851
13. Shakur Y, Shennan KI, Taylor NA, Docherty K (1989) A major C-peptide deletion prevents secretion of a mutant human proinsulin from transfected monkey kidney cells. J Mol Endocrinol 3: 155–162
14. Gross DJ, Villa-Komaroff L, Kahn CR, Weir GC, Halban PA (1989) Deletion of a highly conserved tetrapeptide sequence of the proinsulin connecting peptide (C-peptide) inhibits proinsulin to insulin conversion by transfected pituitary corticotroph (AtT20) cells. J Biol Chem 264: 21486–21490
15. Gross DJ, Halban PA, Kahn CR, Weir GC, Villa Komaroff L (1989) Partial diversion of a mutant proinsulin (B10 aspartic acid) from the regulated to the constitutive secretory pathway in transfected AtT-20 cells. Proc Natl Acad Sci USA 86: 4107–4111
16. Orci L, Ravazzola M, Perrelet A (1984) Proinsulin associates with Golgi membranes of the pancreatic β-cell. Proc Natl Acad Sci USA 81: 6743–6746
17. Chung K-N, Walter P, Aponte GW, Moore H-P (1989) Molecular sorting in the secretory pathway. Science 243: 192–197
18. Rosa P, Weiss U, Pepperkok R, Ansorge W, Niehrs C, Stelzer EHK, Huttner WB (1989) An antibody against secretogranin 1 (chromogranin B) is packaged into secretory granules. J Cell Biol 109: 17–34
19. Hutton JC (1989) The 24th Minkowski lecture: The insulin secretory granule Diabetologia 32: 271–281
20. Hutton JC (1984) Secretory granules Experientia 40: 1091–1098
21. Guest PC, Rhodes CJ, Hutton JC (1989) Regulation of the biosynthesis of insulin secretory granule proteins: co-ordinate translational control is exerted on some, but not all, granule matrix constituents. Biochem J 257: 431–437
22. Guest PC, Bailyes EM, Rutherford NG, Hutton JC (1990) Insulin secretory granule biogenesis: co-ordinate regula-

tion of the biosynthesis of the majority of constituent proteins. Biochem J 274: 73–78
23. Sopwith AM, Hales CN, Hutton JC (1984) Pancreatic B-cells secrete a range of novel proteins besides insulin. Biochim Biophys Acta 803: 342–345
24. Guest PC, Pipeleers D, Rossier J, Rhodes CJ, Hutton JC (1990) Co-secretion of carboxypeptidase H and insulin from isolated rat islets of Langerhans. Biochem J 264: 503–508
25. Fricker LD (1991) Peptide processing exopeptidases: amino and carboxypeptidases involved with peptide biosynthesis. In: Fricker LD (ed) Peptide biosynthesis and processing. CRC Press, Boca Raton, London pp 199–229
26. Winkler H, Hortnagl H, Smith AD (1970) Membranes of the adrenal medulla. Biochem J 118: 303–310
27. Hunter A, Phillips JH (1989) The recycling of a secretory granule membrane protein. Exp Cell Res 182: 445–460
28. Brand SH, Laurie SM, Mixon MB, Castle JD (1991) Secretory carrier membrane proteins 31–35 define a common protein composition among secretory carrier membranes. J Biol Chem 266: 18949–18957
29. Rudnick G (1986) ATP-driven H+ pumping into intracellular organelles. Ann Rev Physiol 48: 403–413
30. Grimaldi KA, Hutton JC, Siddle K (1987) Production and characterisation of monoclonal antibodies to insulin secretory granule membrane. Biochem J 245: 567–573
31. Grimaldi KA, Siddle K, Hutton JC (1987) Biosynthesis of insulin secretory granule membrane proteins: control by glucose. Biochem J 245: 557–566
32. Davidson HW, Rhodes CJ, Hutton JC (1988) Intraorganellar calcium and pH control proinsulin cleavage in the pancreatic β-cell via two distinct site-specific endopeptidases. Nature (London) 333: 93–96
33. Rhodes CJ, Zumbrunn A, Bailyes EM, Shaw E, Hutton JC (1989) The inhibition of proinsulin-processing endopeptidase activities by active-site-directed peptides. Biochem J 258: 305–308
34. Taylor NA, Docherty K (1992) Sequence requirements for processing of proinsulin in transfected mouse pituitary AtT20 cells. Biochem J 286: 619–622
35. Rhodes CJ, Lincoln B, Shoelson SE (1992) Preferential cleavage of des-31, 32-proinsulin over intact proinsulin by the insulin secretory granule type 2 endopeptidase: implications of a favoured route for prohormone processing. J Biol Chem 267: 22719–22727
36. Mizuno K, Nakamura T, Oshima T, Tanaka S, Matsuo H (1988) Yeast KEX2 gene encodes an endopeptidase homologous to subtilisin like serine proteases. Biochem Biophys Res Commun 156: 246–254
37. Roebroek AJM, Schalken JA, Bussemakers MJG et al. (1986) Characterisation of human c-fes/fps reveals a new transcription unit (fur) in the immediately upstream region of the proto-oncogene. Mol Biol Rep 11: 117–125
38. Molloy SS, Bresnahan PA, Leppla SH, Klimpel KR, Thomas G (1992) Human furin is a calcium-dependent serine endoprotease that recognises the sequence Arg-X-X-Arg and efficiently cleaves anthrax toxin protective antigen. J Biol Chem 267: 16396–16402
39. Wise RJ, Barr PJ, Wong PA, Kiefer MC, Brake AJ, Kaufman RJ (1990) Expression of human proprotein processing enzyme: correct cleavage of the von Willebrand factor precursor at a paired basic amino acid site. Proc Natl Acad Sci USA 87: 9378–9382
40. Bresnahan PA, Leduc R, Thomas L et al. (1990) Human fur gene encodes a yeast KEX-2-like endoprotease that cleaves pro-β-NGF in vivo. J Cell Biol 111: 2851–2859
41. Misumi Y, Oda K, Fujiwara T, Takami N, Tashiro K, Ikehara Y (1991) Functional expression of furin demonstrating its intracellular localisation and endoprotease activity for processing of proalbumin and complement pro-C3. J Biol Chem 266: 16954–16959
42. Smeekens SP, Avruch AS, La Mendola J, Chan SJ, Steiner DF (1991) Identification of a cDNA encoding a putative prohormone convertase related to PC2 in AtT20 cells and islets of Langerhans. Proc Natl Acad Sci USA 88: 340–344
43. Seidah NG, Gaspar L, Mion P, Marcinkiewicz M, Mbikay M, Chretien M (1990) cDNA sequence of two distinct pituitary proteins homologous to Kex2 and furin gene products: tissue-specific mRNAs encoding candidates for pro-hormone processing proteinases. DNA Cell Biol 9: 415–424
44. Fricker LD, Das B, Hogue-Angeletti R (1990) Identification of the pH-dependent membrane anchor of carboxypeptidase E (EC 3.4.17.10). J Biol Chem 265: 2476–2482
45. Thomas L, Leduc R, Thorne BA, Smeekens SP, Steiner DF, Thomas G (1991) Kex2-like endoproteases PC2 and PC3 accurately cleave a model prohormone in mammalian cells: Evidence for a common core of neuroendocrine processing enzymes. Proc Natl Acad Sci USA 88: 5297–5301
46. Nakayama K, Hosaka M, Hatsuzawa K, Murakami K (1991) Cloning and functional expression of a novel endoprotease involved in prohormone processing at dibasic sites. J Biochem 109: 803–806
47. Nakayama K, Watanabe T, Nakagawa et al. (1992) Consensus sequence for precursor processing at mono-arginyl sites: evidence for the involvement of a Kex2-like endoprotease in precursor cleavages at both dibasic and mono-arginyl sites. J Biol Chem 267: 16335–16340
48. Shennan KIJ, Smeekens SP, Steiner DF, Docherty K (1991) Characterisation of PC2, a mammalian Kex2 homologue, following expression of the cDNA in microinjected Xenopus oocytes. FEBS Lett 284: 277–280
49. Bailyes EM, Shennan KIJ, Seal AJ et al. (1992) A member of the eukaryotic subtilisin family has the enzymatic properties of the type 1 proinsulin-converting endopeptidase. Biochem J 285: 391–394
50. Bennett DL, Bailyes EM, Nielsen E, Guest PC, Rutherford NG, Arden SD, Hutton JC (1992) Identification of the type 2 proinsulin processing endopeptidase as PC2, a member of the eukaryote subtilisin family. J Biol Chem 267: 15229–15236
51. Smeekens SP, Montag AG, Thomas G et al. (1992) Proinsulin processing by the subtilisin-related proprotein convertases furin, PC2 and PC3. Proc Natl Acad Sci USA 89: 8822–8826
52. Nakagawa T, Hosaka M, Torii S, Watanabe T, Murakami K, Nakagawa K (1993) Identification and functional expression of a new member of the mammalian Kex2-like processing endoprotease family: its striking structural similarity to PACE4. J Biochem 113: 132–135
53. Seidah NG, Day R, Hamelin J, Gaspar A, Collard MW, Chretien M (1992) Testicular expression of PC4 in the rat: molecular diversity of a novel germ cell-specific Kex2/subtilisin-like proprotein convertase. Mol Endocrinol 6: 1559–1570
54. Nakayama K, Kim W-S, Torii S et al. (1992) Identification of the fourth member of the mammalian endoprotease family homologous to the yeast Kex2 protease: its testis-specific expression. J Biol Chem 267: 5897–5900
55. Seidah NG, Day R, Marcinkiewicz M, Benjannet S, Chretien M (1991) Mammalian neural and endocrine proprotein and pro-hormone convertases belonging to the subtilisin family of serine proteinases. Enzyme 45: 271–284

56. Christie DL, Batchelor DC, Palmer DJ (1991) Identification of Kex2-related proteases in chromaffin granules by partial amino acid sequence analysis. J Biol Chem 266: 15679–15683
57. Mackin RB, Noe BD, Speiss J (1991) Identification of a somatostatin-14-generating propeptide converting enzyme as a member of the Kex2/furin/PC family. Endocrinology (Baltimore) 129: 2263–2265
58. Guest PC, Arden SD, Bennett DL, Clark A, Rutherford NG, Hutton JC (1992) The post-translational processing and intracellular sorting of PC2 in the islets of Langerhans. J Biol Chem 267: 22401–22406
59. Shennan KIJ, Seal AJ, Smeekens SP, Steiner DF, Docherty K (1991) Site-directed mutagenesis and expression of PC2 in microinjected Xenopus oocytes. J Biol Chem 266: 24011–24017
60. Vindrola O, Lindberg I (1992) Biosynthesis of the prohormone convertase mPC1 in AtT20 cells. Mol Endocrinol 6: 1088–1094
61. Steiner DF, Smeekens SP, Ohagi S, Chan SJ (1992) The new enzymology of precursor processing endoproteases. J Biol Chem 267: 23435–23438
62. Alarcon C, Lincoln B, Rhodes CJ (1993) The biosynthesis of the subtilisin-related proprotein convertase PC3, but not that of the PC2 convertase, is regulated by glucose in parallel to proinsulin biosynthesis in rat pancreatic islets. J Biol Chem 268: 4276–4280