Philippe Halban Minkowski Award, 1990, Copenhagen

Philippe Halban was born in Oxford in 1950, and received both his B.A. and Ph.D. in biochemistry from that University. In 1975 he moved to the Institut de Biochimie Clinique in Geneva, where he spent the next 9 years working under the direction of Professor Albert Renold, first on insulin pharmacokinetics and then on insulin production by the beta cell. This latter theme has been a major focus of his research to this day. Dr. Halban next moved to the Joslin Diabetes Center, Harvard Medical School in Boston, Mass., USA where he worked for 3 years. Since 1987 he has been Louis Jeantet Professor of Medicine and Director of the Jeantet Research Laboratories at the Geneva University Medical Centre. Dr. Halban's group in Geneva is studying the cell and molecular biology of both insulin production and islet-cell organisation, with the long-term goal of understanding these events in fine detail, and identifying changes responsible for altered beta-cell function in diabetes.

Proinsulin processing in the regulated and the constitutive secretory pathway

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Summary Proinsulin is converted to insulin in beta-cell granules. Conversion involves endoproteolytic cleavage at the two pairs of basic residues linking the insulin A- and B-chains to C-peptide. The sequence of events leading to complete conversion differs from one proinsulin species to the next. In man, the structure of the proinsulin molecule is such as to favour cleavage at the B-chain/C-peptide junction leading to the generation of des-31,32 split proinsulin as the predominant, naturally occurring conversion intermediate. Under normal circumstances, proinsulin conversion is largely completed before secretion, and neither the intact prohormone nor conversion intermediates are thus encountered in large quantities

in the circulation. In some pathological situations, including non-insulin-dependent diabetes, insulinoma and familial hyperproinsulinaemia, unusually high ratios of des-31,32 split proinsulin and/or proinsulin to insulin have been reported. As we understand the biochemistry of proinsulin conversion in increasingly fine molecular detail, it should become possible to make use of such unusual ratios to provide insight into lesions underlying altered beta-cell function in disease states. [Diabetologia (1994) 37 [Suppl 2]: S65–S72]

Key words Proinsulin, conversion, conversion intermediates, insulin, beta cell, secretory pathways.

Fig. 1. The two routes for proinsulin conversion. The arrows represent the combined action of an endoprotease and carboxypeptidase to generate conversion intermediates, or insulin and C-peptide, without residual C-terminal basic amino acids. The preference for one of the two possible conversion routes for human, rat I or rat II proinsulin is shown next to the arrows. Thus, for rat II proinsulin, des-64,65 split proinsulin is the predominant intermediate whereas for human proinsulin it is the des-31,32 split form. For rat I proinsulin, cleavage at both the proinsulin junctions occurs with approximately the same probability

Proinsulin: the exemplar proprotein

Although the primary sequence, and two-chain structure, of insulin had been known since the 1950's, it was not until 1967 that Donald Steiner and his co-workers demonstrated that the two chains were initially linked by a connecting peptide [1, 2]. This was the first direct experimental evidence for the generation of a bioactive protein by intracellular post-translational proteolysis, although examination of the primary sequence of other biologically active peptides had suggested their generation from a higher molecular weight precursor [3]. Steiner must also be given credit for proposing the enzymatic pathway for proinsulin conversion [4], which, with hindsight, has proved to be remarkably accurate. It is thus gratifying to see that his group has played a prominent role

in the identification and characterisation of the conversion endoproteases PC1 and PC2 [5–7].

General features of proinsulin conversion

Alternative routes of proinsulin conversion: As initially proposed by Steiner [4], and now confirmed by studies on proinsulin conversion by the recently identified conversion endoproteases [8], proinsulin conversion is a multi-step process. Depending upon the conversion route taken, discrete conversion intermediates will be generated (Fig. 1). It would seem that endoproteolytic cleavage at either of the two proinsulin junctions is the rate limiting step by comparison with the very rapid and efficient subsequent action of carboxypeptidase H (also known as E) [9]. The conversion intermediates recovered from granules, beta cells, or indeed encountered in the circulation, are thus typically in their trimmed state, without C-terminal basic residues [10, 11]. If the B-chain/C-peptide junction is the first site cleaved and trimmed, des-31,32 split proinsulin is generated as the conversion intermediate. Cleavage at the alternative junction, linking C-peptide and the A-chain, generates des-64,65 split proinsulin. As will become apparent, the conversion route adopted depends upon the cell type, as well as the proinsulin species.

Secretory granules are the conversion compartment of the regulated pathway: Based upon our studies in the rat [12], it is suggested that in the beta cell of healthy individuals, the vast majority of newly-synthesised proinsulin molecules are targetted to the granules of the regulated secretory pathway. Morphological evidence further indicates that the bulk of proinsulin conversion occurs in granules [13–15]. This is perfectly in keeping with the known low pH/high Ca^{++} dependence of the conversion enzymes [8] since this cellular compartment is known to contain millimolar levels of Ca^{++} [16] and to become progressively acidified, as granules mature, to a pH of approximately 5.5 [14, 16, 17]. Although it is impossible to exclude precocious initiation of conversion in the TGN as suggested by others [8], if this does occur it is not of any quantitative significance under normal circumstances.

In the event that proinsulin is not targetted efficiently to the regulated pathway, it will be secreted via the constitutive pathway [18, 19]. This is the default, bulk-flow secretory route shared by all cell types [18–20]. It will be seen below, that proinsulin can also be converted in this pathway, albeit less efficiently than in the regulated pathway.

The conversion endoproteases of the regulated pathway: type 1/PC1 and type 2/PC2: Some 25 years elapsed between the discovery of proinsulin and the

Fig. 2. Proinsulin conversion in the regulated pathway of islets (upper panel) or the constitutive pathway of transfected hepatoma (FAO) cells (lower panel). Upper panel: rat or human islets were labelled for 10 min and then chased for 60 min. After extraction, the labelled products were analysed by reversed phase HPLC. The graph shows the percentage of radioactivity in the form of insulin, conversion intermediates or proinsulin (data taken from [34, 36]). Lower panel: FAO cells were transfected with rat proinsulin I or II or human proinsulin. Immunoreactive products released to the medium during a 2-h incubation were analysed by HPLC. The graph shows the percentage of immunoreactivity in the form of insulin, conversion intermediates or proinsulin (data from [35, 48]). The data are presented as mean + SEM □ human; ■ rat I; ▨ rat II

identification of the enzymes responsible for its conversion. In 1987 Hutton's group [8] identified two enzyme activities in insulinoma granules, termed type 1 and 2. Shortly thereafter, furin, the first member of a mammalian family of subtilisin-like proteases with close homology to the yeast Kex-2 gene product was identified. These enzymes all cleave propeptides at pairs of basic residues, but are distinguished by their tissue distribution, kinetics and substrate specificity [21–23]. Two members of this family of enzymes, PC1 (also known as PC3) and PC2 are restricted in their expression to cells equipped with the regulated secretory pathway, and were as such suggested to be the prohormone conversion endoproteases [5, 6, 24, 25]. It is now believed (and assumed for the purposes of this discussion) that PC1 and PC2 are respectively equivalent to the type 1 and 2 activities purified from insulinoma granules [26–28].

Detailed features of proinsulin conversion

Studies in vitro on the conversion activity of type 1/PC1 and type 2/PC2 endoproteases: The study of proinsulin conversion in vitro has depended upon the use of human insulin as the substrate and semi-purified type 1 and 2 enzymes from rat insulinoma [8, 27, 29, 30], or mammalian PC1 expressed in *Xenopus* oocytes [26, 31]. In order to interpret such studies one must assume that a) the semi-purified insulinoma enzymes do indeed correspond to PC1 and PC2; b) that post-translational modification (glycation) and processing of the endoproteases themselves in insulinoma cells or oocytes is identical to that in beta cells; c) that the in vitro environment used for the study of conversion is comparable to that encountered in the physiological setting, the intra-granular milieu. Given these three assumptions (albeit with some reservations), and considering all the data available, it appears from these studies that type 1/PC1 is responsible for cleavage at the B-chain/C-peptide junction and type 2/PC2 for cleavage at the C-peptide/A-chain junction (although this enzyme can also cleave at the alternative junction with a greatly reduced efficiency [31]). It has also been shown that PC2 only cleaves the C-peptide/A-chain junction of intact proinsulin relatively slowly, preferring des-31,32 split proinsulin as its substrate [30]. It has become apparent from our own studies that the conversion route is in fact dictated not only by such considerations, but above all by the interplay between the proinsulin structure and the relative levels of the conversion enzymes (to be discussed).

In vivo studies on isolated rat or human islets: evidence for the importance of –4 basic residues: In the rat, there are two, non-allelic insulin genes. The sequence of rat proinsulin I differs from that of rat proinsulin II by two residues in the B-chain and two in the C-peptide. Rat proinsulin I is converted to insulin more rapidly than proinsulin II [32–34]. In studies on the kinetics of proinsulin conversion in rat islets, we have shown that this is due to slow cleavage at the B-chain/C-peptide junction of proinsulin II, leading to the accumulation of large amounts of des-64,65 split proinsulin II during the conversion process [34]. Examination of the sequence differences between the two rat proinsulins shows that a basic residue, lysine, at residue 29 of proinsulin I is changed to a methionine in proinsulin II. This residue lies close to the B-chain/C-peptide junction. Indeed, it is four residues N-terminal to the point of cleavage (between arginine 32 and glutamate 33, the first residue of C-peptide), the so-called –4 position. As we shall see later, –4 basic residues are also favoured for effective conversion of propeptides, including proinsulin, in the constitutive pathway.

The comparison of both rat proinsulins with human proinsulin again reveals interesting differences in sequence, and notably at the C-peptide/A-chain junction [35]. Both rat proinsulin I and II present a -4 basic residue (arginine 62) at this junction, whereas in human proinsulin there is a leucine. Human proinsulin, however, like rat proinsulin I, does have a -4 basic residue (lysine 29) at the B-chain/C-peptide junction. If the -4 basic residue is truly playing a role in facilitating conversion in the regulated pathway, we could predict that the conversion kinetics of human proinsulin should be somewhat slower than that of rat proinsulin I, with the accumulation of greater amounts of the des-31,32 split form. This is precisely what we found when the kinetics of conversion of human proinsulin were followed in isolated human islets [36]. The direct comparison of the kinetics of conversion of rat and human proinsulins is shown in Figure 2 (upper panel). Human or rat islets were pulse-labelled for 10 min and then incubated for a 2-h chase [34, 36]. Islets were harvested at set times during the chase, extracted, and the percentage of radioactivity in the form of proinsulin, conversion intermediates or insulin analysed by reversed phase HPLC. In Figure 2, only the data for the 60-min time point are shown. Although the amount of radioactivity found in fully processed insulin was not significantly different for the three species at this time point, the differential accumulation of radioactivity in des-31,32 split human proinsulin and des-64,65 split rat proinsulin II is quite striking, and confirms rapid cleavage at the B-chain/C-peptide and C-peptide/A-chain junction of these two proinsulin species, respectively. Taken together the data provide strong evidence for a role of proinsulin structure, and in particular -4 basic residues, in conversion. This interpretation of our data, however, depends upon the as yet untested assumption that the conversion machinery of rat and human islets is identical. Given the importance of intrinsic levels of PC1 and PC2 in conversion (to be discussed) it will be necessary to compare such levels directly in rat and human beta cells. Another interesting approach towards resolving this issue will be to express one of the proinsulins in the alternative tissue (i.e. human proinsulin in rat islets). A recently described method for obtaining high levels of expression of foreign genes in primary cells using adenovirus [37] may be promising in this context, and we are currently initiating such a study.

Comparison of proinsulin conversion kinetics and levels of PC1 and PC2: evidence for the dominant role of PC1: The studies described above on the kinetics of conversion of proinsulin by type 1/PC1 and type 2/PC2 in vitro, suggested distinct and discrete roles for the two enzymes in the cleavage of the B-chain/C-peptide and C-peptide/A-chain junctions, re-

spectively. We were concerned, however, by the fact that these studies were performed on human proinsulin as the unique substrate, since our own work showed that the structure of the proinsulin molecule could significantly affect conversion kinetics (Fig. 2). It is also important to recall that the type 1 and type 2 enzymes were purified from rat insulinoma tissue. Although their identity as PC1 and PC2 (respectively) appears well founded, their dual role in proinsulin conversion in primary beta cells, as opposed to transformed insulinoma cells, remains to be established. We therefore extended our studies to the direct comparison of proinsulin conversion in transformed or primary beta-cells.

For this purpose we used the highly differentiated insulin-producing INS cell line [38], which was derived from a transplantable rat insulinoma similar in origin to that used by Davidson et al. [8] for the isolation of the conversion enzymes. Unlike RIN cells, these cells produce both rat insulin I and II and therefore provide a useful model for comparing proinsulin conversion kinetics with primary (rat islet) beta-cells. The conversion of both rat proinsulins in these cells was much slower than in the primary cells and was accompanied during the chase by the striking accumulation of the des-64,65 split intermediate of both proinsulin I and II [39]. This suggested unusually slow cleavage at the B-chain/C-peptide junction of both proinsulins, yet in primary cells this junction was cleaved with great efficiency in proinsulin I (Fig. 2 and [39]). There are several possible explanations for this difference in cleavage kinetics. Perhaps the intra-granular environment (acidity, Ca^{++} etc.) in INS cells is not as well adapted for PC1 activity as it is in primary beta-cell granules. Alternatively, the transfer of proinsulin from the TGN to the granules may be retarded, with precocious conversion at the C-peptide/A-chain junction by PC2 occurring in the TGN [8]. Finally, it appeared possible that the intrinsic activity of PC1, if it was implicated in conversion at the B-chain/C-peptide junction, may be lower in INS cells than in beta cells. We therefore compared the protein levels of PC1 and PC2 in INS cell and rat islet extracts by Western blot analysis, and in INS cells we did indeed find less PC1 [39]. Always assuming that the protein levels measured in this way faithfully reflect enzymatic activity, the relatively lower levels of PC1 could thus explain the sluggish conversion of proinsulin in INS cells, and these data lend support to the role of PC1 in the conversion process in the living cell. One possible error in this study was the use of islet extracts, with their mixed population of cells, rather than pure beta cells, as the reference tissue for primary cells. We therefore measured PC1 and PC2 levels by Western blot analysis of extracts of beta and non-beta cell populations purified by autofluorescence activated flow cytometry. The data show that primary beta cells have very high ratios of

PC1 to PC2, whereas in non-beta cells the opposite is true [40]. This makes the difference between INS cells and primary beta cells even more striking than we had supposed when using islets rather than the purified primary cells as the reference tissue.

It is known from the work of others, that in some transformed cells only one of the two regulated pathway convertases can be detected. One such line, AtT20 cells, was of particular interest. These transformed mouse pituitary corticotrophs had been shown to have high levels of PC1 but essentially no detectable PC2 [41]. Although we had been using these cells to express proinsulin and study its trafficking and processing [42–45], we had not made any direct comparison of proinsulin conversion kinetics in this cell and primary beta-cells. Such a comparison has now been made for both rat proinsulins and for human insulin (Irminger et al. unpublished data). Strikingly, the conversion of all three proinsulin species is more rapid in the transfected AtT20 cells than in islet cells, despite the almost total absence of PC2. Thus, in contrast to results obtained *in vitro* [41], our data suggest that PC1 may be able to convert proinsulin completely to insulin in AtT20 cells, and indeed similar data have been reported for proinsulin conversion in COS cells co-transfected with the prohormone and PC1 [41].

We are left to conclude from all these studies that PC1 is certainly implicated in proinsulin conversion. The role of PC2 is, however, not clear. Indeed, the rapid conversion of proinsulin in AtT20 cells, taken with the low levels of PC2 in native beta cells, suggest that this enzyme, at least in the rat, may not play any significant part in proinsulin conversion.

Proinsulin conversion in the constitutive pathway: One hallmark of beta-cell differentiated function is insulin secretion via the regulated pathway. There are certain circumstances, however, in which the normally effective targetting of proinsulin to this pathway is perturbed, leading to its secretion via the constitutive pathway [18, 19]. Although such diversion from the regulated to the constitutive pathway may be limited to cases of insulinoma [10, 46, 47] and very rare examples of familial hyperproinsulinaemia [42], it is nonetheless of some interest to understand how proinsulin is handled in the constitutive pathway.

In order to follow proinsulin processing in the constitutive pathway without any interference from the regulated pathway, we elected to express proinsulin in rat hepatoma (FAO) cells [35, 48]. All three proinsulin species of interest, rat I, rat II and human, have been expressed by stable transfection in these cells. In each case, there was limited proteolytic processing of proinsulin, as indicated by the secretion not only of proinsulin itself, but also of conversion intermediates and insulin. The kinetics of conversion and release from FAO cells are, of course, quite different

from regulated cells (be they primary beta cells, transformed INS cells, or transfected AtT20 cells). Thus, in these “constitutive” cells, there is no granular storage compartment, and proinsulin or its conversion products are released within some 60 min of their synthesis [35, 48]. Indeed, no conversion intermediates or insulin were ever found within the cells, suggesting that conversion was a very late event arising just before release. Given these observations, we have elected to compare the percentage of *immunoreactive* proinsulin, intermediates or insulin released to the medium by transfected FAO cells (Fig. 2 lower panel; data derived from [35, 48]), with the percentage of *radioactive* products generated during a chase in islets (Fig. 2 upper panel). In both cases, we are thus focusing our attention on the fate of newly-synthesised products. This comparison shows that conversion in the constitutive pathway of FAO cells obeys the same rules as those observed in the regulated pathway of islet cells. Rat proinsulin I is most extensively converted to insulin. For rat proinsulin II, the major immunoreactive product is des-64,65 split proinsulin, whereas for human proinsulin it is the des-31,32 split form. These data again focus attention on the importance of a –4 basic residue preceding the two proinsulin cleavage sites [35, 48], and indeed it is known for other proteins converted in the constitutive pathway that both pairs of basic residues in the –1 and –2 site, and a –4 basic residue facilitate cleavage [49].

The conversion of proinsulin in the constitutive pathway of hepatoma cells may not, needless to say, be representative of that occurring in the beta-cell after diversion from the regulated to the constitutive pathway. Unfortunately, we do not have access to an appropriate model for studying this directly. We have, however, observed that in transfected AtT20 cells, which can be taken as a good model for beta cells in that they are richly granulated and express high amounts of PC1 (as discussed), some clones are less efficient at targetting human proinsulin to the regulated pathway than others. This seems to be an example of spontaneous and partial dedifferentiation and does not appear to be related to the transfection of the cells with proinsulin *per se*. We have studied one of these clones in some detail [45] since it provides a unique situation in which proinsulin processing in the regulated and the constitutive pathway can be monitored in parallel in the very same cell. It is comforting to note that the data for conversion in the constitutive pathway are in complete agreement with those obtained in the transfected FAO cells.

In conclusion, proinsulin conversion is dictated by a) the structure of the proinsulin molecules, and in particular the presence of a –4 basic residue (i.e. 4 residues N-terminal to the point of cleavage) at both of the proinsulin junctions; b) the level of conversion endoproteases, and in particular that of PC1. As it happens, the pattern of conversion intermediates

generated during conversion in the regulated or the constitutive pathway is the same for any given species of proinsulin.

Proinsulin and conversion intermediates in NIDDM

The detection and measurement of conversion intermediates in the circulation depended for many years upon HPLC analysis of serum samples [10]. Although this method allows for the unambiguous separation of proinsulin, both conversion intermediates and insulin, it is so time-consuming as to be inappropriate for the screening of large numbers of subjects. Furthermore, this analytical approach depends upon the pre-purification of proinsulin-related peptides from large volumes of serum, and this raises questions of possible differences in the recovery of individual products. More recently, a method has been described for the quantification of these products by two-site IRMA [50], allowing for the assay of numerous samples from different patient populations of interest (see [51] for a recent review). This method depends upon a panel of highly specific antibodies not necessarily available to all interested scientists, and cannot discriminate between all the products of interest (and notably between the split and split and trimmed forms of the conversion intermediates [11]). It will be important in the future to directly compare these two analytical approaches. The only such comparison in the literature is that between HPLC analysis and a less sophisticated immunoassay approach in which the conversion intermediates were not resolved from proinsulin itself [52].

Using these techniques, and above all the IRMA method of Sobey et al. [50], it has become apparent that the ratio of proinsulin and/or des-31,32 split proinsulin to insulin is frequently elevated in NIDDM (see [19, 51] for review). The explanation for these unusually elevated ratios remains to be found. The comparison of circulating levels of proinsulin and conversion intermediates in the basal and stimulated states (reviewed in [51]) indicates that these products, like insulin, are being released by the regulated pathway in these patients. Although it has been suggested that there may be a fundamental impairment of proinsulin processing in NIDDM [53, 54], there is only circumstantial evidence for this. When one considers the available data, it seems more likely that increased demand upon the beta cell, and in particular an imbalance between rates of synthesis, intracellular degradation and release could simply lead to granular contents being discharged before conversion is complete. This more conservative explanation is, however, flawed [53, 54] since not all situations in which there is increased demand upon the beta cell leads to such aberrant ratios of proinsulin/conversion intermediates to insulin.

Now that the detailed kinetics of proinsulin processing have been documented, and armed with a more intimate understanding of the enzymology of proinsulin conversion, it should be possible to pinpoint the lesion(s) responsible for the appearance of inappropriate amounts of proinsulin and conversion intermediates. This will certainly provide much needed insight into the pathophysiology of pancreatic function in NIDDM and possibly pave the way for improved and innovative therapy.

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