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Mini-Review

Proinsulin: recent observations and controversies

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Abstract. Proinsulin has generally been regarded as an inert precursor to insulin. However, over the past few years, proinsulin has established itself as a useful research tool for understanding how cells synthesize and secrete peptide hormones. Last year, proinsulin attracted renewed interest for its role as the precursor to C peptide, which may prove useful in the treatment of patients suffering from insulin-dependent diabetes mellitus. This mini-review focuses on three aspects of proinsulin, each of which attracted attention in 1997. These three aspects illustrate how this peptide hormone precursor may yet prove to be more important than its primary role as a prohormone, with only one bioactive product, would suggest.

Key words. Proinsulin; protein sorting; secretory granule; posttranslational processing; diabetes mellitus; C peptide.

Introduction

The hormone insulin plays a central role in regulating metabolism. Because of this, it has been the subject of numerous studies, resulting in its association with a number of scientific milestones. A short list of these achievements indicates that insulin was among the first peptides and/or hormones to be discovered [1], completely sequenced [2, 3], to have its structure determined by X-ray crystallography [4, 5], chemically synthesized [6, 7], shown to be synthesized as a larger precursor [8, 9], cloned [10] and synthesized by genetically modified bacteria [11].

Many review articles have subsequently been published in which the biology of insulin was examined. In addition, its physiologic role in diabetes has recently been discussed in this journal [12]. In contrast, less attention has focused on its precursor, proinsulin, whose properties have been reviewed on several other occasions [13–22]. Therefore, why write another article focusing on a propeptide which was discovered 30 years ago and which possesses little or no biological activity of its own? The answer is that this propeptide still has the potential to shed significant light on the field of endocrinology. Indeed, during 1997 three articles stood out as examples of how proinsulin may yet lead us to significant basic scientific and clinical insights. Specifically, the following questions have been addressed. How is proinsulin sorted to the regulated secretory pathway? What information within proinsulin and its converting enzymes dictates how it should be converted to insulin? And, is there additional biological information, besides that contained in insulin, to be found within this prohormone?

Cell biology

The mechanism by which peptides and proteins destined for regulated secretion are selected and packaged into secretory granules is still not completely understood. It appears that most endocrine cells have at least two pathways by which proteins leave the cell, a constitutive secretory pathway and a regulated secretory pathway (RSP). Proteins destined for the RSP appear to be initially sorted away from constitutively secreted proteins in the *trans*-Golgi network (TGN) [23]. A second level of selection may take place during the conversion of immature secretory granules to mature secretory granules [24]. In contrast to transmembrane domain-containing proteins, which can be localized to a particular subcellular compartment by virtue of information contained in their cytosolic domains, secretory proteins are generally soluble at physiologic pH. How are peptide hormones selectively isolated from a sea of soluble proteins for inclusion in the RSP?

Two mechanisms have been proposed. One hypothesis is that regulated secretory proteins may undergo an aggregation phenomenon, possibly mediated by changes in both pH and Ca^{++} concentration within the TGN [25, 26]. The aggregated proteins are somehow selected for enclosure within secretory granules. Alternatively, the regulated secretory proteins may selectively bind to a membrane-associated receptor that possesses the transmembrane and cytosolic domains needed for sorting the ligand into the RSP. These proposed models are not mutually exclusive.

Last year two articles appeared which suggested that a protein which acts as sorting receptor had been identified in anterior pituitary cells [27, 28]. It was proposed that carboxypeptidase E (CPE, also known as carboxypeptidase H or CPH) is, in addition to its role as a known exoprotease, also responsible for sorting proopiomelanocortin (POMC) to the RSP (see also ref. 29). This finding engendered significant discussion on theoretical grounds [30], and prompted another group to use proinsulin to reexamine this issue [31].

The initial findings indicated that POMC is able to bind to CPE, and that pituitary cells derived from animals without functional CPE (*Cpe*^{fat} mice) release more POMC via the constitutive pathway than do cells containing CPE [27, 28]. In contrast, the follow-up study, using pancreatic islet cells derived from the same strain of mice, did not show a decrease in regulated secretion of proinsulin-derived products [31]. While these observations appear to contradict one another, it should be pointed out that the sorting mechanism does not have to be the same for all proteins destined for the RSP.

Because this controversy remains to be resolved, we are left with a number of unanswered questions:

1) While POMC appears to have a specific association with CPE, CPE is not a transmembrane protein. So, is CPE an adaptor protein located between POMC and the true sorting receptor? Or, does it simply coaggregate with POMC? (N.B. CPE does exist in a membrane-associated form [32, 33]. However, it is a peripheral membrane protein and therefore would not possess the extraluminal domain believed to be required to signal receptor clustering and initiate vesicle budding.)

2) As both POMC and CPE are destined for the RSP, they may simply be travelling together. Therefore, if aggregation of proteins in the TGN is the sorting mechanism, do all RSP-directed proteins aggregate together? Or can they aggregate individually, in pairs or in many different combinations that are all equally suitable for RSP-directed sorting to occur?

3) POMC has an RSP-sorting signal in the form of the Cys-Cys loop [34, 35]. So do chromogranins A and B [36, 37]. Does this imply that all three proteins are sorted to the RSP using the same mechanism?

4) Proinsulin does not appear to have a Cys-Cys loop sorting signal. Does the observation that proinsulin does not require CPE for sorting to the RSP invalidate any conclusion from the study of POMC sorting?

5) Proinsulin forms homogeneous hexameric crystals around two zinc atoms. Is this proinsulin's functional equivalent of the Ca^{++} and pH-dependent aggregation observed for some of the other RSP-directed proteins?

6) Is there any one protein sorted to the RSP that can serve as a model for determining the mechanism by which all regulated secretory proteins are directed to maturing secretory granules?

I will only offer to answer the last question, with a moderately confident no. Given the number of different proteins destined for the RSP and the lack of an apparent consensus sorting sequence in these proteins, combined with the presumed difficulty of evolutionarily engineering a common physical property into all of the RSP-directed proteins sufficient to segregate them from all other proteins in the TGN, I believe that there are multiple mechanisms involved in sorting these proteins. This is the most likely reason why a single RSP-sorting mechanism has not yet been defined, and why continued examination of the RSP-sorting phenomenon should continue, using POMC, chromogranin, proinsulin and other RSP-targeted proteins as model systems.

Biochemistry

Many peptide hormones are initially synthesized as larger precursors that undergo posttranslational processing to achieve their final biologically active form. A family of subtilisin-like serine proteases, the furin/PC

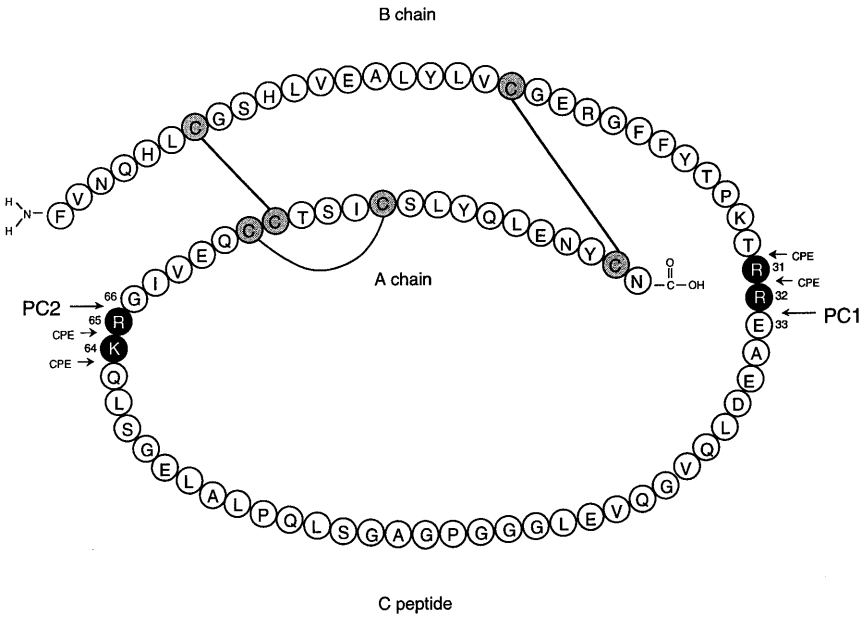

Figure 1. Diagram of human proinsulin. Cleavage sites for PC1, PC2 and CPE are indicated. Amino acid residues used in the nomenclature of conversion intermediates are numbered. Black circles represent the two pairs of basic amino acids used for proteolyitc processing, while the grey circles represent cysteine residues which participate in disulphide bonding.

family, has been identified as the enzymes responsible for cleaving many of these precursors at sites containing basic amino acids [38, 39]. However, not all sites containing basic amino acids (either one or more basic residues) are used as processing sites. Indeed, some precursors have multiple potential cleavage sites, some of which are used in certain cell types, while other potential cleavage sites may be used in a different cell type. The net effect of this differential processing is that a single precursor can be used as the source of different biologically active peptides. Examples of this type of precursor include POMC [40, 41] and proglucagon [42].

A number of laboratories have chosen to study the specificity of posttranslational proteolytic processing by using proinsulin as a model substrate. Proinsulin is an almost ideal model for two reasons. First, it has only two cleavage sites, and these sites are cut by different members of the furin/PC family. Proinsulin is cleaved by PC1 at the B chain/C peptide junction and by PC2 at the C peptide/A chain junction (fig. 1) [43, 44] (see ref. 45 for review). Second, although its three-dimensional structure has not been completely described, what is known suggests that both of the processing sites are accessible for cleavage [46]. This result, and the presence of three disulphide bonds in the structure of both insulin and proinsulin, implies that the structure of proinsulin should be similar in vivo and in vitro. Thus, studies aimed at delineating those elements of the enzymes responsible for the specific cleavage of propeptides could be conveniently executed using proinsulin as a model substrate.

The exact mechanisms involved in processing proinsulin are important both from a scientific perspective, related to the study of protease specificity, and from a clinical view, because of alterations which occur in non-insulin-dependent diabetes mellitus (NIDDM). Normally, proinsulin is believed to be processed first by PC1 to generate 32,33-split proinsulin, whose exposed basic amino acids are then removed by CPE to generate des-31,32-split proinsulin. This intermediate is cleaved by PC2 and CPE to generate mature insulin [47]. The converse series of reactions, PC2 cleavage to generate 65,66-split proinsulin coupled with CPE to produce des-64,65-proinsulin, followed by PC1 and CPE to generate mature insulin, is rarely observed. A number of studies have indicated that levels of intact proinsulin, des-31,32-split proinsulin, or both, are elevated relative to insulin in individuals suffering from NIDDM, or in experimental model systems mimicking NIDDM [48–61]. While alteration of the processing scheme does not seem to be the direct cause of this disease [62–64], further study of the insulin biosynthetic pathway may help illuminate the underlying β-cell defect responsible for this condition.

Originally, investigators utilized conventional approaches of incubating propeptide-converting enzymes with proinsulin to examine different aspects of proteolytic processing [43, 44, 65–70]. More recently, researchers have examined the specificity of proinsulin conversion by expressing proinsulin either by itself, or in combination with the propeptide-converting enzymes, in eukaryotic cells [71–83]. Unfortunately, this type of system suffers from the problem that other proteases, be they members of the furin/PC family or not, have the opportunity to cleave proinsulin in situ. Therefore, it is extremely difficult to assign observed proteolytic events to specific propeptide-converting enzymes.

An alternative approach to correlating converting enzymes with their respective substrates is to examine instances of deficient furin/PC enzyme activity. This can be done either in vitro using antisense technology [84–86], or by examining either naturally occurring [87, 88] or experimentally induced gene knockouts [89–91]. Using these approaches, PC1 has been shown to be involved in the processing of POMC and proinsulin [84, 85, 87, 88], while PC2 is involved in the processing of proinsulin, proglucagon and prosomatostatin [86, 90, 91]. Examination of PC4 gene knockout mice has not yet identified a propeptide substrate for this enzyme, but has indicated that PC4 plays an important role in male reproductive function [89].

While these studies have provided strong support for assigning particular converting enzymes to specific propeptide substrates, these systems do not provide a mechanism to examine the enzyme-substrate interactions which dictate PCE specificity. We have proposed using an alternative model system in which the propeptide substrate and the converting enzyme are individually expressed using recombinant DNA technology, and then incubated together in vitro. The advantages of this system are that the products can be obtained in sufficient quantities for structural characterization, and the observed proteolytic activity can be traced to a single expressed PCE.

While members of the furin/PC family have been expressed using a variety of eukaryotic protein expression systems, the production of proinsulin has been more difficult. However, this past year a procedure for producing proinsulin using prokaryotic protein expression followed by subsequent purification and refolding was published [92]. This means that it is now possible for academic laboratories to generate laboratory-scale amounts of proinsulin, and, by manipulating the sequence of the complementary DNA (cDNA), to produce small amounts of altered proinsulin as well.

This development provides researchers with a tool to dissect those members of the furin/PC family, initially PC1 and PC2, which are able to cleave proinsulin at only one of the two potential cleavage sites. Therefore, we are now able to ask, are there microdomains within individual members of the furin/PC family that are responsible for dictating cleavage specificity? And, if these putative microdomains can be delineated using proinsulin as the substrate, are these microdomains responsible for selecting specific cleavage sites on other propeptides as well? Finally, does the structure of the propeptide substrate itself play a role in dictating cleavage specificity? It is in this last area that the proinsulin model becomes most important, because its constrained structure in solution suggests that the protein-protein recognition events that occur in vitro will reflect the recognition process which takes place in vivo. Proinsulin provides an outstanding model for determining how members of the furin/PC family can perform differential propeptide processing.

Physiology

Following the discovery of proinsulin, it was determined that many peptide hormones are initially synthesized as larger precursors (for reviews see refs 93, 94). Of particular interest was the observation that some of these precursors contained, within their sequence, additional peptide hormones. In some cases, the relative positioning of potential basic amino acids first suggested that these stretches of amino acids could be removed from their precursors during posttranslational processing, and possess previously unknown biological activities. As a group, these biologically active molecules have been called cryptic peptides.

Located between those parts of proinsulin that make up the mature B and A chains is an example of a potential cryptic peptide which was given the name C peptide (connecting peptide). Despite many years of effort, only a few reports have suggested that C peptide may indeed be physiologically active (for a recent review see ref. 95). Last year brought us one of the most interesting of these reports [96].

In the most recent study, it was found that injection of C peptide reversed some of the abnormal effects observed in diabetic rats. Prominent among these were normalization of blood flow, vascular permeability and sodium/potassium adenosine triphosphatase (ATPase) activity. While it should be noted that these effects were observed with pharmacologic doses of C peptide (plasma levels reached 10 times control levels following injection), what is particularly interesting is the relationship between the structure of C peptide and its biological effect. The sequence of the amino acids within the C peptide could be reversed, or, alternatively, the stereochemistry of each amino acid could be changed from the normal L isomer to the corresponding D isomer, yet the peptide retained its biological effect.

This observation implies that the peptide does not confer its biological activity through a conventional receptor. Alternatively, it was proposed by the authors that C peptide may mediate its effects via interactions with the plasma membrane of the target cells. It was pointed out in an accompanying commentary that C peptide does not possess the hallmarks of a pore-forming peptide [97]. The authors of the commentary suggested that, instead, C peptide may interact with membranes in an alternative manner, or it may interact more directly with the cell's machinery for regulating gene expression.

This study leaves us with three major unanswered questions. First, does the C peptide have a biological activity in nondiabetic organisms, or does it only have an effect at supraphysiologic levels in animals suffering the effects of diabetes? Second, could patients suffering from insulin-dependent diabetes mellitus (IDDM) benefit from injecting pharmacologic doses of C peptide in conjunction with insulin? Finally, what is the mechanism by which C peptide reversed some of the vascular and neurologic damage caused by the diabetic state? The excitement generated by the observation of these novel effects of C peptide raises the possibility that additional treatments aimed at ameliorating some of the damage caused by IDDM may eventually be available to patients suffering from this disease.

Summary

Far from being an inert polypeptide which serves only as a precursor to one of the most important peptide hormones, proinsulin has recently been recognized as an important tool that can be exploited to increase our understanding of some of the fundamental principles of endocrinology. As a model for understanding protein sorting and substrate recognition, and for its potential as the source of a clinically useful therapeutic agent (despite our current inability to understand its mechanism of action), proinsulin has attracted the interest of cell biologists, biochemists, physiologists, pharmacologists and clinicians. Now that these challenges have been recognized, it is hoped that we will be able to take advantage of proinsulin's somewhat mysterious properties to increase our understanding of human biology.

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