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Processing-independent analysis of peptide hormones and prohormones in plasma

Jens Peter Goetze[1], Ingrid Hunter[1,2], Solvej Koelvraa Lippert[1], Linda Bardram[3], Jens Frederik Rehfeld[1]

[1]Department of Clinical Biochemistry, Rigshospitalet, Copenhagen, Denmark, [2]Department of Small Animal Clinical Sciences, University of Copenhagen, Denmark, [3]Department of Gastrointestinal Surgery, Rigshospitalet, Copenhagen, Denmark

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1. ABSTRACT

Peptide hormones are post-translationally matured before they reach a structure in which they can fulfill their biological functions. The prohormone processing may encompass a variety of endoproteolytic cleavages, N- and C-terminal trimmings, and amino acid derivatizations. The same prohormone can be variably processed in different cell types and, in addition, diseased cells often change the processing of a given precursor. The translational process is often either increased or decreased in diseased cells, which renders the ensuing modifications of the prohormone incomplete. Consequently, a variable mixture of precursors and processing-intermediates accumulates in plasma. In order to exploit disturbed posttranslational processing for diagnostic use and at the same time provide an accurate measure of the translational product, a simple analytical principle named "processing-independent analysis" (PIA) was designed. PIA-methods quantitate the total mRNA product irrespective of the degree of processing. PIA-methods have now been developed for a number of prohormones and proteins, and their diagnostic potential appears promising in diagnosis of cardiovascular disease and in several malignancies.

2. INTRODUCTION

The mapping and identification of the human genome has resulted in a paradigmatic shift in diagnosis, therapy and prevention of disease. The understanding of the pathogenesis of diseases at an exact molecular and cellular level and the implementation of molecular biology with its vast toolbox of technologies has led to the concept of molecular medicine.

In the last decades, particularly DNA techniques have attracted attention with an exponential increase in the number of recognized mutated and deleted disease genes. More recently, DNA-array technologies have shown disease-specific patterns of gene expression that influence biomedical research, drug development and, not least, diagnosis. In the wake of gene diagnostics, new and improved diagnosis at protein level has now also been established under headings such as post- or functional genomics and proteomics. Early on, advanced mass spectrometry of proteins and peptides was surrounded by a great diagnostic promise (1). The rapid development of protein-array techniques was also followed with great expectations (2, 3). Nevertheless, the proteomic era in peptide biomarker research has largely failed and left the

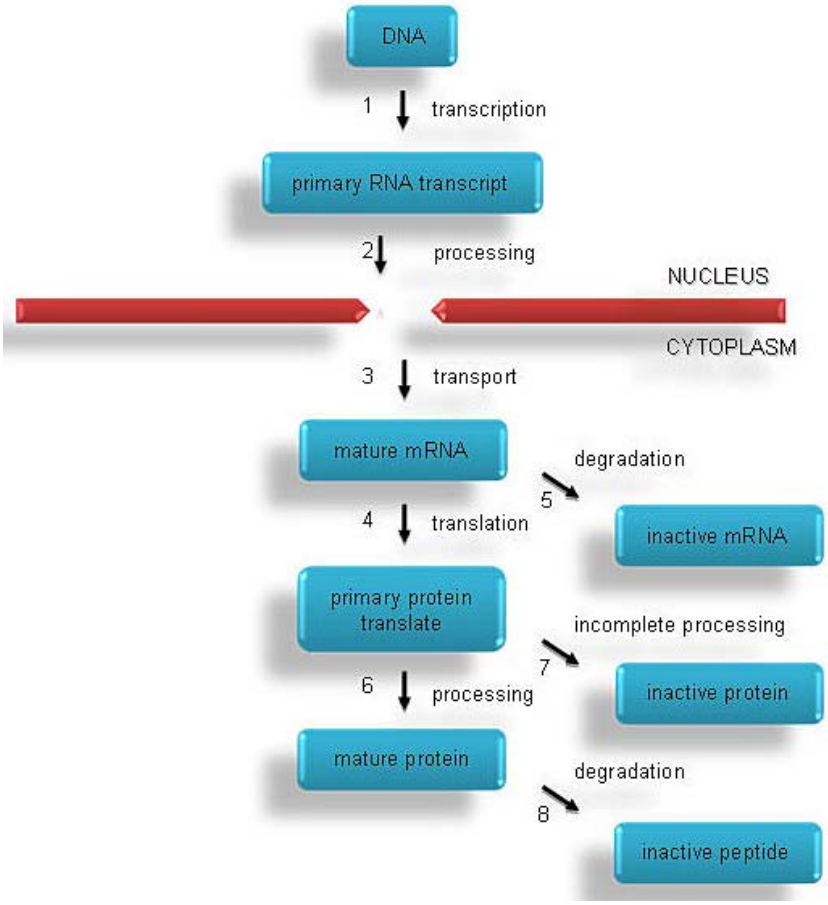

Figure 1. Main phases of the cellular gene expression cascade. The posttranslational phases (phases 6 and 7 on the figure) are often overlooked in standard textbooks of cell and molecular biology.

scenario relying on immunoassays for accurate measurement of peptide products in plasma (4).

The functional or bioactive structure of a protein is defined not only by the gene and mRNA encoding the protein. Proteins, and among them secretory proteins, often undergo multiple covalent modifications before they reach the structure that is required in order to exert their function. The posttranslational modifications of proteins are often affected in diseased cells in a way that determines not only the amount of the functional protein or peptide synthesized in the cell. Disturbed processing also changes the concentration pattern of precursor, processing-intermediates, and the degradation products. Recognition of the pathological significance of posttranslational processing and measurement of processing changes in specific tissues open new possibilities for diagnosis of diseases. In the following, the significance will be illustrated with secretory proteins and peptides belonging to the neuroendocrine system.

3. THE POSTTRANSLATIONAL PHASE OF GENE EXPRESSION

Reviews on peptide measurement in clinical diagnosis have usually paid little attention to the complex and elaborate maturation of the product of ribosomal translation, the proprotein (Figure 1). Removal of the N-terminal signal sequence from prepropeptides is often acknowledged without emphasis of the fact that removal generally occurs in the endoplasmatic reticulum before the translation of the C-terminal sequence of the proprotein is terminated. Accordingly, most cDNA-deduced preproproteins are theoretical structures that almost never occur in living cells. With a few exceptions [for instance apolipoprotein M and haptoglobin-related protein (5, 6)], they exist only in theory or *in vitro* in cell-free translational extracts. It is, however, important to know the exact cleavage site of the signal sequence in order to define the N-terminus of the proprotein. Precise knowledge of the proprotein structure is again necessary for understanding of posttranslational processing mechanisms.

3.1. Endoproteolytical cleavage

The classical or primary sites for endoproteolytic cleavage are located at the peptide bond of the C-terminus of the basic amino acid residues, Arg and Lys. As pointed out by Steiner in his fundamental studies on the biosynthesis and conversion of proinsulin to insulin (7, 8), cleavage often occurs at dibasic sites. Of these, Lys-Arg sites are most commonly cleaved, and Arg-Arg constitutes

Table 1. Posttranslational modifications

Endoproteolytic cleavages	1. Dibasic sites 2. Monobasic sites 3. Post (poly) Glu/Asp sites 4. Post Phe-sites 5. Tri- and tetrabasic sites
Exoproteolytic trimmings	1. Carboxyamidation 2. Glutaminyl cyclation 3. N-terminal dipeptidyl cleavage
Amino acid derivatizations	1. Acylations 2. Disulfide bridgings 3. Gamma carboxylation 4. Glycosylations 5. Isomerisations 6. Phosphorylations 7. Sulfations

the second most common dibasic cleavage site. In contrast, Arg-Lys and especially Lys-Lys sites are rarely cleaved (9). Also cleavages at monobasic and tri- or tetrabasic sites are common (10-12). On the other hand, many peptide bonds after Arg and Lys residues are never processed. Accordingly, it is difficult to predict cleavage at a given site although attempts have been made (11, 12).

The endoproteolytic cleavage at mono- and dibasic sites is achieved by subtilisin-like proprotein convertases of which seven are known today (for reviews, see refs. 13 and 14). The cleavage of most neuroendocrine precursors can be explained by two of these, the prohormone convertases 1/3 and 2 (PC1/3 and PC2) (15-18). After cleavage at the C-terminus of Arg or Lys, the basic residue is removed by a carboxypeptidase. So far, carboxypeptidase E appears responsible for most C-terminal trimming of basic residues in prohormone processing (19). In accordance with the assumed significance of these processing enzymes, spontaneous endocrine syndromes in man and mice (20-21) as well as phenotypes of knock-out mice are seen by deficiency or elimination of the enzymes. The neuroendocrine disturbances comprise obesity, mild diabetes mellitus, and severe Cushing's disease (20-23), and in PC4 deficiency also impaired fertility (24). The symptoms are easily explained by deficient processing of specific prohormones.

In addition to the processing at characteristic basic sites by prohormone or proprotein convertases, protein precursors may also undergo secondary endoproteolytic cleavage at other residues. Hence, systematic cleavage has been reported after Phe-residues (25, 26), after polyacidic Glu/Asp sequences (27-29), and others. Also N-terminal trimming by dipeptidyl peptidases has been reported for a number of protein precursors (for review, see ref. 30). All of the processing enzymes responsible for endoproteolysis at secondary sites are probably not yet identified. Some of these sites resemble those cleaved by lysosomal degradation enzymes. But since the processings observed occur along the secretory pathway in the cells, it has been suggested that perhaps minute amounts of less specific lysosomal enzymes, such as cathepsins, occasionally may be located also along the secretory pathway to contribute to the posttranslational maturation. The correct explanation awaits molecular identification and precise cellular localization of these secondary processing enzymes.

3.2. Exoproteolytic trimming

A characteristic feature of many peptide hormones and neuropeptides is covalent modification of their N- and C-termini. The modifications protect against extracellular degradation by amino- and carboxypeptidases. Some peptides are modified in both ends, others only in one. In half of all regulatory peptides (hormones, peptide transmitters, growth factors and cytokines in their active form), the C-terminal amino acid residue is alpha-amidated (for review, see ref. 31). For most of these peptides this alpha-amidation is a prerequisite for binding to specific receptors and hence for biological activity (32, 33). The carboxyamidation process is accomplished by a now well-characterized enzyme, PAM (peptidylglycine alpha-amidating monooxygenase), which is a complex of two enzymes derived from the same proenzyme present in secretory granules (34, 35). In the presence of copper, ascorbic acid, and at a pH of 5 – as it is – in the mature secretory granules, the PAM complex hydrolyses the C-terminal glycyl residue in the immediate precursor of alpha-amidated peptides and proteins. Hence, glycine is the obligatory amide donor. Accordingly, the sequence –X–Gly–Arg/Lys–Arg/Lys– in a proprotein constitutes a phylogenetically preserved "amidation site" to be cleaved and trimmed sequentially by prohormone convertases (often PC1/3), carboxypeptidase E, and the PAM complex.

N-terminal trimmings are more variable. N-terminal glutaminyl residues are often cyclized to pyroglutamic acid by the specific enzyme, glutaminyl cyclase (36). Also N-terminal or near N-terminal acetylation or other acylations and O-sulfations seem to protect against aminopeptidase degradation (27, 37), and, as mentioned above, some N-termini are trimmed by dipeptidyl peptidases (30).

3.3. Amino acid derivatizations

The list of posttranslational amino acid derivatizations is still growing. Primary and secondary protein structures are nowadays deduced from cDNA sequences. Amino acid derivatizations, however, cannot be deduced. Therefore, mass spectrometry of proteins and peptides is necessary to determine the degree and nature of derivatizations. Moreover, it is likely that mass spectrometry will reveal new amino acid modifications along with identification of the entire human proteom. Table 1 shows a number of the known derivatizations. In these years, many of the genes encoding processing enzymes responsible for the amino acid derivatizations are being cloned, and the enzyme structure and function are being identified. Like several proprotein convertases may participate in the endoproteolytic processing of mono- and dibasic cleavage sites, a certain redundancy is also seen for enzymatic amino acid derivatizations. For instance, mammals express two different tyrosyl-sulfotransferases (for review, see ref. 38).

The exact role of each derivatization is not yet fully elucidated. As mentioned, some seem to protect the protein or peptide from enzymatic degradation before or after cellular release. Other derivatizations increase or determine the interaction with binding proteins such as

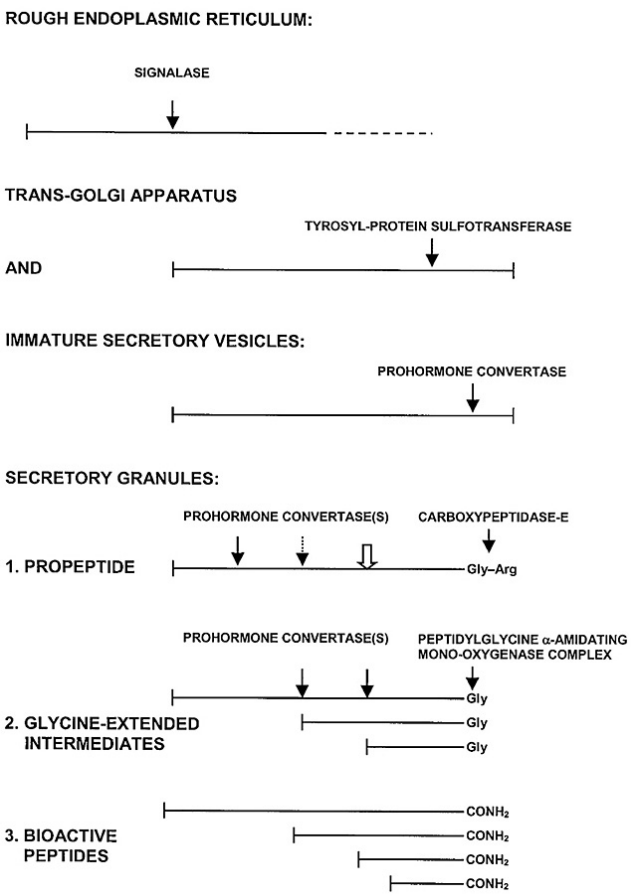

Figure 2. Bioactive processing products of human preprogastrin in antroduodenal G-cells. The mono- and dibasic cleavage sites on progastrin products are indicated in amino acid monoletter code (R, RR and KK). The signal peptide is removed co-translationally at the N-terminus of serine (S) in position 1 of progastrin. The small s and ns indicates whether the peptides are O-sulfated or not (right side of figure).

chaperones, processing enzymes, and receptors. Derivatizations may also serve as signals in the intracellular trafficking. Irrespective of the functional significance of a given derivatization, it influences measurement of the protein or peptide. The methods used for measurement are most often immunochemical: And if a given epitope on the protein or peptide contains one or more derivatizations, they may determine whether the epitope is bound to antibodies.

4. GUT AND HEART: EXAMPLES OF POSTTRANSLATIONAL PROCESSING

4.1. Gastrin

Progastrin is the precursor of the gastrointestinal hormone, gastrin, which regulates gastric mucosal growth and gastric acid secretion (Figure 2). The maturation of progastrin in antroduodenal G-cells involves multiple enzymatic processes. The first is initiated in the transGolgi-network, where progastrin becomes tyrosyl-sulfated and seryl-phosphorylated. Simultaneously or even earlier, cleavage at two double Arg sites begins (Arg_{36}–Arg_{37} and Arg_{73}–Arg_{74}). The cleavages, however, are never complete. They are followed by partial endoproteolytic cleavages at the double Lys site (Lys_{53}-Lys_{54}) and the monobasic Arg_{19} site (17). Minor cleavages also occur after Trp_{58} and the poly-$Glu_{60\text{-}64}$ sequence (27, 38). In order to ensure carboxyamidation, carboxypeptidase E removes the C-terminal Arg-residues so that PAM can use the glycyl residue as amide donor. Along the secretory pathways the free N-terminal glutaminyl residues (Gln_{38} and Gln_{55}) are also cyclized to pyroglutamic acid. The result of the multiple modifications is that the G-cells release a mixture of alpha-carboxyamidated, i.e. acid-stimulatory, gastrins into the circulation (Figure 2). The dominating molecular forms in normal human plasma are gastrin-34 and gastrin-17, which both circulate in tyrosyl-sulfated and unsulfated variants. In addition, longer (gastrin-52 and gastrin-71) and shorter (gastrin-14 and gastrin-6) forms are also released to plasma. On top of the bioactive gastrins, however, the G-cells also secrete processing intermediates as well as N- and C-terminal progastrin fragments that at present are without known bioactivity. The pattern of amidated gastrins and other progastrin products in circulation is influenced by age, gender, meals, and pH in the stomach. The ratio between acid stimulatory gastrin and inactive precursors

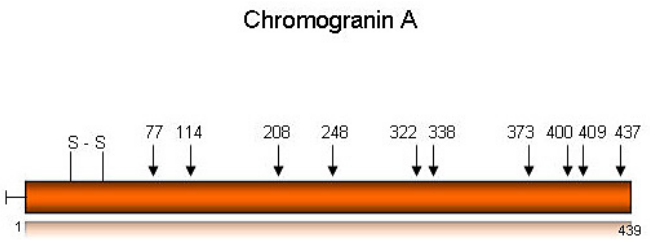

Figure 3. Human chromogranin A with indication of dibasic cleavage sites (arrows) and the N-terminal disulfide bridge (S-S). The numbers (1 and 439) indicate number of amino acid residues.

and precursor fragments therefore vary considerably and individually. As a general rule, the fraction of unprocessed and incompletely processed progastrin fragments increases exponentially with increased cellular synthesis, as seen in tumors (gastrinomas) and in hypo- and achlorhydria (39, 40). Consequently, methods that measure only the concentration of carboxyamidated gastrins provide a false low picture of the cellular synthesis. In contrast, methods that measure the total progastrin product provide a more accurate measure. This distinction has a significant diagnostic impact (39).

4.2. Chromogranin A

Chromogranin A is a highly acidic secretory protein of 439 amino acids expressed in most neuroendocrine cells, where it is packed closely together with hormonal or neurotransmitter peptides as well as monoamines in secretory granules or synaptic vesicles. The precise functions of chromogranin A (CgA) is not yet known, but different roles have been suggested (for review, see ref. 41). They include cellular chaperone function, modulation of prohormone processing, or a precursor role for new bioactive peptides. Irrespective of possible functions, circulating CgA has turned out to be a highly useful general marker for neuroendocrine tumors, including "silent" endocrine tumors and the fairly frequent intestinal carcinoid tumors that have otherwise been difficult to diagnose at an early stage (42). As suggested by the many mono- and dibasic sites in the sequence, CgA is subject to extensive but tissue-specific posttranslational processing in neuroendocrine cells (Figure 3). Accordingly, many endoproteolytic fragments have been identified in tissue and plasma (43, 44), with some fragments claimed to exert independent hormonal functions (for reviews, see refs. 45 and 46). However, solid evidence for such functions, including identification of specific receptors, still remains to be demonstrated. The cellular cleavages of CgA vary considerably and individually. Moreover, the cleavage pattern is tissue and tumor specific. Consequently, measurements of CgA in human plasma are troublesome, not only because the processing of CgA varies between tumors, but also because the epitopes targeted by CgA immunoassays differ markedly and are often not known. In order to avoid false negative results and in order to be able to compare measurements from different laboratories, it is therefore necessary to reach consensus on a method for measurement that in a uniform way quantitates as much as possible of CgA in plasma, irrespective of the degree and individuality of the posttranslational processing.

4.3. Cardiovascular peptides

Natriuretic peptides are primarily synthesized in the mammalian heart and comprise Atrial (ANP) and B-type (BNP) natriuretic peptides (47). C-type natriuretic peptide constitutes the last member of the structural family and is more diffusely expressed in the vasculature and male genital glands (48). The primary structures of the three bioactive end-products are highly related (Figure 4), which also renders their receptor targets almost identical. In contrast, the precursor forms for ANP, BNP and CNP differ in respect to each other, and proANP and proBNP are phylogenetically not so well preserved through evolution (Figure 5). As the biosynthesis for proBNP has received the most attention, the posttranslational processing of this natriuretic peptide will briefly be summarized here. ProBNP is a 108 amino acid residue precursor with the bioactive BNP-32 in the C-terminal region. As other preprostructures, the signal peptide is removed during translation. ProBNP is stored in granules in the atrial myocytes, while the secretion pattern from ventricular myocytes seems constitutive (49). Endoproteolytic cleavage occurs at the Arg-Ala-Pro-Arg motif ($proBNP_{73-76}$), where corin, a cardiac-specific serine protease, seems to be responsible for the cleavage upon peptide secretion (50). Exoproteolytic trimming also occurs in both the N-terminus of proBNP as well as the released BNP product (51, 52). To date, no other proteolytic cleavages have been identified, where the precursor otherwise contains several sites for putative prohormone convertase cleavage (47). The proBNP mid-region of proBNP contains a number of putative sites for O-glycosylation, and this region is indeed variably glycosylated (53). The function of this modification remains unclear, but the glycosylated threonine residue just N-terminal for the Corin cleavage site ($proBNP_{73-76}$) may convey an important regulatory function on post-translational maturation and bioactivation (54).

As mentioned, the posttranslational phase of proANP and proCNP expression has received less attention than the proBNP counterpart, which probably relates to the current interest for clinical proBNP measurement. Nevertheless, the ANP prohormone has been shown to be cleaved into several fragments, and some of these fragments have even been suggested to possess independent hormonal activities (55). The release of ANP from the precursor seems mediated via corin cleavage (50), whereas the remaining endoproteolytical cleavages within the prostructure remain undetermined in

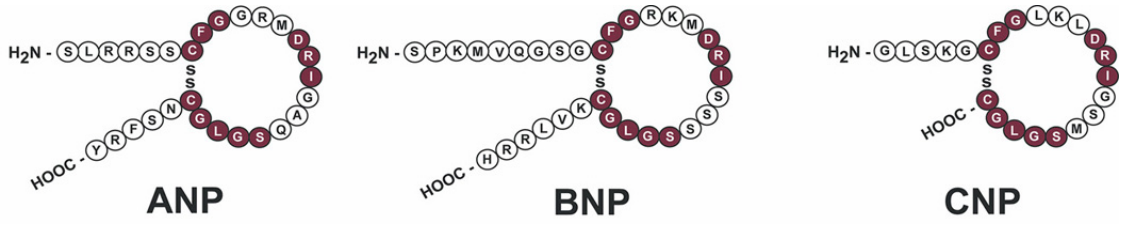

Figure 4. Human natriuretic peptides. Note the bold amino acid residues are preserved between the three peptides.

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                                                  1
PREPROANP: MSSFSTTTVSFLLLLAFQL--LGQTRANPMYNAVSNADL--MDFKNLLDHLEEKMPLEDEVVPPQVLSEPNEEAGAALSP
                                                  1
PREPROBNP: MDPQTAPSRALLLLLFLHLAFLGG-RSHPLGSPGSASDLETSGLQEQRNHLQGKLSELQVEQTS-------------LEP
                                                  1
PREPROCNP: MHLSQLLACALLLTL---LS-LRPSEAKPGAPPKVPRTPP---AEELAEPQAAGGGQKKGDKAP---------GGGGANL


                                                                                        126
PREPROANP: LPEVPPWTGEVSPAQRDGGALGRGPWDSSDRSALLKSKLRALLTAPRSLRRSSCFGGRMDRIGAQSGLGCNSFRY
                                                                                    108
PREPROBNP: LQESPRPTGVWKSREVATEGIRGHRKMV--------LYTLRAPRSPKMVQGSGCFGRKMDRISSSSGLGCKVLRRH
                                                                          103
PREPROCNP: KGDRSRLLRDLRVDTKSRAAWARLLQEH--------PNARKYKGANKKGLSKGCFGLKLDRIGSMSGLGC------
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Figure 5. Aligned primary structures for human preproANP, preproBNP, and preproCNP. The bioactive natriuretic peptides are underlined in each prostructure.

respect to enzymatic activity. Whether the prostructure is glycosylated also remains unsettled, although there is some indirect evidence for this modification also in the proANP structure (56). As for proCNP, even less information is available. The protease furin has been shown to release CNP-22 from its prohormone (57), but CNP also exists in a larger form (CNP-53) where the endoproteolytical catalyst is unknown (58). Chromatographic studies also suggest the existence of a small N-terminal proCNP fragment, which still needs biochemical identification (59).

5. PROCESSING-INDEPENDENT ANALYSIS (PIA) – OR HOW TO QUANTITATE PROTEINS IRRESPECTIVE OF THE DEGREE AND NATURE OF THE POSTTRANSLATIONAL PROCESSING

As illustrated with the above descriptions of progastrin, chromogranin A, and the prohormones of the natriuretic peptides, the processing of propeptides varies in several respects. Not only are there major variations from peptide to peptide, but the processing of the same peptide can vary in the same person in a cell and tissue specific manner. In addition, the processing may change dramatically in a given cell both during the ontologic development and during disease. Finally, the processing of a propeptide varies individually in the same type of tumor in different patients. With the structural clarification of the human gene sequence and with knowledge about the encoded primary structure of all proproteins, it is foreseeable that new patterns and types of posttranslational processing will appear. This again will increase the demands to the diagnostic use of protein and peptide measurements.

In order to overcome the excessive variability, we designed a general analytical principle according to which methods can be developed to measure any protein or peptide in tissue or plasma, irrespective of the nature and degree of posttranslational processing (60-63). The principle of such processing-independent analysis (PIA) is illustrated in Figure 6. PIA measurements are based on three premises:

- Known proprotein structure

- Known or deductible cellular and post-secretory processing in the circulation

- Recognition of the shortcomings of conventional diagnostic methods

The immunochemical version of PIA is then developed in the following way: A sequence of 10 to 12 amino acid residues of the proprotein that on one hand is neither modified nor cleaved during cellular processing, but on the other hand neighbors *in vitro* cleavage sites (typically a trypsin-sensitive basic residue) is synthesized. The peptide is directionally coupled through either its N- or C-terminal residue to a suitable carrier protein (we generally use bovine serum albumin). In order to facilitate coupling, a cystein residue is added during synthesis at the terminus of the peptide through which coupling is going to take place. The peptide carrier complex is mixed in a suitable vehicle and then injected conventionally for production of antibodies specific for the peptide (we use subcutaneous injection over the groins of rabbits). For development of a suitable radioimmunoassay, a variant hapten peptide is also synthesized with a tyrosyl residue inserted in the end opposite to the epitope. The tyrosylated

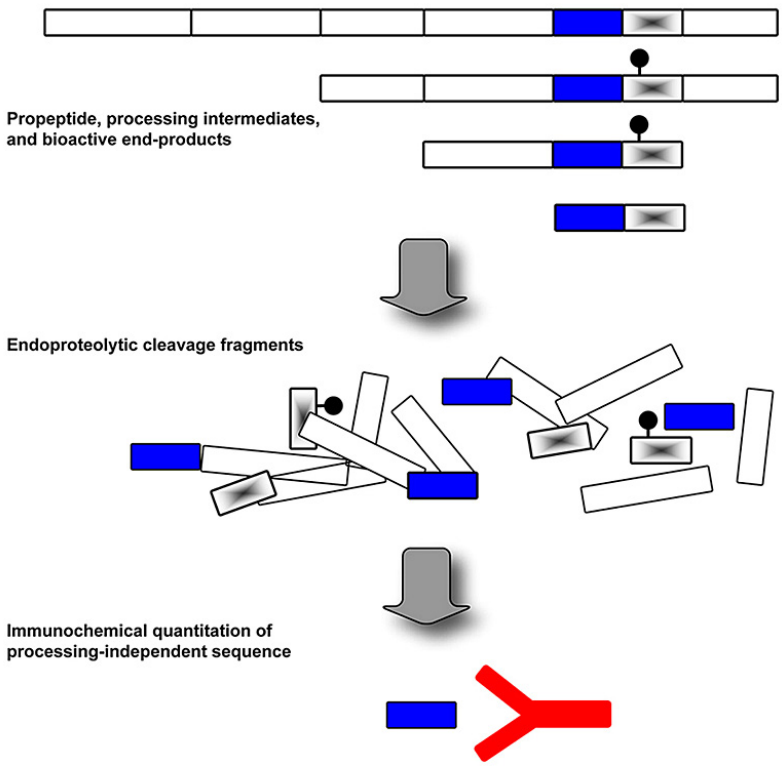

Figure 6. General scheme of posttranslational maturation of secretory proteins showing the principle of "processing-independent" analysis (PIA). The *blue bar* shows a sequence, which does not undergo posttranslational modification, but is present in precursors, processing-intermediates and mature products. *Black dots* indicate different amino acid derivatizations. The processing-independent sequence must be located between suitable cleavage sites, that allow release of one "processing-independent" fragment per molecule translated proprotein by a suitable endoprotease (i.e. trypsin). After inactivation of the endoprotease, *in vitro* quantitation of the "processing-independent" peptide fragment can be performed by a specific assay. The quantity is an accurate measure of "the total translation product" – irrespective of its degree of processing.

peptide is then ^{125}I-iodinated to be used as tracer. In this way a monospecific radioimmunoassay directed against either N- or C-terminus of the processing-independent sequence is established. The sample to be assayed (plasma or tissue extract) is preincubated with a suitable endoprotease (we generally use trypsin). The endoprotease treatment ensures first that the selected epitope is fully exposed for antibody binding, and second that fragments of equal size are released from unprocessed proproteins or partly modified processing intermediates. After inactivation of the protease (by addition of inhibitors or by boiling of the sample), the total translation product is then quantitated by the PIA-designed radioimmunoassay where per definition proprotein, processing intermediates and end-products are included in the quantitation with equimolar potency. Hence, each translated molecule of protein is measured, irrespective of the degree of processing.

6. PITFALLS OF PIA MEASUREMENTS

The first problem is simple and technical. As the initial protease preincubation and inactivation adds analytical steps, it increases the analytical variance and labour-intensiveness of the measurements. This is particularly obvious in comparison with assays that do not require extraction of other pretreatments of the sample; for instance, direct RIA or ELISA measurements on serum or plasma samples. On the other hand, many peptide hormones cannot be measured in plasma without initial extraction or denaturation of plasma proteins and enzymes. Fortunately, treatment with trypsin also degrades common plasma proteins and hence eliminates the necessity of extraction before measurement (64). In this way, PIA measurements may make other preanalytical extractions superfluous, and PIA can be performed without extraction. It is also possible that the protease treatment can be simplified by coupling of the protease to plastic beads or tubes, so that the pretreatment requires only an extra centrifugation and decantation.

The second problem is of more theoretical nature. While PIA measurement on *tissue extracts* affords an accurate and precise quantitative measure of protein-translation, molecule for molecule (provided that the tissue extraction is complete), measurements of *plasma samples* are affected by the large variability in clearance of the different precursors, processing intermediates, and bioactive end-products. Generally, the longer the peptide chain, the slower the clearance. Propeptides and longer processing intermediates consequently accumulate in

plasma in comparison with the shorter and more extensively processed bioactive peptides. In diagnostic terms, this phenomenon is nevertheless an advantage because abnormal cells with disturbed and deteriorated processing mechanisms (as seen in cancer and heart disease) release more unprocessed and partly processed precursors and less fully processed products. Thus, the incomplete processing contributes to increase the plasma concentration as measured with a PIA method, whereas conventional methods that are directed against the bioactive end-product of the cellular processing will measure peptide concentrations that provide a false low picture of the disease. PIA measurements consequently improve both the diagnostic and analytical sensitivity.

A third question to consider is the analytical specificity. Proteins display various degrees of homology and similarity, which may reflect the phylogenetic evolution from common ancestors. Therefore, when a certain epitope (i.e. a sequence of 5 to 8 amino acid residues) is selected – irrespective of whether it is for RIA, PIA, ELISA, IRMA or other antibody based measurements – it is important to ensure from protein sequence data banks that the selected epitope indeed is specific for the proprotein to be measured. This is not only a theoretical question. The history of diagnostic immunoassays and immunocytochemistry abounds with examples of false positive results caused by unforeseen similarity or homology with unknown or known proteins.

7. TWO EXAMPLES OF THE CLINICAL USE OF PIA MEASUREMENTS

7.1. Progastrin

Increased secretion of progastrin products has been encountered by most diseases of the stomach and by cancers in the gastrointestinal tract, pancreas, lungs, and ovaries (64, 65). However, the diagnostic use of measurement of gastrin peptides by conventional assays or by PIA measurements has not been shown for the individual disease, except for gastrinomas (40, 66). Gastrinomas occur mostly in the pancreas and the duodenum. Less frequent are ovarian gastrinomas, and exceptionally gastrinomas have been found in the antrum, lungs and heart (67). When gastrin assays became generally available in the nineteen-seventies, large and metastatic gastrinomas with clear clinical symptoms were frequently discovered. They are easy to diagnose, but today they are rarely found in countries with an organized gastrin assay service. Most gastrinomas detected today are pinhead-sized and contribute only moderately to the circulating concentrations of amidated gastrin. Patients with small gastrinomas may even periodically have normal gastrin concentrations in plasma (67, 68). Consequently, today's gastrinomas show borderline concentrations – not least in the increasing population of people with occasional dyspepsia that take proton pump inhibitors and other inhibitors of gastric acid, which also increase the gastrin concentrations in plasma. In that situation PIA measurements of the total progastrin concentration in plasma may help to ensure correct diagnosis in patients with recurrent dyspepsia, when they are fasting and not taking acid inhibitors for a week (69). In metastatic gastrinomas PIA measurements show larger abnormalities than conventional measurement of amidated gastrins (39, 70).

7.2. ProBNP

Plasma measurement of proBNP-derived peptides has been eagerly pursued in cardiac dysfunction. As heart failure is a common disease with a ~2% prevalence in Western countries, it is no surprise that these peptides may become the most frequently measured peptides in routine laboratories. The clinical use today is mostly as "rule-out" markers of heart failure, as the diagnostic sensitivity is high but the specificity is low.

We have developed a PIA for human proBNP (64) and applied this method in clinical studies (71-90). Apart from the clinical data stemming from these studies, we also made another important observation, as the plasma concentrations of the proBNP-derived peptide fragments differ. This led us to suggest an "endocrine paradox" in heart failure, where the condition is characterized by sodium retention and edema in the midst of sky-high plasma concentrations of natriuretic hormones (91). Notably, it was concluded early on that cardiac processing of natriuretic peptides was a simple process with only one endoproteolytical cleavage. Since then, it has been firmly established that cardiac natriuretic peptides undergo complex post-translational processing and that this processing is highly variable and associated to disease status. The complete picture of natriuretic peptide processing is still being defined where the PIA method has aided research in elucidating the total concentration of proBNP-derived peptides in plasma, irrespective of post-translational cleavages and amino acid modifications. In fact, we expect that the PIA's for proANP and proCNP measurement may also facilitate a broader understanding of the molecular heterogeneity of these related prostructures in health and disease.

8. PERSPECTIVE

The concept of molecular biology was founded more than 50 years ago in the wake of the discovery of the DNA double helix. From the very beginning, it was never doubted that molecular biology was going to have a major impact on clinical medicine. Over the last decades, the development has proved this prophecy more than true in almost any area of medicine. Hence, the term molecular medicine may soon get a flavor of old, unnecessary tautology because most clinical practice will require insight in molecular biology.

In molecular biology understanding of the gene expression cascade is of central significance. Knowledge of the cascade is also of decisive importance for insight in the pathogenesis of a rapidly growing list of common diseases. Studies of expression cascades have so far, however, given only modest attention to the last phase of expression, the posttranslational phase. The purpose of this review has been to improve the balance by indicating that there are

possibilities for new molecular diagnosis to be explored at closer examination of posttranslational processing phase.

Posttranslational processing of proproteins is sometimes simple and without obvious possibilities for new and improved types of diagnostic measurements. But the postgenomic era of biology will present a host of new deduced protein structures. And among these are secretory proteins which, like many known protein and peptide hormones, have undergone multiple maturation steps along the secretory pathway. In the study of the biosynthesis of these proteins, new diagnostic possibilities are likely to appear and contribute to the development of cardiology, gastroenterology, oncology, and other areas of clinical medicine.

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Send correspondence to: Jens Peter Goetze, DMSc, Department of Clinical Biochemistry, Rigshospitalet, DK-2100 Copenhagen, Denmark, Tel: 45 3545-2202, Fax: 45 3545-2524, E-mail: JPG@dadlnet.dk

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