Hypothesis

PROTEOLYSIS AND THE EVOLUTIONARY ORIGIN OF POLYPEPTIDE HORMONES

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1. Introduction

This communication elaborates the hypothesis that the evolutionary origin of polypeptide hormones is from lysosomal proteolysis. It attempts a novel integration of the cell biology of lysosome formation and function with that of secretion in order to explain some curious features of the latter process, particularly the mechanisms and significance of the cleavage of prohormones. From this basic hypothesis is derived a wider view of the origin, role and regulation of proteolysis at sites of pairs of basic amino acids. Some of my ideas on this subject arose, and were briefly introduced, in a contribution to discussion at a recent symposium [1].

2. Precursors of secreted polypeptides

The chemical modification of the primary translation products of secretory proteins has been reviewed [2] and this has highlighted the unexplained very frequent occurrence of synthetic precursors of secreted polypeptides. Two types of precursor have been identified: the so-called prepolypeptides which are very short-lived, represent the true translation products of the mRNA and contain, relative to the secreted polypeptide (or propolypeptide if one exists), an N-terminal extension of ~ 15–30 mainly hydrophobic amino acids. The propolypeptides are longer-lived and are most often cleaved at a highly characteristic site in the molecule, marked by a pair or larger group of basic amino acids, before secretion but after passage into the lumen of the endoplasmic reticulum (ER). The additional sequences in this type of precursor vary greatly in length and structure and, in endocrine polypeptide secretion, the resulting active sequence may be either the N- or the C-terminal fragment so derived [3]. The site of cleavage of the prepolypeptide is not characterised by the presence of basic amino acids. It seems likely that most secreted polypeptides are produced via a prepolypeptide (although ovalbumin may be an exception [4]) but that not all go through a propolypeptide stage.

The significance of the hydrophobic 'pre-' sequences has been clarified. As proposed independently by Blobel and Sabatini [5] and Milstein et al. [6] the sequences mediate binding of polysomes synthesising secretory polypeptides to the membrane of the ER. However the specificity of the sequence is not as great as was originally supposed there being considerable variation in length and sequence [7–12]. Subsequent work on bacterial synthesis of membrane proteins has revealed a similar phenomenon mediating the attachment of polysomes to the cell membrane and extracellular release of enzymes [13,14]. Therefore it now seems likely that the hydrophobic 'pre-' sequences are fairly non-specific peptides which mediate the insertion of polypeptides into membranes. Subsequent cleavage may allow part of the sequence to cross the membrane but the mechanism of this is far from clear. Indeed it should be pointed out here that there are other (non-synthetic) situations in which polypeptides are presumed to cross membranes. For example specific, as opposed to general autophagic, degradation of cytosol proteins should involve the transfer of at least part of the protein into the lumen of the lysosome for complete degradation. Such proteins may therefore also contain the hydrophobic sequences necessary to mediate this transfer.

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The significance of the 'pro-' sequences remains completely obscure although a number of possibilities have been considered [3]. A simple mechanical explanation based upon the minimum length of polypeptide needed to traverse the ribosome and the membrane of the ER has been proposed [3]. However this cannot account for the existence of proalbumin [15]. The suggestion that 'pro-' sequences might be required for a transport process within the ER [2] also seems unlikely since many polypeptides undergoing essentially similar steps of processing prior to secretion do so without the intermission of a 'pro'-stage.

3. Hypothesis to explain propolypeptides

The close relationship of lysosomal function and secretion has been discussed by de Duve and Wattiaux [16]. As an extension of this concept I propose that the mechanisms and pathway of polypeptide secretion have developed from the more primitive process of lysosomal degradation of protein and that this evolutionary relationship provides the basic explanation for the existence of propolypeptides. I suggest that in endocrine polypeptides these 'pro-' sequences have no general functional significance but are related to, and provide an essential clue of, the evolutionary origin of these polypeptides. In making this suggestion it is not intended to rule out the possibility that some 'pro-' sequences may be found to have specific endocrine actions. Whilst the importance of the insulin C-peptide for the correct folding of proinsulin is well recognised, the wide species variations in the sequence of C-peptide compared with the conservation of structure of insulin would suggest that in this example the former does not have an additional specific endocrine function.

Although hypothetical evolutionary relationships are of intellectual interest, they cannot be subjected to experimental examination. The reason for presenting the hypothesis lies not in the rather negative dismissal of the importance of 'pro-' sequences but as a means of developing new theoretical and practical approaches to a number of biological systems. Before doing this the ultrastructural and enzymological evidence indicating a close relationship between lysosomal digestion and secretion will be briefly reviewed.

4. Evidence supporting the hypothesis

4.1. *Ultrastructural*

Ultrastructural studies of the production of lysosomes and their fusion with phagocytic vacuoles [16, 17] and of the production and storage of secretory vesicles [18,19] reveal a number of common features. Phagocytic vacuoles produced by an invagination of the plasma membrane fuse with primary lysosomes derived from the Golgi apparatus to form secondary lysosomes within which degradation of phagocytosed material occurs. Novikoff et al. [20] have recently suggested that the process of formation of a secretory vesicle involves the fusion of a vesicle derived from the rough ER with an element of the Golgi which is closely related to the primary lysosome and which provides enzymes for the processing of prohormones. Furthermore the process of secretion appears to involve a phagocytosis-like reclamation of membrane. Orci et al. [21] have incubated islets of Langerhans in horse radish peroxidase with and without glucose stimulation of insulin secretion and shown glucose-stimulated uptake of peroxidase into small vesicles. This and other ultrastructural evidence supports Douglas's suggestion [22] of an exocytosis–vesiculation cycle in secretion. The cycle has obvious similarities with the phagocytosis–defecation cycle of unicellular organisms such as amoeba [23].

4.2. *Enzymological*

Enzymological evidence relating lysosomes to secretory granules comes from several sources. Lysosomal-type acid phosphatase has been localised in many different types of secretory granule [24–27]. Enzymes capable of degrading proinsulin are very similar both in terms of specificity and pH optimum to those known to exist in lysosomes [31]. Lysosomes [28] and secretory chromaffin granules [29] appear to maintain an internal pH of about 5 and which is important for their function. In both cases this may be achieved by an ATP-driven proton pump [30] although the evidence for this in lysosomes is only circumstantial. 'Lysosomal' enzymes are released in parallel with secretion from a number of tissues including the adrenal medulla [32,33] and may even form the main products of secretion [34,35]. In the latter situation it is not clear whether the enzymes released are genuinely from organelles which also func-

tion in the cell as lysosomes or whether this is the ultimate example of the close relationship between the two processes.

4.3. *Specific examples of the close relationship between secretion and pinocytosis/phagocytosis*

An example of phagocytosis coupled to lysosomal fusion and secretion is provided by the secretion of thyroid hormones by the thyroid gland [36]. This may be a specific and unique example of such coupling. However it should be noted that the relationship postulated here between secretion–vesiculation and phagosome production in phagocytosis presents the theoretical possibility that the substance forming the secretory precursor of one cell-type could be provided by the secretion of a different cell-type. Such a process appears indeed to exist in the example of phosvitin and lipovitellin production by the chicken ovary. Chicken liver synthesises and secretes the polypeptide vitellogenin which is taken up and split in the ovary to produce phosvitin and lipovitellin. The latter polypeptides are incorporated into yolk granules in a molecular ratio of one to one [37].

The exchange of proteins between cells is certainly not restricted to endocrine systems. Thus the addition of normal fibroblasts to a culture of enzyme-deficient fibroblasts can lead to the pinocytotic uptake and incorporation of the deficient enzyme into lysosomes of the abnormal cells [38]. Glial cells transfer proteins to adjacent neurones by a Ca^{2+}-requiring process consistent with exocytosis coupled to pinocytosis [39]. It is possible that similar processes occur in the uptake, action and destruction of polypeptide hormones. It has been found that the structural requirements for insulin binding and degradation are indistinguishable [40]. Growth hormone binding studies have shown a time-dependent generation of bound hormone which is unable to re-equilibrate rapidly with hormone free in solution [41]. Nerve growth factor is taken up by axons and undergoes retrograde transport [42]. The existence of these processes indicates the possibility that polypeptide hormones may act and be destroyed at sites remote from the plasma membrane and that activity may be exerted by fragments of the original hormone [40]. Indeed the possibility of further exocytotic release of such fragments cannot be ignored. Thus the apparent entry of insulin into cells [43] may not in fact represent intact insulin free in the cytoplasm.

Further variation on the theme of secretion of precursor by one cell type followed by processing by another cell type could be achieved by secretion of the processing enzyme(s) as well as the precursor by separate cell-types. In this variation the active substance is produced by extracellular proteolysis rather than by intracellular proteolysis followed by secretion. An example of this method of production of an endocrinologically active polypeptide is provided by the renin–angiotensin system [44]. Here the proteolytic enzyme renin is secreted by the kidney to act on a substrate secreted by the liver to generate the polypeptide angiotensin in the circulation.

5. Theoretical extensions of the hypothesis

5.1. *The existence and regulation of transport processes in secondary lysosomes*

Current understanding of the process of formation of the secondary lysosome implies that part of the membrane is derived from the plasma membrane in such a way that what was previously part of the external surface of the plasma membrane comes to face the contents of the secondary lysosome. This implies that the agents and products of lysosomal digestion have direct access to external plasma membrane components, i.e., components otherwise situated anatomically where polypeptide hormone receptors are found. Furthermore elements of the membrane of the secondary lysosome must be responsible for the transport of the products of digestion within this organelle into the cytosol unless this is accomplished by a process of simple diffusion. Hence by this physical proximity the agents and products of lysosomal digestion are presented with the possibility of modulating the transport of nutrients into the cytosol, an action often observed in polypeptide hormone function. Thus one could envisage with this close approximation of effector and receptor the beginnings of endocrine regulatory processes in a unicellular organism.

If endocrine polypeptides originated from the contents of the secondary lysosome it is interesting to consider which elements might be involved. The phagocytosed material seems an unlikely source since it could not be subject to the continual genetic variation which would be required during evolution. Thus

it is likely that the regulatory factors arose from the lysosomal enzymes or from membrane components. Indeed since the lysosomal enzymes themselves probably originated from membrane enzymes, as they exist in bacteria, the distinction is probably unnecessary. Like many if not all proteins on the outer surface of the plasma membrane, a number of endocrine polypeptides are glycoproteins as is the recently described precursor of ACTH [45]. If some of the effectors arise by partial degradation of membrane proteins then a structural relationship could exist between some effectors and receptors. One model in which a fragment of a parent molecule can restore the properties of the original intact molecule on addition to the residue of the molecule is provided by the S-peptide and S-protein of ribonuclease [46]. Another way in which polypeptide–polypeptide interaction can lead to a change in properties is by oligomeric aggregation of structurally related or unrelated subunits. The changed properties conferred by aggregation can be produced by quite small peptides derived from the monomer. A 14 residue phosphorylated peptide derived from phosphorylase *b* can react with intact phosphorylase *b* and confer on it properties like those of phosphorylase *a* [47]. This therefore provides another model for polypeptide hormone action in one of which variations the agonist and receptor contain closely related structural features.

Certain interesting theoretical consequences may follow from the idea that some effectors and receptors may be structurally related. Firstly the structural properties which are required for effector–receptor interaction may be very similar to those mediating effector aggregation for storage, allowing for simultaneous evolutionary conservation of both properties. It has been pointed out that similar regions of the insulin molecule are involved in dimerisation and in receptor binding [48]. Secondly the crystalised effector may then provide a crude but informative model for the action of the hormone itself. In this context it has been shown that dramatic and reversible changes take place in one of the insulin molecules in the dimer when 2 Zn insulin crystals are converted to a 4 Zn structure in the presence of halide ions [49].

The structural relationships between endocrine polypeptides and their receptors will only emerge when the formidable problems of receptor isolation are overcome. Nevertheless in the meantime it is possible that sequence analyses will provide evidence for the pathway of evolution of polypeptide hormones and their relationship to the contents of secondary lysosomes. A particularly promising situation would appear to exist in relation to nerve growth factor. Recent investigations of the large quantities of nerve growth factor present in mouse salivary glands have shown that this is not an important source of the endogenous hormone. Further work has shown that the activity actually resides in a single large polypeptide of mol. wt 114 000 and that smaller peptides previously described are probably artefacts of degradation [50]. The large polypeptide is a normal constituent of salivary exocrine secretion. When the function of this polypeptide is understood it may provide an insight into the relationship which can exist between an endocrine-like polypeptide sequence and a larger, possibly enzymatically active, polypeptide.

5.2. *The significance of proteolytic cleavage at pairs of basic amino acids*

If propolypeptide cleavage is related to more general processes of proteolysis as I have proposed then the points of cleavage of propolypeptides may have a much wider significance as recognition sites for endoproteolytic cleavage. To date, cleavage at sites of pairs or groups of basic amino acids is by far the most common but not the exclusive [12] means of initial processing of prohormones. The dominant position of the former site of cleavage raises the question as to whether proteolysis of some non-secreted proteins may proceed via the action of an endoprotease recognising sequences containing pairs of basic amino acids. One might then expect the frequency of existence of such pairs to depart from that predicted by a random association consequent upon the frequency of the amino acids within a given polypeptide sequence. We have examined this frequency statistically for a number of cytosol enzymes and failed to observe any significant deviation [51]. However if these sites served the function of mediating specific proteolysis in a regulatory sense it will be essential to examine regulatory enzymes. Since few complete sequences of regulatory enzymes are available this has not yet proved possible. Another approach to examining the significance of pairs of basic amino acids outside secretion is to determine to

what extent such pairs or groups have been conserved during evolution. Striking examples of conservation are provided by the sequences of cytochrome *c*, myoglobin, haemoglobin, glyceraldehyde 3-phosphate dehydrogenase and triose phosphate isomerase [52]. It is worth noting that the pair of basic amino acids highly conserved in the α-chain of haemoglobin forms part of the haem pocket and would be expected to be protected by haem from proteolytic attack. It is well known that haem protects the globin chains from degradation. Thus controlled access to such sites of endoprotease attack could be important in regulating globin degradation.

A much more intriguing example of the consistent appearance of pairs of basic amino acids in polypeptides is in positions adjacent to sites of serine phosphorylation in proteins subject to cycles of phosphorylation and dephosphorylation [53]. It is known that phosphorylation of the serine reduces the sensitivity of the intact polypeptide to tryptic digestion at these sites in vitro [54]. Whilst the three dimensional structures of most of these proteins are not known it is clear that in most of the possible local configurations such as α-helix, β-pleated sheet and β-bend there could be close enough approximation between the negatively-charged phosphate and positively-charged basic amino acids for a strong ionic interaction to occur. An α-helical model of the phosphoserine sequence of the β-subunit of phosphorylase kinase [55] shows that ionic interaction could occur with both basic amino acids simultaneously. If these pairs of basic amino acids provide sites for the initiation of proteolysis then it seems likely that the phosphorylation–dephosphorylation cycle in some proteins could not only rapidly control the activity of the proteins but also regulate proteolysis. Hence a single process could mediate both short-term and long-term control of enzyme activity. However in proteins in which phosphorylation produces inactivation presumably other processes are involved.

The role of pairs of basic amino acids in determining the ultimate sequence of an active polypeptide is most dramatically shown in the family of peptides including ACTH, α+β MSH, β+γ lipotropin, the endorphins and the encephalins which all apparently occur within the larger precursor molecule [45]. In this situation pairs of basic amino acids seem to be acting as 'punctuation marks' allowing proteolytic mechanisms to determine the polypeptide sequence produced. This type of signal may also be involved in polypeptide hormone inactivation since both ACTH and PTH contain groups of basic acids at sites where cleavage would destroy biological activity. It is worth pointing out that there is a close genetic relationship between Lys–Lys as a polypeptide sequence determinant and one of the 'stop' and the 'start' codons since these latter two codons (UAG and AUG, respectively) are each a single mutation from one of the lysine codons (AAG). Thus this type of proteolytic sequence determination is reversibly related by two mutations to the established genetic codes for polypeptide chain-length determination.

It is important also to recognise that the close genetic relationship possible between the stop and start and Lys–Lys codons can allow mutations of the stop and start codons to produce potentially reversible (i.e., by proteolysis) chain-length extensions. If this occurred a certain amount of natural selection of such extensions could go on in the cell itself, since extensions which were not stabilised by some process within the cell would be rapidly removed.

This interesting interrelationship naturally raises the question as to whether proteolytic determinations of polypeptide chain-length evolutionarily pre- or post-dates length determination by the genetic code. An attractive feature of considering proteolysis to be the more primitive mechanism is that it suggests the possibility of a pre-biological natural selection of polypeptide sequences through resistance to proteolysis. It is a well recognised fact that proteins are stabilised and more resistant to proteolysis when bound to substrates, cofactors, prosthetic groups and other polypeptides. Thus the presence of 'predator' proteolytic mechanisms in a pre-biological solution of randomly-growing polypeptide chains could give apparent direction of evolution towards binding sites by allowing the natural selection of those 'best fitted'.

6. Novel experimental approaches resulting from the hypothesis

6.1. *Transport in lysosomes and its regulation*

The experimental possibilities for examining the existence, properties and regulation of transport systems in primary and secondary lysosomes from a

variety of tissues and organisms are obvious. Very few investigations, outside simple permeability studies, seem to have been carried out in this area. In studies initiated as a result of these ideas we have produced evidence for a stereospecific glucose-transport system in rat liver lysosomes which is inhibitable by competition with other sugars and by phloridzin and cytochalasin [56].

6.2. *Sites of pairs or groups of basic amino acids as points for the initiation and regulation of proteolysis*

Examples have been given of a number of proteins in which pairs or groups of basic amino acids have been highly conserved. In other proteins undergoing phosphorylation–dephosphorylation cycles pairs of basic amino acids are close to the site of phosphorylation. Phosphorylation is already known to protect some of these proteins in vitro from proteolytic cleavage by trypsin. In all these situations it is now possible to examine experimentally whether enzymes exist in vivo which attack these sites and whether their effect can be reduced by factors such as phosphorylation or substrate, regulator and prosthetic-group binding.

7. Conclusion

De Duve and Wattiaux [16] in their comprehensive discussion of the functions of lysosomes raised the possibility that secreted proteins such as plasma proteins and a number of polypeptide hormones were late evolutionary descendants of digestive enzymes. Since this suggestion was made, a large amount of additional information has become available concerning the process of secretion. The present hypothesis modifies and extends the ideas considered by de Duve and Wattiaux. It proposes that the existence of propolypeptides may be explained by the fact that secreted proteins and endocrine polypeptides have been evolutionarily derived from the process of proteolysis of membrane and/or enzyme components of secondary lysosomes; that the latter have transport processes for inorganic and organic products of lysosomal digestion and that these may be regulated by some intermediate polypeptide products of lysosomal digestion. Experiments have been initiated to look for such transport processes and have already yielded positive results. The possible structural relationships between effector and receptor and between hormone action and storage have been discussed and may also provide a theoretical basis for the study of the properties of hormone crystals as models of hormone action.

The discovery that pairs of basic amino acids are recognition sites for endoproteolytic cleavage of propolypeptides has been used to consider whether these pairs may function similarly in other proteolytic processes. Attention has been drawn to the evolutionary conservation of such pairs in some proteins and their possible protection from proteolysis by ligands as well as by phosphorylation of adjacent serines. The close genetic relationship between one of the lysine codons and the 'start' and one of the 'stop' codons has been pointed out. The wider possibilities of proteolytic determination of polypeptide chain-length and its evolutionary significance has been considered. Again experiments designed to evaluate the role of such sites in proteolysis generally and its regulation are feasible and, it is suggested, worthwhile.

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