Polypeptide Growth Factors: Some Structural and Mechanistic Considerations

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Polypeptide growth factors are substances that stimulate an increase in cell size and/or cell number during embryonic development. In some cases, they have a similar effect on tissues in the mature organism where they function as "maintenance" factors to sustain cell viability. While their profound impact on cell behavior is well recognized, their relationship to other regulators of cell function has remained generally ill-defined. However, the developing appreciation of their hormone-like behavior suggests that they may be conveniently grouped with many other endocrine agents to form a broader group of secondary hormones. The utility of the classification is illustrated by the insulin-related family of molecules. It also serves to emphasize the similarities in function shared by many of these substances including trophic stimulation and modulation of gene expression. Internalization, though, appears to be another common feature. However, whether the uptake of the growth factor mediates an intracellular action or is designed solely to regulate responsiveness at the cell surface and/or degradation remains an important unanswered question. A brief review of two growth factors (nerve growth factor and epidermal growth factor) serves to outline the possible functions that may be served by this endocytotic process.

Growth is defined as an increase in the size of the body or any of its tissues which results from an increase in cell size (hypertrophy) or cell number (hyperplasia). Such changes in cell populations are not only important features of development but also are evident in the regenerative processes of wound healing and in the routine turnover of cells that characterizes most tissues. Regulation of these growth processes is complex, involving hormones, neural elements, and especially proximal contacts by both heterologous and homologous cells [67]. In all instances, there is a transfer of information that is usually mediated by some type of chemical messenger.

Among the agents functioning in this capacity are the polypeptide growth factors, hormone-like substances that are released from many different cell types and reach their destination by a variety of routes ranging from local diffusion to systemic transport. In general, they can induce both hypertrophic and hyperplastic responses in their target tissues. The increase in cell size is closely linked to an array of metabolic changes generally defined as a positive pleiotypic response [40], which includes stimulation of metabolite uptake and polysome formation leading to increased protein and nucleic acid synthesis. Hyperplasticity can result from a reduction in the extent of programmed cell death or an increase in the rate of mitosis. Substances acting in the latter capacity are also referred to as mitogens, and many of the growth factors with this property have been grouped into two broad subcategories based on the point in the cell cycle when they appear to act. Agents such as platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) prime a significant portion of a quiescent cell population (G_0) to enter a state of readiness or "competence," while other agents, notably the somatomedins, stimulate these competent cells to enter the S phase and proceed through mitosis [75]. The perception of distinct roles for these agents emphasizes an important insight into the mechanism of growth control: The stimulation of cell division is a sequential process that is often regulated at different steps and, in many cases, the coordinated action of different factors is required for a maximal response [66].

An important aspect of the action of growth factors may be inferred from the definitions given above: Insofar as growth involves only an increase in size or number of cells, there is no reason to attribute to these agents a role in the differentiation of their target tissues, although in most cases such a function has not been rigorously excluded either. Clearly, they do have an impact on gene expression, which is manifested in the display of specialized traits that characterize maturing, responsive cells. Thus, they can be viewed as modulators of the phenotypic profile, amplifying the distinctive characteristics of a committed cell, as opposed to differentiating agents which switch on (or off) previously unexpressed genes [81]. As modulators, they behave like the classical hormones (vide infra). However, not all of the polypeptide growth factors necessarily fit this description; for example, erythropoietin and the colony-stimulating factors (CSFs) may direct the differentiation as well as the proliferation of their target cells [1, 52]. Thus, although the role of growth factors in differentiation remains largely unresolved, it is not at the present level of understanding a requisite part of their action and will not be further considered in this article.

In addition to the hypertrophic and hyperplastic effects associated with growth and repair processes, polypeptide growth factors have a distinct role as maintenance or survival factors which is not inherent in the definition of a growth-promoting substance. It is clear that most of these substances are normally present in the mature organism, and, based on tissue culture experiments, deprivation in many instances causes responsive cells to be seriously affected or even die. This has also been observed in vivo with nerve growth factor (NGF) where the implementation of a passive- or autoimmune state provides antibodies directed against NGF causing atrophy of neurons in the sympathetic and sensory nervous systems [33, 50]. For some growth factors, this maintenance of cell viability may represent a more fundamental characteristic than their hypertrophic or hyperplastic activities as it would be manifested throughout the lifetime of the target cell, even in situations where growth phenomena had abated.

RELATION TO "CLASSICAL" HORMONES

The first interaction of polypeptide growth factors with their target cells is, at our present level of knowledge, exclusively with the exterior face of the plasma membrane, placing them in a broad category of substances that initiate their biological activity in this manner. For the most part, this contact is a highly specific one because of the presence of membrane-bound receptors that recognize and bind the external ligands both avidly and selectively [12, 47]. Although a myriad of different events is subsequently triggered by the formation of these complexes, these agents share one other common denominator; namely, they transfer information to the cell from its environment. In some cases, such as with toxins of plant or bacterial origin [17], the exchange is of deleterious value, but usually it represents a positive influence, resulting in the stimulation of metabolic machinery with the overall effect of regulating the designated physiological function of that cell. Certain classes of these external regulators, such as hormones and neurotransmitters, are well defined conceptually and are easily recognized by name and, in most cases, function. However, many other such substances are less well categorized, although they have demonstrated physiological significance, often because existing definitions are too rigid to accommodate them in established groupings. Largely because of their diverse nature and limited characterization, the polypeptide growth factors suffer this fate. Although clearly hormone-like in their action, they are, nonetheless, rarely listed as such. In addition, many substances, classically listed as hormones, are now appreciated to act as growth factors as well, which adds further ambiguity.

This problem of hormonal definition and classification is not of recent origin. Many discussions of the subject have been published, one of the most detailed being that of Huxley [43]. He chose to emphasize the transfer of positive information as a basis for definition rather than mode of transport, which would have redefined a wide variety of substances as hormones. Robison et al [64] presented a more limited version of these ideas that subdivided those substances, considered hormones under the classical definition, into two major categories. A logical extension of these ideas provides a convenient means for viewing the polypeptide growth factors in relation to other hormones without the ambiguities engendered by placing them in separate categories.

The principal characteristics of the two classes defined by Robison et al [64] are summarized in Table I. The first group, referred to as messengers, show rapid responses immediately following formation of the hormone-receptor complex. These almost always initially involve an increase in the level of intracellular cyclic AMP produced by the stimulation of membrane (and ultimately receptor) associated adenyl cyclase. In such cases, the cyclic nucleotide becomes a second messenger and is the agent that further dictates the majority of the remaining hormonal responses in that cell. The duration of enhanced cAMP production, and thus the response, are basically proportional to the receptor occupancy, which is typically limited to a short interval because of the relatively rapid turnover of this type of hormone. Members of the second class, which have been variably called maintenance, permissive, or developmental and designated here as secondary hormones,* share some of the features of the primary hormones but are distinguished by

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*The two classes of hormones are designated primary and secondary in this article, as suggested by Bradshaw and Niall [8], to denote the relative development of their distinguishing characteristic responses (with respect to time). The term "messenger" was found to be less satisfactory because members of both classes can properly be viewed as serving that role.

TABLE I. Classification Scheme for Hormones and Hormone-Like Substances*

Property	Primary (messenger)	Secondary (maintenance, permissive, developmental)
Response	Rapid (sec, min)	Rapid: Slow (hr, day)
Duration of effect	Short	Short: Long
Turnover	Fast	Slow
Principal effect	Production of cAMP	Pleiotypic stimulation: changes in protein synthesis
Mechanism of action	External	External: Internal (?)
Examples	Epinephrine	Thyroid hormones
	Glucagon	Glucocorticoids
	Parathyroid hormone	Insulin
		Growth hormone

*After Robison, Butcher, and Sutherland [64].

several important differences. Principal among these is the extended period of response, often approaching hours or days, that can continue long after hormone-receptor interactions are no longer demonstrable. While the early effects are frequently associated with general anabolic stimulation, the long-term responses, being, for the most part the distinctive features of this group, are clearly related to specific changes in protein synthesis. However, the mechanism by which either the short or long-term responses are produced is not well understood. The rapid effects are certainly initiated by the association with the plasma membrane but have not as yet been shown to involve the generation of a second messenger like the receptor occupancy-dependent production of cyclic nucleotides induced by primary hormones. Nonetheless, a messenger molecule of undetermined chemistry may well be produced that regulates, among other things, the flux of ions and metabolites and, at the same time, initiates the events leading to the modulation of gene expression. However, it is also possible that the two classes of temporal responses are basically separate events that develop independently following formation of the hormone-receptor complex. That is, the "messenger" generated to initiate the general anabolic responses may be unrelated to the stimulus required for the long-term effects. In this regard, the growing evidence that hormones of this class are readily internalized by endocytosis provides a feasible mechanism for the complementation of such a dual mechanism (vide infra). This uptake allows for lysosomal degradation following the appropriate fusion events, but would also permit the hormone or its receptor to act as a new second messenger for events not triggered by the formation of the original hormone-receptor complex. Evidence in support of this mechanism (or variations of it) for one system, NGF, is described below. However, the extent to which internalization is an important feature of the mechanism of other secondary hormones is presently highly speculative.

As noted in Table I, examples of primary hormones are epinephrine and parathyroid hormone, whereas the second class includes such substances as thyroid hormone, glucocorticoids, insulin, and growth hormone. However, Robison et al [64] noted that the distinction between these two groups of hormones "is not always as clear as the human urge to classify things might like it to be." That is, many primary hormones exert maintenance effects despite the fact that they appear, by other criteria, to be classed with the first group, and it

is possible that they may, in some circumstances, be internalized as well. Chief among these are the gonadotrophins, thyroid-stimulating hormone and adrenocorticotropin [76]. Nonetheless, this classification remains useful, particularly in dealing with growth factors, because it logically associates them with other substances of similar structural and functional properties.

PROPERTIES OF GROWTH FACTORS

General Considerations

An alphabetical listing of many of the polypeptide growth factors is given in Table II. The entries have been limited to those substances that have been more extensively characterized (see for example, Gospodarowicz and Moran [36]); the existence of many other factors has been inferred from observed biological activities, but has not yet been associated with a unique molecular species. The sources and target cells listed represent the principal tissues used for purification and bioassay, respectively. For many of the substances, a complete knowledge of the physiologically relevant tissues, with respect to either origin or response, is unknown and, in some instances, those reported in Table II may not be significant in the living organism. For example, the site of synthesis of the insulin-like growth factors and somatomedins is uncertain, although liver is strongly suspected [80], and the cells responsive to fibroblast growth factor in vivo have not been firmly established [36].

More extensive data for each of the factors can be found in the references cited in Table II. However, a few general aspects deserve further comment here. First, there exists the distinct possibility that some of the factors listed in Table II represent different names for the same substance. For example, the relationship of the insulin-like growth factors (IGF) I and II, the somatomedins A and C, and multiplication-stimulating activity is presently obscure, but it appears likely that at least some of these will represent the same entity when purification and structural analyses are complete on all of the factors [86].

A second and related problem, in cases where structural analyses are not available, is the association of the biological activity with the correct molecular entity. Recent observations with fibroblast growth factor obtained from bovine brain illustrate this point. Following the initial description by Gospodarowicz et al [34] of the purification and characterization of brain FGF, Westall et al [83] reported that the factor was identical to various fragments derived from the carboxyl terminal region of myelin basic protein (MBP) which had previously been sequenced by Eylar [21]. While preparations of bovine brain FGF made by the procedure of Gospodarowicz et al [34] clearly contain these fragments as their major constituents, Thomas et al [79] have shown that the mitogenic activity of these preparations (the assay originally used to describe FGF) is not associated with the MBP peptides but with an acidic protein present as less than 5% of the sample. This material is clearly distinct from pituitary FGF, which was not reported to be related to MBP [83], as judged by isoelectric focusing criteria.*

Finally, as judged by the molecular weight values shown in Table II, there is no obvious molecular similarity indicative of the group as a whole. However, comparison of amino acid sequences has revealed a relatedness among some of the factors of the kind associated with proteins that have evolved from a common precursor. Such a relationship has been appreciated for some time for the two pituitary hormones, growth hormone and

*S.K. Lemmon, M.C. Riley, and R.A. Bradshaw (unpublished observations).

TABLE II. Polypeptide Growth Factors

Factor	Source[a]	Target tissue	Mitogenicity[b]	M_r	Primary sequence	Ref.
Colony-stimulating factor (= macrophage-stimulating factor)	Many human tissues	Immature myeloid precursors, macrophages	Yes	1,300–150,000	No	[52]
Epidermal growth factor	Mouse submaxillary, human urine	Epidermal cells, fibroblasts	Yes	6,045[c]	Yes	[10]
Erythropoietin	Human plasma or urine	Red blood cell precursors	Yes	46,000	No	[1]
Fibroblast growth factor	Bovine pituitary	Mesodermal cells	Yes	13,000[d]	No	[37]
Growth hormone	Pituitary	Liver, muscle, adipose	No	22,005[e]	Yes	[55]
Insulin	Pancreas	Liver, adipose, muscle	Yes	5,796[e]	Yes	[18]
Insulin-like growth factor I	Human plasma	Liver, adipose muscle, cartilage fibroblasts	Yes	7,649	Yes	[86]
Insulin-like growth factor II	Human plasma	Same as IGF-I	Yes	7,471	Yes	[86]
Multiplication stimulating activity	Rat serum, liver	Same as IGF-I	Yes	8,000	No	[58]
Nerve growth factor	Mouse submaxillary, snake venoms	Sympathetic and certain sensory neurons	No	13,259[c]	Yes	[6]
Ovarian cell factor	Bovine pituitary	Ovarian tumor cells	Yes	10,000–13,000	No	[35]
Placental lactogen	Placenta	Mammary gland, liver	ND	22,125[e]	Yes	[55]
Platelet-derived growth factor	Human platelets	Arterial smooth muscle cell, fibroblasts	Yes	13,000[f]–38,000	No	[65]
Prolactin	Pituitary	Mammary gland, ovary, liver	ND	22,500[d]	Yes	[55]
Relaxin	Porcine corpa lutea	Pubic symphysis, cervix, uterus	ND	6,056[g]	Yes	[70]
Somatomedin A	Human plasma	Same as IGF-I	Yes	7,000	No	[80]
Somatomedin C	Human plasma	Same as IGF-I	Yes	7,000	No	[80]
Thrombin	Plasma	Fibroblasts	Yes	30,030[h]	Yes	[59]
Thymosin	Thymus	Lymphoid cells	ND	12,200	No	[84]

[a]From many species unless specified.
[b]ND, not determined.
[c]From mouse.
[d]From bovine pituitary.
[e]From human.
[f]Molecular weight from studies of Antoniades et al [4].
[g]Molecular weight from the sequence of James et al [45].
[h]From cow.

prolactin, and placental lactogen [55]. A second group that has been elucidated more recently is the subset related to insulin [7, 8]. Of the structurally defined members, NGF was the first to be so identified [24], followed by the two closely related insulin-like growth factors I and II [62, 63] and relaxin [45, 69]. Because of the functional similarities and receptor cross-recognition [51], other somatomedins are also expected to resemble insulin. The relationships of this subset are a particularly good illustration of the value of classifying polypeptide growth factors as secondary hormones.

Insulin-Related Subset

The gross biological properties of the insulin-related molecules belie the observed similarities in structure and potentially in mechanism. The prototype of this family, insulin, is a pancreatic hormone more commonly recognized for its profound effects on carbohydrate, lipid, and amino acid metabolism, although it is well known to support growth in vivo and in tissue culture [27] and was selected as the model for defining positive pleiotypic effectors [40]. The IGFs were first defined as the substances displaying insulin-like activity in human serum that were not suppressed by antibodies to insulin and were designated nonsuppressible insulin-like activities (NSILA) [26]. Following fractionation by acid ethanol extraction [44], the soluble component (NSILAs) was found to consist of two closely related polypeptides that were renamed insulin-like growth factors I and II when sequence analysis demonstrated that they were structurally quite similar to the pancreatic hormone [62, 63]. These molecules also act as promoters of sulfate incorporation into the proteoglycans of cartilage (as judged by in vitro assay), and therefore may act in the capacity of somatomedins, the mediators of growth hormone [86].

In contrast to insulin and the IGFs, NGF and relaxin are without effect in the typical target tissues of insulin, ie, liver, adipose tissue, and muscle; rather, they act on the more specialized tissues of the peripheral nervous system [49] and the female reproductive tract [70], respectively. In the embryo, NGF acts as a trophic stimulator of developing sympathetic and selected sensory neurons leading to the proliferation of axonal processes and the ultimate formation of functional synapses. The maintenance of these neurons remains an essential activity of NGF in the adult state [49]. Relaxin appears to exert its effects primarily at parturition when it stimulates the loosening of the pubic symphysis rendering the birth canal more pliable for the passage of the fetus. It is also alleged to soften the cervix, inhibit uterine muscle contraction, and affect mammary gland development [70]. However, unlike the other insulin-related substances, analysis of these biological responses has not been carried out at the molecular or cellular level as yet, and it is therefore uncertain to what extent it exerts these effects through pleiotypic activation.

The structural similarities of the insulin-related molecules underlying these apparently diverse biological activities is summarized in Figure 1. The relatedness, which is manifested in amino acid residues occupying identical positions when the factors are appropriately aligned for comparison, is confined to the A and B chains of insulin that are formed from the biosynthetic single polypeptide chain precursor, proinsulin, by specific proteolytic excision of the intervening C peptide [74]. As shown by the solid boxes, the IGFs exhibit the greatest number of identities with the insulin chains, whereas relaxin and NGF are more distantly related. There are, however, some additional identities found between the factors themselves, not seen in the comparison to insulin, that are denoted by the lined and stippled positions. The boxes containing single bars represent deletions arbitrarily introduced to maximize the identities and should be viewed as nonidentities in the context of this comparison.
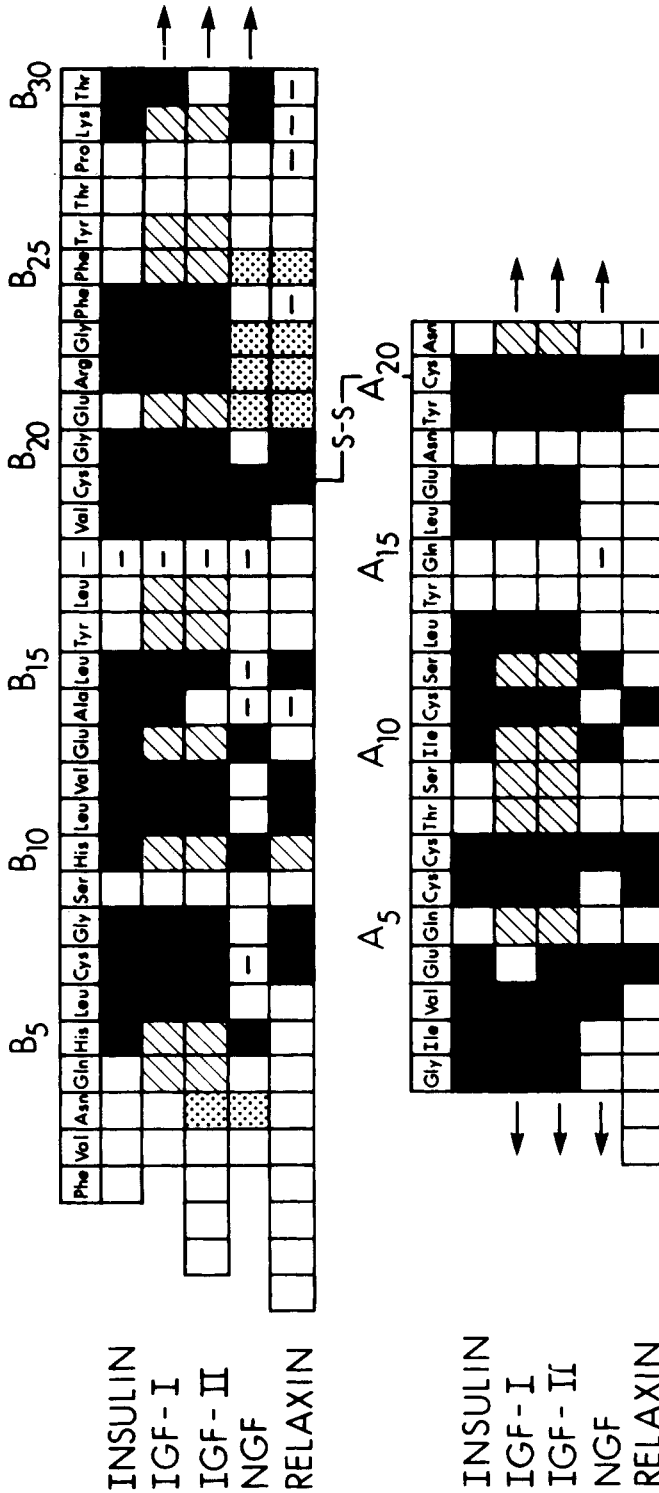
Fig. 1. Diagrammatic comparison of the amino acid sequences of the insulin-related polypeptides in the regions corresponding to the A and B chains of insulin. The actual sequence given in the top line of both segments is that of human insulin [57]. In the block diagram of the insulin structure (second line), solid squares indicate residues in the insulin sequence also found at the same position in one or more of the other factors. Blank squares represent positions in the insulin sequence occupied by different amino acids in the other factors, whereas lined or stippled squares indicate identities in two or more of the growth factors that are not identical to the corresponding residue of insulin. A few deletions, indicated by squares containing dashes, have been introduced arbitrarily to maximize the homology. The single disulfide identically paired in all molecules (B19-A20) is shown. The arrows indicate that the segments listed are part of continuing sequences in the active form of the hormone (see Fig. 2). The polypeptide sequences are human insulin-like growth factor-I (IGF-I) [62], human insulin-like growth factor-II (IGF-II) [63], mouse nerve growth factor (NGF) [6], and porcine relaxin [45].

Further insight into the relationship of these factors as well as the manner in which they have diverged to form unique entities is derived from a consideration of their secondary structures. As shown schematically in Figure 2 (as well as by the arrows in Figure 1), NGF and the IGFs contain the A and B segments shown in Figure 1 as parts of a larger, single polypeptide chain. In this sense they are comparable to proinsulin. In fact, NGF contains a C-peptide region of identical length to the connecting peptide of proinsulin, whereas this segment is foreshortened by about two-thirds in the IGFs. There is no significant homology (identities) with the proinsulin C-peptide in either case. Relaxin, however, is isolated as a two-chain structure comparable to insulin. Preliminary evidence [25] suggests that a prorelaxin molecule analogous to proinsulin is the progenitor of relaxin, but no information about the putative C-peptide is presently available.

A striking additional feature of relaxin and the IGFs is the complete conservation of the disulfide bond pattern of insulin. NGF contains only one of the three disulfide bonds of insulin with the other two being replaced by ones unique to NGF located elsewhere in the molecule (Fig. 2). However, the single conserved disulfide found in all five proteins (emphasized in Fig. 1) is especially important in insulin, as it is located in the re-
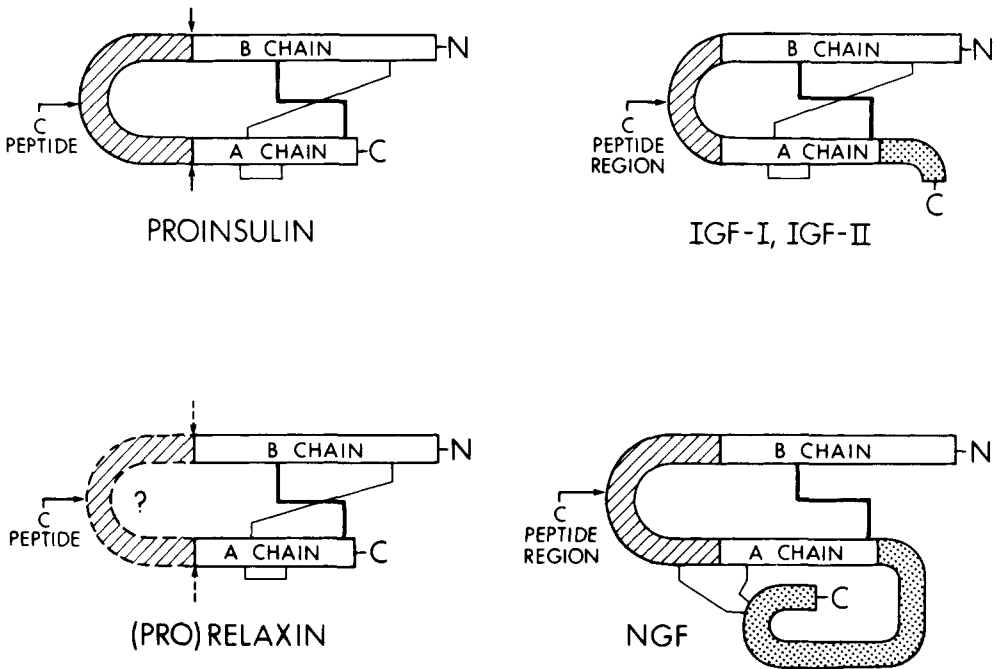

Fig. 2. Schematic comparison of the three-dimensional structure of the five insulin-related growth factors. The diagonally lined segments indicate the connecting "C-bridge" regions (presumed in relaxin) that are not excised in IGF-I, IGF-II, and NGF. The stippled areas represent carboxyl-terminal extensions. The single conserved disulfide is emphasized by the heavy line connecting the A and B chains; other disulfides are shown by lighter lines. For the purposes of these diagrams, the small differences between the IGFs in the length of their C-peptide and the carboxyl-terminal extensions have been ignored [62, 63].

gion of that molecule thought to comprise the receptor-binding site [61]. Both NGF and the IGFs also contain carboxyl terminal extensions that presumably reflect features peculiar to their own unique molecular interactions.

The conversion of insulin and relaxin to two-chain forms and the absence of such processing in NGF and the IGFs may occur as a result of the need to store insulin and relaxin in high concentrations that can be readily released in a large bolus to meet physiological demands [7, 20]. The low steady-state release of NGF and presumably of the IGFs, as judged by the absence of any tissue stores of these molecules, has apparently resulted from evolutionary modifications in the sequence that have eliminated the cleavage points as they became unnecessary. Conversely, this property may have been acquired by insulin and relaxin.

The structural relatedness of the insulin subset is an important illustration of the development of new physiological function through evolutionary change. In addition, it has been instrumental in the emerging ideas concerning the relationship of growth factors and hormones and, most importantly, has provided new views about the mechanism of action of secondary hormones, which are summarized in the ensuing section.

MECHANISM OF ACTION

General Considerations

Although it would be naive to expect that such a diverse group of substances as listed in Table II would share a single mechanism of action, it is not unreasonable to anticipate that some common features will be found. Nonetheless, such generalities have not been easy to identify. On the whole, the polypeptide growth factors stimulate the anabolic metabolism of their target cells following formation of the complex with the cell surface receptor. However, the manner in which this is accomplished is still rather obscure. It does not appear to involve the prolonged production of cyclic nucleotides (although a role for these agents, compatible with transitory changes in concentration, has not been eliminated) and may occur through more than one type of alternative mechanism including the production of other kinds of "second messengers." A more universal feature of these agents appears to be the receptor-mediated endocytosis that ultimately follows the initial interaction with the receptor. However, this is a general phenomenon, found with a large variety of macromolecules [30, 54], and may not be important for function other than as a means for degradation through fusion with lysosomes. On the other hand, it may be of primary importance in the expression of the long-term effects associated with the growth-related processes. The relative merit of this hypothesis is described in the ensuing sections.

Perhaps the most important feature shared by these substances, exemplified by the listing in Table II, is the initiation of both rapid and slow responses. While these are variably expressed in individual cases, they are, nonetheless, a constant feature throughout, and a description of the mechanism of action of any growth factor will necessarily have to account for both phases of activity.

External vs Internal Sites of Action

Although considerable attention is often focused on the long-range effects of growth factors and related substances, particularly those with mitogenic activity, the initial or rapid events that are clearly triggered by the hormone while it is on the cell surface are of

equal importance. The relationship of these two phenomena represents the basic enigma that must be resolved before a clear understanding of the principal mechanistic features can be achieved. Several possibilities are currently plausible:

1) The factor interacts with the cell surface to generate a signal that is responsible for both the rapid and slow responses. A close corollary of this hypothesis is that one of the rapid responses in turn produces the signal for the long-term effects, ie, the slow responses could, in theory, be generated without all of the rapid ones if the second signal were introduced separately. In either case, the entire process would not involve internalization (for other than degradative purposes).

2) The factor interacts with the cell surface to generate two independent signals, one producing the rapid effects and the other producing the long-term, growth-related responses. As above, this hypothesis does not require internalization for any part of the activity.

3) The factor interacts with the cell surface to produce only a signal for the rapid events. Internalization is then required to produce the signal for the slow responses.

In any of the models with more than one signal, a concerted mechanism requiring both to act before one or both temporal responses is initiated is also possible. Furthermore, only a single class of receptors is required for any of the models, although two independent receptor types are equally compatible with the last two hypotheses. Also, the models do not specify the nature of the "signal." These could range from second messengers to changes in metabolite or ion concentrations and may well vary from one system to another. Finally, the models do not place emphasis on the relative importance of either phase of activity. In fact, the surface-mediated events have been more extensively studied and in many cases, such as insulin, are probably more important physiologically.

The possibility that internalization by absorptive pinocytosis (or receptor-mediated endocytosis) plays a decisive role in the mechanism of action of any of the secondary hormones is a relatively new idea. Bulk phase pinocytosis, which is simply the engulfment of extracellular fluid by invagination of the plasma membrane and subsequent fusion resulting in the release of a vesicle into the cytoplasm, allows the cell to sample its environment and, therefore, in a general sense, mediates communication between the cell and its surroundings. When this process is modified to include an initial complexation of soluble components to specific receptor molecules on the cell surface, it becomes appreciably more sensitive as the agent can be concentrated as much as 1,000-fold prior to internalization [71]. This feature is certainly of importance for the uptake of some metabolites that enter the cell via this pathway, and may well be of significance in endocrine systems too.

The diversity of substances that are internalized by absorptive pinocytosis underscores the importance of this phenomenon in the overall interaction between cells and their environment. In addition to secondary hormones, toxins, carrier proteins — such as low-density lipoproteins and transferrin — various glycoproteins, lysosomal enzymes, antibodies, and viruses are, at least in part, transported across the plasma membrane by this process [30, 54]. In fact, the detailed studies [see for example, 31, 32] with low-density lipoprotein have been among the most revealing in establishing the essential features of receptor-mediated endocytosis, which appear to have broad applicability to other ligands. Of particular note is the participation of specialized areas in the membrane called "coated pits" because of the bristle coat composed primarily of the protein clathrin underlying these regions, the formation of "coated vesicles" from invagination and fusion of the membrane, and their subsequent association with intracellular organelles, in particular, lysosomes [31].

A fundamental element in this scheme is the concomitant internalization of the receptor along with the ligand. At least in experimental situations, this can lead to a major reduction in the number of receptor molecules on the cell surface. The extent and duration of this effect seem to depend largely on whether or not the internalized receptors are recycled back to the plasma membrane. Receptors for endocrine substances do not appear to be reutilized and must be replaced by de novo protein synthesis, whereas receptors for carrier molecules such as LDL are extensively reused. As a result of the greater time required for the new synthesis of the former type, the deficit is more readily demonstrable. In such cases, the process has been designated receptor *down regulation* [13, 48, 72].

The two categories of receptor treatment following endocytosis may signify an important physiological distinction. Those systems in which receptors are recycled generally involve the transfer of the ligand to the cell for further processing and utilization. In contrast, irreversible receptor consumption is found in those instances where information transfer may be viewed as the overall purpose of the original interaction. This suggests the possibility that the receptor itself might play a major role in any signal resulting from the internalization event. Such a hypothesis has been suggested by Fox and Das [23] in their "Endocytotic Activation Model" for EGF. They proposed that after the agent binds to its surface receptor and enters the cell by endocytosis, it is transported to the lysosome where the contents of the endocytotic vesicle come in contact with proteolytic enzymes. The digestion of the receptor would result in the generation of a new "messenger" or perhaps an enzyme which, in turn, would catalyze the production of an agent responsible for the subsequent long-term effects.

The principal roles that internalization might play in hormone action are summarized in Table III. The first three entries list the basic possibilities that might apply to systems in which internalization is a fundamental part of the mechanism (see model 3 above). In the first two possibilities, lysosomal fusion, and subsequent proteolysis, to produce an active peptide to act as the second signal is a major alternative. However, the hormone or acceptor can be envisioned to act without such modification, too. The third possibility suggesting that the receptor can act in some fashion after translocation to an intracellular structure is consistent with the hypothesis that it is the receptor that must be internalized for long-term effects to develop and that the hormone serves only to initiate this event. The final two entries of Table III, degradation and desensitization, have been shown to occur in experimental systems but still must be established to be of importance physiologically.

Evidence in support of either roles 1 or 3 (Table III) is provided by several reports identifying intracellular receptors for a number of secondary hormones (Table IV). These entities, which have been identified by binding assays, have been found to be associated with elements of the nucleus, Golgi membranes, and other organelles. However, in no case has any functionality been associated with them, and it has been suggested by one group [5] that they represent solely biosynthetic precursors of the plasma membrane receptors. However, it is difficult to conceive how nuclear receptors are related to biosynthetic precursors. At present, no relationship between a cell surface and intracellular receptor has been demonstrated and, in the case of the insulin receptor, naturally occurring antibodies directed against it, which are characteristic of type B syndrome insulin-resistant diabetes, do not cross-react with nuclear receptors [29]. However, either the translocation process or the new intracellular environment could materially alter or screen the antigenic determinants of the nuclear entity. More definitive characterization of both plasma membrane and nuclear receptors will be required to determine if any relationships exist.

TABLE III. Potential Roles of Internalization in Hormone Action

1. Interaction of hormone or receptor or fragment thereof with intracellular receptors.
2. Processing of hormone or receptor to generate "second messenger."
3. Direct action of receptor after translocation to intracellular organelle.
4. Degradation.
5. Regulation of cell sensitivity to hormone by decreasing number of surface receptor.

TABLE IV. Intracellular Receptors for Polypeptide Hormones/Factors

Hormone/factor	Tissue	Location	Ref.
Insulin	Liver	Golgi	[60]
	Heart	Mitochondria	[22]
	Liver	Microsomes	[42]
	Liver	Nucleus	[28, 41]
Nerve growth factor	Dorsal root ganglia	Nucleus	[3]
	Pheochromocytoma (PC12)	Nucleus	[85]
Growth hormone	Liver	Golgi	[5]
	Liver	Microsomes	[56]
Prolactin	Liver	Golgi	[82]
Epidermal growth factor	Liver	Nucleus	[53]

Specific Examples

Nerve growth factor. NGF is one of the most extensively characterized polypeptide growth factors and represents the single case in which there exists considerable data to support a mechanism of action involving the internalization of a peptide hormone [6]. In fact, the idea that polypeptide growth factors or related hormones are taken up by their target cells first received experimental support in studies on NGF [39] and was only subsequently established for other related substances [9, 77]. It should be noted that the demonstration of internalization was, as with other polypeptide hormones, preceded by a period characterized by the prevailing view that hormones acted solely at the external surface of their target cells, a theory engendered in large measure by experiments utilizing insolubilized hormones [6, 16]. While this methodology clearly was not without value, it obscured the discovery of internalization which, at least in the case of NGF, appears to be of fundamental importance.

To gain a proper appreciation of the proposed mechanism of NGF, one must view it in the context of its overall physiological role. It is particularly important in this regard to recognize that the target tissues for this substance, ie, sympathetic and selected sensory neurons, have a unique morphology that changes dramatically during development and have certain specific requirements such as the establishment of appropriate contacts allowing constant communication with their periphery. These unique problems have been effectively managed by modifying the classical endocrine scheme. Rather than having a single site of synthesis, storage, and release, the hormone is elaborated in multiple sites throughout the organism. These tissues ultimately form the end organs that are innervated by the

responsive neurons. Systemic transport is replaced by interstitial diffusion, which has the added advantage of providing tropic stimulation that aids in the development of the neuronal network [14] as the neurites presumably grow along gradients of NGF. This delivery system is also highly flexible with the length of the diffusion pathway depending on the extent of axonal maturation; the final limiting distance is the transsynaptic passage.

The mechanism of trophic stimulation initiated when NGF reaches the neuron and binds to its plasma membrane receptor also remains unaltered throughout development, despite the changes that occur in cell shape. The principal destination of NGF is the cell body which it reaches after endocytotic uptake at any stage in the development of the cell. However, as the growth cone of the axon approaches its end organ and the extracellular transit of NGF from its source thereby decreases, the length of the intracellular flow of NGF, in terms of both time and distance, increases. As with other features of the system, this process subserves an additional function for the neurons; namely, it allows the NGF to act as a messenger between the synapse and the perikaryon. It appears that neurons that have formed the correct synaptic junctions are assured a continuous supply of NGF, whereas those that do not are somehow deprived and subsequently expire. Agents acting in this capacity are collectively referred to as chromatolytic messengers, because the interruption of their flow results in the state of neuronal chromatolysis which, if not corrected, will lead to cell death [15]. It seems appropriate to view this chromatolytic function as being synonymous with the survival or maintenance activity of NGF.

The message that NGF brings to the cell body is manifested by changes in specific transcriptional events among which are the induction of tyrosine hydroxylase and dopamine β-hydroxylase [78]. While the manner in which this is accomplished is unknown, it appears probable that the nuclear receptors identified in responsive neurons are somehow involved [3]. These entities display binding and solubility characteristics that clearly distinguish them from plasma membrane receptors of the same tissue, and they are found to be highly concentrated in responsive neurons. Subcellular fractionation experiments suggest that neurons maximally loaded with ^{125}I-NGF introduced by retrograde axonal transport contain about 15–30% the labeled hormone in the nucleus [46]. As judged by EM autoradiography, much of the tracer appears in lysosomal structures [68].

Recently, nuclear receptors for NGF have also been identified in the PC-12 cell line, derived from a pheochromocytoma [85], which adopt a differentiated phenotype in response to NGF [38]. The properties of their receptors are similar to the characteristics of the nuclear receptors identified in the normal target tissues of NGF but were found to be localized in the nuclear envelope. This result is intriguing in view of the finding that initiation of microtubule orientation preceding neurite outgrowth in neuroblastoma cells occurs at or in close proximity to the nuclear envelope [73]. As the stimulation of neurite outgrowth is one of the major effects of NGF which has a delayed onset [49], the interaction of NGF with these receptors may be necessary for this biological effect.

EGF. Unlike NGF, epidermal growth factor (EGF) has mitogenic activity, both in vivo and in vitro [10]. Given the availability of ample quantities, it has been used extensively as a prototype for mitogenic agents in studies on the control of cell division. In addition, biological activities such as precocious eyelid opening and incisor eruption, and inhibition of gastric acid secretion, have been associated with the peptide. However, a complete spectrum of its physiological role encompassing its site of synthesis, means of transport, and target tissues remains substantially undefined. Nonetheless, data collected from experiments in tissue culture have provided considerable information about the mode of action of EGF. As with other polypeptide growth factors, it complexes with

high affinity to cell surface receptors, a process which is followed by endocytosis [9]. However, there appear to be significant differences from the internalization of NGF: The process is much more rapid with respect to both uptake and proteolytic degradation [10]. Virtually all of the internalized EGF is extensively degraded in a few hours in contrast to retrogradely transported NGF, which remains in the cell soma for well over a day. Despite the efficacy of internalization, there appears to be compelling support for an "external" mechanism. Indeed, Aharonov et al [2] have shown that the continued occupation of at least a small portion of cell surface receptors during the period of incubation necessary to produce cell division (~18 hrs) is a prerequisite for mitogenicity, and Das [19] has provided evidence for the production of a second messenger, which stimulates DNA synthesis when transferred to nuclei not exposed to EGF. However, it was not established in this study whether the putative messenger was generated from an external or internal location of the EGF. Alternatively, Carpenter et al [11] have demonstrated a specific phosphorylation of the receptor by a kinase that is either closely associated with the receptor or is an integral part of it. Although no functional significance has yet been attached to this finding, it might represent an event triggering the uptake of the complex, which would suggest a mechanistic role for it. It might also be noted that the observations of Aharonov et al [2] are equally compatible with a mechanism that requires a controlled uptake of EGF or its receptor, as opposed to the controlled production of a second messenger at the plasma membrane. The recent observations of Moriarty and Savage [53] of the presence of apparent EGF receptors in the nuclei of hepatocytes would be consistent with such a role for internalization.

CONCLUDING REMARKS

In the last several years polypeptide growth factors have been transformed from biological curiosities to agents of prime importance recognized for their in vivo activities as well as their usefulness in tissue culture and in the study of cell division. Central to this rise in stature has been the progressive realization of their hormonal character. As expounded in this article, it now seems fitting to group these substances in a broad category of secondary hormones organized according to principles that transcend the classical definitions. This has not only served to develop further our understanding of their mode of action but has also expanded our concepts about the action of classical hormones. Among other things, it has brought considerable attention to the phenomenon of receptor-mediated endocytosis, particularly as a potential mechanistic feature. This exciting concept, which still requires substantial clarification and development, has been a primary focus of this article. As with all discussions that are heavily dependent on hypotheses and speculations, it may be anticipated that substantial revision will be necessary when the results of future experimentation become available. In the interim, it may be hoped that these ideas will stimulate that research.

ACKNOWLEDGMENTS

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