EPIDERMAL GROWTH FACTOR

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by
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Introduction

Upon the foundations provided by experimental embryology, endocrinology, cell biology, biochemistry, and molecular biology, the intricacies of the regulatory processes that occur during embryonic development are slowly coming to light. While the importance of "classical" hormones in the control of growth and development has long been recognized, we now know that many more intercellular signals are involved in these highly complex processes. The recent advances in this area have, somewhat unexpectedly, also provided mechanisms that may lead to a more detailed understanding of important biomedical questions, such as the growth behavior of malignant cells.

My own efforts in this area of research over the past thirty years have been directed toward the understanding, on a biochemical level, of two biological observations, both initially made in the Department of Zoology, directed by Dr. Viktor Hamburger, at Washington University.

The first observation was that of Dr. Rita Levi-Montalcini who noted that certain mouse tumors, when implanted into chick embryos, released a factor that stimulated the growth of specific embryonic neurons. The second biological observation was made during my study of the nerve growth factor detected in male mouse submaxillary glands. It was noted [l] that when crude submaxillary gland preparations were injected into newborn mice, unexpected "side effects" not related to the activities of nerve growth factor, were produced. These effects included precocious eyelid opening (6-7 days, compared with 12-14 days for controls) and precocious tooth eruption (5-6 days, compared with 8-10 days for controls).

After I transferred to the Biochemistry Department at Vanderbilt University in 1959, these "side effects" were to become the focus of my research. From my training in embryology, I felt that any substance that altered the timing of specific developmental processes would be of biological significance. I, of course, did not foresee that the biochemical mechanism by which these extracts induced precocious eyelid opening would be related to those involved in oncogenic transformation by one class of retroviruses. This lecture summarizes briefly some of the thoughts and key experiments that have led to our present understanding of epidermal growth factor (EGF).

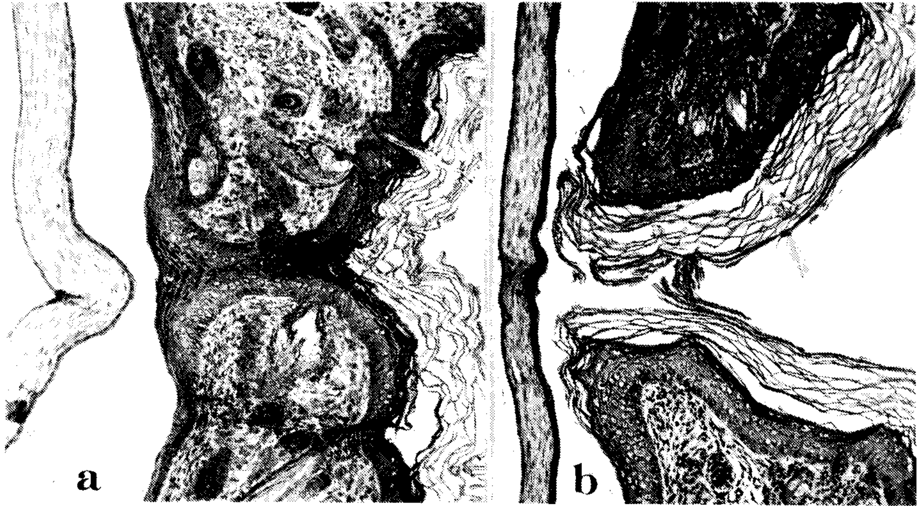

Figure la and b. Cross sections of the eyelid area from control, la, and experimental, lb, 8-day-old rats. The experimental animal had received daily injections (1 μg per 1 gm body weight) of the active protein. × 100. (reprinted from *Journal of lnvestigative Dermatology* (1963) 40, l-5.)

The First Decade. By employing precocious eyelid opening as an assay, the factor, a small protein, responsible for these effects was isolated from murine submaxillary glands in the early 1960's [2]. Histological examination (Fig. 1) of control and EGF-treated newborn animals (mouse, rat, rabbit) revealed that the observed precocious eyelid separation was the consequence of a more generalized biological effect, namely, an enhancement of epidermal growth and keratinization [3]. The apparent precocious incisor eruption induced by EGF was, in reality, caused by an enhanced differentiation of the lids of the treated animals.

Since these were whole animal experiments, we were faced with the problem of whether the factor operated directly on epidermal cells or whether growth was induced indirectly, possibly by the increased production of a more "classical" hormone.

The techniques of tissue and organ culture seemed ideally suited for resolving this problem. A preliminary organ culture study was carried out during a sabbatical at the Istituto Superiore di Sanità in Rome in collaboration with Drs. Rita Levi-Montalcini and Domenica Attardi, and the study subsequently was continued at Vanderbilt University. The name epidermal growth factor, or EGF, was first used in the initial reports of these studies [4]. The results demonstrated that EGF directly stimulated the proliferation of epidermal cells in organ cultures of chick embryo skin; this mitogenic action of EGF, therefore, did not necessarily depend on other systemic or hormonal influences. During these experiments, the range of responsive animals was widened to include birds as well as mammals, suggesting that knowledge of the evolutionary origins of EGF would contribute to our understanding.

By 1970 we had accumulated a spectrum of information regarding many aspects of the physiology of EGF:

1. We described a series of metabolic alterations (enhancement of polysome formation, induction of ornithine decarboxylase, etc.) that accompany the growth stimulating effects of EGF on epidermal cells. Many of these changes are now known to take place in a variety of cells when a growth stimulus is applied.
2. We identified the tubular cells of the submaxillary gland, which in the mouse exhibit sexual dimorphism, as the major site of synthesis of EGF in this species and noted, with the aid of a radioimmunoassay, that the synthesis of EGF, especially in female mice, was markedly enhanced by the administration of testosterone.
3. We demonstrated that, in crude homogenates of the mouse submaxillary gland, EGF existed as a high molecular weight noncovalent complex (-75,000 daltons) consisting of two molecules of EGF and two molecules of an EGF-binding protein that possessed arginyl esterase activity.
4. On a more practical level, we and others found that the topical application of EGF accelerated cornea1 re-epithelialization in rabbits with wounded corneas.

The reader is referred to a number of early review articles wherein this information is detailed and references provided [5, 6]. By the end of the first decade, I was convinced that EGF plays a normal physiological role in many species, either during embryonic development or in homeostasis; what this role was at the whole animal level and how EGF interacted with cells at the molecular level were problems for the future.

The Second Decade. The development of a rapid, essentially two-step procedure for isolation of milligram quantities of EGF from murine submaxillary glands in the early 1970's [7] permitted the purification of sufficient quantities of mEGF (mouse-derived EGF) for a thorough characterization. This single technical advance opened the door to the application of many biochemical methodologies and insights. Amino acids analysis revealed that mEGF is a 53-residue polypeptide, entirely devoid of alanyl, phenylalanyl, or lysyl residues [8]. The primary sequence of mEGF [9] and the position of the three internal disulphide bonds [10] were determined and are depicted in Fig. 2. Though mEGF has yet to be crystallized and subjected to X-ray diffraction analysis, considerable spectroscopic data have been accumulated suggesting that the hormone has little periodic secondary structure; the presence of ß-sheet structures have been detected (reviewed in (11)).

At about this time (1973) a new facet of the biology of EGF was uncovered. Armelin [12] and Hollenberg and Cuatrecasas [13] were the first to report that fibroblasts in culture responded to EGF with enhanced DNA synthesis. These findings were corroborated in our laboratory with human fibroblasts [14, 15].

The finding that mouse-derived EGF was a potent mitogen for human cells indicated that receptors for EGF were present on human cells and, therefore, a polypeptide similar to EGF might be found in human tissue. We took two approaches in an attempt to isolate EGF-like molecules from human urine.

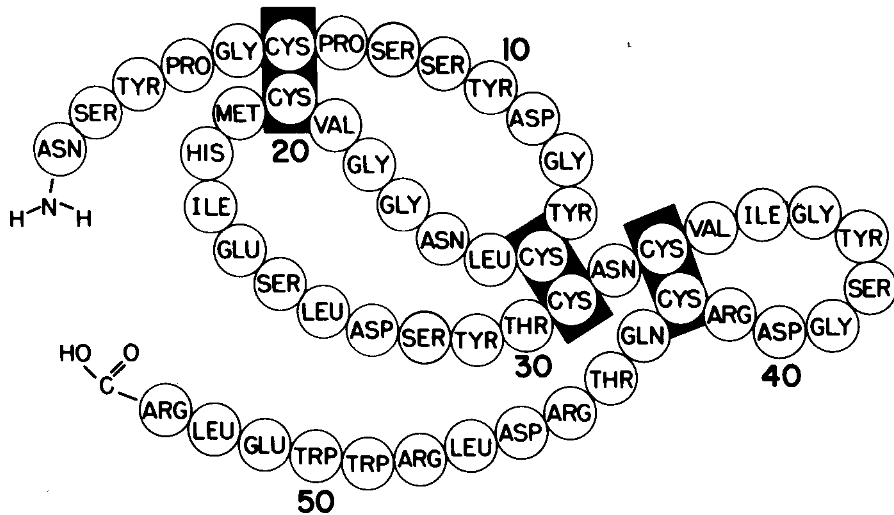

Figure 2. The amino acid sequence of EGF with placement of disulfide bonds. (Reprinted from *Journal of Biological Chemistry* (1973) 248, 7669-7672.)

First, an immuno-affinity column procedure (using anti-mouse EGF antibodies) was used to purify partially a substance from human urine that was similar to the mouse hormone in its biological activity [16]. In another approach, we developed a sensitive and specific radioreceptor competitive binding assay for EGF-related polypeptides, using cultured human fibroblasts and ^{125}I-labelled mouse EGF, that permitted the isolation of microgram quantities of pure growth factor from protein concentrates of human urine [17]. The biological effects of the purified human polypeptide were qualitatively identical to those previously described for the mouse growth factor. These included the stimulation of the proliferation *in vitro* of fibroblasts and corneal epithelial cells, as well as the induction of precocious eyelid opening in the newborn mouse, which still remains the most specific biological assay for EGF. The amino acid compositions of the human and mouse polypeptides differed, but clear similarities were noted. Both polypeptides apparently competed for the same site on the cell membrane and antibodies to the mouse polypeptide crossreacted with the human hormone. We concluded that we had isolated the human counterpart of murine EGF.

As is usual in science, an unexpected and completely new aspect of the biology of EGF emerged with the report by Gregory [18] that urogastrone, a gastric antisecretory hormone isolated from human urine, appeared to be identical to human EGF and closely related to murine EGF. Human EGF (urogastrone) and murine EGF are now believed to invoke identical response in all target cells. The relationship between human EGF and urogastrone could only have been detected from a structural comparison of these molecules; even today, no rationale is available to connect inhibition of acid secretion and stimulation of cell growth.

Given a cell culture system (human fibroblasts) in which EGF acted as a "growth factor" we were faced in 1975 with a rather formidable task: how does EGF stimulate cell growth? Although neuronal uptake and retrograde transport of nerve growth factor had been demonstrated in 1974 [19], almost all endocrinologists were of the opinion that peptide hormones, after binding to their receptors on the plasma membrane, were released into the extracellular environment.

Our initial experiments [20] utilizing ^{125}I-EGF and human fibroblasts confirmed the presence of plasma membrane receptors for EGF. Two additional and significant observations were made. First, the binding of ^{125}1-EGF to the cell surface of intact fibroblasts was rapidly followed by proteolytic degradation of the growth factor by a cell-mediated process. Secondly, it was noted that NRK cells lost their ability to bind ^{125}1-EGF following transformation by the Kirsten virus. The former observation directed us to an examination of the possibility that cell-bound EGF was internalized prior to degradation [21]. The latter observation was later generalized to include a variety of cells transformed by retroviruses [22] and eventually led George Todaro and others to isolate the EGF-related polypeptide, a-transforming growth factor, and to propose the autocrine hypothesis [62].

As a step in defining the biochemical events that occur during and subsequent to the interaction of EGF with the cell surface, we examined the metabolic fate of the bound hormone. We came to the conclusion (2 1) that subsequent to the initial binding of ^{125}1-EGF to specific plasma membrane receptors, the EGF:receptor complex is internalized and the hormone is ultimately degraded in lysosomes. These conclusions, drawn from studies of the interaction of ^{125}1-EGF with human fibroblasts, were based on the following series of observations:

1. Cell-bound ^{125}1-EGF was rapidly degraded to [^{125}I]monoiodotyrosine at 37°. At 0°, however, cell-bound ^{125}1-EGF was not degraded, but slowly dissociated from the cell surface.
2. When the binding of ^{125}1-EGF was first carried out at 37° and the cells then incubated at 0°, almost no release of cell-bound radioactivity was detected.
3. Degradation of ^{125}1-EGF, but not binding, required metabolic energy.
4. The degradation was blocked by drugs that inhibit lysosomal function, such as chloroquine and ammonium chloride.
5. When ^{125}1-EGF was bound to cells at 0°, the hormone was readily accessible to surface reactive agents, such as trypsin and antibodies to EGF. However, when the hormone was bound to cells at 37° it was much less accessible to either of these reagents.
7. Exposure of fibroblasts to EGF resulted in an apparent loss of plasma membrane receptors for EGF.

Taken together, these observations, which have subsequently been extented by others to a number of polypeptide hormones, provided quantitative biochemical evidence for a complex mechanism through which cells interact with extracellular regulatory signals.

The challenge of direct visualization of the internalization of EGF was

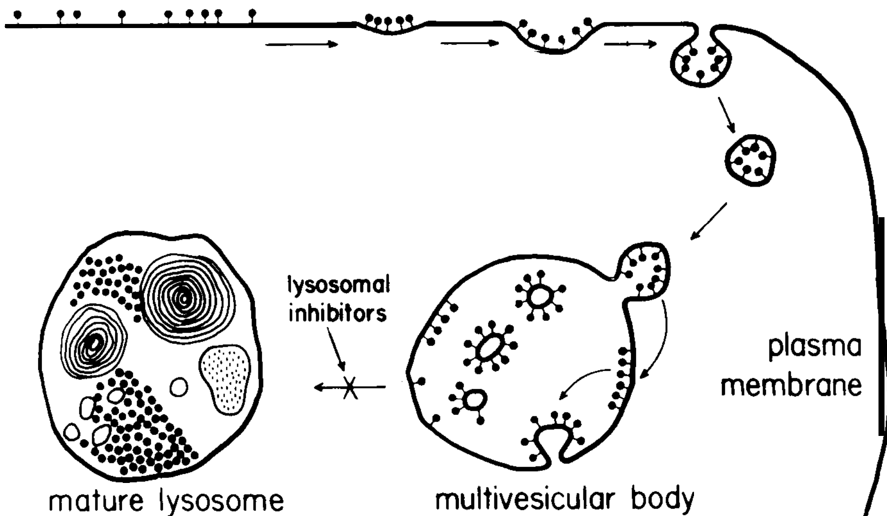

Figure 3. Diagram of F-EGF interaction with A-431 cells. F-EGF- receptor complexes, identified by the characteristic spatial relationship of particles and membrane (4- to 6-nm separation), are apparent at initial binding and are preserved through the processes of clustering, pinocytosis, and incorporation into MVBs. Further incubation at 37°C allows disruption of the F-EGF-receptor complex (attested by pools of free ferritin), a process blocked by the presence of amines. (reprinted from *Proceedings of National Academy of Science USA* (1979) 76, 5689-5693.)

approached using three general procedures: the preparation and tracing of fluorescent derivatives of EGF [23, 24] the tracing of ^{125}1-EGF by electron microscopic autoradiography [25], and the preparation and tracing of EGF-ferritin conjugates by electron microscopy [26, 27].

Although all three morphological approaches confirmed the original biochemical studies with ^{125}1-EGF [21], the use of the biologically active EGF-ferritin conjugate (F-EGF) provided the clearest and most direct picture of the metabolic fate of EGF. At 4° the EGF-ferritin conjugate specifically bound to the plasma membrane of cells and appeared to be randomly distributed on specific receptor sites. When the cells were warmed to 37°, the EGF-ferritin redistributed on the surface of the plasma membrane within one minute to form many small clusters. The clusters of receptor-bound EGF-ferritin molecules were then rapidly internalized into endocytic vesicles. Within 30 min approximately 84% of the ferritin was seen in multivesicular bodies that were considered to be lysosome-related, These data also provided morphological evidence for the hypothesis that "down-regulation" of surface receptors for EGF involves internalization of intact hormone-receptor complexes. A diagram that illustrates our conclusions is presented in Fig. 3. It was subsequently demonstrated, by metabolic labelling and immunoprecipitation with anti-receptor antibodies, that EGF-mediated internalization of the EGF:receptor complex is associated not only with the degradation of EGF but also with enhanced degradation of the receptor [28].

A critical question in this area of hormone research is whether the intracellular processing of hormones and their receptors is related to, or necessary for, the generation of biological responses to the hormone. My opinion is that no clear experimental evidence exists to answer this very important question.

In view of our inability to define the relevance of receptor-mediated internalization to the growth factor's biological activity and our belief that cellular alterations induced by EGF result from the amplification and propagation of a series of "signals" generated during the binding and internalization of the hormone, we sought, in the late 1970's, to obtain a cell-free system that responded *in vitro* to the addition of EGF. Since the A-431 human epidermoid carcinoma cell line had been shown to have an extraordinarily high concentration of EGF receptors, 2-3 X 10^6 receptors/cell [29, 24], we utilized a membrane preparation from these cells to look for an EGF-dependent alteration of membrane structure and/or function. Like the technical turning point that the rapid purification of milligram quantities of EGF provided (see above), the identification of the A-431 cell line as an enriched source of EGF receptors was instrumental for both biochemical and molecular biological studies of the mechanism of action of EGF.

As expected, membranes from these cells were able to specifically bind relatively large quantities of ^{125}I-EGF. Since phosphorylation and dephosphorylation reactions participate in the control of many metabolic pathways and membranes contain endogenous protein kinases and phosphatases, a study [30] was initiated to assess the possible role of EGF as a modulator of these regulatory processes. Aliquots of the A-431 membrane preparation were examined for their ability to phosphorylate endogenous membrane components and to determine whether the binding of EGF resulted in a perturbation of this biochemical system. The incubation of A-431 membranes at 0° with [γ-^{32}P]ATP in the presence of Mg^{++} or Mn^{++},resulted in the incorporation of radioactivity into trichloroacetic acid-insoluble material. Of key importance was the discovery that the prior addition of EGF to the reaction mixture resulted in a 3-fold enhancement of the phosphorylation of endogenous membrane-associated protein (Fig. 4).

The enchanced incorporation of ^{32}P into the membrane preparations was specific for EGF; the major phosphorylated membrane components detected were proteins having molecular weights of ~ 170,000 and 150,000. The addition of EGF to A-431 membrane preparations stimulated the phosphorylation of not only endogenous membrane proteins, but also a number of exogenously added protein substrates.

It was suggested at that time (1978) that the phosphorylation of membrane or membrane-associated components might be an initial event in the generation of intracellular signals that regulate cell growth. The reader is referred to a review (31) that summarized our knowledge as of 1979.

By the end of this second decade, I was encouraged and excited by the prospect that we had made a significant inroad into the understanding of the mechanism of action of EGF at the cellular and biochemical level.

The Third Decade. The detection of a direct effect of EGF on a chemical

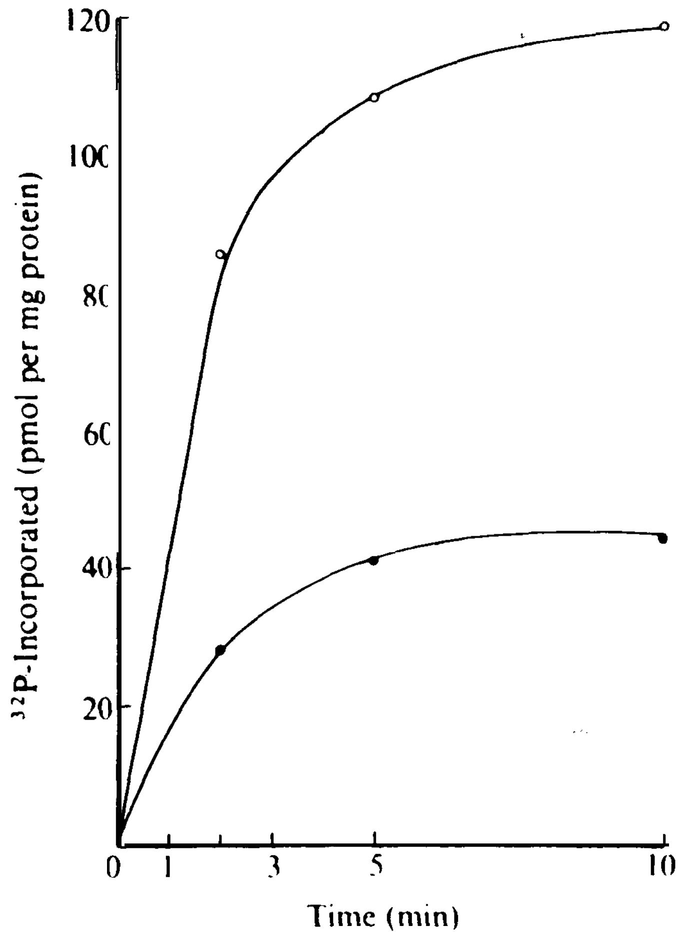

Figure 4. Stimulation of EGF of the incorporation of ^{32}P-hosphate from [γ-^{32}P] ATP into cell membranes. The reaction mixtures contained A-431 membrane (27 μg protein), HEPES buffer (20 mM, pH 7.4), MnCI, (1 mM), [γ-^{32}P]ATP (15 μM, 8X lO^{5}c.p.m.), EGF (40 ng) and BSA (7.5 μg) in a final volume of60 μ1. The reaction tubes were placed on ice and preincubated for 10 min in the presence (0) or absence (●) of EGF. The reaction was initiated by the addition of labelled ATP and incubation at 0°C was continued for the indicated times. The reaction was terminated by pipetting 50-μl aliquots on to squares (2 cm) of Whatman 3 MM filter paper and immediately dropping the paper into a beaker of cold 10% TCA containing 0.01 M sodium pyrophosphate. The filter papers were washed extensively with pyrophosphate-containing 10% TCA at room temperature, extracted with alcohol and ether dried, and the radioactivity measured in a Nuclear Chicago gas-flow counter. (reprinted from *Nature* (1978) 276, 408-410.)

reaction in a cell-free system led to a more detailed biochemical characterization of the reaction in A-431 membranes and to its extension to membrane preparations from normal human placenta and cultured human fibroblasts [32, 33, 34]. The EGF-stimulated kinase activity of A-431 membranes was not removed by extraction of the membranes with a variety of solutions, such as high salt or urea, suggesting that the kinase, receptor, and substrates were

integral membrane proteins. We, at this time, were aware of reports from several laboratories [35, 36, 37] that the molecular weight of the putative receptor for EGF, as detected by crosslinking with ^{125}1-EGF, was in the range of our major phosphorylated membrane glycoproteins, i.e. 150-170 kDa.

Our studies provided the following data concerning the mechanism by which EGF regulated protein phosphorylation in the cell-free A-431 membrane system:

1. Activation of the membrane-associated kinase activity by EGF was a rapid process, even at 0°.
2. Dephosphorylation reactions in the membrane also occurred with great rapidity, but were not affected by the presence of EGF.
3. EGF does not cause the release from the membrane of either a soluble protein kinase or modulator of the kinase.
4. The EGF-induced activation of the membrane kinase could be reversed by removal of the hormone from the membrane by anti-EGF IgG, indicating that proteolytic activation was not involved.

We originally assumed, based on data that clearly indicated different heat sensitivities of the receptor and the kinase [32], that at least two separate entities were involved. However, the possibility that the receptor and the kinase activities were present in the same molecule was raised by two unexpected observations. First, A-431 membranes could be solubilized by detergents with retention of EGF-enhanced phosphorylation activity as well as ^{125}1-EGF binding activity. Second, the EGF-receptor, the EGF-dependent kinase activity, as well as the substrate, were co-purified by EGF-affinity chromatography as a major 150 kDa protein.

We originally had reported [32] that the EGF-stimulated protein kinase phosphorylated mainly threonine residues, and in this and other regards it resembled the kinase activity of the transforming protein of Rous sarcoma virus (RSV). Soon thereafter, however, it was reported that the RSV-associated protein kinase and tumor virus-associated protein kinases [38, 39] actually phosphorylated tyrosine residues, originally mistakenly identified as threonine due to co-migration of the two phosphorylated amino acids in the electrophoretic system employed. Since we had employed a similar electrophoretic system, we reinvestigated the nature of the EGF-stimulated protein kinase reaction and discovered that the affinity purified EGF-activated receptor-kinase also phosphorylated tyrosine residues [40].

To determine whether the EGF receptor associated kinase activity might be due to trace contamination with $pp60^{src}$ we looked for an interaction of the EGF receptor kinase preparations with $pp60^{src}$ antisera. All though the receptor kinase was able specifically to phosphorylate these anti-*src* antibodies, the receptor-kinase was not precipitated by such antisera [41, 42]. We interpreted these results to mean that while the EGF receptor-kinase might be related to $pp60^{src}$, the two kinases were not identical.

We then attempted to purify further the EGF receptor [43]. When the previous affinity purification procedure [34] was applied to A-431 membrane vesicles in the absence of Ca^{2+}, the receptor-kinase was isolated as a 170 kDa

protein. The 150 kDa receptor protein initially observed in preparations from scraped membranes has been shown to be a proteolytic fragment of the 170 kDa native species, produced by the action of a Ca^{2+}-dependent neutral protease [44, 45].

The receptor properties of both the 170 kDa and 150 kDa preparations were demonstrated not only by their capacities to bind ^{125}I-EGF, but also by covalent crosslinking to ^{125}1-EGF. The major functional difference between the 170 and 150 kDa preparations appeared to be the ability of the former to "autophosphorylate" at a rate of 5 to 10 times greater than the latter. This observation is understandable since it is now known that the major autophosphorylated tyrosine residues are located near the carboxy-terminus of the 170 kDa receptor and are not present in the 150 kDa proteolytic fragment.

We addressed the question of whether the three domains present in our receptor preparation (binding, kinase, and substrate) reside in one or more than one molecule by applying more stringent purification procedures. The three domains remained associated not only following EGF affinity chromatography but also lentil lectin-Sepharose chromatography, indicating that both the receptor and the kinase were associated with lectin-reactive carbohydrate groups. More importantly, the three detectable domains remained associated following electrophoresis in nondenaturing gels and immunoprecipitation using antisera to the purified receptor. Although these results were not considered definitive, they encouraged speculation that all of- the domains were present in the same molecule.

The question was resolved by a series of experiments designed to identify the EGF-stimulated kinase by affinity labelling [46, 47]. When A-431 membrane vesicles were treated with 5^1-p-FSO_2BzAdo, a reagent previously shown to affinity label ATP or ADP binding sites in a variety of enzymes, the EGF-stimulated kinase was irreversibly inhibited. When A-431 membrane vesicles were labelled with 5^1-p-FSO_2Bz[^{14}C]Ad o and then subjected to SDS-polyacrylamide gel electrophoresis and autoradiography, most of the covalently attached affinity label migrated with the 170 kDa receptor and its 150 kDa proteolytic fragment. The labelling observed was at an ATP binding site, as it was inhibited by AMP-PNP, a hydrolysis-resistant ATP analog, but not by adenosine, AMP, ADP, GTP, or NAD. Furthermore, after labelling A-431 vesicles or scraped membranes with 5^1-p-FSO_2Bz[^{14}C]Ado, the receptor was affinity purified under conditions previously shown to co-purify the receptor and the kinase. The receptor was the only component of the purified preparation which contained detectable affinity label. Thus, we concluded that the receptor and the kinase are two domains of the same polypeptide. Lastly, if the EGF-sensitive kinase activity was inactivated, by mild heating or exposure to n-ethylmaleimide, the 150 kDa and 170 kDa receptor species could not be labelled with the ATP affinity reagent. The mechanism by which binding of EGF to the external domain of the receptor activates the cytoplasmic catalytic domain is not yet known [11, 48].

That the EGF receptor is a glycoprotein was first suggested by observations that a variety of lectins inhibit the binding of ^{125}1-EGF to cultured human

fibroblasts (49) or to human placental membranes [50] and that the receptor may be purified by lectin chromatography [51]. The biosynthesis and glycosylation of the receptor in A-431 cells have recently been addressed in several studies in which cells have been metabolically labelled and the receptor species identified by immunoprecipitation [48, 52].

It is not possible to consider here the thousands of reports regarding EGF and its receptor in biology and medicine. The reader is referred to several recent reviews that summarize various aspects of this ever burgeoning area [11, 48, 53, 54].

I now, very briefly, indicate some of the major advances made in other laboratories throughout the world that I believe will lead to a more complete understanding of the role of EGF and its receptor/kinase in growth regulation.

(1) The elucidation of the amino acid sequence of the EGF receptor, deduced from the nucleotide sequence of cDNA clones, and the discovery that the *erb*-B transforming gene of avian erythroblastosis virus probably is derived from the avian EGF receptor [55].

(2) The elucidation of the nucleotide sequence of the cDNA for prepro EGF which predicts a 128,000 molecular weight protein precursor [56, 57]. The EGF precursor may be a membrane-spanning protein, conceivably a receptor for an as yet unknown ligand. Of great interest in this regard has been the detection of preproEGF in the kidney [58], the detection of two EGF-related loci (in *Drosophila* and in *Cuenorhabditis)* that regulate development [59, 60], and the detection of an EGF-related sequence in the genome of the vaccinia virus (61). These findings suggest that EGF is of ancient origin and may have been used for a variety of functional roles.

(3) The discovery that both fetal and malignant cells produce an EGF-related protein (α-TGF) that appears to interact with the EGF receptor and mimics the biological activity of EGF [62].

(4) The discovery that the receptors for insulin as well as a number of other growth factors are ligand activated tyrosine kinases [48, 54].

Although our current working hypothesis is that the initial functional signal transmitted by EGF is related to the tyrosine kinase activity of its receptor, the exact pathway of growth activation, expecially between the receptor and cell nucleus, remains elusive. This is true not only for EGF, but also for the other growth factors whose receptors are tyrosine kinases as well as those oncogenes whose products are tyrosine kinases.

Where do we go from here? - Do we look for specific cellular proteins whose functions are altered by tyrosine phosphorylation? Is the intracellular translocation of tyrosine kinases of physiological significance? Is it possible that autophosphorylated receptors or related oncogene proteins serve some still unidentified regulatory role? What are the mechanisms for sending stimulator-y or inhibitory signals to the nucleus? What is the normal physiological role of EGF during development and homeostasis? The answers to these and a host of other questions must be found before we can fully comprehend this important regulatory system.

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