Journal of Cellular Biochemistry 23:191–202 (1983)

Biology of the A-431 Cell: A Useful Organism for Hormone Research

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Progress in understanding the scientific basis of biological processes is often accelerated by the discovery and study of a model system in which the events of interest are amplified and more amenable to analysis. Studies concerned with the biochemical basis of hormone-receptor interactions and the transmission of hormone-induced signals have been slowed by the technical difficulties involved with measuring and characterizing biological events that occur rapidly and with very low concentrations of reactants. Many years of effort have been devoted to the difficult task of purifying hormones with success, often depending on the choice of a hormone 'rich' starting material. Similarly, the isolation of hormone receptors requires an appropriate source of starting material. As a general rule, however, target tissues are not as enriched for receptors as secretory tissues are for hormones.

Although it may not be generally applicable, certain tumor cell lines appear to have unusually high concentrations of receptors for hormones. These cell lines represent an attractive choice for receptor studies since one can deal with a homogeneous population of cells, compared to the heterogeneity of a tissue, and the interactions between hormone and intact cell can be controlled with cell culture methodology. Of course, an obvious disadvantage is that there are practical limits to the numbers of cells that can be grown in vitro. The A-431 cell line, which has a high number of receptors for the mitogenic polypeptide epidermal growth factor (EGF), perhaps is the tumor cell line that has been most exploited for hormone receptor studies. The objective of this article is to review the biology of this interesting cell line and to examine its usefulness and limitations for hormone-receptor investigations. It should be noted at the outset that there is no known biological necessity for the large number of EGF receptors in this cell line and no known role of EGF in the physiology of the cell in vivo. It is not known to what extent the interactions of EGF with A-431 cells are representative of the interactions between hormones and natural target cells. Therefore, care must be exercised in extrapolating the consequences of EGF interaction with A-431 cells to the responses that occur in normal target cells.

Received May 1, 1983; revised and accepted July 11, 1983.

ESTABLISHMENT AND GROWTH CHARACTERISTICS

Ten years ago Giard et al [1] established 13 different human tumor cell lines representing kidney, lung, and epidermoid carcinomas, rhabdomyosarcoma, glioblastoma, astrocytoma, and melanoma. These lines were produced from a total of 200 attempts to culture cells from individual tumors. One of these successful attempts resulted in a cell line designated A-431 which was derived from an epidermoid carcinoma of the vulva of an 85-year-old female. The tumorigenicity of the A-431 cell line was ascertained by growth in soft agar and the formation of rapidly growing subcutaneous tumors in antithymocyte serum-treated mice. After being maintained in cell culture for six years, the cells have retained their tumorigenicity as judged by the formation of tumors in nude mice [G. Carpenter and W. Mitchell, unpublished results].

The A-431 cells grow well in culture, usually doubling every 24 hr depending on the cell density, serum concentration, and schedule of medium changes. When initially seeded in stationary cultures, the cells form small islands of epidermoid cells and eventually become confluent with some multilayering. When grown in roller bottles the cells do not become confluent but rather grow in multilayered clumps which appear as white streaks on the side of the culture vessel. In stationary cultures cell densities of up to 4×10^5 cells per cm^2 are obtained and in roller bottles, the yield of cells is approximately the same. The population doubling time (nearly 72 hr) of A-431 cells in roller bottle cultures, however, is considerably slower than the growth rate for stationary cultures. A-431 cells were established in Dulbecco's modified Eagle's medium (high glucose) containing 10% fetal calf serum. Fetal calf serum, however, is not a strict requirement, and the cells grow equally well in the presence of calf serum. Since the cells produce large amounts of acid, our laboratory has included an organic buffer (20 mM HEPES, pH 7.5) in the growth medium. We have found that the A-431 cells can be grown in low serum (0.5%) by adding 10% milk [G. Carpenter, unpublished observations]. Low-fat commercial bovine milk (pasteurized and homogenized milk is suitable) is centrifuged at 20,000 rpm for 40 min and the translucent fluid is separated from the casein which is found in the pellet and the lipid which rises to the surface. The clarified fluid is filtered directly or is diluted in medium and then sterilized by filtration. The presence of low serum is required in the medium only when the cells are first plated and can be omitted in subsequent medium changes. We find that the cells grow as well in this milk medium as they do in 10% serum. Also, the high concentration of EGF receptors in these cells is not diminished when the cells are grown in milk supplemented media. In fact, the amount of ^{125}I-EGF bound per cell is higher (25%–50%) in the milk grown cells than in cells grown in serum supplemented medium.

Conditions for the growth of A-431 cells in serum-free media have been described. Barnes [2] used a 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium supplemented with bovine insulin (10 μg/ml), human transferrin (10 μg/ml), human cold-insoluble globulin (5 μg/ml), and ethanolamine (0.5 mM) to obtain growth of A-431 cells equivalent to that obtained in presence of 10% serum. Barka and Van Der Noen [3] employed the same nutrient medium base, but supplemented with insulin (50 μg/ml), fetuin (0.5 mg/ml), transferrin (10 μg/ml), biotin (1 μg/ml), and oleic acid (5 μg/ml) in fatty acid-free bovine serum albumin.

RECEPTORS FOR EPIDERMAL GROWTH FACTOR

In 1977 Fabricant et al [4] surveyed a number of cell lines, including several of those established by Giard et al [1], for their capacity to bind radiolabeled EGF or nerve growth factor. The A-431 cell line bound considerably more ^{125}I-EGF than any of the other cell lines tested. The actual number of EGF receptors per cell in the A-431 line was underestimated in this study, probably because saturating concentrations of ligand were not used. Data published by Haigler et al [5] and Sawyer and Cohen [6] show that saturation of ^{125}I-EGF binding to A-431 cells, in a 60-min incubation at 37°C, occurs at growth factor concentrations of 100 to 200 ng/ml. At these concentrations an average of 2.6×10^6 molecules of EGF were bound per cell, suggesting that there are at least this number of EGF receptors per cell [5]. At saturating levels of ^{125}I-EGF (200 ng/ml) approximately 10% of the added hormone is cellbound after a 1 hr incubation at 37° [5]. Under the same incubation conditions, but in the presence of a low hormone concentration (1.5 ng/ml) nearly 50% of the added ligand is cell-bound.

The time course of ^{125}I-EGF binding to A-431 cells at 37° indicates that maximal binding is achieved after 60–90 min. At later times, up to 12 hr, the amount of cell-bound radioactivity does not decline but remains nearly equal to that reached at 60–90 min. Even when binding is carried out in the presence of high ^{125}I-EGF concentrations, a decline in cell-bound radioactivity will not be seen until 15–24 hr. At these later times, a large percentage of the free hormone in the medium has been degraded which can account for the decrease in cell-bound ^{125}I-EGF. Based simply on the time course of ^{125}I-EGF one might conclude that down-regulation of EGF receptors by EGF does not occur in A-431 cells. The down regulation of receptors in other cell lines is characterized by a time course of labeled ligand binding that shows a decline in cell-bound radioactivity after the initial maximal binding is achieved [7]. However, other methods for studying the influence of EGF on the total binding capacity of EGF receptors in A-431 cells or the effect of EGF on the amount of EGF receptor protein present demonstrate that down regulation does occur, [8, 9, 51, 52, Stoscheck and Carpenter, manuscript submitted]. The reason that the down regulation of receptors in A-431 cells is not reflected by a decrease in cell-bound radioactivity during the time course of ^{125}I-EGF binding is not entirely clear.

Two studies have quantitated the effect of EGF on the half-life of the EGF receptor. Krupp et al [9] have employed density shift methodology and Scatchard analyses to determine the half-life of EGF-receptor binding activity. They report that in the absence of EGF receptor activity in A-431 cells has a half-life of 16 hr. In A-431 cells cultured in the presence of EGF, the receptor half-life was 4.5 hr. This increase in the loss of receptor activity was presumed to reflect increased receptor degradation induced by EGF. Stoscheck and Carpenter (manuscript submitted) have measured receptor turnover more directly by quantitating the rate of loss of metabolically labeled EGF receptor protein during a "chase" in non-radioactive media. They report half-lives for receptor protein of 20 hr in the absence of EGF and 8.9 hr in the presence of EGF. These measurements which are independent of receptor activities provide conclusive evidence that EGF leads to an enhanced rate of degradation of its receptor.

FATE OF CELL-BOUND EGF

Studies of fibroblasts have clearly demonstrated that cell-bound ^{125}I-EGF is rapidly internalized and degraded. The fate of EGF bound to A-431 cells has been studied by several different techniques. The formation of EGF:receptor clusters on the cell surface has been examined morphologically with ferritin or fluorescein conjugated EGF [5, 10, 11] by time-resolved phosphorescent emission and anisotropy of an eosin derivative of EGF [12] and by fluorescence photobleach recovery of EGF labeled with tetramethylrhodamine [13]. The latter two studies indicated that lateral diffusion coefficient of the EGF:receptor complex was 3×10^{-10} cm^2/sec at 4° and 8.5×10^{-10} cm^2/sec at 37°. Rotational correlation times for the hormone:receptor complex were estimated to be 25–90 μs at 4° and up to 350 μs at 37°. It was concluded that the longer rotational correlation time at 37° reflected the formation of clusters of EGF:receptor complexes within which rotational movement of receptors was restricted. The observed change in the diffusion coefficient is, according to the author's interpretation, due mainly to the influence of temperature. They conclude from theoretical considerations that individual EGF:receptor complexes and micro-clusters of these complexes diffuse at the same rate. It is not clear whether one can assume that receptors or receptor clusters diffuse freely throughout the lateral plane of the membrane or whether diffusion is restricted. For example, clusters formed at coated pit regions in the membrane might be restricted. Clustering of EGF:receptor complexes occurs rapdily at 37°, but not at 5°, and the clusters are calculated to contain 10 or more EGF:receptor complexes [10–12]. It is not known whether unoccupied receptors are present in the clusters.

The internalization of clustered EGF:receptor complexes by A-431 cells has been studied by several laboratories [5–7, 10–12] and is similar qualitatively to that described for the processing of EGF and other ligands by fibroblasts [7]. An essential quantitative difference seems to be that EGF:receptor internalization in A-431 cells is relatively inefficient. At low concentrations of EGF (2 ng/ml) that produce a 7% occupancy of available receptors, A-431 cells internalize nearly all the bound EGDF rapidly [5]. However, when the ligand concentration is raised to produce occupancy of nearly all receptors, the rate of internalization does not correspondingly increase.

A-431 cells contain approximately 5- to 10-fold more receptors for low density lipoproteins (LDL) than other cell lines [14]. At high ligand occupancy, internalization of LDL receptors is relatively inefficient in A-431 cells. In other cell lines LDL receptors (over 60%) are concentrated in coated regions of the plasma membrane, but in the A-431 cell only 4% of the receptors are found in coated regions. Since LDL receptors seemed to be internalized only through coated pit regions of the membrane the inefficient internalization of LDL receptors may be due to the inability of A-431 cells to incorporate LDL receptors into coated pits. Thus, the limited metabolism of cell-bound EGF may reflect a general inability of A-431 cells to internalize ligands efficiently. It is not clear whether EGF:receptor complexes must interact with coated pits in A-431 cells to be internalized. The relationship of coated pits to the internalization of EGF receptors is unclear. The studies of Haigler et al [10] suggest that internalization of EGF receptor may occur through coated and non-coated regions of the membrane. However, coated structures may form and become uncoated rapidly, making the analysis difficult.

The interpretation of quantitative studies of EGF:receptor internalization in A-431 is complicated by this inefficient internalization process. As the data of Haigler et al [10] show, a large fraction of EGF bound to cells may dissociate as intact molecules. At saturating levels of EGF approximately 40% of cell-bound growth factor will dissociate as intact molecules, while 60% will be degraded. Also, the dissociated intact molecules may rebind to the cell and be degraded. Quantitative studies of the fate of cell-bound ligand can be substantially in error if these parameters are not taken into account.

After internalization, EGF:receptor complexes are found in multivesicular bodies and are degraded by a lysosomal mechanism in A-431 cells that is similar to the process by which other cells metabolize EGF:receptor complexes. This degradation process is the point of action of various amines [11], such as ammonium chloride, which inhibit degradation and not internalization as previously reported [15].

BIOLOGICAL RESPONSES OF A-431 CELLS TO EGF

A number of biological responses of A-431 cells to EGF have been reported and are listed in Table I.

A very rapid effect of EGF that is observed in live cells (within 1–2 min) is a large increase in the rate of calcium influx [6] and efflux [16]. Although it is not clear whether there is a change in total intracellular calcium, calcium influx was found to be essential for EGF-stimulated phosphatidylinositol turnover [6]. Thus, whether EGF alters the intracellular calcium concentration or alters intracellular calcium pools, EGF-stimulated calcium transport is biologically significant.

An increased rate of turnover of certain phospholipids is also rapidly induced by EGF in A-431 cells. Enhanced phosphatidic acid turnover was observed within 5 min, and an increase in phosphatidylinositol turnover was observed within 30 min after exposure of the cells to EGF [6]. This alteration of phospholipid metabolism was specific to phosphatidic acid and phosphatidylinositol: No change in turnover was observed for phosphatidylcholine, phosphatidyl ethanolamine, or phosphatidylserine. However, no change in the intracellular levels of the different phospholipids was observed at either 1 or 24 hr after exposure of the cells to EGF.

TABLE I. Biological Responses of A-431 Cells to EGF

Response	Reference
Inhibition of growth	[2,20]
Stimulation of growth	[24]
Increased calcium flux	[6,16]
Increased phosphoinositol turnover	[6]
Redistribution of actin and α-actinin	[19]
Membrane ruffling	[17]
Increased fluid-phase pinocytosis	[18]
Cell rounding	[16]
Enhanced tyrosine kinase activity	[28,34]
Increased C-kinase activity	[47]

One of the initial morphological effects of EGF on A-431 cells is the induction of ruffling of the plasma membrane and the extension of filopodia that occurs within 5 min [17]. This activity coincides with EGF-induced fluid phase pinocytosis [18]. By 10 min after the exposure of the cells to EGF, most of the lamellipodia and filopodia have retracted with small blebs or microvilli remaining on some of the cells. At this time, the cells are observed to begin to retract from the substrate and move towards each other; by 1 hr the cells have clumped together forming colonies several cell layers thick. This process may continue for several hours [17].

When A-431 cells are exposed to EGF in calcium-free medium, another rapid morphological effect is observed, that of cell rounding [16]. The cell rounding phenomenon looks like an exaggeration of the contraction observed in medium containing calcium. Thus, it is likely that the effect of the absence of calcium may be to loosen the constraints of the cell's adhesion to the culture dish so that cellular contraction is magnified in the presence of EGF. Studies involving treatment of the cells with cytochalasin B or colchicine indicated that microfilaments, but not newly formed microtubules, are required for the rounding process that is induced in A-431 cells by EGF [16].

At approximately the same time after EGF exposure that cell rounding begins (30 min), actin and α-actinin filaments begin to disaggregate whereas no change in the organization of microtubules and intermediate filaments are observed [19]. The effect is transient; after incubation in the presence of EGF for 8 hr normal patterns of actin and α-actinin organization are observed. This phenomenon may be associated with the cell rounding process. However, reaggregation of the filaments is observed after 8 hours whereas the cells remain rounded as long as EGF is present.

The most pronounced long term effect of EGF on A-431 cells is the inhibition of cell proliferation [2, 20]. This inhibitory effect of EGF on cell division is reversable by removal of EGF from the medium. The inhibition of A-431 cell proliferation by EGF is rapid and can be detected within 12 hr by the direct determination of cell number. Interestingly, the sensitivity of the cells to the growth inhibitory effects of EGF is dependent on cell density, tissue culture substratum, and culture medium [2,20]. Clones of A-431 cells which grow in the presence of EGF have been selected and analyzed [9,21,22]. These A-431 variants have reduced numbers of receptors for ^{125}I-EGF and coordinately reduced EGF-sensitive tyrosine kinase activity. The percent reduction of EGF receptors was not strictly correlated with the ability to grow in the presence of EGF. These variant lines do exhibit morphological responses, cell-rounding, and aggregation in response to EGF but are not growth inhibited by EGF. In fact, EGF stimulates the growth of the variant A-431 cell lines. This would indicate that the morphological effects of EGF on the parental A-431 cells are probably not the cause or effect of growth inhibition by EGF. The biochemical mechanism which produces the growth inhibition of A-431 cells in response to EGF is not clear. Morphological changes or "excessive" stimulation, for example by high tyrosine kinase activity, do not seem to be sufficient explanations. It should be noted that A-431 cells are not the only cell type in which EGF has an inhibitory effect on cell proliferation. EGF has been found to also inhibit the cell proliferation of rat pituitary tumor cells which do not have unusual levels of EGF receptors [23]. In contrast to effect of EGF on the morphology of A-431 cells, EGF induces the spherically shaped pituitary cells to assume a more elongated, flattened shape. In a recent report, a

stimulatory effect of EGF on the proliferation of A-431 cells has been indicated [24]. The data indicated that although nanomolar concentrations of EGF inhibit cell growth, picomolar levels of EGF actually produced a slight (25%) but consistent stimulation of A-431 cell multiplication. Interpretation of these results is difficult, however. Since A-431 cells internalize and degrade large quantities of EGF, the cells in these experiments should have depleted EGF from the medium in a relatively short time.

The effects of EGF or protein phosphorylation in A-431 cells will be considered in a later section.

ISOLATION OF THE EGF RECEPTOR FROM A-431 CELLS

The current interest in the A-431 cell line stems from two related points. Since EGF receptors are present at a relatively high level, the receptor can be purified [25,26]. Second, it is possible to measure, with membrane preparations from A-431 cells, a biochemical response, ie, activation of protein phosphorylation, to the formation of EGF:receptor complexes in vitro from A-431 cells [27,28]. The discovery of this EGF-sensitive protein phosphorylation system was made in A-431 membrane preparations, in part, because the high concentration of receptors help to amplify the phosphorylation reactions. As these two topics (receptor isolation and activation of protein phosphorylation) are intertwined, they will be treated together. Extensive reviews on each topic are available elsewhere [29,30].

The initial step in receptor isolation has been the production of a crude membrane fraction. Of the two procedures which have been used, the induced-vesiculation method [26] is preferable to the original method which involved scraping, hypotonic lysis, and separation of the membrane fraction on a discontinuous sucrose gradient [25]. The A-431 cells apparently contain a calcium activated protease which, in cell extracts, will convert the native 170,000 dalton receptor to a slightly lower molecular weight form of 150,000 daltons [31,32]. This protease activity on the receptor is minimized with the vesiculation procedure. The original scraping method [25] has been modified by the omission of calcium and the inclusion of an appropriate protease inhibitor to allow preservation of the 170,000-dalton receptor [32]. This revised method for membrane preparation may be of use for receptor purification, particularly in the case of cell lines or conditions that do not permit vesiculation.

A-431 cells grown in roller bottles (850 cm^2) are routinely used for production of membrane vesicles according to the procedure of Cohen et al [26]. The roller bottles are seeded with 30–50 $\times$ 10^6 cells and 10–11 days later the vesicles are prepared. At this point each roller bottle will contain approximately 300 $\times$ 10^6 cells and about 225 mg of cell protein. As the EGF receptor constitutes approximately 0.1% of the total cell protein, there is about 200 μg of receptor present in each roller bottle. Cohen et al [26] reported that the yield of vesicles from a roller bottle containing 200 $\times$ 10^6 cells is about 5 mg of protein. The vesicles contain approximately 25% of the total EGF receptor population as judged by the recovery of ^{125}I-EGF binding activity. Since the receptor should be more concentrated in the vesicle preparation, perhaps up to 1% of the total protein, the amount of receptor present at this step would be about 50 μg in the vesicles produced from one roller bottle. Isolation of the receptor is achieved by solubilizing the membrane vesicles in Triton and passing this material over an EGF agarose column [25, 26]. The absorbed receptor is eluted with either free EGF or with 5 mM ethanolamine, pH 9.0. The

yield at this point, however, is difficult to quantitate. On a practical basis the production of 100 μg of purified receptor can be achieved with a reasonable level of effort. However, to purify mg quantities of receptor would require an extremely large amount of time and supplies. Alternative sources, such as mouse liver [33], may be more efficient for large scale receptor isolation.

The purified EGF receptor has a molecular size of 170,000 daltons and is composed of one polypeptide chain with at least two types of posttranslational modifications. Carbohydrate is present on the receptor in the form of N-linked oligosaccharides [Soderquist and Carpenter, manuscript in preparation]. It is not known whether O-linked oligosaccharides are present. The second type of modification present is phosphate groups covalently linked to serine, threonine, and tyrosine residues [34,35].

From studies of protein phosphorylation in A-431 membrane preparations it was shown that EGF:receptor formation increased the activity of an endogenous protein kinase in the membrane. Several lines of evidence from studies of the purified EGF receptor or membrane vesicles are consistent with the presence of this protein kinase activity within the EGF receptor molecule [26,36]. The receptor, therefore, is bifunctional in that it contains an EGF binding site and a protein kinase site that is activated by the binding of EGF to the receptor site. The kinase is specific for the phosphorylation of tyrosine sites in substrate proteins or peptides [37]. The kinase is also cyclic nucleotide and calcium independent [27,38]. Membranes prepared from A-431 cells have very active tyrosine phosphatase activity which can be inhibited by vanadate [49] or zinc [50].

EGF-INDUCED TYROSINE PHOSPHORYLATION IN A-431 CELLS

The capacity of EGF to activate a tyrosine protein kinase activity in vitro has also been studied with intact A-431 cells. The amounts of phosphorylated tyrosine residues in cell protein is exceedingly low compared to the phosphorylation of serine and threonine residues. Phosphotyrosine is usually less than one per cent (1%) of the total phosphoamino acids, approximately 0.03% in non-transformed cells [39]. Depending upon the cell density the relative abundance of phosphotyrosine in A-431 cells is between 0.05% and 0.27%. The addition of EGF to A-431 cells results in a rapid increase of approximately three-fold in the amount of phosphotyrosine [34].

The increase in phosphotyrosine levels in A-431 cells is maximal within 1–2 min following the addition of EGF. If EGF is removed from the medium by the addition of antibodies to EGF, the increased concentration of phosphotyrosine rapidly (within 5 min) returns to the basal level. In the continuous presence of EGF, the increased amount of intracellular phosphotyrosine is maintained for 6 hr and then slowly declines, which probably reflects decreased levels of EGF in the media due to intracellular metabolism of the hormone.

Since the biological responses to EGF may be mediated by tyrosine phosphorylations, an objective of intense efforts in several laboratories is the identification of cellular phosphoproteins that show increased levels of phosphotyrosine in response to EGF. However, it is a tacit assumption, without direct experimental evidence, that tyrosine phosphorylation has causal relationship to the actions of EGF. If this assumption is correct, then substrates of the EGF receptor-kinase may be intracellular mediators of EGF action. There are two practical obstacles involved in these studies,

particularly when carried out in intact cells. First, it is not possible to identify an EGF sensitive, phosphotyrosine-containing protein as a direct substrate of the EGF receptor-kinase. There might, in fact, be a cascade of kinases each with its own substrates. It has been reported that treatment of A-431 cells with EGF increases nearly twofold the activity of the calcium, phospholipid-dependent C-kinase [47]. A second obstacle concerns the specifity, or lack of specifity, associated with protein kinases. It is very difficult to decide whether the phosphorylation of a given protein has a physiological function or whether it is a so-called "promiscuous" phosphorylation that has no biological consequence. An example of this problem is the extensive tyrosine phosphorylation of proteins in E coli by the cloned tyrosine kinase activity of the transforming gene of the Abelson murine leukemia virus [40].

In intact A-431 cells three proteins have been identified that exhibit enhanced phosphotyrosine levels in the presence of EGF. These include the receptor-kinase which undergoes an autophosphorylation reaction typical of most all protein kinases, an 81,000-dalton protein, and a protein that is referred to as the 36K protein [34,41,42]. This latter protein is apparently one protein that is identified in different gel systems as a 34,000–39,000 dalton molecular weight protein. Nothing is known concerning the identity or physiological role of either the 36K or 81K proteins. They appear to be abundant cell proteins; particularly the 36K protein. No function can as yet be assigned to the tyrosine autophosphorylation of the EGF receptor.

The 36K protein is also a substrate in A-431 cells for the protein kinase activity associated with the transforming gene (src) of the Rous sarcoma virus [41,42]. Peptide mapping studies indicated that the same sites on the 36K protein are phosphorylated in A-431 cells treated with EGF or infected with Rous virus [42]. Infection of A-431 cells with the Rous virus also increases the relative abundance of phosphotyrosine, but does not stimulate phosphorylation of the 81K protein or the EGF receptor.

Related studies carried out in vitro with the EGF receptor-kinase from A-431 and the src kinase indicated that these enzymes share substrate specificities in several cases. In early studies which indicated that protein kinase activity was associated with or was an intrinsic property of Rous transforming gene, antibody to the src was employed as a phosphorylation substrate. Interestingly, when these anti-src antibodies are added to the EGF receptor-kinase they, but not control sera, are utilized as phosphorylation substrates in an EGF-dependent manner [43,44]. The anti-src antibodies, however, do not precipitate the EGF receptor-kinase. Apparently there exists sufficient recognition or affinity between the EGF kinase and the anti-src IgG to permit enzyme-substrates interactions to occur, but there is insufficient affinity for precipitation reactions to take place. Another example of common substrate specificities for the EGF receptor-kinase and the src kinase is their similar utilization of synthetic peptide substrates which are related to the tyrosine autophosphorylation site in the src kinase [45]. The small polypeptide hormone gastrin has a tyrosine phosphorylation site similar to that present in the src protein and related synthetic peptide substrates. Gastrin [46] and angiotensin analogues [48] are utilized as efficiently as the src peptides as phosphorylation substrates by the EGF receptor-kinase from A-431 cells.

The similarities of action of EGF and the Rous sarcoma virus through tyrosine protein kinase activities has generated a "common ground" between these diverse growth regulating agents. Future studies may confirm and elaborate this exciting area.

ANTIBODIES TO THE EGF RECEPTOR

Several types of antibodies have been produced to the EGF receptor of A-431 cells. Haigler and Carpenter [53] prepared a polyclonal antiserum to A-431 membranes which contained antibodies to the EGF receptor. Stoscheck and Carpenter [54] have raised rabbit antibodies to the purified EGF receptor in its native and denatured form. While some of thesse polyclonal sera inhibit ^{125}I-EGF binding and others do not, none of the antisera have exhibited EGF-like activity in the assays employed, including the induction of DNA synthesis.

Two groups have made monoclonal antibodies to the A-431 EGF receptor. Waterfield et al [55] have described an IgG monoclonal antibody which immunoprecipitates the EGF receptor, does not block ^{125}I-EGF binding to intact cells, and displays no mitogenic activity. On the other hand Schreiber et al [56,57] have reported a monoclonal IgM antibody to the A-431 EGF receptor which inhibits ^{125}I-EGF binding and has agonist-like effects on terms of its ability to induce DNA synthesis in normal, quiescent cells and to stimulate EGF receptor phosphorylation in vitro. They also describe an IgG monoclonal which does not block growth factor binding and which by itself does not possess mitogenic activity. However, when cells were incubated with this null monoclonal and then a second antibody (goat anti-mouse IgG) was added, presumably to increase the degree of receptor clustering, mitogenic activity was observed. The interpretation of these antibody effects is important. Schreiber et al [56,57] suggest that since the mitogenic effects of EGF can be induced by antibodies to the receptor, the role of EGF, therefore, is to induce receptor clustering and that receptor clustering, whether brought about by the hormone or antibodies to the receptor, is sufficient to induce a mitogenic response. The accuracy of this interpretation remains to be determined. One of the main problems is that neither the induction of DNA synthesis nor the stimulation of protein phosphorylation may have the requisite specificity. DNA synthesis can be induced in cell culture by agents as nonspecific as inorganic precipitates [58,59] or brief alkaline pH exposure [60]. Indeed *unbalanced* cell division can result from these maneuvers giving the appearance of mitogenicity [60]. In regard to protein phosphorylation in vitro, organic solvents such as dimethylsulfoxide are potent activators of EGF receptor kinase activity [61]. Therefore, the true agonist properties of these monoclonals, while potentially significant, are not yet well enough developed to be regarded as real agonists of EGF.

ACKNOWLEDGMENTS

The authors acknowledge the support of research grants from the American Cancer Society (BC-294B) and National Institutes of Health (CA 24071) during the preparation of this manuscript. C.S. is a postdoctoral fellow supported by training grant HD 07043 from the National Institutes of Health. G.C. is an Established Investigator of the American Heart Association.

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