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The Epidermal Growth Factor Receptor as a Multifunctional Allosteric Protein†

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Epidermal growth factor (EGF) is a polypeptide growth factor of 53 amino acid residues (Carpenter & Cohen, 1979), which mediates its biological responses by binding to and activating a specific cell surface receptor termed the EGF receptor. As a growth factor receptor, the EGF receptor is unique because it is specifically recognized and activated by three distinct growth factors encoded by separate genes (EGF, TGF-α, and VVGF). These growth factors have similar disulfide backbone structures, but their overall sequence is only 24% identical. Nevertheless, all three growth factors inhibit the binding of each other to the EGF receptor with similar affinities, suggesting that they bind to a similar region of the EGF receptor.

The EGF receptor is a glycoprotein of M_r 170 000, and it possesses intrinsic protein tyrosine kinase activity (Ushiro & Cohen, 1980). Growth factor receptors with intrinsic protein kinase activity can be divided into at least three distinct receptor classes, all of which have probably evolved from common ancestral genes. Generation of various transfected cell lines expressing native, mutant, and chimeric receptors has allowed the dissection of common and distinct aspects of receptor structure, as well as elucidating the mode of receptor action and regulation (Yarden & Ullrich, 1988). In this paper, we will describe recent studies that provide new insights into the structure of the EGF receptor, the role of various domains of the EGF receptor, the mechanism of receptor activation, and the potential role of the EGF receptor in oncogenesis.

Following the purification of EGF receptor (Yarden et al., 1985) and its partial sequencing (Downward et al., 1984a,b), the complete primary structure of the EGF receptor was deduced from the sequence of cDNA clones (Ullrich et al., 1984). The mature EGF receptor is composed of three major regions: a large glycosylated extracellular EGF binding region, which is anchored to the plasma membrane by a single membrane spanning region of 23 hydrophobic amino acids; a cytoplasmic portion containing the kinase domain, which contains consensus residues typical of the tyrosine kinase gene family [reviewed in Hunter and Cooper (1985) and Yarden and Ullrich (1988)]. The Lys-721 residue and consensus sequence Gly-X-Gly-X-Phe-Gly-X-Val, located 15 residues upstream to the lysine residue, probably function as part of the ATP binding site (Russo et al., 1985) in the kinase domain. The binding of EGF to the receptor induces activation of the protein tyrosine kinase (Ushiro & Cohen, 1980) leading to self-phosphorylation and phosphorylation of various cellular substrates. In intact cells, autophosphorylation occurs mainly on Tyr-1173 while at least two additional tyrosine residues located at the C-terminal end of the EGF receptor are also phosphorylated when EGF is added to solubilized membranes or to pure EGF receptor (Downward et al., 1984a). It has been suggested that autophosphorylation of EGF receptor releases a negative constraint leading to enhanced phosphorylation of exogenous substrates by the EGF receptor (Betrics & Gill, 1985).

Quantitative binding experiments with radiolabeled EGF indicate that the stoichiometry of ligand binding to the EGF receptor is 1:1 (Weber et al., 1984). Nevertheless, analyses of binding experiments of ^{125}I-EGF to intact cells according to the method of Scatchard reveal nonlinear plots, which are interpreted as an indication of the presence of different receptor classes with distinct affinities toward EGF. Hence, high-affinity EGF receptors with an apparent K_d of (1–3) $\times 10^{-10}$ M comprise 5–10% of the total receptors while the remaining low-affinity receptors have an apparent K_d of (2–15) $\times 10^{-9}$ M (King & Cuatrecasas, 1982). The treatment of cells expressing EGF receptor with the tumor promoter phorbol myristate acetate (PMA) or with growth factors such as PDGF or bombesin, each binding to its own distinct receptor, abolishes the high-affinity state of the EGF receptor and also reduces the tyrosine kinase activity of the EGF receptor (Shoyab et al., 1979; Cochet et al., 1984; Iwashita & Fox, 1984; Wrann et al., 1980). This process termed "receptor transmodulation" is probably mediated by the Ca^{2+}-sensitive kinase C, which has been shown to phosphorylate the EGF receptor on several sites [reviewed in Schlessinger (1986) and Hunter and Cooper (1985)]. One of these sites is Thr-654 (Hunter et al., 1984; Davis & Czech, 1985), which is located 10 amino acid from

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the plasma membrane region. It has been proposed that the phosphorylation of Thr-654 allosterically regulates ligand binding affinity and protein tyrosine kinase activity of the EGF receptor (Hunter & Cooper, 1985). However, it was recently shown that PDGF is able to stimulate phosphorylation on Thr-654 of the EGF receptor in protein kinase C deficient fibroblasts, suggesting that kinases other than kinase C may be involved in the "transmodulation" of EGF receptor by PDGF (Davis & Czech, 1987).

EGF receptors are randomly distributed on the cell surface (Schlessinger et al., 1978; Haigler et al., 1978, 1979), and they undergo rapid lateral (Hillman & Schlessinger, 1982) and rotational (Zidovezki et al., 1981, 1985) diffusion. Following ligand binding, however, the occupied receptors cluster in coated pits, and after internalization both EGF and the receptor are degraded by lysosomal enzymes (Carpenter & Cohen, 1979). In the absence of EGF the half-life of the EGF receptor is 10–12 h. EGF binding decreases EGF receptor half-life to approximately 1 h (Stoscheck et al., 1985; Honegger et al., 1987a). Two reports provide evidence suggesting that receptor degradation may occur after several rounds of receptor recycling (Dunn et al., 1986; Murthy et al., 1986).

An allosteric oligomerization model was proposed to explain how ligand binding to the extracellular domain can activate the tyrosine kinase domain which is on the cytoplasmic side of the plasma membrane (Schlessinger et al., 1983; Schlessinger, 1986; Yarden & Schlessinger, 1987a,b). According to this model monomeric EGF receptors are in equilibrium with oligomeric receptors. It is postulated that the oligomeric receptors have higher ligand binding affinity than receptor monomers and, therefore, the binding of EGF will stabilize the oligomeric state. This leads to the activation of the catalytic properties of the kinase domain by subunit interaction between neighboring cytoplasmic domains. Such a mechanism bypasses the requirement for an energetically unfavorable conformational change to be transmitted through the transmembrane region connecting the two functional portions: an essential feature of every model, which assumes an intramolecular mechanism for the activation of the tyrosine kinase by the binding of EGF to the extracellular domain (Weber et al., 1984; Bertics & Gill, 1985; Staros et al., 1985). It is well established that EGF stimulates receptor oligomerization on the cell surface of living cells and in plasma membrane preparations. Receptor oligomerization induced by EGF has been demonstrated by various independent methods including morphological (Haigler et al., 1979), biophysical (Hillman & Schlessinger, 1982; Zidovezki et al., 1981, 1985), and biochemical (Boni-Schnetzler & Pilch, 1987; Cochet et al., 1988; Fanger et al., 1986; Yarden & Schlessinger, 1987a,b) approaches. The capacity of EGF receptors to undergo oligomerization is an intrinsic property of the EGF receptor since pure EGF receptor undergoes rapid ligand-induced, temperature-sensitive, reversible receptor dimerization (Yarden & Schlessinger, 1987b).

A versatile approach for the analysis of the various domains of the EGF receptor and their potential role in signal transduction is to use site-directed in vitro mutagenesis combined with transfection experiments into cultured animal cells. This approach was used to generate various cell lines that express a functional human EGF receptor and various receptor mutants. In the remaining portion of this paper we will describe recent studies aimed to elucidate the role of the protein tyrosine kinase of the EGF receptor and to provide new insights into the mechanism of receptor transmodulation and the role of the EGF receptor in oncogenesis.

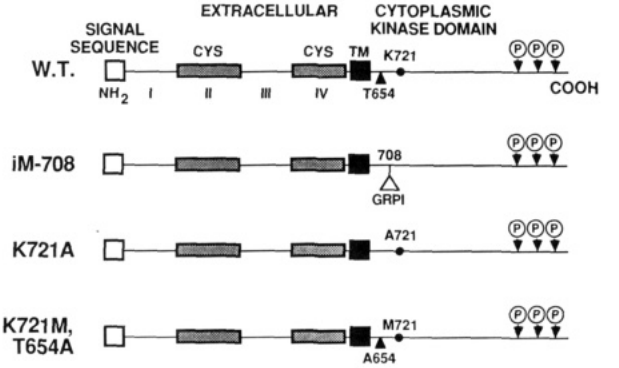

FIGURE 1: Receptor mutants generated to explore the role of the protein tyrosine function of EGF receptor. (1) iM-708 is an insertional mutant in the kinase domain containing an additional four amino acids after amino acid 708 of the EGF receptor (Prywes et al., 1986; Livneh et al., 1987, 1988). (2) K721A is a point mutant in which Lys-721, which is part of the ATP binding site, was replaced by an alanine residue (Honegger et al., 1987a,b; Moolenaar et al., 1988). (3) K721M, T654A is EGF receptor containing two mutations: Lys-721 was replaced by a methionine residue and Thr-654 was replaced by an alanine residue (Chen et al., 1987).

Table I: Properties of Various Kinase Negative Mutants of EGF Receptor

properties	W-T[a]	K721A	iM-708	K721M, T654A
kinase activity	+	–	–	–
high- and low-affinity surface receptors	+	+	+	+
transmodulation mediated by phorbol ester	+	+	–	–
ligand internalization	+	+	+	ND[b]
ligand degradation	+	+	ND	ND
receptor down regulation	+	–	ND	–
receptor degradation	+	–	ND	ND
$[Na^+]/[H^+]$ exchange	+	–	ND	ND
Ca^{2+} influx	+	–	ND	–
inositol phosphate formation	+	–	ND	ND
S-6 ribosome phosphorylation	+	–	–	ND
c-*fos* expression	+	–	ND	–
c-*myc* expression	+	–	ND	ND
DNA synthesis and cell proliferation	+	–	–	–
morphological changes and foci formation	+	–	–	ND

[a] W-T, wild type. [b] ND, not determined.

The Role of the Protein Tyrosine Kinase Function of the EGF Receptor. Several different EGF receptor mutants were generated in order to explore the role of the protein tyrosine kinase function of the EGF receptor (Figure 1). These include the following: a point mutant in which Lys-721 was replaced by an alanine residue (K721A; Honegger et al., 1977a,b); an EGF receptor containing two mutations in which Lys-721 was replaced by a methionine residue and Thr-654 was replaced by an alanine residue (Chen et al., 1987); and an insertional mutant containing four additional amino acids in the kinase domain after residue 708 (iM-708; Prywes et al., 1986; Livneh et al., 1987, 1988). The most complete analysis was performed with the point mutant (K721A), and therefore, the biological properties of this mutant will be described in detail. The biological properties of all three mutants are summarized in Table I.

The wild-type human EGF receptor was expressed in various cell lines including NIH-3T3 cells that contain undetectable amounts of endogenous EGF receptors. The expressed wild-type EGF receptor behaves like the native EGF receptor and is able to stimulate all the known responses to EGF so far

tested (Table I). Hence, these transfected cells provide a reliable system for exploring the role of the various domains of the EGF receptor.

Unlike wild-type EGF receptor, which exhibits EGF-dependent protein kinase activity, the mutant receptor expressed in K721A lacks protein tyrosine kinase activity in vitro (Honegger et al., 1987a) and in vivo (Honegger et al., 1987b). Despite this deficiency, the mutant receptor is normally processed and expressed on the cell surface where it exhibits typically both high- and low-affinity binding sites. Moreover, as found with the wild-type receptor, phorbol ester treatment abolished the high-affinity binding sites and induced phosphorylation of both wild-type and mutant EGF receptors on serine and threonine residues (Honegger et al., 1987b). It is concluded, therefore, that the kinase activity of the EGF receptor is not required for processing and expression of EGF receptors on the cell surface, for the display of high- and low-affinity EGF receptors, or for transmodulation mediated by phorbol ester (Table I). In spite of having normal ligand binding characteristics, the kinase-defective mutant is unable to stimulate various responses of EGF, suggesting that the tyrosine kinase activity is essential for EGF receptor signal transduction.

The kinase negative mutant is unable to stimulate both early responses such as $[Na^+]/[H^+]$ exchange, Ca^{2+} influx, inositol phosphate formation (Moolenaar et al., 1988), protooncogene c-*fos* and c-*myc* expression, and S-6 ribosome phosphorylation and delayed responses such as DNA synthesis and "foci formation" (Honegger et al., 1987b). Hence, ligand binding alone provides a necessary, but insufficient, step while protein tyrosine kinase activity induced by EGF is essential for signal transduction. The inability of the kinase-defective receptor mutant to stimulate inositol lipid hydrolysis is of special interest. This result suggests a possible linkage between the protein tyrosine kinase function and the phosphoinositol signaling pathway. Regulatory proteins in this pathway, such as G-proteins or phospholipase C, may serve as substrates and thus be regulated by protein tyrosine kinases.

The kinase activity is also essential for normal receptor cellular routing (Honegger et al., 1987b). Although the rate of internalization and degradation of ^{125}I-EGF was similar for both wild-type and mutant EGF receptors, the mutant receptors were not down regulted and degraded in response to EGF but were instead recycled to the cell surface for reutilization. It was concluded, therefore, that the protein tyrosine kinase activity of EGF receptor is not essential for ligand internalization and degradation (Table I). However, the degradation of EGF receptor after endocytosis is dependent on the kinase activity of the EGF receptor (Honegger et al., 1987a). On the basis of these results, we propose a model for the cellular trafficking of EGF receptors (Figure 2). It is proposed that EGF receptors are internalized and recycled continuously under normal conditions and that activated receptor is trafficked to lysosomes for degradation following several rounds of recycling so that the efficiency of receptor degradation is approximately 30%. As a consequence, in every three rounds of recycling, on average, activated receptor is trafficked to lysosomes where it is degraded. However, in the kinase-defective mutant the efficiency of the degradation pathway (step 3 in Figure 2) is greatly reduced, and therefore, the mutant receptors undergo continuous recycling while continuing to deliver ^{125}I-EGF to lysosomes for degradation (step 4 in Figure 2). The kinase activity serves the active role of specifically targeting the EGF receptor for degradation probably by phosphorylation of specific, as yet unidentified,

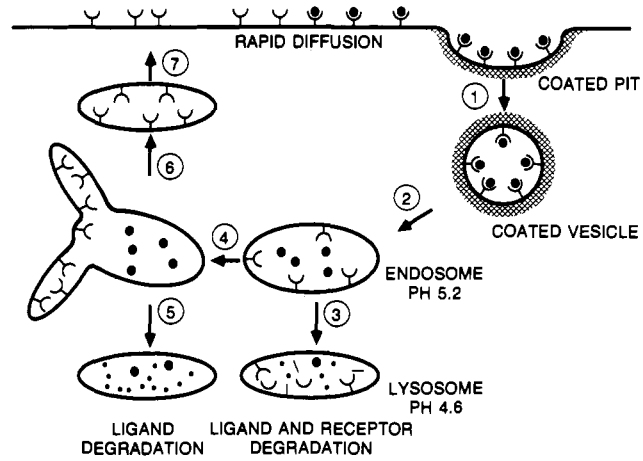

FIGURE 2: Cellular trafficking of the EGF receptor. A model describing the various steps involved in recycling and degradation of EGF receptor. Step 1: Formation of coated vesicles containing occupied EGF receptors. Step 2: Migration to endosomes where EGF molecules dissociate from receptors because of low pH. Step 3: Both EGF and EGF receptor delivered to lysosomes where they become degraded. Steps 4–7: EGF molecules delivered to lysosomes where they become degraded while EGF receptor molecules return to the cell surface for reutilization. According to this model, following several rounds of recycling (steps 1, 2, 4, 6, and 7) EGF receptors are delivered to lysosomes where both EGF and EGF receptors are degraded (step 3). However, for mutant receptors the rate of step 3 is much less than the rate of step 4, and therefore, mutant receptors undergo continuous recycling while the internalized EGF molecules undergo degradation.

substrates involved in receptor trafficking.

Hence, both signal transduction and normal receptor trafficking depend on a functional protein tyrosine kinase, suggesting that both processes are regulated by tyrosine phosphorylation of cellular substrates, which may act as internal stimuli for the pleiotropic response of EGF.

Mechanism of EGF Receptor Transmodulation by Heterologous Ligands. The ligand binding affinity and the protein tyrosine kinase activity of the EGF receptor can be regulated by the phorbol ester PMA or by growth factors such as bombesin or PDGF, which bind to specific receptors [reviewed in Schlessinger (1986)]. Since both PMA and PDGF also induce phosphorylation of Thr-654 of the receptor by activating the Ca^{2+}-dependent protein kinase C, it was suggested that the phosphorylation state of this residue may play a role in receptor transmodulation [reviewed in Hunter and Cooper (1985) and Schlessinger (1986)]. This hypothesis was examined by generating cell lines that express EGF receptor mutants in which Thr-654 was substituted either by an alanine residue (Lin et al., 1986) or by a tyrosine residue (Livneh et al., 1987, 1988). Lin et al. (1986) have shown that the Ala-654 mutant of EGF receptor does not internalize in response to PMA, while in contrast, EGF induces characteristic EGF receptor internalization and degradation. It was proposed, therefore, that two independent mechanisms play a role in the internalization of the EGF receptor, one mechanism using Thr-654 phosphorylation and a second EGF-dependent mechanism.

In the second EGF receptor mutant Thr-654 was replaced by a tyrosine residue, creating a potential phosphate acceptor site that cannot be phosphorylated by kinase C. As observed for the wild-type receptor, the mutant receptor exhibited characteristic high- and low-affinity binding sites when expressed on the cell surface of NIH-3T3 cells. Moreover, PMA abolished the high-affinity binding sites of both wild-type and mutant receptors and stimulated receptor phosphorylation on serine and threonine residues other than Thr-654. The addition of PMA to NIH-3T3 cells expressing a wild-type human EGF

receptor blocked the mitogenic capacity of EGF. However, this inhibition did not occur in cells expressing the Tyr-654 EGF receptor mutant. In the latter cells EGF was able to stimulate DNA synthesis even in the presence of inhibitory concentrations of PMA. While phosphorylation of sites other than Thr-654 may regulate ligand binding affinity, the phosphorylation of Thr-654 by kinase C appears to operate as a negative control mechanism for EGF-induced mitogenesis in mouse NIH-3T3 fibroblasts.

Further insight into the mechanism of EGF receptor transmodulation was obtained from the analysis of the properties of the insertional mutant iM-708 (Figure 1 and Table I) (Prywes et al., 1986; Livneh et al., 1987, 1988). Like wild-type EGF receptor, this mutant receptor is also expressed on the cell surface and exhibits both high- and low-affinity binding sites [Table I and Livneh et al. (1987, 1988)]. However, unlike the wild-type receptor or the K721A kinase-negative mutant, PMA was unable to modulate the affinity or induce the phosphorylation of this EGF receptor mutant (Livneh et al., 1987, 1988). The inability of the iM-708 mutant to undergo phosphorylation in response to PMA may indicate that the insertion of amino acids after residue 708 alters the structure of the EGF receptor in a way that abolishes its specific interactions with protein kinase C. This association between the receptor and the substrate binding domain of kinase C is essential for receptor phosphorylation. These interactions are probably maintained in the K721A mutant, which can be phosphorylated by kinase C on serine and threonine residues and can be transmodulated by PMA (Honegger et al., 1987a). Thus, it appears that phosphorylation of the EGF receptor by kinase C is essential for transmodulation of EGF receptor functions by PMA and by growth factors and that the phosphorylation state of, as yet unidentified, serine and threonine residues regulates ligand binding affinity and kinase activity. In addition, the phosphorylation of Thr-654 of EGF receptor provides a negative control mechanism for EGF-induced mitogenesis.

The Role of the EGF Receptor in Oncogenesis. The v-*erb*B oncogene of avian erythrobastosis virus encodes a truncated EGF receptor devoid of most of the extracellular ligand binding region and also 34 amino acids at the C-terminus (Downward et al., 1984a,b; Ullrich et al., 1984; Yamamoto et al., 1983; Nilsen et al., 1985). Hence, we have proposed that the v-*erb*B oncogene transforms by acting as a constitutively activated EGF receptor (Downward et al., 1984a,b; Ullrich et al., 1984; Kris et al., 1985; Lax et al., 1985). However, Riedel et al. (1987) have shown that a chimeric receptor composed of the extracellular and transmembrane regions of the human EGF receptor attached to the cytoplasmic region of avian v-*erb*B was still able to transform cultured rat-1 cells. It appears that the v-*erb*B protein in the chimeric receptor retains its transforming activity. Moreover, the binding of EGF to the chimeric receptor augments its transforming activity. This suggests that, in addition to the extracellular and C-terminal deletions in v-*erb*B, structural differences between v-*erb*B and the cytoplasmic region of the EGF receptor may be major factors that influence the transforming potential of v-*erb*B (Riedel et al., 1987). The EGF receptor gene was found to be amplified in several types of human tumors including gliomas and epidermoid carcinomas (Libermann et al., 1984, 1985; Hendler & Ozanne, 1984; Yamamoto et al., 1986). Structural alterations were also observed (Libermann et al., 1985), but the most frequent lesion found was gene amplification with concomitant overexpression of the EGF receptor. Overexpression of EGF receptor may be important in oncogenesis since EGF is able to induce transformation in transfected NIH-3T3 cells that overexpress the EGF receptor (Velu et al., 1987; Riedel et al., 1988; DiFiore et al., 1987b). Similarly, transfected NIH-3T3 cells were transformed by overexpression of the HER2/*neu* protooncogene (Hudziak et al., 1987; DiFore et al., 1987a). This gene encodes for a putative growth factor receptor highly homologous to the EGF receptor (Bargmann et al., 1986). The EGF receptor may also play a role in oncogenesis through both autocrine or paracrine mechanisms since various animal and human tumor cells produce TGF-α, respond to EGF or TGF-α, and also possess elevated levels of EGF receptors. These important mechanisms of transformation may offer the physician potential novel therapeutic approaches for certain neoplasias.

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