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REVIEW

Dissection of Functional Sites on the Receptor for Epidermal Growth Factor

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The epidermal growth factor receptor is a protein of 1186 amino acyl residues, which appears to be disposed as a 621-residue extracytoplasmic domain, a 23-residue transmembrane segment, and a 542-residue cytoplasmic domain. The binding of EGF to the extracytoplasmic domain of the receptor causes the dimerization of the receptor and the activation of a tyrosyl residue-specific protein kinase that forms part of the cytoplasmic domain of the receptor. We have applied a variety of techniques to identify individual residues within the receptor with specific receptor functions. Within the extracytoplasmic domain, residues adjacent to bound EGF are being identified by a strategy involving affinity cross-linking and protein microsequencing. Murine EGF (mEGF), which contains no Lys and therefore has the α-amino group as its only primary amine, and site-directed mutants of mEGF, which contain a single Lys and an Asn1 → Gln substitution in which the resulting Gln1 is chemically cyclized to pyroglutamate, are each modified on their single amino group with sulfo-*N*-succinimidyl 4-(fluorosulfonyl) benzoate. The resulting *p*-fluorosulfonylbenzoyl derivative of the EGF species is allowed to bind to the receptor, whereupon it reacts covalently with it. Limit proteolysis and sequencing are used to identify the modified residue of the receptor, e.g., mEGF modified on the α-amino terminus cross-links with Tyr101. Within the cytoplasmic domain, two residues essential for kinase activity have been identified. Lys721, identified by affinity labeling with 5′-(*p*-fluorosulfonylbenzoyl) adenosine and protein sequencing, appears to position the α- and β-phosphates of ATP within the active site and is essential for phosphoryl transfer. Asp813 appears to function as the catalytic base for phosphoryl transfer, as site-directed mutagenesis of Asp813 → Ala abolishes kinase activity.

INTRODUCTION

The binding of epidermal growth factor (EGF), a 6-kDa mitogenic polypeptide hormone, to its plasma membrane receptor initiates a cascade of intracellular events that ultimately leads to cell division (reviewed in *1*, *2*). One of the most rapid of the events that occurs after EGF binds to its receptor is the activation of a protein kinase (*3*) specific for tyrosyl residues (*4*). Affinity labeling studies carried out with the ATP analog 5′-(*p*-fluorosulfonylbenzoyl) adenosine (5′-FSBA) showed that the EGF receptor and the kinase that it activates are parts of the same molecule (*5*, *6*), a result subsequently confirmed when cDNA to the receptor was cloned and sequenced, revealing a region in the carboxyl-terminal half of the EGF receptor with striking homology to the tyrosyl residue-specific protein kinase $pp60^{src}$ (*7*).

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Translation of the receptor cDNA sequence revealed that the receptor is a protein of 1186 amino acyl residues, which appears to be disposed as a 621-residue extracytoplasmic domain, a 23-residue transmembrane segment, and a 542-residue cytoplasmic domain (*7*; see Fig. 1). The extracytoplasmic, ligand-binding domain of the receptor contains 12 non-overlapping consensus sites for *N*-linked glycosylation (*7*) and has been subdivided into four subdomains, each of 150–160 residues: subdomains II and IV are Cys-rich regions bracketing subdomain III, which possesses a significant degree of homology to the amino-terminal subdomain I (*8*).

In the first attempt at defining the portion(s) of the extracytolasmic domain of the receptor involved with EGF binding, A431 cells, a human epithelioid cell line that overproduces EGF receptors (*12*), were incubated with ^{125}I-EGF, cross-linked, and solubilized, and the receptors were purified by immunoaffinity chromatography (*8*). Cyanogen bromide cleavage of the ^{125}I-EGF–receptor complex resulted in a fragment of $M_r \approx 50{,}000$ containing most of the radioactivity, which was recognized by an anti-peptide antibody to a sequence in subdomain III (*8*). The subsequent discovery that the chicken EGF receptor bound murine EGF (mEGF) with ~250-fold lower affinity than does the human receptor (*13*) led to a series of subdomain switching experiments. Substituting subdomain III from the human EGF receptor into the chicken receptor resulted in a chimeric receptor that bound mEGF with an affinity similar to that of the human receptor, confirming the importance of domain III in EGF binding (*14*). For a series of human/chicken chimeric receptors, the authors noted contributions to the formation of a high-affinity EGF binding site from subdomain I, as well as from both the amino and carboxyl halves of subdomain III (*15*).

Other attempts to localize the EGF binding site within the receptor have focused on monoclonal antibodies that compete with EGF for binding. The epitopes of three such monoclonal antibodies were mapped, and it was concluded that residues between Ala351 and Asp364 in subdomain III contribute to EGF binding (*16*). Four of the 14 residues within this sequence differ between the human and chicken receptors; however, when the human receptor was mutated to the chicken residues at these four sites, there was no effect on the binding of EGF, though it eliminated the epitopes for three tested monoclonal antibodies (17). This study suggests that this sequence does not contribute to the EGF binding site, and that the monoclonal antibodies that compete for EGF binding do so by sterically hindering EGF binding, not by recognizing the same binding site (*17*). We have developed an approach to the identification of residues immediately adjacent to the bound hormone, based on a new step-wise affinity cross-linking reagent (*vide infra*).

The cytoplasmic domain of the receptor consists of a juxtamembranous region, the kinase domain, and the carboxyl terminal tail that contains five sites for autophyosphorylation (Fig. 1). Studies from our laboratory utilizing protein microsequencing of 5′-FSBA-labeled receptor demonstrated that 5′-FSBA modifies Lys721 within the kinase domain (*18*), a residue that has since been shown to be highly conserved in protein kinases (*19*). Site-directed mutagenesis of Lys721 to Met (*20*), Ala (*21*), or Arg (*22*) was shown to abolish the kinase activity of the receptor. Further, it was shown that EGF does not elicit the full range of biochemical responses in cells that express these mutant receptors, which has been interpreted to suggest that

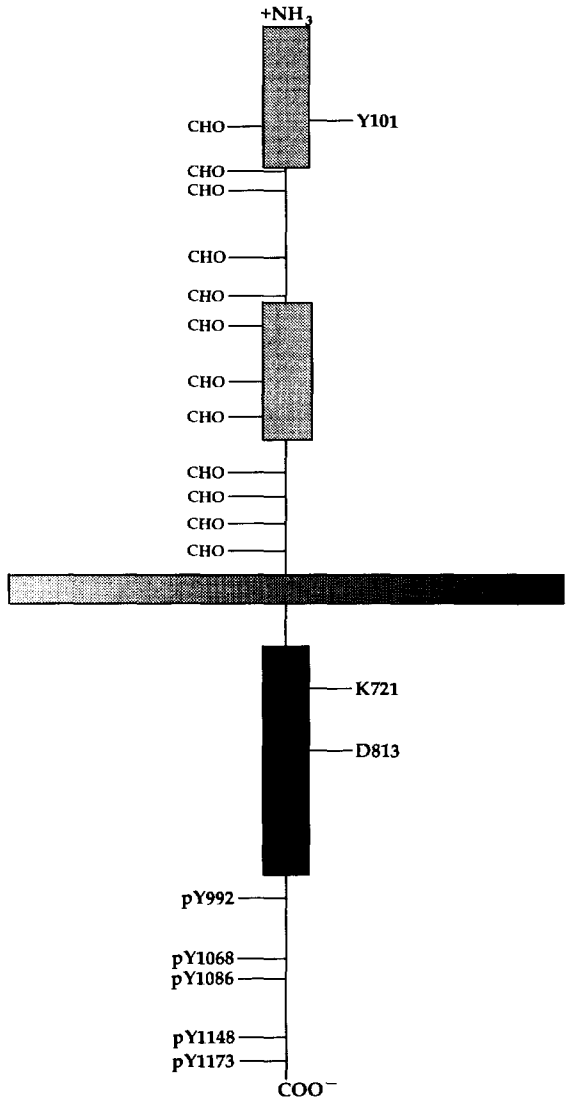

FIG. 1. Schematic representation of the EGF receptor sequence. In this linear representation of the receptor, the amino-terminal, extracytoplasmic domain is divided into four subdomains (*8*). Subdomains I and III, which contribute to the EGF binding site, are represented as rectangles, and the Cys-rich subdomains II and IV are represented as lines. Within the carboxyl-terminal cytoplasmic domain, the kinase subdomain is represented as a rectangle and the juxtamembrane segment and the carboxyl terminal tail that contains the sites of autophorphorylation are represented as lines. On the left of the figure are marked the five known sites of autophosphorylation (*pY*) (*9–11*) and potential sites of *N*-linked glycosylation (CHO) (*7*).

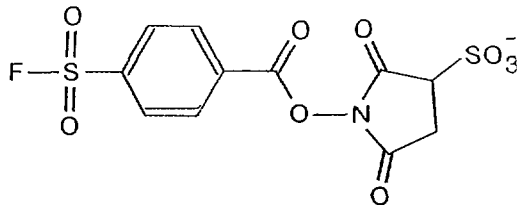

FIG. 2. The structure of sulfo-*N*-succinimidyl 4-(fluorosulfonyl)benzoate (SSFSB). The compound is isolated as the Na^+ salt (*26*). Reprinted with permission from (*26*) (Copyright 1992, American Chemical Society).

receptor kinase activity is required for signal transduction leading to mitogenesis (*20, 21, 23*).

The crystal structure of the catalytic subunit of the cAMP-dependent protein kinase with Mg^{2+} ATP and a peptide inhibitor bound (*24*) has provided important insights into potential catalytic roles of active-site residues, not only for that enzyme, but for the family of protein kinases. In the crystal structure, Lys72, which is homologous to Lys721 of the EGF receptor (*19*), interacts with the α- and β-phosphates of the bound ATP (*24*). The essentiality of Lys721 for kinase activity suggests that it may play more than a role in positioning, perhaps helping to promote the leaving group function of the ADP β-phosphate after attack of the phosphate-accepting hydroxyl on the γ-phosphate.

In the crystal structure of the cAMP-dependent protein kinase, Asp166 of the cAMP-dependent protein kinase, which is homologous to Asp813 of the EGF receptor (*19*), appears correctly positioned to serve as the catalytic base, accepting the proton from the attacking hydroxyl (*24, 25*). Based on this observation, together with previous ones from chemical modification and mutational studies of the cAMP-dependent protein kinase, we mutated Asp813 of the EGF receptor to Ala to test the importance of this residue in kinase activity.

RECEPTOR RESIDUES ADJACENT TO BOUND EGF

To identify residues of the receptor proximal to bound EGF, we devised an affinity cross-linking strategy around a new reagent, sulfo-*N*-succinimidyl 4-(fluorosulfonyl)benzoate (SSFSB, Fig. 2) (*26*). The properties of this reagent are that at physiological pH, (1) the more rapidly reacting group, the sulfo-*N*-succinimidyl ester, is relatively specific for primary amino groups, yielding stable amide products (*27*), and (2) the aromatic fluorosulfonyl group reacts orders of magnitude more slowly, but is relatively promiscuous, forming adducts with a variety of nucleophilic side chains in proteins. Further, (3) both functional groups of SSFSB hydrolyze slowly in comparison with their rates of reaction with nucleophilic residues in proteins, and (4) the short span and relatively hindered structure of SSFSB impart a spatial selectivity to the reagent. The application of SSFSB to this problem exploits

the fact that in murine EGF (mEGF), that are no Lys residues (*28*), so that the only primary amino group is the α-amino terminus, and that mammalian EGFs are recognized by the receptors of other mammalian species with high specificity.

Brief treatment of mEGF with SSFSB, followed by separation of the modified hormone from excess reagent by gel filtration, yields N_α-*p*-fluorosulfonylbenzoyl mEGF, as confirmed by laser desorption time-of-flight mass spectrometry of products of the SSFSB–mEGF reaction mixture separated by HPLC (*26*). Incubation of SSFSB-modified ^{125}I-labeled mEGF with membrane vesicles from A431 cells, which overexpress the human EGF receptor, results in specific binding of the hormone to the receptor and covalent linkage to the receptor (Fig. 3), via a reaction driven by the high local concentration of an appropriate nucleophilic residue in the vicinity of the fluorosulfonyl group of the bound hormone (*29*). The observation that excess unlabeled mEGF competed with both the covalently linked and noncovalently linked hormone suggested that both were specifically bound to the receptor. Comparison of covalently linked EGF with that specifically bound but not covalently linked indicated that 44–69% of the specifically bound hormone was covalently linked to receptor, meeting the requirement for high yield of incorporation necessary for sequencing.

Though the competition experiments shown in Fig. 3 suggest that the covalent modification of the receptor by N_α-*p*-fluorosulfonylbenzoyl mEGF is specific, it is important to ascertain whether the hormone is in its native configuration within the binding site. In Fig. 4, the ability of covalently linked mEGF to stimulate

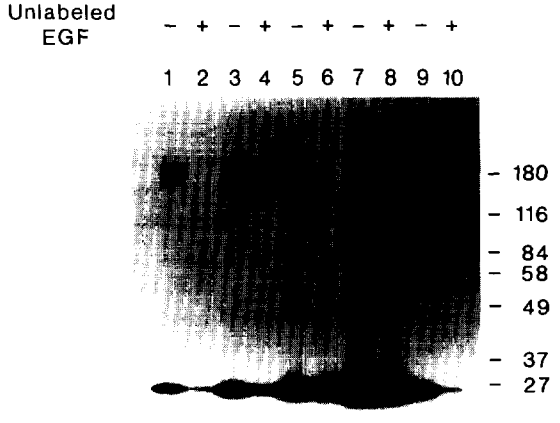

FIG. 3. Step-wise affinity cross-linking of ^{125}I-mEGF to its receptor with SSFSB. Various concentrations of SSFSB-modified ^{125}I-mEGF were incubated with membrane vesicles from A431 cells in the absence or presence of excess (5.5 μM) nonradiolabeled, unmodified mEGF, as indicated. The concentration of SSFSB-reacted ^{125}I-mEGF was 0.03 μM for lanes 1 and 2, 0.1 μM for lanes 3 and 4, 0.3 μM for lanes 5 and 6, and 1 μM for lanes 7 and 8. As a control, 1μM ^{125}I-mEGF of the same specific activity which had not been modified by SSFSB was incubated with A431 vesicles in lanes 9 and 10. Samples were separated by electrophoresis in sodium dodecyl sulfate-containing 7.5% polyacrylamide gels. The figure is an autoradiograph of the gel, which was dried without staining. Molecular weight standards are indicated on the right. Reprinted with permission from (*26*) (Copyright 1992, American Chemical Society).