Biochemical Pharmacology, Vol. 60, pp. 1217–1223, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00425-1

Role of Conformational Alteration in the Epidermal Growth Factor Receptor (EGFR) Function

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ABSTRACT. This mini-review addresses the effect of glycosylation and phosphorylation on the conformational alterations of the epidermal growth factor receptor (EGFR). Based on studies with full-length and truncated EGFRs, we propose a model to suggest that receptor–receptor self-association, which occurs in the truncated receptor and depends on core glycosylation, is prevented in intact receptor by a certain extracellular domain and that the function of the ligand is to remove the negative constraint. We also propose, based on works with a conformation-specific antibody directed to an unphosphorylated peptide, that the interactions among negatively charged phosphotyrosine residues in the receptor molecule result in bringing two epitopes separated by a long stretch of amino acids close to each other to form an antibody-binding site. The implications of these posttranslational modifications on receptor functions are also discussed in this article. BIOCHEM PHARMACOL **60**;8:1217–1223, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. conformation-specific antibody; EGF receptor; N-linked glycosylation; phosphorylation sites; receptor tyrosine kinases; type III truncated EGFR

The growth of cells in culture and, most likely, in the animal, is subject to control by various agents, such as extracellular signaling proteins. These growth factors mediate their pleiotropic actions by binding to high-affinity cell surface receptors. A number of growth factor receptors, such as receptors for EGF,† PDGF, insulin, colony-stimulating factor-1, and hepatocyte growth factor, belong to the tyrosine kinase family of proteins. These receptor tyrosine kinases are multisited and multifunctional proteins with similar structural features that include a single hydrophobic transmembrane region of 20–25 amino acids which separates the large extracellular domain from the cytoplasmic region. The exoplasmic domain contains the ligand-binding site, whereas the intracellular portion contains the tyrosine kinase domain and the C-terminal tail that are important for signal transduction [reviewed in 1].

Upon ligand binding, a receptor tyrosine kinase undergoes a series of changes, the earliest one being the conversion of the monomeric form of the receptor to the dimeric form. This leads to activation of the kinase, resulting in phosphorylation of its own tyrosine residues, a phenomenon known as autophosphorylation. The phosphotyrosine residues of the activated receptor then act as docking sites for target molecules, such as signal transducers. This association initiates a cascade of events leading to DNA synthesis and cell division. In the case of the EGFR, the autophosphorylation sites are clustered in the last 194 amino acids in the C-terminal tail of the receptor [2]. In addition to being docking sites for Src homology-2 domain-containing proteins involved in signal transduction, the EGFR C-terminal tail is also important in receptor internalization, down-regulation, and endocytosis [3]. This article will focus on how functioning of the EGFR is influenced by conformational alteration of the receptor induced by posttranslational modifications such as glycosylation and phosphorylation.

EXTRACELLULAR DOMAIN OF THE EGFR

The EGFR (ErbB1) and the other three members (ErbB2/neu, ErbB3, and ErbB4) of this family have similar structural features: a cysteine-rich extracellular region, an uninterrupted kinase domain, and multiple autophosphorylation sites clustered at the C-terminal tail [1]. Among these receptors, ErbB3 has virtually no kinase activity, although it is capable of binding ATP. The ligands for ErbB1 include EGF, transforming growth factor-α, heparin-binding EGF, betacellulin, amphiregulin, and epiregulin. Neuroregulins (NRG), which are predominantly expressed in parenchymal organs and in embryonic central and nervous systems, bind both ErbB3 and ErbB4. No ligand has yet been identified for ErbB2. The human EGFR is a transmembrane glycoprotein with 1186 amino acids. The extracellular domain of the mature receptor contains 621 amino acids followed by a single transmembrane domain (amino acids 622–644) and a juxtamembrane domain (amino acids 645–682). The tyrosine kinase domain extends from amino

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† *Abbreviations:* Ab P2, antibody to peptide 2; EGF, epidermal growth factor; EGFR, EGF receptor; Erb, erythroblastosis; PDGF, platelet-derived growth factor; and PDGFR, PDGF receptor.

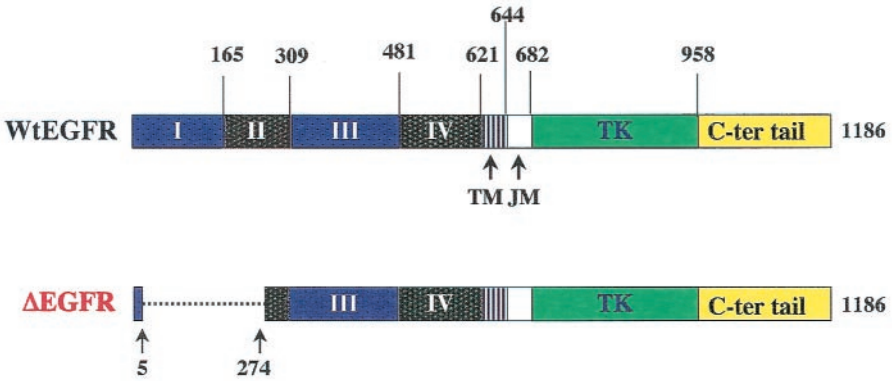

FIG. 1. Schematic structures of the extracellular domains of the wild-type (Wt) EGFR and ΔEGFR. The extracellular region of the EGFR is divided into four domains: domain I (amino acids 1–165), cysteine-rich domain II (166–309), domain III (310–481), and cysteine-rich domain IV (482–621). The scheme also shows the locations of the transmembrane domain (TM: 622–644), juxtamembrane domain (JM: 645–682), tyrosine kinase domain (TK: 683–958), and C-terminal tail (959–1186). The ΔEGFR lacks amino acids 6–273 and the missing part is denoted by ·····.

acids 683 to 958, whereas all the autophosphorylation sites are located between amino acids 992–1186 (see Fig. 1). On the basis of internal sequence identity, the extracellular portion of the EGFR has been subdivided into four domains: domain I (amino acids 1–165) and domain III (310–481) have 37% sequence identity, whereas domain II (166–309) and IV (482–621) are rich in cysteines [4]. Each of domains I and III contains four conserved cysteines with disulfide-pairing patterns of 1-2 and 3-4. Domains II and IV contain 22 and 20 cysteines, respectively. The 11 disulfide bonds in domain II are arranged into three units of a 1-3, 2-4 pattern and five units of a 1-2 pattern, and the 10 disulfides in domain IV are arranged into three units of a 1-3, 2-4 pattern and four units of a 1-2 pattern [5]. Biochemical and biophysical studies suggest that domain III, which is flanked by two cysteine-rich domains, binds directly with EGF with a K_d of 100–400 nM and that two molecules of the monomeric receptor–ligand complex then interact to form a dimeric complex. Domain I is believed to be involved in the second interaction [4,5]. The involvement of domain III in ligand binding is further supported by studies with monoclonal antibodies to domain III; these antibodies in general compete with EGF for receptor binding [6,7]. The participation of domain I in EGF binding was confirmed by studies with a truncated EGFR. Nearly 50% of grade IV gliomas (glioblastoma multiforme) have amplified *EGFR* genes. In the majority of such cases, the *EGFR* gene amplification is correlated with structural rearrangement of the gene, resulting in in-frame deletions that preserve the reading frame of the receptor message. To date, three truncated forms of EGFR have been identified. The type III deletion mutant which occurs in 17% of the glioblastomas appears to be the most prevalent. This deletion mutant is characterized by an 801-bp in-frame deletion resulting in the removal of NH_2-terminal amino acid residues 6 through 273 from the extracellular domain of the intact 170-kDa EGFR (Fig. 1). This receptor, known as EGFRvIII/ΔEGFR, lacks the entire domain I and a part of domain II. Although domains III and IV are intact in the receptor, the ΔEGFR fails to bind EGF [reviewed in 1]. This suggests that, in addition to domain III, domain I, which is separated from domain III by a cysteine-rich region of 144 amino acids, is also important in EGF binding. Thus, the ligand-binding domain of the EGFR is not contiguous. Although the truncated receptor lacks EGF-binding activity, it is capable of receptor–receptor self-association. We have been able to isolate the dimeric receptor in a biologically active form and demonstrate that the autokinase activity per molecule of the dimeric ΔEGFR is comparable to that of the ligand-activated wild-type receptor. In addition, the dimeric form of the ΔEGFR is extremely stable and does not revert back to the monomeric form under *in vivo* conditions.* Thus, the transforming activity of the truncated receptor is due to hyperactivation of the receptor resulting from receptor–receptor self-association.

ROLE OF CORE GLYCOSYLATION IN THE EGFR FUNCTION

The EGFR contains both complex-type and high-mannose-type Asn-linked oligosaccharides; however, no O-linked sugar could be detected in the receptor expressed in the human carcinoma cell line A431 [8]. There are 12 potential N-linked glycosylation sites in the receptor, i.e. 2 in each of domains I and II and four in each of domains III and IV [9]. Core glycosylation plays an important role in EGF binding and hence in kinase activation. Aglyco-EGFRs synthesized in the presence of tunicamycin, an inhibitor of N-linked glycosylation, lack ligand-binding activity and hence kinase activation [8, 10]. However, the enzymatic removal of the carbohydrate chains from the glycosylated receptor does not have any negative effect on EGF binding or kinase activation. This has been demonstrated by synthesizing the high-mannose form of the receptor by growing cells in the presence of swainsonine, an inhibitor of mannosidase II. The modified receptor is as active as the control receptor.

* Fernandes H, Cohen S and Bishayee S, Manuscript in preparation.

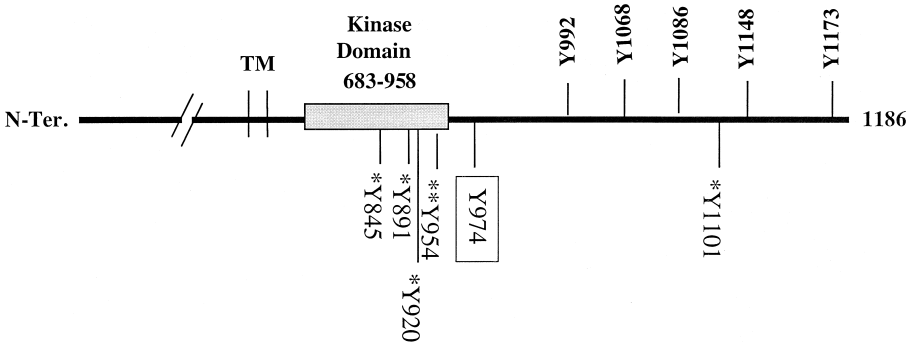

FIG. 2. Locations of tyrosine phosphate acceptor sites and also of a tyrosine required for interaction with clathrin adaptors in the EGFR. The autophosphorylation sites are marked by bold letters at the top, whereas tyrosines phosphorylated by c-Src kinase are marked by single asterisk. Y845 and 1101 in C3H10T1/2 murine fibroblasts, Y891 and 920 in MCF7 human breast cancer cells, and Y845 in A431 human carcinoma cells and in MDA-MB-468 human breast cancer cells are phosphorylated by c-Src. The putative autophosphorylation site Y954 is marked by double asterisks. Clathrin adaptor-interacting tyrosine (Y974) is boxed.

Unlike the aglyco-receptor synthesized in the presence of tunicamycin, the complete removal of the carbohydrate chains from the high-mannose receptor by endoglycosidase H does not abolish the ligand-binding or autokinase activity of the receptor. This strongly suggests that the initial requirement for N-linked oligosaccharides is for proper folding of the receptor to generate an EGF-binding active conformation; once such a conformation is attained, the carbohydrate chains are dispensable. Although the ΔEGFR undergoes dimerization and kinase activation in the absence of EGF binding, interestingly our recent studies revealed that core glycosylation is also needed for receptor–receptor self-association. In addition, as with the wild-type receptor, the glycosylation-induced conformation of the truncated receptor is highly stable, since removal of the carbohydrate chains from the glycosylated receptor does not have any negative effect on receptor dimerization or kinase activation†

The similar characteristics with respect to sugar requirements for self-dimerization and EGF binding appear to suggest that glycosylation positively regulates receptor–receptor association and that this is a prerequisite for ligand binding. The facts that unliganded intact receptor preexists in monomeric form whereas the truncated receptor undergoes self-dimerization suggest that there are two opposing interactions in the EGFR. One is involved in receptor–receptor self-association and is dependent on glycosylation. This site is probably located in domain III and/or domain IV. The other exerts a negative influence on protein–protein interaction and is located in domain I. In full-length receptor, the protein is in a monomeric state, since the negative influence of domain I is much stronger than the positive influence exerted by domain III. However, EGF binding to domain III somehow overcomes the inhibitory effect of domain I. Thus, although we think of activation by ligand as a positive step, it may, instead, simply reflect the ability of a ligand to remove a negative constraint. Future studies with the receptor in which potential glycosylation sites are mutated will enable us to understand the influence of individual oligosaccharide chains located in domains III and IV in receptor conformation.

PHOSPHORYLATION SITES IN THE EGFR

Under normal circumstances, the kinase activity of a receptor is dormant. Ligand-induced receptor dimerization results in bringing the kinase site and other parts of a receptor molecule close to the corresponding sites in another molecule. The residual kinase activity of the dimeric receptor induces cross-phosphorylation and this somehow activates the kinase. For a number of receptor kinases, the initial phosphorylation takes place in a domain known as activation loop and this phosphorylation is obligatory for kinase activation, resulting in phosphorylation in additional sites of its own receptor molecule as well as of other substrates. For example, phosphorylation of Tyr1146, 1150, and 1151 in the insulin receptor, Tyr653 and 654 in the fibroblast growth factor receptor 1 (FGFR1), Tyr674 and 675 in TrkB (receptor for brain-derived neurotropic factor, BDNF), Tyr857 in βPDGFR, and Tyr849 for αPDGFR is important for kinase activation [11–14]. This phosphorylation-induced kinase activation is not restricted to receptor kinases. A number of non-receptor kinases, such as ZAP70 (zeta-associated protein of 70 kDa) kinase as well as Src-related kinases, are also activated by a similar mechanism. During T-cell activation, ZAP70 is recruited to the zeta-chain of the T-cell receptor complex and is phosphorylated on Tyr392 and 393 located in the kinase domain by Lck (lymphocyte-specific protein tyrosine kinase; p56), an Src-related kinase. This initial phosphorylation results in the activation of ZAP70 kinase and autophosphorylation of Tyr315 [reviewed in 15]. In this respect, the members of the EGFR family differ from other receptor tyrosine kinases. Five autophosphorylation sites have so far been identified in the EGFR, all of which are clustered at the extreme C-terminal 194 amino acids. Among these sites, Tyr1068, 1148, and 1173 are major sites, whereas Tyr992 and 1086 are minor sites (Fig. 2). It should be mentioned in this context that we could not

detect any phosphorylation of the EGFR in an F5 mutant (MI41) in which all five autophosphorylation sites were substituted with Phe [2]. Unlike the other receptor kinases discussed above, phosphorylation of a particular site or sites in the EGFR is not required for full enzymatic activity. Similarly, no activation loop-mediated kinase activation has been demonstrated for ErbB2/neu and ErbB4. ErbB3 has virtually no kinase activity. It should be mentioned in this connection that a number of tyrosine residues, including Tyr845, 891, 920, 954, 974, and 1101, are also present in the cytoplasmic domain of the EGFR (Fig. 2). Some of these tyrosines are phosphorylated by c-Src kinase in a cell-specific manner. For example, Tyr845 and 1101 in C3H10T1/2 murine fibroblasts, Try891 and 920 in MCF7 human breast cancer cells, and Tyr845 in A431 human carcinoma cells and in MDA-MB-468 human breast cancer cells have been identified [reviewed in 16]. As the phosphorylation is highly cell-specific, it is highly likely that a third component, in addition to the receptor and c-Src, must be involved in the phosphorylation at some of these sites. Phosphorylation of Tyr845 is of special importance because of its location in the putative activation loop of the receptor kinase. Studies from different laboratories appear to suggest a direct correlation between phosphorylation at Tyr845 and EGFR functioning: (i) increased phosphorylation of EGFR substrates Shc (Src homology collagen-like) and PLC_γ (phospholipase C_γ) as well as enhanced growth and tumor formation; and (ii) failure of a Y845F mutant to synthesize DNA in response to EGF or serum, either in the presence or absence of overexpressed c-Src [16, 17]. More studies should be done to ascertain the role of Tyr845 in normal functioning of the EGFR. Another tyrosine residue that appears to be involved in signal transduction is Tyr954. Phosphopeptide-containing Tyr954 inhibits the interaction of SH-PTP2, an SH2 (Src homology 2) domain-containing phosphatase, with the phosphorylated EGFR, suggesting Tyr954 as a putative phosphate acceptor site [18].

In addition to the tyrosine residues mentioned above, Tyr974 also plays an important role in receptor functioning. Tyr974 is located in an internalization motif (^{973}FYRAL^{977}) needed for the interaction of the EGFR with AP-2 (clathrin adaptor protein complex 2). Mutation of this tyrosine essentially eliminates the high-affinity interaction between the receptor and clathrin adaptors; however, there is no evidence to suggest the phosphorylation of Tyr974 [3]. It is highly likely that the hydroxyl group of Tyr974 is important for the three-dimensional structure of the AP-binding domain.

AUTOPHOSPHORYLATION-INDUCED CONFORMATIONAL CHANGES IN THE EGFR

As phosphorylation introduces negative charges to a protein molecule, it is highly likely that autophosphorylation should have a profound effect on receptor conformation. In fact, such conformational changes have been well documented with different receptor kinases. However, the susceptible epitopes and the tyrosine residue(s) involved in particular structural alteration mostly remain to be determined. In this respect, we have not only identified one such domain, but also identified the phosphate acceptor sites that are responsible for its conformational change. While working with antipeptide antibodies, we made a serendipitous finding that allowed us to identify such a site. We have previously reported the generation of a conformation-specific antibody to the β-type PDGFR. This antipeptide antibody (Ab P2) is directed to an intracellular domain (amino acid residues 964–979; EGYKKKYQQVDEEFLR) of the human β-type receptor [19]. Although the antibody is directed to an unphosphorylated peptide epitope, it recognizes only the tyrosine-phosphorylated receptor in immunoprecipitation. In addition to the PDGFR, Ab P2 also binds to the EGFR, and interestingly, its recognition is also phosphorylation-dependent [20]. However, the antibody is not directed to phosphotyrosine since phenyl phosphate, an analog of phosphotyrosine, has no effect on the immunoprecipitation of either the PDGFR or the EGFR, suggesting that the antibody recognizes the phosphorylated protein and not phosphotyrosine. Our subsequent studies revealed that the phosphorylation of the EGFR results in bringing two epitopes close to each other to form an antibody-binding site. A detailed report of this work has been published [2, 21].

In P2 peptide, there are two tripeptide sequences—YQQ and DEE—that are also present in the cytoplasmic domain of the EGFR as is shown in Fig. 3, panel a. Among the P2-derived peptides, one containing DEE (C-terminal peptide) blocks the immunoprecipitation of the ^{32}P-labeled EGFR, whereas a peptide containing YQQ (N-terminal peptide) has no such effect on immunoprecipitation (Table 1). This suggests that Asp-Glu-Glu is probably responsible for the recognition of the EGFR by the antibody. However, for a peptide to be immunogenic, it should have a chain length of at least five amino acids; secondly, the affinity of the inhibitory peptide, i.e. Asp-Glu-Glu-containing peptide, is extremely low compared to the full-length peptide. In fact, it is more than 13,000-fold lower at the molar level. Accordingly, we made a systematic study to identify the high-affinity Ab P2-binding site in the EGFR and also to identify the tyrosine residues that regulate the conformation of this site. As shown in Fig. 3a, all five autophosphorylation sites are clustered at the extreme C-terminal 194 amino acids. Among these sites, three are located between the two tripeptide sequences, one is at the extreme C-terminal tail of the receptor, and another is part of a tripeptide. To identify the tyrosine residues involved in regulation, we used single Tyr->Phe mutants of the EGFR, labeled the receptor with ^{32}P, and then equal amounts of purified receptor were subjected to immunoprecipitation with Ab P2. As shown in Fig. 4, antibody binding was not affected by substitution at either of the tyrosines at 1173 and 1148; however, substitution of tyrosines at any of the other three autophosphorylation sites located at 992, 1068,

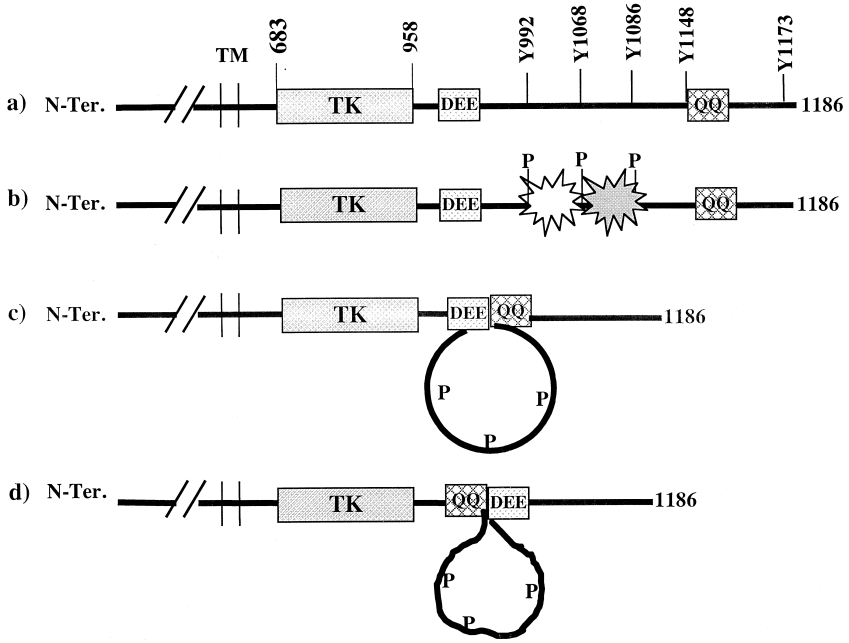

FIG. 3. A model to explain the recognition of the phosphorylated and not the unphosphorylated EGFR by Ab P2. (a) The two peptide sequences QQ and DEE (boxed) that form a single antigenic determinant for Ab P2 are separated by 169 amino acids and neither of these epitopes is capable of high-affinity binding with the antibody. (b) Phosphorylation of the three tyrosine residues located between the two epitopes imparts very high negative charge to the peptide backbone. (c) This results in the bending of the intervening sequence in such a way that QQ and DEE come closer to each other to form a high-affinity antibody-binding site. (d) The bending due to charge–charge interaction possibly results in changing the orientations of the peptides, with QQ positioning itself upstream of DEE. TK, tyrosine kinase domain; TM, transmembrane domain.

and 1086 drastically reduced the immunoprecipitation by 80–90%. Our phosphopeptide analysis revealed that a single Tyr->Phe substitution has no negative effect on the phosphorylation of the other tyrosine residues [2]. In addition, the extent of immunoprecipitation of the ^{32}P-labeled EGFR from the double-mutant Y(1173–1148)F was similar to that of the wild-type receptor, suggesting that these two tyrosines, either alone or in combination, play no role in antibody binding.

We also studied the binding characteristics of a deletion mutant, Dc63, with Ab P2. In addition to Tyr 1173 and 1148, 63 amino acids from the extreme C-terminal tail of the receptor are missing from the mutant. This mutant also lacks the tripeptide YQQ which is part of the P2 peptide. As shown in Fig. 4, the antibody failed to recognize the ^{32}P-labeled Dc63. Since all three tyrosines needed for antibody recognition are efficiently phosphorylated in the Dc63 mutant [2] and the mutated receptor still failed to recognize the antibody, this suggests that such a lack of antibody binding is due to the absence of the tripeptide sequence YQQ. Since Tyr->Phe substitution at 1148 has no effect on antibody binding, these studies together with peptide inhibition results suggest that Gln-Gln in combination with Asp-Glu-Glu forms a high-affinity complex with Ab P2 and that such a complex formation is dependent on tyrosine phosphorylation at 992, 1068, and 1086 as a unit.

TABLE 1. Effect of P2-derived peptides on the immunoprecipitation of the EGFR by Ab P2

Peptide	Concentration for 50% inhibition of immunoprecipitation
P2 peptide (EGYKKKYQQVDEEFLR)	3 ng/mL
N-ter peptide (EGYKKKYQQV)	No detectable inhibition
C-ter peptide (VDEEFLR)	40 μg/mL

The ^{32}P-labeled EGFR was immunoprecipitated with Ab P2 in the absence or presence of increasing concentrations of the indicated peptide. Following electrophoresis and autoradiography, the EGFR band was densitometrically scanned and the peptide concentration for 50% inhibition of immunoprecipitation was calculated from the plot. The values are the average of two experiments.

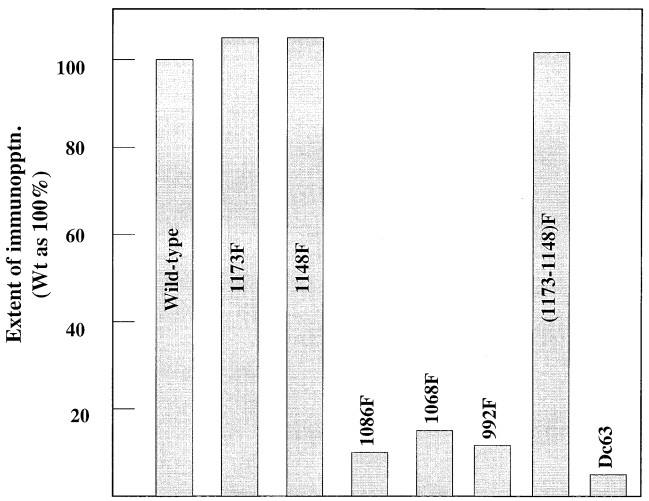

FIG. 4. Binding of Ab P2 with the ^{32}P-labeled EGFR mutants. Detergent-solubilized membranes from the wild-type (Wt) and the EGFR mutants were phosphorylated with labeled ATP in the presence of EGF. After purification of the phosphorylated receptor by antiphosphotyrosine antibody, 1.25 fmol of the receptor was incubated overnight at 4° with 33 pmol of protein A-purified Ab P2 in a total volume of 15 μL. Following isolation of the immune complexes with formaldehyde-fixed *Staphylococcus aureus*, the labeled proteins were analyzed by SDS–PAGE/autoradiography, and the region containing the EGFR band was densitometrically scanned. The amino acid phenylalanine is identified by its single letter code F. Dc63 is a deletion mutant lacking 63 amino acids from C-terminal tail and missing tyrosines 1148 and 1173.

These two sites in the EGFR are separated by more than 150 amino acids (Fig. 3). It is an open question how QQ and DEE come close to each other to form a high-affinity Ab P2-binding site. Our studies revealed that the antibody binding is highly dependent on the phosphorylation as a unit on the three tyrosine residues at 992, 1068, and 1086 located between these two peptides. Since phosphorylation-induced modification imparts a very high negative charge to a peptide backbone, it is then possible that the interactions among the negatively charged phosphotyrosine residues in the receptor molecule might result in the bending of the peptide chain surrounding these amino acids in such a way that QQ and DEE come close to each other to form an antibody-binding site (Fig. 3c). If phosphorylation is needed just to bring QQ and DEE close to each other, then a deletion mutant lacking the intervening amino acids should be recognized by the antibody in a phosphorylation-independent fashion. Furthermore, in the P2 peptide, QQ is N-terminus with respect to DEE; however, the orientation is reversed in the EGFR, i.e. QQ is C-terminus. If the proper orientations of the peptides are a prerequisite for antibody binding, then our model predicts that the conformational alteration of the receptor due to charge–charge interaction takes place in a highly ordered fashion. The phosphorylation-induced bending not only brings DEE and QQ closer to each other, but such bending also results in positioning QQ upstream of DEE as in the P2 peptide (Fig. 3d).

Our model also predicts that phosphorylation-induced conformational change should be reversible, i.e., once the phosphate moieties are removed, the receptor should return to its original structure. To test this hypothesis, we investigated the effect of receptor dephosphorylation on the Ab P2-binding activity [2]. For this purpose, the EGFR from ^{35}S-labeled cells was phosphorylated with unlabeled ATP in the presence of EGF, and then the receptor was purified by antiphosphotyrosine antibody followed by wheat germ agglutinin agarose. The highly purified receptor was then incubated with solid-phase alkaline phosphatase. As shown in Table 2, alkaline phosphatase treatment resulted in the complete loss of Ab P2-binding activity of the receptor. The next column shows that alkaline phosphatase treatment indeed resulted in the removal of the phosphate groups from the receptor since the enzyme-treated receptor and not the untreated receptor failed to bind to the antiphosphotyrosine antibody.

TABLE 2. The phosphorylation-induced conformational change of the EGFR is reversible

	Extent of immunoprecipitation with control as 100%	
Receptor type	Ab P2	Anti-phos.tyr. Ab.
Control	100	100
Alkaline phosphatase-treated	15	5

The EGFRs in the detergent-solubilized cell lysates from A431 cells labeled with ^{35}S-methionine and cysteine were phosphorylated with unlabeled ATP in the presence of EGF. Following purification of the EGFRs by antiphosphotyrosine antibody and wheat germ agglutinin, an aliquot of the purified receptor was left untreated (control) or treated with solid-phase alkaline phosphatase in the presence of BSA and the supernatants then subjected to immunoprecipitation in the presence of vanadate with Ab P2 or antiphosphotyrosine antibody. Following electrophoresis, the EGFR band was quantified by densitometric scanning.

AB P2 AS A BIOLOGICAL TOOL

The significance of the phosphorylation-induced conformational change that we have observed on the receptor function and intracellular signaling remains to be elucidated. Because of the close proximity of the kinase domain to the phosphate acceptor sites, it is possible that such a conformational change might influence the kinase activity of the receptor. A number of investigators have compared the kinase activity of the phosphorylated receptor with that of the unphosphorylated receptor. However, EGFR phosphorylation is highly heterogeneous. Our peptide analysis of the ^{32}P-labeled EGFR revealed that out of the total radioactivity present in five peptides, 38% is in Tyr1148, 26% in Tyr1173, 20% in Tyr1068, 10% in 1068, and 6% in Tyr992 [2]. Because of the heterogeneous nature of the phosphorylated EGFR, it is then highly likely that the kinase activity in these studies reflects the activity of the mixed population of receptors. Since Tyr1086 and 992 are not efficiently phosphorylated in the EGFR, the effect of such phosphorylation on kinase activity could not be determined in the mixed receptor population. Thus, by using Ab P2, which interacts only with the receptor population that is phosphorylated as a unit on all three tyrosines at 992, 1068, and 1086, we will be able to isolate such a receptor population and study its kinase activity.

A number of antipeptide antibodies that specifically recognize the activated receptor have been reported; however, all these antibodies are directed to phosphotyrosine-containing peptides [22–24]. In this respect, Ab P2 is one of the two conformation-specific antireceptor antibodies directed to an unphosphorylated peptide that recognizes the activated receptor. Because of the high-affinity interaction of Ab P2 with the EGFR phosphorylated on certain tyrosine residues, this antibody can also be used as a biological tool in studying the structure–function relationship of receptors and also for screening different EGFR mutants in which phosphorylation is affected. Furthermore, since this antibody recognizes the EGFR phosphorylated on specific tyrosine residues, Ab P2 has the potential as a diagnostic tool in detecting activated EGFRs in human tumor biopsies.

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