# The EGF Receptor Transmembrane Domain: $^2$H NMR Study of Peptide Phosphorylation Effects in a Bilayer Environment†

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ABSTRACT: Phosphorylation events are considered to be key control points in receptor tyrosine kinase function. We have used wide-line $^2$H NMR spectroscopy to look for physical effects of phosphorylating a threonine residue within the cytoplasmic domain of the human EGF receptor, as sensed at a distant site in the transmembrane portion. Modifications were made to $Thr_{654}$ (a cytoplasmic residue suggested to be involved in regulation of EGF binding and of cytoplasmic domain function), and effects were sought at $Ala_{623}$ (near the extracellular membrane surface but within the membrane-spanning region). The study was carried out on synthetic peptides corresponding to the EGF receptor transmembrane domain plus 10 or 11 residues of the cytoplasmic domain, assembled into lipid bilayer membranes. Three peptides were compared that differed only at $Thr_{654}$. This residue was alternately: nonphosphorylated but left as a (−)-charged C-terminus (-$Thr_{654}COO^-$), nonphosphorylated and with a neighboring amidated glycine residue as the C-terminus (-$Thr_{654}GlyCONH_2$), or phosphorylated and with a neighboring amidated glycine residue as the C-terminus (-$Thr_{654}PO_4^-GlyCONH_2$). Bilayer membranes were composed of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) or 2:1 POPC/cholesterol, containing 6 mol % peptide relative to phospholipid. The deuterated site, $Ala_{623}$, was intrinsically conformationally sensitive; yet spatial orientation and motional order of the probe location were found not to be obviously influenced by phosphorylation.

The epidermal growth factor receptor is an important Class I receptor tyrosine kinase (RTK),[1] and is seen as a prototypical example of a membrane protein which can transduce signals when stimulated by specific cell surface contact events (*1*−*5*). It is a 170 kDa species possessing an external glycosylated portion responsible for the earliest events in recognition, a single-α-helix transmembrane domain, and an intracellular portion exhibiting protein kinase activity as well as phosphorylation and docking sites (*6, 7*). Insight as to the submolecular details of signal transduction by such species has generally been inferred from indirect experiments because of the technical difficulties of working with membrane proteins. Wide-line $^2$H NMR spectroscopy offers the possibility of direct measurement since it uses nonperturbing nuclear probes to provide information on motional behavior, interactions, and conformation at selected locations in molecules even when they are minor constituents of multicomponent bilayer membrane assemblies (*8*−*10*). In the present work, we addressed the issue of what physical signal might pass between a site of phosphorylation and a topographically distinct site within a transmembrane receptor. This was done using NMR techniques developed on model peptides and prokaryotic systems by other workers (reviewed in *11*−*18*).

Suggested mechanisms for transmembrane signaling by ligand-regulated tyrosine kinases tend to focus on homo- and heterodimer/oligomer relationships, and on conformational changes (*4, 5, 19*−*25*). The possibility of contributions from altered dynamics has been noted (*26*−*28*). Phosphorylation of the receptor cytoplasmic domain appears to be important: in the case of the EGF receptor, tyrosine phosphorylation occurs as a very early stimulatory event, and phosphorylation at threonine 654 is a later (negative-regulatory) step. It is the latter that we have examined in the present work. $Thr_{654}$, located 10 residues from the putative cytoplasmic membrane surface, is thought to be phosphorylated by protein kinase C (*6, 29, 30*). This event has been suggested to exert control over the activated EGF receptor by contributing to regulation of the tyrosine kinase site and of the EGF binding site. Thus, phosphorylation at $Thr_{654}$ has been considered to exert physical effects in both directions within the receptor. However, straightforward interpretations have been called into question (*31, 32*).

Lin et al. (*33*) have pointed out that, while protein phosphorylation is a central mechanism in cell regulation, the structural basis of phosphorylation-initiated functional modification of proteins has been demonstrated only for glycogen phosphorylase and isocitrate dehydrogenase, two soluble enzymes. Effects of phosphorylation in general have been considered to arise from the resultant charge, and from phosphate-specific effects. We previously reported $^2$H NMR spectra of a nonphosphorylated peptide containing the transmembrane domain of the human EGF receptor, probe-labeled (as here) at $Ala_{623}$ (*26, 28*). It appeared that $Ala_{623}$, near the amino terminus of the peptide, was a site of significant conformational sensitivity. Thus, this peptide from a receptor in which the juxtamembrane region is seen

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[1] Abbreviations: EGF, epidermal growth factor; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; TFE, trifluoroethanol; [$d_4$]$Ala_{623}$, deuterated amino acid corresponding to the indicated position in the human EGF receptor; RTK, receptor tyrosine kinase.

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as a "prime mediator of heterologous modulation of receptor activities" (*6*) seems to lend itself to examination of signaling mechanisms in a membrane environment. For the present work, three transmembrane peptides were prepared by solid-phase synthesis. The sequences included the putative transmembrane region (residues 622−644) plus a 10 or 11 residue stretch of the cytoplasmic domain. The first peptide was left with a free C-terminal nonphosphorylated threonine residue (-$Thr_{654}COO^-$), the second was also nonphosphorylated but possessed a neighboring amidated glycine residue as C-terminus (i.e., was uncharged) (-$Thr_{654}GlyCONH_2$), and the third was phosphorylated at $Thr_{654}$ in addition to having an amidated glycine residue as C-terminus (-$Thr_{654}PO_4^-$-$GlyCONH_2$). Deuterated alanine was inserted at the position corresponding to $Ala_{623}$ within the putative transmembrane region to permit examination of the transmitted effects of (−) charge and of phosphorylation.

## MATERIALS AND METHODS

1-Palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC) was obtained from Avanti Polar Lipids, Birmingham, AL, and was used without further purification. Cholesterol was from Sigma, St. Louis, MO. Peptides were synthesized by Chiron Technologies, Raleigh, NC, following the protocol described previously for the peptide with free carboxy terminus, and had a purity of >85−95% (*26*). The phosphorylated peptide was associated with significant difficulties of synthesis and purification.

Samples were prepared as unsonicated liposomes via the following general protocol. TFE (2,2,2-trifluoroethanol, 4 mL, Aldrich, NMR grade, bp 77−80 °C) was added to dry peptide (up to 10 mg), and appropriate amounts of dry lipid were then weighed in and dissolved with warming to 55 °C. Samples were allowed to sit at this temperature for at least 30 min after visually apparent complete dissolution. Solvent was rapidly removed under reduced pressure at 45 °C on a rotary evaporator to leave thin films in 50 mL round-bottom flasks. These were subsequently held for 18 h at 23 °C under high vacuum. Hydration was with 30 mM HEPES containing 20 mM NaCl and 5 mM EDTA, pH 7.1−7.3, made up in deuterium-depleted water. Samples were warmed to 35 °C without vortexing during hydration to minimize production of small vesicles.

$^2$H NMR spectra were acquired at 76.7 MHz on a Varian Unity 500 spectrometer using a single-tuned Doty 5 mm solenoid probe, with temperature regulation to ±0.1 °C. A quadrupolar echo sequence ("SSECHO" from the Varian pulse library) was employed with full phase cycling and $\pi/2$ pulse length of 5−6 $\mu$s. Pulse spacing was 20−30 $\mu$s. A recycle time of 100 ms was chosen to optimize the final S/N ratio while avoiding spectral distortion and saturation after experimentation with longer and shorter values. Spectral sweep width was 100 kHz.

## RESULTS AND DISCUSSION

Amino acid sequences for the 34-mer and 35-mers studied are shown in Figure 1. In each case, they comprise the putative transmembrane domain of the EGF receptor plus 10 or 11 residues of the cytoplasmic domain. The suggested transmembrane portion calculated using the method of Rost and colleagues (*34*) has been underlined.

*K|**A**$_{623}$TGMVGALLLLLVVALGIGLFMRRRHIVRKR**T**$_{654}$-$COO^-$

*K|**A**$_{623}$TGMVGALLLLLVVALGIGLFMRRRHIVRKR**T**$_{654}$G-$CONH_2$

*K|**A**$_{623}$TGMVGALLLLLVVALGIGLFMRRRHIVRKR**T**$_{654}$G-$CONH_2$
|
$PO_4^-$

FIGURE 1: Amino acid sequences of the peptides studied. The perdeuterated alanine residue corresponding to alanine at position 623 of the human EGF receptor is indicated in boldface, as is the phosphorylation site, threonine at position 654. The putative transmembrane domain has been underlined. In each case, the cytoplasmic domain (carboxy terminus) is on the right. Note that both the uppermost and lowermost peptides carry negative charges at $Thr_{654}$, while this site is uncharged in the middle peptide. A biotinylated lysine residue (*) replaced the extracellular $Ser_{621}$.

Elongated amphiphiles dispersed in fluid membranes tend to undergo rapid symmetric rotation about axes perpendicular to the bilayer. For such molecules containing deuterium nuclei, eq 1 is useful in relating the NMR spectral splitting ($\Delta\nu_Q$) for a given deuterium nucleus to the orientation and motional characteristics of the C−D bond.

$$\Delta\nu_Q = (3/8)(e^2Qq/h)S_{mol}|3\cos^2\Theta_i - 1| \qquad (1)$$

$e^2Qq/h$ is the nuclear quadrupole coupling constant (165−170 kHz for a C−D bond), $S_{mol}$ is the molecular order parameter (assuming axially symmetric order) describing orientational fluctuations of the C−D bond relative to the bilayer normal, and $\Theta_i$ is the average orientation of each C−D bond relative to the bilayer normal (*8*−*10*). For deuterated methyl groups, which undergo rapid rotation about the attaching bond axis even at temperatures well below 0 °C, it is convenient to consider a 'resultant' C−D vector directed along the C−$CD_3$ bond. This can be dealt with in eq 1 by considering $\Theta_i$ to be the angle between the C−$CD_3$ vector and the molecular long axis, and introducing an additional factor of 1/3. Dominant nonaxially symmetric rotation about an axis [e.g., all 2-fold rotational jumps, and 3-fold (or higher) jumps with unequal population weighting of available conformers] can cause a shift in intensity toward the spectral center, leading to significant obscurement of the Pake splitting, although quite commonly intensity persists at frequencies corresponding to those predicted for symmetric motions about the same axis (*11*, *35*−*40*).

Spectra of the three EGF receptor peptides deuterated at $Ala_{623}$ are shown in Figure 2 for membranes of POPC. The sharp peak in the middle of each spectrum could be separately resolved on an expanded frequency axis into two components (not shown here). One of these, offset about 0.3 kHz downfield from the powder spectrum midpoint, represents residual HOD; the other, about which the powder pattern is symmetric, reflects a transmitter spike and the presence of some vesicles with high curvature for which the quadrupole splittings are motionally averaged to zero. These common features of $^2$H NMR powder spectra of amphiphiles in liposomes will not be further considered. The primary spectral peaks of interest are readily interpreted in terms of features associated with the $CD_3$ group. Equation 1 dictates that a $CD_3$ group on an immobilized peptide backbone should

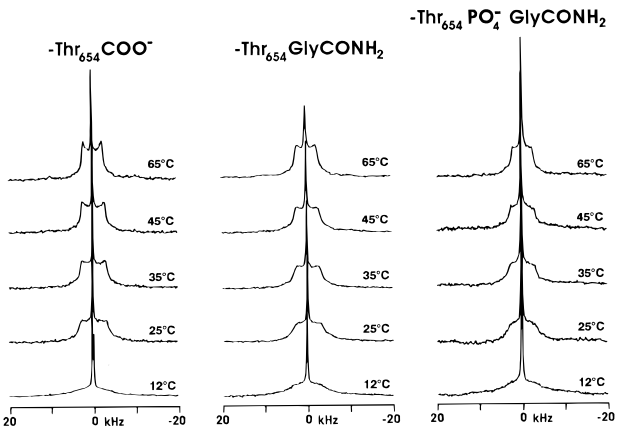


FIGURE 2: $^2$H NMR spectra corresponding to EGF receptor transmembrane peptides in POPC bilayers. Peptide/phospholipid mole ratio was 6:100. Spectra were recorded from high to low temperature without repositioning a given sample within the probe. The number of accumulated transients represented by each spectrum ranged from 200 000 to 1 200 000—in several cases, spectral intensity has been adjusted to account for differences in the number of transients within a given column.

give rise to a single Pake doublet of about 40 kHz splitting. The (deutero)methyl group of alanine 623 is firmly fixed to the peptide backbone. Thus, the only motions accessible to it are rapid rotation about the $C_\alpha-C_\beta D_3$ bond (a motion which is present unchanged in all of our experiments), rotational diffusion of the entire peptide about the membrane perpendicular, 'wobble' of the peptide long axis, and conformational instability of the peptide backbone. As a result of the $S_{mol}$ term in eq 1, 'wobble' of the entire peptide within the membrane and finite rapid conformational fluctuations of the peptide backbone will contribute to reduction of the $CD_3$ splitting to a value less than the 40 kHz maximum. Rotational diffusion of the transmembrane helix axis is the other modifier of spectral splitting (via the term containing $\Theta_i$). The values seen for $\Delta\nu_Q$ in POPC liposomes ranged about 5 to 6 kHz, demonstrating the existence of a significantly preserved average conformation. The $C_\alpha$ deuteron of alanine was expected to make only minor contributions to spectral intensity due to its low relative numbers and to unfavorable relaxation effects (*41*), as seen by us previously for related peptides (*26, 28*). Spectral peaks which seem likely to be associated with the α-deuteron were only observed for the most fluid membranes (POPC at 65 °C): a very low intensity outer doublet of 21−23 kHz splitting.

For each peptide, the spectral splittings have been measured and are listed in Table 1. They are remarkably similar among the three peptides under a given set of conditions. Theoretically this similarity could result from a fortuitous combination of alteration in one parameter (e.g., spatial orientation), with compensatory alteration in another (e.g., backbone 'wobble') such that the two exactly offset one another and the spectral splitting remains unchanged; however, this seems highly improbable, particularly given the persistence of the observation as a function of temperature.

The symmetry of the overall rotational diffusion of the peptide in the membrane is reflected in spectral shape as mentioned in association with eq 1. In particular, loss of 'smooth' rotation (as might occur with peptide aggregation) can lead to 'infilling' of the Pake doublet and loss of the sharp Pake spectral edges. Given the presence of more impurities in the case of the phosphorylated peptide (arising from difficulties in purification), it would be inappropriate to place undue emphasis on subtle differences in line shape; however, it should be noted that the sharpness and near-vertical nature of the Pake doublet spectral edges are similar as a function of temperature in all three peptides examined. Slowed or axially asymmetric rotational diffusion of the peptide within the bilayer can cause these outer edges to slope inward (making the spectrum more 'triangular' as seen at 12 °C). We have suggested that this obscurement of the Pake splitting by central intensity shift reflects enthalpy-driven reversible homodimer/oligomer formation of the transmembrane peptide, with resultant slowing and asymmetry of motion (*28*, see also *42*). In keeping with the concept that some peptide−peptide interaction is occurring leading to peptide immobilization, there was some spectral intensity loss (10−45%) at 12 °C in spite of the fact that the host matrix is fluid above −3 °C (*43*). Overall, temperature effects on the spectra are very similar within the group studied.

Table 1: $^2$H NMR Spectral Splittings ($\Delta\nu_Q$) Corresponding to $CD_3$ Groups in [$d_4$]Ala$_{623}$[a]

| | | spectral splittings ($\Delta\nu_Q$) ±0.1 to ±0.3 kHz[b] at temp (°C) | | | | | |
|---|---|---|---|---|---|---|---|
| deuterated peptide | lipid | 12 | 25 | 35 | 45 | 55 | 65 |
| -Thr$_{654}$COO$^-$ | POPC | *c* | 6.4 | 6.0 | 5.6 | 5.1 | 4.7 (22.75) |
| | POPC/chol | *c* | 9.4 | 8.8 | 8.8 | – | 8.3 |
| -Thr$_{654}$GlyCONH$_2$ | POPC | *c* | 6.5 | 5.85 | 5.7 | – | 5.0 (22.8) |
| | POPC/chol | *c* | 9.0 | 8.2 | 8.3 | | 8.2 |
| -Thr$_{654}$PO$_4^-$GlyCONH$_2$ | POPC | *c* | 6.5 | 6.0 | 5.7 | – | 4.85 (22.4) |
| | POPC/chol | *c* | 8.7 | 8.6 | 8.1 | | 8.1 |

[a] In each case, the peptide was assembled into bilayers of POPC, or POPC containing 33 mol % cholesterol. All samples were prepared by hydration of films dried down from TFE solution, studied as a function of decreasing temperature. Each peptide had [$d_4$]Ala at the position corresponding to Ala$_{623}$ of the human EGF receptor (Figure 1). Values in parentheses are for the presumed α-deuteron ($C^\alpha$-D) on [$d_4$]Ala$_{623}$: these peaks were only measurable in the most fluid membranes (POPC at 65 °C). [b] The quoted estimated uncertainty reflects typical maximum variability found for samples prepared on different occasions and interobserver variability. Estimated experimental error was greater (±0.3 kHz) in the amidated and phosphorylated peptides particularly at 25 and 35 °C. [c] At 12 °C, spectra of [$d_4$]Ala$_{623}$ samples had become sufficiently characteristic of axially asymmetric motion that 90° edges were not readily identifiable, and there was some intensity loss.

Representative spectra of the three peptides are shown in Figure 3 for POPC bilayers containing a cholesterol concentration that might be seen as typical of cell plasma membranes. Spectral splittings are listed in Table 1. As was the case in the cholesterol-free membranes, the spectra of the three peptides are remarkable primarily for their similarity under a given set of conditions. Also as with the peptides in POPC alone, the spectra are Pake doublet in nature, reflecting rapid peptide rotational diffusion within the bilayer. Only at low temperature do the spectra develop clear features that are associated with axially asymmetric motion (described above with regard to eq 1 and for the membranes without cholesterol). The spectral splittings in the membranes containing cholesterol range from 8 to 9 kHz over the temperature range studied. Once again, the temperature effects on spectral shape are remarkably similar among the three peptides, extending even to the pattern of intensity loss which was some 65−80% at 12 °C.

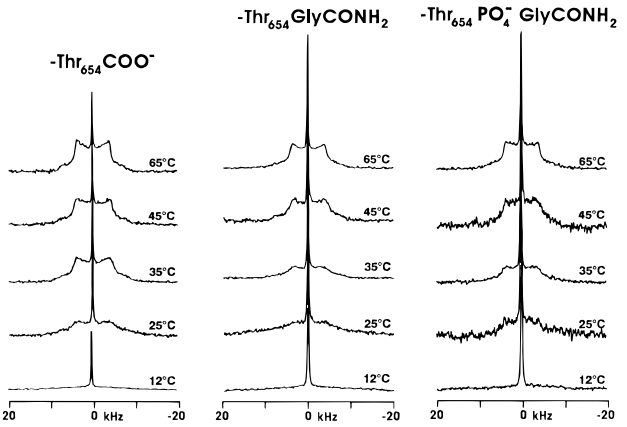


FIGURE 3: $^2$H NMR spectra corresponding to EGF receptor transmembrane peptides in 2:1 (mole ratio) POPC/cholesterol bilayers. Peptide/phospholipid mole ratio was 6:100. Spectra were recorded from high to low temperature without repositioning a given sample within the probe. The number of accumulated transients represented by each spectrum ranged from 200 000 to 1 200 000; in several cases, spectral intensity has been adjusted to account for differences in the number of transients within a given column.

The question addressed in the current experiments is whether some measurable physical signal is transmitted by phosphorylation or charge alteration at $Thr_{654}$ (just external to the 'cytoplasmic' surface), as sensed by a site near the opposite side of the membrane. The basic answer is that we observed little evidence of physical change. An examination of eq 1 shows that the technique itself has high potential for sensitivity to orientation of the probed site (via quadrupole splitting changes), an orientation change of as little as a few degrees having the potential to alter splitting by 1 kHz if $S_{mol}$ approaches 1 as expected (*44*, *45*). Moreover, local motional properties of $Ala_{623}$ and motions of the peptide as a whole should be sensitively reflected in spectral splitting and line shape.

We noted previously for the -$Thr_{654}COO^-$ form of the peptide that the spectral splitting associated with the $CD_3$ group of $Ala_{623}$ is about 6 kHz at 35 °C for 6 mol % peptide in a POPC membrane but rises upon addition of cholesterol to 9 kHz (*26*, *28*). $Ala_{623}$ is the third residue in the peptide sequence, and it is known that there can be a tendency to fraying/unraveling of the ends of transmembrane helical peptides (*46*−*48*). High-resolution $^1$H NMR studies of the -$Thr_{654}COO^-$ form of the peptide in the lipomimetic solvent trifluoroethanol demonstrated the relatively less structured nature of this portion of the peptide and the α-helical nature of the immediately downstream region beginning at $Met_{626}$ (*26*). Hence, we have suggested that the cholesterol effect noted above is associated with adoption of a more stable α-helical array at the 'unravelled' amino terminus in the more ordered (though still fluid) cholesterol-containing membrane. This now seems very likely to be the case since in more recent experiments with the same peptide, in which deuteration was at both $Ala_{623}$ and $Ala_{637}$ (the latter in an almost certain region of stable α-helix; see Figure 1 and *46*, *48*, *49*), we have found that prior to cholesterol addition the $CD_3$ spectral splitting of $Ala_{623}$ is about half the $Ala_{637}$ splitting while with addition of cholesterol to the membrane the $Ala_{623}$ splitting increases to become equal to that of $Ala_{637}$ (9 kHz) (unpublished observation). Thus, the probed site is neither conformationally locked and unable to change, nor so unstructured as to be insensitive to local conditions.

Phosphorylation of protein threonine/serine residues is a major mechanism of cell regulation and represents the great majority of protein phosphorylation events (*50*, *51*). It has been well documented that phosphorylation of $Thr_{654}$ in the human EGF receptor is associated with regulatory control over some aspects of EGF signaling pathways. Thus, phosphorylation of this residue by PKC has been implicated in reduction of both tyrosine kinase activity and high-affinity EGF binding (reviewed in *6*, *29*, *30*). However, recent experiments have brought into question the mechanistic connection between $Thr_{654}$ phosphorylation and tyrosine kinase activity, and have not demonstrated effects on EGF binding (*31*, *32*, and references cited therein). Thus, while the latter studies conclude that $Thr_{654}$ is important for tyrosine kinase regulation, the mechanism is unclear. We were curious therefore to test for effects on conformation and behavior of the transmembrane domain as measured *directly* in a dissected membrane system that has potential for conformational and dynamic sensitivity. No physical effect of phosphorylation was noted. It is important to recognize that the probe location was geographically distinct from the site of phosphorylation/charge alteration: $Ala_{623}$ is close to the 'extracellular' membrane surface within the hydrophobic interior, while $Thr_{654}$ is outside the membrane and some 10 residues removed from the 'cytoplasmic' surface. Thus, our results say little about the local conformation of the cytoplasmic domain—for instance, there could have been a local effect on the conformation or dynamics of the C-terminus brought about by interaction of the phosphate group or charged C-terminus with one of the five basic arginines on the same (or a neighboring) peptide (*52*). However, if so, there was apparently no major resultant alteration in overall peptide orientation or motion since this would have shown up in the spectral line shape of $Ala_{623}$.

In glycogen phosphorylase (GP) and isocitrate dehydrogenase (ID), where structural effects of phosphorylation have been demonstrated by X-ray crystallography, a key effect of threonine phosphorylation was seen to be altered physical proximity and relative orientation of interacting molecules (reviewed in *33*). Thus, in mammalian GP, serine phosphorylation causes ionic interaction-mediated shift of the monomeric subunits relative to one another in the homodimer, with resultant modification of the dimer catalytic site. In ID, the phosphate physically and electrostatically blocks binding of the substrate molecule. In yeast GP, it is proposed that threonine phosphorylation causes a local folding change in a hydrophobic site which in turn changes the relative orientation of the monomeric subunits that make up the catalytic dimer interface. Hence, one might postulate that the primary event induced by $Thr_{654}$ phosphorylation in the EGF receptor is not so much direct induction of a major conformational change but rather alteration of a binding site on its cytoplasmic domain. Certainly it is claimed that the juxtamembrane region of the EGF receptor interacts with a number of proteins important for signaling (e.g., *53*, *54*). Within a crowded environment at a membrane surface, forcing sites on two relatively stiff macromolecules close together might well be expected to have a major 'transmitted' effect on the orientation and accessibility of their distant ends, without any major conformational alteration in either participant. This concept is essentially a variant of the "scissors model" of tyrosine kinase activation [that hormone binding

to the extracellular ends of a receptor dimer could cause a scissoring motion about the transmembrane fulcrum, which would be directly transmitted to the catalytic intracellular domains and alter their reactive potential without conformational change of the individual proteins (*22*)]. Our observation would clearly be consistent with signaling mechanisms that invoke relatively 'stiff' receptors and in which subunit orientation relative to a neighbor is regulated in distance or time. The design of the current experiment did not permit assessment of whether a conformational signal might pass in the *opposite* direction (downstream of $Thr_{654}$): it would be interesting to make related measurements with probes on the other (i.e., cytoplasmic) side of the phosphorylation site in longer versions of the peptides studied here.

## CONCLUSIONS

The mechanistic role of protein phosphorylation in biological systems has been suggested to range from simple charge effects and salt bridge formation, to conformational changes and recognition site determination. In the present experiments with the EGF receptor transmembrane domain, no direct alterations were detected in conformation/orientation, local dynamics, or peptide self-association after phosphorylation or simple charge alteration of a threonine residue. The probe site was near the 'extracellular' membrane surface, within the hydrophobic domain, while the site of phosphorylation was within the 'cytoplasmic' domain 10 residues from the membrane surface. The observation of relative conformational insensitivity at one site in a receptor, to a physical change at a distant site, is reminiscent of our previous observations surrounding glycosphingolipids as receptors (*55* and references cited therein) and those of Gullick et al. surrounding the transmembrane domain of Neu/erbB-2 (*56*)(see also *26*).

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