Ca\textsuperscript{2+}/Calmodulin-dependent Kinase II Phosphorylates the Epidermal Growth Factor Receptor on Multiple Sites in the Cytoplasmic Tail and Serine 744 within the Kinase Domain to Regulate Signal Generation*

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Down-regulation of receptor tyrosine kinase activity plays an essential role in coordinating and controlling cellular growth/differentiation. Ca\textsuperscript{2+}/calmodulin-dependent kinase II (CaM kinase II) phosphor-ylation of threonine 1172 in the cytoplasmic tail of HER2/c-erbB2 can modulate tyrosine kinase activity and consensus phosphorylation sites are also found at serines 1046/1047 in the structurally related epidermal growth factor receptor (EGFR). We show that serines 1046/1047 are sites for CaM kinase II phosphorylation, although there is a preference for serine 1047, which resides within the consensus -R-X-X-S-. In addition, we have identified major phosphorylation sites at serine 1142 and serine 1057, which lie within a novel -S-X-D-consensus. Mutation of serines 1046/1047 in full-length EGFR enhanced both fibroblast transformation and tyrosine autokinase activity that was significantly potentiated by additional mutation of serines 1057 and 1142. A single CaM kinase II site was also identified at serine 744 within sub-kinase domain III, and autokinase activity was significantly affected by mutation of this serine to an aspartic acid making this site appear constitutively phosphorylated. We have addressed the mechanism by which CaM kinase II phosphorylation of the EGFR might regulate receptor autokinase activity and show that this modification can hinder association of the cytoplasmic tail with the kinase domain to prevent an enzyme-substrate interaction. We postulate that the location and greater number of CaM kinase II phosphorylation sites in the EGFR compared with HER2/c-erbB2, leading to differential regulation of autokinase activity, contributes to differences in the strength of downstream signaling events and may explain the higher relative transforming potential of HER2/c-erbB2.

The human epidermal growth factor receptor (EGFR) family comprise four members, EGFR/HER1, erbB2/HER2, erbB3/HER3, and erbB4/HER4, which transduce distinct signals for proliferation and differentiation (1). EGFR signaling is initiated by ligand binding, receptor dimerization, and autopho-
We have previously mapped a novel CaM kinase II phosphorylation site to threonine 1172 in erbB2/HER2 and shown that this site can contribute to the regulation of the RTK activity (20). Here, we have extended the same experimental approach to the EGFR and have identified new CaM kinase II sites in the cytoplasmic tail, which lie within a consensuS -S-D- phosphorylation sequence. Parallel analyses of site-specific mutant receptors have revealed pronounced differences in the potential to transform mouse NIH3T3 fibroblasts and to up-regulate tyrosine autokinase activity. We identify serine 1142 (-S-L-D-) as a preferred site compared with serines 1046/1047, and phosphorylation of these sites together with serine 1057 (-S-V-D-) in the kinase domain provides a clearer explanation of the regulation of EGFR signaling by CaM kinase II. Furthermore, we present evidence that the mechanism for controlling receptor tyrosine autophosphorylation may involve inhibition of cytoplasmic tail interactions with the EGFR kinase domain, thereby preventing an enzyme-substrate interaction.

EXPERIMENTAL PROCEDURES

Construction of EGFR Mutants—Site-directed mutagenesis of the human EGFR cDNA cloned into the pCMV-1 expression plasmid was performed as described previously (21). Mutations were confirmed by dyeoxy sequencing and restriction enzyme analysis. Other mutants used in this study have been described previously; HER-1/2-T1172A (20) and HERAC-T (21).

Preparation and Phosphorylation of GST Fusion Proteins—Amino acid residues tyrosine 974 to alanine 1186 of the human EGFR receptor (HERWT, 212 amino acids; HER15, 197 amino acids), serine 1013 to proline 1084 of the chicken EGF receptor (CER, 72 amino acids), and serine 1013 to proline 1084 (v-erb-B, ES4, 50 amino acids) were amplified from appropriate cDNAs by polymerase chain reactions (numbering of receptor residues is based on equivalent residues in the human EGFR as in Ref. 22). Smaller EGFR GST fusion proteins were prepared in the same way to generate the following sequences; GST-1, threonine 1022 to tyrosine 1045; GST-2, phenylalanine 1041 to aspartic acid 1056; GST-3, aspartic acid 1048 to proline 1095; GST-4, phenylalanine 1062 to histidine 1105; GST-5, proline 1130 to glycine 1165; GST-III, alanine 731 to leucine 758. Amplified fragments were subcloned into the bacterial expression vector pQE-2T (Amersham Pharmacia Biotech) and GST fusion proteins isolated as described previously (20). Rat CaM kinase II overexpressed in HEK-293 cells was purified by calmodulin-Sepharose affinity chromatography and used for in vitro phosphorylation of purified GST proteins as described (20).

Generation of Recombinant EGFR Retrovirus and Focus Formation Assays—EGFR mutants were subcloned into the retroviral pLNC vector (23). Constructs were transfected into the helper virus-free packaging cell line H9S09 (ATCC) by calcium phosphate precipitation (24). After 2 days, ecotropic virus-containing supernatants were each transfected with 100 μg of pCMV-1-HERAC-T (21). Purified HER-WT was efficiently phosphorylated by CaM kinase II at 4°C (30 min) to ensure complete and efficient phosphorylation of receptors. Immune complexes were washed three times in wash buffer and resuspended in 100 μl of wash buffer containing 5 mM MnCl₂ and 5 mM MgCl₂. Phosphorylation was initiated by addition of 5 μCi of [γ-³²P]ATP and after 5 min at room temperature terminated by addition of EGTA to a final concentration of 10 mM. Immunoprecipitates were washed once with wash buffer containing 10 μg EGTA and resuspended in 100 μl of SDS-sample buffer. Samples were separated by SDS-7.5% PAGE, and gels were dried and exposed to x-ray film. Radioactive spots corresponding with the migration of the full-length receptor were excised and quantitated by scintillation counting. To examine receptor autophosphorylation by Western blotting, cells were transfected as above, and immunoprecipitated receptors were separated by 7.5% SDS-PAGE and transferred onto nitrocellulose. Filters were incubated with anti-phosphotyrosine monoclonal antibody (4G10; Upstate Biotechnology) or anti-EGFR monoclonal antibody (Sigma; catalog number E3138), and immunoreactive proteins were visualized using the ECL system (Amersham Pharmacia Biotech).

Association of GST Fusion Proteins with Wheat Germ Agglutinin-purified HERAC-T—Four plates of HEK-293 cells (1 × 10⁶ cells/10-cm dish) were each transfected with 100 μg of pCMV1-HERAC-T. Lysates were incubated by rotation with 50-μl packed volume of wheat germ agglutinin-Sepharose beads (1 h; 4°C). GST-HER-WT (amino acids 974–1186) was either left unphosphorylated or phosphorylated (1 h; 4°C) with 0.2 μg of CaM kinase II in the presence of 100 μM ATP under the same conditions described above for GST fusion protein phosphorylation. After washing wheat germ agglutinin-Sepharose-bound protein six times with wash buffer, pre- or nonphosphorylated GST-HER-WT reactions were added to beads and incubated by rotation (1 h; 4°C) followed by a further six washes with wash buffer. Beads were resuspended in SDS-sample buffer and bound protein-separated SDS-10% PAGE followed by staining with Coomassie Blue or immunoblotting against an anti-phosphotyrosine monoclonal antibody (Sigma; catalog number E3138). Immunoreactive proteins were visualized using the ECL system (Amersham Pharmacia Biotech).

RESULTS

Phosphorylation of EGFR-GST Fusion Proteins with CaM Kinase II—To test whether the EGFR cytoplasmic tail is a substrate for CaM kinase II, we constructed a pGEX plasmid in which the DNA coding sequence representing amino acids 974–1186 (HER-WT) was fused to the DNA encoding GST. Purified HER-WT was efficiently phosphorylated by CaM kinase II in vitro in contrast to purified GST alone (Fig. 1a). HER-WT contains a region corresponding with a synthetic peptide (RRFLQRyySSDTPGAL; EGFR1041–1053), which has been shown previously to be a substrate for CaM kinase II and has an optimal consensus -R-X-X-S/T- phosphorylation sequence at serine 1047 (18). Serines 1046/1047 within this region are also major in vivo sites of phosphorylation in the EGFR (25).
We therefore prepared a GST fusion protein that has a deletion corresponding with residues 1039–1053 (HER-del- 

ta15), and *in vitro* phosphorylation of this protein with CaM kinase II was only partially reduced relative to HER-WT, indi-
cating that there are additional major sites of phosphoryla-
tion in the EGFR cytoplasmic tail (Fig. 1a). Shorter fusion proteins were also examined using the same approach; how-
ever, in this instance peptide sequences were generated from the highly homologous CER and also the structurally related v-erbBES4 oncogene. The v-erbBES4 oncoprotein is derived from the CER, and as well as having a large deletion of the ligand binding domain, also has several cytoplasmic domain mutations, including a deletion of residues equivalent to 1040–
1060 in the human EGFR (26).

The short 72-amino acid CER protein comprises residues serine 1016 to glutamine 1087, and this can also be efficiently phosphorylated *in vitro* by CaM kinase II in contrast to the short 50-amino acid ES4 fusion protein that underwent only background phosphorylation (Fig. 1a). These data taken to-
gether therefore suggest that the cytoplasmic tail of the EGFR is an excellent substrate for CaM kinase II in a region that includes serines 1046/1047 as well as at other unidentified sites. Phosphoamino acid analysis of HER-WT after CaM ki-

nase II phosphorylation also indicated that these sites were only at serine residues (Fig. 1b).

**Mapping of Novel CaM Kinase II Sites in the EGFR Cyto-

plasmic Tail—Serine 1047 resides within the traditional -R-X-

-X-S(T)- for CaM kinase II phosphorylation; however, scanning of residues 974–1186 in the EGFR reveals no other consensus sites. Several other physiological substrates for CaM kinase II have been described that do not have recognizable consensus sites (27). For example, the intermediate filament protein vimen-
tin has a phosphorylation site in which the critical deter-

minant of site specificity is an acidic residue in the second position on the C-terminal side of the phosphorylation site (28). Analysis of vimentin and other peptide substrates have sug-
ggested that the sequence -S-X-D- may serve as a novel recog-
nition site for phosphorylation by CaM kinase II with the possibility of additional preference for a hydrophobic residue at position X (27, 28). Support for this observation comes from the identification of a CaM kinase II site at serine 142 of the cAMP response element-binding protein, which negatively regulates transcriptional activity and fits this alternative consensus (29).

As a starting point to mapping the other sites in HER-WT, we examined residues 974–1186 for -S-X-D- sequences. Pros-
pective sites were found at serine 1057 (-S-I-D-), serine 1096 (-S-R-D-), and serine 1142 (-S-T-D-). Previously reported sites at serine 1046 and serine 1047 lie within -S-X-D- and -R-X-S-

consensus sites, respectively. In addition, serine 1040 (-K-E-D-S-)

resides within the -R-X-S- consensus but has a basic lysine instead of arginine at the third position C-terminal to the serine, and it has been reported that a limited number of substrates have this site preference for CaM kinase II (27). In an effort to identify any of the potentially new sites in the EGFR as targets for CaM kinase II, EGFR-GST fusion proteins were prepared that spanned these serines, which were also individually mutated to alanine (Fig. 2a). *In vitro* phosphorylation identified the major site of CaM kinase phosphorylation at serine 1142 and confirmed that serines 1046/1047 can also be phosphorylated, albeit to a much lesser extent, with a clear preference for serine 1047 (Fig. 2b). Serine 1057 also under-
grew a significant degree of phosphorylation comparable with serine 1047; however, serine 1096 and serine 1040 were very poorly phosphorylated (Fig. 2b). Quantitation of *in vitro* phos-

phorylation reactions indicated that serine 1142 incorporated about 5-fold more phosphate than serines 1046/1047 and about 10-fold more than serine 1057 (data not shown). The consensus sequence and location of individual phosphorylation sites in the cytoplasmic tail of the EGFR are depicted in Fig. 2c.

**Potentiation of EGFR Transforming Activity by Mutation of CaM Kinase II Phosphorylation Sites Reveals Their In Vivo Functional Significance—**Previously we have mapped a single CaM kinase II site in HER2/c-erbB2 and by two-dimensional mapping showed that this site could be hyperphosphorylated in *in vivo* (27). Support for this conclusion comes from the identification of a CaM kinase II site in HER2/c-erbB2 and by two-dimensional mapping showed that this site could be hyperphosphorylated in *in vivo* (27). Support for this observation comes from the identification of a CaM kinase II site at serine 142 of the cAMP response element-binding protein, which negatively regulates transcriptional activity and fits this alternative consensus (29).

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We have therefore assessed the *in vivo* biological conse-
quence of serine phosphorylation site-specific mutation by compar-
ing the ability of EGFR mutants to induce transformed foci in 3T3 fibroblasts. In this regard, mutation of serines 1046/1047 to alanine in the EGFR increases both signaling strength and oncogenic potential (19). We have used retroviral co-infec-
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Fig. 2. Identification of CaM kinase II phosphorylation sites in the EGFR cytoplasmic tail. Smaller HER GST fusion proteins, both wild-type and site-specific alanine mutants, were prepared and phos-

phorylated with CaM kinase II as described under “Experimental Pro-
ductions.” GST-1, threonine 1022 to tyrosine 1045; GST-2, phenylala-

nine 1041 to aspartic acid 1056; GST-3, aspartic acid 1048 to proline 1095; GST-4, phenylalanine 1062 to histidine 1105; GST-5, proline 1130 to glycine 1165. Samples were separated by SDS-12.5% PAGE, and gels were either stained with Coomassie Blue (a) or dried and exposed to x-ray film (b). c, regions covered are depicted diagramati-
cally, and proposed consensus phosphorylation sites are underlined.
Regulation of EGFR Signaling by CaM Kinase II

FIG. 3. Focus formation of NIH3T3 fibroblasts expressing EGFR receptor mutants. Passage 10 NIH3T3 fibroblasts (8 \times 10^4 cells) were infected (4 h) with HER mutant retroviruses (m.o.i. 0.004) and then infected (4 h) with transforming growth factor- \alpha virus (m.o.i. 0.02) as described under “Experimental Procedures.” Cells were grown in Dulbecco’s modified Eagle’s medium, 4% fetal calf serum and after 14 days foci were stained with crystal violet. Data are representative of two independent experiments.

FIG. 4. CaM kinase II phosphorylation of sub-kinase domain III in the EGFR. a, amino acid sequence alignment of sub-domain III in the kinase domains of HER-1 (EGFR), HER-2/c-erbB2, HER-3, and HER-4 is based on the classification of conserved regions found in all tyrosine kinases (30). Serine 744 in the HER-1 is highlighted, and the proposed consensus CaM kinase phosphorylation site in HER-1, HER-3, and HER-4 is underlined. b, GST-III-WT comprises residues alanine 731 to leucine 758 of HER-1, and GST-III-S744A has an alanine mutation at serine 744. Proteins were phosphorylated by CaM kinase II as described under “Experimental Procedures” and separated by SDS-12.5% PAGE. Gels were either stained with Coomassie Blue or dried and exposed to x-ray film. c, HER-293 cells expressing wild-type or mutant receptors were starved in 0.5% fetal calf serum, Dulbecco’s modified Eagle’s medium for 24 h and stimulated for 10 min with 100 ng/ml EGF. After lysis, EGF receptors were immunoprecipitated, separated by SDS-7.5% PAGE, and immunoblotted against anti-phosphotyrosine antibody (anti-PY) or anti-EGFR antibody (anti-HER).

FIG. 5. Autokinase activity of EGFR and HER-2 receptor mutants. Receptor immunoprecipitates from transfected HEK-293 cells in the presence (\( \square \)) or absence (\( \blacksquare \)) of CaM kinase II were assessed for autokinase activity as described under “Experimental Procedures.” Data are expressed relative to the wild-type receptors and are equalized at serine 744. Proteins were phosphorylated by CaM kinase II (Fig. 6b), and this effect was prevented by mutation of major phosphorylation sites at serines 1046, 1047, 1057, and 1142 to alanine (data not shown).

Mechanism of EGFR Kinase Regulation; CaM Kinase II Phosphorylation of the EGFR Cytoplasmic Tail Inhibits Its Association with the Tyrosine Kinase Domain—Since the EGFR cytoplasmic tail is a substrate for CaM kinase II for other -S-X-D- consensus sequences. One additional site was found at serine 744, which, based on sequence alignment of known kinases, is located within sub-domain III or helix C of the tyrosine kinase domain (30). This site is conserved between the type I RTKs, except for HER3, and there is almost complete identity in the whole of sub-domain III between EGFR and HER2/c-erbB2 apart from substitution of the EGFR equivalent of serine 744 for glycine in HER2/c-erbB2 (Fig. 4a). A GST fusion protein comprising sub-domain III of the EGFR can be phosphorylated by CaM kinase II, and this can be prevented by mutation of serine 744 to alanine (Fig. 4b). In addition, when the serine 744 → alanine mutation is incorporated into the full-length receptor, there is approximately a 2-fold increase in receptor autophosphorylation assessed by overexpression in HEK-293 cells and anti-phosphotyrosine blotting (Fig. 4c). We then attempted to mimic hyperphosphorylation of serine 744 by mutation to a negatively charged aspartic acid and found that receptor tyrosine autophosphorylation is significantly impaired (Fig. 4c). This loss of activity is unlikely to be due to an effect on overall domain structural integrity, since mutation of the same site to alanine up-regulates kinase activity.

Regulation of EGFR and HER2/c-erbB2 Tyrosine Kinase Activity by CaM Kinase II—We examined whether phosphorylation of the EGFR by CaM kinase II could inhibit receptor tyrosine kinase activity by immune complex kinase assay. The kinase activity of the wild-type EGFR is reduced to about 40% by co-expression in HEK-293 cells with a constitutively active form of CaM kinase II lacking a C-terminal auto-inhibitory domain (Fig. 5). Mutation of serines 1046/1047 increased kinase activity by about 1.5-fold, which was enhanced slightly by additional mutation of serine 1057, and significantly increased by approximately 3.5-fold compared with wild-type EGFR when serine 1142 was also mutated (Fig. 5). Mutation of serine 744 to aspartic acid, making this site appear constitutively phosphorylated, severely affected kinase activity, and mutation to alanine caused a 2-fold increase relative to wild-type EGFR; however, kinase activity was not significantly inhibited by co-expression with constitutively active CaM kinase II in contrast with wild-type, S1046A/S1047A, S1046A/S1047A/S1057A, and S1046A/S1047A/S1142A (Fig. 5). The tyrosine autokinase activity of HER2/c-erbB2, in the form of a chimeric receptor comprising the extracellular domain of EGFR, was increased about 1.3-fold by mutation of threonine 1172 to alanine, and this activity could not be significantly inhibited by CaM kinase II (Fig. 5; see also Ref. 20).
FIG. 6. Effect of CaM kinase II on EGFR kinase domain and cytoplasmic tail interactions. a, schematic representation of experimental design to assess interactions between the EGFR kinase domain and cytoplasmic tail. b, wheat germ agglutinin precipitates of HERACT were incubated with GST-HERCT (20, 8, or 3 μg), which had been prephosphorylated in the presence or absence of purified CaM kinase II as described under "Experimental Procedures." Samples were separated by SDS-10% PAGE and stained with Coomassie Blue or immunoblotted against anti-EGFR antibody (Sigma; E3138, anti-HERCT).

DISCUSSION

In this study, we provide evidence that CaM kinase II contributes significantly to the control of EGFR signaling and attenuation of tyrosine autokinase activity. Two serine residues at positions 1046 and 1047 have been implicated previously in EGFR-induced receptor desensitization (18). We now confirm that these are phosphorylated by CaM kinase II with a marked preference for serine 1047 and find additional sites at serines 1142 and 1057 that reside within an alternative -S-X-D-consensus phosphorylation sequence. Serine 1142 is a preferred site of phosphorylation and combined site-directed mutagenesis of this, together with other CaM kinase II sites in the EGFR cytoplasmic tail, leads to up transformation of NIH3T3 fibroblasts and increases receptor tyrosine autokinase activity about 3-fold.

The tyrosine-specific phosphorylation function of RTKs is indispensable for the engagement of intracellular effector systems that govern cellular responses such as proliferation, differentiation, and migration. While receptor-specific substrate recruitment provides some definition in the transmission of distinct signals, differences in amplitude and duration of receptor tyrosine kinase activity can make important contributions to the nature of the biological response (31). For instance, transient MAP kinase activation downstream of the EGFR mediates a proliferative response in PC12 cells, while sustained activity from an activated EGFR mutant results in transformation in regulating normal EGFR function. Deletion of these regulatory sites may in turn partly explain the conflicting results. Removal of 202 C-terminal residues enhanced fibroblast transformation (34); however, truncation of the C-terminal tail at residue 973 does not result in a constitutively active kinase (35). Naturally occurring C-terminal truncations and mutations in the EGFR receptor have been found in a number of oncogenic products of avian erythroblastosis viruses (37). Sequence alterations responsible for their increased oncogenic capacity include deletions in the carboxytail and point mutations in the kinase domain (26). An internal deletion of 21 amino acids in v-erbB, corresponding to residues 1040–1060 in the human EGFR, were found to be essential for transformation (38, 39). This region includes the negative regulatory CaM kinase II phosphorylation sites at serines 1046/1047 and serine 1057. These observations support an important role for CaM kinase II site-specific phosphorylation in regulating normal EGFR function. Deletion of these three regulatory sites in v-erbB could contribute to defects in desensitization followed by an accumulation of an active tyrosine kinase within the cell that is manifest as a transformed phenotype.

Activation and regulation of the EGFR kinase is clearly complex and involves covalent modification as well as a number of critical protein-protein interactions. Binding of EGF leads to receptor dimerization and simultaneous activation of the kinase domain (40). Interactions within the kinase domain then contribute to the stabilization of an active dimeric conformation (21). The recent crystal structure of the fibroblast growth factor receptor tyrosine kinase domain has been determined, and a comparably large buried interface was found between two conserved regions comprising helix C of the kinase domain (41). The crystal structure of the EGFR kinase domain is not known; however, a recent model predicts a dimeric contact between helices C that is responsible for maintaining an active conformation in both a symmetric and asymmetric dimer (42). Interestingly, we find an additional CaM kinase II phosphorylation site at serine 744 (-S-V-D-consensus), which is located at the C-terminal end of helix C in the kinase domain (Fig. 4). This represents a region of the kinase domain that is well conserved in the type I RTK family with the exception of HER-3, which displays no kinase activity. Substitution of Serine 744 to a negatively charged aspartic acid residue to mimic phosphorylation at this site abolishes activity of the kinase, and conversely, mutation to alanine up-regulates kinase about 2-fold (Figs. 4 and 5). It is also notable that both HER2/c-erbB2 and the EGFR have identical sequences in helix C, with the exception of a substitution to glycine in HER2/c-erbB2 of the position equivalent to serine 744 in the EGFR (Fig. 4a). The absence of this site in HER2/c-erbB2 may therefore...
also contribute to it being refractory to regulation by CaM kinase II (Fig. 5).

There are several plausible explanations for the mechanism by which serine/threonine phosphorylation mediates inhibition of RTK activity. Receptors are known to be activated by dimerization (21, 43); however, no marked effect of PKC or CaM kinase II on EGF receptor aggregation was observed in experiments using covalent cross-linking analysis (18, 44). Alternative possibilities include the recruitment of tyrosine phosphatases that is sensitive to serine/threonine phosphorylation of the receptor, or that activation of serine/threonine kinases leads directly to cellular phosphatase activation and thereby indirectly modulates RTK activity (17). A model involving fold-back inhibition by the carboxyl tail of the EGF receptor has been proposed (45). We have, for the first time, been able to detect interactions between the EGFR cytoplasmic tail and its own kinase domain and show that this interaction can be disrupted by prephosphorylation of the EGFR cytoplasmic tail with CaM kinase II (Fig. 6). These data raise the intriguing possibility that the mechanism by which CaM kinase II regulates EGFR signaling and autokinase activity is through a direct effect on a substrate interaction with an enzyme active site. Additional regulation and complete shutdown of the kinase might then be achieved by the subsequent phosphorylation of serine 744 in helix C of the EGFR kinase domain, which would disrupt the helix C interactions required for stabilization of an active kinase configuration. Serine 744 phosphorylation may also be a dominant regulatory modification, since the S744A mutant EGFR no longer retains sensitivity to kinase regulation in the presence of constitutively active CaM kinase II, in contrast with the alanine mutants in the EGFR cytoplasmic tail (Fig. 5). In conclusion, the studies reported here highlight the importance of CaM kinase II in the feedback regulation and differential control of RTK signaling. We have shown that the number and location of CaM kinase II sites within important structural domains can account for the degree of tyrosine kinase regulation and ultimately contribute to the strength of signal emanating from activated cell surface receptors such as HER2/c-erbB2 and the EGFR. An important goal for future research will be to fully understand the structural aspects of these events and, through the use of phosphopeptide-specific antisera raised against individual phosphorylation sites, examine spatial and temporal signal activation of these key receptor regulatory pathways in vivo.

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