

# The Human Epidermal Growth Factor Receptor Contains a Juxtamembrane Calmodulin-Binding Site<sup>†</sup>

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**ABSTRACT:** A ligand-insensitive form of the human epidermal growth factor receptor (EGFR) was enriched by  $\text{Ca}^{2+}$ -dependent calmodulin-affinity chromatography purification. The basic amphiphilic segment Arg<sub>645</sub>-Arg-Arg-His-Ile-Val-Arg-Lys-Arg-Thr<sub>654</sub>-Leu-Arg-Leu-Leu-Gln<sub>660</sub>, located within the cytoplasmic juxtamembrane domain of this receptor, was purified as a fusion protein with glutathione *S*-transferase and shown to bind calmodulin in a  $\text{Ca}^{2+}$ -dependent manner. An apparent dissociation constant of 0.4  $\mu\text{M}$  calmodulin ( $K_d'(\text{CaM})$ ) and an apparent affinity constant of 0.5  $\mu\text{M}$  free  $\text{Ca}^{2+}$  ( $K_a'(\text{Ca})$ ) were measured for this binding process. Binding of calmodulin at the juxtamembrane site prevented the phosphorylation of residue Thr-654 by protein kinase C, and an apparent inhibition constant of 0.5–1  $\mu\text{M}$  calmodulin ( $K_i'(\text{CaM})$ ) was determined. Conversely, phosphorylation of this site by protein kinase C prevented its subsequent interaction with calmodulin. We therefore propose that cross talk between signaling pathways mediated by calmodulin and protein kinase C occurs at the juxtamembrane domain of the EGFR. This calmodulin-binding sequence is highly conserved among protein tyrosine kinases of the vertebrate EGFR family.

The epidermal growth factor receptor is an integral transmembrane glycoprotein of 170 kDa responsible for the initiation of mitogenic signaling after binding of its ligand. This receptor exhibits an extracellular region containing the EGF<sup>1</sup>-binding domain, a single transmembrane region, and a cytoplasmic region containing the catalytic site responsible for its intrinsic protein tyrosine kinase (EC 2.7.1.112) activity (1–3). Ligand binding promotes receptor dimerization and its transphosphorylation at five C-terminal tyrosine residues. This process activates the receptor and enables it to interact with, and phosphorylate, a set of intracellular target proteins, which in turn commits the cell to its mitotic division (4, 5). Upon prolonged ligand exposure, the EGFR becomes finally internalized and degraded within lysosomes (2, 4).

The cytoplasmic segment of the EGFR comprised between the inner membrane boundary and its tyrosine kinase domain, denoted as the juxtamembrane domain, contains the residue Thr-654, which is the target of phosphorylation by protein

kinase C (EC 2.7.1.37) (6). This phosphorylation results in inhibition of the EGFR tyrosine kinase activity, as well as in the prevention of its long-term internalization and down-regulation (7–9). The EGFR juxtamembrane domain is additionally responsible for the specificity of mitogenic signaling and receptor turnover (10–13).

Calmodulin is a  $\text{Ca}^{2+}$ -binding protein involved in the regulation of multiple intracellular processes by binding to target proteins upon increase in the concentration of cytoplasmic free  $\text{Ca}^{2+}$  (14, 15). This protein bears two globular domains, each containing two  $\text{Ca}^{2+}$ -binding sites, connected by a long flexible  $\alpha$ -helical segment (16). By virtue of this flexible helical tether, the two lobes of calmodulin get closer in such a way that they interact simultaneously with the target polypeptide. The two globular domains exhibit large hydrophobic patches flanked by negatively charged regions, which become exposed upon  $\text{Ca}^{2+}$  binding. This enables calmodulin to bind to positively charged amphiphilic  $\alpha$ -helices present in calmodulin-regulated proteins by means of both hydrophobic and electrostatic interactions (17, 18). In addition, calmodulin-binding sites often contain phosphorylation sites for protein kinases in their C-terminal half (18).

One of the early events occurring during cell stimulation by EGF is an increase in the concentration of cytosolic free  $\text{Ca}^{2+}$  (19, 20). Therefore, the  $\text{Ca}^{2+}$ -calmodulin complex might play some role in EGFR-mediated signaling. In this context, we have demonstrated the existence of a number of interactions between calmodulin and the EGFR from rat liver (21–23). First, a ligand-sensitive EGFR was purified from solubilized plasma membranes by  $\text{Ca}^{2+}$ -dependent calmodu-

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<sup>1</sup> Abbreviations: CaM, calmodulin; DSS, suberic acid bis(*N*-hydroxysuccinimide ester); EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EGTA, [ethylenbis(oxyethylenitrilo)]-tetraacetic acid; GST, glutathione *S*-transferase; GST-HER<sub>(645–660)</sub>, fusion protein between GST and the EGFR calmodulin-binding site; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IgG, immunoglobulin G; IPTG, isopropyl  $\beta$ -D-thiogalactopyranoside; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; TGF $\alpha$ , transforming growth factor- $\alpha$ ; Tris, tris(hydroxymethyl)aminomethane.

lin-affinity chromatography. Second, the isolated receptor phosphorylates exogenous calmodulin in an EGF-stimulated manner at residue Tyr-99 with a stoichiometry close to 1 (mol/mol), in a process that requires the absence of  $\text{Ca}^{2+}$  and the presence of a cationic polypeptide as cofactor. Third, calmodulin inhibits the EGFR tyrosine kinase activity - in a  $\text{Ca}^{2+}$ -dependent manner, preventing both its transphosphorylation and the phosphorylation of exogenous substrates. In addition, the human EGFR, overexpressed in stably transfected EGFR-T17 fibroblasts, phosphorylates exogenous calmodulin with the same requirements found for the rat liver receptor when permeabilized cells are used (24). Therefore, calmodulin might act as an intracellular regulator of the EGFR activity according to the oscillations in cytosolic free  $\text{Ca}^{2+}$  following ligand-induced receptor stimulation.

In this work we have identified a basic amphiphilic site within the cytosolic juxtamembrane domain of the human EGFR, spanning amino acids 645 through 660, and characterized its calmodulin-binding properties using a fusion protein between glutathione *S*-transferase (EC 2.5.1.18) and this receptor segment. We have also demonstrated the mutually exclusive occurrence of calmodulin-binding at, and protein kinase C-phosphorylation of, this regulatory domain. The physiological implications and possible cross talk between both processes are discussed.

## MATERIALS AND METHODS

**Reagents.** Radiolabeled [ $\gamma$ - $^{32}\text{P}$ ]ATP (triethylammonium salt) (3000–4500 Ci·mmol $^{-1}$ ) and [ $^{125}\text{I}$ ]calmodulin (50–150  $\mu\text{Ci}\cdot\mu\text{g}^{-1}$ ) (1 Ci = 37 GBq) were purchased from New England Nuclear and ICN. Calmodulin–agarose, EGF (from mouse submaxillary glands), and alkaline phosphatase-conjugated anti-mouse and anti-rabbit IgGs developed in goat were products of Sigma. Pig brain calmodulin was purified essentially as described (25). Bovine brain calmodulin, protein kinase C (from rat brain), and alkaline phosphatase-conjugated rabbit anti-sheep IgG were purchased from Calbiochem. A polyclonal antiserum directed to GST was obtained after rabbit immunization with the purified protein. Purified sheep polyclonal IgGs against the human EGFR were purchased from Cambridge Research Biochemicals. The properties of the mouse monoclonal antibody to calmodulin used in this work have been described (26). Other reagents used in this work were of analytical grade.

**Purification of the EGFR.** EGFR-T17 fibroblasts, a stably transfected NIH-3T3 derived cell line overexpressing the human EGFR (20), were grown to confluence in ten 150 mm dishes on Dulbecco's-modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, and 40 mg·mL $^{-1}$  gentamicin, and the fibroblasts were starved for serum overnight. The cells were washed with PBS (137 mM NaCl, 2.7 mM KCl, 12 mM Na/K-phosphate, pH 7.4), gently scraped from the plates, harvested by centrifugation, and lysed in 3 mL of an ice-cold hypotonic buffer containing 15 mM Hepes–Na (pH 7.4), 1 mM EGTA, and a cocktail of protease inhibitors made of 0.6 mM PMSF, 0.3  $\mu\text{M}$  aprotinin, 4.5  $\mu\text{M}$  leupeptin, and 1.5  $\mu\text{M}$  pepstatin A (cocktail 1). After 10 min on ice, the lysate was centrifuged at 130000g $_{\text{max}}$  for 30 min at 4 °C. The supernatant was discarded, and the pellet was resuspended in the same buffer

and centrifuged again as above. The new pellet was washed with the same buffer without EGTA, centrifuged again, resuspended in 3 mL of 25 mM Hepes–Na (pH 7.4) containing protease inhibitors (cocktail 1), frozen in liquid  $\text{N}_2$ , and stored at –70 °C until use.

The EGFR was solubilized from this particulate fraction at 0 °C for 10 min upon addition of 1% (w/v) Triton X-100 and 5% (w/v) glycerol. The preparation was centrifuged as above and 0.1 mM  $\text{CaCl}_2$  was added to the supernatant. This solubilized fraction was subjected to calmodulin-affinity chromatography as described earlier (21, 22) in a 2 mL bed-volume calmodulin–agarose column equilibrated with 25 mM Hepes–Na (pH 7.4), 1% (w/v) Triton X-100, 5% (w/v) glycerol, 0.1 mM  $\text{CaCl}_2$ , and protease inhibitors (cocktail 1). After the column was washed with 20 bed volumes of this buffer, elution was carried out with the same buffer but in which the  $\text{CaCl}_2$  was substituted by 1 mM EGTA. The presence of the EGFR in the eluted fractions was monitored by phosphorylation assays and immunoblots, as described below.

**Recombinant Protein Expression and Purification.** A DNA fragment encoding the human EGFR amino acids 645–660 was amplified by PCR from the corresponding cDNA (1) present in plasmid cvsHERc, using *Taq* DNA polymerase (EC 2.7.7.7) and the oligonucleotides 5'-CGG-GATCCCGAAGGCGCCACATC-3' and 5'-GGAATTCACG-CAGCAGCTCCG-3' as forward and reverse primers, respectively. The forward primer contains a *Bam*HI site (underlined) prepended to nucleotides 2191–2205 of the EGFR cDNA. The reverse primer corresponds to antisense nucleotides 2226–2238 to which a stop codon and an overlapping *Eco*RI site (underlined) were appended. The 64 base pair PCR product was cleaved with *Bam*HI and *Eco*RI and inserted into the *Escherichia coli* expression vector pGEX-2T (27) at the 3'-end of the GST-encoding sequence. The resulting construct was verified by restriction analysis and automated DNA sequencing by PCR using fluorescent dideoxy chain terminators. This plasmid directs the IPTG-inducible expression of a fusion protein composed of amino acids 645–660 of the human EGFR in frame with GST, hereafter denoted as GST-HER<sub>(645–660)</sub>.

The GST-HER<sub>(645–660)</sub> and GST recombinant proteins were purified from 100 mL cultures of plasmid-transformed *E. coli* BL21(DE3) after induction with 0.1 mM IPTG for 5 h by a procedure modified from that previously described (27). The bacteria were incubated at 30 °C for 20 min in 2 mL of a medium containing 50 mM Tris-HCl (pH 8), 100 mM NaCl, 1 mM EDTA, and 1 mg·mL $^{-1}$  lysozyme (EC 3.2.1.17) and then lysed by 3–4 freezing/thawing cycles using liquid  $\text{N}_2$ . The lysates were treated with 125  $\mu\text{g}\cdot\text{mL}^{-1}$  deoxyribonuclease I (EC 3.1.21.1) at 37 °C for 20 min in the presence of 10 mM  $\text{MgCl}_2$  and 2 mM ATP, and thereafter, 15 mM EDTA and 1% (w/v) Triton X-100 were added. After incubation at room temperature for 10 min, the lysate was spun at 10000g $_{\text{max}}$  for 10 min at 4 °C. The supernatant was subjected to glutathione–Sephacrose affinity chromatography at room temperature, and after extensive wash with 1% (w/v) Triton X-100 in PBS, elution was achieved with 10 mM glutathione in 100 mM Tris-HCl (pH 8). All the purification steps included the protease inhibitors 0.6 mM PMSF, 0.3  $\mu\text{M}$  aprotinin, and 4.5  $\mu\text{M}$  leupeptin (cocktail 2).

**Calmodulin Crosslinkage Assays.** Binding of either GST-HER<sub>(645–660)</sub> or GST recombinant proteins (0.35  $\mu$ M) to 0.1–0.3 nM (25 nCi) [<sup>125</sup>I]calmodulin was assayed in 100  $\mu$ L of a reaction mixture containing 50 mM Hepes–Na (pH 7.4), the concentrations of CaCl<sub>2</sub> and/or EGTA indicated in the legends of the figures, and protease inhibitors (cocktail 2). Where indicated, non-radiolabeled calmodulin (from pig or bovine brain) was included at a 10-fold molar excess (3.5  $\mu$ M) with respect to the recombinant protein (0.35  $\mu$ M). After incubation at 37 °C for 30 min, the binding mixture was treated with 3 mM DSS at 0–4 °C for 20 min, and the cross-linkage reaction was stopped upon addition of 250 mM glycine and further incubation at 0–4 °C for 5–10 min. Proteins were precipitated upon addition of ice-cold 10% (w/v) trichloroacetic acid and analyzed by electrophoresis and autoradiography as described below. For assays aimed to a direct visualization of the cross-linked complexes by Coomassie Blue staining or immunoblot, 3.5  $\mu$ M GST-HER<sub>(645–660)</sub> or GST and non-radiolabeled 3.5  $\mu$ M calmodulin (1:1 molar ratio) were used in the binding reactions.

**Phosphorylation Assays.** Phosphorylation by the EGFR was assayed as follows: First, the receptor-containing fraction was incubated on ice for 30 min in the absence or presence of 1  $\mu$ M EGF. Thereafter, the phosphorylation was performed at 37 °C for 2.5 min in 100  $\mu$ L of a reaction mixture containing 25 mM Hepes–Na (pH 7.4), 5 mM MgCl<sub>2</sub>, 0.2% (w/v) Triton X-100, 1% (w/v) glycerol, 100  $\mu$ g·mL<sup>−1</sup> poly-L-(Glu·Tyr) (when added), and protease inhibitors (cocktail 2). The reaction was started upon addition of 10  $\mu$ M (0.5  $\mu$ Ci) [ $\gamma$ -<sup>32</sup>P]ATP and stopped with ice-cold 10% (w/v) trichloroacetic acid.

Unless indicated otherwise, phosphorylation assays with protein kinase C were carried out at 37 °C in 100  $\mu$ L of a medium containing 50 mM Hepes–Na (pH 7.4), 5 mM MgCl<sub>2</sub>, 1.5 mM CaCl<sub>2</sub>, 1.2 mM dithiothreitol, 0.025% (w/v) Triton X-100, 250  $\mu$ g·mL<sup>−1</sup> L- $\alpha$ -phosphatidyl-L-serine, 2.5  $\mu$ g·mL<sup>−1</sup> 1,3-diolein(C18:1,[*cis*]-9), 4 milliunits of protein kinase C, 0.35  $\mu$ M of either GST-HER<sub>(645–660)</sub> or GST, and protease inhibitors (cocktail 2). The reaction mixtures were sonicated before substrate addition. The reaction was initiated upon addition of 10  $\mu$ M (2  $\mu$ Ci) [ $\gamma$ -<sup>32</sup>P]ATP and arrested with ice-cold 10% (w/v) trichloroacetic acid. The phosphorylation of the fusion protein by protein kinase C was found to be linear at least during the first 3 min of reaction. Therefore, for the experiments in which critical kinetics parameters were determined the assays were carried within this time frame as indicated in the legends of the figures.

In both EGFR and protein kinase C phosphorylation assays, the samples were finally centrifuged and the pellets containing the precipitated proteins were analyzed by electrophoresis and autoradiography as described below.

**Immunoblots.** The proteins resolved by electrophoresis were transferred to polyvinylidene difluoride membranes at 300 mA for 2 h in a buffer containing 48 mM Tris (base) and 39 mM glycine (pH 8.3), 1.3 mM sodium dodecyl sulfate, and 20% (v/v) methanol. Then they were fixed for 45 min by treatment with 0.2% (v/v) glutaraldehyde in TBS (25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2.7 mM KCl) and stained with Fast Green FCF. Thereafter, the membranes were blocked by incubation with 5% (w/v) fat-free powdered milk in TBS (MTBS) for 2 h at room temperature, washed

extensively with 0.1% (v/v) Tween-20 in TBS (TTBS), and probed overnight with either an anti-GST polyclonal anti-serum (1/1000 dilution), an anti-EGFR polyclonal antibody (0.2  $\mu$ g·mL<sup>−1</sup>), or an anti-calmodulin monoclonal antibody (0.2  $\mu$ g·mL<sup>−1</sup>) in MTBS. The membranes were washed with TTBS and incubated for 1–1.5 h with the corresponding alkaline phosphatase-conjugated secondary antibody (diluted 1/1000) in MTBS. Color development took place at 37 °C with 0.32 mg·mL<sup>−1</sup> nitro blue tetrazolium and 0.16 mg·mL<sup>−1</sup> 5-bromo-4-chloro-3-indolyl phosphate in a medium containing 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 5 mM MgCl<sub>2</sub>.

**Other Analytical Procedures.** Slab-gel electrophoresis was performed as described (28) at 7.5 mA overnight at room temperature using 5–20% (w/v) polyacrylamide linear gradients in the presence of 0.1% (w/v) sodium dodecyl sulfate at pH 8.3. Unless stated otherwise, 10 mM EGTA was present in the sample buffer to obtain migration of calmodulin as a single band at 21 kDa. After electrophoresis, the gels were stained with Coomassie Blue and dried under vacuum at 80 °C on Whatman 3MM Chr filter paper. Autoradiographs were obtained by exposure of X-ray films at −20 °C. The intensities of the radiolabeled bands in the autoradiographs were quantified using a scanning photodensitometer, and the values obtained were directly proportional to the amounts of <sup>125</sup>I or <sup>32</sup>P contained in the bands within the exposure times used. Phosphoamino acid analysis was carried out essentially as described (29). Protein concentrations were quantified by the protein–dye binding assay (30) using bovine serum albumin as a standard. The concentrations of free Ca<sup>2+</sup> in the assays were calculated by a computer program similar to that previously described (31).

## RESULTS

**Purification of the Human EGFR by Ca<sup>2+</sup>-Dependent Calmodulin-Affinity Chromatography.** Previous reports from our laboratory have shown the purification of the EGFR from rat liver by calmodulin-affinity chromatography (21–23). In order to ascertain whether the human receptor also exhibits the property of interacting with calmodulin in a Ca<sup>2+</sup>-dependent manner, we have used EGFR-T17 fibroblasts, a stably transfected cell line which overexpresses this receptor, to purify the EGFR. After the cells were lysed in a hypotonic buffer, a particulate fraction was obtained after repeated wash with EGTA aimed at removing the pool of calmodulin associated to the membranes in a calcium-dependent manner (32). The receptor was solubilized from the particulate fraction in the presence of Triton X-100 and glycerol and applied to a calmodulin–agarose column equilibrated with a buffer containing CaCl<sub>2</sub>. Elution was performed with an EGTA-containing buffer, and the purification of the EGFR was monitored by assaying its transphosphorylation activity and by immunoblot using a specific antibody against this receptor.

When the transphosphorylation of the EGFR present in the particulate fraction was assayed, we observed a strong stimulation of its tyrosine kinase activity induced by EGF (results not shown). Similar stimulation by EGF was observed in this fraction after solubilization (Figure 1, panel A), as well as in the receptor present in the column flow-through (results not shown). In contrast, and as a difference



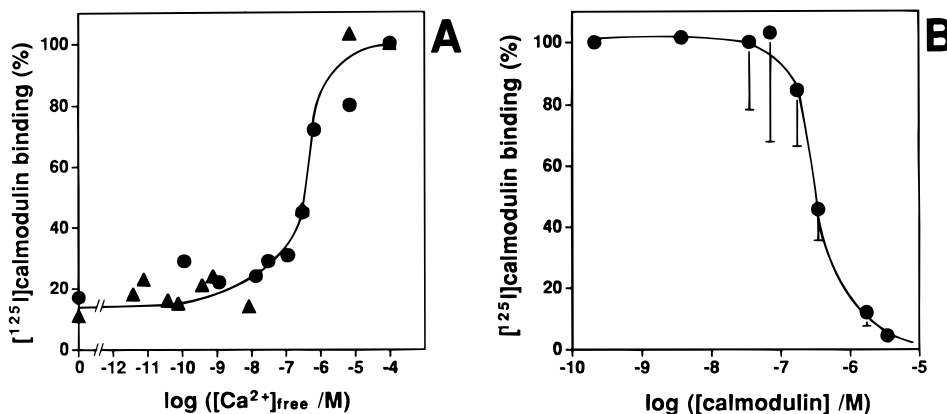


FIGURE 4: Affinities of the calmodulin-binding process. (Panel A) GST-HER<sub>(645-660)</sub> fusion protein (0.35  $\mu\text{M}$ ) and 0.1–0.3 nM (25 nCi)  $[^{125}\text{I}]$ calmodulin were incubated at 37  $^{\circ}\text{C}$  for 30 min in 100  $\mu\text{L}$  of a medium containing 50 mM HEPES–Na (pH 7.4), 1 mM  $\text{MgCl}_2$ , 1 mM EGTA, and different amounts of  $\text{CaCl}_2$  to obtain the indicated concentrations of free  $\text{Ca}^{2+}$ . The cross-linkage reaction was carried out as indicated in Materials and Methods. The GST-HER<sub>(645-660)</sub>– $[^{125}\text{I}]$ calmodulin complex was quantified by scanning densitometry after electrophoresis and autoradiography from two separate experiments (triangles and circles), and the apparent affinity constant for free calcium ( $K'_a(\text{Ca}^{2+})$ ) was calculated from the inflection point of the curve attained at 50% labeling. (Panel B) Crosslinkage assays were carried out as indicated in Materials and Methods in a medium containing 50 mM HEPES–Na (pH 7.4), 0.1 mM  $\text{CaCl}_2$ , and the indicated concentrations of non-radiolabeled pig or bovine brain calmodulin. Each point and associated error bars represent the mean  $\pm$  SEM relative intensity of the GST-HER<sub>(645-660)</sub>– $[^{125}\text{I}]$ calmodulin complex formed in 3–4 separate experiments. The apparent dissociation constant for calmodulin ( $K'_d(\text{CaM})$ ) was calculated from the inflection point of the curve attained at 50% labeling.

calmodulin complex was visualized as a double band by Coomassie Blue staining after electrophoresis run in the presence of either  $\text{Ca}^{2+}$  or EGTA in the sample buffer. In contrast, recombinant GST did not bind calmodulin and only the formation of GST dimers was faintly detected as previously reported (33), which also occurred in the absence of added calmodulin (results not shown).

Calmodulin is known to experience a  $\text{Ca}^{2+}$ -dependent electrophoretic mobility shift, running as a double band at 16.5 kDa (main component) in the presence of calcium and as a single 21 kDa band in the absence of calcium (presence of EGTA) (34). This property of calmodulin is also noticeable in Figure 3 (panel A). Similarly, the GST-HER<sub>(645-660)</sub>–calmodulin complex was also found to undergo a  $\text{Ca}^{2+}$ -induced mobility shift, migrating as a double band of approximately 44 kDa in the presence of  $\text{Ca}^{2+}$  and as a double band of approximately 50 kDa in the presence of EGTA (see arrows). This property was thus most likely conferred by the calmodulin moiety of this complex. To further ascertain the formation of the GST-HER<sub>(645-660)</sub>–calmodulin complex, cross-linkage assays were carried out using  $[^{125}\text{I}]$ calmodulin, and the complex was visualized by autoradiography after electrophoresis in the presence of EGTA (see arrow in Figure 3, panel B). Fainter bands of higher molecular mass, presumably corresponding to complexes containing more than one unit of the two interacting molecules, were also detected (see Figure 3, panel B). In contrast, no significant complex formation was detected when the carrier protein GST was used. The interaction between calmodulin and the EGFR juxtamembrane sequence occurred in a  $\text{Ca}^{2+}$ -dependent manner, since complex formation was drastically diminished in the presence of EGTA. As expected, non-radiolabeled calmodulin efficiently competed the association between  $[^{125}\text{I}]$ calmodulin and the GST-HER<sub>(645-660)</sub> fusion protein (Figure 3, panel B). These results were confirmed by cross-linkage experiments using non-radiolabeled calmodulin and subsequent complex detection by immunoblot using a specific monoclonal antibody against calmodulin (results not shown).

To further analyze the effect of calcium on the binding of calmodulin to the EGFR juxtamembrane sequence,  $[^{125}\text{I}]$ calmodulin cross-linkage to the GST-HER<sub>(645-660)</sub> fusion protein was assayed in the presence of different concentrations of free  $\text{Ca}^{2+}$ . As depicted in Figure 4 (panel A), only low levels of  $[^{125}\text{I}]$ calmodulin binding were attained in the absence of this cation, most likely attributable to residual, nonspecific binding to the GST moiety occurring in the presence of EGTA (see Figure 3, panel B), and maximum  $[^{125}\text{I}]$ calmodulin binding levels were attained at 10–100  $\mu\text{M}$  free  $\text{Ca}^{2+}$ . From these experiments, a  $K'_a(\text{Ca})$  of 0.5  $\mu\text{M}$  free  $\text{Ca}^{2+}$  was determined for calmodulin binding to the EGFR juxtamembrane sequence.

Also, we carried out experiments in which  $[^{125}\text{I}]$ calmodulin was cross-linked to GST-HER<sub>(645-660)</sub> in the presence of different concentrations of non-radiolabeled calmodulin. Radiolabeled calmodulin was maximally displaced by non-radiolabeled 2–5  $\mu\text{M}$  calmodulin (see Figure 4, panel B), and from these experiments a  $K'_d(\text{CaM})$  of around 0.4  $\mu\text{M}$  calmodulin was calculated.

**Calmodulin Prevents the Phosphorylation of Thr-654 by Protein Kinase C.** Since protein kinase C is known to phosphorylate the residue Thr-654 of the EGFR (6), which is contained within the juxtamembrane calmodulin-binding sequence, we set to analyze the effect of calmodulin on this phosphorylation process. As evident from Figure 5 (panel A), protein kinase C efficiently phosphorylated the GST-HER<sub>(645-660)</sub> fusion protein, whereas it failed to phosphorylate the GST carrier protein. Phosphoamino acid analysis of the radiolabeled band revealed that incorporation of phosphate took place on threonine (Figure 5, panel B), which corresponds to Thr-654 of the EGFR sequence appended to GST. When phosphorylation by protein kinase C was assayed after preincubation of the fusion protein with calmodulin, the extent of Thr-654 phosphorylation was drastically reduced (see Figure 5, panel A). When the GST-HER<sub>(645-660)</sub> fusion protein was incubated with different amounts of calmodulin prior to phosphorylation by protein kinase C, the levels of phosphate incorporation were progressively reduced, with a

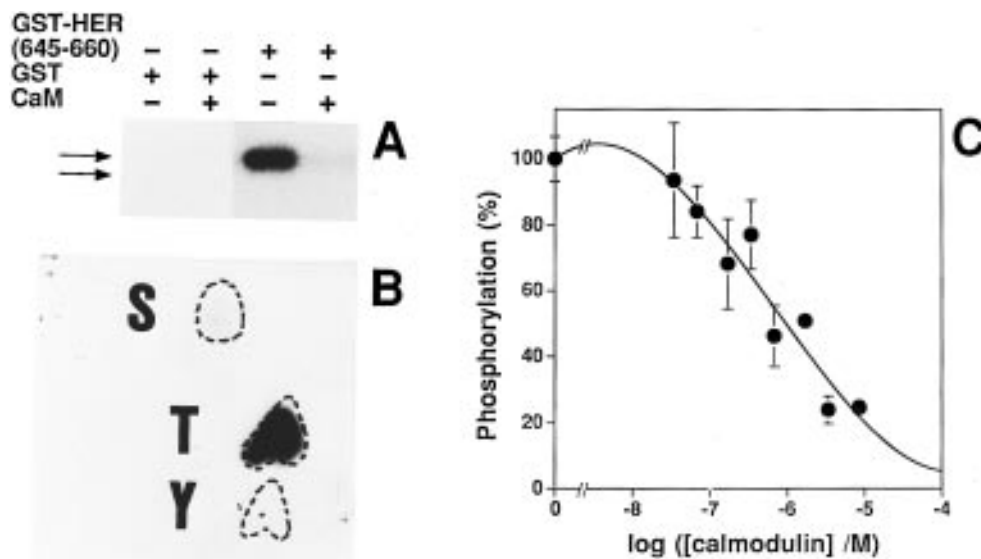


FIGURE 5: Calmodulin inhibits the phosphorylation of Thr-654 by protein kinase C at the EGFR juxtamembrane sequence. (Panel A) GST-HER<sub>(645-660)</sub> and GST recombinant proteins (0.35  $\mu$ M) (where indicated) were incubated at 37 °C for 30 min in the absence (–) and presence (+) of 3.5  $\mu$ M bovine brain calmodulin (CaM) in 50  $\mu$ L of a medium containing 50 mM Hepes–Na (pH 7.4) and 0.1 mM CaCl<sub>2</sub>. Thereafter, phosphorylation by protein kinase C was assayed in a volume of 100  $\mu$ L at 37 °C for either 5 min (GST) or 2 min (GST-HER<sub>(645-660)</sub>), and electrophoresis followed by autoradiography was performed as described in Materials and Methods. Arrows point to the positions of both recombinant proteins. (Panel B) The GST-HER<sub>(645-660)</sub> fusion protein (0.35  $\mu$ M) was phosphorylated by protein kinase C at 37 °C for 15 min but using 10  $\mu$ M (5  $\mu$ Ci) [ $\gamma$ -<sup>32</sup>P]ATP. Thereafter, the phosphorylated band was excised from the dried gel and processed for phosphoamino acid analysis. The positions of migration of standard phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) stained with ninhydrin are indicated on the autoradiograph by dashed lines. (Panel C) The GST-HER<sub>(645-660)</sub> fusion protein (0.35  $\mu$ M) was incubated at 37 °C for 30 min with the indicated concentrations of bovine brain calmodulin in 50  $\mu$ L of a medium containing 50 mM Hepes–Na (pH 7.4) and 0.1 mM CaCl<sub>2</sub>. Thereafter, phosphorylation by protein kinase C was assayed in a volume of 100  $\mu$ L for 1 min at 37 °C as described in Materials and Methods. Each point and associated error bars represent the mean  $\pm$  SEM relative levels of phosphorylation of GST-HER<sub>(645-660)</sub> from 3 to 4 separate experiments. The apparent inhibition constant for calmodulin ( $K'_i(\text{CaM})$ ) was calculated from the inflection point of the curve attained at 50% phosphorylation.

75–80% inhibition attained at the highest calmodulin concentrations assayed (5–10  $\mu$ M) (Figure 5, panel C). From this plot, a  $K'_i(\text{CaM})$  of 0.5–1  $\mu$ M calmodulin was calculated.

**Phosphorylation of Thr-654 by Protein Kinase C Prevents Calmodulin Binding.** Since binding of calmodulin to its target proteins relies on electrostatic and hydrophobic interactions, we assessed the effect of phosphorylation of the EGFR juxtamembrane sequence by protein kinase C on subsequent calmodulin binding. With this purpose, the GST-HER<sub>(645-660)</sub> fusion protein and the carrier protein GST, used as a control, were incubated with protein kinase C in the absence and presence of non-radiolabeled ATP. Thereafter, the formation of the GST-HER<sub>(645-660)</sub>–calmodulin complex was detected by autoradiography using [<sup>125</sup>I]calmodulin (Figure 6, left panel) or by immunoblot with a specific antibody against calmodulin using non-radiolabeled calmodulin (Figure 6, right panel). In both cases, the GST-HER<sub>(645-660)</sub>–calmodulin complex was clearly visible when ATP was omitted. However, when phosphorylation of the fusion protein by protein kinase C was allowed by the presence of ATP, subsequent calmodulin binding was drastically hampered (Figure 6, left and right panels). As expected, in the absence of protein kinase C, the GST-HER<sub>(645-660)</sub>–calmodulin complex was detected in both the absence and presence of ATP (results not shown). This excluded the possibility that ATP itself could prevent the binding of calmodulin to the fusion protein. As previously shown, GST did not form a complex with calmodulin in either the absence or presence of ATP (Figure 6, left and right panels). Therefore, not only binding of

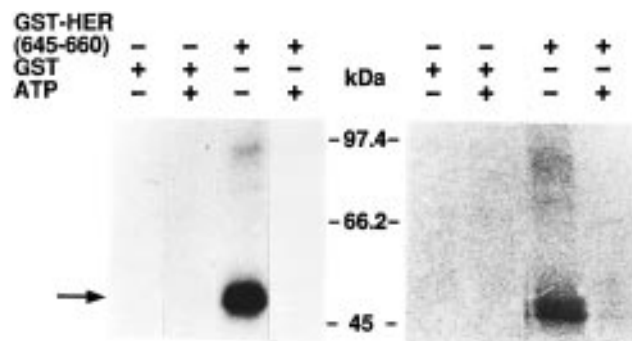


FIGURE 6: Phosphorylation of Thr-654 by protein kinase C inhibits calmodulin binding to the EGFR juxtamembrane sequence. (Left panel) GST-HER<sub>(645-660)</sub> and GST recombinant proteins (0.35  $\mu$ M) (where indicated) were incubated at 37 °C for 15 min in the presence of 20 milliunits of protein kinase C as described in Materials and Methods but in the absence (–) and presence (+) of non-radiolabeled 1 mM ATP. Thereafter, the cross-linkage of the recombinant proteins to 0.1–0.3 nM (25 nCi) [<sup>125</sup>I]calmodulin was assayed in the presence of 0.75 mM CaCl<sub>2</sub>, followed by electrophoresis and autoradiography as described in Materials and Methods. (Right panel) The experiment was performed as indicated in the left panel, except that 3.5  $\mu$ M GST-HER<sub>(645-660)</sub> or GST recombinant proteins were used, and [<sup>125</sup>I]calmodulin was substituted by non-radiolabeled 3.5  $\mu$ M pig brain calmodulin in the cross-linkage assays. Thereafter, electrophoresis followed by immunoblot using a specific monoclonal antibody against calmodulin was performed as described in Materials and Methods.

calmodulin to the EGFR juxtamembrane sequence inhibited subsequent phosphorylation by protein kinase C but this phosphorylation prevented the binding of calmodulin to this sequence.

## DISCUSSION

We have first demonstrated in this work using  $\text{Ca}^{2+}$ -dependent calmodulin-affinity chromatography that the human EGFR, overexpressed in stably transfected murine fibroblasts, binds calmodulin. Similarly, calmodulin-binding capacity was first observed by us for the EGFR from rat liver (21–23). Nevertheless, the receptor isolated from EGFR-T17 fibroblasts appears to be in an EGF-insensitive state, in contrast to the receptor isolated from rat liver which was readily activatable by EGF or  $\text{TGF}\alpha$ , although the latter receptor also exhibited a strong basal activity in the absence of ligands (21). The reason why these receptors isolated by calmodulin-affinity chromatography behave differently toward their ligand is not clear at present. Moreover, as expected, the yield of receptor isolated from the fibroblasts by calmodulin-affinity chromatography was rather low. Two reasons could account for this observation: (i) the presence of a pool of calmodulin tightly bound to the particulate fraction in a  $\text{Ca}^{2+}$ -independent manner that it is not removed by EGTA (32) and (ii) the partial phosphorylation of the receptor by cellular protein kinase C before its purification.

Calmodulin-binding sites are extremely variable and do not exhibit an established consensus sequence. However, these sites generally consist of basic amphiphilic amino acid sequences which fold into an  $\alpha$ -helical structure (17, 18, 35). In this conformation, interactions between the basic amino acids of these sites and acidic amino acids of calmodulin, as well as interactions between hydrophobic residues of both molecules, contribute to stabilize the binding of calmodulin to its target site. Thus, an useful approach to recognize calmodulin-binding sites in proteins has become the search for stretches in which positively charged amino acids are interspersed with some hydrophobic residues and in which both types of amino acids segregate onto opposite sides when plotted on a helical-wheel projection.

Following this strategy, we have previously noticed (21) that the human EGFR cytosolic juxtamembrane sequence Arg<sub>645</sub>-Arg-Arg-His-Ile-Val-Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu-Leu-Gln<sub>660</sub> was a good candidate to constitute a calmodulin-binding site. Therefore, we have constructed a fusion protein in which this EGFR sequence was appended to the C-terminal end of GST, and we have shown binding of calmodulin in a  $\text{Ca}^{2+}$ -dependent manner. A single calmodulin molecule appears to predominantly bind to the fusion protein forming a GST-HER<sub>(645–660)</sub>-calmodulin complex, although traces of higher order complexes were also detected. The calculated  $K'_{d(\text{CaM})}$  of 0.4  $\mu\text{M}$  is consistent with that inferred for the receptor from rat liver by measuring the inhibition of its tyrosine kinase activity by calmodulin, a process with a  $K'_{i(\text{CaM})}$  of 1  $\mu\text{M}$  (21). Since the  $K'_{d(\text{CaM})}$  values determined for calmodulin-binding sites in different proteins range between 0.1 nM and 2  $\mu\text{M}$  (17, 35, 36), the binding of calmodulin to the human EGFR juxtamembrane site falls within the low-affinity part of the spectrum.

The propensity of the EGFR juxtamembrane site to form an  $\alpha$ -helical structure was evaluated by computer-assisted analysis using 10 distinct algorithms. Very good agreement was found between the different programs in predicting a helical conformation for the C-terminal half of this sequence, whereas a helical structure was assigned with less certainty to its N-terminal half (results not shown). Nevertheless, a

preadopted  $\alpha$ -helical structure does not appear to be a strict requirement for a calmodulin-binding site, but rather binding of calmodulin to these regions appears to increase the  $\alpha$ -helical content of the interacting polypeptide, as inferred from circular dichroism analyses (see refs (17, 35, and 36) and references therein). The EGFR sequence could thus become further stabilized into an  $\alpha$ -helix once in its calmodulin-bound state. Furthermore, the necessary conformational change could be favored by the proximity of the membrane phospholipids, as it has been proposed to occur for the calmodulin-binding domain of neuromodulin (37).

A couple of parameters have been used to diagnose the calmodulin-binding ability of protein segments. These are the helical hydrophobic moment ( $\langle\mu_{\text{H}}\rangle$ ) and the average hydrophobicity ( $\langle\text{Hb}\rangle$ ). Most calmodulin-binding sites fall within the area between 0.1 and 0.7 for  $\langle\mu_{\text{H}}\rangle$  and between  $-1$  and 0.5  $\text{kcal}\cdot\text{mol}^{-1}$  ( $-4.2$  to 2.1  $\text{kJ}\cdot\text{mol}^{-1}$ ) for  $\langle\text{Hb}\rangle$  in plots of  $\langle\mu_{\text{H}}\rangle$  versus  $\langle\text{Hb}\rangle$  (38). A calculation of  $\langle\mu_{\text{H}}\rangle$  and  $\langle\text{Hb}\rangle$  for the human EGFR calmodulin-binding site (35) gave values of 0.37 for  $\langle\mu_{\text{H}}\rangle$  and  $-0.93$   $\text{kcal}\cdot\text{mol}^{-1}$  ( $-3.89$   $\text{kJ}\cdot\text{mol}^{-1}$ ) for  $\langle\text{Hb}\rangle$ . Therefore, high hydrophobicity does not appear to be an obligatory feature for a sequence to have calmodulin-binding capacity (38, 39), although its absence may well result in diminished binding affinity, as it is the case for the EGFR. The EGFR calmodulin-binding site has nine net positive charges and an isoelectric point of 12.8, which contrasts with the isoelectric point of 6.2 of the whole receptor.

Binding of calmodulin to the EGFR juxtamembrane sequence occurs in a  $\text{Ca}^{2+}$ -dependent manner with a  $K'_{a(\text{Ca})}$  of 0.5  $\mu\text{M}$  free  $\text{Ca}^{2+}$ . Considering that the binding of  $\text{Ca}^{2+}$  to calmodulin exhibits two high-affinity ( $K_{a(\text{Ca})} \approx 0.07$ –0.17  $\mu\text{M}$ ) sites and two low-affinity ( $K_{a(\text{Ca})} \approx 0.6$ –0.9  $\mu\text{M}$ ) sites (40), it is expected that the four  $\text{Ca}^{2+}$ -binding sites of calmodulin should be occupied before calmodulin could bind to the EGFR juxtamembrane sequence with maximum efficiency. Since growth factors modulate the concentration of cytosolic free  $\text{Ca}^{2+}$ , changing from lower than  $10^{-7}$  M at resting state to higher than  $10^{-5}$  M after mitogenic stimulation (15, 19, 20), it is likely that binding of calmodulin to the EGFR could follow up the oscillations of intracellular free  $\text{Ca}^{2+}$  occurring under physiological conditions.

We have also shown that protein kinase C phosphorylated the Thr-654 residue of the EGFR present in the GST-HER<sub>(645–660)</sub> fusion protein and that this process was efficiently inhibited when binding of calmodulin was allowed to occur prior to phosphorylation. Calmodulin has been reported not to inhibit the phosphorylation of diverse substrates by protein kinase C of the same origin (rat brain) as that used in this work, such as syntide (41) and histone (42), or its self-phosphorylation, as observed by us (results not shown) and other authors (42, 43). Hence, binding of calmodulin to the EGFR juxtamembrane sequence could sterically hinder the interaction of protein kinase C with its phosphorylation site at Thr-654, hence accounting for the observed inhibition. This contention is supported by the high similarity between the  $K'_{d(\text{CaM})}$  and the  $K'_{i(\text{CaM})}$  values of 0.4  $\mu\text{M}$  and 0.5–1  $\mu\text{M}$ , respectively, determined using the fusion protein.

Reciprocally, phosphorylation of Thr-654 by protein kinase C prevented subsequent binding of calmodulin to the EGFR juxtamembrane sequence. Here a steric hindrance effect can



be ruled out, since (i) protein kinase C was present at a much lower molar ratio (150–3000 fold) than the fusion protein substrate, (ii) the same amount of protein kinase C used in the experiments of Figure 6 failed to inhibit calmodulin binding in standard cross-linkage assays, and (iii) protein kinase C in the absence of ATP did not prevent subsequent calmodulin binding (see Figure 6). Therefore, the incorporation of phosphate at Thr-654 appears to prevent the interaction of calmodulin with this site. The introduction of the phosphate negative charge on Thr-654 could form an ionic bond with an adjacent positively charged residue, which could induce a conformational change rendering this site less accessible by calmodulin.

Protein kinase C-phosphorylatable threonine and serine residues are often found within or near to calmodulin-binding sites. This obeys the fact that the consensus for protein kinase C phosphorylation contains basic amino acids on both sides of the phosphorylatable hydroxyamino acid (44). In cases where such circumstances concur, either or both inhibition of protein kinase C phosphorylation by calmodulin and inhibition of calmodulin binding by phosphorylation have been reported. In this context, calmodulin interferes with phosphorylation of calcineurin (EC 3.1.3.16) (41), neurogranin (45), and p68 RNA helicase (43) by protein kinase C. Furthermore, these mutual exclusion effects between calmodulin binding and protein kinase C phosphorylation have been described for the plasma membrane  $\text{Ca}^{2+}$ -ATPase (EC 3.6.1.38) (46), the myosin light chain kinase (EC 2.7.1.37) (47, 48), neuromodulin/P-57 (49), and the myristoylated alanine-rich protein kinase C substrate (50). Moreover, these antagonistic effects have also been reported for other protein kinases, as it is the case for the phosphorylation of the myosin light chain kinase by cAMP-dependent protein kinase (EC 2.7.1.37) and calmodulin binding to this protein (47).

The mutual exclusion between calmodulin-binding and protein kinase C phosphorylation occurring at the human EGFR juxtamembrane sequence raises the paradox that both processes are expected to become activated upon the increase in the cytosolic  $\text{Ca}^{2+}$  concentration that occurs upon EGF stimulation and to negatively modulate the activation of this receptor. Phosphorylation of the EGFR at Thr-654 by protein kinase C results in inhibition of its intrinsic tyrosine kinase activity, both *in vitro* and *in vivo* (7, 8) and in prevention of its long-term internalization and down regulation (9), which are two EGF-promoted processes. As for calmodulin, this protein has been well established by our group to act *in vitro* as a  $\text{Ca}^{2+}$ -dependent inhibitor of the tyrosine kinase activity of the EGFR from rat liver purified by calmodulin-affinity chromatography (21, 23). Therefore, we have hypothesized that binding of calmodulin to this juxtamembrane site could be responsible for the inhibition of the EGFR tyrosine kinase activity. However, we have so far failed to demonstrate this inhibitory effect of calmodulin on the human EGFR isolated from the fibroblasts, either purified by calmodulin-affinity chromatography or after immunoprecipitation from cell lysates (results not shown). One possibility to explain this discrepancy could be that the receptor could be prephosphorylated by protein kinase C in the cells before isolation. An additional possibility could be that the role of calmodulin in these cells were instead to prevent the negative effects of protein kinase C on the receptor intracellular processing. In this line of

thought, the EGFR–calmodulin complex could represent a species labeled to be targeted for its internalization, whereas the protein kinase C-phosphorylated receptor could constitute a standby desensitized form amenable to undergo a new EGF-mediated activation after its dephosphorylation by intracellular phosphatases (EC 3.1.3.16). The differential distribution of calmodulin and protein kinase C along the plasma membrane and/or their different affinities for  $\text{Ca}^{2+}$  and for the EGFR calmodulin-binding site would determine which of the two processes act first on this site during the EGF-mediated signaling cycle.

A number of additional regulatory interactions has been reported to occur within the EGFR juxtamembrane domain at amino acid stretches overlapping, or immediately following, the calmodulin-binding site described in this work: (i) the amino acid segment 660–667, and especially Arg-662, has been found to determine substrate recognition and hence the specificity of the mitogenic signaling by the receptor (11); (ii) two dileucine motifs at positions 658–659 and 679–680 are important for normal receptor expression and turnover (12); (iii) the stretch 644–666 is involved in the association of the EGFR with phosphoinositide kinases (EC 2.7.1.68), which become phosphorylated at tyrosine by the receptor (10); and (iv) the stretch 645–657 contacts with, and activates, the  $\alpha$ -subunit of the adenylyl cyclase (EC 4.6.1.1) GTP-binding regulatory protein  $G_{s\alpha}$  (13). Thus, the presence of a calmodulin-binding site and the possibility of cross talk between calmodulin and protein kinase C at this region opens a new level of complexity for the multiregulatory function of the EGFR juxtamembrane domain.

A search for sequence homologs of the human EGFR juxtamembrane calmodulin-binding site in available databases revealed a number of proteins containing identical or similar basic amphiphilic sequences (see Table 1). Interestingly, all the proteins with conserved cytosolic juxtamembrane sequences are vertebrate receptor tyrosine kinases and related oncogene products belonging to the EGFR family. As shown in Table 1, this sequence is 100% identical in the EGFRs from human, mouse, and chicken, the product of a fusion gene between parts of the chicken *c-erbB* and avian leukemia virus (ALV), and the product of the avian erythroblastosis virus (AEV) *v-erbB* oncogene. The calmodulin-binding sites of the rat and human EGFRs appear to be identical except for a single amino acid substitution introducing a negative charge in the former. Lower but significant homologies were found at this site for the platyfish *Xiphophorus maculatus* melanoma receptor tyrosine kinase Xmrk (75%), the rat and hamster Neu (69%), and the human ErbB-2 (63%), ErbB-4 (56%), and ErbB-3 (50%) proteins. Nevertheless, the conservation of basic amino acid residues at this site must be emphasized. The fact that the homology of this site in vertebrates is significantly higher than that found when the whole protein sequences are matched probably underlines its physiological relevance. To establish (i) whether the ability to bind calmodulin is maintained in the homolog sequences showing lower identities, (ii) whether the threonine residue present in most of them is phosphorylated by protein kinase C, and (iii) whether cross talk between protein kinase C phosphorylation and calmodulin binding exists should require experimental testing. On the other hand, the cytosolic juxtamembrane site in EGFR homologs among invertebrates, such as *Schistosoma man-*



Table 1: Sequence Homology between the Human EGFR Calmodulin-Binding Site and Other Proteins of the EGFR Family<sup>a</sup>

protein	calmodulin-binding site homolog	identity (%)	
		CaM-binding sequence	whole sequence
human EGFR (ErbB-1)	RRRHIVRKRTLRLQLQ		
mouse EGFR	.....	100	91
chicken EGFR	.....	100	77
ALV:chicken EGFR	.....	100	83
AEV v-ErbB	.....	100	83
rat EGFR <sup>b</sup>	...E.....	94	nd
platyfish Xmrk	...R•K•...I•C•...	75	56
rat Neu	...QKI•Y•M•...	69	52
hamster Neu	...QKI•Y•M•...	69	49
human ErbB-2	••QKQI•Y•M•...	63	52
human ErbB-4	••KS•KK•A•...F•-	56	50
human ErbB-3	•G•R•QN•AM•Y•E	50	43
<i>Schistosoma</i> SER	K•KAEAE•-I•EQ•R	25	25
<i>Caenorhabditis</i> LET-23	•CQR•GK•LKIAEMVD	19	33
<i>Drosophila</i> DER	C•QKQKA•KETVKMTM	13	41

<sup>a</sup> Multiple sequence alignment between the human EGFR and protein sequences of the EGFR family was carried out. Amino acid stretches aligning with the human EGFR calmodulin-binding site are shown using the single-letter code. Dots denote identity with the residue in the human EGFR, dashes denote unpaired residues, and amino acid replacements are indicated. The percentage of pairwise sequence identity is given for the various calmodulin-binding site counterparts and for the whole protein sequences. <sup>b</sup> Amino acid sequence derived from a cDNA sequence kindly provided by Dr. H. S. Earp from the University of North Carolina, Chapel Hill, NC. nd indicates not determined.

*soni*, *Caenorhabditis elegans*, and *Drosophila melanogaster*, lacks significant homology with their human counterpart, although a significant number of basic amino acids are present in this sequence (see Table 1).

The insulin receptor is the only receptor tyrosine kinase, in addition to the EGFR, for which binding of calmodulin is known to occur (51), although the calmodulin-binding site in the insulin receptor has not yet been identified. Future work should elucidate to which extent additional segments of the EGFR, if any, contribute to the binding of calmodulin to the receptor *in vivo*, if calmodulin-binding sites are present in other members of the tyrosine kinase receptor superfamily, and which is the regulatory role of calmodulin, if any, on these receptors.

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