Regulatory Interaction between Calmodulin and the Epidermal Growth Factor Receptor[a]

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Growth factor-stimulated cell proliferation is preceded by a transient increase in the cytoplasmic Ca^{2+} concentration,[1] and calmodulin, an intracellular calcium receptor protein,[2] appears to intervene in the modulation of this process.[3] Calmodulin binds to nuclear proteins and could therefore affect their functions.[4] However, calmodulin does not control cell proliferation by acting exclusively at the nuclear level. Several lines of evidence obtained in our laboratory indicate that the epidermal growth factor receptor (EGFR) is a calmodulin-binding protein and that calmodulin acts as a modulator of its intrinsic tyrosine kinase activity.[5,6]

We have demonstrated that the EGFR can be isolated from solubilized rat liver plasma membranes by calmodulin-affinity chromatography.[5,6] Binding of the EGFR to calmodulin-agarose occurs in the presence of Ca^{2+}, and its elution is achieved upon addition of EGTA. The preparation obtained contains a set of calmodulin-binding proteins associated with the plasma membrane. However, the major autophosphorylated protein in this preparation corresponds to the EGFR. This autophosphorylation is stimulated by epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) with essentially the same efficiency.[5] Furthermore, the isolated receptor presents both EGF- and TGFα-stimulated tyrosine kinase activity towards the exogenous substrate poly-L-(Glu : Tyr).[5,6] The identity of the isolated EGFR

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was confirmed by immunoblot analysis and immunoprecipitation using a polyclonal antibody against a human EGF receptor/c-*erb*B-2 product-common epitope.[5] FIGURE 1 shows the proteins that are phosphorylated in the absence (–) and presence (+) of EGF, using a solubilized plasma membrane preparation (panel 1) and the EGTA-eluted material from the calmodulin-affinity chromatography (panels 2 and 3), in assays carried out in the absence (panels 1 and 2) and presence (panel 3) of poly-L-(Glu : Tyr). The autophosphorylated EGFR is observed as a minor band in the solubilized plasma membrane fraction before calmodulin-affinity chromatography (panel 1). By contrast, the EGF-stimulated autophosphorylation of the EGFR (panel 2) and the EGF-stimulated phosphorylation of poly-L-(Glu : Tyr), which migrates in the gels as a smear (panel 3), are readily observable in the material eluted with EGTA from the calmodulin-agarose column.

Bovine brain calmodulin, which contains two tyrosine residues (Tyr99 and Tyr138), becomes phosphorylated in Tyr99 in the absence of Ca^{2+} and in an EGF-stimulated manner by the isolated EGFR.[6] Low concentrations of Ca^{2+} (around 1 μM) strongly inhibit the phosphorylation of calmodulin.[6] The phosphorylation of calmodulin is absolutely dependent on the presence of a polycation or a basic protein, and its stoichiometry is close to 1 mol of phosphate per mol of calmodulin.[5,6] However, calmodulins isolated from *Trypanosoma cruz,*[7] which contains a single tyrosine residue (Try138), and from *Leishmania mexicana* are phosphorylated in tyrosine by the EGFR only in trace amounts (results not shown). FIGURE 2 (panel 1) shows the phosphorylation of bovine brain calmodulin by the EGFR in the absence (–) and presence (+) of EGF and in the presence of poly-L-(Lys). The addition of EGF stimulates the phosphorylation of calmodulin 2.7-fold. As we have shown previously,[6] the presence of poly-L-(Lys) in the assay system causes the phosphorylation of other unidentified proteins present in the EGFR preparation (FIG. 2, panel 1).

Further evidence of the interaction of the EGFR with calmodulin was obtained by analyzing the effect of calmodulin on both the autophosphorylation of this receptor and its tyrosine-kinase activity toward exogenous substrates. We have demonstrated that calmodulin in the presence of Ca^{2+} inhibits both processes in a concentration-dependent mode.[5,6] FIGURE 2 (panel 2) shows the results of assays of phosphorylation of poly-L-(Glu : Tyr) by the isolated receptor in the absence (lane a) and presence (lanes b and c) of calmodulin. Calmodulin inhibits the phosphorylation of poly-L-(Glu : Tyr) by 42% and 72% after 1.5 min (lane b) and 40 min (lane c) of incubation, respectively. The inhibition of the autophosphorylation of the EGFR is also noticeable (lanes b and c). We have excluded the presence of calmodulin-dependent phosphatase activity in these preparations.[5] Therefore, calmodulin acts as a negative modulator of the tyrosine-kinase activity of the EGFR.

We have also demonstrated that when calmodulin is allowed to be phosphorylated by the tyrosine kinase of the EGFR prior to the phosphorylation of the substrate poly-L-(Glu : Tyr), the phosphorylation of this substrate is strongly enhanced (*results not shown*). This observation is compatible with a model in which phosphocalmodulin acts as a positive modulator of the tyrosine-kinase activity of the EGFR. However, the complexity of this two-step assay system justifies a cautious interpretation of these results.

In conclusion, we have demonstrated that calmodulin interacts, at least *in vitro,* with the EGFR in both Ca^{2+}-dependent and Ca^{2+}-independent modes. The actual

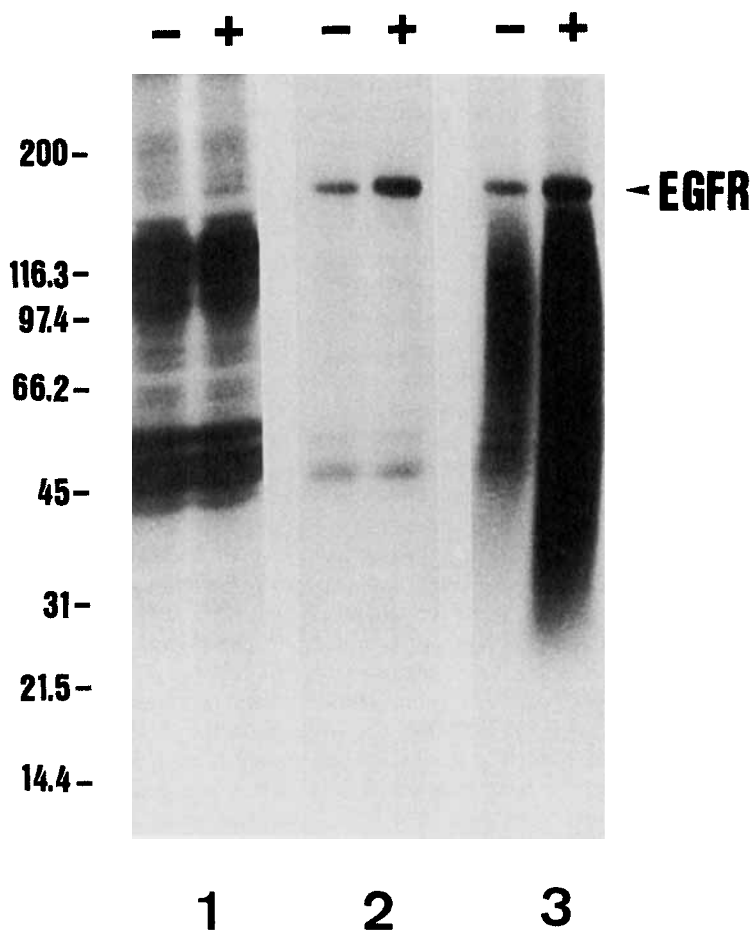

FIGURE 1. Activity of the EGFR isolated by calmodulin-affinity chromatography. A Triton X-100-solubilized plasma membrane fraction (30 μL) (panel 1) and the EGTA-eluted fraction (40 μL) from the subsequent calmodulin-affinity chromatography (panels 2 and 3) were assayed at 37°C for 3 min in 100 μL of a medium containing 15 mM Na-Hepes (pH 7.4), 6 mM $MgCl_2$, 0.4 mM EGTA, 0.4% (w/v) Triton X-100, 2% (w/v) glycerol, and 10 μM (2 μCi) $[\gamma - {}^{32}P]ATP$ in the absence (–) and presence (+) of 1 μM EGF, and in the absence (panels 1 and 2) and presence (panel 3) of 0.1 mg/mL poly-L-(Glu : Tyr). The fractions were incubated with EGF for 30 min on ice before the addition of radiolabeled ATP. The reactions were stopped with ice-cold 10% (w/v) trichloroacetic acid, and the precipitated proteins were analyzed by SDS-PAGE and autoradiography. The arrowhead points to the EGFR radiolabeled band. Molecular mass standards (kDa) are also indicated. Additional methodological information is given in refs. 5 and 6.

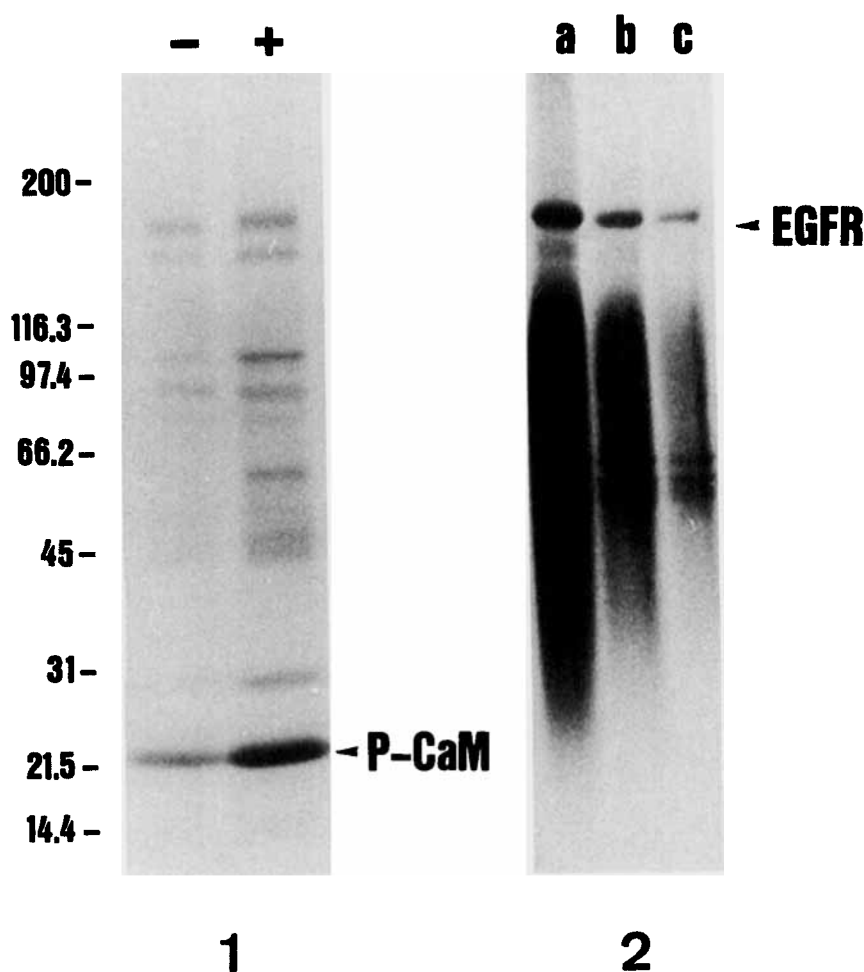

FIGURE 2. Calmodulin is phosphorylated by the isolated EGFR in the absence of Ca^{2+} but inhibits its tyrosine-kinase activity in the presence of Ca^{2+}. Panel 1: Calmodulin (1.2 μM) was phosphorylated using the EGFR-containing EGTA-eluted fraction (50 μL) from the calmodulin-affinity chromatography at 37°C for 5 min in 100 μL of a medium containing 15 mM Na-Hepes (pH 7.4), 6 mM $MgCl_2$, 0.5 mM EGTA, 0.5% (w/v) Triton X-100, 2.5% (w/v) glycerol, 0.5 μM poly-L-(Lys), and 10 μM (2 μCi) [γ-^{32}P]ATP in the absence (–) and presence (+) of 1 μM EGF. Panel 2: Poly-L-(Glu : Try) (0.1 mg/mL) was phosphorylated using the EGTA-eluted fraction (40 μL) from the calmodulin-affinity chromatography at 37°C for 1 min in 100 μL of a medium containing 15 mM Na-Hepes (pH 7.4), 6 mM $MgCl_2$, 0.4 mM EGTA, 0.5 mM $CaCl_2$ (100 μM free Ca^{2+}), 0.4% (w/v) Triton X-100, 2% (w/v) glycerol, 1 μM EGF, and 10 μM (2 μCi) [γ-^{32}P]ATP in the absence (lane a) and presence (lanes b and c) of 3 μM calmodulin. The EGFR-containing fraction was incubated with EGF for 40 min (lanes a–c) on ice, and calmodulin was added 1.5 min (lane b) or 40 min (lane c) before the addition of radiolabeled ATP. The reactions (panels 1 and 2) were stopped with ice-cold 10% (w/v) trichloroacetic acid, and the precipitated proteins were analyzed by SDS-PAGE and autoradiography. EGTA (10 mM) was added to the electrophoresis sample buffer in order to obtain migration of phosphocalmodulin at 21 kDa. Arrowheads point to the phosphocalmodulin (P-CaM) (panel 1) and to the autophosphorylated EGFR (panel 2) radiolabeled bands. Molecular mass standards (kDa) are also indicated. Additional methodological information is given in refs. 5 and 6.

calmodulin-binding site(s) in the EGFR has not yet been identified. However, an amphiphilic basic domain, a candidate to constitute an alpha-helical calmodulin-binding site, can be found in the juxtamembrane region of the cytoplasmic domain of the human EGFR.[5]

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