Evidence for the direct interaction between calmodulin and the human epidermal growth factor receptor

Hongbing LI[1] and Antonio VILLALOBO[2]

Instituto de Investigaciones Biomédicas, Consejo Superior de Investigaciones Científicas and Universidad Autónoma de Madrid, c/Arturo Duperier 4, E-28029 Madrid, Spain

Previous work from our laboratory has demonstrated that the Ca^{2+}–calmodulin complex inhibits the intrinsic tyrosine kinase activity of the epidermal growth factor receptor (EGFR), and that the receptor can be isolated by Ca^{2+}-dependent calmodulin-affinity chromatography [San José, Benguría, Geller and Villalobo (1992) J. Biol. Chem. **267**, 15237–15245]. Moreover, we have demonstrated that the cytosolic juxtamembrane region of the human receptor (residues 645–660) binds calmodulin in a Ca^{2+}-dependent manner when this segment forms part of a recombinant fusion protein [Martín-Nieto and Villalobo (1998) Biochemistry **37**, 227–236]. However, demonstration of the direct interaction between calmodulin and the whole receptor has remained elusive. In this work, we show that calmodulin, in the presence of Ca^{2+}, forms part of a high-molecular-mass complex built upon covalent cross-linkage of the human EGFR immunoprecipitated from two cell lines overexpressing this receptor. Although several calmodulin-binding proteins co-immunoprecipitated with the EGFR, suggesting that they interact with the receptor, we demonstrated using overlay techniques that biotinylated calmodulin binds directly to the receptor in a Ca^{2+}-dependent manner without the mediation of any adaptor calmodulin-binding protein. Calmodulin binds to the EGFR with an apparent dissociation constant (K_d') of approx. 0.2–0.3 μM. Treatment of cells with epidermal growth factor, or with inhibitors of protein kinase C and calmodulin-dependent protein kinase II, or treatment of the immunoprecipitated receptor with alkaline phosphatase, does not significantly affect the binding of biotinylated calmodulin to the receptor.

Key words: biotinylated calmodulin, calcium, covalent cross-linkage, overlay.

INTRODUCTION

The epidermal growth factor (EGF) receptor (EGFR) belongs to the superfamily of tyrosine kinase receptors located in the plasma membrane that upon ligand binding dimerizes and trans-phosphorylates. The resulting phosphotyrosine residues in the receptor recruit adaptor proteins and transduction systems which initiate signalling events to the cell interior to attain proliferative, differentiation, cell-survival and even cell-motility responses (for review, see [1–4]).

Signalling by the EGFR is accompanied in many cell types by a transient increase in the cytosolic concentration of free Ca^{2+} (for review, see [5]). This early calcium signal mediates the retro-inhibition of the intrinsic tyrosine kinase activity of the EGFR using regulatory Ca^{2+}-dependent serine/threonine-protein kinases that phosphorylate the receptor, such as protein kinase C [6–10] and calmodulin (CaM)-dependent protein kinase II [11–13]. In addition, the Ca^{2+}–CaM complex also regulates the tyrosine kinase activity of the EGFR acting on the receptor itself [5,14–16].

Thus in previous work from our laboratory we have demonstrated that the EGFR from rat liver and from murine fibroblasts overexpressing the human receptor can be isolated by Ca^{2+}-dependent CaM-affinity chromatography [14–16]. Furthermore, we have also shown that the Ca^{2+}–CaM complex directly inhibits the intrinsic tyrosine kinase activity of the receptor [5,14,15]. Moreover, using a recombinant fusion protein, we proved that the cytosolic juxtamembrane region (residues 645–660) of the human EGFR binds CaM in a Ca^{2+}-dependent fashion, and that there is cross-talk between the binding of CaM to this receptor segment and the phosphorylation of Thr^{654} by protein kinase C [16]. Hence, the binding of CaM to the fusion protein prevents Thr^{654} phosphorylation, and, conversely, the phosphorylation of this residue by protein kinase C prevents the CaM-binding process [5,16]. The CaM-binding site under consideration appears to be highly conserved in EGFRs from different species, as it is identical in human, mouse and chicken, which underscores its physiological significance [16]. Moreover, although there are some differences in their amino acid sequences, this site may retain its CaM-binding capacity in other ErbB receptor family members.

Despite the accumulated evidence, the direct interaction of CaM with the intact receptor has remained elusive and has not been demonstrated to date. In this study, we fill this gap by reporting the direct binding of CaM to the human EGFR isolated from two cell lines overexpressing this receptor. Moreover, we have demonstrated that the EGFR interacts with other CaM-binding proteins.

EXPERIMENTAL

Reagents

Monoclonal anti-CaM antibody (α-CaM) developed in mouse was obtained from Upstate Biotechnology, and monoclonal anti-EGFR antibodies (α-EGFRs) from clone 528 (recognizing

Abbreviations used: CaM, calmodulin; α-CaM, anti-CaM antibody; α-P-Tyr, anti-phosphotyrosine antibody; BS^3, bis(sulphosuccinimidyl) suberate; EGF, epidermal growth factor; EGFR, EGF receptor; α-EGFR, anti-EGFR antibody; EZ-link™ NHS-LC-biotin, succinimidyl-6-(biotinamido) hexanoate; GF109203X, 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide.

[1] Permanent address: Science and Research Centre, Shijiazhuang Medical School, 16 Zhongshan Xi Road, Shijiazhuang, Hebei 05008, People's Republic of China.

[2] To whom correspondence should be addressed (e-mail antonio.villalobo@iib.uam.es).

the extracellular domain of the human receptor) and from clone 13 (recognizing the intracellular segment 996–1022 of the human receptor) developed in mouse were obtained from Oncogene and Transduction Laboratories, respectively. The monoclonal anti-phosphotyrosine antibody RC20 (α-P-Tyr) conjugated to horseradish peroxidase was also obtained from Transduction Laboratories. Anti-mouse IgG (Fc-specific) developed in goat and conjugated to horseradish peroxidase, alkaline phosphatase from bovine intestinal mucosa, deoxycholic acid (sodium salt), sodium orthovanadate, leupeptin, pepstatin A, aprotinin, PMA and PMSF were purchased from Sigma. The prestained molecular-mass standards for electrophoresis were obtained from Bio-Rad. From Pierce were purchased bis(sulphosuccinimidyl) suberate (BS^3), EZ-link™ NHS-LC-biotin [succinimidyl-6-(biotinamido) hexanoate] and streptavidin conjugated to horseradish peroxidase. BioTrace™ PVDF membranes were purchased from Pall Gelman Laboratory, and ECL™ was obtained from Amersham Bioscience. Mouse IgG serum fraction, GF109203X {2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide} and KN93 were obtained from Calbiochem. *Escherichia coli* cultures expressing recombinant rat CaM lacking its N-terminal methionine and post-translational modifications [17] was a kind gift of Professor Nobuhiro Hayashi (Fujita Health University, Aichi, Japan). Other chemicals were of analytical grade.

Cell cultures

Human epidermoid carcinoma A431 cells and EGFR-T17 murine fibroblasts overexpressing the human EGFR were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal bovine serum, 2 mM L-glutamine and 40 μg/ml gentamicin in a humidified atmosphere of 5% (v/v) CO_2 in air at 37 °C. The cells were maintained overnight in a foetal bovine serum-free medium before performing the experiments.

Purification of recombinant CaM and preparation of biotinylated CaM

Recombinant rat CaM was purified from *E. coli* as described in [17], except that the soluble cell extract was heated at 95 °C for 5 min before the heat-resistant proteins of the supernatant were subjected to phenyl-Sepharose chromatography. The concentration of CaM was determined spectrophotometrically at 276 nm using a 1% (w/v) absorption coefficient of 1.8. Purified CaM was biotinylated using EZ-link™ NHS-LC-biotin as described in [18].

EGFR immunoprecipitation

Serum-starved confluent cells grown in dishes of 15 cm diameter were incubated in the absence and presence of 10 nM EGF for 1–5 min at room temperature. Thereafter, the medium was removed and 1 ml of a lysis buffer, containing 50 mM Tris/HCl (pH 8), 150 mM NaCl, 1 mM EGTA, 1% (w/v) Triton X-100, 0.5% (w/v) deoxycholic acid, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin A and 10 μg/ml aprotinin, was added and incubated for 30 min on ice. If not indicated otherwise, the cell lysate was prepared in the presence of EGTA. The cell extract was collected by centrifugation on a bench-top centrifuge at 15600 $\boldsymbol{g}_{max}$ for 35 min, and 2–8 mg of solubilized proteins was incubated for 3–12 h in 200 μl of a medium containing 50 mM Tris/HCl (pH 8), 150 mM NaCl, 1 mM sodium orthovanadate, 1 mM PMSF and 1.5 μg of α-EGFR (recognizing the extracellular domain) precoupled to 30 μl of a slurry of Protein A–agarose. The beads were collected by low-speed centrifugation and washed six times in 50 mM Tris/HCl (pH 8), 150 mM NaCl, 1 mM sodium orthovanadate and 1 mM PMSF (TNOP buffer). The samples were boiled for 5 min in Laemmli sample buffer, the beads were removed by low-speed centrifugation, and the supernatant was processed by SDS/PAGE as described below. Control mock immunoprecipitations were performed using a non-relevant mouse IgG fraction instead of the α-EGFR, with negative results.

Western-blot analysis

Proteins in the gel were electrotransferred to a PVDF membrane in a medium containing 48 mM Tris-base, 36.6 mM glycine, 20% (v/v) methanol and 0.04% (w/v) SDS (TGMS medium), and the proteins were fixed with 0.2% (v/v) glutaraldehyde for 45 min in a medium containing 0.1% (w/v) Tween 20, 100 mM Tris/HCl (pH 8.8), 500 mM NaCl and 0.25 mM KCl (T-TBS medium). The PVDF membrane was blocked with 5% (w/v) BSA in T-TBS medium, and probed with α-P-Tyr conjugated to horseradish peroxidase, α-EGFR (recognizing the intracellular domain) or α-CaM at dilutions of 1/1000 to 1/3000. Appropriate secondary antibodies coupled to horseradish peroxidase at a dilution of 1/3000 were used as required. The positive bands were developed using the enhanced chemiluminescence Luminol (ECL™) method following instructions from the manufacturer. When required, re-probing the PVDF membrane with a different antibody was performed after stripping the membrane in a medium containing 100 mM β-mercapthoethanol, 2% (w/v) SDS and 62.5 mM Tris/HCl (pH 6.7) at 50 °C for 30 min or after overnight incubation at room temperature.

CaM cross-linking experiments

Protein A–agarose/α-EGFR beads containing immunoprecipitated EGFR were washed four times in TNOP buffer and resuspended in a medium containing 20 mM Hepes/NaOH (pH 7.5), 150 mM NaCl, 0.2 mM $CaCl_2$, 1 mM sodium orthovanadate and 1 mM PMSF (linkage buffer). Free CaM or biotinylated CaM (0.45 μg) was added to 1 ml of the immunoprecipitated receptor preparation and incubated for 3 h at 4 °C. Thereafter, 1 mM BS^3 was added and incubated for 1 h at 4 °C. Controls containing 1 mM EGTA instead of $CaCl_2$ were performed. The beads were washed four times in linkage buffer, boiled for 5 min in Laemmli sample buffer and processed for electrophoresis. The proteins were electrotransferred to a PVDF membrane using TGMS medium, and fixed with 0.2% (v/v) glutaraldehyde for 45 min in T-TBS medium. The PVDF membrane was blocked with 5% (w/v) BSA in T-TBS medium and probed with the different antibodies as described above.

When biotinylated CaM was used, streptavidin conjugated to horseradish peroxidase at a 1/3000 dilution was added and incubated for 30 min at 37 °C or for 1 h at room temperature. The positive bands were developed using the ECL™ method following instructions from the manufacturer. Controls in the absence of CaM or biotinylated CaM, and in the absence of any primary antibody, were developed with horseradish peroxidase-conjugated streptavidin.

CaM overlay experiments

The whole-cell extract or the immunoprecipitated EGFR prepared as described above was subjected to electrophoresis, and the proteins were electrotransferred to a PVDF membrane using TGMS medium and fixed with 0.2% (v/v) glutaraldehyde for 45 min in T-TBS medium. The PVDF membrane was blocked with 5% (w/v) BSA in T-TBS medium and incubated with

0.45 μg/ml biotinylated CaM in the presence of 0.2 mM $CaCl_2$ for 30 min at 37 °C or overnight at 4 °C. Controls containing 1 mM EGTA instead of $CaCl_2$ were performed. After extensive washing of the PVDF membrane in the same medium, streptavidin conjugated to horseradish peroxidase (1/3000 dilution) was added and incubated for 30 min at 37 °C or for 1 h at room temperature. The positive bands were developed using the ECL™ method.

Other analytical procedures

Slab gel electrophoreses were performed according to Laemmli [19] at 12 mA overnight in a linear gradient 5–20 % (w/v) polyacrylamide gel in the presence of 0.1 % (w/v) SDS at pH 8.3. Protein concentrations were determined using bicinchoninic acid and copper sulphate (BCA method; Sigma) following the manufacturer's instructions and using BSA as a standard. The intensity of the bands present on the X-ray films after ECL™ development was quantified by a computer-assisted scanner using the NIH Image 1.59 program.

RESULTS

Detection of CaM-binding proteins in whole-cell extracts by overlay with biotinylated CaM

To detect the major CaM-binding proteins present in cell-free extracts from two cell lines overexpressing the human EGFR, we performed overlay experiments using biotinylated CaM. Figure 1 shows that A431 tumour cells (Figure 1, lanes 1) and EGFR-T17 fibroblasts (Figure 1, lanes 2) contain different proteins that bind CaM in a Ca^{2+}-dependent manner (Figure 1, Ca^{2+}). Particularly interesting is a set of high-molecular-mass CaM-binding proteins in the range of approx. 140–220 kDa, as the EGFR has an apparent molecular mass of 170 kDa. All the CaM-binding proteins detected in these experiments require Ca^{2+} for the binding process. Indeed, the bands observed in the absence of calcium (Figure 1, EGTA) bound unspecifically to peroxidase-conjugated streptavidin, as they were also detected when biotinylated CaM was not used (Figure 1, Control).

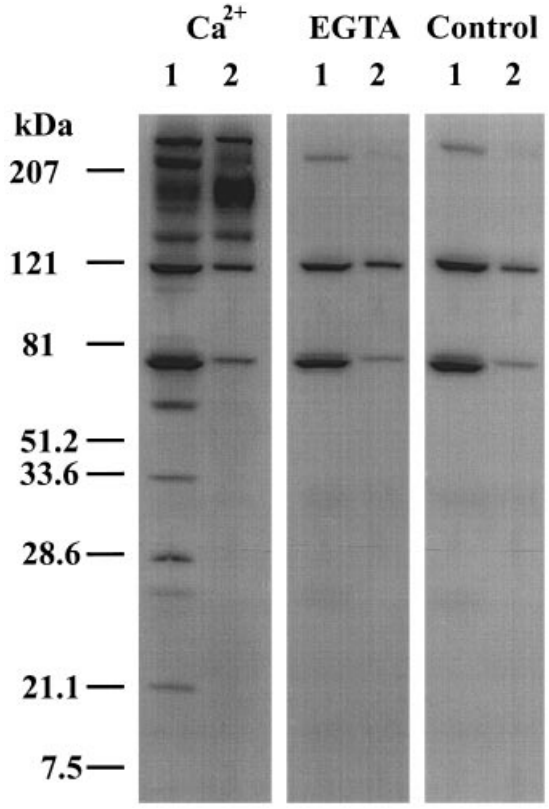

Figure 1 CaM-binding proteins in A431 tumour cells and EGFR-T17 fibroblasts

Whole-cell extracts (50 μg of protein) of A431 tumour cells (lanes 1) and EGFR-T17 fibroblasts (lanes 2) were subjected to overlay with biotinylated CaM in the presence (Ca^{2+}) and absence (EGTA) of calcium as described in the Experimental section. Non-specific binding was detected in the absence of biotinylated CaM (Control).

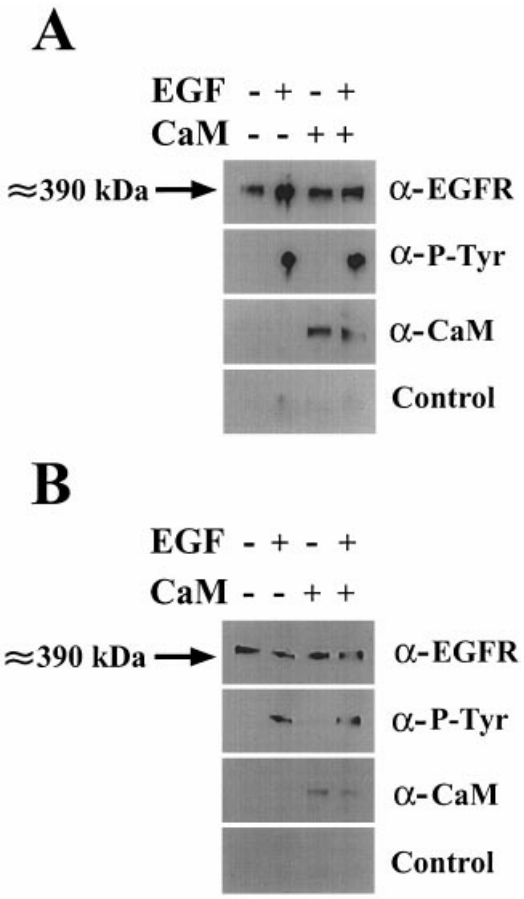

Figure 2 Covalent cross-linkage of CaM to a human EGFR complex

The immunoprecipitated EGFR from 3.5 mg of protein cell extract of A431 tumour cells (**A**) and from 2 mg of protein cell extract of EGFR-T17 fibroblasts (**B**) in non-stimulated cells (−) or cells treated with 10 nM EGF for 5 min (+), was subjected to covalent cross-linkage with BS^3 in the absence (−) and presence (+) of CaM as indicated. Separate samples were probed with an α-EGFR, an α-P-Tyr or an α-CaM as described in the Experimental section. Experiments performed in the absence of any primary antibody (Control) are also presented. The arrow points to the approx. 390 kDa complex.

Covalent cross-linkage of CaM to a human EGFR complex

To determine whether CaM could be associated with the EGFR, we performed cross-linkage experiments using the immunoprecipitated receptor from A431 tumour cells (Figure 2A) and EGFR-T17 fibroblasts (Figure 2B). Treatment of the immunoprecipitated receptor with the cross-linking agent BS^3 induced the formation of a high-molecular-mass complex of approx.

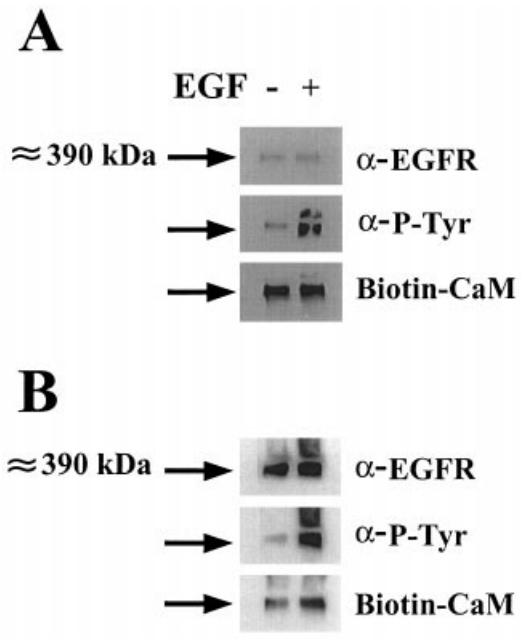

Figure 3 Covalent cross-linkage of biotinylated CaM to a human EGFR complex

The immunoprecipitated EGFR from 3 mg of protein cell extract of A431 tumour cells (**A**) and from 2.5 mg of protein cell extract of EGFR-T17 fibroblasts (**B**) in cells treated (+) or not (−) with 10 nM EGF for 5 min, were subjected to covalent cross-linkage with BS^3 in the presence of 0.45 μg/ml biotinylated CaM. Separate samples were probed with α-EGFR, α-P-Tyr or streptavidin conjugated to peroxidase (Biotin-CaM) as described in the Experimental section. The arrow points to the approx. 390 kDa complex.

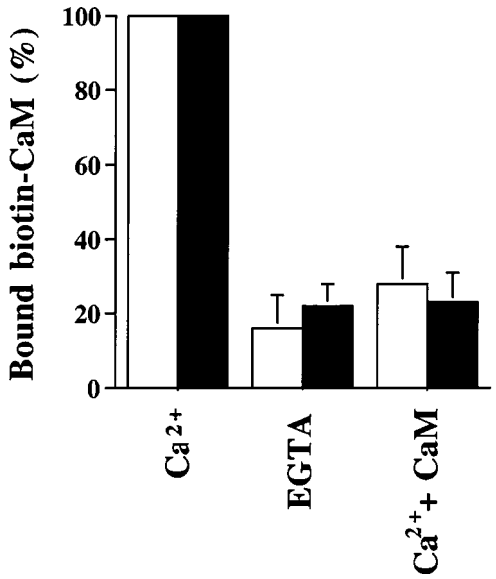

Figure 4 Ca^{2+}-dependent binding of biotinylated CaM to the human EGFR complex is chased by free CaM

The immunoprecipitated EGFR from 4–7 mg of protein cell extract of A431 tumour cells (open bars) and from 2.3–2.5 mg of protein cell extract of EGFR-T17 fibroblasts (filled bars) in non-stimulated cells was subjected to covalent cross-linkage in the presence of 0.45 μg/ml biotinylated CaM in the presence (Ca^{2+}) and absence (EGTA) of calcium, and in the presence of calcium plus 45 μg/ml free CaM (Ca^{2+} + CaM) as indicated. The PVDF membrane was probed with peroxidase-conjugated streptavidin to detect the binding of biotinylated CaM. Thereafter, the membrane was stripped and re-probed with an α-EGFR, to determine the amount of receptor present, as described in the Experimental section. The plot shows the mean ± S.E.M. densitometric intensities of the signals obtained for the biotin-CaM/α-EGFR ratio from three independent experiments.

390 kDa that was recognized when probed with α-EGFR and α-P-Tyr, when the cells were pre-treated with EGF, and with α-CaM, when CaM was added. However, no CaM signal was detected in the complex when this regulatory protein was absent in the assay system. Experiments performed in the absence of any primary antibody gave no detectable signals (Figure 2, Control).

We have also performed the experiments using biotinylated CaM instead of free CaM (Figure 3). Thus the high-molecular-mass complex of approx. 390 kDa was detected when probed with α-EGFR and α-P-Tyr, most significantly when the cells were treated with EGF, and when using steptavidin conjugated to peroxidase, which detects biotinylated CaM. Similar results were obtained with the immunoprecipitated receptor from A431 tumour cells (Figure 3A) and EGFR-T17 fibroblasts (Figure 3B).

Figure 4 shows that the relative amount of biotinylated CaM present in the approx. 390 kDa complex formed upon covalent cross-linkage of the immunoprecipitated EGFR in the presence of calcium (Figure 4, Ca^{2+}) diminishes dramatically when the experiments were performed in the absence of calcium and presence of a chelating agent (EGTA), or when an excess of free CaM in the presence of calcium (Figure 4, Ca^{2+} + CaM) was added before the cross-linking agent BS^3. The results were essentially identical using the immunoprecipitated receptor from A431 tumour cells (Figure 4, open bars) and EGFR-T17 fibroblasts (Figure 4, filled bars).

Direct binding of CaM to the human EGFR

The immunoprecipitated EGFR preparations used to perform the cross-linkage experiments could have contained some co-immunoprecipitated adaptor proteins or proteins involved in signalling mediated by this receptor. Therefore it was difficult to evaluate the real composition of the high-molecular-mass (approx. 390 kDa) complex formed upon covalent cross-linkage, besides the fact that the EGFR and CaM (when added) form part of this complex. Moreover, it is possible that the presence of

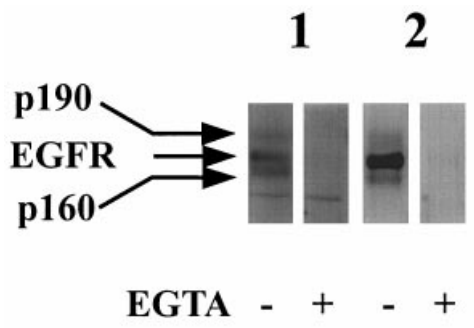

Figure 5 Direct binding of biotinylated CaM to the human EGFR and to the EGFR-associated p190 and p160 CaM-binding proteins

The immunoprecipitated EGFR from 4 mg of protein cell extract of A431 tumour cells (lanes 1) and from 2 mg of protein cell extract of EGFR-T17 fibroblasts (lanes 2) of non-stimulated cells, was subjected to overlay with 0.45 μg/ml biotinylated CaM in the presence of 0.2 mM $CaCl_2$ (lanes 1 and 2, left-hand lanes) as described in the Experimental section. Controls in the presence of EGTA are also presented (lanes 1 and 2, right-hand lanes). The arrows point to EGFR and the p190 and p160 CaM-binding proteins.

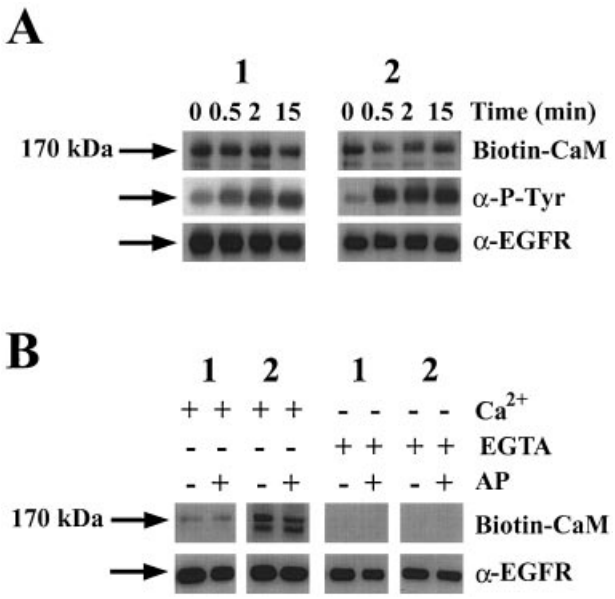

Figure 6 Binding of biotinylated CaM to the human EGFR was not affected by EGF or alkaline phosphatase treatment

(**A**) The immunoprecipitated EGFR from 7 mg of protein cell extract of A431 tumour cells (panel 1) and from 1.8 mg of protein cell extract of EGFR-T17 fibroblasts (panel 2) of non-stimulated cells or cells stimulated with 10 nM EGF for the indicated periods of time, was subjected to overlay with 0.45 μg/ml biotinylated CaM in the presence of 0.2 mM $CaCl_2$. The PVDF membrane was sequentially stripped and re-probed first with an α-P-Tyr and thereafter with an α-EGFR, as described in the Experimental section. The arrow points to the 170 kDa EGFR. (**B**) The immunoprecipitated EGFR from 4 mg of protein cell extract of A431 tumour cells (lanes 1) and from 2.5 mg of protein cell extract of EGFR-T17 fibroblasts (lanes 2) in non-stimulated cells was incubated in the absence (−) and presence (+) of 3 units of alkaline phosphatase (AP) for 30 min at 30 °C in a medium containing 50 mM Tris/HCl (pH 9), 5 mM $MgCl_2$ and 135 mM NaCl. The samples were subjected to overlay with 0.45 μg/ml biotinylated CaM in the presence of 0.4 mM $CaCl_2$ or 1 mM EGTA as indicated. The PVDF membrane was stripped and re-probed with an α-EGFR as described in the Experimental section. The arrow points to the 170 kDa EGFR.

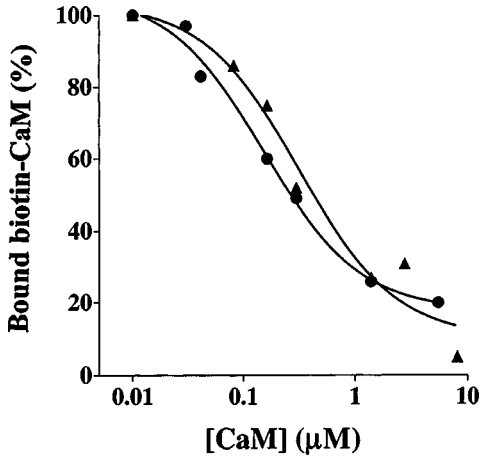

Figure 7 Apparent affinity constant of the human EGFR for CaM

The immunoprecipitated EGFR from 4 mg of protein cell extract of A431 tumour cells (●) and from 2 mg of protein cell extract of EGFR-T17 fibroblasts (▲) in non-stimulated cells was subjected to overlay with 0.45 μg/ml biotinylated CaM in the presence of 0.2 mM $CaCl_2$ and the indicated concentrations of free CaM as described in the Experimental section. The percentage of biotinylated CaM bound to the EGFR is plotted against free CaM concentration.

unidentified EGFR-associated CaM-binding protein(s) were responsible for the binding of CaM to this complex. Therefore, it was necessary to use a different technique to see whether CaM binds directly to the receptor. For this purpose, we overlaid the immunoprecipitated EGFR using biotinylated CaM (see Figure 5). Upon development with streptavidin conjugated to horseradish peroxidase, which detects biotinylated CaM, we indeed demonstrated the presence of several positive bands of 190 kDa (Figure 5, p190), 170 kDa (Figure 5, EGFR) and 160 kDa (Figure 5, p160), in both A431 tumour cells (Figure 5, lane 1, left) and EGFR-T17 fibroblasts (Figure 5, lane 2, left). Control experiments performed in the presence of EGTA (Figure 5, lanes 1 and 2, right) are also shown. Moreover, additional controls performed in the absence of biotinylated CaM only detected the faint approx. 130 kDa band present in A431 tumour cells (results not shown).

p190 and p160 appear to be co-immunoprecipitated CaM-binding proteins associated with the EGFR. However, to ascertain that the prominent 170 kDa CaM-binding protein observed in these experiments was indeed the EGFR, we performed similar overlay experiments with biotinylated CaM, and re-probed the PVDF membrane to look for the presence of the receptor itself. Figure 6(A) shows that the 170 kDa CaM-binding protein also gave a positive signal when probed with α-EGFR. Moreover, the binding of biotinylated CaM to the receptor was not affected significantly by the treatment of A431 tumour cells (Figure 6A, panel 1) or EGFR-T17 fibroblasts (Figure 6A, panel 2) with 10 nM EGF for increasing periods of time. As expected, however, the incubation of cells with EGF increased the trans-phosphorylation of the receptor, as detected using α-P-Tyr. Experiments performed using increasing concentrations of EGF gave similar results (results not shown).

The EGFR can also be phosphorylated by regulatory serine/threonine-protein kinases, such as protein kinase C [6–10] and CaM-dependent protein kinase II [11–13]. Moreover, we have shown previously that the binding of CaM to a fusion protein containing the juxtamembrane region (residues 645–660) of the human EGFR was prevented by the phosphorylation of Thr^{654} by protein kinase C [16]. Therefore, we decided to test whether the binding of biotinylated CaM to the whole receptor could be affected by its phosphorylation mediated by these regulatory serine/threonine-protein kinases.

Experiments performed in the presence of GF109203X and KN93, cell-permeant inhibitors of protein kinase C and CaM-dependent protein kinase II, respectively, demonstrated that the binding of biotinylated CaM to the receptor immunoprecipitated from both A431 tumour cells and EGFR-T17 fibroblasts was not affected significantly by the presence of these inhibitors (results not shown). Moreover, the chronic treatment of A431 tumour cells with the phorbol ester PMA, used to down-regulate protein kinase C, did not affect the binding process significantly (results not shown). Nevertheless, as the basal phosphorylation state of the immunoprecipitated EGFR catalysed in the cells by regulatory serine/threonine-protein kinases was not known, it was of interest to test whether the treatment of the immunoprecipitated receptor with alkaline phosphatase modified the binding of biotinylated CaM. Figure 6(B) shows that this was not the case in the receptor from A431 tumour cells (Figure 6B, lanes 1) or EGFR-T17 fibroblasts (Figure 6B, lanes 2). Treatment with alkaline phosphatase dephosphorylated the EGFR when probed with α-P-Tyr (results not shown). Moreover, the same Figure also shows that the binding of biotinylated CaM to the receptor was

dependent on the presence of Ca^{2+}, as EGTA totally prevented this process.

Determination of K'_d for CaM in the human EGFR

Using the overlay technique with biotinylated CaM described above, we determined the apparent dissociation constant (K'_d) of CaM for the whole human EGFR. Figure 7 shows that the amount of biotinylated CaM bound to the receptor decreased progressively in the presence of increasing concentrations of free CaM. From this plot we determined a K'_d value for CaM of 0.17 μM in the receptor from A431 tumour cells (Figure 7, ●), and of 0.34 μM in the receptor from EGFR-T17 fibroblasts (Figure 7, ▲). We also determined that the K'_d values for CaM of the p190 and p160 CaM-binding proteins co-immunoprecipitated with the EGFR from both cell types were below 0.1 μM (results not shown).

DISCUSSION

The calcium signal generated by the EGFR is of paramount importance for the regulation of the activity and fate of the receptor [5]. In this study we demonstrate for the first time that CaM binds directly to the EGFR. This could explain the inhibitory action of this modulator on the tyrosine kinase activity of the receptor [5,14,15].

The covalent complex formed upon incubation of the EGFR with the cross-linking agent BS^3 had an apparent molecular mass of approx. 390 kDa. However, this complex had no detectable differences in electrophoretic mobility when the experiments were performed in the absence or presence of CaM (see Figure 2). This result is somewhat surprising. However, it is important to keep in mind that the molecular mass of CaM (16.5 kDa) is less than 10% of the molecular mass of an EGFR monomer (170 kDa), and less than 5% of the molecular mass of the dimeric form of the receptor (340 kDa). Therefore, the expected change in electrophoretic mobility is near the limits of the resolution of the SDS/PAGE system used. Nevertheless, the covalent complex appeared to be significantly larger than the one expected to be formed by one EGFR dimer bound to one or two CaM molecules (approx. 356 and 370 kDa, respectively). This suggests that some additional unidentified proteins may form part of this complex. However, the IgG used to immunoprecipitate the receptor does not form part of the approx. 390 kDa complex formed upon treatment with the cross-linkage agent, as no signal was detected in the immunoblots performed in the presence of anti-IgG antibodies and in the absence of any primary antibody (see Figure 2).

The cross-linkage experiments, therefore, did not answer the question of whether or not CaM binds directly to the EGFR. To solve this problem, we overlaid the immunoprecipitated EGFR with biotinylated CaM. The experiments performed with this technique showed unequivocally that CaM indeed binds directly to the receptor. Moreover, we detected the presence of additional CaM-binding proteins in the immunoprecipitated EGFR preparations. Thus we observed the presence of p190 and p160, two conspicuous Ca^{2+}-dependent CaM-binding proteins of unknown nature, which probably interact with the EGFR. We noticed that the relative proportions of the signal intensities for biotinylated CaM bound to p190 and p160 CaM-binding proteins and to the EGFR varied somewhat between preparations. In general, when the binding of biotinylated CaM to p190 and p160 was very strong the binding of this modulator to the receptor was weaker. Conversely, a strong binding of biotinylated CaM to the EGFR was accompanied by weaker binding to the p190 and p160 CaM-binding proteins. This was independent of the amount of immunoprecipitated EGFR used in each experiment. Moreover, we occasionally obtained certain preparations where the binding of biotinylated CaM to the EGFR was very weak or absent. We also occasionally detected the presence of additional CaM-binding proteins co-immunoprecipitated with the receptor; particularly, an approx. 210 kDa protein phosphorylated at tyrosine residues, which decreases its phosphorylation upon activation of the receptor with EGF (results not shown). The variability in the association of CaM with the different targets suggests that there could exist very dynamic interactions between the triad formed by this regulator, the EGFR and the receptor-associated CaM-binding proteins. The reasons for these changes are not yet clear. However, it is tempting to speculate that it could be due to subtle variations in the physiology of the cell, such as those imposed by the extent of cellular confluence in the cultures, the actual number of intercellular contacts established among the cells, and/or the ageing of the cell cultures.

In this study we have shown that the binding of CaM to the EGFR was not affected by the activation of the receptor by EGF, the treatment of cells with inhibitors of regulatory protein-serine/threonine kinases acting on the EGFR or the addition of alkaline phosphatase to the immunoprecipitated receptor. However, it is difficult to ascertain whether or not the phosphorylation state of the receptor could affect the binding of CaM. This is due to the fact that the proportion of high- and low-affinity receptors that are activated on the cell surface upon ligand binding in our experimental conditions is not known. Recently it has been shown by fluorescence single-molecule imaging that the great majority of the high-affinity receptors at the surface of living cells are activated upon addition of EGF [20]. However, the presence of a large number of low-affinity non-active receptors on the cell surface could mask the effect of phosphorylation on the binding of CaM to the receptor. Moreover, the simple determination of the relative number of high- and low-affinity receptors at the cell surface may not be any help, as frequently the non-linear Scatchard plots obtained may hinder analysis of the data [21].

Interestingly, the K'_d value for CaM determined in this work for the whole human EGFR (approx. 0.2–0.3 μM) was very close to the K'_d determined (0.4 μM) for the cytosolic juxtamembrane region of the human receptor when forming part of a fusion protein [16]. Moreover, the apparent inhibition constant of CaM for the tyrosine kinase activity of the EGFR from rat liver was demonstrated to be close to 1 μM [14]. These observations suggest that the cytosolic juxtamembrane segment (residues 645–660) of the EGFR could indeed be responsible for the binding of CaM in the intact receptor, and that the inhibitory action exerted by the Ca^{2+}–CaM complex was mediated by its direct binding to the receptor at this site.

The modulation of the EGFR by CaM may be more complex than expected, as this calcium modulator not only controls the activity of CaM-dependent protein kinase II, which phosphorylates and inhibits the intrinsic tyrosine kinase of the receptor [11–13], but also performs this inhibition by binding directly to the receptor ([5,14–16], and this study). Moreover, CaM may possibly have additional roles to do with the activity and/or fate of the receptor by interacting with p190 and p160, two EGFR-associated CaM-binding proteins detected in this work. Therefore, it is expected that the trio formed by CaM, regulatory CaM-dependent protein kinases and EGFR-associated CaM-binding proteins may have physiological significance in the modulation of the receptor in intact cells.

We wish to thank Professor Nobuhiro Hayashi (Fujita Health University, Aichi, Japan) for the generous gift of bacteria expressing recombinant CaM, and Dr José Martín-

Nieto (Universidad de Alicante, Alicante, Spain) for helpful discussion. This work was financed by grants (to A.V.) from the Comisión Interministerial de Ciencia y Tecnología (SAF99-0052) and the Agencia Española de Cooperación Internacional (99CN0011).

REFERENCES

1 Ullrich, A. and Schlessinger, J. (1990) Signal transduction by receptors with tyrosine kinase activity. Cell **61**, 203–212
2 Weiss, A. and Schlessinger, J. (1998) Switching signals on or off by receptor dimerization. Cell **94**, 277–280
3 Zwick, E., Hackel, P. O., Prenzel, N. and Ullrich, A. (1999) The EGF receptor as central transducer of heterologous signalling systems. Trends Pharmacol. Sci. **20**, 408–412
4 Schlessinger, J. (2000) Cell signaling by receptor tyrosine kinases. Cell **103**, 211–225
5 Villalobo, A., Ruano, M. J., Palomo-Jiménez, P. I., Li, H. and Martín-Nieto, J. (2000) The epidermal growth factor receptor and the calcium signal. In Calcium: The Molecular Basis of Calcium Action in Biology and Medicine (Pochet, R., Donato, R., Haiech, J., Heizmann, C. and Gerke, V., eds.), pp. 287–303, Kluwer Academic Publishers, Boston
6 Hunter, T., Ling, N. and Cooper, J. A. (1984) Protein kinase C phosphorylation of the EGF receptor at a threonine residue close to the cytoplasmic face of the plasma membrane. Nature (London) **311**, 480–483
7 Davis, R. J. (1988) Independent mechanisms account for the regulation by protein kinase C of the epidermal growth factor receptor affinity and tyrosine-protein kinase activity. J. Biol. Chem. **263**, 9462–9469
8 Livneh, E., Dull, T. J., Berent, E., Prywes, R., Ullrich, A. and Schlessinger, J. (1988) Release of a phorbol ester-induced mitogenic block by mutation at Thr-654 of the epidermal growth factor receptor. Mol. Cell. Biol. **8**, 2302–2308
9 Countaway, J. L., McQuilkin, P., Girones, N. and Davis, R. J. (1990) Multisite phosphorylation of the epidermal growth factor receptor. Use of site-directed mutagenesis to examine the role of serine/threonine phosphorylation. J. Biol. Chem. **265**, 3407–3416

Received 14 September 2001/29 November 2001; accepted 3 January 2002

10 Lund, K. A., Lazar, C. S., Chen, W. S., Walsh, B. J., Welsh, J. B., Herbst, J. J., Walton, G. M., Rosenfeld, M. G., Gill, G. N. and Wiley, H. S. (1990) Phosphorylation of the epidermal growth factor receptor at threonine 654 inhibits ligand-induced internalization and down-regulation. J. Biol. Chem. **265**, 20517–20523
11 Countaway, J. L., Nairn, A. C. and Davis, R. J. (1992) Mechanism of desensitization of the epidermal growth factor receptor protein-tyrosine kinase. J. Biol. Chem. **267**, 1129–1140
12 Theroux, S. J., Latour, D. A., Stanley, K., Raden, D. L. and Davis, R. J. (1992) Signal transduction by the epidermal growth factor receptor is attenuated by a COOH-terminal domain serine phosphorylation site. J. Biol. Chem. **267**, 16620–16626
13 Feinmesser, R. L., Wicks, S. J., Taverner, C. J. and Chantry, A. (1999) Ca^{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. J. Biol. Chem. **274**, 16168–16173
14 San José, E., Benguría, A., Geller, P. and Villalobo, A. (1992) Calmodulin inhibits the epidermal growth factor receptor tyrosine kinase. J. Biol. Chem. **267**, 15237–15245
15 Benguría, A., Martín-Nieto, J., Benaim, G. and Villalobo, A. (1995) Regulatory interaction between calmodulin and the epidermal growth factor receptor. Ann. N.Y. Acad. Sci. **766**, 472–476
16 Martín-Nieto, J. and Villalobo, A. (1998) The human epidermal growth factor receptor contains a juxtamembrane calmodulin-binding site. Biochemistry **37**, 227–236
17 Hayashi, N., Matsubara, M., Takasaki, A., Titani, K. and Taniguchi, H. (1998) An expression system of rat calmodulin using T17 phage promoter in *Escherichia coli*. Protein Expression Purif. **12**, 25–28
18 Billingsley, M. L., Pennypacker, K. R., Hoover, C. G., Brigati, D. J. and Kincaid, R. L. (1985) A rapid and sensitive method for detection and quantification of calcineurin and calmodulin-binding proteins using biotinylated calmodulin. Proc. Natl. Acad. Sci. U.S.A. **82**, 7585–7589
19 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**, 680–685
20 Sako, Y., Minoghchi, S. and Yanagida, T. (2000) Single-molecule imaging of EGFR signalling on the surface of living cells. Nat. Cell Biol. **2**, 168–172
21 Holbrook, M. R., Slakey, L. L. and Gross, D. J. (2000) Thermodynamic mixing of molecular states of the epidermal growth factor receptor modulates macroscopic ligand binding affinity. Biochem. J. **352**, 99–108