C-kinase Phosphorylates the Epidermal Growth Factor Receptor and Reduces Its Epidermal Growth Factor-stimulated Tyrosine Protein Kinase Activity*

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The Ca**+- and phospholipid-dependent protein kinase (C-kinase) binds tightly in the presence of Ca**+ to purified membranes of A431 human epidermoid carcinoma cells. The major membrane substrate for C-kinase is the epidermal growth factor (EGF) receptor. Phosphorylation of the EGF receptor is Ca**+-dependent and occurs at threonine and serine residues. After tryptic digestion of the receptor, three major phosphothreonine-containing peptides were identified. These are identical with three new phosphopeptides present in the EGF receptor isolated from A431 cells treated with either of the tumor promoters 12-O-tetradecanoylphorbol 13-acetate or teleocidin. C-kinase catalyzes phosphorylation at these same sites in purified EGF receptor protein. These results indicate that, in A431 cells exposed to tumor promoters, C-kinase catalyzes phosphorylation of a significant population of EGF receptor molecules. This phosphorylation of EGF receptors results in decreased self-phosphorylation of the EGF receptor at tyrosine residues both in vivo and in vitro and in decreased EGF-stimulated tyrosine kinase activity in vivo.

Tumor promoters, such as 12-O-tetradecanoylphorbol 13-acetate and teleocidin, have many direct effects on cells, inducing a pleiotropic growth response whose manifestations include increased 2-deoxyglucose transport, protein, RNA and DNA synthesis, membrane phospholipid turnover, and plasminogen activator production and induction of morphological changes (1, 2). Many of the biological effects of tumor promoters appear to be mediated by a protein kinase, which is dependent on Ca**+, phospholipid, and diacylglycerol. Nishi-zuka and co-workers have suggested that C-kinase is activated in cells by phosphatidylinositol turnover leading to diacylglycerol production (3, 4). TPA can substitute for unsaturated diacylglycerol to increase the affinity of C-kinase for Ca**+ and phosphatidylserine in activating the enzyme (5). The phosphor diester receptor and C-kinase from rat brain extensively co-purify, suggesting that the two activities reside in the same or closely associated molecules (6, 7). C-kinase can also be irreversibly activated by a specific Ca**+-dependent protease (8).

Addition of TPA to parietal yolk sac cells increases the fraction of C-kinase associated with the plasma membrane (9), suggesting that initial substrates for phosphorylation may be located there. Because tumor promoters alter epidermal growth factor binding (10-13) and because tumor promoters and growth factors produce several similar biological effects, the EGF receptor has been examined as a substrate for C-kinase. We have found that C-kinase phosphorylates membrane-associated as well as purified EGF receptors at threonine residues at sites identical with those phosphorylated when intact cells are exposed to tumor promoters. The EGF receptor protein itself possesses protein kinase activity with specificity for tyrosine residues in substrate proteins (14). This tyrosine protein kinase activity is reduced after phosphorylation of the EGF receptor by C-kinase.

EXPERIMENTAL PROCEDURES

Materials

[y-32P]ATP (3000 Ci/mmol; 1 Ci = 3.7 X 1012 Bq) was from Ameraham; tosylphenylalanyl chloromethyl ketone-trypsin, papain, phospholipids, TPA, and phorbol were from Sigma; teleocidin was prepared by Dr. T. Sugimura (National Cancer Center Research Institute, Tokyo) and supplied by Dr. I. B. Weinstein (Columbia University, New York). Protein A containing formalin-fixed Staphylococcus aureus (Pansorbin) was from Calbiochem. DEAE-cellulose (DE52) was from Whatman; ACA 34 Ultragel was supplied by LKB. EGF was purified from adult male mouse submaxillary glands obtained from Pel-Freeze (15). Human epidermoid carcinoma A431 cells were grown as described previously (16) and membranes were isolated by a modification of the method of Thom et al. (17) in which the homogenization buffer contained 0.5 mM EDTA and 0.5 mM EGTA. 528 and TLS monoclonal antibodies against the EGF receptor were kindly provided by Drs. Tomoyuki Kawamoto (18) and Joseph Schlessinger (19), respectively.

Methods

Protein Phosphorylation—C-kinase was purified from fresh rat brain by modification of procedures described by Kikkawa et al. (20). For most experiments enzyme was purified by DE52 chromatography, ammonium sulfate fractionation, and filtration on Ultragel ACA 34. Enzyme solutions containing 0.2 mg/ml of soybean trypsin inhibitor and 10% glycerol were stable for 1 month at 4 °C. At this stage, 105 nmol of phosphate were incorporated into histone I/min/mg of enzyme protein at 22 °C (units/mg). This enzyme fraction was completely dependent on Ca**+ with a K0 of less than 20 μM and was stimulated 6- to 10-fold by a combination of phosphatidylserine and diacylglycerol. TPA at 10 ng/ml could substitute for diacylglycerol.
For some experiments C-kine was further purified on a hydroxyapatite column which was eluted with a linear phosphate gradient (50-500 mM). The enzyme eluting between 120 and 250 mM was brought to 1 M NaCl and loaded on a phenyl-Sepharose column. The column was then developed with a decreasing sodium chloride gradient from 1 M to 0 M. The enzyme was eluted between 0.2 and 0 M NaCl. The specific activity of the purified enzyme was not determined because stabilizing soybean trypsin inhibitor protein was present.

For purification of the EGF receptor, monoclonal anti-EGF receptor antibody 528 was coupled to Sepharose 4B with 98% yield by the cyanogen bromide procedure (21) to provide an affinity matrix containing 10 μg/ml antibody. A431 cells were homogenized in buffer containing 20 mM HEPES-NaOH, pH 7.2, 2 mM EDTA, 1 mM dithiothreitol, 3 mM benzamidine HCl, 3.6% aprotinin, 14% glycerol, and 1% Triton X-100, then diluted with 10 vol of 20 mM HEPES, pH 7.2, containing 160 mM NaCl, 0.15% 2-mercaptoethanol, and 10% glycerol. The homogenate was clarified by centrifugation, and the supernatant was applied directly to the affinity matrix. After extensive washing with buffers of increasing ionic strength and 1 M urea, the EGF receptor was eluted with either 1 M acetic acid or with 6 M urea and was dialyzed against 20 mM HEPES-NaOH, pH 7.2/0.25% Triton X-100. The purified receptor preparation contained a single band of approximately 170,000 M, on SDS-polyacrylamide gel electrophoresis and a single NH₂-terminal leucine by Edman degradation.

In Vitro Phosphorylation Reactions—Assays for C-kine activity were similar to those described by Takai et al. (22). Standard reaction mixtures contained 20 mM HEPES-NaOH buffer, pH 7.5, 10 mM MgCl₂, 20 μg/ml of antibodies, 40 μg/ml of antibody, 100 μg/ml of histone H1, 50 μM [γ-³²P]ATP (specific activity, 5000 cpm/pmol), and the enzyme solution to be assayed. Reactions were carried out for 10 min at 30 °C and phosphorylated protein collected on phosphocellulose squares in 30% acetic acid. Paper squares were washed twice with 15% acetic acid, once with acetone, dried, and radioactivity determined. For phosphorylation of EGF receptor protein by C-kine, reactions contained 20 mM HEPES-NaOH, pH 7.2, 10 mM MgCl₂, 0.5 mM CaCl₂, 0.1 mM Na₃VO₄, 0.5-2.0 μM [γ-³²P]ATP, 60 μg/ml of phosphotyrosine, 6 μg/ml of dlein, the indicated amounts of C-kine, and C-kine and EGF receptor protein in A431 membranes (17), in Triton X-100 solubilized membranes (14), or as affinity purified material. Reactions were incubated for 5-10 min at 22 °C and terminated by addition of 5X Laemmli sample buffer (23) or RIPA buffer (24) to solubilize EGF receptors.

Immunoprecipitation of EGF Receptors—EGF receptor was isolated using TL5 monoclonal antibody (19), from [²²P]-labeled cell extracts, made with RIPA buffer (25) containing 2 mM EDTA. Lysate corresponding to ~10⁶ A431 cells was incubated with 12 pmol of TL5 for 30 min, followed by 5 μl of goat antiserum to mouse immunoglobulin for 30 min then 1.5 mg of Pansorsbin for 60 min, all at 0 °C. Immunoprecipitates were washed twice with 1% Triton X-100, 0.5% 2-mercaptoethanol, 0.1% sodium deoxycholate, and 10% glycerol, then diluted with 10 vol of 20 mM HEPES-NaOH, pH 7.2, containing 10% sucrose, 1 μM CaCl₂, and 1 μM MgCl₂ for 3 min at 0 °C followed by addition of 1 μM calmodulin (data not shown).

Two-dimensional Peptide Mapping—Phosphorylated EGF receptor was isolated with 10 μg/ml of histone H1 as substrate. The reaction was strongly inhibited by 0.5 mM chlorpromazine but was not affected by 1 μM calmodulin (data not shown).

Two-dimensional maps of tryptic digests of [²²P]-labeled EGF receptor revealed that C-kine catalyzed phosphorylation of 3 peptides (X, Y, Z in Fig. 2F). All contained phosphothreonine. The sites of serine phosphorylation were not identified. In addition, a peptide and presumably the phosphoserine is distributed among many minor sites. The additional peptide phosphorylated at tyrosine (spot J in Fig. 2F) corresponded to the major site of self-phosphorylation in EGF receptor (Fig. 2E and Refs. 31 and 32). To confirm the specificity of C-kine catalyzed phosphorylation of EGF receptor, the latter was purified to homogeneity by affinity chromatography on Sepharose immobilized anti-EGF receptor IgG. Purified EGF receptor retained ¹²⁵I-EGF and ¹²⁴I-anti-receptor IgG binding activity but not C-kine-dependent protein kinase activity. C-kine catalyzed phosphorylation of this purified EGF receptor at phosphothreonine and phosphoserine and the same threonine-containing phosphopeptides were observed (Fig. 2H). The phosphothreonine-containing peptide was not detected (Fig. 2H). An additional phosphorylation site was noted which was apparently less accessible in the native protein (Fig. 2H versus 2F).

Similar experiments were performed after the C-kine had been submitted to two additional purification steps. After the final step, the enzyme was able to phosphorylate membrane-bound EGF receptor in a calcium-dependent manner. In this phosphorylation this was strongly inhibited by a specific inhibitor of C-kine prepared from placenta termed phorbol ester-binding inhibitory factor which was kindly provided by I. Vilgrain. The presence of this inhibitor during the reaction blocked phosphorylation of the EGF receptor on threonine residues, but allowed autophosphorylation of the EGF receptor on tyrosine residues (data not shown). Although a requirement for phospholipid and diacylglycerol was observed with A431 membranes as substrate, phosphorylation of purified EGF receptor protein showed a strong requirement for phospholipid and diacylglycerol (data not shown).

Addition of TPA or tacrolin to A431 cells labeled to equilibrium with [³²P]orthophosphate resulted in a 2- to 3-fold increase in phosphorylation of the EGF receptor at both threonine and serine residues. Analysis of tryptic digests revealed 2 major and 1 minor phosphopeptides not present in
EGF receptor from control cells or cells treated with the inactive parent phorbol (Fig. 2, A, B, and C) or cells treated with carrier dimethyl sulfoxide (data not shown). These tryptic peptides, which together contained about 10% of the total radioactivity as phosphothreonine, were well resolved from the phosphotyrosine-containing tryptic peptide observed in EGF-treated cells (Fig. 2E) and from several other phosphopeptides whose pattern was unaffected by treatment of cells with tumor promoters. New peptides containing phosphoserine were not detected. The new sites phosphorylated in the EGF receptor isolated from tumor promoter-treated cells were identical with the sites phosphorylated in the EGF receptor by C-kinase in vitro (Fig. 2G). These results support the idea that tumor promoters activate C-kinase which catalyzes phosphorylation of EGF receptor at 3 specific sites (Fig. 2D). The increase in phosphorylation is rapid, being detected within 3 min of tumor promoter addition and persists for at least 1 h.

When EGF is added to A431 cells, increased phosphorylation of the EGF receptor occurs because of increased labeling of several serine and threonine phosphopeptides as well as appearance of a single phosphotyrosine peptide (31, 32). Under these conditions, EGF did not increase labeling of the specific phosphopeptides labeled by C-kinase (Fig. 2E), while tumor promoters did not increase labeling of the EGF-specific phosphotyrosine-containing peptide (Fig. 2, B and C).

To characterize C-kinase-catalyzed phosphorylation of EGF receptors further, A431 membranes were incubated in the absence or presence of C-kinase in buffer containing either CaCl2 or EGTA. The membranes were then washed in NaCl-containing buffer and incubated for different times with [γ-32P]ATP, MgCl2, and CaCl2. It can be seen in Fig. 3A that, in the absence of C-kinase, the EGF receptor undergoes rapid phosphorylation followed by rapid dephosphorylation which is almost complete in 5 min. This dephosphorylation is due to the presence in the membrane of an active tyrosine-specific protein phosphatase (33).
with C-kinase in the presence of CaCl₂, a phosphoprotein of 83,000 M₀ corresponding to the self-phosphorylated C-kinase was found associated with the membrane (Fig. 3B, lane c). The C-kinase was tightly bound to membrane and could not be removed by washing with 0.5 M NaCl. When membranes were incubated with enzyme in the absence of Ca²⁺, C-kinase failed to bind to the membranes (Fig. 3B, lane b). Once bound to the membrane, C-kinase was able to phosphorylate the EGF receptor rapidly; but, like the autophosphorylation process, the EGF receptor was then dephosphorylated by membrane protein phosphatases which hydrolyze phosphothreonine and phosphoserine (Fig. 3).

Because Rosner et al. (34) recently reported that tumor promoters blocked phosphorylation of EGF receptors on tyrosine residues, we examined the effect of tumor promoters on EGF-induced tyrosine phosphorylation of the EGF receptor/kinase and other cell proteins in intact A431 cells. Addition of EGF increased the relative content of phosphotyrosine in total cell proteins from 0.04 to 0.21% (Table I). Treatment with TPA alone had no effect, but TPA reduced the EGF-stimulated increase in protein phosphotyrosine content by ~45%. TPA also reduced the EGF-stimulated increase in phosphotyrosine content of the EGF receptor by ~60% (Table I).

EGF receptor from EGF- or TPA-treated cells contains 2.2-3.6 mol of phosphate/mol of polypeptide (data not shown). One can therefore calculate that about 20–35% of the molecules were phosphorylated at tyrosine in EGF-treated cells and at peptides X, Y, or Z in TPA-treated cells.

To evaluate the role of C-kinase in this effect, EGF receptor/kinase was phosphorylated by C-kinase prior to self-phosphorylation. A431 membranes containing EGF receptor/kinase were incubated with C-kinase, CaCl₂, MgCl₂, and unlabeled ATP for 5 min at 22 °C to phosphorylate the EGF receptor/kinase (Fig. 3, A and B). EGF at 100 nM was then added for 2 min at 22 °C followed by addition of [γ-³²P]ATP and MnCl₂. Fig. 3C shows that ³²P incorporation into alkali-stable phosphoamino acids was markedly reduced in EGF receptor/kinase phosphorylated by C-kinase (Fig. 3C), indicating a reduced ability of EGF receptor/kinase to self-phospho-

![Phosphopeptide maps of EGF receptor. A-E, subconfluent 5-cm diameter dish cultures of A431 cells were labeled with 5 mCi of ³²P in 2 ml of Dulbecco-Vogt-modified Eagle's medium lacking phosphate but supplemented with 5% complete calf serum. After 16 h, 2 μl of dimethyl sulfoxide containing 0.5% phorbol, teleocidin, or TPA were added, and 1 h later the cells were lysed and the EGF receptor was recovered by immunoprecipitation ("Experimental Procedures"). In a similar but separate experiment, EGF was added to labeled cells, in the absence of dimethyl sulfoxide, for 1 h before lysis and immunoprecipitation. EGF receptor was digested with trypsin and an equal fraction of each sample was analyzed ("Experimental Procedures"). Autoradiographs were exposed with the aid of a fluorescent screen. Arrowheads, points of sample application. A, 100 ng/ml of 4β-phorbol (1070 cpm Cerenkov analyzed, 20-h exposure). B, 100 ng/ml of teleocidin (TC) (1460 cpm Cerenkov analyzed, 20-h exposure). C, 100 ng/ml of TPA (1560 cpm Cerenkov analyzed, 20-h exposure). D, diagram showing positions of phosphopeptides of EGF receptor with their component phosphoamino acids indicated (SER, phosphoserine; THR, phosphothreonine; TYR, phosphotyrosine). Peptides (X, Y, and Z) whose phosphorylation is stimulated by tumor promoters are shown as closed ovoids. A peptide (No. 1) whose phosphorylation is stimulated by incubation with EGF is shown hatched. E, 60 ng/ml of EGF (640 cpm Cerenkov analyzed, 48-h exposure). F–H, EGF receptor, phosphorylated by incubation of A431 membranes or immunopurity affinity-purified EGF receptor with C-kinase, was extracted from gels similar to that shown in Fig. 1A, lane c. Phosphotryptic peptide maps are shown. F, EGF receptor phosphorylated in membranes (13.5 μg) by C-kinase (0.12 unit) (2750 cpm Cerenkov analyzed, 48-h exposure). G, mixture of sample analyzed in F (460 cpm) with sample analyzed in B (980 cpm) (20-h exposure). H, immunopurity affinity-purified EGF receptor (3 μg) phosphorylated by C-kinase (1.2 units) (300 cpm Cerenkov analyzed, 6-day exposure).]
Phosphorylation of the EGF Receptor by C-kinase

FIG. 3. Binding of C-kinase to A431s cell membranes and its effect on EGF receptor phosphorylation. A. aliquots of membranes from A431s cells (40 μg of protein) were incubated in a final volume of 0.16 ml for 5 min at 22 °C with 1 mM CaCl₂ in the absence (Δ) or presence (○) of 3.8 units of C-kinase purified through the hydroxypatite step. One sample was incubated in the same way with C-kinase but CaCl₂ was replaced by 1 mM EGTA. At the end of the incubation, each sample was diluted in a 1.5 ml of 20 mM HEPES-NaOH buffer, pH 7.2, containing 0.5 M NaCl and spun at 10,000 × g for 5 min. Membrane pellets were redissolved in the initial volume of 20 mM HEPES-NaOH buffer, pH 7.2, and incubated at 22 °C with 5 μM [γ-32P]ATP (30,000 cpm/pmol), 10 mM MgCl₂, and 1 mM CaCl₂. At different time intervals, aliquots of 20 μl were withdrawn and boiled for 3 min in Laemmli sample buffer. Each sample was analyzed on 10% SDS-polyacrylamide gels and autoradiographed. The bands corresponding to the EGF receptor were cut out and radioactivity was determined by liquid scintillation counting. B, autoradiograph of SDS-polyacrylamide gel of membranes phosphorylated for 3 min at 22 °C. Lane a, membrane incubated without C-kinase; lane b, membranes incubated with C-kinase plus 1 mM EGTA; lane c, membranes incubated with C-kinase plus 1 mM CaCl₂. EGF was not present in any reactions. R, EGF receptor; C, C-kinase. The gel was autoradiographed with exposure for 1 h at −70 °C. C, A431s membranes (13.5 μg) were incubated in a final volume of 20 μl containing 3 μM unlabeled ATP, 10 mM MgCl₂, 1 mM CaCl₂, 0.2 mM Na₃VO₄, without or with 0.12 unit of C-kinase for 5 min at 22 °C. EGF (100 nM) was added for 2 min followed by [γ-32P]ATP (2 × 10⁶ cpm) and 1.5 mM MnCl₂ for 5 min. Reactions were terminated by addition of 5X Laemmli sample buffer, products separated on an 8% SDS-polyacrylamide gel which was treated with 1 M KOH for 2 h at 55°C (31), and autoradiographed.

DISCUSSION

In 32P-labeled A431 cells, the EGF receptor is constitutively phosphorylated at several serine and threonine residues, and a tyrosine residue is phosphorylated in response to EGF (31). When either of two active tumor promoters with two distinct structures are added, two new major and one minor phosphotyrosine-containing tryptic peptides are observed. Iwashita and Fox have also found that active tumor promoters stimulate phosphorylation of EGF receptors at threonine-containing peptides (38). In vitro, C-kinase catalyzes specific phosphorylation at these same sites in the EGF receptor, indicating that the in vivo phosphorylation, which is stimulated by tumor promoters, is most likely mediated by this enzyme. In vitro phosphorylation requires Ca²⁺; phospholipid and diacylglycerol are not required with A431s cell membranes, presumably because of the lipid present, but phosphorylation of purified EGF receptor protein required phospholipid, and either TPA or diacylglycerol.

A431s cell membranes isolated in the presence of EGTA contain little C-kinase although the enzyme is readily detected in the cytosol. Added C-kinase binds strongly to such membranes and binding as well as activation of the enzyme is dependent on Ca²⁺. Tumor promoters and Ca²⁺ are likely to cooperate in vivo to fix C-kinase at the membrane (9). Isolated membranes contain phosphatase activities which rapidly re-

| Additions | Phosphotyrosine content |
|-----------|------------------------|
| Cell protein | EGF receptor protein |
| None | % | % |
| EGF | 0.04 | 0 |
| TPA | 0.21 | 8 |
| TPA + EGF | 0.04 | 1 |
| TPA + EGF | 0.12 | 3 |

move not only tyrosine-linked phosphate but also threonine- and serine-linked phosphate. In vivo the state of phosphorylation of the EGF receptor therefore depends on the balance between kinase activation in response to exogenous signals such as EGF and tumor promoters and these phosphatase activities.
The EGF receptor is a major membrane-bound substrate for tumor promoter activated C-kinase in A431 cells which contain abundant EGF receptors. In different cell types tumor promoters are reported to decrease the affinity of EGF receptors for EGF (13), to decrease the number of EGF receptors (10, 11), or to alter the metabolism of receptors with differing affinities for EGF (12). Tumor promoters are also reported to mimic (35) or oppose (36) the biological effects of EGF. In A431 cells, TPA, by itself, does not affect total cell protein phosphorylation content nor does it induce tyrosine phosphorylation of the EGF receptor or the 81,000 M₀, or 36,000 M₀, proteins the phosphorylations of which are increased by EGF. TPA does inhibit EGF-stimulated tyrosine kinase activity as measured by total cell protein phosphorylation content. TPA also blocks EGF-stimulated self-phosphorylation of the EGF receptor/kinase (Ref. 34 and present results) and C-kinase may be activated as a result of these studies.

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Note Added In Proof—We have recently performed experiments in which A431 cells were labeled with ³²P for only 2 h prior to exposure to EGF for 10 min. Under these conditions, incorporation of ³²P into peptides X and Z was detected. The extent of incorporation into peptide X was similar to the incorporation into peptide No. 1 (the phosphotyrosine-containing peptide). With cells labeled for 16-24 h and then exposed to EGF for periods from 1 min to 24 h, we had previously only detected increased phosphorylation of peptide No. 1 (see Fig. 2F). Our present results are in agreement with Iwashita and Fox (38) and with the model that EGF could lead to C-kinase activation by accumulated diacylglycerol from phosphatidyl inositol degradation.

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