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Epidermal Growth Factor (EGF) Stimulates Association and Kinase Activity of Raf-1 with the EGF Receptor

HARALD APP,[1] RACHEL HAZAN,[2] ASHER ZILBERSTEIN,[2] AXEL ULLRICH,[3] JOSEPH SCHLESSINGER,[4] AND ULF RAPP[1]*

Laboratory of Viral Carcinogenesis, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, Maryland 21702[1]; Rorer Biotechnology, Inc., King of Prussia, Pennsylvania 19406[2]; Max Planck Institut für Biochemie, Department of Molecular Biology, Martinsried, Federal Republic of Germany[3]; and Department of Pharmacology, New York University Medical Center, New York, New York 10016[4]

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Raf-1 serine- and threonine-specific protein kinase is transiently activated in cells expressing the epidermal growth factor (EGF) receptor upon treatment with EGF. The stimulated EGF receptor coimmunoprecipitates with Raf-1 kinase and mediates protein kinase C-independent phosphorylation of Raf-1 on serine residues. Hyperphosphorylated Raf-1 has lower mobility on sodium dodecyl sulfate gels and has sixfold-increased activity in immunocomplex kinase assays with histone H1 or a Raf-1 sequence-derived peptide as a substrate. Raf-1 activation requires kinase-active EGF receptor; a point mutant lacking tyrosine kinase activity is inactive in Raf-1 coupling and association. It is noteworthy that tyrosine phosphorylation of c-Raf-1 induced by EGF was not detected in these cells. These observations suggest that Raf-1 kinase may act as an important downstream effector of EGF signal transduction.

Raf serine- and threonine-specific protein kinases are cytosolic enzymes that stimulate cell growth in a variety of cell systems (34, 36, 37). Three isozymes have been characterized: c-Raf (Raf-1) (5), A-Raf (2), and B-Raf (18, 41). These enzymes differ in their expression in various tissues. Raf-1 is expressed in all organs and in all cell lines that have been examined, and A- and B-Raf are expressed in urogenital and brain tissues, respectively (44). Raf genes are proto-oncogenes: they can initiate malignant transformation of cells when expressed in specifically altered forms. Genetic changes that lead to oncogenic activation generate a constitutively active protein kinase by removal or interference with an N-terminal negative regulatory domain of the protein (14, 35). Microinjection into NIH 3T3 cells of oncogenically activated but not wild-type versions of the Raf-1 protein prepared with *Escherichia coli* expression vectors results in morphological transformation and stimulates DNA synthesis (35, 43). Thus, activated Raf-1 is an intracellular activator of cell growth. Raf-1 protein serine kinase is a candidate downstream effector of mitogen signal transduction, since Raf oncogenes overcome growth arrest resulting from a block of cellular *ras* activity due either to a cellular mutation (*ras* revertant cells) or microinjection of anti-*ras* antibodies (34, 42). c-Ras function is required for transformation by a variety of membrane-bound oncogenes and for growth stimulation by mitogens contained in serum (42). Raf-1 protein serine kinase activity is regulated by mitogens via phosphorylation (30), which also effects subcellular distribution (33, 36). Raf-1-activating growth factors include platelet-derived growth factor (PDGF) (31), colony-stimulating factor 1 (1), insulin (3, 24), epidermal growth factor (EGF) (31), interleukin 2 (46), and interleukin 3 and granulocyte-macrophage colony-stimulating factor (7). Upon mitogen treatment of cells, the transiently activated Raf-1 protein serine kinase translocates to the perinuclear area and the nucleus (33, 36). Cells containing activated Raf are altered in their pattern of gene expression (13), and Raf oncogenes activate transcription from Ap-1/PEA3-dependent promoters in transient transfection assays (19, 22, 49).

There are at least two independent pathways for Raf-1 activation by extracellular mitogens: one involving protein kinase C (PKC) and a second initiated by protein tyrosine kinases (3, 24, 31, 40, 46). In either case, activation involves Raf-1 protein phosphorylation. Raf-1 phosphorylation may be a consequence of a kinase cascade amplified by autophosphorylation or may be caused entirely by autophosphorylation initiated by binding of a putative activating ligand to the Raf-1 regulatory domain, analogous to PKC activation by diacylglycerol (32). In the latter scheme, production of such a ligand would be under control of PKC or the receptor tyrosine kinase. Support for a kinase cascade mechanism of activation of Raf-1 comes from experiments with the PDGF-β receptor. High levels of tyrosine phosphorylation of Raf-1 were observed in SF-9 insect cells doubly infected with baculoviruses expressing the PDGF-β receptor and Raf-1. Moreover, direct tyrosine phosphorylation, correlating with Raf-1 kinase activation, could be achieved with partially purified enzymes in vitro (30). However, the fraction of Raf-1 molecules that were tryosine phosphorylated in NIH 3T3 or BALB 3T3 cells in a ligand (PDGF)-dependent manner is very low (<1%), and its role in Raf-1 activation remains to be established.

We wished to further examine the mechanisms involved in Raf-1 activation by the EGF receptor (EGF-R), which is structurally distinct from the PDGF–colony-stimulating factor 1 class and the insulin class of receptors (47). The effect of EGF stimulation was studied in two cell systems—the human epidermoid carcinoma cell line A431 and previously described transfected NIH 3T3 cells overexpressing either wild-type or a kinase-negative mutant form of the human EGF-R (15, 16, 25, 26). These cell lines have previously been used for the identification of phospholipase Cγ (PLCγ) as an EGF-R substrate (27–29, 48) and have been extensively characterized with regard to receptor physiology.

* Corresponding author.

MATERIALS AND METHODS

NIH 3T3 clone 2.2 cells devoid of endogenous EGF-R were transfected with wild-type (HER14) or kinase-negative (DK721A) receptors as described previously (15, 16, 28). In the case of the kinase-negative receptor mutant, the putative ATP binding lysine was substituted by an alanine (15, 16). Cells were maintained in Dulbecco modified Eagle medium (DMEM) containing 10% (vol/vol) calf serum.

Preparation of cytosolic cell extracts and immunoprecipitations. Cells were grown in 75-cm^2 flasks in DMEM containing 10% calf serum until confluency and starved overnight in 0.05% calf serum. Before lysis, cells were exposed to 40 nM EGF for 10 min at 37°C and rinsed three times in phosphate-buffered saline. Control cells were not exposed to EGF. Cells were lysed in TBST buffer (50 mM Tris hydrochloride [pH 7.3], 150 mM NaCl, 0.5% Triton X-100) or in RIPA buffer (50 mM Tris hydrochloride [pH 7.3], 150 mM NaCl, 1% Triton X-100, 0.5% desoxycholate, 0.1% sodium dodecyl sulfate [SDS], 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 0.2 mM sodium orthovanadate, 25 mM sodium fluoride, 10 mM sodium pyrophosphate, 25 mM glycerophosphate). Insoluble material was removed by centrifugation at 4°C for 30 min at 12,000 × *g*. Protein concentrations were determined by the method of Bradford (6). Immunoprecipitations were performed by incubating lysates with polyclonal rabbit antiserum against the v-Raf 30-kDa protein (23) or a polyclonal rabbit antiserum against a synthetic peptide (SP63) corresponding to the last 12 carboxy-terminal amino acids of the Raf-1 protein and protein A for 3 h at 4°C.

Western immunoblotting. The immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis (PAGE). The gels were electroblotted on nitrocellulose, and the blots were blocked with 5% (wt/vol) gelatin in TBST buffer and incubated with polyclonal antiserum against Raf-1 or EGF-R. After extensive washing with TBST buffer, the blot was labeled with ^{125}J-staph protein A (Dupont NEN). Nonbound ^{125}J-staph was removed by washing the blots with TBST buffer, and the dried membrane was exposed to X-ray film.

Immunocomplex kinase assay. Immunoprecipitates were washed three times with cold RIPA buffer and twice with kinase buffer (50 mM Tris hydrochloride [pH 7.3], 150 mM NaCl, 12.5 mM $MnCl_2$, 1 mM dithiothreitol, and 0.2% Tween 20). Immunocomplex kinase assays were performed by incubating immunoprecipitates from 10^6 cells in 80 μl of kinase buffer with 20 μCi of [γ-^{32}P]ATP (10 mCi/ml) and 20 μl of the Raf-1 substrate peptide (5 mg/ml) for 30 min at 25°C. The sequence of the Raf-1 substrate peptide is IVQQFGFQ RRASDDGKLTD. A control peptide had tyrosine in position 5, as does wild-type Raf-1, and alanine in place of serine in position 12. The assay was linear for at least 40 min. The phosphorylation reaction was terminated by spotting 15-μl aliquots of the assay mixture on a 2- by 2-cm Whatman P81 phosphocellulose filter. The filters were washed four times for 30 min in 1% orthophosphoric acid and air dried, and the amount of ^{32}P incorporated was determined by the Cerenkov method. No differences were observed when counts were compared between filters on which the whole reaction mix or only the supernatant was spotted. Peptide phosphorylation in this assay was verified by running the reaction products on 20% SDS gels.

Phosphoamino acid analysis. One-dimensional phosphoamino acid analysis was carried out as described by Cooper et al. (8). Phosphoamino acids were separated at pH 1.8 (6% formic acid and 15% acetic acid) for 4 h at 750 V.

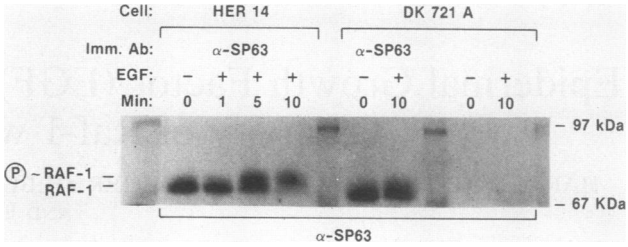

FIG. 1. Time course of Raf-1 mobility shift upon growth factor treatment. Cells (10^7) expressing either wild-type (HER14) or kinase-negative (DK721A) EGF-R were stimulated at 37°C with 40 nM EGF for the times indicated, lysed, and subjected to immunoprecipitation with anti-SP63 polyclonal antiserum. Immunoprecipitated proteins were separated by 7.5% SDS–PAGE, transferred to nitrocellulose, and probed with the same antiserum. Immunoreactive proteins were detected with ^{125}I-labeled protein A, and autoradiographs were exposed for 12 h. Each lane represents immunoprecipitates from 10^7 cells. Lanes: 2 through 5, HER14 cells; 7 and 8, DK721A cells; 9 and 10, DK721A cells with competing SP63 peptide (10 μg/ml); 1, 6, 9, and 11, marker proteins of 97 and 67 kDa.

RESULTS

To determine whether EGF induces the shift in migration in SDS gels that is typical for phosphorylation activation of Raf-1 protein kinase, lysates of treated and control cells were subjected to immunoprecipitation and immunoblotting with Raf-1-specific antiserum. NIH 3T3 cells lacking endogenous EGF-R but expressing approximately 3 × 10^5 human wild-type (HER14) EGF-R or kinase-negative mutant K721A EGF-R were transferred to starvation medium (0.05% calf serum) at early confluency and stimulated with EGF at 40 nM for 0 to 10 min. The effect of EGF on Raf-1 mobility is shown in Fig. 1. In the absence of EGF treatment, Raf-1 migrates as a single polypeptide of 72 kDa, corresponding to the expected molecular mass of Raf-1 protein kinase (5). The addition of EGF to HER14 but not to K721A cells resulted in a small increase in apparent mass of Raf-1 to 74 kDa. This shift first became detectable by 5 min, when approximately 50% of Raf-1 protein was affected, and continued to spread so that by 10 min the entire pool of Raf-1 protein had been modified. The inability of EGF to induce the Raf mobility shift in NIH 3T3 cells expressing the kinase-negative mutant of EGF-R demonstrates that receptor dimerization is not sufficient for Raf-1 modification, since the point mutation in K721A does not affect this event (47). It therefore seemed likely that the kinase activity of the EGF-R was important in mediating induction of the mobility shift in Raf-1.

The increase in apparent molecular mass of Raf-1 protein upon EGF treatment was due to phosphorylation, since incubation with potato acid phosphatase completely reversed the gel retardation (data not shown). To evaluate the effect of EGF-stimulated Raf-1 protein phosphorylation on its serine- and threonine-specific protein kinase activity, immune complex kinase assays were performed that utilized a synthetic peptide (IVQQFGFQRASDDGKLTD) or histone H1 as a substrate. The peptide corresponds to a potential autophosphorylation site in the Raf-1 kinase, which has been altered by substitution of phenylalanine for ty-

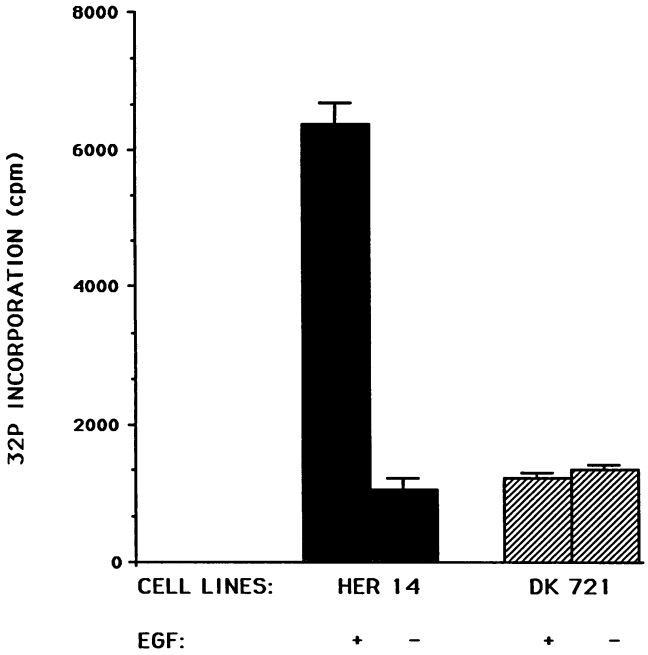

FIG. 2. Kinase activity upon EGF treatment of HER14 and DK721A cells. Monolayer cultures of HER14 or K721A cells were incubated in the presence or absence of 40 nM EGF for 10 min at 37°C. Lysates were centrifuged, and the resulting supernatants were immunoprecipitated with Raf-1 antiserum. Immunocomplexes were assayed for kinase activity using peptide (IVQQFGFQRRASDDGK LTD) as substrate. In the absence of peptide, immune complex kinase assays with unstimulated cells yielded ≤5% of counts observed in the peptide assay with stimulated cells. No counts were incorporated when a modified version of this peptide was used, in which serine in position 12 was replaced by alanine, and position 5 retained the Raf-1-specific tyrosine.

rosine in position 7 so as to restrict it from tyrosine phosphorylation.

For kinase assays, lysates of HER14 and K721A cells were prepared before and after stimulation with 40 nM EGF for 10 min. Comparison of the levels of kinase activity in Raf-containing immunoprecipitates showed a sixfold stimulation in HER14 cells upon EGF treatment (Fig. 2). Similar data were obtained when histone H1 was used as a substrate (data not shown). Consistent with the absence of the EGF-induced mobility shift of Raf-1 in NIH 3T3 cells expressing the kinase-negative mutant form of the EGF-R, no stimulation of Raf-1 protein kinase activity was observed in K721A cells (Fig. 2). When Raf-1 kinase activity was assayed with a modified version of the substrate peptide in which Ser-12 was replaced by alanine and Tyr-5 was retained, no counts were detected on the spotted filters (data not shown). This indicates that the kinase activity measured in our assay did not include a contribution of a contaminating tyrosine kinase activity.

Activated EGF-R associates with the candidate signal transducing enzyme PLCγ (27–29, 48). Similarly, Raf-1 was

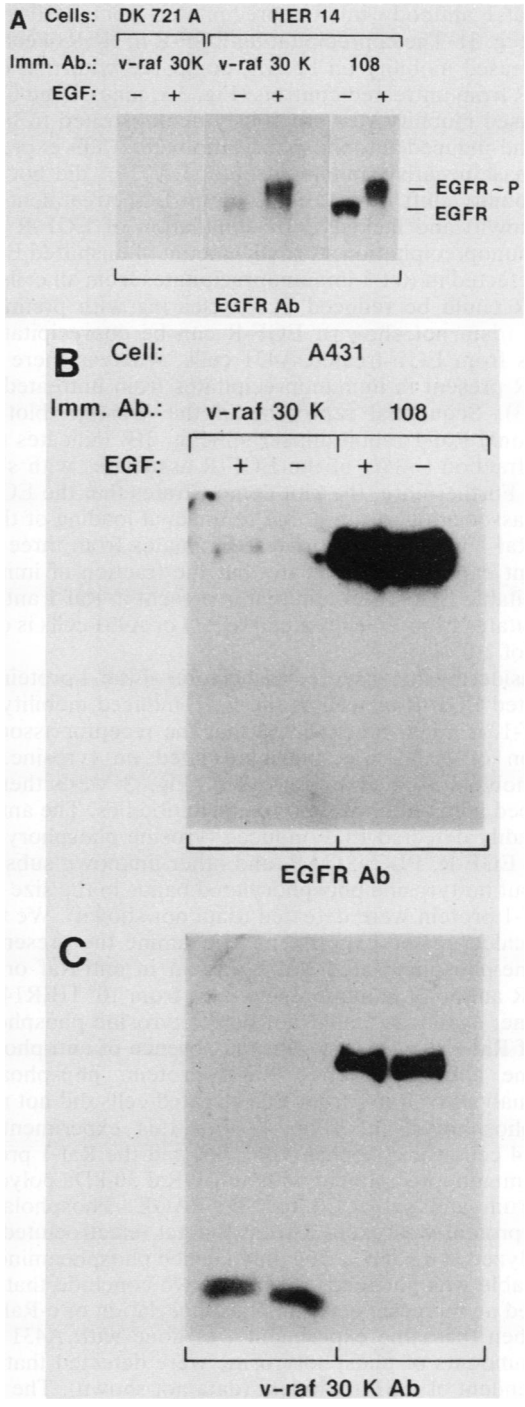

FIG. 3. Association of Raf-1 with ligand-activated EGF-R in HER14, DK721A, and A431 cells. Density-arrested and serum-starved HER14, DK721A, or A431 cells were stimulated for 10 min with 40 nM EGF at 37°C before lysis with RIPA buffer and immunoprecipitation. (A) Immunoprecipitates from HER14 and DK721A cells with anti-v-Raf 30K polyclonal antiserum or with a monoclonal EGF-R antibody (108) were subjected to 7.5% SDS–PAGE and transferred to nitrocellulose. EGF-R was detected by incubating with a polyclonal antiserum (RK2) against the EGF receptor (28), followed by ^{125}I-labeled protein A labeling. Exposure times for immunoblots were 3 days (lanes 1 through 4) or 1 day (lanes 5 and 6). (B and C) Immunoprecipitates from EGF-treated and control A431 cells with Raf-1-specific anti-v-Raf 30K antiserum or monoclonal anti-EGF-R antibody 108 were blotted, and the blots were developed sequentially with EGF-R antiserum RK2 (B) and anti-v-Raf 30K (C). Exposure times were 3 days and 1 day for panels B and C, respectively.

shown to coimmunoprecipitate with activated PDGF-β receptor in cell lines expressing high levels of receptors (30). To evaluate whether ligand-induced activation of Raf-1 protein kinase by the EGF-R correlated with receptor association, two cell systems were used: the NIH 3T3 cells expressing wild-type and mutant receptors (Fig. 3A) and human A431 cells (Fig. 3B) expressing approximately 2×10^6 EGF-R per cell (15, 16). Serum-starved cells were stimulated with 40 nM EGF for 10 min, and lysates from cells were immunoprecipitated with Raf-1- or EGF-R-specific antibodies. After separation by SDS-PAGE and transfer to nitrocellulose, immunoblotting was performed with either anti-EGF-R or anti-Raf-1 antibodies. EGF-R is present in anti-Raf-1 antibody immunoprecipitates from EGF-treated cells (Fig. 3). The coprecipitating EGF-R in HER14 cells has a decreased mobility on PAGE, compared with that of the EGF-R from untreated controls (Fig. 3A, lanes 4 and 6); this decreased mobility was previously demonstrated to be due to ligand-induced autophosphorylation (28). Cells expressing the kinase-negative mutant receptor DK712A did not show the mobility shift in the EGF-R upon EGF treatment (data not shown) and lacked EGF stimulation of EGF-R Raf-1 coimmunoprecipitation. A small amount of unshifted EGF-R was detected in Raf-1 immunoprecipitates from all cells; this EGF-R could be reduced by preclearing with preimmune serum (data not shown). EGF-R can be coprecipitated in lysates from EGF-treated A431 cells, whereas there is no EGF-R present in immunoprecipitates from untreated cells (Fig. 3). Sequential reprobing of the Western blot with polyclonal Raf-1 rabbit antiserum (Fig. 3B) indicates that a small fraction (~1%) of the EGF-R associates with shifted Raf-1. Furthermore, the blot demonstrates that the EGF-R–Raf-1 association was not due to unequal loading of the gel with Raf-1 immunoprecipitates. Estimates from three independent experiments indicate that the fraction of immunoprecipitable EGF-R protein that is present in Raf-1 antibody precipitates from EGF-treated HER14 or A431 cells is on the order of 1.0%.

Considering the observed association of Raf-1 protein with activated EGF-R as well as the EGF-induced mobility shift of Raf-1, it might be expected that the receptor-associated fraction of Raf-1 was phosphorylated on tyrosine. The immunoblots from experiments in Fig. 3 were therefore reprobed with antiphosphotyrosine antibodies. The antibodies readily detected EGF-induced tyrosine phosphorylation of the EGF-R, PLCγ, GAP, and other unknown substrates (47), but no tyrosine phosphorylated bands in the size range of Raf-1 protein were detected (data not shown). We therefore scaled up the experiment to examine the presence of tyrosine-phosphorylated Raf-1 protein in anti-Raf or anti-EGF-R antibody immunoprecipitates from 10^8 HER14 cells per lane; again, we could not detect tyrosine phosphorylation of Raf-1. Consistent with the absence of anti-phosphotyrosine antibody-reactive Raf-1 protein, phosphoamino acid analysis of Raf-1 from EGF-treated cells did not reveal any phosphotyrosine (Fig. 4). For this experiment, 10^7 HER14 cells were labeled with ^{32}Pi, and the Raf-1 proteins were immunoprecipitated with anti-v-Raf 30-kDa polyclonal antiserum and subjected to SDS-PAGE. Phospholabeled Raf-1 protein was excised from the gel, electroeluted, and hydrolyzed in 6 N HCl. The only labeled phosphoamino acid detectable was phosphoserine; thus we conclude that EGF induced an increase in serine phosphorylation of c-Raf (Fig. 4). When the same experiment was done with A431 cells, trace amounts of phosphotyrosine were detected that were independent of EGF treatment (data not shown). The lower

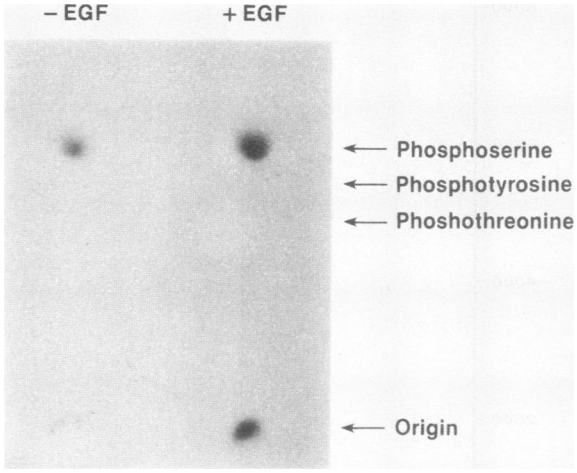

FIG. 4. Phosphoamino acid analysis of the immunoprecipitated Raf-1 protein from EGF-treated and untreated HER14 cells. HER14 cells (10^7) were phosphate starved for 16 h, labeled with 1 mCi of [^{32}P]phosphate for 3 h at 37°C, and treated with 40 nM EGF for 10 min at 37°C. Cells were lysed in RIPA buffer and immunoprecipitated. Proteins were separated by 7.5% SDS–PAGE, the Raf-1 bands were cut out of the gel, and the protein was electroeluted. From the electroeluted Raf-1 protein 1,960 cpm was recovered from the EGF-treated cells and 1,111 cpm was recovered from the untreated cells. The proteins were hydrolyzed for 2 h at 110°C in 6 N hydrochloric acid. Phosphoamino acid analysis was performed at pH 1.8 as described by Cooper et al. (8). The Raf-1 protein showed a shift in mobility when part of the electroeluted protein was rerun on 7.5% SDS–PAGE.

limit for detection of phosphotyrosine in Raf-1 in these experiments was on the order of 1% of the amount of phosphoserine.

The absence of tyrosine phosphorylation of Raf-1 protein in response to EGF in HER14 cells raises the possibility that serine protein kinase(s) acts as an intermediate in a kinase cascade connecting the stimulated EGF-R to activation of Raf-1 kinase. One candidate for this role is PKC, since this enzyme has previously been shown, upon treatment of cells with tetradecanoylphorbol-13-acetate (TPA), to trigger Raf-1 phosphorylation and kinase activation (31, 40). We therefore examined whether EGF induction of the Raf-1 mobility shift was dependent on the presence of PKC (Fig. 5). HER14 cells were pretreated with 200 ng of TPA for 72 h for complete downregulation of PKC and then tested for their ability to

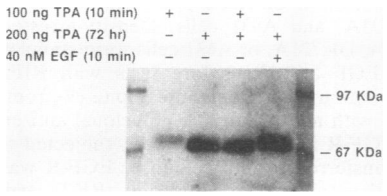

FIG. 5. Independence of EGF-mediated Raf-1 activation from PKC. HER14 cells (10^7) were incubated for 48 h with or without 200 ng TPA and stimulated with either 100 ng of TPA for 20 min at 37°C or with EGF (40 nM) for 10 min at 37°C. Cells were lysed in RIPA buffer, equal amounts of protein were immunoprecipitated with anti-v-Raf 30-kDa antiserum and electrophoresed, and the separated proteins were blotted onto nitrocellulose. The blot was incubated with the same antibody and then labeled with ^{125}I-labeled protein A.

respond to EGF with Raf-1 retardation. The PKC downregulation by pretreatment with TPA was effective in eliminating the TPA-induced Raf-1 retardation. In contrast, EGF-induced Raf-1 mobility shift was not blocked by downregulation of PKC.

DISCUSSION

Raf-1 protein kinase is a candidate transducer of mitogenic signals originating at the cell membrane (31, 34–36). Comparison of different classes of transmembrane tyrosine kinase receptors for their ability to couple to Raf protein kinases is likely to yield insight into receptor structures that are critical for Raf-1 interaction. Such a comparison will also establish whether Raf-1 activation is a common component of receptor signaling and whether there are distinct mechanisms for Raf-1 coupling. In the present study we report that one of the intracellular consequences of EGF receptor stimulation by EGF is activation of Raf-1 protein kinase. Treatment of HER14 cells with EGF stimulated Raf-1 phosphorylation, resulting in retarded mobility of SDS gels. This modification was apparent within 5 min and affected the total pool of Raf-1 molecules by 10 min. The shifted Raf-1 protein was active as a serine kinase as determined by use of a synthetic peptide or histone H1 as a substrate, and the retarded mobility of *raf-1* protein and kinase activity were abolished by treatment with potato acid phosphatase (data not shown). Phosphoamino acid analysis revealed that EGF stimulated serine but not tyrosine phosphorylation of Raf-1. The serine phosphorylation did not depend on the presence of PKC, since it also occurred in HER14 cells in which PKC was downregulated after prolonged treatment with TPA. All three EGF-induced events, namely, stimulation of Raf-1 serine phosphorylation, receptor association, and kinase activation, required the presence of active EGF receptor and did not take place in cells that overexpressed a tyrosine kinase-negative point mutant of EGF-R.

The time course of Raf-1 phosphorylation in EGF-treated HER14 cells separates this event from the very early tyrosine phosphorylations of several cellular substrate proteins, including that of PLCγ, which occurs within 1 min after EGF treatment (28, 29, 48). Raf-1 phosphorylation and concomitant protein kinase activation are also slower than ligand-induced receptor endocytosis, which is stimulated within less than 5 min after EGF binding (10). However, EGF-induced Raf-1 hyperphosphorylation in HER14 cells persists for at least 2 h (data not shown), a period that encompasses the entire endocytotic schedule, including receptor recycling and degradation (10–12, 15, 16). Nevertheless, it is not likely that Raf-1 kinase activation is involved in endocytosis, since the kinase-negative EGF-R mutant Dk721, which is blocked in Raf-1 coupling, still undergoes efficient ligand-induced internalization (10, 15).

The mechanism of Raf-1 serine phosphorylation and protein kinase activation is unclear. In the case of the previously characterized PDGF receptor interaction with Raf-1, it is thought that direct phosphorylation of Raf-1 by the receptor on tyrosine residues is a primary event that triggers Raf-1 autophosphorylation on serine, which in turn would stabilize the protein in the active conformation (30). However, the fraction of Raf-1 protein that is tyrosine phosphorylated upon PDGF treatment of BALB 3T3 cells is exceedingly low, and there are no data that establish a requirement for tyrosine phosphorylation of Raf-1 for PDGF-stimulated Raf-1 kinase activation. For the EGF-R, we did not detect EGF-stimulated tyrosine phosphorylation of Raf-1, and therefore we have to consider other mechanisms for activation. If autophosphorylation were solely responsible for EGF-induced Raf-1 hyperphosphorylation, then activation might be triggered by the binding interaction of Raf-1 protein to the activated receptor or by binding of an activating putative intracellular ligand to the Raf-1 regulatory domain. The latter might be generated by an EGF-induced lipid metabolite, similar to the PLC-mediated production of diacylglycerol, which is the natural activator of PKC (32). Alternatively, there might be other serine kinases involved in Raf activation, such as MAP-2 kinase (38) or S6 kinase (9, 21, 45), both of which are known to be growth factor regulated (4, 20, 38, 39, 45). The ability of EGF to stimulate Raf-1 phosphorylation in PKC-downregulated HER14 cells eliminates this enzyme as a necessary upstream activator. Determination of regulatory phosphorylation sites of Raf-1 protein should facilitate the analysis of potential upstream activators.

There are multiple pathways for Raf-1 recruitment, as described originally for NIH 3T3 and BALB 3T3 cells (31) and more recently in related work on Raf-1 coupling to other receptor systems. For example, the T-cell receptor in mouse 2B4 T cells depends entirely on PKC for Raf-1 coupling (40), whereas activation by interleukin 2 (46) and interleukin 3 and granulocyte-macrophage colony-stimulating factor (7) correlates with high-stoichiometry tyrosine phosphorylation of Raf-1. Other transmembrane tyrosine kinase receptors, which lead to PKC- and tyrosine phosphorylation-independent Raf-1 activation, include receptors for insulin (3, 24) and colony-stimulating factor 1 (1). Such redundancy in pathways for Raf-1 activation is consistent with a central role for Raf-1 as a downstream effector of a common output, growth stimulation, from a functionally and structurally diverse group of receptors. In regard to regulatory phosphorylation sites on Raf-1 protein, the apparent position of Raf-1 as a signal integrator predicts multiple such sites.

The activated EGF-R interacts with more than one intracellular candidate signal transducer enzyme. The most extensively studied in this system is PLCγ, which becomes phosphorylated on tyrosine and serine in response to treatment of A431 cells with EGF and may consequently be altered in activity (27–29, 48). Comparison of different classes of transmembrane tyrosine kinases that share PLCγ coupling suggests that kinase activity and autophosphorylation are essential for the association between EGF-R and PLCγ. Autophosphorylation appears to be required also for the association of PDGF receptor with Raf-1 (30). This raises the possibility of binding competition between Raf-1 and PLCγ. Thus, the relative levels of PLCγ and Raf-1 protein in cells may be a determinant of the quality of the signal output from activated growth factor receptors. In regard to induction of proliferation from the activated receptor, Raf-1 and GAP appear so far to be the only signal transducers that are shared between all protein tyrosine kinase growth factor receptors (47). Since Raf-1 is well established as a proto-oncogene with broad tissue specifity, it is likely to be essential, if not sufficient, for mediation of the proliferation response to growth factors in many cell types.

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