The serine/threonine-specific protein kinase Raf-1 plays a key role in mitogenic signal transduction by coupling Ras to the mitogen-activated protein (MAP) kinase cascade. Ras-mediated translocation to the plasma membrane represents a crucial step in the process of serum-stimulated Raf-1 kinase activation. The exact role of the multisite phosphorylation in Raf regulation, however, is not clear. We have previously reported that the mobility shift-associated hyperphosphorylation of Raf correlates with a reduction of serum-stimulated Raf kinase activity (Wartmann, M., and Davis, R. J. (1994) J. Biol. Chem. 269, 6695–6701).

Here we show that incubation of serum-starved CHO cells with D609, a purported inhibitor of phosphatidylinositol-specific phospholipase C, also results in a mobility shift of Raf-1 that is due to hyperphosphorylation on sites identical to those observed following mitogen stimulation. Subcellular fractionation analyses revealed that D609-induced mobility shift-associated hyperphosphorylation was paralleled by a decreased membrane association of Raf-1. Similar results were obtained in an in vitro reconstitution system. Furthermore, PD98059, a specific inhibitor of activation of the MAP kinase kinase MEK, prevented D609-induced Raf hyperphosphorylation and restored the amount of membrane-bound Raf to control levels. Taken together, these data suggest that mobility shift-associated hyperphosphorylation of Raf-1, by virtue of reducing the amount of plasma membrane-bound Raf-1, represents a negative feedback mechanism contributing to the desensitization of the MAP kinase signaling cascade.

Raf-1 is a ubiquitously expressed serine/threonine protein kinase that assumes a critical role in relaying proliferative and developmental signals initiated by cell-surface receptors to the nucleus (1–4). Genetic studies in the nematode Caenorhabditis elegans and the fruit fly Drosophila melanogaster as well as biochemical studies in vertebrate cells have elucidated that Raf-1 couples Ras to the MAP kinase cascade consisting of Raf-1 itself, the dual specificity MAP kinase kinases MKK1 and MKK2 (also termed MEK-1 and MEK-2), and the extracellular signal-regulated protein kinases (ERKs) or MAP kinases (5). The MAP kinases carry the signal to the nucleus, where they phosphorylate transcription factors capable of mediating changes in gene expression (6, 7).

While the regulation of MEK and MAP kinases by phosphorylation is well understood (8–10), the molecular mechanism(s) involved in Raf-1 regulation remain more obscure. The best understood aspects of Raf-1 kinase regulation are the initial events that precede its mitogen stimulation. Thus, growth factor receptor-induced activation of the mammalian nucleotide exchange factor mSOS, mediated by adapter proteins such as Shc and Grb2, stimulates the conversion of Ras from the inactive, GDP-bound state to the active, GTP-bound state. Activated Ras in turn directly interacts with Raf-1, resulting in the translocation of Raf from the cytoplasm to the plasma membrane. These conclusions are based on the findings that Raf-1 physically interacts with Ras (11–14) and that Raf-1 is associated with the plasma membrane in cells expressing oncogenic Ras (15–17). Importantly, Raf and Ras transiently interact in mammalian cells upon extracellular stimulation (18), providing a potential molecular basis for the transient membrane translocation of Raf observed in serum-stimulated cells (17). The physical interaction of Raf-1 with activated Ras in vitro, however, is insufficient for stimulation of Raf-1 kinase activity (Ref. 19; data not shown). Artificial plasma membrane localization, on the other hand, is sufficient for Raf kinase activation in a Ras-independent manner (15, 20). Taken together, these observations suggest that membrane localization of Raf-1 is necessary for its activation and that the role of Ras is to recruit Raf-1 to the membrane for activation by an as yet elusive mechanism.

Hyperphosphorylation of Raf-1 is a cellular response common to a wide range of physiological stimuli that activate the Raf-1/MEK/MAP kinase pathway and may be relevant to the process of Raf activity regulation (1, 3, 4). Phosphorylation of tyrosine residues 340 and 341 has been reported to be involved in the stimulation of Raf kinase activity in some cellular systems (21–23). However, in many cellular systems, Raf kinase activation occurs in the apparent absence of Raf-1 tyrosine phosphorylation. In fact, even under circumstances when tyrosine phosphorylation is observed, the majority of phosphorylation events occur on serine residues (1). Thus, Raf-1 is phosphorylated in vivo at Ser-43, Ser-259, Ser-499, and Ser-621 (24–26). While constitutive phosphorylation at Ser-621 might be necessary for Raf functionality, phosphorylation at Ser-259 and Ser-499 has been implicated in the protein kinase C-mediated activation of Raf (24, 26, 27). Raf phosphorylated at cyclic AMP-dependent protein kinase; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; Shc, Src homologue 2-containing protein; SOS, son-of-sevenless; PAGE, polyacrylamide gel electrophoresis.
Ser-43 by cAMP-dependent protein kinase displays a decreased affinity for Ras in vitro. This could contribute to the negative regulation of the Raf-1/MEK-1/MAP kinase by cAMP-elevating agents observed in some cellular systems (28). Furthermore, phosphorylation of Raf on Thr-269 mediated by a ceramide-activated protein kinase has been implicated in tumor necrosis factor-induced Raf kinase activation (29).

The exact molecular relationship between these phosphorylation events and those that are associated with the characteristic retardation of the electrophoretic mobility of Raf-1 following stimuli that activate the Ras/Raf-1/MEK/MAP kinase pathway is not clear. Initial experiments employing serine/threonine-specific phosphatases suggested a causal relationship between serine/threonine phosphorylation and Raf mobility shift as well as Raf kinase activation (30). However, recent evidence argues against such a positive relationship between these two events (17, 31–34). We have previously observed that the kinetics of mitogen-stimulated Raf kinase activation and Raf mobility shift are poorly correlated events. Thus, while mitogen-stimulated Raf-1 kinase activation occurs rapidly and is transient in nature, the decrease in Raf-1 protein mobility only becomes apparent at later times and coincides with a marked attenuation of mitogen-stimulated Raf-1 kinase activity (17).

Here we show that the mobility shift-associated hyperphosphorylation of Raf is associated with a decreased affinity of this form of Raf for the plasma membrane. Since plasma membrane localization is a positive determinant for Raf kinase activity, this post-translational modification might represent a molecular mechanism accounting, at least in part, for the attenuation of Raf kinase activity following mitogen stimulation. We further demonstrate that hyperphosphorylation of Raf can be blocked by a specific inhibitor of MEK activation and that this correlates with restoration of plasma membrane-bound Raf to control levels. Thus, the mobility shift-associated hyperphosphorylation of Raf is likely a consequence of activating the downstream components in the MAP kinase cascade and might represent a negative feedback mechanism contributing to the desensitization of this signaling pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**—[32P]Orthophosphate and γ-[32P]ATP were purchased from Amersham. FCS and Ham’s F-12 medium were from Life Technologies, Inc. Restriction enzymes were from Boehringer Mannheim. Polyvinylidene difluoride membranes (Imobilon-P) were obtained from Millipore Corp. Protein A-Sepharose and Protein G-Sepharose were from Sigma. Recombinant kinase-inactive human MKK1 carrying an N-terminal hexahistidine tag (His6-MKK/K97M) was prepared by Ni2+-NTA-agarose (Pharmacia Biotech Inc.) affinity chromatography followed by DEAE-Sepharose (Pharmacia) chromatography as described previously (35). The monocular α-Flag monoclonal antibody M2 was obtained from Kodak Scientific Imaging Systems, while the rabbit α-Raf (C-12) antiserum sc-133 was from Santa Cruz Biotechnologies, Inc. Restriction enzymes were from Boehringer Mannheim. FCS and Ham’s F-12 medium were from Life Technologies, Inc.) according to the manufacturer’s protocol. After 24 h, the transfected cells were placed in medium containing 2 µg/ml puromycin (Fluka). Recipient CHO cells were incubated for 20 h with tunicamycin (0.3 µg/ml) to abrogate the resistance of these cells to retroviral infection (38). Virus-containing medium collected from pools of puromycin-resistant OE cells was then used to infect the tunicamycin-treated CHO cells in the presence of 8% FCS. Two days after infection, the infected CHO cells were subjected to selection in medium containing 20 µg/ml puromycin. In contrast to the previously described CHO/pCMV/Flag-Raf cell line (17), the expression of the different Flag-Raf-1 proteins was maintained at a constant level in the continuous presence of selective pressure, as judged by Western blot analysis using the rabbit α-Raf-1 (C-12) antiserum sc-133. All experiments reported here were performed with pools of CHO cells either stably transfected with pBabe-Puro (CHO) or pBabe-Puro/Flag-Raf-1 (CHO/Flag-Raf-1).

**Phosphorylation of Raf-1 in Intact Cells**—CHO cells grown in 100-mm dishes were preincubated for 60 min in phosphate- and serum-free Ham’s F-12 medium at 37 °C. The cells were then incubated for 12 h in 10 ml of phosphate- and serum-free Ham’s F-12 medium containing 0.2 mM orthovanadate (17). The end of the labeling period, the cells were treated with 20% FCS or 10 µg/ml D609 for defined times. The cells were then washed quickly in phosphate-buffered saline and harvested in 500 µl of lysis buffer (20 mM Tris (pH 8.0), 137 mM NaCl, 2 mM EDTA, 2 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 25 mM dithiothreitol) at 4 °C. Precleared supernatant was prepared by sedimenting insoluble material by centrifugation at 14,000 × g for 15 min at 4 °C. The precipitate was preincubated for 10 min with 20 µl of protein G-Sepharose for 30 min at 4 °C. The precleared supernatant was then incubated for 1 h at 4 °C with 2 µg of α-Flag monoclonal antibody M2 immobilized on 20 µl of protein G-Sepharose. The immunoprecipitates were washed once with lysis buffer, twice with 0.5 M LiCl, 0.1 M Tris (pH 7.4), 25 mM β-glycerophosphate, and once with 10 mM Tris (pH 7.4), 25 mM β-glycerophosphate. The samples were boiled for 5 min supplemented with 50 µl of 2× sample buffer containing 100 mM dithiothreitol and analyzed by SDS-PAGE (7% gel).

**Phosphopeptide Mapping**—Phosphorylated proteins were detected by autoradiography, and the proteins of interest were consecutively excised from the dried gel. Trypsin digestion and two-dimensional phosphopeptide analysis of Raf phosphoproteins were performed basically as described by Stover et al. (39). Briefly, dried gel slices were rehydrated in 50% acetonitrile, air-dried for 4 h, and then soaked in 100 µl of 100 mM NaHCO3 containing 5 µg sequencing grade trypsin (Boehringer Mannheim). The digestion was left to proceed overnight at 30 °C, and the phosphopeptides were then eluted by rocking in 50% acetonitrile for 4 h. After lyophilization, phosphopeptides were mapped using the HTLLE-7000 peptide mapping system according to the manufacturer’s protocol (CBS Scientific Co.). Briefly, lyophilized peptides were resuspended in 10 µl of pH 1.9 buffer (2.2% formic acid and 7.5% acetic acid), 2 µl (1000–1500 cpm) of the sample was spotted on cellulose TLC plates (Merck), and electrophoresis at pH 1.9 and 1000 V proceeded for 45 min. After drying, the plates were placed in a chromatography tank containing phosphochromatography buffer (37.5% n-butanol, 25% pyridine, and 7.5% acetic acid) for 12–16 h for separation in the second dimension. Phosphopeptides were detected by autoradiography. Phosphoamino acid analysis was performed by partial acid hydrolysis (1 h at 110 °C in 6 N HCl) and thin layer electrophoresis as described (40).

**In Vitro Protein Kinase Assays**—CHO cells grown in 100-mm dishes, starved for 18 h in serum-free Ham’s F-12 medium prior to the experiment, and then treated as indicated in the figure legends. The cells were then washed quickly with ice-cold phosphate-buffered saline and harvested in 500 µl of lysis buffer. Clarified supernatant was prepared by sedimenting insoluble material by centrifugation at 14,000 × g for 10 min at 4 °C. Raf protein kinase activity was measured using an immunocomplex protein kinase assay with recombinant kinase-inactive human MKK1, His6-MKK/K97M, as an exogenous substrate basically as described previously (17). Clarified supernatant was incubated for 1 h with 2 µg of the α-Flag monoclonal antibody M2 immobilized on 20 µl of protein G-Sepharose for 30 min at 4 °C. The immunoprecipitates were then washed three times with lysis buffer and twice with kinase buffer (25 mM Hepes (pH 7.5), 25 mM β-glycerophosphate, 1 mM dithiothreitol, 5 mM MnCl2, 15 mM MgCl2). The washed Flag-Raf-1 immunoprecipitates were incubated with 50 µl [γ-32P]ATP (10 Ci/mmol) and 200 ng of His6-MKK/K97M (70–80% pure) in a final volume of 50 µl for 30 min at 30 °C. The reactions were terminated by the addition of 50 µl of 2×...
sample buffer and then boiled for 5 min prior to analysis by SDS-PAGE (7% gel). The gel was dried, and the phosphorylation of MKK was quantitated using a PhosphorImager and ImageQuant software (Molecular Dynamics Inc., Sunnyvale, CA).

MAP Kinase (ERK2) activity was measured in an immunocomplex kinase assay using myelin basic protein as an exogenous substrate as described previously (36) with minor modifications. ERK2 was immunoprecipitated from clarified cell lysates obtained as described above using 2 μl of specific antiserum (36) adsorbed to Protein A-Sepharose. Immunocomplexes were washed three times with lysis buffer and once with kinase buffer (30 mM Tris-HCl (pH 8.0), 20 mM MgCl2, and 2 mM MnCl2) and then incubated in a final reaction volume of 30 μl together with 15 μg of myelin basic protein, 10 μM unlabeled ATP, and 0.1 μM γ-32P-ATP (1200 Ci/mmol) for 30 min at 37°C. Reactions were terminated by the addition of sample buffer, proteins were subjected to SDS-PAGE (15% gel), and phosphorylation of myelin basic protein was quantified with a PhosphorImager and ImageQuant software (Molecular Dynamics Inc., Sunnyvale, CA).

PKA activity in crude cell extracts was assayed using kemptide as a substrate basically as described (41).

**In Vitro Dephosphorylation of Raf and Determination of Phosphatase Activities**—PP1 and PP2A were partially purified from rabbit skeletal muscle as described previously (42). 15 min prior to use, PP1 and PP2A were diluted 10-fold into dephosphorylation buffer (50 mM Tris-HCl (pH 7.5), 0.1% β-mercaptoethanol, 3 mM MnCl2, 0.1 mM EDTA, 50 mM NaCl). In some assays, dephosphorylation buffer was supplemented with 1 μM okadaic acid to inactivate phosphatasases. Flag-Raf-1 was immunoprecipitated from CHO cells treated for 60 min with 10 μg/ml D609 as described above. Immunoprecipitates were washed twice with lysis buffer and twice with phosphate buffer (50 mM Tris- HCl (pH 7.5), 0.1 mM EDTA, and 50 mM NaCl). The washed immunoprecipitates were incubated for 60 min at 30°C in a final volume of 50 μl (50 mM Tris-HCl (pH 7.5), 0.1% β-mercaptoethanol, 1 mM MnCl2, 0.1 mM EDTA, 50 mM NaCl) with or without okadaic acid-pretreated, diluted PP1 and PP2A.

The effects of D609 on the catalytic activity of PP1 and PP2A in vitro were determined using clarified cell lysates prepared in the absence of phosphatase inhibitors, while the in vitro effects of D609 were measured using partially purified PP1 and PP2A catalytic subunit. Assays were performed with phosphorylase a as a substrate as described (42).

**Western Blot Analysis**—Total cellular proteins or immunoprecipitates were resolved by SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes (Immobilon-P) and analyzed by Western blotting using the polyclonal α-Raf-1C-12) antiserum sc-153 (Santa Cruz Biotechnology, Inc.). Immunocomplexes were visualized with corresponding secondary antibodies using the enhanced chemiluminescence procedure (Amersham International PLC).

**Cell Fractionation and In Vitro Reconstitution**—Subcellular fractionation was performed essentially as described (43) with minor modifications. Cells were washed with ice-cold phosphate-buffered saline, disrupted by sonication in hypotonic buffer (25 mM Hepes, pH 7.5, 25 mM β-glycerophosphate, 2 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, and 10 μg/ml leupeptin), and centrifuged for 30 min at 100,000 × g yielding the cytosol (S100) and the membrane pellet (P100). The latter was resuspended in hypotonic buffer containing 1% Triton X-100, sonicated, and subsequently recentrifuged for 30 min at 100,000 × g in order to obtain a clarified Triton X-100-extractable membrane fraction. When analyzing the subcellular distribution of Raf in vitro, the membrane pellet obtained from parental CHO cells was resuspended in hypotonic buffer lacking Triton X-100. These resuspended membranes were then incubated for 30 min at 4°C with the cytosolic (S100) fraction obtained from Flag-Raf-expressing CHO cells. Subsequently, the reconstituted hypotonic extract was subjected to subcellular fractionation as described above to obtain the cytosolic and clarified Triton X-100-extractable membrane fractions.

**RESULTS**

The PC-PLC-specific Inhibitor D609 Induces a Raf-1 Protein Mobility Shift That Does Not Correlate with Raf Kinase Activation—Raf-1 shares general structural features with members of the protein kinase C family, including the presence of a zinc-binding cysteine-rich domain termed the “zinc finger” (44). The corresponding motifs in protein kinase C mediate the binding of diacylglycerols and phorbol esters necessary for its activation (45). This structural similarity has led to the hypothesis of allosteric regulation of Raf-1 by a lipid co-factor (44).

In order to investigate a potential role for lipid second messengers in mitogen-stimulated Raf-1 kinase activation, we tested the effect of inhibitors of different phospholipases on serum-stimulated Raf-1 kinase activity. Ligand-induced activation of the Ras/Raf-1/MEK/MAP kinase pathway is tightly associated with a hyperphosphorylation-mediated decrease in the electrophoretic mobility of Raf-1 upon SDS-polyacrylamide gel electrophoresis. Thus, in our initial experiments, we examined the effect of the various inhibitors on the electrophoretic mobility of Raf-1 in a CHO cell line overexpressing an epitope-specific antibody and kinase-inactive MKK1 as a substrate (see “Experimental Procedures”). Raf-dependent phosphorylation of MKK1 was quantified with a PhosphorImager and ImageQuant Software and is expressed as mean ± S.D. (D609, n = 11; FCS, n = 3) relative to levels measured in untreated cells (control).

![FIG. 1. The PC-PLC-specific inhibitor D609 induces a Raf-1 protein mobility shift that does not correlate with Raf kinase activation.](image-url)

Serum-starved CHO/Flag-Raf-1 cells were incubated for 30 min with increasing concentrations of D609 (A), D609 (10 μg/ml) or potassium ethyl xanthate (6 μg/ml) (B), or fetal calf serum (20%) (C) for the indicated times. The electrophoretic mobility of the Raf-1 protein was investigated by Western blotting using a c-Raf-1 specific antiserum. D, serum-starved CHO/Flag-Raf-1 cells were incubated with D609 (10 μg/ml) or with fetal calf serum (20%) for 30 min. Raf kinase activity was measured in an immunocomplex kinase assay employing an epitope-specific antibody and kinase-inactive MKK1 as a substrate (see “Experimental Procedures”). Raf-dependent phosphorylation of MKK1 was quantified with a PhosphorImager and ImageQuant Software and is expressed as mean ± S.D. (D609, n = 11; FCS, n = 3) relative to levels measured in untreated cells (control).
cells (Fig. 1D). However, the extent of reduction varied among the experiments (0-89%); see “Discussion”). Potassium ethyl xanthate (PEX), a biologically inactive structural homologue of D609, affected neither Raf-1 mobility (Fig. 1B) nor Raf-1 protein kinase activity when applied at equimolar concentration (data not shown).

These results clearly demonstrate that the marked electrophoretic mobility shift of Raf-1 observed in response to mitogens and other physiological stimuli is not required for Raf-1 kinase activation and, hence, that the Raf mobility shift is an inadequate monitor for the activation state of Raf.

D609- and Serum-induced Raf-1 Mobility Shift Is Due to Hyperphosphorylation and Occurs on Identical Sites—The molecular relationship between the multiple Raf phosphorylation events and the Raf mobility shift is not known. Thus, it is possible that differences in the phosphorylation pattern, which do not translate into an altered electrophoretic mobility of Raf, account for the differential modulation of Raf kinase activity by serum and D609. We therefore analyzed the phosphorylation state of Raf following serum or D609 treatment. To this end, Flag-Raf-1 was immunopurified from CHO/Flag-Raf-1 cells metabolically labeled with [32P]orthophosphate. Raf-1 phosphoproteins were then analyzed by gel electrophoresis and autoradiography. As shown in Fig. 2A, serum as well as D609 elicited an increase in the total phosphate content of Raf-1 as compared with the untreated control (1.5- and 1.4-fold, respectively). Phosphoamino acid analysis revealed that both basal and serum-induced hyperphosphorylation occurred predominantly on serine and to a minor extent on threonine, while tyrosine phosphorylation was not detected (data not shown). Raf-1 phosphoproteins were further analyzed by tryptic two-dimensional phosphopeptide mapping (Fig. 2B). In serum-starved cells, two major phosphopeptides were present (peptides 3 and 4). Serum treatment of cells resulted in the enhanced phosphorylation or de novo appearance of several peptides (peptides 5 and 6, and peptides 1, 2, and 7, respectively). The pattern of phosphopeptides induced by D609 was virtually identical to that induced by serum. Taken together, the absence of detectable differences in the hyperphosphorylation pattern of Raf induced by serum and D609, in contrast to the differential effect of these treatments on Raf kinase activity (Fig. 1), strongly implies that these phosphorylation events are not per se involved in the regulation of the enzymatic activity of Raf.

D609-induced Hyperphosphorylation of Raf-1 Correlates with Decreased Membrane Localization of Raf-1 in Vivo and in Vitro—Plasma membrane localization represents an important determinant in Raf-1 kinase activation. It is thus conceivable that mobility shift-associated hyperphosphorylation could be involved in Raf kinase activity attenuation by negatively modulating plasma membrane localization of Raf. In order to test this hypothesis, we first investigated the effect of D609 on the subcellular localization of Raf-1 in vivo. As shown in Fig. 3A, incubation of cells with D609 for times that lead to a Raf mobility shift (see Fig. 1B) induced a decrease in plasma membrane-associated Raf-1. Although this correlation implies a direct mechanistic relationship between these two phenomena, one cannot rule out from these experiments the possibility that the decrease in plasma membrane localization of Raf-1 is due to effects of D609 on plasma membrane components regulating the association of Raf with this subcellular compartment rather than being a direct consequence of Raf hyperphosphorylation.

In order to discount a contribution of modulated plasma membrane components in the observed phenomenon, we analyzed the membrane association of Raf-1 in vitro by employing a cell-free reconstitution system. Serum-starved parental CHO cells were subjected to subcellular fractionation as described above, except that the initial P100 pellet was resuspended in hypotonic buffer devoid of detergents. Portions of these “naive” membranes were then mixed with cytosolic fractions obtained from either quiescent or D609-treated CHO/Flag-Raf-1 cells. After incubation on ice for 30 min, these “reconstituted” hypotonic extracts were subjected to standard subcellular fractionation, and distribution of Raf-1 between the cytosolic and the membrane fractions was analyzed as described above. However, in order to facilitate visual quantification, proteins were separated on 12% gels to prevent resolution of individual mobility-shifted Raf species observed on 7% gels. In addition, exposure times during Western blot analysis using the enhanced chemiluminescence detection method were adjusted to give similar intensities for immunodetected Raf-1 recovered from the cytosolic fraction and the particulate fraction. Judging from the difference in exposure times during enhanced chemiluminescence-based immunodetection of Raf-1, about 5% of Flag-Raf-1 was recovered from the Triton X-100-extractable membrane fraction in this assay. This ratio is in good agreement with that estimated for the in vivo distribution of Raf-1 based on subcellular fractionation of whole cells (Ref. 18; Fig. 3A). As shown in Fig. 3B, the amount of Flag-Raf-1 recovered in the membrane fraction was reduced when cytosolic extracts

---

**Fig. 2.** D609-induced Raf-1 mobility shift is due to hyperphosphorylation and occurs on sites identical to those found in serum-stimulated cells. CHO/Flag-Raf-1 cells were metabolically labeled with [32P]orthophosphate in serum-free medium for 12 h and either left untreated or incubated at the end of the labeling period for 15 min with 20% fetal bovine serum or for 30 min with 10 μg/ml D609. A, Flag-Raf-1 proteins were isolated by immunoprecipitation using an epitope-specific antibody and then analyzed by SDS-PAGE and autoradiography. B, Raf-1 phosphoproteins were further analyzed by two-dimensional tryptic phosphopeptide mapping. A schematic representation of the observed phosphopeptides is shown in the lower left part. Induced and constitutively phosphorylated phosphopeptides are represented by open and closed symbols, respectively.
Mobility shift-associated hyperphosphorylation of Raf-1 correlates with decreased plasma membrane localization of Raf-1 in vivo and in vitro. A, CHO/Flag-Raf-1 cells were incubated for 30 min with D609 (10 μg/ml) or left untreated and then subjected to subcellular fractionation (see "Experimental Procedures"). Flag epitope-tagged Raf-1 protein in the 100,000 × g supernatant (Cytosol) and pellet (Membrane) was immunoprecipitated with α-Flag epitope mAb M2 and then analyzed by Western blotting using a Raf-specific antiserum. In order to facilitate visual quantification, proteins were separated on 12% gels to prevent resolution of individual mobility-shifted Raf species observed on 7% gels. In addition, exposure times during Western blot analysis using the enhanced chemiluminescence detection method were adjusted to give similar intensities for immunodetected Raf-1 recovered from the cytosolic fraction and the particulate fraction. Comparison of the relative exposure times suggested a subcellular distribution of 90–95% cytosolic Raf and 5–10% membrane-bound Raf. B, serum-starved CHO/Flag-Raf-1 cells were treated for 30 min with D609 (10 μg/ml) or left untreated. Cytosolic extracts (Input) were incubated for 30 min at 4 °C with the membrane-containing fraction prepared from serum-starved parental CHO cells. Subsequently, the subcellular localization (Cytosol, Membrane) of Flag-Raf-1 protein following cellular fractionation was analyzed by immunoprecipitation and Western blotting as described above. C, serum-starved CHO/Flag-Raf-1 cells were treated for 30 min with fetal calf serum (20%) or left untreated. Cytosolic extracts were prepared from starved or serum-treated CHO/Flag-Raf-1 cells and analyzed as described for B.

from D609-treated cells were compared with those of control cells. This was not due to differences in the amount of Raf-1 present in the S100 fractions of serum-starved and D609-treated cells (Input). Importantly, as shown in Fig. 3C, a similar reduction of Raf recovered from the particulate fraction was observed when cytosolic extracts obtained from serum-treated cells were analyzed in this reconstitution assay. Thus, these data strongly suggest that mobility shift-associated hyperphosphorylation in response to mitogen stimulation of cells negatively regulates plasma membrane localization of Raf-1. Since the kinetics of appearance of mobility-shifted Raf correlate with a reduction in Raf-1 kinase activity following an initial maximal stimulation in response to serum treatment (17), the proposed mechanism may account, at least in part, for the attenuation of Raf kinase activity following mitogen stimulation.

Inhibition of D609-induced MAP Kinase Activation by PD98059 Restores Membrane-associated Raf Protein to Control Levels—The molecular events that lead to hyperphosphorylation of Raf-1 in response to serum- or D609-treatment of CHO cells are enigmatic. It is conceivable, however, that these treatments result either in the activation of a latent Raf-1 kinase or inhibition of a constitutive Raf-1 kinase phosphatase. We therefore initially investigated the effect of D609 on the cellular activity of several protein kinases that had been reported to phosphorylate Raf in vitro. While PKA activity was not altered in response to D609 (data not shown), surprisingly, ERK2 activity was strongly stimulated with kinetics and to an extent similar to that induced by FCS (Fig. 4). Incubation of cells with potassium ethyl xanthate, a biologically inactive D609 homologue, did not result in ERK2 activation. The kinetics of stimulation of ERK2 activity in response to D609 and FCS preceded those of the appearance of the Raf mobility shift, which ensued with slightly different lag times (Fig. 1B; see also "Discussion"). Furthermore, Western blot analysis of lysates prepared from parallel dishes using a MEK-specific antiserum revealed a time-dependent retardation of the electrophoretic mobility of MEK in response to D609 (data not shown). This mobility shift is believed to correlate with MEK activation (47). These observations thus raise the intriguing possibility that hyperphosphorylation of Raf-1 in response to D609 is the consequence of activation of the MAP kinase cascade at a step distal to Raf, probably at the level of MEK.

We therefore investigated the effect of blocking activation of the MAP kinase pathway on the D609-induced mobility shift and subcellular relocalization of Raf. Pretreatment of CHO/Flag-Raf-1 cells with the MEK activation inhibitor PD98059 (48) reduced the basal activity of ERK2 and completely blocked D609-induced ERK2 activation (Fig. 5C). Analysis of the subcellular localization of Raf revealed that preincubation of cells with PD98059 completely prevented the D609-induced reduction of membrane-associated Raf (Fig. 5A). Significantly, this correlated with a reversion of the D609-induced mobility shift of Raf (Fig. 5B). Furthermore, analysis of the relative levels of the different forms of hyperphosphorylated Raf species revealed an inverse correlation between the degree of mobility shift-associated hyperphosphorylation and membrane association of Raf (Fig. 5B, lane 3).

In summary, the results presented here strongly suggest that mobility shift-associated hyperphosphorylation of Raf (a) is a consequence of activation of components in the MAP kinase pathway downstream from Raf and (b) reduces its association with the plasma membrane. Since membrane localization represents a crucial role in Raf kinase activation and since D609- and serum-induced hyperphosphorylation of Raf-1 occur on identical sites, hyperphosphorylation-mediated reduction of plasma membrane association of Raf may represent a molecular mechanism accounting for the “down-regulation” of Raf-1 kinase activity that follows its initial mitogen stimulation.
The molecular mechanism(s) involved in the regulation of Raf-1 protein kinase activity are complex and still incompletely understood. The initial events leading to activation of Raf-1 seem to involve Ras-mediated membrane translocation and phosphorylation on tyrosine residues (21–23). Despite the obvious physiological importance, little is known about the mechanism(s) involved in turning Raf-1 kinase “off.” Treatment of cells with a wide range of physiological stimuli results in hyperphosphorylation of Raf. While phosphorylation and activation of Raf by protein kinase C (24, 27) and/or tyrosine kinases such as Src and Lck (22, 23) might represent early receptor-stimulated events, these post-translational modifications are unlikely to account for the characteristic retardation of the mobility of Raf upon SDS-PAGE. The nature and significance of these mobility shift-associated hyperphosphorylation events have been controversial. Although experiments with serine/threonine-specific phosphatases initially suggested a stimulatory role for these post-translational modifications of Raf-1, recent evidence has challenged this hypothesis (17, 31–34). It has previously been demonstrated in NIH3T3 cells that inhibition of endogenous PC-PLC by D609 blocks activation of Raf-1 in response to mitogenic growth factors (56). However, in contrast to our results obtained with CHO cells, Cai et al. (56) failed to observe a Raf-1 mobility shift in response to treatment of serum-starved NIH3T3 cells with D609. This is probably due to the different cell system or the higher concentration of D609 (35 μg/ml) used in their experiments. While we observed a pronounced Raf protein mobility shift at low concentrations of D609, the shift was less pronounced at higher concentrations (>35 μg/ml; Fig. 1A). Thus, the inhibition of serum-stimulated Raf-1 kinase activation at concentrations of D609 higher than

**DISCUSSION**

The molecular mechanism(s) involved in the regulation of Raf-1 protein kinase activity are complex and still incompletely understood. The initial events leading to activation of Raf-1 seem to involve Ras-mediated membrane translocation and phosphorylation on tyrosine residues (21–23). Despite the obvious physiological importance, little is known about the mechanism(s) involved in turning Raf-1 kinase “off.” Treatment of cells with a wide range of physiological stimuli results in hyperphosphorylation of Raf. While phosphorylation and activation of Raf by protein kinase C (24, 27) and/or tyrosine kinases such as Src and Lck (22, 23) might represent early receptor-stimulated events, these post-translational modifications are unlikely to account for the characteristic retardation of the mobility of Raf upon SDS-PAGE. The nature and significance of these mobility shift-associated hyperphosphorylation events have been controversial. Although experiments with serine/threonine-specific phosphatases initially suggested a stimulatory role for these post-translational modifications of Raf-1 (30), recent evidence has challenged this hypothesis (17, 31–34). First, the kinase activity negatively affected by phosphatase treatment in the experiments of Kovacina et al. (30) is unlikely to reflect Raf-1, since the peptide used, Syntide-2, is not a Raf-1 substrate (49). While the more recent demonstration of Raf-1 kinase inactivation following phosphatase treatment was performed using the physiological Raf-1 substrate MEK, it was not demonstrated whether this correlated with corresponding changes in the electrophoretic mobility of Raf-1 (50). Furthermore, only highly purified Raf-1 devoid of the putative chaperone proteins hspr90 and 14–3–3, which form a complex with Raf-1 in vivo (17, 51–54), was susceptible to inactivation by phosphatase treatment. Second, a comparison of the time course of activation and mobility shift of Raf in response to various agonists has shown that these processes are poorly correlated (17, 33, 55). Thus, we have previously demonstrated that the transient activation of Raf protein kinase in response to serum stimulation of cells correlated with a transient membrane translocation of Raf and occurred in the absence of a Raf mobility shift. On the contrary, in accordance with the the kinetics of serum-stimulated Raf hyperphosphorylation presented here, the appearance of the Raf mobility shift correlated with an attenuation of Raf kinase activity and was paralleled by a reduction in the amount of membrane-associated Raf (17).

Here we show that mobility shift-associated hyperphosphorylation of Raf induced by treatment of cells with D609, a PC-PLC inhibitor, correlates with a decrease in membrane-localized Raf below that observed in untreated cells. The results obtained in the cell-free reconstitution experiments demonstrated that this phenomenon is not due to effects of D609 on the plasma membrane. Importantly, hyperphosphorylated Raf from serum-treated cells displayed a similar decreased tendency to localize to the membrane fraction in this *in vitro* assay. Furthermore, comparison of the phosphopeptide maps revealed no significant differences in the phosphorylation state of Raf in D609- and mitogen-stimulated cells. Taken together, these results suggest that negative modulation of membrane localization mediated by mobility shift-associated hyperphosphorylation of Raf represents an important molecular mechanism that could account, at least in part, for the attenuation of Raf kinase activity that follows its initial mitogen activation.

The results presented here imply that mobility shift-associated hyperphosphorylation does not directly regulate the catalytic activity of Raf, but rather exerts its effect indirectly, by modulating the ability of Raf to associate with the plasma membrane, the residence of the elusive Raf—“activating principle.” Consistent with this hypothesis, dephosphorylation of mobility-shifted Raf, isolated from D609 or serum-treated cells, by incubation with either of the serine/threonine-specific protein phosphatases PP1 or PP2A, did not significantly alter the "activating principle," such as a putative lipid cofactor. Following serum starvation, the levels of this "activating principle" are likely low, but not necessarily absent. Fluctuations in the levels of the Raf activator in serum-starved cells might explain the varying degrees of reduction (0–89%) below basal Raf kinase activity observed in D609-treated cells. In serum-stimulated cells, on the other hand, the level of the Raf activator is likely significantly increased and might account for the intermediate, rather than basal or reduced, Raf kinase activity of mobility-shifted Raf observed at later times following mitogen stimulation.

It has previously been demonstrated in NIH3T3 cells that inhibition of endogenous PC-PLC by D609 blocks activation of Raf-1 in response to mitogenic growth factors (56). However, in contrast to our results obtained with CHO cells, Cai et al. (56) failed to observe a Raf-1 mobility shift in response to treatment of serum-starved NIH3T3 cells with D609. This is probably due to the different cell system or the higher concentration of D609 (35 μg/ml) used in their experiments. While we observed a pronounced Raf protein mobility shift at low concentrations of D609, the shift was less pronounced at higher concentrations (>35 μg/ml; Fig. 1A). Thus, the inhibition of serum-stimulated Raf-1 kinase activation at concentrations of D609 higher than

**FIG. 5.** The MEK activation inhibitor PD98059 blocks D609-induced MAP kinase activation and restores membrane-associated Raf protein to control levels. Serum-starved CHO/Flag-Raf-1 cells were pretreated with PD98059 (50 μM) or vehicle for 60 min and then incubated with D609 (10 μg/ml), or left untreated, for an additional 60 min. A, cells were subjected to subcellular fractionation followed by immunoprecipitation and visualization of Flag-Raf-1 as described in the legend to Fig. 3. B, same as A except for the omission of Flag-Raf-1 immunoprecipitation and separation of proteins on 7% rather than 12% gels. C, protein kinase activity of ERK2 was determined in detergent lysates from parallel cultures as described in the legend to Fig. 4.
The clearest evidence arguing for an involvement of MEK or a downstream component in the hyperphosphorylation of Raf-1 is PD98059-sensitive strongly accompanied by a decrease in the MEK kinase activity of Raf-1. Moreover, the use of an agent that activates MAP kinase pathway components downstream of Raf-1, such as PD98059, shifts Raf-1 to a hypophosphorylated state. The hypophosphorylated state of Raf-1 is known to exist in a latent, activation-competent state, whereas Raf-1 becomes hyperphosphorylated on sites that decrease its affinity for the plasma membrane as a consequence of activation of downstream components in the MAP kinase signaling cascade. In this turn reduces the amount of Raf-1 available for further activation by the elusive Raf-activating principle. Following this desensitization period, a hypothetical phosphatase might be responsible for converting Raf-1 to an activation-competent state, thus resetting the system to a latent state ready to respond to new signals.}

Research suggests the involvement of a component of the MAP kinase pathway at a point distal to Raf and, to a lesser extent, MEK kinase. While MEK-1 is unable to phosphorylate recombinant Raf-1 in vitro (data not shown) and thus is an unlikely Raf-1 kinase, the classical MAP kinase isoforms are able to phosphorylate Raf-1 in vitro (Refs. 58 and 59; data not shown). While this is consistent with MAP kinase being a potential mediator of Raf-1 hyperphosphorylation in vivo, phosphorylation of Raf-1 by the classical MAP kinases in vitro fails to cause the characteristic mobility shift of Raf-1 (data not shown). Thus, it is likely that the putative Raf kinase responsible for mobility shift-associated hyperphosphorylation of Raf lies downstream of ERK2. The molecular mechanism of how D609 leads to the activation of ERK2 (and MEK; data not shown) and whether D609 affects additional components in this pathway remains to be established. Differential modulation of component(s) downstream of ERK2 by D609 and FCS might explain the observation that, while D609 and FCS stimulated ERK2 activity with similar kinetics, the time course of appearance of mobility-shifted Raf elicited by D609 was somewhat slower than that induced by FCS.

PKA phosphorylates Raf-1 on sites that either negatively affect the Raf-1/Ras interaction (i.e., Ser-43) or inhibit the catalytic activity of Raf (28). PKA is thus a potential Raf kinase whose activation might account for the phenomenon reported here. Indeed, comparison of the phosphopeptide maps presented here with those reported by Morrison and co-workers (28) suggests that D609 as well as serum induce the phosphorylation of Raf on Ser-43 (Fig. 2, phosphopeptide 1 and/or 2). However, the in vivo phosphorylation of this site is probably not mediated by PKA, since we did not observe activation of PKA in response to either stimulus (data not shown). Furthermore, it remains to be established whether mobility shift-associated hyperphosphorylation interferes with membrane localization of Raf at the level of interaction with Ras or other membrane components. Interestingly, it has been suggested that membrane anchoring of Raf, in contrast to its initial translocation, occurs in a Ras-independent manner (20).

An alternative mechanism that could account for Raf-1 hyperphosphorylation is the negative modulation of phosphatases...
involves the regulation of MAP kinase signal transduction. PP2A has been implicated in the negative regulation of MEK and MAP kinases (60) and is able to dephosphorylate Raf-1 in vitro (data not shown). Cellular inactivation of PP2A might therefore not only result in enhanced phosphorylation and activation of MEK and MAP kinase, but might at the same time allow more efficient Raf-1 hyperphosphorylation. Interestingly, it has been reported that PP2A is inhibited in response to growth factor or insulin stimulation of cells, likely as a consequence of tyrosine and/or threonine phosphorylation of the catalytic subunit of PP2A (61, 62). While D609 failed to inhibit the catalytic activity of PP2A or PP1 in vitro as well as in vitro (data not shown), it might alter the substrate specificity or subcellular localization of PP2A, two parameters that might be controlled by the association of the catalytic subunit with different regulatory subunits (63, 64). Furthermore, the involvement of other serine/threonine-specific phosphatases in the regulation of Raf hyperphosphorylation cannot be ruled out.

The strong amplification potential of the MAP kinase cascade (48), together with the inhibition of a serine/threonine-specific phosphatase(s), might explain the apparent paradox of D609-mediated MEK and MAP kinase activation in the absence of Raf kinase stimulation. Alternatively, other MEK kinases, such as MEKK-1 (65) or Tpl-2 kinase (66), might be involved in the D609-induced response.

Resetting signaling cascades to “default values” following stimulation is crucial for any cellular system to be able to appropriately respond to changes in its environment. Attenuation of signal transduction via the MAP kinase cascade is achieved at various levels and by different mechanisms. Some of the desensitization mechanisms are built into the signal transducer itself. Thus, Ras can revert to its inactive state by dissociation from the adapter protein Grb2 and its functional Raf-1 becomes activated in a nascent signaling complex that is recruited to the plasma membrane, where Raf-1 becomes activated in an as yet ill-defined fashion that might include stimulatory phosphorylation events, putative lipid-second messengers, or both.

A later stage following serum stimulation, the majority of Raf-1 becomes hyperphosphorylated on sites that decrease its affinity for the plasma membrane as a consequence of activation of downstream components in the MAP kinase signaling cascade. This in turn reduces the amount of Raf-1 available for further activation at the plasma membrane. Following this desensitization period, a hypothetical phosphatase might be responsible for converting Raf-1 to an activation-competent state, thus resetting the system to a latent state ready to respond to new signals.

**Acknowledgments**—Dr. Roger J. Davis (Howard Hughes Medical Institute, Program in Molecular Medicine, Worcester, MA) is thanked for plasmids PSV/Plag-Raf-1 and J. J. St. John’s antiserum 2880. Escherichia coli strain BL21(DE3)plysS transformed with pKH-1 encoding human MMK1 fitted with an N-terminal hexahistidine tag was generously provided by Dr. N. Ahn (University of Colorado, Boulder, CO). Drs. Kurt Ballmer-Hofer, George Thomas and Xu-Fen Ming (Friedrich Miescher Institute, Basel) are thanked for helpful discussions and critical reading of the manuscript.

**REFERENCES**

1. Daum, G., Eisenmann, Tappe, L., Fries, H. W., Tropfmaier, J., and Rapp, U. R. (1994) Trends. Biochem. Sci. 19, 474–480
2. Marshall, C. J. (1994) Curr. Opin. Genet. Dev. 4, 82–89
3. Rapp, U. R. (1991) Oncogene 6, 495–500
4. Li, P., Wood, K., Marnon, H., Haser, W., and Roberts, T. C. (1991) Cell 64, 479–482
5. Aqvist, J., Zhang, X. F., and Kyriakis, J. M. (1994) Trends. Biochem. Sci. 19, 579–583
6. Davis, R. J. (1993) J. Biol. Chem. 268, 14553–14556
7. Blumer, K. J., and Johnson, G. L. (1994) Trends. Biochem. Sci. 19, 236–240
8. Aqvist, J. D., Sirot, Y., Campbell, D. G., Cohen, P., Sathianandam, R., Rapp, U., Ashworth, A., Marshall, C. J., and Cowley, S. (1994) EMBO J. 13, 1610–1619
9. Payne, D. M., Rossmann, A. J., Martino, P., Erickson, A. K., Her, J. H., Shabanowitz, J., Hunt, O. F., Weber, M. J., and Sturgill, T. W. (1991) EMBO J. 10, 885–892
10. Zheng, C. F., and Guan, K. L. (1994) EMBO J. 13, 1123–1131
11. Moolde, S. A., Wullienscurn, B. M., Weber, M. J., and Wolfinb, A. (1993) Science 260, 1658–1661
12. Van Aelst, L., Barr, M., Marcus, S., Polverino, A., and Wigler, M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6211–6217
13. Vojik, A. B., Healelamb, S. M., and Cooper, A. J. (1993) Cell 74, 205–214
14. Warne, P. H., Vicania, P. R., and Downward, J. (1993) Nature 364, 352–355
15. Leever, S. J., Paterson, H. F., and Marshall, C. J. (1994) Nature 369, 411–414
16. Traverse, S., Cohen, P., Paterson, H. F., Marshall, C., Capp, U., and Grand, R. J. (1993) Oncogene 8, 3175–3181
17. Wartmann, M., and Davis, R. J. (1994) J. Biol. Chem. 269, 6695–6701
18. Hallberg, B., Rayer, S. I., and Downward, J. (1994) J. Biol. Chem. 269, 6913–6916
19. Zhang, X. F., Settleman, J., Kryia, J., Makue, S., Takuei-Suzuki, E., Elledge, S. J., Marshall, M. S., Bruder, J. T., Rapp, U. R., and Avruch, J. (1993) Nature 364, 308–313
20. Stokoe, D., Macdonald, S. G., Cadwallader, K., Symons, M., and Hancock, J. F. (1993) Science 261, 1463–1467
21. Morrison, D. K., Kaplan, D. K., Escobedo, J. A., Rapp, U. R., Roberts, T. M., and Williams, L. T. (1989) EMBO J. 8, 457–464
22. Fabian, J. C., Don, I. O., and Morrison, D. K. (1993) Mol. Cell. Biol. 13, 7170–7179
23. Marta, R., Light, Y., Paterson, H. F., and Marshall, C. J. (1995) EMBO J. 14, 3136–3145
24. Kelch, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finckenueller, G., Mame, D., and Rapp, U. R. (1993) Nature 364, 249–252
25. Mcgrae, R. B., Nichols, D. W., Stanton, V. P., Jr., Cai, H., Whorff, R. C., Patel, V., Cooper, G. M., and Laudane, A. P. (1992) Oncogene 7, 33–42
26. Morrison, D. K., Heidecker, G., Rapp, U. R., and Copeland, T. D. (1993) J. Biol. Chem. 268, 17309–17316
27. Soderi, O., Voller, K., Liyanage, M., Frith, D., Kour, G., Mark, G. E., and Stabel, S. (1992) Oncogene 7, 2259–2262
28. Burger, M. B., and Bolle, L. (1995) Trends. Biochem. Sci. 20, 18–22
29. Yao, B., Zhang, Y., Delitak, S., Mathias, S., Bausa, S., and Kolesnik, R. (1995) Nature 379, 307–310
30. Kovacina, K., Nye, K., Kusugaki, B., Brautigan, D. L., Tonks, N. K., Rapp, U. R., and Roth, R. A. (1990) J. Biol. Chem. 265, 12115–12118
31. Crespo, P., Xu, N., Dianotti, J. L., Troppmair, J., Rapp, U. R., and Gutkind, J. S. (1994) J. Biol. Chem. 269, 21103–21109
32. Samuels, M. L., Wehler, M. J., Bishop, J. M., and McMahon, M. (1993) Mol. Cell. Biol. 13, 6241–6252
33. Ueki, K., Matsuda, S., Tuse, K., Gotoh, Y., Tamemoto, H., Yachi, M., Akanuma, Y., Yaxoki, Y., Nishida, E., and Kadowaki, T. (1994) J. Biol. Chem. 269, 15756–15761
34. Williams, N. G., Paradis, H., Agarwal, S., Charest, D. L., Pelech, S. L., and Roberts, T. M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5772–5776
35. Manseau, S. J., Reising, K. A., Candi, J. M., Hermann, A. S., Gloor, J. W., Herskind, K. R., Wartmann, M., Davis, R. J., and Ahn, N. G. (1994) J. Biochem. (Tokyo) 116, 304–314
36. Marte, B. M., Graus Porta, D., Jeschke, M., Fabbro, D., Hynes, N. E., and
Taverna, D. (1995) *Oncogene* 10, 167–175
37. Morgenstern, J. P., and Land, H. (1990) *Nucleic Acids. Res.* 18, 3587–3596
38. Miller, D. G., and Miller, A. D. (1992) *J. Virol.* 66, 78–84
39. Stover, D. R., Becker, M., Liebetanz, J., and Lydon, N. B. (1995) *J. Biol. Chem.* 270, 15591–15597
40. Gould, K. L., and Hunter, T. (1988) *Mol. Cell Biol.* 8, 3345–3356
41. Roskoski, R. (1983) *Methods Enzymol.* 99, 3–6
42. Cohen, P., Alemany, S., Hemmings, B. A., Resink, T. J., Stralfors, P., and Tung, H. Y. (1988) *Methods Enzymol.* 159, 390–408
43. Borner, C., Filipuzzi, I., Wartmann, M., Eppenberger, U., and Fabbro, D. (1989) *J. Biol. Chem.* 264, 13902–13909
44. Ghosh, S., Xie, W. Q., Quest, A. F. G., Mabrouk, G. M., Strum, J. C., and Bell, R. M. (1994) *J. Biol. Chem.* 269, 10000–10007
45. Nishizuka, Y. (1992) *Science* 258, 607–614
46. Schutze, S., Potthoff, K., Machleidt, T., Berkovic, D., Wiegmann, K., and Kronke, M. (1992) *Cell* 71, 765–776
47. Grinstein, S., Butler, J. R., Furuya, W., L’Allemain, G., and Downey, G. P. (1994) *J. Biol. Chem.* 269, 18313–18320
48. Alesci, D. R., Cuendan, A., Cohen, P., Dudley, D. T., and Saltiel, A. R. (1995) *J. Biol. Chem.* 270, 27489–27494
49. Force, T., Bonventre, J. V., Heidecker, G., Rapp, U., Avruch, J., and Kyriakis, J. M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 1270–1274
50. Chen, J., Parsons, S., and Brautigan, D. L. (1994) *J. Biol. Chem.* 269, 7957–7962
51. Stancato, L. F., Chow, Y.-H., Hutchissen, K. A., Perdw, G. H., Jove, R., and Pratt, W. B. (1995) *J. Biol. Chem.* 268, 21711–21716
52. Freed, E., Symons, M., Mardenald, S. G., McCormick, F., and Ruggieri, R. (1994) *Science* 265, 1713–1716
53. Irie, K., Gotoh, Y., Yashar, B. M., Errede, B., Nishida, E., and Matsumoto, K. (1994) *Science* 265, 1716–1719
54. Fu, H., Xia, K., Pallas, D. C., Cui, C., Conroy, K., Narasiman, R. P., Mamom, H., Collier, R. J., and Roberts, T. M. (1994) *Science* 266, 126–129
55. Ishibana, C., Alemey, S., Calvo, V., and Fernandez-Renart, M. (1994) *Eur. J. Immunol.* 24, 2746–2754
56. Cai, H., Erhardt, P., Trompmaier, J., Diaz Meco, M. T., Sihanamndam, G., Rapp, U. R., Moscat, J., and Cooper, G. M. (1993) *Mol. Cell Biol.* 13, 7645–7651
57. Waters, S. B., Hkt, K. H., Ross, S. E., Syu, L.-J., Guan, K.-L., Sabiel, A. R., Koretzky, G. A., and Pessin, J. E. (1995) *J. Biol. Chem.* 270, 29883–29886
58. Lee, R., Cobb, M. H., and Blackshear, P. J. (1992) *J. Biol. Chem.* 267, 20888–20902
59. Anderson, N. G., Li, P., Maroden, L. A., Williams, N., Roberts, T. M., and Sturgill, T. W. (1991) *Biochem. J.* 277, 573–576
60. Sontag, E., Fedorov, S., Kamibayashi, C., Robbins, D., Cobb, M., and Mumbry, M. (1993) *Cell* 73, 887–897
61. Chen, J., Parsons, S., and Brautigan, D. L. (1994) *J. Biol. Chem.* 269, 7957–7962
62. Guo, H., and Damuni, Z. (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90, 2500–2504
63. Wera, S., and Hemmings, B. A. (1995) *Biochem. J.* 311, 17–29
64. Mumbry, M. C., and Walter, G. (1993) *Physiol. Rev.* 73, 673–699
65. Lange-Carter, C. A., Pleiman, C. M., Gardner, A. M., Blumer, K. J., and Johnson, G. L. (1993) *Science* 260, 315–319
66. Salmon, A., Ahmad, T. B., Carlile, G. W., Pappin, D., Narasiman, R. P., and Ley, S. C. (1996) *EMBO J.* 15, 817–826
67. Bourne, H. R., Sanders, D. A., and McCormick, F. (1991) *Nature* 348, 117–127
68. Cherniack, A. D., Klarlund, J. K., and Czech, M. P. (1994) *J. Biol. Chem.* 269, 4717–4720