Phorbol ester stimulation of the MAPK cascade is believed to be mediated through the protein kinase C (PKC)-dependent activation of Raf-1. Although several studies suggest that phorbol ester stimulation of MAPK is insensitive to dominant-negative Ras, a requirement for Ras in Raf-1 activation by PKC has been suggested recently. We now demonstrate that in normal, quiescent mouse fibroblasts, endogenous c-N-Ras is constitutively associated with both c-Raf-1 and PKCs in a biochemically silent, but latent, signaling module. Chemical inhibition of novel PKCs blocks phorbol 12-myristate 13-acetate (PMA)-mediated activation of MAPKs. Down-regulation of PKCα protein levels by antisense oligodeoxyribonucleotides blocks MAPK activation in response to PMA stimulation, demonstrating that PKCα activity is required for MAPK activation by PMA. c-Raf-1 activity in immunoprecipitated c-N-Ras-c-Raf-1-PKCα complexes is stimulated by PMA and is inhibited by GF109203X, thereby linking c-Raf-1 activation in this complex to PKC activity. These observations suggest that in quiescent cells Ras is organized into ordered, inactive signaling modules. Furthermore, the regulation of the MAPK cascade by both Ras and PKC is intimately linked, converging at the plasma membrane through their association with c-Raf-1.

The regulation of the Raf-1 serine/threonine kinase is a highly complex and poorly understood process (1, 2). Raf-1 activation is a critical component of the proliferative response. Raf-1 activation regulates the MAPK3 cascade, a linear kinase cascade comprising the MAPKs, ERK1, and -2, which are the physiological targets for MEK1, the substrate for Raf-1 (3–5). Raf-1 can be activated in response to both oncogenes and receptor tyrosine kinase activation. Raf-1 activity is regulated by phosphorylation on serine and tyrosine residues (6–8), with both PKC and Src implicated as Raf-1 regulatory kinases. Raf-1 activity is also subject to negative regulation by PKA-mediated phosphorylation of serine 621 in the kinase domain (9, 10) and serine 43 in the Ras-binding domain (11), in response to elevations in intracellular cAMP levels. The direct interaction of Raf-1 with Ras (12, 13), plasma membrane phospholipids (14, 15), and molecular chaperone proteins of the hsp90 (16) and 14-3-3 families (17) also regulates Raf-1 activity possibly through contributions to Raf-1 dimerization and the masking of critical regulatory phosphorylation and phosphatidylerine-binding sites (reviewed in Ref. 2). Other protein-directed mechanisms of Raf-1 regulation have been identified recently. These appear to function through a poorly understood mechanism of membrane targeting and the organization of Raf-1 with its effectors. These include KSR (18–20), which may function as a scaffolding protein in organizing the interaction of Raf-1 with its substrate MEK1, CNK (21), which targets Raf-1 to cell-cell contacts, and RKIP (22) which inhibits Raf-1 signaling by disrupting the interaction of Raf-1 with its substrate, MEK1.

The Raf kinases contribute to the complex process of cell proliferation through their regulation of the MAPK cascade. Persistent stimulation of this cascade by activated Raf protein can result in cellular transformation (reviewed in Ref. 23). Recent work by Mason et al. (8) demonstrated that the expression of oncogenic Ras in cells induced phosphorylation of Raf-1 at serine 338, a consensus site for PKC-mediated phosphorylation, whereas activated Src induced Raf-1 phosphorylation predominantly at tyrosine 341; Raf-1 phosphorylation was also dependent upon its association with the plasma membrane. Mitogen-induced Raf-1 activity required phosphorylation on both Ser-338 and Tyr-341, which cooperate to induce full Raf-1 activation. Under specific conditions, Tyr-341 phosphorylation appeared to direct Ser-338 phosphorylation, since Raf-1 harboring a Y341A substitution was not phosphorylated on Ser-338 in cells expressing activated Ras and Src. Although co-expression of activated Ras and Src induced Ser-338 phosphorylation that was dependent upon phosphorylation of Tyr-341, mitogen-induced (PMA or EGF) Ser-338 phosphorylation could occur in the absence of Tyr-341 phosphorylation. This observation suggested that two distinct Raf-1 kinases might phosphorylate Raf-1 on Ser-338 and contribute to its activation in vivo. Recently, in addition to PKC, PAK3, a kinase downstream of the Ras-related Rho GTPases, Cdc42 and Rac, was also identified as a putative Raf-1 kinase by its ability to phosphorylate Raf-1 on Ser-338 in vivo (24).

PKC activation by phorbol esters or diacylglycerol (DAG) acti-
vates the MAPK cascade, presumably through stimulating Raf-1 activity (25, 26). Several PKC family members phosphorylate and activate Raf-1 in vitro indicating that the actions of PKC may directly impinge upon the MAPK cascade. Ser-338 has been identified as a putative PKC phosphorylation site on Raf-1 (7, 8). Both classical (cPKC) and novel (nPKC) PKCs have the potential to phosphorylate and activate Raf-1. Both cPKC and nPKC respond to DAG or phorbol ester and have been suggested as Raf-1 regulatory kinases (25–28). There is little evidence, however, as to whether Raf-1 activity is directly regulated by distinct PKC isoforms. Schonwasser et al. (26) demonstrated that although cPKCζ and the atypical (DAG- and Ca2+ -independent) PKCζ stimulate MEK1 and ERK2 activity, only PKCζ and cPKCζ were able to directly stimulate Raf-1 activity. However, a study by Ueda et al. (29) suggested that the expression of activated nPKCδ, but not activated cPKCζ or nPKCζ, activated the MAPK pathway in a c-Raf-1-dependent manner. Although these studies demonstrated some degree of PKC isotype specificity in Raf-1 regulation, the use of activated PKC constructs did not clearly define an unequivocal role for distinct PKC isotypes in Raf-1 regulation.

The interaction and regulation of Raf-1 by Ras (30, 31) is the best characterized Ras-dependent signaling cascade. In vivo, activated Ras binds Raf-1, localizing the kinase to the plasma membrane (14) where it is subsequently phosphorylated and activated (6, 8). The physical interaction of Ras with Raf-1 also provides a structural requirement for efficient Raf-1 activation. Tamada et al. (13) described a Ras activator region mutant, RasV45E, which, although it bound efficiently to Raf-1 and recruited the kinase to the plasma membrane, failed to stimulate efficiently Raf-1 activity. These data indicate that not only did Ras interact with the Ras-binding domain (RBD) of Raf-1 but that the interaction of Ras with the Raf-1 cysteine-rich domain was also critical in Raf-1 activation.

Previous studies in this laboratory demonstrated a Ras isoform dependence in the regulation of c-Raf-1 activation in C3H10T1/2 mouse fibroblasts transformed by the minimal expression of oncogenic Ha-Ras. These studies demonstrated that N-Ras has a higher affinity for c-Raf-1 than did Ha-Ras in vivo (32). Cells transformed with oncogenic N-Ras had a persistently elevated MAPK activity that was resistant to the actions of growth factor and receptor tyrosine kinase antagonists, unlike those transformed by oncogenic Ha-Ras. This suggested that distinct aspects of an Ha-Ras-transformed cell phenotype, including persistent stimulation of MAPK activity, were the consequence of autocrine growth factor signaling (32, 33) and not the physical association of c-Raf-1 with Ha-Ras. Indeed, contrary to expectations, oncogenic Ha-Ras was not associated with c-Raf-1. Oncogenic N-Ras, however, was sufficient in itself to stimulate the MAPK cascade through its stable association with c-Raf-1. Further analysis demonstrated that in Ha-Ras-transformed cells, c-Raf-1 was associated with wild-type c-N-Ras and that antisense mediated down-regulation of c-N-Ras protein levels in these cells ablated their elevated MAPK activity and inhibited their serum-independent growth. These observations supported the concept that in this cell background, c-N-Ras was the Ras isoform that specifically regulated c-Raf-1 and the MAPK cascade.

We have now identified the existence of signaling complexes containing c-N-Ras, c-Raf-1, and PKCζ that are biochemically silent, insofar as possessing no significant Raf-1 activity, in quiescent, normal, non-transfected C3H10T1/2 mouse fibroblasts. In light of the recent observations of Marais et al. (34), who demonstrated that the interaction between Raf-1 and Ras was necessary for PKC to activate Raf-1, we have now extended those observation to show that these biochemically silent complexes of c-N-Ras, c-Raf-1, and PKCζ are latent and are directly activated by phorbol esters. We postulate that these latent complexes represent physiological signaling modules that are a target for the phorbol ester-mediated activation of c-Raf-1 through the activation of PKCζ.

**EXPERIMENTAL PROCEDURES**

**Materials—**PVDF membrane, enhanced chemiluminescence (ECL) reagent, [γ-32P]ATP (3000 Ci/mmol), and [α-32P]cAMP were purchased from Amersham Pharmacia Biotech. PKC inhibitors GF109203X and Go6976 and the cell-permeable Ca2+ chelator, BAPTA-AM, were purchased from Calbiochem. Kinase-dead GST-MEK1(K97A) was purchased from StressGen Biotechnologies Corp. The phorbol ester, phorbol 12-myristate 13-acetate (PMA), Percoll, protein G-Sepharose, and the Sephacryl S200HR gel filtration media were purchased from Sigma. Protein A-Sepharose was purchased from Repligen. Mouse EGF was purchased from Austral Biologicals. Recombinant PKCe and PKCη were purchased from Vanderbilt Corp. and Upstate Biotechnology, Inc., respectively. The PKCs and PKCζ substrate peptides were purchased from BIOSOURCE International and Santa Cruz Biotechnology, respectively. PKCs phosphorohistoic-modified sense and antisense oligodeoxynucleotides (ODN) were synthesized and purified by high pressure liquid chromatography (Sigma). Tissue culture reagents were purchased from Meditech. Fetal calf serum and newborn calf serum were purchased from Atlanta Biologicals. All other reagents were molecular biology grade.

**Antibodies—**Raf-1 and PKCζ-specific monoclonal antibodies were purchased from Transduction Laboratories. N-Ras and phosphoERK monoclonal antibodies were purchased from Santa Cruz Biotechnology. The anti-MAPK rabbit polyclonal antibody was purchased from Upstate Biotechnology, Inc. The N-Ras-specific polyclonal antisera used for immunoprecipitation studies have been described previously (32). Horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies were purchased from Transduction Laboratories and Kirkegaard & Perry Laboratories, respectively. The phospho-Ser-338 Raf-1 rat monoclonal antibody was a kind gift from Richard Marais, Institute of Cancer Research, London, UK.

**Cell Lines—**Normal mouse C3H10T1/2 fibroblasts and their (G12V/Ha-Ras-transformed counterparts, 11A cells, were maintained in Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal calf serum in a 5% (v/v) CO2/air environment. Cells were made quiescent by serum deprivation in serum-free Dulbecco’s modified Eagle’s medium for 48–72 h.

**Western Blot Analysis—**Immunoblot analysis was performed according to standard protocols. After resolution on SDS-PAGE gels, proteins were electroblotted to PVDF membranes and blocked using 10% (w/v) nonfat dry milk in TBS containing 0.1% (v/v) Tween 20 (TBST). Membranes were washed with TBST and incubated with the appropriate dilution of primary antibody in TBST containing 10% (v/v) newborn calf serum. Membranes were then extensively washed with TBST before being incubated with the appropriate horseradish peroxidase-conjugated secondary antibody. The membranes were again extensively washed, and the immunoreactive bands were detected by ECL.

**Immunoprecipitations—**Equal amounts of whole cell lysate or solubilized plasma membrane proteins were incubated with 50 μl of anti-N-Ras sera or non-immune sera or with 2.5–5 μg of purified antibodies at 4 °C. After 2 h, the lysates were microcentrifuged at 14,000 rpm on 4 °C, and the clarified lysates were transferred to clean tubes containing either protein A-Sepharose or protein G-Sepharose, in excess, and centrifuged for 30 min at 4 °C. The antibody complexes were precleared for an additional hour at 4 °C. Immunoprecipitates were washed extensively with TBST, denatured by the addition of Laemmli buffer, and resolved on SDS-PAGE gels.

**Preparation of Plasma Membranes—**Plasma membranes were prepared using a modified protocol as described by Smart et al. (54). Briefly, cells were washed, scraped, and pelleted by low speed centrifugation in Tricine buffer (20 mM Tricine, 250 mM sucrose, 1 mM EDTA, pH 7.8). The cell pellet was resuspended in 0.75 ml of Tricine buffer and disrupted with 20 strokes in a 7-ml Dounce. The cell debris was pelleted by centrifugation at 5,000 rpm in a refrigerated microcentrifuge at 4 °C for 10 min. The supernatant was removed and stored on ice. The cell debris was resuspended in 0.75 ml of Tricine buffer, homogenized, and recentrifuged as described. The supernatant was removed and combined with the first supernatant. The supernatant was then layered on a 30% (v/v) Percoll gradient prepared in Tricine buffer and centrifuged at 90,000 × g for 30 min at 4 °C. Following centrifugation through the 30% Percoll gradient, the distinct, plasma membrane band was collected and resuspended in Tris-buffered saline (TBS), pH 7.8, and...
pelleted by centrifugation at 100,000 × g for 1 h at 4 °C. The plasma membrane pellet was collected and washed twice with TBS followed by centrifugation at 14,000 rpm in a microcentrifuge at 4 °C. The pelleted plasma membranes were solubilized in P21 buffer (20 mM MOPS, 200 mM sodium succinate, 5 mM MgCl2, 1 mM EDTA, pH 7.6) containing 1% (w/v) CHAPS. GST-MEK1 was visualized on a Molecular Dynamics StormImager. Phorbol Ester-Mediated Activation of Raf-1—c-N-Ras immunoprecipitates were washed as described above. Immunoprecipitates were then washed once in Buffer A (50 mM Tris-Cl, pH 7.5, 15 mM MgCl2, 10 mM phosophate inhibitors, 0.1% ml leupeptin, 2 mM MnCl2 and 1 mM CaCl2) containing 2.5 μg of Rottlerin (15 mM), and 100 μg of kinase Buffer B containing 25 μM ATP, 30 μCi of [γ-32P]ATP, 2.5 μg of GST-MEK1(K97A), 25 μg/ml phosphatidyserine, and 50 ng/ml PMA for 30 min at 30 °C. Additional calcium was not added to the buffers. 10 μl GF109203X was included in some reactions to inhibit PKC activity. Kinase reactions were terminated by the addition of 50 μl of 2× Laemmli buffer and phosphorylated proteins were resolved on 8% SDS-PAGE gels and visualized on a Molecular Dynamics StormImager.

PKC Activity toward Kinase-dead GST-MEK1(K97A)—1–5 ng of purified, recombinant human PKCε was incubated with 2.0 μg of GST-MEK1(K97A) in Kinase Buffer B with or without phosphatidyserine and PMA for 30 min at 30 °C. In parallel reactions, 2 ng of recombinant human PKC ε was incubated with 10–100 ng of PKCε peptide substrate (NH2-ERMPPPRKK(QGSRRR)-OH) in kinase buffer containing PMA for 30 min at 30 °C. After 30-min reactions were transferred to 0.22 μm GS filters (Millipore), prewashed with P21 buffer containing 1 mM ATP, and washed extensively with the same buffer. [32P]Phosphate incorporation was determined by scintillation counting.

N-Ras-1 Destabilization Conditions—CHAPS-solubilized rat brain extracts (30) were used as a source of N-Ras. These lysates were depleted of GTP-bound N-Ras with 25 mM EDTA and 100 μM GTP. These assays were done in two different ways. First we tested whether the exchange was inhibited at buffer pH values ranging from 4–9. This was done simply by altering the pH of the assay buffer and determining the GTP binding assay as described previously (30). Second, we tested whether the Ras-GTP complex itself was stable at pH values ranging from 4 to 9. In this assay the GTP binding was performed at pH 7.4 in the standard binding buffer described above. Excess Mg2+ was added to stabilize the complex; the pH was altered, and excess unlabeled GTP was added to quench any labeled GTP released by the change in pH. After 1 h at 4 °C, the reactions were then filtered through 0.22-micron filters as described above. Bound radioactivity was determined by scintillation counting.

Determination of the Guanine Nucleotide State of c-N-Ras in Purified Plasma Membranes—Purified plasma membranes were prepared as described earlier (54). The plasma membrane pellet was solubilized in p21 containing 1% CHAPS and clarified by centrifugation. The supernatant was divided into 5 aliquots. One aliquot was directly incubated with prebound GST-RBD. The remaining aliquots were incubated in pH 7.4 buffer for 1 h. At this point the GTP binding was performed at pH 7.4 in the standard binding buffer described above. Excess Mg2+ was added to stabilize the complex; the pH was altered, and excess unlabeled GTP was added to quench any labeled GTP released by the change in pH. 1 h at 4 °C, the reactions were then filtered through 0.22-micron filters as described above. Bound radioactivity was determined by scintillation counting.

RESULTS
c-N-Ras and c-Raf-1 Are Stably Associated in Quiescent, Normal Mouse C3H10T1/2 Fibroblasts—We had previously identified a Ras isoform-specific association of c-Raf-1 with N-Ras in cells transformed by either oncogenic Ha-Ras or N-Ras (32). This association of c-Raf-1 with c-N-Ras was also observed when c-N-Ras was immunoprecipitated from quiescent, normal mouse C3H10T1/2 fibroblasts under conditions of serum starvation (Fig. 1A). Complexes of c-N-Ras and Raf-1 were also

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c-Raf-1 in c-N-Ras/PKC-e Complexes Activated by Phorbol Ester

The current perception of Ras-regulated Raf-1 signaling is that upon Ras activation, whether through point mutation or the actions of receptor-driven guanine nucleotide exchange factor (e.g. SOS) activity, active Ras binds cytosolic c-Raf-1, thereby recruiting the kinase to the plasma membrane (14). The physical interaction of Ras with c-Raf-1 and its juxtaposition at the plasma membrane facilitates c-Raf-1 activation. However, Raf-1 was detected in purified plasma membranes isolated from serum-starved, quiescent C3H10T1/2 cells (Fig. 1C), and this plasma membrane association is in the absence of any significant MAPK activity. Upon subsequent PMA or EGF stimulation there is an increase in plasma membrane c-Raf-1 levels, indicating that mitogen stimulation induces some recruitment of Raf-1 to the plasma membrane.

The observations that Raf-1 is already membrane-associated in quiescent cell plasma membranes and that it is present in a biochemically inactive complex with Ras might poise the Raf-1 in this signaling module in a unique position to be rapidly activated prior to, or in tandem with, mitogen-induced Ras activation and subsequent Raf-1 recruitment and activation. Since the association of Raf-1 with Ras alone is not sufficient to stimulate Raf-1 activity, the inactive c-N-Ras-Raf-1 complexes were analyzed further for putative Raf-1 activators.

PKC Co-immunoprecipitates with c-N-Ras and c-Raf-1—Further immunoblot analysis of plasma membranes from quiescent C3H10T1/2 cells also identified the presence of both PI3-OH kinase and PKC (data not shown). Both PKC and PI3-OH kinase have been described as having significant effects on c-Raf-1 activity. Several PKC isoforms are able to phosphorylate Raf-1 in vitro, stimulating its activity (25, 26, 35). PI3-OH kinase appears to be able to exert both positive and negative regulatory effects on Raf-1 activity through the phosphatidylinositol 3,4,5-trisphosphate-stimulated activation of phosphoinositide-dependent kinase (reviewed in Ref. 36) and protein kinase B/Akt (37), respectively. Since the PKC-mediated activation of Raf-1 appears to require Ras, we were prompted to further investigate the relationship between PKC and the inactive c-N-Ras-Raf-1 complex.

Since both EGF and PMA stimulate the MAPK cascade, we analyzed the status of the c-N-Ras-Raf-1 complexes from unstimulated and EGF- and PMA-stimulated cells. Again, c-Raf-1 co-immunoprecipitated with c-N-Ras from serum-starved control and EGF-stimulated cells. c-Raf-1 was also present in the c-N-Ras immunoprecipitates from PMA-stimulated cells (Fig. 2A, upper panel). PMA stimulation, like EGF stimulation, can induce a reduction in c-Raf-1 electrophoretic mobility. Overnight treatment with PMA down-regulates the levels of DAG-dependent cPKC and nPKCs. This chronic PMA treatment blocks MAPK activation in serum-starved C3H10T1/2 cells stimulated with PMA but not with EGF (see Fig. 4A). Therefore, to determine whether PKC was a component of the inactive c-N-Ras-Raf-1 signaling complex, c-N-Ras immunoprecipitates were probed for PKC using a mixture of PKC isoform-specific monoclonal antibodies. Western blot analysis detected a band of ~90 kDa in c-N-Ras immunoprecipitates from both quiescent and EGF-stimulated cells (Fig. 2A, lower panel). This 90-kDa band was also present in c-N-Ras immunoprecipitates from PMA-stimulated cells, although at a much reduced level. c-N-Ras immunoprecipitates from chronic PMA-treated cells did not contain the 90-kDa PKC band (Fig. 2B, upper panel), although c-Raf-1 still co-immunoprecipitated with c-N-Ras (Fig. 2B, lower panel). These data suggest that a phorbol ester-down-regulatable PKC isozyme(s) associates with c-N-Ras-Raf-1 complexes. The reduction in the level of PKC that co-immunoprecipitates with c-N-Ras after PMA stimulation may reflect a rapid down-regulation of PKC in the complexes. The absence of the 90-kDa PKC in the c-N-Ras immunoprecipi-

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A PKC and c-Raf-1 co-immunoprecipitate with c-N-Ras. c-N-Ras was immunoprecipitated from whole cell lysates prepared from control, serum-starved C3H10T1/2 cells (Con) and from cells stimulated with either 10 ng/ml EGF or 5 μM PMA for 3 min. Immunoprecipitates were resolved on a 6% SDS-PAGE gel and immunoblotted for Raf-1 (upper panel). Immunoprecipitates were also probed for PKC using a mixture of PKC isoform-specific monoclonal antibodies (lower panel). The positions of Raf-1 and PKC are indicated. The data are representative of two independent experiments. B, chronic PMA treatment down-regulates c-N-Ras-associated PKC. As described above, parallel cell cultures were treated with 5 μM PMA overnight (chronic PMA treatment) to down-regulate cPKC and nPKC isoforms. Cells were then stimulated with either 10 ng/ml EGF or 5 μM PMA for 3 min. c-N-Ras was immunoprecipitated from cell lysates and the immunoprecipitates immunoblotted for c-Raf-1 and PKC. C, PMA treatment results in serine 338 phosphorylation of Raf-1 within the c-N-Ras complex. Cells were untreated or treated with 5 μM PMA + the MEK inhibitor U0126 (10 μM) for 3 min. Cell lysates were prepared as described above. Lysates were immunoprecipitated for N-Ras and probed with an antibody directed against the phosphorylated serine at position 338 of Raf-1. D, classical and novel PKC expression in C3H10T1/2 cells. Cell lysates were prepared from 72-h starved cells with or without overnight treatment with 5 μM PMA. Equal amounts of protein were resolved on 8% SDS-PAGE gels, transferred to PVDF membranes, and immunoblotted for distinct PKC isoforms as indicated. E, c-Raf-1 Western blot was included as a protein loading control. E, c-Raf-1 co-immunoprecipitates with membrane-associated PKCδ and PKCe. (i) c-Raf-1 and the indicated PKCs were immunoprecipitated (2.5 μg of antibody per immunoprecipitation) from 50 μg of membrane-enriched or cytosolic fractions prepared from serum-starved cells. The immunoprecipitates were resolved on 8% SDS-PAGE gels, transferred to PVDF membrane, and immunoblotted for Raf-1. The position of Raf-1 is indicated. IP, immunoprecipitating antibody; NI, non-immune IgG. The immunoprecipitation of PKCδ and PKCe from the membrane-enriched fraction in E(i) was confirmed by Western blot in E(ii). (ii) Raf-1 is readily immunoprecipitated from cytosolic but not membrane fractions. Cytosolic and membrane-enriched fractions (50 μg) were immunoprecipitated with the c-Raf-1-specific monoclonal antibody (2.5 μg). Washed immunoprecipitates were resolved on 8% SDS-PAGE gels, transferred to PVDF membrane, and immunoblotted for Raf-1. F, c-N-Ras and PKCe co-immunoprecipitate. Upper panel, c-Raf-1, PKCδ, and PKCe were immunoprecipitated from membrane-enriched fractions as above. Immunoprecipitates were resolved on a 16.5% SDS-PAGE gel and immunoblotted for c-N-Ras. The position of Ras (21 kDa) is indicated. Lower panel, c-N-Ras was immunoprecipitated from 30 μg of plasma membrane prepared from serum-starved C3H10T1/2 cells. The immunoprecipitate was resolved on a 8% SDS-PAGE gel and immunoblotted for PKCe. Immunoreactive bands were visualized by ECL. The position of PKCe is indicated. IP, immunoprecipitating antibody; NI, non-immune antibody.
were treated with PMA, lysed, and immunoprecipitated with antisera against c-N-Ras. The immunoprecipitates were then analyzed for the presence of phosphoserine 338 on Raf-1. Fig. 2C demonstrates a clear increase in the amount of Ser(P)-338-Raf-1 associated with c-N-Ras following treatment with phorbol esters. Inclusion of the MEK inhibitor U0126 failed to reduce the level of Raf-1 phosphorylation, suggesting that the phosphorylation does not result from a MAPK feedback loop. Similar to the results reported by Marias et al. (34), PKC-mediated activation of Raf-1 does occur in the context of a pre-existing Ras-Raf-1 complex.

Since PKC appears to be a component of inactive c-N-Ras-Raf-1 complexes, PKC and nPKC expression in C3H10T1/2 cells was determined by Western blot analysis. C3H10T1/2 cells express cPKCa, neither cPKCb nor cPKCγ was detected in the lysates. nPKCδ and nPKCe were also present in C3H10T1/2 cell lysates (Fig. 2D), whereas nPKCη was poorly expressed, if at all. PKCa, -δ, and -e have each been implicated as Raf-1 activators. Overnight treatment with PMA, as expected, down-regulated the expression of PKCa, -δ, and -e isoforms but had no effect upon c-Raf-1 expression.

It has been reported previously that PKCe (90 kDa) is associated with Raf-1 in Rat6 fibroblasts transformed by the overexpression of PKCe (28, 38). Since a 90-kDa PKC appeared to co-immunoprecipitate with c-Raf-1 and c-N-Ras, we sought to determine whether endogenous PKCe was constitutively associated with endogenous c-Raf-1 in C3H10T1/2 cells; c-Raf-1 was found associated with both PKCe and PKCe immunoprecipitates from membranes isolated from serum-starved C3H10T1/2 cells (Fig. 2E(i)). The amounts of PKCe and PKCe immunoprecipitated from the membrane preparation is indicated in Fig. 2E(ii). PKCε immunoprecipitates did not appear to co-immunoprecipitate c-Raf-1. In contrast, PKCe and PKCe immunoprecipitates from a cytosolic fraction did not appear to be associated with a significant amount of c-Raf-1 (Fig. 2E(iii)), although weak Raf-1 signals could be detected after longer film exposures (data not shown). The data, however, strongly suggest that in quiescent cells, PKCe and -e are only associated with membrane-bound Raf-1. PKCs were not detected in c-Raf-1 immunoprecipitates from membranes. This may, however, reflect significant differences in the ability of the Raf-1 antibodies to immunoprecipitate endogenous c-Raf-1 from cytosolic and membrane fractions. Endogenous c-Raf-1 could be readily immunoprecipitated from cytosolic extracts but not membrane extracts (Fig. 2E(iii)) and may reflect differences in the macromolecular nature of cytosolic and membrane-associated c-Raf-1 or the masking of the c-Raf-1 epitope upon plasma membrane association.

When PKCe, and PKCe immunoprecipitates from membranes were immunoblotted for c-N-Ras, only the PKCe immunoprecipitate contained a 21-kDa immunoreactive band consistent with c-N-Ras (Fig. 2F, upper panel). Parallel analysis of the purified antibodies confirmed that the immunoreactive 21-kDa band in the PKCe lane does not correspond to variable mobility of the IgG light chain (data not shown). Conversely, PKCe was detected in c-N-Ras immunoprecipitates (Fig. 2F, lower panel). Similar experiments, however, failed to detect PKCe in c-N-Ras immunoprecipitates (data not shown). These observations imply that in quiescent cell membranes, both endogenous PKCe and PKCe are closely associated with c-Raf-1. However, only PKCe appears to be associated with c-Raf-1 in a complex that contains c-N-Ras.

Antibodies to PKCeIncrease the Molecular Mass of Plasma Membrane-associated c-Raf-1 and c-N-Ras—The previous co-immunoprecipitation data suggest that there might be ternary complex between c-N-Ras, Raf-1, and PKCe in serum-starved C3H10T1/2 cells. These data, however, do not exclude the possibility that there are individual complexes composed of c-N-Ras-Raf-1, PKCe-Raf-1, and c-N-Ras-PKCe. To address this issue, the interaction between PKCe and the c-N-Ras-Raf-1 complex was further analyzed by Sephacryl S200HR gel filtration chromatography. Purified plasma membrane proteins were prepared from serum-starved C3H10T1/2 cells and immobilized for c-N-Ras and c-Raf-1 and visualized by ECL. The fractions in which c-N-Ras and Raf-1 eluted are indicated. The open arrow indicate where apoferritin (440 kDa) and IgG (150 kDa) were found to elute from the Sephacryl S200HR column. The data are representative of two independent experiments. B, densitometric analysis of eluted c-Raf-1 and c-N-Ras. The immunoreactive c-Raf-1 and c-N-Ras bands from A were quantitated using the image analysis program, NIH Image, and plotted against their elution point from the column.
class-specific inhibitors was confirmed, evidently of cPKC or nPKC activation. The specificity of the PKC ii (B) activation (Fig. 4) MAPK activation in response to either PMA or EGF stimulation (Fig. 4A). Pretreatment of serum-starved cells with the specific PKC inhibitor, Go6976 (41), failed to block MAPK activation in response to either PMA or EGF stimulation (Fig. 4B). EGF also stimulated ERK activation in chronic PMA-treated cells that were preincubated with either GF109203X or Go6976 (Fig. 4Bii), demonstrating that EGF-mediated activation of the MAPK cascade occurred independently of cPKC or nPKC activation. The specificity of the PKC class-specific inhibitors was confirmed, in vitro, using recombinant PKCα and PKCe. GF109203X completely blocked PKCα and PKCe activity, whereas Go6976 inhibited only PKCα (Fig. 4C). Similar experiments were performed using the PKCζ-specific inhibitor, Rottlerin (42) (Fig. 4D). Pretreatment with Rottlerin failed to block PMA-mediated MAPK activation in serum-starved C3H10T1/2 cells. From these studies we can conclude that PMA-mediated stimulation of the MAPK cascade occurs through a Ca2+-independent, phorbol ester-dependent, and GF109203X-sensitive novel PKCζ that is not PKCζ. These observations agree with the previous data in implicating PKCζ as the specific PKC isoform that activates Raf-1 upon phorbol ester stimulation. The role of PKCs in regulating the MAPK cascade was directly addressed through the specific down-regulation of PKCζ protein by antisense ODNs. Cationic liposomes (43, 44) were used to transiently transfect C3H10T1/2 cells with 1 μM PKCζ sense or antisense ODNs for 5 h. The cells were then serum-starved for 48 h. Cells were lysed following 3 min of stimulation with PMA, and the activity of immunoprecipitated MAPK was measured using MBP as a substrate. PMA stimulated a robust activation of MAPK in cells incubated with PKCζ sense ODN (Fig. 4Ei), identical to that seen in untreated cells (data not shown). In contrast, MAPK activation was severely abrogated in cells treated with the PKCζ antisense ODN where the levels of PKCζ protein were reduced by the antisense ODN (Fig. 4Eii). Western blots of cell lysates confirmed the specificity of the PKCζ ODN. PKCζ protein levels were not significantly affected by PKCζ ODN treatment nor were the levels of c-Raf-1 protein, a PKC-related kinase (data not shown). There was a strong correlation between the degree of reduction in PKCζ protein levels and PMA-stimulated MAPK activity; a 50–76% ODN-mediated reduction in PKCζ protein levels correlated with a 59–87% reduction in PMA-stimulated MAPK activity (Fig. 4Eiii). These data would appear to confirm PKCζ as a critical component of the mechanism by which PMA activates MAPK. Phorbol Ester Stimulates Raf-1 Activity and Its Phosphorylation on Ser-338 in c-N-Ras Immunoprecipitates—In order to address the functional significance of the endogenous, signaling complexes of c-N-Ras, c-Raf-1, and PKCe, PMA was directly added to immunoprecipitated complexes from serum-starved cells. c-Raf-1 activity in the complex was then determined using a kinase-dead GST-MEK1(K97A) substrate in a direct kinase assay. As observed previously, c-N-Ras immunoprecipitates from serum-starved cells contained no detectable c-Raf-1 activity (Fig. 5A). However, the inclusion of PMA in the reaction mixture stimulated Raf-1 activity. The PMA-mediated activation of c-Raf-1 could be inhibited by pre-incubating the immunocomplexes with GF109203X. In contrast, GF109203X failed to block c-Raf-1 activity in c-N-Ras immunoprecipitates from cells stimulated with PMA prior to cell lysis (data not shown). In control experiments, recombinant human PKCe did not phosphorylate the GST-MEK1 substrate, even in the presence of PMA (Fig. 5B), demonstrating that the PKCe present in the c-N-Ras-Raf-1-PKCe immunocomplexes cannot use GST-MEK1 as a substrate. The activity of the recombinant PKCe used in the in vitro assay was confirmed using a PKCe-specific peptide substrate in parallel assays (Fig. 5B). In subsequent experiments, the c-Raf-1 associated with c-N-Ras immunoprecipitates was analyzed, by Western blot, for phosphorylation at serine 338, which is phosphorylated upon PMA stimulation in vivo (7, 8) and by PAK3 in vitro (24). C3H10T1/2 cells were made quiescent by serum deprivation, and c-N-Ras was immunoprecipitated as described previously. The washed immunoprecipitates were either left untreated, incubated with PMA, or preincubated with PMA plus 10 μM GF109203X on ice before incubation at 30 °C for 30 min. The c-N-Ras-associated c-Raf-1 was then analyzed for Ser-338 phosphorylation by using a phospho-(Ser-338)Raf-1-specific antibody (8). Western blot analysis demonstrates that the c-Raf-1 that co-immunoprecipitates with c-N-Ras from quiescent cells is not phosphorylated on Ser-338. Incubation of these c-N-Ras immunoprecipitates with PMA resulted in a strong phospho-(Ser-338)Raf-1 signal, and this phospho-(Ser-338)Raf-1 signal was almost completely inhibited by the inclusion of GF109203X in the reaction mixture (Fig. 5C). Therefore, PMA stimulates c-Raf-1 activity in inactive c-N-Ras-Raf-1 complexes by inducing the phosphorylation of Raf-1 on the critical, PKC consensus, Ser-338 phosphorylation site. Taken together, the data support the hypothesis that these biochemically inactive, constitutive, complexes of c-N-Ras, c-Raf-1, and PKCe are latent and represent physiologically relevant signaling entities awaiting selective activation signals.

Determination of the Guanine Nucleotide(s) Bound to c-N-Ras within the Latent c-N-Ras-Raf-1-PKCe Complex—There are two currently used protocols to determine the amount of Ras-GTP within a cellular environment. The first involves metabolically labeling cells with [32P]orthophosphate, immunoprecipitating the Ras proteins and analyzing the bound nucleotides by TLC analysis. Whereas this method has been useful for determining the relative ratio of GTP to GDP bound for total Ras proteins, we have found that this method does not give a sufficient signal to noise ratio when examining the GTP-bound state of specific Ras isoforms (data not shown). The second method involves using the Ras-binding domain (RBD) of Raf-1...
FIG. 4. A, PMA-mediated MAPK activation is inhibited by chronic phorbol ester treatment. (i) 72-h serum-starved control and chronic PMA-treated C3H10T1/2 cells were stimulated with 5 μM PMA or 10 ng/ml EGF for 3 min. Cell lysates were prepared, and equal amounts of protein were resolved on 10% SDS-PAGE gels and immunoblotted for activated MAPK using an antibody that recognizes phosphorylated, activated MAPK (Santa Cruz Biotechnology). Immunoreactive bands were visualized by ECL. Activated MAPK proteins (pERK1 and -2) are indicated (lower panel). Equal protein loading was confirmed by immunoblotting for ERK1 protein levels (upper panel). The data are representative of three independent experiments. (ii) a calcium-independent PKC mediates MAPK activation in response to PMA stimulation. Serum-starved C3H10T1/2 cells were incubated for 1 h with 25 μM BAPTA-AM, a cell-permeable calcium chelator, prior to stimulation with 5 μM PMA for 3 min. Equal amounts of cell lysates were resolved on SDS-PAGE gels and immunoblotted for activated MAPKs as described. The data are representative of three independent experiments. B, a novel PKC mediates MAPK activation in response to PMA stimulation. (i) 72-h serum-starved cells were incubated with 10 μM GF109203X (GFX) or 10 μM G68976 (G6) 1 h prior to stimulation with either 10 ng/ml EGF or 5 μM PMA for 3 min. Cell lysates were prepared and analyzed for activated MAPKs as described above. The positions of activated pERK1 and pERK2 are indicated (lower panel). Equal protein loading was confirmed by immunoblotting for ERK1 protein levels (upper panel). (ii) EGF stimulates MAPK activation independently of nPKC or cPKC. Chronic PMA-treated C3H10T1/2 cells were preincubated with 10 μM of either GF109203X or G68976. Cells were then stimulated with 10 ng/ml EGF for 3 min, and cell lysates were prepared and resolved on 8% SDS-PAGE gels. Lysates were analyzed for phosphorylated ERK1 and ERK2 as described above (lower panel). Equal protein loading was confirmed by immunoblotting for ERK1 protein levels (upper panel). C, G68976 is a specific inhibitor of Ca<sup>2+</sup>-dependent PKCs. Recombinant cPKCa and nPKCe (200 ng) were incubated in kinase buffer containing 10 μCi of [γ-<sup>32</sup>P]ATP and 400 ng of PKC peptide substrates in the presence or absence of either 10 μM GF109203X or G68976 for 30 min at 30 °C. The reaction was stopped, and the incorporation of radiolabeled phosphate into the substrates was quantitated by scintillation counting. The data are representative of two independent experiments performed in triplicate. D, a PKCe-specific inhibitor does not block PMA-mediated MAPK activation. 72-h serum-starved C3H10T1/2 cells were pretreated with 15 μM Rottlerin prior to treatment with PMA for 3 min (5 μM). Cells were harvested and lysed, and the level of phosphorylated MAPK was determined as described previously. Raf-1 was blotted to confirm equal loading. E(i), antisense-mediated down-regulation of PKCe protein inhibits PMA-stimulated MAPK activation. C3H10T1/2 cells transiently transfected with 1 μM sense (S) or antisense (AS) PKCe-specific ODN were allowed to recover overnight and then serum-starved for 48 h before being stimulated with 5 μM PMA for 3 min. Cells were lysed in 400 μl of lysis buffer, and MAPK was immunoprecipitated from 300 μl of lysate. The activity of the immunoprecipitated MAPK was measured in a direct kinase assay using MBP as a substrate, and MBP phosphorylation was visualized and quantitated on a Molecular Dynamics StormImager. AS(1) and AS(2) represent two independent determinations with PKCe antisense ODN. The antisense experiments were performed twice, each in triplicate. The results shown are representative of these replicates. In each experiment the percent reduction in the MAPK activity was always higher than the percent reduction in PKCe expression. E(ii), PKCe...
to trap Ras-GTP. This is then analyzed by Western analysis for the amount of Ras bound to the GST-RBD fusion protein. The problem with this particular approach is that this method will not detect Ras-GTP bound to effector proteins, i.e. Ras-GTP has only one effector-binding site. To determine whether the c-N-Ras within the latent c-N-RasPKC\text{e} complex was bound with either GTP or GDP, we set up a model system using c-N-Ras from rat brain lysates and the GST-RBD to destabilize the Ras-Raf interaction without altering the guanine nucleotide associated with the N-Ras protein. We tested two different conditions, increasing salt concentrations (Fig. 6A) and pH extremes (Fig. 6B). The interaction between N-Ras and Raf-1 is not destabilized by increasing salt concentrations (Fig. 6A). We did find, however, that the c-N-RasPKC\text{e} complex could be destabilized by exposure to either pH 9 or pH 4 buffer (Fig. 6B).

We then tested whether Ras-GTP itself was stable under either of these pH extremes. This was tested using two slightly different protocols. In Fig. 6C, we analyzed the ability of recombinant c-N-Ras to exchange nucleotides in buffer at the indicated pH values. Although there is some variability in the

antisense ODNs specifically down-regulate PKC\text{e} protein levels. Two 40-μl aliquots of cell lysate from the PKC\text{e} ODN-treated cells as described above were resolved on 8% SDS-PAGE gels and immunoblotted for PKC\text{e} (upper panel) and PKC\text{a} (lower panel). Immunoreactive bands were visualized by ECL. E(iii), quantitation of PKC\text{e} antisense ODN effects on PKC\text{e} expression and PMA-stimulated MAPK activity. The expression of PKC\text{e} and PKC\text{a} protein in sense and antisense ODN-treated cells was quantitated by densitometry using the image analysis program, NIH Image. PKC\text{e} expression in sense ODN-treated cells was given a value of 100%. PMA-stimulated MAPK activity in sense and antisense ODN-treated cells was quantitated using a Molecular Dynamics StormImager, with the MAPK in sense ODN-treated cells represented as 100% activity. The data are representative of duplicate experiments, each performed in triplicate.

FIG. 5. A, phorbol ester stimulates Raf-1 activity in c-N-Ras immunoprecipitates in a PKC-dependent manner. c-N-Ras immunoprecipitates from 72-h serum-starved C3H10T1/2 cells were incubated in 50 μl of kinase buffer containing [γ-32P]ATP and the Raf-1 substrate, GST-MEK1(K97A), in the absence or presence of phosphatidylinerine (PS) and PMA. In parallel reactions, c-N-Ras immunoprecipitates were incubated in kinase buffer containing 10 μg GF109203X. All reactions were incubated on ice for 10 min prior to incubating at 30°C for 30 min. The kinase reaction was stopped by the addition of 50 μl of 2× Laemmli buffer. Reactions were resolved on 8% SDS-PAGE gels, and phosphorylated GST-MEK1 was visualized on a Molecular Dynamics StormImager. The data are representative of at least three independent experiments. B, GST-MEK1 is not a PKC\text{e} substrate. (i) 2 μg of kinase-dead GST-MEK1 was incubated with 1–5 ng of recombinant PKC\text{e} in a kinase reaction, with or without PMA and phosphatidylerine for 30°C for 30 min. (ii) in parallel, 10–100 ng of PKC\text{e} substrate was incubated with 2 ng of recombinant PKC\text{e} in a kinase reaction to confirm the activity of the recombinant PKC\text{e} kinase. After the reactions were terminated, the incorporation of [32P]phosphate into substrate peptide was determined by scintillation counting. C, PMA stimulates the phosphorylation of Raf-1 on serine 338. Cell lysates prepared from serum-starved C3H10T1/2 cells were split over several c-N-Ras immunoprecipitates, and c-N-Ras was immunoprecipitated as described previously. In identical reactions as described above, the c-N-Ras immunoprecipitates were then incubated in kinase buffer with ATP but without [γ-32P]ATP or kinase-dead GST-MEK1(K97A) for 30 min at 30°C. Reactions were terminated and resolved on 6% SDS-PAGE gels, transferred to PVDF membrane, and immunoblotted for phosphorylated Raf-1 using a phospho-(Ser-338)Raf-1-specific rat monoclonal antibody. Immunoreactive bands were visualized by ECL.
A. NaCl (M) 0 0.1 0.2 0.5 1
N-Ras -

B. pH 4 5 7 8 9 10
N-Ras -

C. pH change
GST-RBD + - + + +
GMPPNP

D. cpm bound (x 10^3)

FIG. 6. Determination of the guanine nucleotide bound to c-N-Ras within the latent c-N-Ras-Raf-1-PKCe complex. A, stability of c-N-Ras-GST-RBD complexes to increasing NaCl concentrations. Membranes from a rat brain extract were loaded with GMPPNP in the presence of excess EDTA. Mg2+ was added to stabilize the Ras-GMPPNP complexes. This was then incubated with glutathione-agarose beads precoupled with the GST-RBD fusion protein. Following a 1-h incubation, the glutathione-agarose GST-RBD beads were washed and then incubated with the indicated NaCl concentrations for 1 h at 4 °C. The c-N-Ras-GST-RBD complexes immobilized to glutathione-agarose beads were washed and analyzed for the presence of N-Ras by Western analysis. B, stability of c-N-Ras-GST-RBD complexes to changes in pH. Similar to the experiments described in A except the c-N-Ras-GST-RBD immobilized to glutathione-agarose beads was treated with buffers at the indicated pH values for 1 h at 4 °C. The beads were then washed and analyzed for the presence of N-Ras by Western analysis. C, stability of recombinant c-N-Ras to changes in pH. c-N-Ras, purified from Escherichia coli, was incubated in GTP-binding buffers containing [α-32P]GTP at the indicated pH values. After 30 min at room temperature the reactions were added to quench buffer and subsequently filtered through 0.22-micron nitrocellulose filters. The amount of radioactivity associated with the c-N-Ras was determined by scintillation counting. D, determination of the guanine nucleotides associated with c-N-Ras in the latent c-N-Ras-Raf-1-PKCe complex. Purified plasma membranes were prepared and solubilized in 1% CHAPS as described previously. The solubilized proteins was separated into 5 equal aliquots

numbers, it is clear that extended incubation of c-N-Ras at pH values 4 or 9 did not inactivate the Ras protein with respect to its ability to exchange guanine nucleotides. We also tested whether Ras-GTP-Mg2+ complexes were stable to these pH conditions. In this experiment, c-N-Ras-[α-32P]GTP was preformed in pH 7.4 buffer. The pH was then changed, and excess unlabeled GTP was added. After 1 h under these conditions, the reactions were analyzed for the amount of bound [α-32P]GTP. We observed no differences in the off-rates of the labeled GTP at the extreme pH values compared with the control assays performed at pH 7.4 (data not shown).

 Plasma membranes from 72-h serum-starved C3H10T1/2 cells were prepared and solubilized with 1% CHAPS as described under “Experimental Procedures.” As expected, direct incubation of the solubilized plasma membranes with the GST-RBD fusion protein did not result in detectable amounts of c-N-Ras association with the RBD moiety. This is in agreement with our observations that the c-N-Ras in the plasma membranes are in latent, pre-existing complexes. Upon treatment of the solubilized plasma membranes with pH 4 buffer, we then detected significant amounts of c-N-Ras capable of binding the GST-RBD protein. We observed no increase in the signal upon exchange with GMPPNP, suggesting that all the c-N-Ras in the plasma membranes of serum-starved C3H10T1/2 cells is associated with GTP. As expected, exchanging the solubilized plasma membrane with GDP resulted in no detectable association of c-N-Ras in the GST-RBD pull-down assay. These data support the conclusion that the c-N-Ras in the latent c-N-Ras-Raf-1-PKCe complex is bound with GTP, even after 72 h of serum starvation.

**DISCUSSION**

There is considerable evidence supporting the concept that the components of signaling cascades are organized into ordered signaling modules through their association with scaffolding, adapter, or chaperone proteins. This juxtaposition of effector proteins and their substrates facilitates their tight regulation, specificity of action, and their compartmentalization within the cell into discrete environments. Examples of ordered signaling modules includes the AKAP complex that co-localizes PKA with its effectors and substrates (45, 46), the JIP scaffold protein that organizes the components of the c-Jun NH2-terminal kinase pathway (47, 48), and MP1 that organizes and regulates the interaction of MEK with ERK1 (49). Similarly, proteins that regulate the subcellular localization and organization of Raf-1 have been identified; the association of connector enhancer of KSR (21) with Raf-1 targets the kinase to cell-cell contacts, whereas the association of KSR with Raf-1 is postulated to regulate both Raf-1 activity and its interaction with MEK (18, 20, 50).

We have generated several pieces of data that support the hypothesis that c-N-Ras, Raf-1, and PKCe exist in a latent ternary complex in serum-starved mouse fibroblasts. First, all the co-immunoprecipitation data are consistent with the exist-

(5 μg of protein per sample). One aliquot was incubated with the GST-RBD coupled to glutathione-agarose in the absence of any further treatment. The remaining protein samples had their pH lowered to 4 with acetate buffer. After 1 h at 4 °C, one sample had glutathione-agarose beads added (without the GST-RBD, indicated by the minus sign) and the pH readjusted to 7.4 with MOPS buffer. A second sample was incubated with the glutathione-agarose beads precoupled with GST-RBD at pH 7.4. The remaining two samples were exchanged with either GMPPNP or GDP in the presence of excess EDTA for 1 h. Mg2+ was added followed by the addition of glutathione-agarose beads precoupled with GST-RBD at pH 7.4. All the samples were washed and analyzed for the presence of c-N-Ras by Western analysis. The data are representative of two separate experiments.
ence of such a ternary complex. Second, the gel filtration analysis confirms that both c-N-Ras and Raf-1 are associated with at least one additional protein of roughly 70–100 kDa. The PKCe antibody-dependent shift in molecular weight of both c-N-Ras and Raf-1 implicates PKCε as the additional protein within the c-N-Ras-Raf-1 complex. These data are further supported by the spatial orientation implicated by the in vitro phosphorylation of Raf-1 by PKCε in c-N-Ras immunoprecipitates. The cumulative data do not completely rule out the possibility that rather than a ternary complex between c-N-Ras, Raf-1, and PKCε, there are a series of other complexes that coincidentally move at the same molecular weight upon gel filtration analysis. We feel, however, the number of different complexes that must exist to explain our observations in these terms is very unlikely. Given these considerations, we feel our data support the existence of a ternary, latent complex between c-N-Ras, Raf-1, and PKCε in quiescent, serum-starved C3H10T1/2 cells.

Our data suggest that components of Ras-regulated signaling pathways may be ordered into signal modules prior to activation. Here we demonstrate that, even in quiescent cells, endogenous c-N-Ras, c-Raf-1, and PKCε are found in a latent signaling complex and that c-Raf-1 activity in the complex can be directly stimulated by PMA. This complex, in situ, may therefore represent a physiological target for DAG-mediated MAPK activation. Although PKCδ was also found to be constitutively associated with membrane-bound c-Raf-1, c-N-Ras did not appear to be a component of this complex. However, this does not completely exclude a role for Ras in PKCδ-regulated c-Raf-1 signaling since the precise role of the four endogenous Ras isoforms (Ha-, N-, Ki-Ras A, and B) in Raf-1 regulation, in vivo, has not been fully determined. These data, however, might suggest that the regulation of Raf-1 activity by PKCδ and PKCε may be in response to distinct agonists. Similarly, the regulation of Raf-1 activity by distinct Ras isoforms may also occur in response to distinct agonists or biological events, in order to elicit specific responses such as proliferation, migration, or differentiation.

The PKCε antisense data presented here would indicate that endogenous PKCε (but not PKCδ) is required for PMA (and consequently DAG)-mediated stimulation of the MAPK cascade in C3H10T1/2 cells. The data also provide further evidence regarding the convergence of the Ras and PKC pathways in c-Raf-1 regulation. More importantly, signaling modules containing components from supposedly distinct signal transduction pathways and common effector proteins, such as c-Raf-1, have the potential to be rapidly activated and regulated by a diverse range of stimuli.

The role of Ras in the activation of Raf-1 by PKC has proven to be controversial, partly through the contradictory results obtained using dominant-negative (S17N)Ras to study the role of Ras in this process. Several studies suggested that PKCε-mediated activation of the MAPK cascade was independent of Ras activation, since PKCε-stimulated MAPK activity appeared to be insensitive to dominant-negative (S17N)Ras (29, 51). Fucini et al. (52) recently demonstrated that the activation of MAPK by PMA, unlike insulin, appeared to occur in the absence of any significant Ras activation. By using the Ras-binding domain of Raf-1 to trap newly formed Ras-GTP, they found that PMA did not result in a significant level of Ras activation, unlike the efficient trapping of Ras-GTP by the RBD domain in insulin-stimulated cells. This contrasted the findings of Barnard et al. (7) and Marais et al. (34) who demonstrated that the association between Ras and Raf-1 was required for PKC to activate Raf-1. These reports, however, can now be reconciled by the possibility that PMA stimulates MAPK in the context of activating the PKCε in the inactive, latent c-N-Ras-Raf-1-PKCε complexes. The presence of preformed c-N-Ras-GTP-Raf-1 complexes would preclude the requirement for the generation of de novo c-Ras-GTP (which is blocked by (S17N)Ras) in response to PMA stimulation. Since Ras effector binding is a mutually exclusive process, where Ras can only associate with one effector when active, the c-N-Ras-GTP present in the inactive c-N-Ras-Raf-1-PKCε signaling complex would be resistant to trapping by the RBD domain. This was clearly demonstrated by our ability to destabilize the c-N-Ras-Raf-1 complex at pH 4.0 and then detect Ras-GTP using the GST-RBD pull-down assay. Interestingly, a specific role for the c-N-Ras isoform in the regulation of the MAPK cascade by phorbol ester was also suggested by Malumbres and Pellicer (53) who reported that cells deficient in N-Ras had a decreased response to PMA. Moreover, the critical role for PKCε in the activation of MAPK by PMA supports the hypothesis that these inactive c-N-Ras-Raf-1-PKCε complexes are unique, physiological targets for phorbol ester and therefore, putatively, diacylglycerol-mediated MAPK activation.

This is the first report to document a functional Ras-GTP-effector complex in quiescent cells. In fact, our observation that there exists a pool of complexed Ras-GTP in quiescent cells highlights the deficiencies in the current tools to make an accurate assessment of Ras-GTP levels. This complex, because of its stability, does not bind to the GST-RBD in the new commonplace pull-down assays. In addition, as demonstrated by Marias et al. (34), this preformed complex would not be blocked by expression of the typical dominant-negative RasAsn-17 protein, which ties up exchange factors and prevents the generation of newly formed Ras-GTP. The identification of a latent c-N-Ras-GTP-dependent signaling complex raises a number of questions relative to the current Ras paradigm. Is it the generation of new Ras-GTP that is important or possibly the cycling of Ras with successive GTP molecules? It is not clear from published work whether the generation of a single molecule of Ras-GTP, in this case c-N-Ras GTP, results in the activation of a single Raf-1 molecule or many Raf-1 molecules. It will be interesting to examine the relationship between this latent c-N-Ras-Raf-1-PKCε and the generation of newly formed Ras-GTP in response to extracellular factors.

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