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Destabilization of Raf-1 by Geldanamycin Leads to Disruption of the Raf-1–MEK–Mitogen-Activated Protein Kinase Signalling Pathway

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The serine/threonine kinase Raf-1 functions downstream of Ras in a signal transduction cascade which transmits mitogenic stimuli from the plasma membrane to the nucleus. Raf-1 integrates signals coming from extracellular factors and, in turn, activates its substrate, MEK kinase. MEK activates mitogen-activated protein kinase (MAPK), which phosphorylates other kinases as well as transcription factors. Raf-1 exists in a complex with HSP90 and other proteins. The benzoquinone ansamycin geldanamycin (GA) binds to HSP90 and disrupts the Raf-1–HSP90 multimolecular complex, leading to destabilization of Raf-1. In this study, we examined whether Raf-1 destabilization is sufficient to block the Raf-1–MEK–MAPK signalling pathway and whether GA specifically inactivates the Raf-1 component of this pathway. Using the model system of NIH 3T3 cells stimulated with phorbol 12-myristate 13-acetate (PMA), we show that GA does not affect the ability of protein kinase Cα to be activated by phorbol esters, but it does block activation of MEK and MAPK. Further, GA does not decrease the activity of constitutively active MEK in transiently transfected cells. Finally, disruption of the Raf-1–MEK–MAPK signalling pathway by GA prevents both the PMA-induced proliferative response and PMA-induced activation of a MAPK-sensitive nuclear transcription factor. Thus, we demonstrate that interaction between HSP90 and Raf-1 is a sine qua non for Raf stability and function as a signal transducer and that the effects observed cannot be attributed to a general impairment of protein kinase function.

The serine/threonine kinase Raf-1 is the central component of a highly conserved signalling cascade that consists of receptor tyrosine kinases, Ras, Raf-1, MEK, and mitogen-activated protein kinase (MAPK) (16, 29, 31). Raf-1 is targeted to the cell membrane by Ras-GTP and becomes attached to the membrane by an unknown mechanism (46, 48). The mechanism of Raf-1 activation is complex and involves phosphorylation at serine and tyrosine residues (15, 17, 30, 35). Raf-1 integrates extracellular signals and phosphorylates the dual-specificity kinase MEK (MAPKK), which in turn phosphorylates the extracellular signal-regulated kinases (ERKs or MAPKs) ERK-1 and -2 (1, 14, 32). Activation of ERKs leads to activation of other downstream kinases such as the kinases $p90^{rsk}$ (6, 13, 47) and MAPKAP (40, 45), as well as several transcription factors such as Elk-1 (18, 50), Jun (9, 38), and c-myc (20, 42, 43). Activation of the Raf signalling pathway can lead to proliferation as well as differentiation (22, 23, 49, 54).

Raf-1 exists as a part of a multimolecular complex that includes HSP90, p50, and other proteins (44, 52). We have previously shown that the benzoquinone ansamycin geldanamycin (GA) disrupts the Raf-1–HSP90 molecular complex, resulting in destabilization of Raf-1 with a significant decrease in protein level secondary to a decreased half-life (41).

Since it has been shown that low amounts of active Raf-1 are sufficient to activate the signalling cascade (1), we examined whether GA disrupts the Raf-1–MEK–MAPK pathway. We also examined whether other members of the cascade, including protein kinase C (PKC; the principal receptor for phorbol esters), MEK, and MAPK were directly affected by GA. Our data reveal that Raf-1 is the only component of this signalling pathway that is depleted by GA treatment and, further, that Raf-1 depletion by GA is sufficient to interdict signalling through this pathway.

MATERIALS AND METHODS

Cells and reagents. GA was obtained from the Developmental Therapeutics Program, National Cancer Institute, Bethesda, Md. It was dissolved at a concentration of 5 mM in 100% dimethyl sulfoxide and stored at −20°C. Phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, Mo.) was also dissolved in dimethyl sulfoxide (1 mM stock concentration) and stored at −20°C. Other chemicals were of highest available grade. The antibodies used were Raf-1 antibody (C12), MEK-1 antibody (C18), ERK-2 antibody (C14) (Santa Cruz Biotechnology, Santa Cruz, Calif.), PKCα antibody (Transduction Laboratories, Lexington, Ky.), Ras antibody (pan-ras Ab-3; Oncogene Science, Cambridge, Mass.), MEK-2 antibody (NT; Upstate Biotechnology, Lake Placid, N.Y.), pan-ERK antibody (Transduction Laboratories), and phospho-specific MAPK antibody (New England Biolabs, Beverly, Mass.). Cell lines were purchased from the American Type Culture Collection (Rockville, Md.). NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium and Jurkat cells were maintained in RPMI 1640, both supplemented with 10% fetal bovine serum and 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES).

Cell lysis and Western blotting (immunoblotting). Cells growing in log phase were lysed with TENSV buffer (50 mM Tris HCl [pH 7.5], 2 mM EDTA, 100 mM NaCl, 1 mM sodium orthovanadate, 1% Nonidet P-40) containing protease inhibitors (aprotinin [20 μg/ml], leupeptin [20 μg/ml], and 1 mM phenylmethylsulfonyl fluoride). Thirty micrograms of total protein was separated on sodium dodecyl sulfate (SDS)–8% or 10% polyacrylamide gels and analyzed by Western blotting as described previously (41). Films were scanned into a Macintosh computer using a Foto/Eclipse gel analysis system (Fotodyne) and processed by using Adobe Photoshop software.

PKC assay. Whole cell lysates were prepared from 10^7 cells. Cells were washed three times in phosphate-buffered saline (PBS), lysed in buffer A (10 mM Tris HCl, [pH 7.6], 0.5 mM EDTA, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl

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fluoride) containing 0.1% Triton X-100, and sonicated for 10 s. PKC activity was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into a PKC substrate (RFARKGSLRQKNV) in the presence of phospholipid vesicles (20% phosphoserine–80% phosphocholine) at 30°C for 30 min. One micromolar PMA was used to activate PKC in this in vitro assay, allowing for functional evaluation of PMA-responsive isozymes. Specific incorporation of ^{32}P into substrate was determined as the difference of values obtained in the presence and absence of the peptide. Activity values were normalized to the protein concentration determined by using the bicinchoninic acid protein assay (Pierce, Rockford, Ill.).

MEK and MAPK activity assay. NIH 3T3 cells were grown to 80% confluence in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 10 mM HEPES (pH 7.3). After pretreatment with or without GA (2 μM for 16 h), cells were stimulated with 100 nM PMA for 10 min before lysis with TENSV buffer containing protease inhibitors and 50 mM NaF. One milligram of total protein was immunoprecipitated with 1 μg of MEK-1 antibody and protein A–Sepharose CL-4B beads (Pharmacia, Uppsala, Sweden). The beads were washed three times with TENSV buffer containing EGTA instead of EDTA and twice with 50 mM HEPES (pH 7.5)–10 mM magnesium acetate. The beads were resuspended in 50 μl of kinase assay buffer (50 mM HEPES [pH 7.5], 10 mM magnesium acetate, 1 mM dithiothreitol, 1 μM okadaic acid, 1 μg of leupeptin per ml) containing 100 μM ATP and 400 ng of MAPK p42 of *Xenopus* origin (Santa Cruz Biotechnology). In some experiments, GA was added to the kinase buffer in various concentrations and immunoprecipitates were preincubated with drug on ice for 15 min. After incubation at 30°C for 20 min, 10 μCi of [γ-^{32}P]ATP and 10 μg of myelin basic protein (MBP; Sigma) were added. After additional incubation at 30°C for 20 min, the kinase reaction was stopped by addition of SDS sample buffer and boiling for 5 min. The proteins were separated on an SDS–12.5% polyacrylamide gel and visualized by autoradiography. Films were scanned into a Macintosh computer using a Foto/Eclipse gel analysis system (Fotodyne). Bands were quantified by using Collage Analysis software (Fotodyne). Pictures were processed by using Adobe Photoshop software.

In the case of MAPK, 1 mg of total protein was immunoprecipitated with ERK-2 antibody coupled to agarose beads (Santa Cruz Biotechnology), and lysates were washed as described above. Immunoprecipitates were resuspended in kinase assay buffer containing 10 μCi of [γ-^{32}P]ATP and 10 μg of MBP. After 20 min of incubation at 30°C, the samples were boiled in SDS sample buffer and analyzed as described above.

Oligonucleotide suppression of Raf-1. Synthesis and purification of phosphorothioate oligonucleotides were performed as previously described (11, 34). NIH 3T3 cells growing at 50 to 75% confluency were treated with 200 nM antisense (ATGCATTCTGCCCCCAAGGA) or mismatched control (ATGCAGTCTTC CACCACGGA) oligonucleotide in the presence of Lipofectin (Gibco-BRL) (5, 11, 34). Because of the long half-life of Raf-1, the cells received a second treatment of oligonucleotides after 24 h and were lysed after 48 h. Twenty-five micrograms of total protein was used for Western blotting. Raf-1 was detected by a monoclonal antibody (Transduction Laboratories) followed by incubation with 5 μCi of ^{125}I-goat anti-mouse antibody (ICN Radiochemicals, Costa Mesa, Calif.). Labeled proteins were visualized and quantitated by PhosphorImager (Molecular Dynamics) analysis. A MAPK assay using ERK-2 immunoprecipitated from treated and untreated cells was performed as described above.

Plasmids and transfections. Plasmids containing cDNAs of wild-type and mutant MEK-1 tagged with the hemagglutinin (HA) antigen (pHA MEK-1 WT and pHA MEK-1 S218/222A ΔN) were a generous gift of M. J. Weber (University of Virginia, Charlottesville) (10). NIH 3T3 cells (2×10^6) were transfected with 8 μg of plasmid by using LipofectAMINE (Gibco BRL, Gaithersburg, Md.). After 6 h of incubation with the plasmid-lipid suspension, cells were grown in medium for 16 h with or without GA. The cells were lysed, and 800 μg of total protein were immunoprecipitated with an HA antibody (Boehringer Mannheim, Indianapolis, Ind.). The immunoprecipitates were used for in vitro MEK kinase assay as described above. Equal amounts of MEK protein in the precipitates were confirmed by Western blotting.

The TRE-Luc reporter construct (36) was a gift of C. M. Zacharchuk (National Cancer Institute). Two tetradecanoyl phorbol acetate-responsive element (TRE) consensus sequences were cloned into the pGL2-Promoter vector (Promega, Madison, Wis.). Vectors were prepared by using a Maxiprep kit (Qiagen Inc., Chatsworth, Calif.) according to the manufacturer's instructions. Jurkat cells (12×10^6) were transfected with 20 μg of either TRE-Luc or pGL2-Promoter by electroporation (Cell-Porator; Gibco BRL, Gaithersburg, Md.) with or without pretreatment with GA (2 μM for 16 h). The cells were then cultured for 2 h to allow for cell recovery and protein expression. Cells were then divided, and one fraction was stimulated with 100 nM PMA. After 6 h, cells were lysed and analyzed for luciferase activity as recommended by Promega Corp., using a Microlite ML1000 luminometer (Dynatech Laboratories, Chantilly, Va.). Luciferase activity of TRE-Luc-transfected cells was normalized for protein concentration of the lysates and by the luciferase activity generated by transfection of the enhancerless plasmid. Experiments were performed in triplicate.

Immunofluorescence. NIH 3T3 cells were grown on coverslips following transfection with pHA MEK-1 S218/222A ΔN as described above. After treatment with or without GA (2 μM for 16 h), cells were fixed with 3.7% formaldehyde in PBS for 10 min at room temperature and permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature. After blocking with 2% bovine serum albumin–0.2% Tween 20 in PBS for 1 h at 4°C, the cells were incubated with HA antibody (Boehringer Mannheim) and phospho-specific MAPK antibody (New England Biolabs) at 5 μg/ml in PBS. After three washes with PBS, fluorescein isothiocyanate-labeled goat anti-rabbit antibody (Molecular Probes, Eugene, Ore.) and rhodamine-labeled goat anti-mouse antibody were applied at 20 μg/ml in PBS for 1 h at 4°C. After three washes in PBS, the slides were mounted with SlowFade (Molecular Probes). Hoechst 33258 (Molecular Probes) was used at 0.4 μg/ml in PBS to visualize cell nuclei. Control experiments were performed to ascertain antibody specificity and to rule out crossover of the fluorophore signal. Images were obtained with a Zeiss Axioskop microscope and an Optronics charge-coupled device camera system.

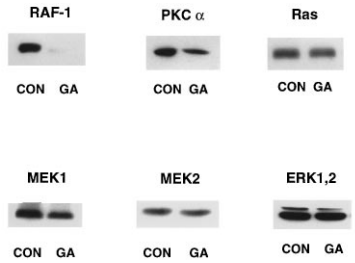

FIG. 1. GA decreases the steady-state level of Raf-1 but not of PKCα, Ras, MEK, or MAPK. Western blots that depict steady-state levels of proteins involved in the Raf-1–MEK–MAPK pathway are shown. Lysates were derived from untreated (CON) or 16-h GA-treated (GA) NIH 3T3 cells.

Cell proliferation assay. NIH 3T3 cells (5×10^3) were plated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum in a 96-well microtiter plate and allowed to equilibrate for 1 day. Serum was then reduced to 0.1%, and cells were further incubated for 2 days. At that time, either serum (final concentration, 10%) or PMA (250 nM) was added in quadruplicate, in the presence or absence of GA (100 nM). Four hours later, [*methyl*-^3H]thymidine (1 μCi, 6.7 Ci/mmol) was added, and plates were allowed to incubate for an additional 16 h. Cells were harvested, and radioactivity was determined with a Packard Micromate 196 Cell Harvester.

RESULTS

GA decreases steady-state level of c-Raf-1 but not Ras, PKCα, MEK, or MAPK. NIH 3T3 cells were treated for 16 h with GA (2 μM). As previously reported (41), this led to a nearly complete loss of Raf-1 protein (Fig. 1). Neither PKCα, the predominant PKC isoform in NIH 3T3 cells (19, 41a), nor MEK, MAPK, or Ras protein levels were significantly affected by GA (Fig. 1).

GA does not block the PMA-induced stimulation of PKC. Phorbol esters are known to activate several PKC isoforms, including PKCα (4). In NIH 3T3 cells, PMA has been shown to stimulate Raf-1 through PKC activation (26, 27). To test whether GA inhibited PKC activation, we lysed NIH 3T3 cells that had been pretreated with GA (2 μM) for 16 h or left untreated. The lysates were used for an in vitro PKC assay using PMA activation. GA did not block the ability of PKC to be activated by PMA in vitro (Fig. 2).

GA blocks the Raf-1–MEK–MAPK pathway. We assayed activation of MEK-1 in NIH 3T3 cells after PMA stimulation. Cells were pretreated with or without GA (as described above) and then stimulated with PMA. MEK-1 was immunoprecipitated and incubated with purified MAPK and ATP. After 20 min, [γ-^{32}P]ATP and MBP were added for an additional 20 min. Stimulation of MEK-1, measured by its ability to activate exogenously added, purified MAPK, was inhibited by GA (Fig. 3A). To distinguish between an indirect effect on MEK due to inactivation of Raf-1 and a direct effect on MEK kinase activity, we preincubated immunoprecipitated MEK with increasing concentrations of GA for 15 min prior to initiating the in vitro assay. There was no effect of GA on MEK activity at the GA concentrations used in cells, although we observed some inhibition of MEK at higher GA concentrations (Fig. 3A, left panel).

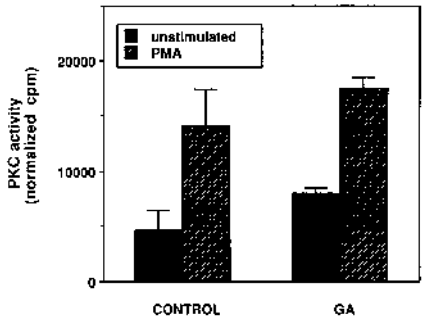

FIG. 2. GA does not block the PMA-induced stimulation of PKC. NIH 3T3 cells were grown to 80% confluence. After treatment with or without GA (2 μM, 16 h), cells were lysed and PKC assays were performed as described in Materials and Methods. PKC activity, normalized for background and total protein, is shown with and without PMA activation.

We also analyzed the direct effects of GA on endogenous MAPK activation in NIH 3T3 cells after PMA. ERK-2 was immunoprecipitated in NIH 3T3 cells before and after PMA stimulation. Again, MBP was used as the substrate in an in vitro kinase assay. In GA-treated cells, stimulation of kinase activity by PMA was greatly reduced (Fig. 3B). In contrast, addition of GA to immunoprecipitated MAPK 15 min prior to the kinase assay did not result in significant inhibition of kinase activity at the concentrations used (2 to 100 μM).

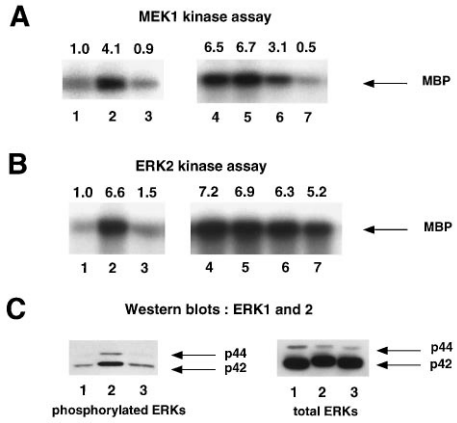

FIG. 3. GA blocks PMA-dependent activation of MEK and MAPK. (A) NIH 3T3 cells were grown to 80% confluence. After pretreatment with or without GA (2 μM, 16 h), cells were stimulated with PMA (100 nM, 10 min) and lysed in TENSV buffer. MEK-1 was immunoprecipitated from 1 mg of total protein and incubated with purified MAPKinase and ATP in kinase reaction buffer. After 20 min, [γ-^{32}P]ATP and MBP were added for an additional 20 min. The reaction was stopped by boiling with SDS-gel loading buffer; proteins were separated on SDS–12.5% polyacrylamide gels and visualized by autoradiography. In some PMA-stimulated conditions, GA was added in increasing concentrations directly to the immunoprecipitates 15 min prior to initiation of the kinase assay (left panel). (B) In a similar assay, ERK-2 was immunoprecipitated from 1 mg of total protein and incubated for 20 min with [γ-^{32}P]ATP and MBP. In some PMA-stimulated conditions, GA was added in increasing concentrations directly to the immunoprecipitates 15 min prior to initiation of the kinase assay (left panel). (C) Activation of endogenous MAPK was also assessed by using an antibody that specifically recognizes phosphorylated MAPK. Western blots show a block of phosphorylation of ERK-1 and -2 in lysates prepared from GA-treated cells. Lane 1, unstimulated cells; lane 2, cells stimulated with PMA; lane 3, cells stimulated with PMA after GA pretreatment (2 μM, 16 h); lanes 4 to 7, cells stimulated with PMA, with GA added in vitro to immunoprecipitates at 0 μM (lane 4), 2 μM (lane 5), 10 μM (lane 6), and 100 μM (lane 7). The results were quantitated by measuring optical densities and are presented above the corresponding autoradiograph as a comparison with the control. One of three similar experiments is shown.

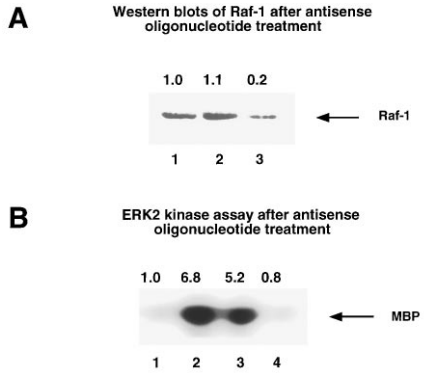

FIG. 4. Antisense oligonucleotide treatment blocks MAPK activation in NIH 3T3 cells. NIH 3T3 cells were treated for 48 h with Raf-1 antisense or mismatch control oligonucleotides. (A) Twenty-five micrograms of total protein was used for a Western blot of Raf-1 protein level. Lane 1, untreated control; lane 2, mismatch control oligonucleotide; lane 3, antisense oligonucleotide. (B) After stimulation with PMA (100 nM, 10 min), ERK-2 was immunoprecipitated from 1 mg of total protein and used for a kinase assay using MBP as a substrate. Lane 1, no PMA stimulation, no oligonucleotide; lane 2, PMA stimulation, untreated control; lane 3, PMA stimulation, mismatch control oligonucleotide treated; lane 4, PMA stimulation, antisense oligonucleotide treated. The results were quantitated by PhosphorImager analysis and are presented above the corresponding lane as a comparison with the control. One of two similar experiments is shown.

As an additional assessment of endogenous MAPK activation, we used a monoclonal antibody that recognizes only phosphorylated MAPK, thus allowing for direct analysis of activated ERKs. This assay also demonstrated an in vivo block of activation of both ERK-1 and ERK-2 by GA (Fig. 3C).

The block of MAPK activation by PMA in NIH 3T3 cells can be mimicked by antisense oligonucleotides directed against Raf-1. Phosphorothioate antisense oligodeoxynucleotides targeted against Raf-1 have been shown to specifically inhibit Raf-1 gene expression in cell culture as well as in xenograft tumor models (33). We used a phosphorothioate 20-mer that targets a sequence in the 3′ untranslated region of the murine Raf-1 message and has a strong antisense effect on murine Raf-1. NIH 3T3 cells were treated with an antisense or control oligonucleotide at 200 nM in the presence of a cationic lipid. Western blots after 48 h of treatment showed a 75% decrease in Raf-1 protein in antisense-treated but not mismatched-control-treated cells (Fig. 4A). We also used lysates from untreated, antisense-treated, and mismatched-control-treated cells for a MAPK immunoprecipitation assay. MAPK activation by PMA was specifically blocked only in antisense oligonucleotide-treated NIH 3T3 cells (Fig. 4B).

GA does not block constitutively active MEK downstream of Raf-1. If GA specifically targets Raf-1 and does not affect the activity of MEK and MAPK, constitutively active MEK should relieve the inhibition downstream of Raf-1. We tested this hypothesis by transiently transfecting either wild-type MEK-1 or mutated, constitutively active MEK-1 into NIH 3T3 cells. After treatment with or without GA, cells were stimulated with PMA and lysed. The transfected MEK-1 was immunoprecipitated by using an HA tag and used in an in vitro kinase assay as described above. Although transfected wild-type MEK-1 behaved similarly to the endogenous protein, constitutively active MEK-1 was neither activated by PMA nor inhibited by GA (Fig. 5A).

We also assessed the sensitivity to GA of signal transduction events downstream of Raf-1 intracellularly by using an immunofluorescent technique. NIH 3T3 cells were transfected with constitutively active MEK, thereby obviating a requirement for

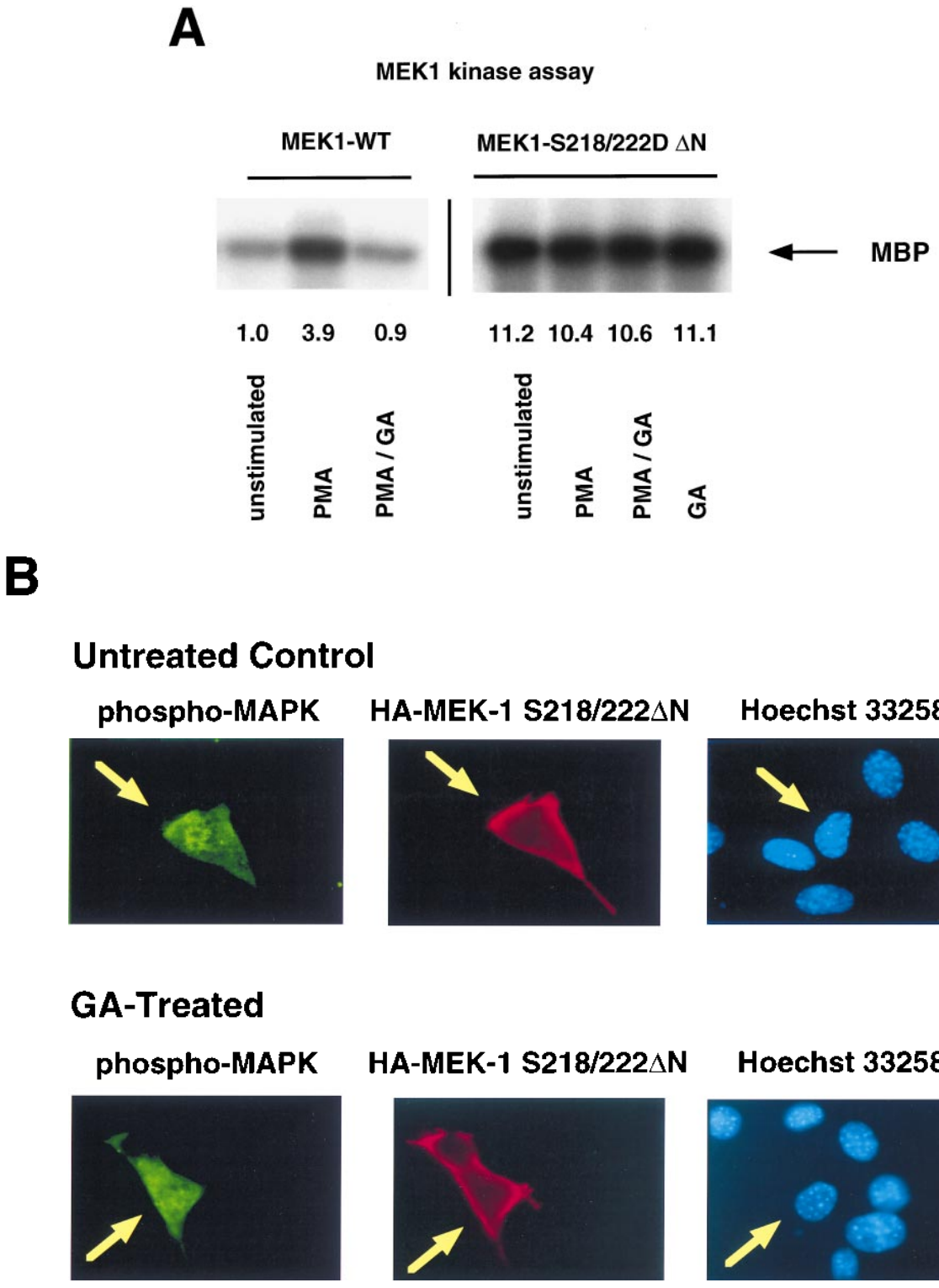

FIG. 5. GA does not block constitutively active MEK. (A) NIH 3T3 cells were transiently transfected with HA-tagged MEK-1 as wild-type protein (MEK1-WT) or as a constitutively active mutant (MEK1-S218/222D ΔN). After pretreatment with or without GA (2 μM, 16 h) and stimulation with PMA (100 nM, 10 min), cells were lysed and MEK-1 was immunoprecipitated with an HA-specific antibody. Precipitates were incubated with exogenous MAPK and ATP for 20 min; MBP and [γ-^{32}P]ATP were added, and incubations continued for an additional 20 min. Phosphorylated MBP was visualized by autoradiography. The results were quantitated by measuring optical densities and are presented below the corresponding autoradiograph as a comparison with the control. One of three similar experiments is shown. (B) NIH 3T3 cells were transiently transfected with HA-tagged MEK-1 S218/222D ΔN and treated with or without GA for 16 h. Anti-HA antibody was used to visualize transfected cells (red), and antibody specific for phosphorylated MAPK (green) was used for two-color immunofluorescence. Hoechst 33258 stains nuclei (blue) in both transfected (arrow) and untransfected cells. Note that only those cells positive for HA-MEK S218/222D ΔN were also positive for phosphorylated MAPK. One of two similar experiments is shown.

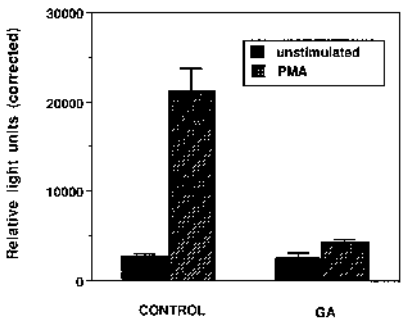

FIG. 6. GA blocks transactivation of a TRE enhancer element in Jurkat cells. Jurkat cells were transfected with a reporter plasmid containing two copies of the TRE consensus sequence linked to the luciferase reporter gene. Cells were stimulated with PMA after pretreatment with or without GA. Luciferase activity secondary to TRE transactivation was determined; values were corrected by comparison with those obtained with a luciferase reporter plasmid lacking the TRE element.

endogenous Raf-1. The transfected cells were identified by using an HA antibody. Two-color immunofluorescence revealed that only the transfected cells showed activation of MAPK, as documented by reactivity with an antibody specific for phosphorylated MAPK. This effect was seen in control cells as well as in cells treated with GA for 16 h (Fig. 5B). Thus, constitutively active MEK abrogated the effects of GA on MAPK activation both in vitro and in vivo.

GA blocks transactivation of a TRE-reporter construct in Jurkat cells. To test the effects of GA on downstream targets of the Raf-MEK-MAPK pathway, we used a reporter gene construct that has previously been shown to be PMA inducible in the human T-cell leukemia cell line Jurkat (36). Activation of T cells either by mitogens, by engagement of the T-cell receptor, or by PMA leads to induction of the AP-1 transcription complex. AP-1 binds to a consensus TRE enhancer sequence. We transfected a TRE-Luc reporter plasmid into Jurkat cells and monitored luciferase activity with and without PMA stimulation (Fig. 6). In this system, GA blocks PMA transactivation of the TRE enhancer element.

GA blocks the PMA-induced proliferative response in NIH 3T3 cells. We further assessed the influence of GA on cell proliferation. Since the Raf-1–MEK–MAPK pathway serves to transmit mitogenic stimuli to the nucleus, disruption of the pathway by inactivation of Raf-1 should block the proliferative response to PMA and growth factors. To test this hypothesis, we stimulated serum-starved NIH 3T3 cells with 10% serum or PMA and measured the increase in cell proliferation by [^{3}H]thymidine incorporation. As can be seen in Fig. 7, the proliferative response to both serum and PMA was completely blocked by GA.

DISCUSSION

The *raf-1* proto-oncogene plays a central role in signal transduction (29). Stimulation of cells by growth factors and other mitogens leads to activation of Ras (7), which in turn directs Raf-1 to the membrane where it becomes activated (46). The mechanism of Raf-1 activation is complex and not completely understood. Recently, Raf-1 activation by phosphorylation of tyrosine residues 340 and 341 has been described (17, 30), and the involvement of a regulatory lipid has been suggested (15). Stimulation of fibroblasts with platelet-derived growth factor or PMA leads to activation of Raf-1 concurrent with phosphorylation of residues S-259 (35) and S-499 (17, 26). Residue S-621 is constitutively phosphorylated in fibroblasts and Sf9 cells, and mutation of this residue to another amino acid results in a Raf-1 protein that cannot be activated (35). Conversely, phosphorylation of residue S-43 by protein kinase A inhibits Raf-1 activity (21).

Various biologically artificial models that use in vitro techniques or overexpression of Raf-1 in insect or mammalian cells present difficulties in relating the results to the normal function of the protein. For this reason, we have used eukaryotic cell lines that have defined signal transduction pathways to study the disruption of the Raf-1–MEK–MAPK pathway by GA.

Raf-1 exists as a multimolecular complex that contains HSP90, p50, and other proteins (44, 52). This complex may function as a transportosome directing Raf-1 to its proper subcellular localization (37). We have recently demonstrated that the benzoquinone ansamycin GA binds directly to HSP90 (53), disrupting complexes of which it is a part. Disruption of the Raf-1–HSP90 association leads to destabilization of Raf-1 (41). The intracellular steady-state concentration of the protein is dramatically reduced, in spite of ongoing Raf-1 synthesis. The loss of Raf-1–HSP90 association occurs concomitantly with disruption of Raf-1 binding to Ras and with aberrant intracellular trafficking of newly synthesized Raf-1. The half-life of the protein is also markedly reduced.

However, since the signalling cascade represents a mechanism of amplification, low amounts of activated Raf-1 can be sufficient to promote signalling via MAPK (1). Therefore, it has been unclear whether GA-induced reduction in the Raf-1 steady-state level would affect signalling through the Raf-1–MEK–MAPK pathway. GA might also affect other kinases in this pathway. We have addressed these issues by using NIH 3T3 cells, which have been shown to respond to PMA stimulation by PKCα-dependent activation of Raf-1 (26, 27).

While Raf-1 protein levels decreased upon GA treatment, the other members of this pathway were not grossly affected. We also found no major differences in the steady-state protein levels of Ras or the Raf-1-associated 14-3-3 ζ protein, while HSP90 is mildly increased after GA treatment (41a, 53). Although GA led to a minor decrease in level of PKCα, the predominant PKC isoform in NIH 3T3 cells, the activation of PKC after PMA stimulation was not impaired.

In vitro immune complex kinase assay of Raf-1 isolated from cell membranes revealed an approximate fivefold stimulation following PMA, while GA pretreatment reduced basal Raf-1 activity by greater than 90% and completely blocked PMA-stimulated activity (data not shown). However, since the Raf-1 steady-state level becomes very low after GA treatment and a

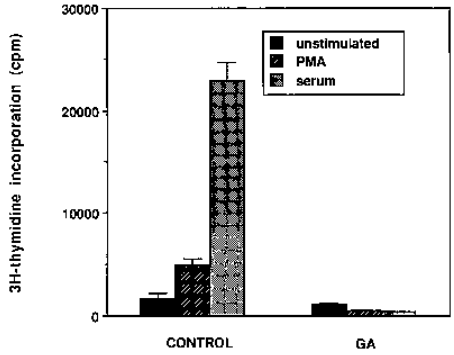

FIG. 7. GA blocks serum- or PMA-induced proliferation of NIH 3T3 cells. Serum-starved NIH 3T3 cells were stimulated by 250 nM PMA or 10% serum in the presence or absence of 100 nM GA. After 4 h, [*methyl*-^{3}H]thymidine was added, and thymidine incorporation was assayed after an additional 16 h.

small amount of highly active, membrane-bound Raf-1 might not be accessible to analysis in cells which do not overexpress Raf-1, we assessed Raf-1 activity indirectly by monitoring the activity of the downstream kinases MEK and MAPK. We found that PMA failed to stimulate both MEK and MAPK in cells pretreated overnight with GA. The inhibitory effect of GA correlated temporally with Raf-1 depletion (41a). We tested for possible direct effects of GA on MEK kinase and MAPK activity in our system. When added directly to the kinase reaction, GA inhibited neither MEK nor MAPK activity at the concentrations used in cells. Furthermore, introduction of Raf-1-independent, constitutively active MEK effectively abrogated the inhibitory effects of GA on MAPK. Taken together with the Raf-1 antisense data (see below), these findings point to Raf-1 protein depletion as the primary mechanism by which GA inhibits PMA-induced activation of the MAPK pathway.

Other MAPKKKs besides Raf-1, including B-Raf and MEKK, as well as a not yet fully characterized MEK activator with a molecular mass of 40 to 50 kDa, have been found in NIH 3T3 cells (39). However, it is unlikely that either B-Raf or MEKK plays a major role in PMA-induced MAPK stimulation in our clone of NIH 3T3 cells. First, we found only very low, barely detectable levels of both B-Raf and MEK kinase protein in our cells. Second, treatment with antisense oligonucleotides specific for a sequence in the 3′ untranslated region of Raf-1 mRNA markedly decreased Raf-1 protein and blocked PMA-stimulated activation of MAPK. Third, in vitro immune complex kinase assay of B-Raf activity revealed minimal induction (less than 50%) following PMA and little inhibitory effect of GA (data not shown). Thus, we conclude that Raf-1 is the predominant MEK activator following PMA treatment in our particular clone of NIH 3T3 cells.

MAPKs are known to activate certain transcription factors, either directly or via activation of additional downstream cytoplasmic kinases (6). Among the transcription factors that respond to serum or PMA stimulation, the transactivator protein AP-1, which consists of a heterodimer of a Jun and a Fos family member, has been described (3, 12). AP-1-binding sequences have been named TREs because they are involved in tetradecanoyl phorbol acetate (i.e., PMA) stimulation (2). Transactivation of TRE-reporter plasmids has been shown to be Raf-1 dependent in NIH 3T3 cells (8). PKC-mediated AP-1 activation has also been shown to be critical for T-cell activation (24, 25, 28, 51). We used a TRE-linked reporter plasmid that was described to be PMA responsive in Jurkat cells (36) in order to show that interruption of the Raf-1–MEK–MAPK pathway at the level of Raf-1 blocked intranuclear activation of the AP-1 transcription factor.

The proliferative response of serum-starved NIH 3T3 cells to both PMA and serum has been well characterized and has been shown to be dependent on an intact Raf-1-initiated signal transduction cascade (26, 27). Both PMA- and serum-dependent stimulation of NIH 3T3 proliferation was completely abrogated by GA.

In summary, our data are consistent with the hypothesis that GA-induced inhibition of the PMA-activated Raf-1–MEK–MAPK signalling pathway in the NIH 3T3 cells that we used is mediated solely via GA-dependent depletion of Raf-1. Additionally, the data suggest that the degree of Raf-1 depletion and disfunction caused by GA is sufficient to interdict signalling via this kinase cascade.

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