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Inhibition of Platelet-Derived Growth Factor- and Epidermal Growth Factor-Mediated Mitogenesis and Signaling in 3T3 Cells Expressing ΔRaf-1:ER, an Estradiol-Regulated Form of Raf-1†

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We have recently described the properties of ΔRaf-1:ER, a fusion protein consisting of an oncogenic form of human Raf-1 and the hormone binding domain of the human estrogen receptor. In this study, we demonstrate that activation of ΔRaf-1:ER in quiescent 3T3 cells (C2 cells), while sufficient to promote morphological oncogenic transformation, was insufficient to promote the entry of cells into DNA synthesis. Indeed, activation of ΔRaf-1:ER potently inhibited the mitogenic response of cells to platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) treatment. Addition of β-estradiol to quiescent C2 cells led to rapid, sustained activation of ΔRaf-1:ER and MEK but only two- to threefold activation of p42 mitogen-activating protein (MAP) kinase activity. Addition of PDGF or EGF to quiescent C2 cells in which ΔRaf-1:ER was inactive led to rapid activation of Raf-1, MEK, and p42 MAP kinase activities, and entry of the cells into DNA synthesis. In contrast, when ΔRaf-1:ER was activated in quiescent C2 cells prior to factor addition, there was a significant inhibition of certain aspects of the signaling response to subsequent treatment with PDGF or EGF. The expression and activation of PDGF receptors and the phosphorylation of $p70^{S6K}$ in response to PDGF treatment were unaffected by prior activation of ΔRaf-1:ER. In contrast, PDGF-mediated activation of Raf-1 and p42 MAP kinases was significantly inhibited compared with that of controls. Interestingly, the mitogenic and signaling responses of quiescent C2 cells to stimulation with fetal bovine serum or phorbol myristate acetate were unaffected by prior activation of ΔRaf-1:ER. It seems likely that at least two mechanisms contribute to the effects of ΔRaf-1:ER in these cells. First, activation of ΔRaf-1:ER appeared to uncouple the activation of Raf-1 from the activation of the PDGF receptor at the cell surface. This may be due to the fact that mSOS1 is constitutively phosphorylated as a consequence of the activation of ΔRaf-1:ER. Second, quiescent C2 cells expressing activated ΔRaf-1:ER appear to contain an inhibitor of the MAP kinase pathway that, because of its apparent sensitivity to sodium orthovanadate, may be a phosphotyrosine phosphatase. It is likely that the inhibitory effects of ΔRaf-1:ER observed in these cells are a manifestation of the activation of some of the feedback inhibition pathways that normally modulate a cell's response to growth factors. 3T3 cells expressing ΔRaf-1:ER will be a useful tool in unraveling the role of Raf-1 kinase activity in the regulation of such pathways.

Members of the Raf family of protein kinases (Raf-1, A-Raf, and B-Raf) are believed to be essential components of mammalian signal transduction pathways (2, 34, 40, 47). Binding of a variety of ligands to their cognate cell surface receptors is believed to lead, through the activation of Ras proteins, to the recruitment of Raf-1 to the plasma membrane and to the activation of kinase activity by a mechanism that is not yet understood (15, 20, 29, 32, 39, 57, 60, 61, 63, 67). Although there is evidence from both in vivo and in vitro studies that phosphorylation of Raf-1 may play a role in the activation of its kinase activity, the precise role of phosphorylation remains unclear (7, 19, 30, 36, 41, 42, 44, 55). It is clear, however, that phosphorylation of Raf-1, as determined by electrophoretic mobility shift, is an unreliable indicator of Raf-1 kinase activity (51).

All three members of the Raf family share a common structural organization, and all are oncogenic when subjected to similar amino-terminal truncation (3, 26). It is not yet clear whether all three Raf protein kinases respond to the same upstream signals or whether they activate the same or different downstream effector pathways.

Raf-1 is capable of activating the enzymes of the mitogen-activated protein (MAP) kinase cascade both in vitro and in the intact cell (14, 15, 25, 31, 35, 51, 59). There is ample evidence that the Ras/Raf-1/MEK/MAP kinase pathway is essential in the proliferation and differentiation of cells in simple eukaryotes such as *Drosophila melanogaster* and *Caenorhabditis elegans* (16, 24, 26, 37, 56). It is unclear, however, what role p42/p44 MAP kinase activation plays in the effects of Raf-1 in mammalian cells, as oncogenic forms of Raf-1 can elicit profound biological effects in the absence of p42/p44 MAP kinase activity (6, 21, 23, 28, 46, 51, 64, 65).

We have previously described the use of a β-estradiol-dependent form of oncogenic human Raf-1, ΔRaf-1:ER (formerly hrafER), to investigate the biological and biochemical events elicited in cells following activation of Raf-1 kinase activity. Activation of ΔRaf-1:ER in C2 cells leads to a rapid, protein synthesis-independent activation of MEK and p42/p44 MAP kinases and to hyperphosphorylation of the resident Raf-1. Whereas the biochemical response to activation of ΔRaf-1:ER requires only a few minutes, the morphological oncogenic transformation of cells is observed 12 to 16 h after the addition of β-estradiol (50).

In this study we wished to determine if the activation of ΔRaf-1:ER in quiescent C2 cells was sufficient to commit the cells to begin DNA synthesis. Second, we wished to determine what effect, if any, the activation of ΔRaf-1:ER in quiescent C2 cells had on the subsequent mitogenic and signaling responses

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† M.M. dedicates this paper to the memory of his father, John McMahon.

of the cells to mitogens such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fetal bovine serum (FBS), or phorbol esters.

MATERIALS AND METHODS

Cell culture. A clone of 3T3 cells expressing ΔRaf-1:ER (C2 cells) was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) FBS at 37°C in a 6% CO_2 humidified atmosphere. β-Estradiol (Sigma) was prepared at 1 mM in ethanol and stored at −20°C and used at a final concentration of 1 μM as described previously (51). C2 cells were made quiescent in DMEM containing 0.5% (vol/vol) FBS for 48 h in the presence or absence of added β-estradiol as described in the text and were subsequently stimulated with 20 ng of PDGF-BB per ml, 25 ng of EGF per ml, 25 ng of basic fibroblast growth factor (bFGF) per ml, 50 ng of phorbol-12-myristate-13-acetate (PMA) per ml (all factors were purchased from UBI Inc.) or 20% (vol/vol) FBS. Rat PC12 cells were treated with 50 ng of mouse nerve growth factor (NGF; 2.5S, Boehringer Mannheim) per ml. All of the factors were used at saturating concentrations.

Preparation of cell extracts. Cells were washed and removed from the surfaces of tissue culture dishes in cold phosphate-buffered saline (PBS) containing 5 mM EDTA and pelleted by centrifugation, lysed in Gold lysis buffer (GLB) containing 20 mM Tris (pH 7.9), 137 mM sodium chloride, 5 mM EDTA Na_2, 10% (vol/vol) glycerol, 1% (vol/vol) Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM aprotinin, 1 mM leupeptin, 1 mM sodium orthovanadate, 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 10 mM sodium fluoride, 1 mM tetrasodium PP_i, and 100 μM β-glycerophosphate. Insoluble material was removed by centrifugation at 12,000 × *g*. The protein concentration of the soluble material was estimated by using the bicinchoninic acid protein assay kit (Pierce). Protein samples were snap frozen, using dry ice-ethanol, and stored at −80°C.

Protein kinase assays and Western blotting. MEK assays were carried out on unfractionated cell lysates as described previously, using recombinant, enzymatically inactive p42 MAP kinase (rp42) as a substrate. In all of the MEK assays the results of the autoradiographic exposure were confirmed by probing the relevant Western blots (immunoblots) with an anti-phosphotyrosine antibody, 4G10 (51). In all of the figures an anti-phosphotyrosine Western blot is presented. Immune complex kinase assays of p42 MAP kinase, ΔRaf-1:ER, and $p74^{raf\text{-}1}$ were carried out as described previously (51). Myelin basic protein (MBP) was used as a substrate for the p42 MAP kinase assays, and MEKB (35), a baculovirus-expressed, enzymatically inactive form of MAP kinase kinase, was used as a substrate for both ΔRaf-1:ER and Raf-1 kinase assays (a gift of Susan Macdonald and Frank McCormick, Onyx Pharmaceuticals). In order to perform the Raf-1 assays, we quantitatively removed all of the ΔRaf-1:ER from 100 μg of C2 cell lysate. This was achieved by using 40 μl of an anti-hbER antiserum (a gift of Steve Robbins and J. Michael Bishop, University of California, San Francisco) and protein A-Sepharose 4B. We confirmed that the ΔRaf-1:ER protein had been quantitatively removed from the cell lysates by Western blotting with the appropriate antisera as described previously (51). We further confirmed that no Raf-1 was immunoprecipitated in complex with ΔRaf-1:ER by Western blotting with the appropriate antisera. All protein kinase assays were analyzed by polyacrylamide gel electrophoresis and Western blotting onto Immobilon P. After autoradiographic quantitation of the protein kinase reaction, the Western blots were probed with the appropriate antisera to ensure that equal amounts of the relevant protein kinase were present in each immunoprecipitate.

Antibodies to p42 MAP kinase, ΔRaf-1:ER, Raf-1, and $p70^{S6K}$ were generously provided by M. J. Weber (University of Virginia), S. Robbins (University of California, San Francisco), R. Schatzman (Syntex), and J. Blenis (Harvard University), respectively. Immunoprecipitation and Western blotting of the PDGF receptor were carried out with a polyclonal antibody that recognizes the A and B forms of the human PDGF receptor (UBI Inc.). Immune complexes were collected by using protein A-Sepharose 4B. Western blotting of the p85 subunit of the phosphatidylinositol 3′-kinase (PI3′ kinase) was carried out by using a polyclonal antiserum raised against rat p85 (UBI Inc.). Anti-phosphotyrosine Western blotting was carried out with a monoclonal antibody (4G10) as described previously (51). Resolution of hypo- and hyperphosphorylated forms of mSOS1 was achieved by electrophoresis of 100 μg of cell lysate through 6% (wt/vol) polyacrylamide gels (Novex) that were run until the 130-kDa marker (Bio-Rad Kaleidoscope markers) reached the bottom of the gel. The gels were Western blotted and probed with an anti-mSOS1 antiserum (UBI Inc.). In all cases protein-antibody complexes were detected with the appropriate secondary antibody coupled to horseradish peroxidase and the enhanced chemiluminescence system (ECL; Amersham). Western blots were stripped for reprobing with other primary antibodies by incubation in a buffer containing 0.2 M glycine (pH 2.5) and 1% (wt/vol) sodium dodecyl sulfate (SDS) for 2 h at 68°C.

DNA synthesis assays. C2 cells plated in 96-well tissue culture trays were made quiescent by culture for 48 h in DMEM containing 0.5% (vol/vol) FBS. To measure the effects of ΔRaf-1:ER activation on entry into DNA synthesis, β-estradiol was added to 1 μM and DNA synthesis was estimated at different times after β-estradiol addition by labeling the cells in media containing 3 μCi of [*methyl*-^{3}H]thymidine per ml for 2 h, at which time the incorporation of [*methyl*-^{3}H]thymidine into DNA was measured by using a Skatron Micro 96 cell harvester and a Betaplate 1205 liquid scintillation counter. Each measurement was performed in triplicate, and an average of the three values is presented.

In order to measure the effect of ΔRaf-1:ER activation on subsequent mitogenic stimulation, C2 cells were made quiescent for 32 h as described above. Cells were then either left untreated or treated for a further 16 h with 1 μM β-estradiol to activate ΔRaf-1:ER as described above. At that time various mitogens were added as described above, and DNA synthesis was measured at different times after the addition as described above.

Analysis of the ability of Raf-1 to bind to Ras · GTP. C2 cells cultured in media containing 0.5% (vol/vol) FBS, either untreated or treated with 1 μM β-estradiol for 24 h, were lysed in GLB that contained 0.1 mM EDTA Na_2 and 1 mM $MgCl_2$. Aliquots (0.5 to 1.0 mg) of cellular proteins were incubated for 1 to 2 h at 4°C, in a total volume of 200 μl, with a silica matrix to which 7.5 μg of c-Ha-Ras had been covalently bound. The Ras-silica matrix had been incubated with either GDP or GMP-PNP (guanidyl-imidodiphosphate, a nonhydrolyzable GTP analog) to mimic Ras in its inactive or active state, respectively, as described previously (39). After the incubation, the silica beads were pelleted and then washed six times in the same buffer containing 0.5 M NaCl. Cellular proteins bound to Ras were liberated by being boiled in SDS sample buffer and were analyzed by polyacrylamide gel electrophoresis (PAGE),

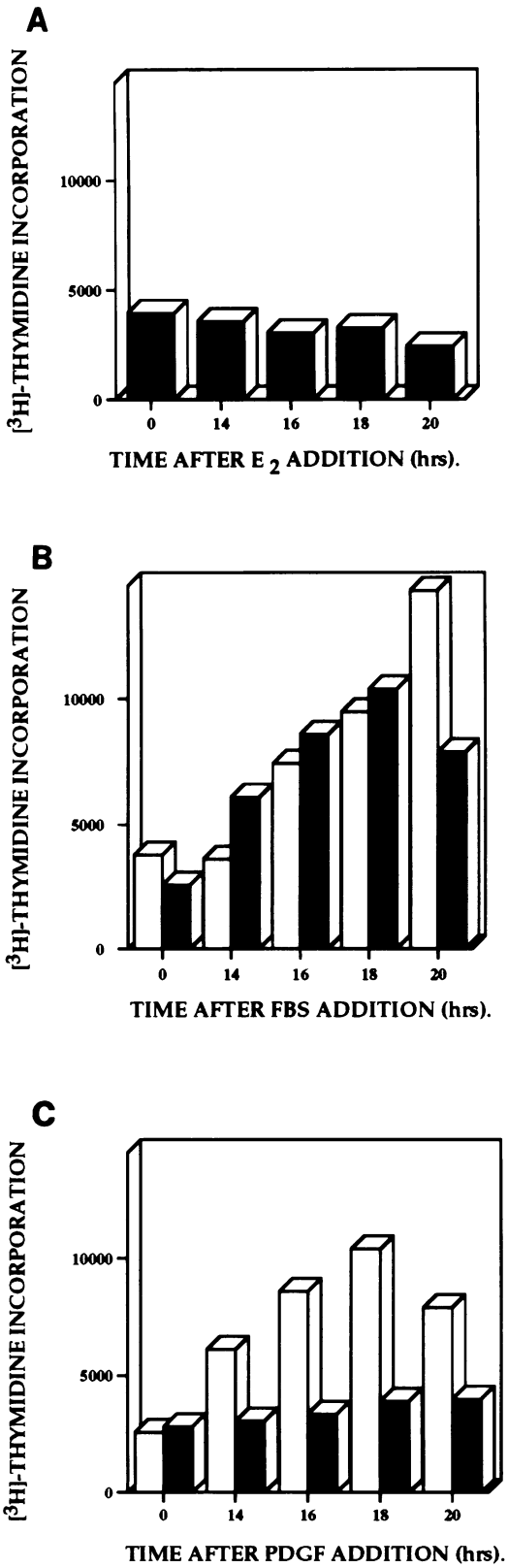

FIG. 1. Measurement of DNA synthesis in C2 cells in response to treatment with β-estradiol, PDGF, and FBS. (A) C2 cells were made quiescent and then treated with 1 μM β-estradiol as described in Materials and Methods. At different times after the addition of β-estradiol, DNA synthesis was estimated by measuring the incorporation of [*methyl*-^{3}H]thymidine into DNA. Cells were labeled for 2 h, at which time [*methyl*-^{3}H]thymidine incorporated into DNA was measured with a Skatron Micro 96 cell harvester and a Betaplate 1205 liquid scintillation counter. Each measurement was performed in triplicate, and an average of the three values is presented. (A) Response of quiescent C2 cells to treatment with 1 μM β-estradiol; (B) response of untreated (open bars) or β-estradiol-treated (filled bars) quiescent C2 cells to treatment with 20% (vol/vol) FBS; (C) response of untreated (open bars) or β-estradiol-treated (filled bars) quiescent C2 cells to treatment with 20 ng of PDGF per ml.

Western blotting, and incubation with a number of antibodies as described in the text.

RESULTS

Activation of ΔRaf-1:ER inhibits the mitogenic response of quiescent C2 cells to PDGF and EGF but not to FBS stimulation. In order to determine if ΔRaf-1:ER activation was sufficient to promote the entry of cells into DNA synthesis, we prepared cultures of C2 cells made quiescent in DMEM containing 0.5% (vol/vol) FBS for 48 h. At that time cells were either left untreated or treated with 1 μM β-estradiol to activate ΔRaf-1:ER. Entry into the S phase of the cell cycle was assessed by measuring the incorporation of [*methyl*-^{3}H]thymidine into DNA after 14, 16, 18, and 20 h of β-estradiol treatment. Activation of ΔRaf-1:ER in quiescent C2 cells led to a marked morphological oncogenic transformation that was detected 10 to 12 h after the addition, similar to that previously described (51). However, activation of ΔRaf-1:ER was insufficient to promote the entry of quiescent C2 cells into DNA synthesis (Fig. 1A). Even prolonged periods of β-estradiol treatment (up to 30 h) had no effect on the entry of the cells into DNA synthesis (data not shown). In addition, when DNA synthesis was measured cumulatively by adding β-estradiol and [*methyl*-^{3}H]thymidine at the same time and labeling the cells for up to 30 h, we observed no effect of ΔRaf-1:ER activation on the entry of the cells into DNA synthesis compared with that in the appropriate controls (data not shown).

In order to assess the effect of ΔRaf-1:ER activation on the response of cells to other mitogens, quiescent C2 cells were either left untreated or treated for 16 h with 1 μM β-estradiol. At this time FBS, PDGF-BB, or EGF was added to the cells, and DNA synthesis was measured at different times as described above and in Materials and Methods. The addition of FBS (Fig. 1B), PDGF (Fig. 1C), or EGF (data not shown) to quiescent C2 cells stimulated their entry into DNA synthesis, which was detected 14 h after factor addition and which remained elevated at all of the time points analyzed. The responses of the cells to serum, PDGF, or EGF were very similar. Activation of ΔRaf-1:ER in quiescent C2 cells for 16 h prior to factor addition had no effect on the mitogenic response of cells to FBS (Fig. 1B) but strongly inhibited the entry of cells into DNA synthesis in response to PDGF (Fig. 1C) or EGF (data not shown). The data demonstrating inhibition of the mitogenic response to PDGF and EGF were almost identical and highly reproducible. Furthermore, the inhibition was evident even when DNA synthesis was measured cumulatively over a 24-h period. In separate experiments, we have demonstrated that activation of ΔRaf-1:ER also inhibited the mitogenic response to bFGF but had no effect on the mitogenic response of cells to lysophosphatidic acid (data not shown).

The addition of ethanol as a solvent control had no effect on the mitogenic response of C2 cells to PDGF, EGF, or serum stimulation. Furthermore, the estrogen receptor antagonists

4-hydroxy-tamoxifen and ICI 164,384, which are potent activators of ΔRaf-1:ER (51), also elicit inhibition of PDGF-, EGF-, and bFGF-mediated mitogenesis in C2 cells. Finally, the addition of β-estradiol to parental 3T3 cells that do not express ΔRaf-1:ER had no effect on the responses of these cells to PDGF, EGF, or serum stimulation (data not shown).

Analysis of the activation of MEK and p42 MAP kinase in response to ΔRaf-1:ER activation in quiescent C2 cells. We have previously demonstrated that activation of ΔRaf-1:ER in C2 cells cultured in 10% FBS leads to constitutive activation of MEK and p42/p44 MAP kinases (51). Since activation of ΔRaf-1:ER was insufficient to promote the entry of quiescent C2 cells into DNA synthesis, we wished to determine the kinetics of MEK and p42 MAP kinase activation in these cells.

Extracts were prepared from quiescent C2 cells either untreated or treated with β-estradiol for 15 or 30 min or 1, 3, 6, 16, or 24 h as described in Materials and Methods. In parallel, we prepared similar lysates from PC12 cells either untreated or treated with NGF for 1, 2, 3, 5, 10, 20, 30, 45, or 90 min or 5 or 17 h. The activities of MEK and p42 MAP kinases were measured as described in Materials and Methods. Under these conditions, ΔRaf-1:ER is activated immediately following the addition of β-estradiol, and total activity in cell lysates increased as the ΔRaf-1:ER protein accumulated in the cell as previously described (data not shown) (51). Activation of ΔRaf-1:ER in quiescent C2 cells led to a rapid activation of MEK that was sustained across the time course (Fig. 2A, lanes 1 to 8). By contrast, the activation of ΔRaf-1:ER led to a two- to threefold activation of p42 MAP kinase. This result is in contrast to the situation in C2 cells cultured in 10% FBS, in which the p42/p44 MAP kinases are activated 10- to 50-fold in response to ΔRaf-1:ER activation (51). As a control, we assessed the activation of MEK and p42 MAP kinase activity in PC12 cells in response to treatment with NGF. Addition of NGF to PC12 cells led to a rapid and sustained activation of MEK activity (Fig. 2C, lanes 1 to 12); however, the activation of p42 MAP kinase was transient, peaking 10 min after factor addition (Fig. 2D, lane 6) and declining thereafter but remaining two- to threefold higher than the starting level of activity (Fig. 2D, lanes 11 and 12). It is important to note that, in estradiol-treated C2 cells and in NGF-treated PC12 cells, at late times after factor addition the level of MEK activity is comparatively high, whereas the level of p42 MAP kinase activity is comparatively low.

ΔRaf-1:ER activation abrogates the PDGF- and EGF-mediated activation of Raf-1 and p42 MAP kinases. Since activated ΔRaf-1:ER inhibited the PDGF- and EGF-mediated entry of cells into DNA synthesis, we decided to analyze the effects of ΔRaf-1:ER activation on the response of the Raf/MEK/MAP kinase cascade in these cells to PDGF and EGF treatment. Quiescent C2 cells, either untreated or treated with 1 μM β-estradiol for 16 h, were stimulated with PDGF for 7 or 15 min, at which time cell extracts were prepared. The enzymatic activities of Raf-1, MEK, and p42 MAP kinases were measured by using recombinant, enzymatically inactive MEK (MEKB), p42 MAP kinase (rp42), and MBP, respectively, as substrates as described in Materials and Methods. The activities of Raf-1, MEK, and p42 MAP kinases were lowest in quiescent C2 cells (Fig. 3C, A, and B, respectively, lanes 1). The addition of PDGF to these cells for 7 or 15 min led to a robust stimulation of the enzymatic activity of all three of these protein kinases (Fig. 3C and 2A and B, respectively, lanes 2 and 3). Quiescent C2 cells treated with 1 μM β-estradiol for 16 h had a large preexisting amount of MEK activity due to the activity of ΔRaf-1:ER (Fig. 3A, lane 4). In fact, the amount of MEK activity in these cells was comparable to that found in PDGF-treated cells (Fig. 3A, compare lanes 3 and 4). Consistent with the observations in Fig. 2, the amount of p42 MAP kinase activity, although slightly larger than in quiescent C2 cells (Fig. 3B, compare lanes 1 and 4), was small compared with that of PDGF-treated cells containing a similar level of MEK activity (Fig. 3B, compare lanes 3 and 4). Subsequent addition of PDGF to these cells had no effect on the already elevated activity of MEK (Fig. 3A, lanes 5 and 6) and gave rise to only a modest activation of p42 MAP kinase that was significantly reduced compared with that of the appropriate controls (Fig. 3B, compare lanes 5 and 6 with lanes 3 and 4). In all cases, the amount of p42 MAP kinase activity observed in immune complex kinase assays correlated with the tyrosine phosphorylation and consequent electrophoretic mobility shift of p42 MAP kinase that has been well documented previously (data not shown) (51). Furthermore, similar inhibition of the PDGF- and EGF-mediated activation of p44 MAP kinase was also observed (data not shown).

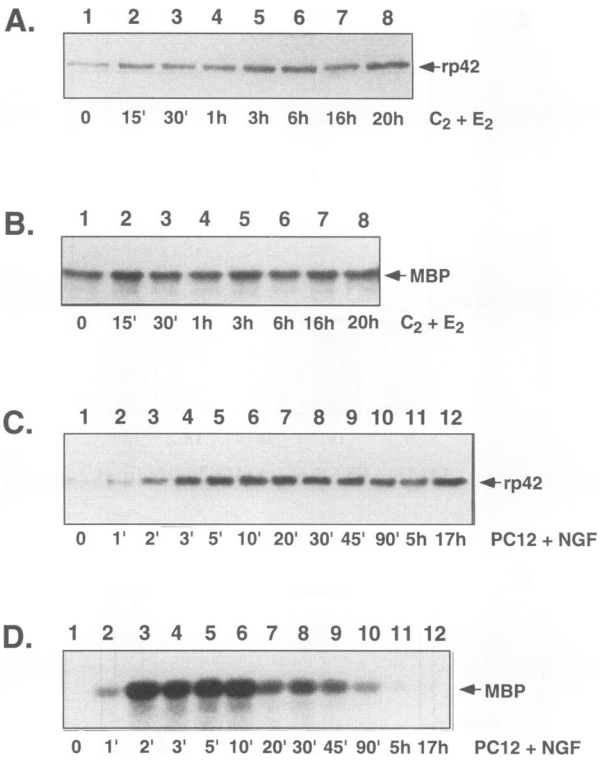

FIG. 2. Activation of MEK and p42 MAP kinases in quiescent C2 cells in response to β-estradiol (A and B) and in PC12 cells in response to treatment with NGF (C and D). C2 cells, made quiescent as described in Materials and Methods, were either left untreated (lanes 1) or treated with 1 μM β-estradiol for the times indicated (lanes 2 to 8). Cells were lysed, extracts were prepared, and protein kinase assays and Western blots were carried out as described in Materials and Methods to measure MEK activity (A) and p42 MAP kinase activity (B). PC12 cells were either left untreated (lanes 1) or treated with 50 ng of NGF for the times indicated (lanes 2 to 12). Cells were lysed, extracts were prepared, and protein kinase assays and Western blots were carried out as described in Materials and Methods to measure MEK activity (C) and p42 MAP kinase activity (D). In each case, MEK activity was measured in unfractionated cell lysates as described in Materials and Methods. After autoradiographic quantitation, the result was confirmed by probing the blots with an anti-phosphotyrosine antibody (A and C). The activity of p42 MAP kinase (B and D) was measured by an immune complex kinase assay, using MBP as a substrate.

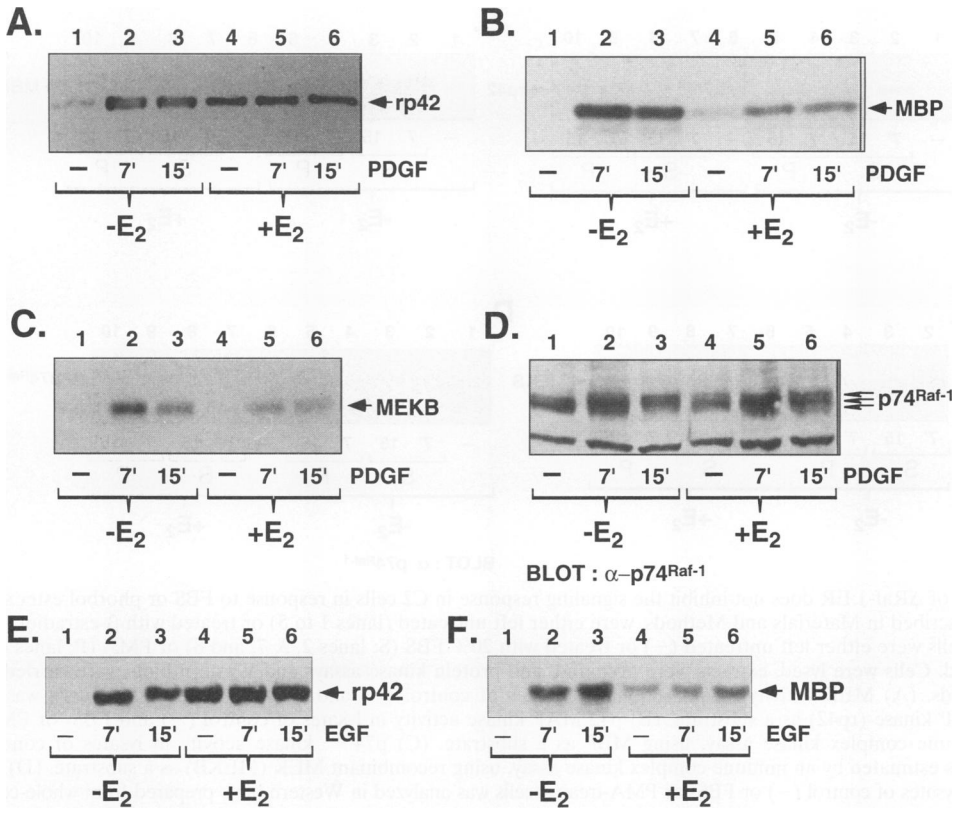

FIG. 3. Inhibition of PDGF- and EGF-mediated signaling events in C2 cells by activation of ΔRaf-1:ER. C2 cells, made quiescent as described in Materials and Methods, were either left untreated (lanes 1 to 3) or treated with β-estradiol (lanes 4 to 6) for 16 h. At this time the cells were either left untreated (−) or treated with PDGF (A to D) or EGF (E and F) for 7 or 15 min as indicated. Cells were lysed, extracts were prepared, and protein kinase assays and Western blots were carried out as described in Materials and Methods. (A) MEK activity in unfractionated lysates of control (−) or PDGF-treated cells was estimated by using recombinant p42 MAP kinase (rp42) as a substrate. (B) p42 MAP kinase activity in lysates of control (−) or PDGF-treated cells was estimated by an immune complex kinase assay, using MBP as a substrate. (C) $p74^{raf\text{-}1}$ kinase activity in lysates of control (−) or PDGF-treated cells was estimated by an immune complex kinase assay, using recombinant MEK (MEKB) as a substrate. (D) Electrophoretic mobility of $p74^{raf\text{-}1}$ from control (−) or PDGF-treated cells was analyzed in Western blots prepared from 100 μg of cell lysate from C2 cells. (E) MEK activity in unfractionated lysates of control (−) or EGF-treated cells was estimated by using rp42 as a substrate. (F) p42 MAP kinase activity in cell lysates of control (−) or EGF-treated cells was estimated by an immune complex kinase assay, using MBP as a substrate.

Quiescent C2 cells treated with β-estradiol contained a low level of Raf-1 kinase activity (Fig. 3C, lane 4). Subsequent addition of PDGF for 7 or 15 min to these cells led to only a modest stimulation of Raf-1 kinase activity that was also significantly reduced compared with that in the appropriate controls (Fig. 3C, compare lanes 5 and 6 with lanes 2 and 3). The addition of PDGF or EGF had no effect on the constitutive activity of ΔRaf-1:ER (data not shown).

Western blot analysis of these cell lysates with an anti-$p74^{raf\text{-}1}$ antiserum (Fig. 3D) demonstrated that PDGF treatment of quiescent C2 cells led to a characteristic phosphorylation-induced mobility shift of Raf-1 (lanes 1 to 3). Raf-1 protein from β-estradiol-treated, quiescent C2 cells was already hyperphosphorylated as a consequence of the activity of ΔRaf-1:ER (lane 4), and subsequent treatment of the cells with PDGF did not significantly augment the observed mobility shift (lanes 5 and 6).

Similar experiments were performed to analyze the effects of ΔRaf-1:ER activation on the response of C2 cells to EGF treatment. The addition of EGF to quiescent C2 cells led to robust activation of MEK (Fig. 3E, lanes 1 to 3) and p42 MAP kinase (Fig. 3F, lanes 1 to 3) activities similar to the activation observed in response to PDGF. C2 cells in which ΔRaf-1:ER had been activated for 16 h contained a high level of MEK activity similar to that seen in an EGF-treated cell (Fig. 3E, lane 4) but a relatively low level of p42 MAP kinase activity (Fig. 3F, lane 4) as demonstrated above. Subsequent addition of EGF to these cells had no effect on the already elevated amount of MEK activity (Fig. 3E, lanes 5 and 6) and led to only a slight increase in p42 MAP kinase activity (Fig. 3F, lanes 5 and 6). These results were essentially identical to those observed with PDGF described above. Indeed, in other experiments we observed similar inhibition of bFGF-mediated activation of p42 MAP kinase (data not shown).

Ethanol treatment of quiescent C2 cells had no effect on Raf-1 or p42 MAP kinase activation in response to PDGF. Similarly, β-estradiol treatment of parental 3T3 cells had no effect on the activation of Raf-1 or p42 MAP kinase in response to subsequent PDGF treatment (data not shown). In all cases, we confirmed by Western blotting that there were equal amounts of protein in each immune complex kinase assay mixture (data not shown).

ΔRaf-1:ER activation has no effect on Raf-1 and p42 MAP kinase activation in response to FBS or PMA. Since ΔRaf-1:ER did not block the mitogenic response of cells to serum stimulation, we wished to assess the effect of activation of

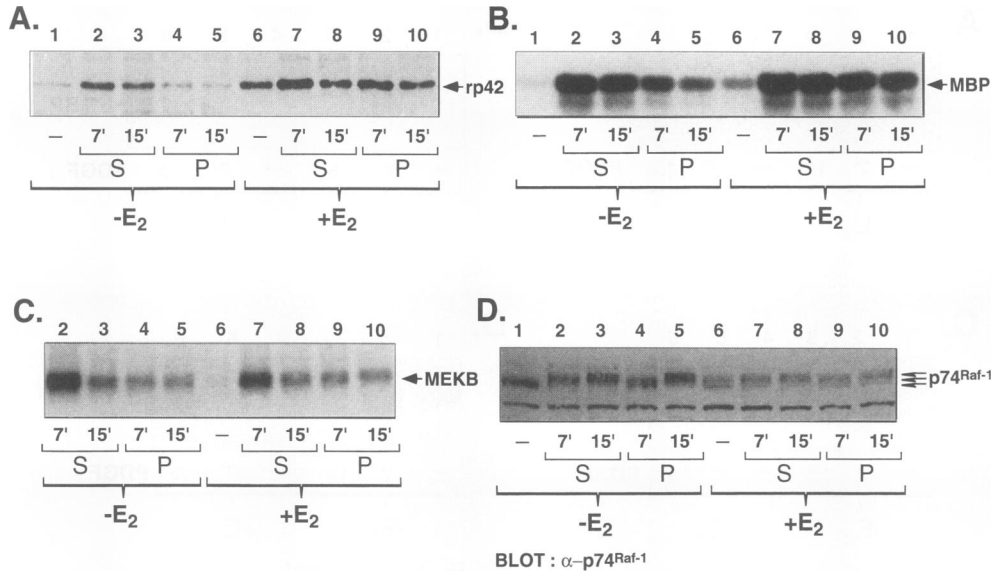

FIG. 4. Activation of ΔRaf-1:ER does not inhibit the signaling response in C2 cells in response to FBS or phorbol ester stimulation. C2 cells, made quiescent as described in Materials and Methods, were either left untreated (lanes 1 to 5) or treated with β-estradiol (lanes 6 to 10) for 16 h. At that time, the cells were either left untreated (−) or treated with 20% FBS (S; lanes 2, 3, 7, and 8) or PMA (P; lanes 4, 5, 9, and 10) for 7 or 15 min as indicated. Cells were lysed, extracts were prepared, and protein kinase assays and Western blots were carried out as described in Materials and Methods. (A) MEK activity in unfractionated lysates of control (−) and FBS- or PMA-treated cells was estimated by using recombinant p42 MAP kinase (rp42) as a substrate. (B) p42 MAP kinase activity in lysates of control (−) and FBS- or PMA-treated cells was estimated by an immune complex kinase assay, using MBP as a substrate. (C) $p74^{raf-1}$ kinase activity in lysates of control (−) or FBS- or PMA-treated cells was estimated by an immune complex kinase assay, using recombinant MEK (MEKB) as a substrate. (D) The electrophoretic mobility of $p74^{raf-1}$ in lysates of control (−) or FBS- or PMA-treated cells was analyzed in Western blots prepared from whole-cell lysates of C2 cells.

ΔRaf-1:ER on the signaling response of quiescent C2 cells to stimulation with serum and the phorbol ester PMA. Quiescent C2 cells, either untreated or treated for 16 h with 1 μM β-estradiol, were stimulated with either 20% (vol/vol) FBS or 50 ng of PMA per ml for 7 or 15 min, at which time the cells were lysed and extracts were prepared. The activities of Raf-1, MEK, and p42 MAP kinases were measured as described in Materials and Methods. As demonstrated in Fig. 4, quiescent C2 cells contained low levels of Raf-1, MEK, and p42 MAP kinase activities (Fig. 4C, lane 6, and Fig. 4A and B, lane 1, respectively). Subsequent addition of FBS or PMA for 7 or 15 min led to activation of all three of these protein kinases (Fig. 4C, A, and B, respectively, lanes 2 to 5). The activation of all of these protein kinases was more robust in response to serum than to PMA stimulation (Fig. 4A, B, and C, compare lanes 2 and 3 with lanes 4 and 5, respectively), which is consistent with numerous observations made for a variety of cell types (50).

For the reasons described above, quiescent C2 cells treated with β-estradiol contained a high preexisting level of MEK activity but a low level of both p42 MAP and Raf-1 kinase activities (Fig. 4A, B, and C, respectively, lanes 6). The subsequent addition of serum or PMA led to a robust stimulation of Raf-1 kinase activity that was similar to that observed in the appropriate controls (Fig. 4C, compare lanes 7 to 10 with lanes 2 to 5). Interestingly, the activation of Raf-1 had little or no effect on the already elevated levels of MEK activity (Fig. 4A, lanes 7 to 10). Most interesting, though, is that while serum and PMA had little or no effect on the level of MEK activity, both treatments significantly increased the activity of p42 MAP kinase, similar to that seen in the controls (Fig. 4B, compare lanes 7 to 10 with lanes 2 to 5). Indeed, the activation of p42 MAP kinase in response to treatment with PMA and β-estradiol was greater than that observed in cells treated with PMA alone, suggesting a synergism between the activities of ΔRaf-1:ER and protein kinase C (Fig. 4B, compare lanes 9 and 10 with lanes 4 and 5). Western blot analysis revealed that serum and PMA treatments of quiescent C2 cells led to an electrophoretic mobility shift of Raf-1 (Fig. 4D, lanes 1 to 5). Although the Raf-1 protein from β-estradiol-treated, quiescent cells already displayed a mobility shift (Fig. 4D, lane 6), the subsequent addition of FBS or PMA augmented this shift (Fig. 4D, lanes 7 to 10), but the effect was subtle. The addition of serum or PMA had no effect on the constitutive activity of ΔRaf-1:ER (data not shown).

PDGF receptor expression and activation are unaffected by the activation of ΔRaf-1:ER. A possible explanation for the specific abrogation of the mitogenic and signaling responses of C2 cells to PDGF could be that activation of ΔRaf-1:ER led to inhibition of expression or activation of the PDGF receptor. In order to address this possibility, we analyzed PDGF receptor expression and activation under conditions similar to those described in the legend to Figure 3. Quiescent C2 cells, either untreated or treated for 16 h with 1 μM β-estradiol, were stimulated with PDGF for 7 or 15 min, at which time cell extracts were prepared as described in Materials and Methods. In order to analyze PDGF receptor expression and activation, we immunoprecipitated the PDGF receptor from these cell lysates and prepared a Western blot. To assess PDGF-dependent activation of receptor tyrosine phosphorylation, the blot was probed first with an anti-phosphotyrosine monoclonal antibody (4G10 [Fig. 5A]). Quiescent C2 cells in the absence or presence of β-estradiol had a low level of PDGF receptor tyrosine phosphorylation (Fig. 5A, lanes 1 and 4, respectively) that was robustly stimulated by the subsequent addition of PDGF to the cells. Prior activation of ΔRaf-1:ER in these cells had no effect on the PDGF-mediated activation of receptor tyrosine phosphorylation (Fig. 5A, compare lanes 2 and 3 with lanes 5 and 6). In addition, we detected the coimmunoprecipi-

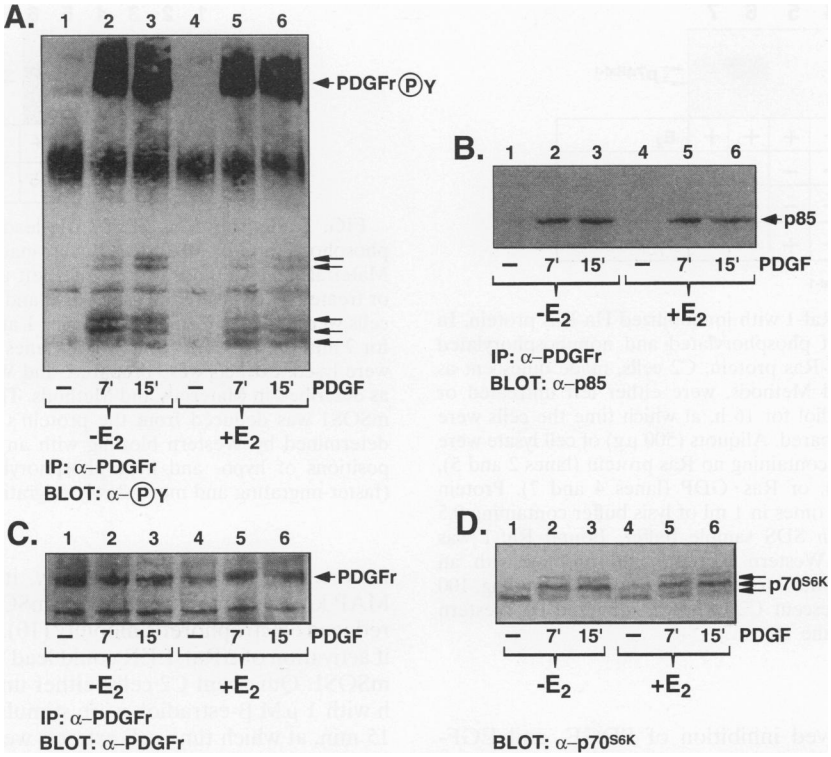

FIG. 5. Expression and activation of PDGF receptors in C2 cells expressing activated ΔRaf-1:ER. C2 cells, made quiescent as described in Materials and Methods, were either left untreated (lanes 1 to 3) or treated with β-estradiol (lanes 4 to 6) for 16 h. At that time the cells were either left untreated (−) or treated with PDGF for 7 or 15 min as indicated. Cells were lysed, extracts were prepared, and immunoprecipitations and Western blots were carried out as described in Materials and Methods. (A) Induction of tyrosine phosphorylation of the PDGF receptor. PDGF receptors were immunoprecipitated from 300 μg of cell lysate with a polyclonal antiserum. Immunoprecipitates were analyzed by SDS-PAGE, Western blotting, and probing with a variety of antibodies. The Western blot described above was probed first with an anti-phosphotyrosine antibody to detect activated PDGF receptors (PDGFr). The presence of tyrosine-phosphorylated cellular proteins in these PDGF receptor immunoprecipitates is indicated by the unlabeled arrows. (B) The Western blot in panel A was stripped as described in Materials and Methods and reprobed with an antibody to the p85 subunit of the PI 3′ kinase. (C) The Western blot in panel B was stripped and reprobed with an antibody to the PDGF receptor. (D) Aliquots (100 μg) of the cell lysates described above were analyzed by SDS-PAGE, Western blotted, and probed with an antiserum that recognizes the 70-kDa ribosomal protein S6 kinase ($p70^{S6K}$).

tation of a number of tyrosine-phosphorylated proteins in complex with the PDGF receptor, as indicated by arrows in Fig. 5A. The coimmunoprecipitation of these proteins with the PDGF receptor was largely unaffected by ΔRaf-1:ER activation.

It has previously been demonstrated that the p85 subunit of the PI3′ kinase forms a complex with the PDGF receptor upon receptor activation. This interaction is mediated by the binding of the two SH2 domains of p85 to phosphorylated tyrosine residues in the kinase insert domain of the PDGF receptor (12, 18, 27, 45). In order to measure the specific recruitment of p85 into complex with the PDGF receptor, the Western blot in Fig. 5A was stripped and reprobed with an antibody against p85 as described in Materials and Methods. As can be seen from Fig. 5B, activation of ΔRaf-1:ER had no effect on the recruitment of p85 into a complex with the PDGF receptor (Fig. 5B, compare lanes 2 and 3 with lanes 5 and 6). In separate experiments, we showed that the association of phospholipase Cγ1 with the PDGF receptor was also unimpaired by the prior activation of ΔRaf-1:ER (data not shown).

In order to confirm that equal amounts of PDGF receptor were present in each immunoprecipitate, the Western blot was probed with an antibody against the PDGF receptor, demonstrating that under these conditions, activation of ΔRaf-1:ER had no effect on the level of expression of the PDGF receptor (Fig. 5C, compare lanes 1 to 3 with lanes 4 to 6).

Independent confirmation of the response of C2 cells to PDGF came from analysis of PDGF-induced phosphorylation of the mitogen-activated 70-kDa ribosomal protein S6 kinase ($p70^{S6K}$). Following mitogenic stimulation, $p70^{S6K}$ displays a reduced electrophoretic mobility in polyacrylamide gels as a consequence of phosphorylation. As can be seen from Fig. 5D, PDGF induced a mobility shift of $p70^{S6K}$ (Fig. 5D, lanes 1 to 3) that was unaffected by the prior activation of ΔRaf-1:ER (Fig. 5D, lanes 4 to 6).

ΔRaf-1:ER-mediated phosphorylation of Raf-1 has no effect on its ability to associate with Ras · GTP. We have previously shown that activation of ΔRaf-1:ER in C2 cells leads to the phosphorylation of the resident $p74^{raf\text{-}1}$ (51). Others have shown that elevation of the intracellular concentration of cyclic AMP (cAMP) in a variety of cell types abrogates the response of the cells to subsequent mitogenic stimulation. It has been suggested that activation of the cAMP-dependent protein kinase leads to phosphorylation of Raf-1 on serine 43. It has been shown that Raf-1 phosphorylated in this manner has a reduced affinity for Ras · GTP, thereby potentially abrogating the response of the Raf-1/MEK/MAP kinase pathway to mitogenic stimulation (5, 11, 22, 66). In order to rule out a role

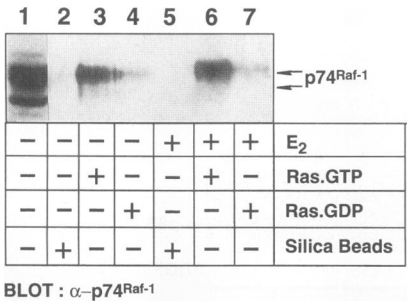

FIG. 6. Association of Raf-1 with immobilized Ha-Ras protein. In order to test the ability of phosphorylated and nonphosphorylated Raf-1 to associate with Ha-Ras protein, C2 cells, made quiescent as described in Materials and Methods, were either left untreated or treated with 1 μM β-estradiol for 16 h, at which time the cells were lysed and extracts were prepared. Aliquots (500 μg) of cell lysate were incubated with silica beads containing no Ras protein (lanes 2 and 5), Ras · GTP (lanes 3 and 6), or Ras · GDP (lanes 4 and 7). Protein complexes were washed six times in 1 ml of lysis buffer containing 0.5 M NaCl and solubilized in SDS sample buffer. Bound Raf-1 was detected by SDS-PAGE, Western blotting, and probing with an anti-Raf-1 antibody. As a control for the efficiency of Ras binding, 100 μg of cell lysate from quiescent C2 cells was analyzed by Western blotting on the same gel (lane 1).

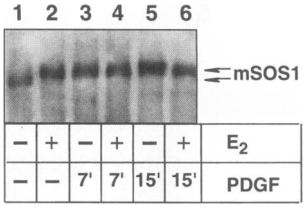

FIG. 7. Activation of ΔRaf-1:ER leads to the constitutive hyperphosphorylation of mSOS1. C2 cells, made quiescent as described in Materials and Methods, were either left untreated (lanes 1, 3, and 5) or treated with β-estradiol (lanes 2, 4, and 6) for 16 h. At that time the cells were either left untreated (lanes 1 and 2) or treated with PDGF for 7 min (lanes 3 and 4) or 15 min (lanes 5 and 6), as indicated. Cells were lysed, extracts were prepared, and Western blots were prepared as described in Materials and Methods. The phosphorylation status of mSOS1 was deduced from the protein's electrophoretic mobility as determined by Western blotting with an anti-mSOS1 antibody. The positions of hypo- and hyperphosphorylated mSOS1 are indicated (faster-migrating and more slowly migrating species, respectively).

for cAMP in the observed inhibition of PDGF- and EGF-induced mitogenesis by ΔRaf-1:ER, we measured the intracellular concentration of cAMP by radioimmunoassay with C2 cells either untreated or treated for 24 h with 1 μM β-estradiol and found no evidence for an elevated level of cAMP (data not shown).

Secondly, we wished to address the possibility that the ΔRaf-1:ER-induced phosphorylation of Raf-1 reduced its ability to associate with Ras · GTP. Cell lysates were prepared from C2 cells made quiescent in the absence or presence of β-estradiol. These lysates were incubated with silica beads onto which the c-Ha-Ras protein had been immobilized, bound to either GMP-PNP (Ras · GTP [Fig. 6, lanes 3 and 6]) or GDP (Ras · GDP [Fig. 6, lanes 4 and 7]) to mimic Ras proteins in their active or inactive state, respectively (39). As a control, we incubated a similar amount of cell lysate with silica beads that contained no immobilized Ras protein (Fig. 6, lanes 2 and 5) as described in Materials and Methods. In addition, we analyzed 100 μg of cell lysate from quiescent C2 cells directly (Fig. 6, lane 1). Proteins in the cell lysate that bound to immobilized Ras were analyzed by Western blotting. Raf-1 protein from quiescent C2 cells showed a specific association with Ras · GTP (Fig. 6, lane 3) and a weaker association with Ras · GDP (lane 4) and did not interact with the silica matrix alone (lane 2). The Raf-1 protein from β-estradiol-treated, quiescent C2 cells showed an identical pattern of association with Ras · GTP (Fig. 6, lane 6) and Ras · GDP (lane 7), indicating that the ΔRaf-1:ER-mediated phosphorylation of Raf-1 did not affect its ability to associate with Ras · GTP in vitro. From the analysis of unfractionated cell lysate (Fig. 6, lane 1) we estimated that 5 to 10% of the Raf-1 protein in the cell lysate interacted with Ras · GTP. As a control, the Western blot was reprobed with the anti-hbER antiserum to demonstrate that the ΔRaf-1:ER protein, which lacks the Ras · GTP binding domain, did not interact with Ras · GTP, Ras · GDP, or the silica matrix (data not shown).

Activation of ΔRaf-1:ER leads to the constitutive hyperphosphorylation of mSOS1. Activation of Ras proteins is promoted by the activity of guanine nucleotide exchange factors such as mSOS1. Recently, it has been reported that MAP kinases can phosphorylate mSOS1 in vitro, giving rise to reduced electrophoretic mobility (10). We wished to determine if activation of ΔRaf-1:ER could lead to the phosphorylation of mSOS1. Quiescent C2 cells, either untreated or treated for 16 h with 1 μM β-estradiol, were stimulated with PDGF for 7 or 15 min, at which time cell extracts were prepared as described in Materials and Methods. Phosphorylation of mSOS1 was assessed by reduction in electrophoretic mobility (Fig. 7). In quiescent C2 cells (lane 1), mSOS1 was hypophosphorylated and the subsequent addition of PDGF for 7 min (lane 3) or 15 min (lane 5) led to reduced electrophoretic mobility, indicating that mSOS1 had been phosphorylated. By contrast, mSOS1 from β-estradiol-treated, quiescent C2 cells already displayed a reduced electrophoretic mobility (lane 2), and the subsequent addition of PDGF did not lead to a further reduction in mobility. In separate experiments, we have shown that mSOS1 became hyperphosphorylated 30 min after the activation of ΔRaf-1:ER in C2 cells (data not shown). These results indicate that hyperphosphorylation of mSOS1 is rapid and sustained following activation of ΔRaf-1:ER in quiescent C2 cells.

The addition of sodium orthovanadate to C2 cells alleviates the ΔRaf-1:ER-mediated inhibition of p42 MAP kinase activity. An interesting observation that emerged from this study was the apparent lack of correlation between the kinase activity of MEK and the activity of p42 MAP kinase. Activation of ΔRaf-1:ER in quiescent C2 cells gave rise to a high level of MEK activity that was similar to that seen in quiescent C2 cells treated with PDGF (Fig. 3A, compare lanes 3 and 4). By contrast, the level of p42 MAP kinase activity in quiescent C2 cells in which ΔRaf-1:ER was activated was significantly less than that observed in C2 cells treated with PDGF (Fig. 3B, compare lanes 3 and 4). We and others have previously shown that the activity of cellular phosphatases plays a significant role in the regulation of the activity of the MAP kinases (9, 51, 58, 62). For example, in rat1a cells, ΔRaf-1:ER activated MEK activity but there was no concomitant activation of p42/p44 MAP kinase unless the cells were treated with phosphatase inhibitors prior to the activation of ΔRaf-1:ER (51). We therefore speculated that the reason why quiescent C2 cells, expressing activated ΔRaf-1:ER, contained a high level of MEK activity but a relatively low level of p42 MAP kinase activity (Fig. 2) is the activation of a phosphatase capable of inactivating the MAP kinases. Candidates for such a molecule include MKP-1/CL100 and PAC-1, whose activities have re-

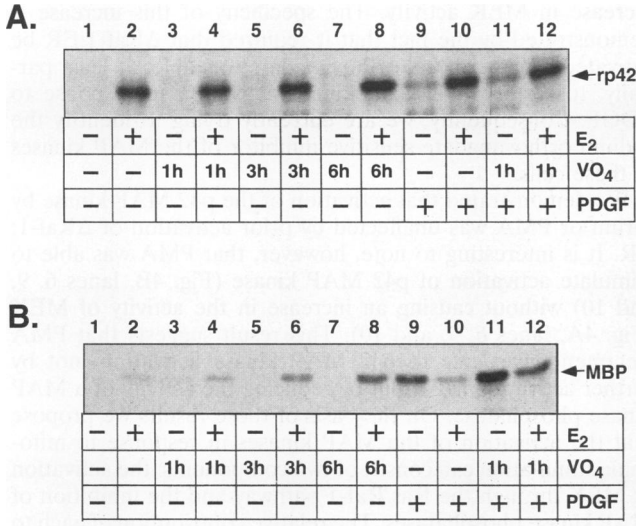

FIG. 8. Orthovanadate treatment of C2 cells expressing activated ΔRaf-1:ER restores the PDGF-mediated activation of p42 MAP kinase. C2 cells made quiescent as described in Materials and Methods were either left untreated (lane 1) or treated with β-estradiol for 16 h (lane 2). Sodium orthovanadate was added to 50 μM to untreated or β-estradiol-treated cells either 1 h (lanes 3 and 4, respectively), 3 h (lanes 5 and 6, respectively), or 6 h (lanes 7 and 8, respectively) prior to the end of the β-estradiol treatment, at which time cells were lysed and extracts were prepared. The activities of MEK (A) and p42 MAP kinase (B) were estimated as described previously. C2 cells made quiescent as described in Materials and Methods were either left untreated or treated with β-estradiol for 16 h and were stimulated with PDGF for 15 min (lanes 9 and 10, respectively). A second set of quiescent C2 cells either untreated or treated with β-estradiol for 16 h were treated with 50 μM sodium orthovanadate for 1 h prior to the addition of PDGF for 15 min (lanes 11 and 12, respectively). As described above, the cells were lysed and extracts were prepared. The activities of MEK (A) and p42 MAP kinase (B) were estimated as described in Materials and Methods.

cently been described (1, 9, 58, 62). To address a possible role for a phosphatase in the regulation of p42 MAP kinase in C2 cells, we utilized sodium orthovanadate as an inhibitor of phosphotyrosine phosphatases. Quiescent C2 cells, either untreated or treated with 1 μM β-estradiol for 16 h to activate ΔRaf-1:ER, were treated with sodium orthovanadate at a final concentration of 50 μM for a further 1, 3, or 6 h, at which time the activities of MEK and p42 MAP kinases in cell lysates were measured as described in Materials and Methods. As seen in previous experiments, quiescent C2 cells contained a low level of MEK activity, whereas those treated with β-estradiol contained high levels of MEK activity (Fig. 8A, compare lanes 1 and 2). Subsequent addition of orthovanadate for 1, 3, or 6 h had little or no measurable effect on the low level of MEK activity in quiescent C2 cells (Fig. 8A, lanes 3, 5, and 7) or on the high level of MEK activity in β-estradiol-treated, quiescent cells (Fig. 8A, lanes 4, 6, and 8). C2 cells made quiescent in the absence of β-estradiol contained a low level of p42 MAP kinase activity, whereas those in the presence of β-estradiol contained a two- to threefold-greater amount of p42 MAP kinase activity (Fig. 8B, lanes 1 and 2, respectively). The addition of sodium orthovanadate to quiescent C2 cells had little or no effect on p42 MAP kinase activity at any time after the addition (Fig. 8B, lanes 3, 5, and 7). By contrast, the addition of sodium orthovanadate to C2 cells containing activated ΔRaf-1:ER led to substantial activation of p42 MAP kinase activity that was detectable after 1 h, was stronger after 3 h, and was most profound after 6 h of treatment (Fig. 8B, lanes 4, 6, and 8, respectively). Indeed, the level of activity seen after 6 h was similar to that observed in quiescent C2 cells treated with PDGF (Fig. 8B, compare lanes 8 and 9).

In conjunction with the previous experiment, we addressed the possibility that sodium orthovanadate could overcome the inhibition of PDGF-induced p42 MAP kinase activation demonstrated in Fig. 3. Sodium orthovanadate was added for 1 h to quiescent C2 cells either untreated or treated with 1 μM β-estradiol for 16 h. These cells were then treated with PDGF for a further 15 min, at which time cell extracts were prepared. PDGF treatment of quiescent C2 cells led to a modest increase in MEK activity (Fig. 8A, compare lanes 1 and 9) and a robust increase in p42 MAP kinase activity (Fig. 8B, compare lanes 1 and 9). Pretreatment of these cells for 1 h with sodium orthovanadate led to a slight potentiation of the PDGF-induced MEK and p42 MAP kinase activities (Fig. 8A and B, compare lanes 9 and 11, respectively). As seen previously, despite the high level of MEK activity (Fig. 8A, lane 10), the addition of PDGF to β-estradiol-treated, quiescent C2 cells led to only a modest increase of p42 MAP kinase activity that was significantly less than that of the appropriate control (Fig. 8B, compare lanes 9 and 10). However, when the cells were pretreated for 1 h with sodium orthovanadate prior to the addition of PDGF, the induction of p42 MAP kinase activity was potentiated to the level observed in quiescent cells treated with PDGF alone (Fig. 8B, compare lanes 9 and 12).

DISCUSSION

The results described here demonstrate that activation of an oncogenic form of Raf-1 (ΔRaf-1:ER) in quiescent 3T3 cells was sufficient to cause morphological oncogenic transformation but was insufficient to promote the entry of 3T3 cells to the S phase of the cell cycle. This observation is similar to that described recently that shows that c-*fos* expression is insufficient to promote mitogenesis but leads to activation of gene expression and morphological transformation (38).

We have found that whilst activation of ΔRaf-1:ER in quiescent 3T3 cells led to the activation of MEK and p42/p44 MAP kinases, it appeared to inhibit the cells' ability to display appropriate mitogenic and signaling responses to subsequent treatment with factors such as PDGF, EGF, or bFGF that signal through transmembrane receptor tyrosine kinases. Specifically, the entry of cells into DNA synthesis was completely inhibited and the growth factor-induced activation of Raf-1 and p42/p44 MAP kinases was significantly abrogated by prior activation of ΔRaf-1:ER. Activation of ΔRaf-1:ER had no effect, however, on the mitogenic and/or signaling response of quiescent 3T3 cells to serum, phorbol ester (PMA), or lysophosphatidic acid stimulation.

From the experiments presented here it appears likely that the mechanism of inhibition of PDGF-induced mitogenesis is mediated by at least two distinct processes. The first point of inhibition occurs either at or upstream of the activation of Raf-1, and the second appears to operate at the level of the p42/p44 MAP kinases.

We observed that the activation of Raf-1 is uncoupled from the activation of the PDGF receptor. It is clear that this observation is not a consequence of the inhibition of expression or activation of the PDGF receptor, as PDGF receptors were appropriately expressed and activated in cells in which ΔRaf-1:ER had been active for 16 h. Furthermore, we detected no abrogation of the PDGF-induced phosphorylation of $p70^{S6K}$ in cells expressing activated ΔRaf-1:ER. These results demon-

strate that activation of ΔRaf-1:ER in C2 cells did not simply lead to a generalized desensitization of cellular signaling pathways.

Although others have demonstrated that an increase in intracellular cAMP can inhibit the subsequent activation of the Raf-1/MEK/MAP kinase cascade, we do not believe that cAMP is involved in the inhibition observed here. First, we detected no increase in intracellular cAMP in cells expressing activated ΔRaf-1:ER. Second, Raf-1 that has been phosphorylated by protein kinase A interacts poorly with Ras · GTP (66), whereas Raf-1 phosphorylated in response to ΔRaf-1:ER activation (51) was not compromised in its ability to interact with Ras · GTP. It is possible, however, that the phosphorylation of Raf-1 mediated by activation of ΔRaf-1:ER abrogates the activation of Raf-1 by inhibiting the Ras-independent step of Raf-1 activation (32, 57). It is important to note that hyperphosphorylated Raf-1 was unimpaired in its activation by treatment of C2 cells with FBS and PMA.

We suspect that the abrogation of Raf-1 activation may reflect inhibition of steps upstream of Ras activation. This inhibition may involve the uncoupling of receptor tyrosine kinases from the activation of Ras proteins by the inhibition of the function of the guanine nucleotide exchange factors such as mSOS1 and mSOS2 that are required to convert Ras proteins from their GDP-bound, inactive state to their GTP-bound, active state (4, 8, 17, 33, 43, 48, 49, 52–54). It has been shown previously that mSOS1 displays reduced electrophoretic mobility as a consequence of phosphorylation following treatment of cells with growth factors. It has been suggested that this phosphorylation is a consequence of feedback phosphorylation from enzymes such as the MAP kinases (10, 48). It has recently been demonstrated that a human MAP kinase can phosphorylate the *Drosophila* SOS protein in vitro (10). Evidence to support this hypothesis comes from the observation that activation of ΔRaf-1:ER in quiescent C2 cells led to the rapid and sustained phosphorylation of mSOS1, as demonstrated by reduced electrophoretic mobility. In order to further investigate this hypothesis, we are currently attempting to measure the effect of prior ΔRaf-1:ER activation on the ability of mSOS1 to associate with GRB2 and activated receptor tyrosine kinases and the subsequent consequences on the activation of Ras proteins in these cells.

It is unlikely, however, that the abrogation of Raf-1 activation can explain the abrogation of MAP kinase activation in response to PDGF, for the following reasons. Activation of ΔRaf-1:ER in quiescent C2 cells led to the robust activation of MEK but only two- to threefold activation of p42 MAP kinase. This situation is similar to that seen in PC12 cells that have been treated with NGF for prolonged periods. It is possible that the relatively low level of p42 MAP kinase activity observed in both cell types following stimulation is due to the activity of MAP kinase phosphatases that play an important role in the regulation of MAP kinase activity in cells (9, 51, 58).

The consequence of the ΔRaf-1:ER-mediated activation of MEK and MAP kinases is that in β-estradiol-treated, quiescent C2 cells there is a large amount of MEK activity, similar to that seen in PDGF-treated cells, but a rather low level of MAP kinase activity compared with that in the same PDGF-treated cells. Subsequent addition of PDGF to β-estradiol-treated, quiescent C2 cells gave rise to only a modest increase in the activity of p42 MAP kinase that was significantly less than that observed in the appropriate controls. The conclusion that a MAP kinase phosphatase is playing an important role in the inhibition of MAP kinase activation is supported by the fact that sodium orthovanadate treatment of cells led to increased p42 MAP kinase activity that occurred without a concomitant increase in MEK activity. The specificity of this increase is demonstrated by the fact that it required that ΔRaf-1:ER be activated. Furthermore, orthovanadate was able, at least partially, to restore p42 MAP kinase activation in response to PDGF. Consequently, we are currently trying to identify the sodium orthovanadate-sensitive inhibitor of the MAP kinases in these cells.

We demonstrated that activation of the p42 MAP kinase by serum or PMA was unaffected by prior activation of ΔRaf-1:ER. It is interesting to note, however, that PMA was able to stimulate activation of p42 MAP kinase (Fig. 4B, lanes 6, 9, and 10) without causing an increase in the activity of MEK (Fig. 4A, lanes 6, 9, and 10). This result suggests that PMA treatment gives rise to p42 MAP kinase activation, not by further activating MEK but by reducing the activity of a MAP kinase phosphatase. On the basis of these results we propose that the activation of the MAP kinases in response to mitogenic stimulation can consist of two components: the activation of MEK through the Ras/Raf-1 pathway and the inhibition of MAP kinase phosphatases. The relative contribution of each to the activation of MAP kinases may depend on the cell type and the particular factor to which the cells are responding.

That an oncogenic form of Raf-1 is inhibitory to the mitogenic response of cells to PDGF, EGF, and bFGF may seem surprising. We believe that the explanation for this phenomenon is that ΔRaf-1:ER is capable of activating some or all of the normal feedback inhibitory loops that are activated in cells following growth factor treatment. Although these feedback controls are insufficient to restrain morphological oncogenic transformation by ΔRaf-1:ER, they are capable of inhibiting the cell's response to certain growth factors. Raf-1-mediated desensitization of cells is not restricted to the cell line that we have described. Recently, Buscher et al. have demonstrated that v-*raf* renders the growth of BAC-1.2F5 macrophages colony-stimulating factor 1 independent. In these cells the expression of v-*raf* renders the p42/p44 MAP kinases insensitive to subsequent stimulation by colony-stimulating factor 1 in a manner that may be analogous to that observed in the experiments described here (6).

Interestingly, the inhibition of mitogenic signaling is not observed in every cell type in which ΔRaf-1:ER is biologically active. We have previously demonstrated that activation of ΔRaf-1:ER in rat1a cells leads to oncogenic transformation with concomitant activation of MEK, but there is no detectable activation of the p42/p44 MAP kinases in these cells (51). Activation of ΔRaf-1:ER in quiescent rat1a cells is insufficient to promote the entry of the cells into DNA synthesis; however, there is no apparent inhibition of PDGF-, EGF-, or bFGF-mediated mitogenesis (50). This result lends support to the idea that activation of the MAP kinases may be important in the inhibition of growth factor-mediated mitogenesis observed in C2 cells.

It is abundantly clear that the Ras/Raf/MEK/MAP kinase signal transduction cascade is subject to a complex pattern of regulation (2). This pathway can be activated by molecules that signal through transmembrane tyrosine kinase receptors such as PDGF and through the serpentine receptors that activate the heterotrimeric G proteins. In addition, the pathway can be activated by phorbol esters that bypass cell surface receptors and activate protein kinase C directly. The pathway is antagonized by cAMP and its agonists (5, 11, 13, 22, 66), and we have demonstrated a different form of antagonism that is manifest as a consequence of the activation of Raf-1 kinase activity in cells. Since this pathway is likely to be essential for both cellular proliferation and differentiation, it is perhaps not surprising that it should be subject to such elaborate control.

Indeed, there may be a variety of regulatory mechanisms that have yet to be identified.

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