Stimulation of Rat-1 cells with lysophosphatidic acid (LPA) or epidermal growth factor (EGF) results in a biphasic, sustained activation of extracellular signal-regulated kinase 1 (ERK1). Pretreatment of Rat-1 cells with either cycloheximide or sodium orthovanadate had little effect on the early peak of ERK1 activity but potentiated the sustained phase. Cycloheximide also potentiated ERK1 activation in Rat-1 cells expressing ΔRaf-1:ER, an estradiol-regulated form of the oncogenic, human Raf-1. Since cycloheximide did not potentiate MEK activity but abrogated the expression of mitogen-activated protein kinase phosphatase (MKP-1) normally seen in response to EGF and LPA, we speculated that the level of MKP-1 expression may be an important regulator of ERK1 activity in Rat-1 cells. Inhibition of LPA-stimulated MEK and ERK activation with PD98059 and pertussis toxin, a selective inhibitor of G_{i}-protein-coupled signaling pathways, reduced LPA-stimulated MKP-1 expression by only 50%, suggesting the presence of additional MEK- and ERK-independent pathways for MKP-1 expression. Specific activation of the MEK/ERK pathway by ΔRaf-1:ER had little or no effect on MKP-1 expression, suggesting that activation of the Raf/MEK/ERK pathway is necessary but not sufficient for MKP-1 expression in Rat-1 cells. Activation of PKC played little part in growth factor-stimulated MKP-1 expression, but LPA- and EGF-induced MKP-1 expression was blocked by buffering [Ca^{2+}], leading to a potentiation of the sustained phase of ERK1 activation without potentiating MEK activity. In Rat-1ΔRaf-1:ER cells, we observed a strong synergy of MKP-1 expression when cells were stimulated with estradiol in the presence of ionomycin, phorbol 12-myristate 13-acetate, or okadaic acid under conditions where these agents did not synergize for ERK activation. These results suggest that activation of the Raf/MEK/ERK pathway is insufficient to induce expression of MKP-1 but instead requires other signals, such as Ca^{2+}, to fully reconstitute the response seen with growth factors. In this way, ERK-dependent and -independent signals may regulate MKP-1 expression, the magnitude of sustained ERK1 activity, and therefore gene expression.

One of the major signal pathways responsible for regulating reentry into the cell cycle leads to activation of the extracellular signal-regulated kinases (ERKs) 1 p44ERK1 and p42ERK2 (also called mitogen-activated protein kinases or MAP kinases) (1–4). Growth factor-induced activation of p74Raf-1 leads to the phosphorylation and activation of the dual specificity protein kinase MEK, which in turn activates the ERK/MAP kinases by phosphorylation of threonine and tyrosine residues in the motif TGY (5–9). p74Raf-1 is recruited to the plasma membrane and regulated by the Ras GTPase proteins (10–12). This cascade is activated by receptor tyrosine kinases such as the EGF receptor (reviewed in Ref. 13), cytokine receptors such as the GMCSF-receptor (14), and G-protein-coupled receptors such as the lysophosphatidic acid (LPA) or thrombin receptors (15, 16, 20).

Several observations demonstrate the importance of this signaling pathway in cell growth. First, activating mutations in Ras (17), Raf (9), or MEK (18) are sufficient to activate ERK, induce gene expression, or cause oncogenic transformation. Second, inhibition in this signaling pathway is associated with inhibition of ERK activation and cell growth (19). Third, among the substrates for ERK1 and ERK2 are transcription factors of the Ets family, Elk-1 and Sap1a (4, 21, 22), which regulate the sustained activation of ERKs (20, 23, 24), which is temporally associated with their accumulation in the nucleus (25–27). In this way, the ERK cascade provides a link between receptor signaling events at the plasma membrane and regulated gene expression in the nucleus.

Whereas much attention has focused on the mechanisms by which this cascade is activated by growth factors and oncoproteins, several studies have turned to the question of which protein phosphatases are responsible for dephosphorylating and thereby inactivating each kinase in the cascade. In the case of ERK, dephosphorylation and inactivation is of considerable interest because the activating phosphorylations are on both threonine and tyrosine (6) and because sustained ERK activation, which seems to be important for proliferative signaling (20, 23, 24), is compartmentalized within the nucleus (25–27).

Recent work has identified a family of dual specificity protein phosphorylation and dephosphorylation.
phosphatases that dephosphorylate both the tyrosine (Tyr\(^{183}\)) and threonine (Thr\(^{185}\)) residues on ERK (28–32). The prototype of this family is MAP kinase phosphatase-1 (MKP-1) (which is encoded by human CL100 and mouse 3CH134 or erk genes, which are immediate early genes induced by oxidative stress and mitogenic stimulation (28, 29, 32)). Overexpression of MKP-1 blocks activation of ERK (31) and cell cycle reentry (33, 34). Furthermore, pretreatment of cells with cycloheximide, to block de novo expression of MKP-1, is able to potentiate sustained ERK activation by serum in NIH3T3 cells (31). Since MKP-1 appears to be expressed exclusively in the nucleus, a simple model envisions growth factor-stimulated activation and nuclear accumulation of ERK leading to increased MKP-1 expression, which in turn accumulates in the nucleus and inactivates ERK, affording exquisite fine tuning of the sustained phase of ERK activation (35). This model has been challenged by the observation that in PC12 cells and endothelial cells MKP-1 is induced but may not be the phosphatase that regulates ERK activity; it was suggested that PP2A and an unidentified PTPase were responsible for dephosphorylation of ERK (36).

In Rat-1 fibroblasts, there is a strong correlation between sustained ERK activation and DNA synthesis in response to mitogenic stimulation with LPA (24); factors that regulate this sustained ERK activation are therefore of great interest. In this report, we demonstrate a role for MKP-1 or a closely related molecule in regulating sustained ERK activity in Rat-1 cells and characterize the pathways regulating expression of MKP-1 in response to growth factor stimulation. Induction of MKP-1 clearly reflects activation of the MEK/ERK cascade, but this pathway alone is insufficient; maximal expression of MKP-1 requires synergistic activation of the ERK pathway and additional ERK-independent signals including Ca\(^{2+}\). In addition, we show that preventing Ca\(^{2+}\)-dependent MKP-1 expression potentiated sustained ERK activation, suggesting that MKP-1 is a focus for regulatory cross-talk to the ERK pathway from other signal pathways.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture reagents were from Irvine Scientific. Prepared SDS-PAGE reagents were from Novex Gel Systems. LPA was obtained from Avanti Polar Lipids. EGF was from Boehringer Mannheim. Okadaic acid was from LC Laboratories. \(\gamma\)-32P\)ATP was from DuPont NEN. Goat-anti-rabbit horseradish peroxidase-conjugated secondary antibodies were from Bio-Rad. All other reagents including myelin basic protein, cycloheximide, and sodium orthovanadate were from Sigma. Antibodies to ERK1 (E12) have been described previously (15). Antibodies to MKP-1 and MKP-2 (Abb-1), generated using a peptide derived from the C-terminal 12 amino acids of mouse CL100 (YLKSPITTSPSC) were the very generous gift of Dr. Fergus McKenzie (15). Antibodies to MKP-1 and MKP-2 (Alb-1), generated using a peptide derived from the C-terminal 12 amino acids of mouse CL100 (YLKSPITTSPSC) were the very generous gift of Dr. Fergus McKenzie and Prof. Jacques Pouyssegur (Center de Biochimie, Université de Nice, Nice, France). Monoclonal antibodies to ERK and MEK were from Pharmingen and Zenmed, respectively. Phosphospecific antibodies for ERK and MEK were from New England Biolabs. The MEK inhibitor PD98059 was prepared by Cheri Blume and Dr. Dan Rogers in the Chemistry Group at ONYX Pharmaceuticals and confirmed by NMR analysis.

**Cells and Cell Culture**—The Rat-1 cells used in this and previous studies (15, 24, 37) were originally provided by Dr. J. L. Bos (Department of Physiological Chemistry, University of Utrecht, The Netherlands). Rat-1 cells were cultured in Dulbecco’s modified Eagle’s medium containing penicillin/streptomycin, glutamine, and 10% fetal bovine serum. Cells were washed once in serum-free medium and then placed in fresh serum-free medium for at least 24 h prior to the experiments described herein. Pretreatments with various agents were as follows: 50 \(\mu\)g/ml cycloheximide for 45 min prior to growth factor addition, 100–1000 \(\mu\)M sodium orthovanadate for 30 min prior to growth factor addition. 40 \(\mu\)g/ml PD98059 for 30 min prior to growth factor addition, and 100 \(\mu\)g/ml pertussis toxin for 18 h prior to growth factor addition.

The derivation and characterization of R1aRaf-1:ER-4 cells will be described elsewhere. These are a clone of Rat-1 cells expressing the conditional form of oncogenic human Raf-1 in which the catalytic domain of Raf-1 is fused to the hormone-binding domain of the human estrogen receptor (38), allowing estrogen-dependent activation of MEK and ERK independently of Ras. R1aRaf-1:ER-4 cells were maintained in the same medium as Ras-Raf-1 cells but supplemented with 400 \(\mu\)g/ml G418. G418 selection was maintained throughout and only removed during serum deprivation for the last 24 h.

**Cell Stimulations**—For ERK1 assays, experiments were performed upon six-well plates of confluent, quiescent cells that had been serum-starved for 24–36 h. Following the addition of the indicated drug or inhibitor, cells were stimulated by the addition of growth factors, and stimulation proceeded at 37 °C for the time indicated. Incubations were terminated by aspiration and the addition of ice-cold TG lysis buffer (20 mM Tris/HCl (pH 8), 1% Triton X-100, 10% glycerol, 137 mM NaCl, 1.5 mM MgCl\(_2\), 1 mM EDTA, 50 mM NaF, 1 mM Na\(_2\)VO\(_3\), 1 mM Pefabloc, 20 \(\mu\)M leupeptin, 10 \(\mu\)g/ml aprotinin). Clarified cell lysates were prepared as described previously (15, 24, 39).

**Immune Complex Kinase Assays for ERK**—Anti-peptide antibodies directed to the extreme C termini of ERK1 (E12) and the assay of immunoprecipitated ERK1 were described previously (15, 24, 37, 39). We have been unable to derive immunoprecipitating antiserum for ERK2. However, we have derived a Rat-1 cell line that stably expresses physiological levels of a Myc epitope-tagged version of ERK2. In all experiments performed, we have noted that ERK1 and MycERK2 are regulated identically to the native ERKs.

**Assay of ERK and MEK Activation by Western Blot Analysis with Phosphospecific Antibodies**—Confluent 10-cm dishes of Rat-1 cells were serum-starved in 5 ml of serum-free Dulbecco’s modified Eagle’s medium for 24 h before being treated and stimulated as indicated. Cells were then washed briefly in ice-cold PBS before the addition of ice-cold TG lysis buffer. Following removal of detergent-insoluble material (TPBS/milk), the clarified supernatant was boiled in sample buffer, and equal amounts of cell lysate were resolved on 10 or 12% SDS-PAGE gels until the 30-kDa marker approached the bottom. Gels were transferred to PVDF using a Bio-Rad transblot apparatus, and the filter was stained with Coomassie Brilliant Blue to confirm equal loading of lanes. Filters were washed thoroughly in 0.1% (v/v) Tween 20 in PBS (TPBS) and then “blocked” overnight in TPBS, 5% (w/v) Carnation powdered milk (TPBS/milk). Filters were then probed at room temperature for 1 h in TPBS/milk with anti-peptide antiserum that specifically recognizes the phosphorylated, activated versions of ERK1 and ERK2 or MEK1 and MEK2. Following five 5-min washes with TPBS, the second antibody, goat anti-rabbit horseradish peroxidase, was used at a 1:5000 dilution in TPBS/milk for 1 h at room temperature. Following five washes in TPBS, the filter was dried and incubated with Amersham ECL reagents according to the manufacturer’s instructions; exposures to hyperfilm were typically 1–2 min. In addition, duplicate blots were probed with conventional, nonphosphospecific monoclonal antibodies to ERK1/2 and MEK1/2 to confirm that equal amounts of these proteins were present in each sample.

**Western Blot Analysis of Endogenous MKP-1 Expression**—Cell samples were fractionated, transferred to PVDF and “blocked” in TPBS/milk as described above. Filters were then probed at room temperature for 1 h in TPBS/milk with a 1:5000 dilution of crude antiseraum Alb-1 raised against the C-terminal peptide of mouse 3CH134 (40). Following five 5-min washes with TPBS, the second antibody, goat anti-rabbit horseradish peroxidase, was used at a 1:4000 dilution in TPBS/milk for 1 h at room temperature. Following five washes in TPBS, the filter was then exposed for 30 min with Amersham ECL reagents according to the manufacturer’s instructions; exposures to hyperfilm were typically 10–30 s.

**Reproducibility of Results**—Results are from single experiments representative of between three and six experiments giving similar results. For ERK1 MBP kinase assays, results are expressed as raw cpm of \(^{32}\)P incorporated into MBP from single point assays. This is a sensitive and highly reproducible assay (15, 20, 23). In addition, some cells were stimulated and assayed in duplicate and gave identical results with errors generally less than 10%. Data were pooled for statistical analysis by student’s t test. Experiments involving Western blotting were performed at least three or four times with identical results; a representative experiment is shown in each case.

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2 S. J. Cook, M. McMahon, and F. McCormick, manuscript in preparation.

3 K. A. Cadwallader and S. J. Cook, unpublished results.
RESULTS

Potentiation of Sustained ERK Activity in Rat-1 Cells by Cycloheximide

Stimulation of Rat-1 cells with 100 μM LPA resulted in a biphasic increase in ERK1 activity, which peaked at 5–10 min before declining rapidly until 30 min, after which a smaller second phase persisted above basal level for up to 3 h (Fig. 1A). Pretreatment of cells with 50 μg/ml cycloheximide for 45 min followed by challenge with LPA had no effect on the magnitude of peak ERK1 activity or the rapid decline after 10 min of stimulation but did potentiate the sustained phase of ERK1 activity from 60 min onward (Fig. 1A). For example, the magnitude of LPA-stimulated ERK1 activity, measured as a percentage of the maximum response at 10 min, was 27 ± 15% after 120 min in control cells but 135 ± 28% after 120 min in cycloheximide-treated cells; the difference between the control and cycloheximide-treated values was statistically significant (p < 0.01). Similar results were obtained when EGF was the stimulus (Fig. 1B). Prolonged treatment with cycloheximide alone had a small, poorly reproducible effect on ERK1 activity (see Fig. 1C).

We also examined the effect of cycloheximide on ERK1 activation by ΔRaf-1:ER (38) in the R1Raf-1:ER-4 cell line. Activation of ΔRaf-1:ER by β-estradiol (β-E2) results in the rapid activation of MEK and ERK in these cells, thereby circumventing the other pathways activated by receptor signaling. The ability of ΔRaf-1:ER to activate ERK1 was greatly potentiated by pretreatment with cycloheximide (Fig. 1C). These results suggest that a labile protein acts as a negative regulator of sustained ERK1 activity, whether stimulated by a receptor...

FIG. 1. Cycloheximide potentiates sustained ERK activation in Rat-1 cells. A, Rat-1 cells were pretreated with sterile water (■) or with 50 μg/ml cycloheximide (○) for 30 min prior to stimulation with 50 μM LPA for various time points between 10 and 180 min (■, ○). Cells were incubated with PBS, 0.01% fatty acid-free bovine serum albumin as a vehicle control (Δ). B, Rat-1 cells were pretreated with sterile water (black bars) or with 50 μg/ml cycloheximide (hatched bars) for 30 min prior to stimulation with 10 nM EGF for 15 or 90 min. C, R1Raf-1:ER-4 cells were pretreated with sterile water (■) or with 50 μg/ml cycloheximide (○) for 30 min prior to stimulation with 1 μM β-estradiol (E2) for various time points between 15 and 120 min (■, ○). Cells were also incubated with cycloheximide alone (○) or 0.1% ethanol in PBS as a vehicle control (●). Following lysis, ERK1 was immune-precipitated and assayed as described under "Experimental Procedures," using MBP as a substrate. Results are from a single experiment representative of four giving similar results and are expressed as radioactivity incorporated into MBP (cpm). D, Rat-1 cells were pretreated with sterile water (control) or with 50 μg/ml of cycloheximide (CHX) for 30 min prior to stimulation with 100 μM LPA for the indicated times. Cell lysates were then resolved by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with antibodies specific for phospho-ERK1 and -ERK2, ERK1 and ERK2, phospho-MEK1 and -MEK2, or MEK1 and MEK2. Results are from a representative experiment.
tyrosine kinase, a G-protein-coupled receptor, or the conditional form of the human c-Raf-1 protooncogene.

This effect was not confined to ERK1. Western blotting with a phosphospecific antibody that only recognizes the activated versions of ERK1 and ERK2 (phosphorylated at Tyr\textsuperscript{185} in the TEY motif) confirmed that both ERK1 and ERK2 isoforms were activated in a strongly sustained manner in the presence of cycloheximide (Fig. 1D). Furthermore, duplicate blots probed with a phosphospecific antibody that only recognizes activated MEK1 and MEK2 confirmed that cycloheximide did not amplify or prolong LPA-stimulated MEK activation under these conditions (Fig. 1D). These results demonstrate that cycloheximide exerts its effect at the level of ERK, not MEK, by preserving the activating phosphorylation sites of ERK1 and ERK2. Since these sites are substrates for MKP family phosphatases, we were interested in seeing if MKPs were expressed in response to growth factors in these cells.

**Induction of MKP-1 Expression by LPA, EGF, and Serum in Rat-1 Cells**

We investigated the expression of MKP-1 in response to LPA, EGF, and serum in Rat-1 cells by Western blotting whole cell lysates with an antibody raised to the C terminus of human 3CH134 (Fig. 2A). This antibody recognizes MKP-1 (CL100/3CH134) and MKP-2 (hVH2) (41, 42) but not MKP-3 (rVH6, Pyst1) (43–45). As a control for the MKP-1 antiserum, we immunoblotted samples from COS cells transfected with empty vector (EXV) or EXV-CL100. Transfected cells were lysed and Western blotted as described under “Experimental Procedures.” We detected a strong immunoreactive band of 39–40 kDa in lysates from CL100-transfected cells that was absent in the cells transfected with empty vector (Fig. 2A). A band of similar molecular weight was strongly induced in quiescent Rat-1 cells stimulated with LPA (Fig. 2A). This protein is consistent with the molecular weight of MKP-1 and indicates that antiserum Alb-1 recognizes both recombinant MKP-1 and endogenous MKP-1 induced by growth factor stimulation.

The ability of LPA, EGF, or FBS to induce MKP-1 protein expression was completely blocked by pretreatment of cells with cycloheximide (Fig. 2B), indicating that MKP-1 expression is a growth factor-stimulated event that requires de novo protein synthesis.

To examine the kinetics of MKP-1 expression, confluent, quiescent Rat-1 cells were stimulated with LPA or FBS for various times from 8 min to 8 h, and lysates were analyzed by Western blot with the MKP-1 antiserum. MKP-1 was barely detectable in quiescent Rat-1 cells but was strongly induced after 30–60 min of LPA stimulation (Fig. 2C, upper panel). This correlated well with the earliest time points at which we could observe a potentiation of ERK1 activation by cycloheximide (Fig. 1A). In response to continuous stimulation with LPA and FBS, MKP-1 expression peaked at 2–4 h before declining, but MKP-1 levels were clearly elevated after 8 h (Fig. 2C). This correlates with the sustained ERK1 activation seen for up to 8 h in response to stimulation with LPA, EGF, or FBS (24).

We were unable to detect MKP-2 expression in quiescent, growth factor-stimulated, or serum-stimulated Rat-1 cells although the antibody used readily detects this enzyme and it is expressed in serum-stimulated CCL39 cells (40). MKP-2 exhibits a slightly reduced mobility on SDS-PAGE, resolving just above MKP-1 with an apparent molecular mass of 42–43 kDa. MKP-2 is expressed in Rat-1 cells, since we have recently shown that it is induced in response to stimulation with the kinase inhibitor Ro-31–8220, which activates the JUN N-terminal kinase (JNK) stress kinase pathway (39). MKP-3/Pyst1 is constitutively expressed and is therefore not likely to account for the effects of cycloheximide in this system (45), whereas PAC1 is restricted hematopoietic cells (46). This antibody is not able to detect hVH3/B23 (47, 48) or hVH5 (49), other members of the family of MKPs, so we cannot rule out the possibility that they may also contribute to the regulation of ERK activity in these cells.

**Sodium Orthovanadate Potentiates Sustained ERK1 Activation in Rat-1 Cells**

To obtain additional evidence for MKP-1 or a related phosphatase playing a role in the sustained phase of ERK1 activation, we examined the effect of sodium orthovanadate, an inhibitor of protein-tyrosine phosphatases including MKP-1 (30). Treatment of Rat-1 cells with sodium orthovanadate alone resulted in activation of ERK1 in a time- and dose-dependent fashion. ERK1 activation by sodium orthovanadate exhibited
the effect of sodium orthovanadate on the EGF response. Importantly, the doses of sodium orthovanadate used did not prevent LPA-stimulated expression of MKP-1 (Fig. 3B), so the potentiation we observed was not due to inhibition of protein synthesis. The simplest conclusion from these results is that the magnitude of sustained ERK1 activity in Rat-1 cells is regulated by the de novo expression of a growth factor-induced, orthovanadate-sensitive phosphotyrosine phosphatase.

**Characterization of the Pathways Regulating Growth Factor-stimulated MKP-1 Expression**

We sought to define which signaling pathways are responsible for MKP-1 expression in response to LPA. Whereas the ERK cascade itself is a likely candidate, both LPA and EGF activate multiple signaling pathways that may be regulated gene expression.

**Activation of MEK/ERK Is Necessary for Maximal MKP-1 Expression in Rat-1 Cells**— Pretreatment of Rat-1 cells with the MEK inhibitor PD98059 (50) resulted in a dose-dependent inhibition of LPA- or EGF-stimulated ERK activation such that ERK activity was completely abolished by 25 or 30 μM PD98059 (Fig. 4A). Pretreatment of Rat-1 cells with 40 μM PD98059 resulted in inhibition of approximately 50% of LPA- and EGF-stimulated MKP-1 induction (Fig. 4B), suggesting that MKP-1 induction is partially dependent upon activation of the Raf-ERK cascade. However, we could not observe complete inhibition of LPA- or EGF-stimulated MKP-1 expression by PD98059 even at doses at which ERK1 was completely inhibited, suggesting that additional pathways are also used by LPA and EGF to regulate MKP-1 expression. This point is further emphasized by the fact that in the same experiments PMA-stimulated MKP-1 expression was completely inhibited by PD98059 (Figs. 4B and 6B).

In Rat-1 cells, LPA activates ERK1 by at least two pathways: (a) a major and sustained pathway, which is mediated by a pertussis toxin-sensitive G<sub>i</sub> protein and involves Ras, and (b) a quantitatively minor and transient pathway, which may involve PKC and Ca<sup>2+</sup> (15, 16, 24, 51, 52). Pretreatment of Rat-1 cells with pertussis toxin inhibited approximately 50% of LPA-stimulated MKP-1 induction (Fig. 4C). Under the same conditions, pertussis toxin inhibits 70% of peak ERK1 activation and all of the sustained response (24). These results together with the PD98059 data are consistent with a G<sub>i</sub>-mediated pathway of ERK1 activation playing a role in MKP-1 expression by LPA. However, we could not rule out the possibility that the “G<sub>i</sub> pathway” for MKP-1 expression was totally independent of that for ERK activation. To address this issue, we utilized Rat-1 cells expressing ΔRaf-1:ER.

**ERK Activation Is Not Sufficient to Induce MKP-1 Expression**— Activation of ΔRaf-1:ER in Rat-1 cells results in a sustained increase in ERK1 activity of similar magnitude to that observed with LPA although the peak of the response is delayed by about 20 min (Fig. 5A); similar results are observed in 3T3 cells expressing ΔRaf-1:ER. Surprisingly, we observed that, despite activation of ERK1 to the same extent as that with LPA, stimulation of ΔRaf-1:ER resulted in a barely detectable increase in MKP-1 expression compared with that observed with serum (Fig. 5B) or LPA (Fig. 5C), even when stimulated for up to 4 h. This result suggested that sustained activation of the ERK cascade may be necessary but is not sufficient to induce MKP-1.

Treatment of R1ΔRaf-1:ER-4 cells with pertussis toxin inhibited LPA-stimulated MKP-1 expression (Fig. 5C, lanes 2 and 3).

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4 J. Beltman and S. J. Cook, unpublished results.
5 S. J. Cook, unpublished results.
However, activation of ΔRaf-1:ER together with LPA bypassed the effect of pertussis toxin on LPA-induced MKP-1 expression so that the maximal response to LPA was fully reconstituted (Fig. 5C, lane 4). Under these conditions, ΔRaf-1:ER activation induced little MKP-1 alone (Fig. 5C, lane 5) but could fully complement for the loss of Gi or Go-mediated signal pathways, whereas LPA was presumably providing non-Gi or -Go signals. These results suggest that of all the pathways possibly regulated by Gi or Go in Rat-1 cells, the ERK cascade is sufficient to complement for their loss and synergize with LPA-stimulated non-Gi signals to reconstitute MKP-1 expression.

PKC Plays a Minor Role in LPA-stimulated MKP-1 Expression—Stimulation of Rat-1 cells with the phorbol ester PMA resulted in a strong induction of MKP-1 protein (Figs. 4B and 6) despite the fact that this agonist elicits only a transient and small ERK activation in these cells (24, 53). The induction of MKP-1 protein by PMA was completely inhibited by PD98059 in experiments in which the responses to LPA and EGF exhibited a significant MEK-independent component (Figs. 4B and 6B), suggesting that induction of MKP-1 by PKC absolutely requires the MEK/ERK pathway, whereas LPA and EGF can use other signal pathways.

To assess the role of PKC in LPA-stimulated ERK activation, we down-regulated PKC expression by pretreating cells with 1 μM PMA for 48 h (39). Under such conditions we observe depletion of essentially all PKC-α, -δ, and -ε but not PKC-ζ (39).
Regulation of MKP-1 Expression by ERK and Ca$^{2+}$

**A**

![MKP-1](image1.png)

**B**

![MKP-1](image2.png)

**Fig. 6.** Activation of PKC plays little role in growth factor-stimulated MKP-1 expression in Rat-1 cells. A, confluent dishes of Rat-1 cells were incubated serum-free in the presence of 1 μM PMA or control for 48 h. Cells were then stimulated for 1 h with LPA (50 μM), EGF (10 nM), PMA (100 nM), or ionomycin (1.5 μM). B, serum-starved Rat-1 cells were pretreated with 50 μM PD98059 for 10 min before stimulating with LPA (50 μM), PMA (100 nM), or ionomycin (1.5 μM) for 1 h. Whole cell detergent lysates were prepared, fractionated by SDS-PAGE, and analyzed for MKP-1 expression with antisera Alb-1 as described under "Experimental Procedures."

Such treatment resulted in complete inhibition of PMA-stimulated MKP-1 expression (Fig. 6A) but had only a small effect upon the LPA response and no effect on the EGF response. We previously observed that the selective PKC inhibitor GF109203X had little effect on LPA-stimulated MKP-1 expression although it completely inhibited the PMA response (39). This suggests a small role for PKC in regulating MKP-1 expression by these growth factors.

**Ca$^{2+}$-dependent MKP-1 Expression Regulates Sustained ERK Activity—**Ca$^{2+}$ plays a major role in regulating gene expression in a variety of systems (54, 55). We used ionomycin to investigate the potential role of Ca$^{2+}$ in the regulation of MKP-1 expression. Ionomycin treatment led to induction of MKP-1 expression (Fig. 6, A and B) although it or A23187 stimulated only a transient activation of ERK1 in Rat-1 cells (24, 53). The induction of MKP-1 expression by ionomycin was not affected by chronic PMA treatment, suggesting that the effect of Ca$^{2+}$ is not mediated via activation of PKC (Fig. 6A). Ionomycin-stimulated MKP-1 expression was strongly inhibited by PD98059, suggesting that ionomycin, like PMA, absolutely requires activation of the MEK/ERK cascade to induce MKP-1 (Fig. 6B).

To assess the requirement for Ca$^{2+}$ in the induction of MKP-1 by growth factors in Rat-1 cells, we used a combination of 3 mM EGTA and 20 μM BAPTA-AM, to buffer agonist-stimulated Ca$^{2+}$ mobilization and Ca$^{2+}$ entry. This treatment completely blocked MKP-1 induction in response to LPA, ionomycin, or EGF (Fig. 7A). This complete abolition of response was not due to toxicity, since, for example, the same treatment did not block growth factor-stimulated ERK1 activation (see below; Fig. 7, B and C). It appears that there is a strong requirement for Ca$^{2+}$ in the induction of MKP-1.

The requirement for Ca$^{2+}$ in regulating MKP-1 expression raised the question of whether Ca$^{2+}$, by increasing MKP-1 levels, might serve to regulate ERK activity by way of a cross-talk pathway. To address this possibility, we pretreated Rat-1 cells with BAPTA-AM and EGTA and then stimulated them with LPA or EGF for various time points up to 2 or 3 h. We found that buffering Ca$^{2+}$ had only a small effect on the early peak of ERK1 activity, but we observed a strong potentiation of the sustained phase of the response to both growth factors (Fig. 7, B and C), as we had previously seen with cycloheximide or sodium orthovanadate.

This effect was not confined to ERK1, since immunoblotting with the phosphospecific ERK1/2 antibody confirmed that both ERK1 and ERK2 were activated in a strongly sustained manner in the BAPTA-AM/EGTA-treated cells compared with untreated cells (Fig. 7D). Furthermore, calcium chelation did not prolong or amplify LPA-stimulated MEK activation, monitored with a phosphospecific MEK antibody (Fig. 7D), suggesting that BAPTA-AM/EGTA exerts its effects by preserving ERK phosphorylation and not by amplifying the upstream signal pathway. Thus, Ca$^{2+}$-regulated MKP-1 expression seems to be functionally relevant to regulation of sustained ERK activity.

**Synergy between ΔRaf-1:ER and Ionomycin or PMA for MKP-1 Expression—**Although activation of ΔRaf-1:ER in Rat-1 cells elicited only a small increase in MKP-1, when we pretreated R1ΔRaf-1:ER-4 cells with okadaic acid the combination of ΔRaf-1:ER and okadaic acid resulted in a strong, synergistic induction of MKP-1 (Fig. 8A).

We also observed synergy between PMA or ionomycin and ΔRaf-1:ER. In R1ΔRaf-1:ER-4 cells, the induction of MKP-1 expression by PMA and ionomycin was reduced compared with normal Rat-1 cells, and this allowed us to look at combinatorial stimulations. In these cells, the combination of PMA and ΔRaf-1:ER clearly gave a synergistic induction of MKP-1 (Fig. 8A). Maximal doses of ionomycin in combination with ΔRaf-1:ER seemed to be synergistic, but to be sure we titrated ionomycin doses from 1 μM down to 125 nM with or without ΔRaf-1:ER activation. Under these conditions, we observed synergy between ionomycin and ΔRaf-1:ER at each dose of ionomycin examined (Fig. 8B). Under the same conditions, PMA and ionomycin did not synergize with ΔRaf-1:ER to activate ERK, and only rarely did we observe an additive response (Fig. 8C).5 These results strongly suggest that the synergy observed between ΔRaf-1:ER and PMA and ionomycin is not due to enhancement of ERK activation but instead is more probably due to the synergistic combination of different signaling pathways.

These results again suggested a role for Ca$^{2+}$ and PKC in regulating MKP-1 levels in Rat-1 cells. To address the question of which is the major signal cooperating with the ERK pathway in the response to LPA, we returned to the synergy between ΔRaf-1:ER and LPA in pertussis toxin-treated R1ΔRaf-1:ER-4 cells. Under these conditions, most of the LPA-stimulated ERK activation is lost, suggesting that some other signal is being provided by LPA to synergize with ΔRaf-1:ER-stimulated ERK activation. This synergy was abolished by BAPTA-AM/EGTA treatment and substantially reduced by BAPTA alone but only weakly inhibited by the selective PKC inhibitor GF109203X.5 These results are consistent with the relative efficacy of inhibiting PKC or Ca$^{2+}$ on MKP-1 expression (Figs. 6 and 7) (39) and suggest that the major signal generated by LPA that regulates MKP-1 levels (albeit in concert with ΔRaf-1:ER) is Ca$^{2+}$ with little role for PKC.

The hormone binding domain of the estrogen receptor contains an estrogen-inducible transactivation domain, which could have contributed directly to the regulation of MKP-1 expression, independently of ERK activation. However, we observed identical results when 4-hydroxytamoxifen was substituted for β-E2.5 Since 4-hydroxytamoxifen is able to inactivate the "protein repressor" function of the hormone binding domain without inducing the transactivation function, these results indicate that the synergy between ΔRaf-1:ER and PMA, ionomycin, or okadaic acid is specifically due to derepression of the Raf kinase moiety and concomitant activation of the ERK cascade.
DISCUSSION

The magnitude and duration of ERK activation appears to be a key determinant in cell fate signaling (3, 20, 23, 24, 27), providing the connection between receptors and nucleus (4, 52). Inactivation of ERKs within the nucleus is catalyzed by MKPs, such as MKP-1, which are induced de novo in response to proliferative and stress signals. These observations lead to a simple model for attenuation of ERK activity in which activation and nuclear accumulation of ERK results in increased expression of MKP-1, which in turn inactivates ERK (35). However, some cells clearly use different mechanisms of ERK dephosphorylation regardless of MKP-1 expression (36).

The sustained phase of ERK1 activation correlates well with proliferation in LPA-stimulated Rat-1 cells (24), and our results suggest that an inducible MKP serves to regulate sustained ERK activity in these cells. The ability of cycloheximide to potentiate the second, sustained phase of ERK activation (Fig. 1) is generally considered to be a hallmark of an MKP-like phosphatase inactivating ERK (31, 36). The effect of cycloheximide on ERK activity is correlated well with the kinetics and cycloheximide-sensitive expression of MKP-1 in response to LPA, EGF, and FBS (Fig. 2). These results, together with the ability of Ca\(^{2+}\)-chelating agents to block MKP-1 expression and potentiate sustained ERK1 activity (Fig. 7), support a role for MKP-1 acting as an ERK phosphatase in Rat-1 cells. In addition, we have recently shown that the kinase inhibitor Ro-31–8220 is able to block MKP-1 acting as an ERK phosphatase in Rat-1 cells. In addition, we have recently shown that the kinase inhibitor Ro-31–8220 is able to block MKP-1 expression and potentiate sustained ERK1 activity (Fig. 7), support a role for MKP-1 acting as an ERK phosphatase in Rat-1 cells.

Significantly, the agents that blocked MKP-1 expression and potentiated sustained ERK activity did not amplify MEK activity, indicating that they exert their effects at the level of ERK by preserving the phosphorylation state and therefore the activity of the enzyme. In fact, both cycloheximide and BAPTA-AM/EGTA treatment caused a reduction in long term MEK activity (Figs. 1D and 7D); this may be due to the amplification of ERK activity under these conditions, since ERK can retrophosphorylate and inhibit MEK1 (56).

In contrast to studies in CCL39 cells (40), we have been unable to demonstrate growth factor-stimulated expression of MKP-2 in Rat-1 cells although we have shown that this molecule is expressed in response to Ro-31–8220, which activates the JNK stress kinase pathway (39). Since many of the MKP family members are able to inactivate ERK in vitro and in cells (40–48), it seems that selectivity is quite low and may be achieved rather by distinct temporal or spatial expression patterns in response to different agonists. Indeed, our preliminary experiments indicate that MKP-2 expression...
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in Rat-1 cells correlates with activation of the JNK and p38 stress kinase pathways rather than with growth factor stimulation.\(^{6}\) The antiserum used in this study will not recognize the other members of the MKP family (MKP-3, hVH3, and hVH5), some of which are inducible immediate early genes, so we cannot presently rule out the possibility that other MKP family members may also contribute to the regulation of sustained ERK activity by cycloheximide in this system.

One significant difference between the effects of cycloheximide and orthovanadate should be pointed out. ERK activity in LPA-stimulated cells peaks at 5–10 min and quickly declines at 15–30 min before persisting in a second phase. In many experiments, this early sharp decline was prevented or delayed by orthovanadate but not by cycloheximide at a time point at which MKP-1 is only barely detectable. This may suggest that the rapid decline in activity after 10 min of LPA treatment may be due to a nonlabile, cytosolic PTPase distinct from MKP-1. Indeed, Keyse and co-workers (45) have recently reported the cloning of an MKP-1-related dual specificity phosphatase called Pyst-1 or MKP-3 that is highly selective for the ERK family, is constitutively expressed, and is located in the cytosol. Perhaps a similar molecule regulates the early peak of ERK activity in Rat-1 cells.

With these caveats in mind, our results suggest that in Rat-1 cells, orthovanadate and cycloheximide potentiate sustained ERK1 activity because they are able to inhibit the activity or the expression of MKP-1 and perhaps other MKPs, which act as ERK phosphatases. MKP-1 Expression Requires the Integration of Coincident ERK- and Ca\(^{2+}\)-dependent Signaling Pathways in Rat-1 Cells—These results are consistent with a model in which activation and nuclear translocation of ERK results in expression of the MKP-1 gene, which in turn serves to negatively regulate ERK activity. However, although ERK activation is required for maximal induction of MKP-1 by LPA and EGF in Rat-1 cells (Fig. 4), it is not sufficient (Fig. 5); maximal MKP-1 expression also requires other signal pathways that are coincident with ERK activation.

Two observations lead us to this conclusion. Blocking activation of the ERK cascade with PD98059 results in complete inhibition of LPA-stimulated ERK activity but can only reproducibly inhibit 50% of LPA-stimulated MKP-1 expression; clearly, ERK-independent signals contribute to this response also. Reexamination of the role of the ERK pathway using \(\Delta\text{Raf-1:ER}\) showed that specific and persistent activation of the ERK cascade alone is not sufficient to induce expression of MKP-1. In this respect, our results differ significantly from those in CCL39 hamster fibroblasts (40), where PD98059 strongly inhibited MKP-1 expression and activation of \(\Delta\text{Raf-1:ER}\) was sufficient to induce MKP-1 expression to the same degree as that with serum. Since we have used the identical antiserum to detect MKP-1, we can only speculate that this disparity represents a cell type-specific difference in the regulation of expression of MKP-1.

A major question arising from our results is why the sustained activation of ERK seen with \(\Delta\text{Raf-1:ER}\) cannot result in MKP-1 expression. Two explanations seemed plausible. First, there is a threshold of ERK activation required to initiate MKP-1 expression, and the sustained response seen with \(\Delta\text{Raf-1:ER}\) is insufficient; perhaps the early burst of ERK activity seen with growth factors at 5–10 min is an important signal. A second explanation is that MKP-1 expression, while requiring ERK activation, also requires other additional signals provided by growth factor receptor signaling, and it is the detection and integration of these coincident signals that serves to induce MKP-1. The former argument seems unlikely, since we observed that whereas ERK1 activity by \(\Delta\text{Raf-1:ER}\) is slightly delayed compared with that for LPA, the magnitude of the response is the same as that for LPA. We might therefore expect to see MKP-1 expression in response to \(\Delta\text{Raf-1:ER}\) but with delayed kinetics. In fact, even after 4 h the expression of MKP-1 is vanishingly low compared with that seen with LPA or FBS.

For a number of reasons, we favor a model in which MKP-1 expression is the result of synergy between coincident signals including Ca\(^{2+}\) and the ERK cascade and in which any one signal is not sufficient for this response. For example, the inhibition of either signal (with PD98059 or BAPTA-AM/
ETGTA) results in the inhibition of MKP-1 expression (Figs. 4 and 7), whereas modest activation of either with δ-Raf:1-ER and ionomycin will give synergistic expression of MKP-1 (Fig. 8). In the case of PMA, PKC can presumably activate the ERK pathway by virtue of activating Raf (57, 58) as well as activating another PKC-dependent pathway; again, synergistic integration of these signals would explain why the PMA response is also dependent upon MEK/ERK and why we see a pronounced synergy between the ERK and Ca$^{2+}$-erased. Both the with what is known about the structure of the MKP-1 promot-
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R. (1994) Biochem J. 301, 407–414
53. Cadwallader, K. A., Beltman, J., McCormick, F. & Cook, S. J. (1997) Biochem. J. 321, 795–804
54. Berridge, M. J. (1995) BioEssays 17, 491–500
55. Rosen, L. B., Ginty, D. D. & Greenberg, M. E. (1995) Adv. Second Messenger Phosphoprotein Res. 30, 225–253
56. Brunet, A., Pagés, G. & Pouységur, J. (1994) FEBS Lett. 346, 299–303
57. Kolch, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenzeller, G., Marme, D. & Rapp, U. (1993) Nature 364, 249–251
58. Marquardt, B., Firth, D. & Stabel, S. (1994) Oncogene 9, 3213–3218
59. Robertson, L. M., Kerppola, T. K., Vendrell, M., Luk, D., Smeyne, R. J., Becchiro, C., Morgan, J. I., Curran, T. (1995) Neuron 14, 241–252
60. Kwak, S. P., Hakes, D. J., Martell, K. J. & Dixon, J. E. (1994) J. Biol. Chem. 269, 3596–3604