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Reciprocal Regulation of Extracellular Signal Regulated Kinase 1/2 and Mitogen Activated Protein Kinase Phosphatase-3

Nichollette A. Zeliadt^a, Laura J. Mauro^b, and Elizabeth V. Wattenberg^{a,*}

^a Division of Environmental Health Sciences, School of Public Health, University of Minnesota, Minneapolis, MN 55455, USA

^b Department of Animal Science-Physiology, University of Minnesota, St. Paul, MN 55108, USA

Abstract

Mitogen activated protein kinase phosphatase-3 (MKP-3) is a putative tumor suppressor. When transiently overexpressed, MKP-3 dephosphorylates and inactivates extracellular signal regulated kinase (ERK) 1/2. Little is known about the roles of endogenous MKP-3, however. We previously showed that MKP-3 is upregulated in cell lines that express oncogenic Ras. Here we tested the roles of endogenous MKP-3 in modulating ERK1/2 under conditions of chronic stimulation of the Ras/Raf/MEK1/2/ERK1/2 pathway by expression of oncogenic Ras. We used two cell lines: H-ras MCF10A, breast epithelial cells engineered to express H-Ras, and DLD-1, colon cancer cells that express endogenous Ki-Ras. First, we found that MKP-3 acts in a negative feedback loop to suppress basal ERK1/2 when oncogenic Ras stimulates the Ras/Raf/MEK1/2/ERK1/2 cascade. ERK1/2 was required to maintain elevated MKP-3, indicative of a negative feedback loop. Accordingly, knockdown of MKP-3, via siRNA, increased ERK1/2 phosphorylation. Second, by using siRNA, we found that MKP-3 helps establish the sensitivity of ERK1/2 to extracellular activators by limiting the duration of ERK1/2 phosphorylation. Third, we found that the regulation of ERK1/2 by MKP-3 is countered by the complex regulation of MKP-3 by ERK1/2. Potent ERK1/2 activators stimulated the loss of MKP-3 within 30 minutes due to an ERK1/2-dependent decrease in MKP-3 protein stability. MKP-3 levels recovered within 120 minutes due to ERK1/2-dependent resynthesis. Preventing MKP-3 resynthesis, via siRNA, prolonged ERK1/2 phosphorylation. Altogether, these results suggest that under the pressure of oncogenic Ras expression, MKP-3 reins in ERK1/2 by serving in ERK1/2-dependent negative feedback pathways.

Keywords

Mitogen activated protein kinase phosphatase-3; extracellular signal regulated kinase; Ras

*Corresponding author. Elizabeth V. Wattenberg. Division of Environmental Health Sciences, School of Public Health, University of Minnesota, Mayo Mail Code #807, 420 Delaware Street SE, Minneapolis, MN 55455, Phone: 612-626-0184, Fax: 612-626-0650, E-mail: watte004@umn.edu.

Conflict of Interest Statement

There are no conflicts of interest.

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Introduction

Extracellular signal regulated kinase (ERK) 1/2 is a major molecular switch in signal transduction (Chen et al., 2001). This serine/threonine kinase and mitogen activated protein kinase (MAPK) family member is activated by various mitogens, regulates several enzymes and transcription factors, and is centrally involved in regulating cell proliferation, differentiation, and gene expression in many systems. Accordingly, aberrant ERK1/2 regulation has been implicated in carcinogenesis (Dhillon et al., 2007). **Typically, ERK1/2 is phosphorylated and activated upon stimulation of the Ras/Raf/MEK1/2/ERK1/2 protein kinase cascade. The magnitude and duration of ERK1/2 activity is determined by the balance between the activity of MEK1/2, the kinases that phosphorylate and activate ERK1/2, and the activity of phosphatases that can dephosphorylate and inactivate ERK1/2. Maintaining the tight regulation of ERK1/2 is critical because the duration and magnitude of its activity can profoundly affect cell fate and function (Marshall, 1995; McCawley et al., 1999; Murphy et al., 2002). The regulation of ERK1/2 activation by phosphorylation has been studied extensively. Far less is known about the role of specific phosphatases in the negative regulation of ERK1/2.**

MAPK phosphatases (MKPs) have emerged as an important class of modulators of protein kinase signaling pathways (Camps et al., 2000; Farooq and Zhou, 2004; Owens and Keyse, 2007). MKPs, which are members of the protein tyrosine phosphatase superfamily and are also known as dual specificity phosphatases, specifically dephosphorylate the threonine and tyrosine residues located within the activation loop of MAPKs. MKP family members can differ with respect to several characteristics, including the following: subcellular location, tissue-specific expression, inducibility by various types of signals, and selectivity for dephosphorylating specific MAPKs, including ERK1/2, c-Jun N-terminal kinase (JNK), and p38.

Among the MKPs that are likely negative regulators of ERK1/2, MKP-3 is a particularly interesting candidate because of its specificity for dephosphorylating ERK1/2 and because of evidence that suggests it may be a tumor suppressor (Groom et al., 1996; Muda et al., 1996; Furukawa et al., 2003). For example, MKP-1 and MKP-2 are nuclear phosphatases that can dephosphorylate ERK, JNK and p38 in a manner that appears to be cell-type specific (Guan and Butch, 1995; Chu et al., 1996; Franklin and Kraft, 1997; Reffas and Schlegel, 2000). In contrast, MKP-3 has been identified as an ERK1/2-specific phosphatase that is localized within the cytoplasm (Muda et al., 1996). MKP-3 has been investigated mainly with the use of exogenous over-expression systems (Kamakura et al., 1999; Castelli et al., 2004; Marchetti et al., 2005). The diverse roles of endogenous MKP-3 in regulating endogenous MAPKs have not been established in most systems. Furthermore, little is known about the regulation of endogenous MKP-3. Interestingly, elevation of MKP-3 mRNA does not always correlate with increases in MKP-3 protein (Reffas and Schlegel, 2000). This suggests that the MKP-3 protein undergoes complex regulation.

Our previous work revealed that MKP-3 protein levels are upregulated in cell lines that express oncogenic Ras (Warmka et al., 2004). **Moreover, we showed that the potent skin tumor promoter palytoxin stimulates the downregulation of MKP-3 in keratinocytes derived from initiated mouse skin, which express oncogenic Ras (Warmka et al., 2002). This further suggests that MKP-3 is an important negative regulator in carcinogenesis. Importantly, the Ras/Raf/MEK1/2/ERK1/2 pathway is frequently deregulated in human carcinogenesis (Downward, 2003). Altogether, these observations led us to investigate the regulation and function of endogenous MKP-3 under conditions where the Ras/Raf/MEK1/2/ERK1/2 pathway is chronically stimulated by expression of oncogenic Ras. Investigating the role of MKP-3 in the regulation of ERK1/2 within the context of**

oncogenic Ras expression is a crucial part of the quest for a better understanding of the various mechanisms by which ERK1/2 activity is modulated during carcinogenesis.

The studies presented here suggest that ERK1/2 and MKP-3 engage in a dynamic interaction that helps rein in the ERK1/2 response under the pressure of oncogenic Ras expression. **We used the following cell lines for these studies: H-ras MCF10A, a human breast epithelial cell line engineered to express oncogenic H-Ras, and DLD-1, a colon cancer cell line that expresses endogenous oncogenic Ki-Ras.** First, we found that MKP-3 is involved in a negative feedback loop that suppresses basal ERK1/2 activity when expression of oncogenic Ras is the primary stimulus for the Ras/Raf/MEK1/2/ERK1/2 cascade. **Second, our studies using siRNA revealed that MKP-3 helps establish the sensitivity of ERK1/2 to extracellular activators by limiting the duration of ERK1/2 phosphorylation. Third, we found that the regulation of ERK1/2 by MKP-3 is countered by the regulation of MKP-3 by ERK1/2, a process that involves modulating the balance between ERK1/2-dependent degradation and resynthesis of MKP-3.** Altogether, the studies presented here suggest that in cells that express oncogenic Ras, ERK1/2 activity is governed, at least in part, by its repartee with MKP-3.

Methods

Materials

U0126 was purchased from Calbiochem (La Jolla, CA). Dulbecco's Modified Eagle Medium/F12 (DMEM/F12), heat-inactivated horse serum, and L-glutamine were purchased from Invitrogen Corporation (Carlsbad, CA). 12-*O*-tetradecanoylphorbol-13-acetate (TPA), cholera toxin, hydrocortisone, insulin, cycloheximide, actinomycin D, and epidermal growth factor (EGF) were purchased from Sigma (St. Louis, MO). Recombinant human tumor necrosis factor alpha (TNF- α) was purchased from R&D Systems, Inc. (Minneapolis, MN).

Cell Culture

H-ras MCF10A cells were the generous gift of Dr. Aree Moon (College of Pharmacy, Duksung Women's University, Seoul, Korea) and were grown in DMEM/F12 supplemented with 5% horse serum, 0.5 μ g/ml hydrocortisone, 10 μ g/ml insulin, 20 ng/ml EGF, 0.1 μ g/ml cholera toxin, and 2 mM L-glutamine. DLD-1 cells were purchased from ATCC (Manassas, VA) and were grown in DMEM supplemented with 10% fetal bovine serum (Intergen Company, Purchase, NY). All cells were grown in a humidified incubator at 37°C with 5% CO₂. For all experiments, cells were plated in complete medium at a density of approximately 3.5×10^5 cells/cm². **H-ras MCF10A cells were incubated for 24–40 hours after plating, and then were incubated in serum-free media without the supplements listed above for an additional 1–24 hours.** DLD-1 cells were incubated for 24 hours after plating, and then were incubated in serum-free media for an additional 24 hours. All experiments were conducted in serum-free media unless otherwise indicated. Incubation of the cells with DMSO (used as a vehicle in some experiments) did not affect protein or RNA levels. The data shown in the figures are representative of at least three independent experiments.

Antibodies and Immunoblotting

Cell lysates were prepared using the following buffer: 50 mM Tris-HCl, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 mM Na₃VO₄, 1 mM NaF. Lysates were cleared by centrifugation (16,000 $\times$ g, 10 min, 4°C). 10–40 μ g of protein were resolved using 10% minigels SDS-polyacrylamide minigels, and then transferred to Immobilon-P PVDF membrane (Millipore, Bedford, MA). After blocking in a TBST/5% milk solution, immunoblots were incubated overnight at 4°C using the following primary antibodies and dilutions: Phospho-p44/42 MAPK (Thr-202/

Tyr-204) (E10) (mouse monoclonal) (1:2000), and phospho-MEK1/2 (Ser-217/221) (rabbit polyclonal) (1:2000) from Cell Signaling (Beverly, MA), and ERK2 (C-14) (rabbit polyclonal) (1:2000), MEK1 (12-B) (rabbit polyclonal) (1:500), MKP-3 (C-20) (goat polyclonal) (1:1000) and β -tubulin (H-235) (rabbit polyclonal) (1:2000) from Santa Cruz Biotechnology (Santa Cruz, CA). The following secondary antibodies were used: anti-mouse IgG horseradish peroxidase-linked antibody and anti-rabbit IgG horseradish peroxidase-linked antibody from Cell Signaling, and bovine anti-goat IgG horseradish peroxidase-linked antibody from Santa Cruz Biotechnology. The use of the anti- β -tubulin antibody in immunoblots has been published in (Legendre et al., 2003). The use of the other antibodies in immunoblots is described in (Warmka et al., 2004). The signal for MKP-3 immunoblots was detected using the SuperSignal West Femto chemiluminescent substrate from Pierce Biotechnology (Pierce Biotechnology, Rockford, IL). All other immunoblots were visualized using the Pierce SuperSignal West Pico substrate. Blots were stripped by incubation in 0.2 M NaOH.

Semi-quantitative RT-PCR

Total RNA was isolated from cells using the RNeasy Plus Mini Kit from QIAGEN (Valencia, CA). 1 μ g of RNA was reverse-transcribed using random hexamers and the RNA PCR Core Kit from Applied Biosystems (Foster City, CA) following the manufacturer's protocol. Negative controls that lacked RNA or reverse transcriptase were included for each experiment. Relative transcript levels of MKP-3 and the endogenous control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were then determined by PCR from cDNA using the following primer pairs: human MKP-3 (accession no. X93920) forward 5'-GCCGCAGGAGCTATACGAGT-3', reverse 5'-CCGTATTCTCGTTCCAGTCG-3'; human GAPDH (accession no. J04038) forward 5'-GTGAAGGTCGGAGTCAACGG-3', reverse 5'-CTCCTGGAAGATGGTGATGG-3'. The following thermal cycling parameters were used: 95°C for 2 min, 25 cycles of 95°C for 30 s, 58.5°C for 30 s, and 72°C for 30 s. PCR reactions were stopped within the linear range of amplification for each primer pair. PCR products were resolved on 2% NuSieve agarose gels containing ethidium bromide. The expected sizes of PCR products were 208 bp and 224 bp for MKP-3 and GAPDH, respectively. No PCR products were observed in negative controls. MKP-3 levels were normalized to GAPDH levels amplified from the same cDNA sample.

Small interfering RNA (siRNA)

A duplex siRNA sequence targeting human MKP-3 was designed using the principles described in (Reynolds et al., 2004) and purchased from Sigma-Proligo (Boulder, CO). A second duplex siRNA sequence, described in (Liu et al., 2005) was also purchased from Sigma-Proligo. The 23-nucleotide duplexes used were (sense strands): duplex 1, AAGUGCGGAAUUGGUUAAUACdTT; and duplex 2, GUGCAACAGACUCGGAUGGUAdTT. Scrambled duplex siRNAs with the same GCAT content as the target siRNAs were used as a negative control: scrambled duplex 1, CAUUACGAAGUGGAUUGAGUAdTT; and scrambled duplex 2, CAUUACGGGAAAGUGCCGAGUdTT. For experiments, H-*ras* MCF10A cells were plated in complete medium approximately 20 hours prior to transfection with a 200 nM mixture of MKP-3 or scrambled duplex siRNAs (with each duplex siRNA represented in equimolar amounts) using HiPerFect Transfection Reagent (QIAGEN Inc., Valencia, CA) according to the manufacturer's standard protocol. 24 hours after transfection, cells were incubated in serum-free media for 1 hour. DLD-1 cells were plated in complete medium and transfected with a 25 nM mixture of MKP-3 or scrambled duplex siRNAs (with each duplex siRNA represented in equimolar amounts) using HiPerFect Transfection Reagent according to the manufacturer's Fast Forward protocol. 24 hours after transfection, cells were incubated in serum-free media for an additional 24 hours. Alexa Fluor 488-labeled negative control siRNA, purchased from QIAGEN, was used to verify transfection efficiency.

Densitometry

Protein levels and PCR products were quantified using a Bio-Rad (Hercules, CA) Fluor-S MultiImager and Bio-Rad Quantity One software, or a Bio-Rad GS-700 Imaging Densitometer and Molecular Analyst software.

Statistical analyses

Statistical analyses were performed using JMP 7.0. To compare treatment means, we used a standard 1-way analysis of variance (ANOVA) model that contained a random factor designating the experiment number to account for between-experiment variation. When 2-way ANOVA was used, this random factor was nested within time. This is important because film from replicate experiments was differentially exposed, resulting in between-experiment variability. Where ANOVA main effects were significant ($p < 0.05$), all pairwise differences between means were assessed using Tukey's Honestly Significant Differences (HSD) test with $\alpha = 0.05$.

Results

Loss of MKP-3 Results in Increased ERK1/2 Phosphorylation

Expression of oncogenic Ras, a frequent occurrence in human carcinogenesis, results in chronic stimulation of the Ras/Raf/MEK1/2/ERK1/2 protein kinase cascade. We previously observed that MKP-3, a negative regulator of ERK1/2, is upregulated in cell lines that express oncogenic Ras (Warmka et al., 2004). This is illustrated in Figure 1A, which shows that MKP-3 protein levels are higher in H-*ras* MCF10A cells, an immortalized human breast epithelial cell line engineered to stably express oncogenic H-Ras, than in parental MCF10A cells cultured under the same serum-starved conditions (Fig. 1A). To test the hypothesis that MKP-3 plays a role in a negative feedback pathway that inhibits ERK1/2 in cells that express oncogenic Ras, we knocked down MKP-3 with siRNA and monitored the phosphorylated, active forms of ERK1/2 by immunoblot in H-*ras* MCF10A cells and DLD-1 cells, a human colon cancer cell line that expresses an endogenous Ki-Ras oncogene (Shirasawa et al., 1993). **Phospho-ERK1/2 was higher in H-*ras* MCF10A cells** (Fig. 1B) and DLD-1 cells (Fig. 1C) in which MKP-3 was knocked down by siRNA than in nontransfected cells or cells transfected with scrambled siRNA (Fig. 1B and C, compare pERK1/2 in lane 2 to lanes 1 and 3, and compare open bars to filled and hatched bars). The siRNA reduced MKP-3 protein by over 90% (Fig. 1B and C, compare MKP-3 in lane 2 to lanes 1 and 3). The siRNA did not cause an increase in the phosphorylated, active forms of MEK1/2, the kinases that phosphorylate and activate ERK1/2 (Fig. 1B and C, *top panel*). We used lysates from TPA- or EGF-stimulated cells as positive controls for the detection of phospho-MEK1/2 (Fig. 1B and C, lane 4). These data indicate that in cells transfected with siRNA, the increase in phospho-ERK1/2 is due to the loss of its negative regulator MKP-3, and not due to an increase in the activity of upstream kinases.

Next, we tested the role of ERK1/2 in driving the upregulation of MKP-3. We blocked ERK1/2 activity by incubating H-*ras* MCF10A cells with the MEK1/2 inhibitor U0126 (Fig. 2A, *top panel*), and then monitored MKP-3 RNA by RT-PCR and MKP-3 protein by immunoblot (Fig. 2B and C). U0126 decreased MKP-3 RNA (Fig. 2B, *top panel*) and protein (Fig. 2C, *top panel*) by nearly 90% within 60 minutes and 8 hours, respectively. Similar results were obtained using DLD-1 cells (data not shown). Altogether, these results indicate that ERK1/2 plays a major role in maintaining the elevation of MKP-3, and support the hypothesis that MKP-3 is involved in an ERK1/2-dependent negative feedback loop that suppresses ERK1/2 under conditions where expression of oncogenic Ras is the primary stimulus of the Ras/Raf/MEK1/2/ERK1/2 pathway.

ERK1/2 is Involved in the Biphasic Modulation of MKP-3

We next investigated the relationship between the modulation of ERK1/2 and MKP-3 under conditions where ERK1/2 is further activated by extracellular stimuli. When we incubated H-*ras* MCF10A cells with TPA, a potent activator of ERK1/2, we observed a striking biphasic modulation of MKP-3 (Fig. 3A, *see panel labeled MKP-3 and hatched bars*). MKP-3 protein levels decreased dramatically by 30 minutes. MKP-3 levels recovered by 120 minutes, however, at which point they were even higher than the initial levels (Fig. 3A). EGF, another potent activator of ERK1/2, stimulated a similar biphasic regulation of MKP-3 in H-*ras* MCF10A cells and DLD-1 cells (Fig. 3B and C). These intriguing results indicate that initially MKP-3 levels decrease while phospho-ERK1/2 is highly elevated, and that later MKP-3 levels increase while phospho-ERK1/2 decreases. These results suggest that MKP-3 acts in a negative feedback pathway that modulates the time course of ERK1/2 phosphorylation.

To understand the biphasic regulation of MKP-3, we began by investigating the initial loss of MKP-3 protein. The modulation of MKP-3 could be due to effects on both MKP-3 RNA and MKP-3 protein. We monitored the effects of TPA on MKP-3 RNA levels by RT-PCR. We found that TPA stimulates an increase in MKP-3 RNA, suggesting that the early loss of MKP-3 is not due to inhibition of MKP-3 gene expression (Fig. 4A, *top panel*, and B). To determine whether the initial decrease in MKP-3 could be due to a change in protein stability, we incubated cells with cycloheximide, an inhibitor of protein synthesis, in the presence or absence of TPA, and monitored the loss of MKP-3 protein over time by immunoblot. Incubation of the cells with TPA resulted in a more rapid decrease in MKP-3 protein than that observed in control cells (Fig. 4C, compare MKP-3 in lanes 2–5 to lanes 6–9, and Fig. 4E, compare open squares to filled squares). To determine whether the effect of TPA on MKP-3 stability was mediated by ERK1/2, we treated cells with a combination of TPA, the protein synthesis inhibitor cycloheximide, and U0126, which inhibits the activation of ERK1/2 by TPA (Fig. 4D, *top panel*). The TPA-stimulated decrease in MKP-3 stability was abolished under these conditions (compare MKP-3 in lanes 1 and 6–9 in Fig. 4D to Fig. 4C, and Fig. 4E, filled circles). Similar results were obtained with EGF in H-*ras* MCF10A cells and DLD-1 cells (data not shown). These results indicate that the initial loss of MKP-3 is due to an ERK1/2-dependent decrease in MKP-3 protein stability.

Next, we investigated the second phase, in which MKP-3 levels rebound. The observation that TPA stimulates an increase in MKP-3 RNA suggested that the ability of MKP-3 levels to rebound involves transcription (Fig. 4A, *top panel*). Accordingly, actinomycin D, an inhibitor of transcription, prevented the recovery of MKP-3 protein (Fig. 5A, *top panel*, compare lane 6 to lane 7, and lane 9 to lane 10, and B, compare open bars to hatched bars). U0126 blocked the ability of TPA to activate ERK1/2 (Fig. 5C, *top panel*) and blocked the TPA-stimulated increase in both MKP-3 protein (Fig. 5C) and MKP-3 RNA (Fig. 5D, *top panel*). Similar results were obtained using EGF in H-*ras* MCF10A cells and DLD-1 cells (data not shown). These data, altogether, support a role for ERK1/2-dependent transcription in the recovery of MKP-3 protein.

MKP-3 Modulates the Magnitude and Duration of ERK1/2 Phosphorylation

The data shown in Figure 3 indicate that phospho-ERK1/2 decreased and remained suppressed as MKP-3 increased, suggesting the MKP-3 may play a role in the temporal regulation of this kinase. In EGF-stimulated DLD-1 cells, the time course of ERK1/2 phosphorylation was close to the time course of MEK1/2 phosphorylation (Fig. 3C). This suggests that once MEK1/2 is inactivated, phosphatases are involved in rapidly reducing the pool of phospho-ERK1/2, and then maintaining ERK1/2 in its dephosphorylated state.

A role for MKP-3 in regulating the duration of ERK1/2 phosphorylation was revealed when we examined the effects of MKP-3 knockdown on EGF-stimulated ERK1/2 in DLD-1 cells (Fig. 6A and B). Blocking the resynthesis of MKP-3 with siRNA prolonged the EGF-stimulated elevation of ERK1/2 phosphorylation (Fig. 6A, *top panel*, and B, compare open bars to filled and hatched bars). Under conditions where the elevation of phospho-MEK1/2 and phospho-ERK1/2 was sustained for prolonged periods (greater than 8 hours in TPA- and EGF-treated H-ras MCF10A cells), we could not detect an effect of MKP-3 knockdown on ERK1/2 phosphorylation (data not shown). Altogether, these results indicate that MKP-3 plays a role in a negative feedback pathway that curtails EGF-stimulated ERK1/2 activity in DLD-1 cells.

Finally, the observation that MKP-3 is involved in regulating basal and EGF-stimulated ERK1/2 phosphorylation in DLD-1 cells led us to determine if MKP-3 plays a role in regulating the sensitivity of these cells to other ERK1/2 activators. Figure 6C illustrates that the loss of MKP-3 sensitizes DLD-1 cells to TNF- α . We observed transient phosphorylation of ERK1/2 by TNF- α in DLD-1 cells (Fig. 6C, *top panel*). Knockdown of MKP-3 by siRNA increased the duration of TNF- α -stimulated ERK1/2 phosphorylation (Fig. 6C, *top panel*, and D, compare open bars to filled and hatched bars). These results suggest that the persistent elevation of MKP-3 helps establish the sensitivity of DLD-1 cells to ERK1/2 activators.

Discussion

Aberrant regulation of the Ras/Raf/MEK1/2/ERK1/2 pathway frequently occurs in human carcinogenesis (Downward, 2003). Our work and the work of others has shown that MKP-3, a putative tumor suppressor and regulator of ERK1/2, is often upregulated in cells and tissues that exhibit abnormal activation of the Ras/Raf/MEK1/2/ERK1/2 pathway (Croonquist et al., 2003; Furukawa et al., 2003; Warmka et al., 2004; Sweet-Cordero et al., 2005). This observation led us to investigate the action of this phosphatase within the context of oncogenic Ras expression. MKP-3 has been mainly studied in *in vitro* and exogenous over-expression systems. Most studies that report on endogenous MKP-3 have focused on understanding the regulation of FGF-stimulated signaling during development *in vivo* (Kawakami et al., 2003; Tsang et al., 2004; Gomez et al., 2005; Li et al., 2007). To our knowledge, the roles and regulation of endogenous MKP-3 in cells that express oncogenic Ras have not been previously explored. This is an important area of investigation because expression of oncogenic Ras results in chronic stimulation of the Ras/Raf/MEK1/2/ERK1/2 pathway; under such conditions, which frequently occur in human carcinogenesis, MKP-3 may be a key modulator.

The results presented here revealed two types of roles for endogenous MKP-3 in suppressing ERK1/2 in cells that express oncogenic Ras. First, MKP-3 is involved in a negative feedback pathway that suppresses ERK1/2 when expression of oncogenic Ras is the primary stimulus of the Ras/Raf/MEK1/2/ERK1/2 pathway (Fig. 7). **We used two tools, the pharmacological inhibitor U0126 and siRNA targeted against MKP-3, to demonstrate that in serum-starved H-ras MCF10A and DLD-1 cells, ERK1/2 drives the upregulation of MKP-3, which in turn dephosphorylates and thus inactivates ERK1/2. This suggests that MKP-3 keeps the basal level of ERK1/2 activity in check even under the pressure of stimulation by oncogenic Ras. Knockdown of MKP-3 by siRNA also results in an increase in ERK1/2 phosphorylation when the cells are maintained in complete media (data not shown), indicating that MKP-3 inhibits ERK1/2 even in the presence of serum.** Second, our studies indicate that MKP-3 can act in a negative feedback pathway that modulates the duration of ERK1/2 activity when cells that express oncogenic Ras undergo further stimulation by extracellular agents. This was revealed when we found that knockdown of MKP-3 by siRNA prolonged the EGF- and TNF- α -stimulated elevation of phospho-ERK1/2 in DLD-1 cells. In EGF-stimulated DLD-1 cells, the decrease in phospho-ERK1/2 preceded the recovery of MKP-3, which suggests that other phosphatases may also be involved in the initial

dephosphorylation of ERK1/2 (see Fig. 3C). We were not able to detect an effect of MKP-3 knockdown on TPA- or EGF-stimulated ERK1/2 phosphorylation in H-*ras* MCF10A cells under the conditions of our studies. TPA stimulated prolonged, highly elevated MEK1/2 activity in H-*ras* MCF10A cells (see Fig. 3A), which could counteract and mask the action of MKP-3 and thus explain the prolonged, elevated activation of ERK1/2. Further research is required to determine why the regulation of EGF-stimulated ERK1/2 differs so dramatically between H-*ras* MCF10A cells and DLD-1 cells, however. The two cell lines could certainly differ with respect to the balance between and access to ERK1/2 regulators because of differences in the following: 1) mechanism of oncogenic Ras expression (engineered overexpression in H-*ras* MCF10A cells versus endogenous expression in DLD-1); 2) expression of the oncogenic Ras family member (H-Ras versus Ki-Ras); and 3) the cell type (breast versus colon). Altogether, our studies indicate that the persistent elevation of MKP-3 can help restrict the ERK1/2 response of cells that express oncogenic Ras. The differences we observed between H-*ras* MCF10A and DLD-1 indicate, however, that the effectiveness of MKP-3 in suppressing ERK1/2 is likely to vary depending on many conditions.

The various roles of MKP-3 in regulating ERK1/2 are countered by the multiple mechanisms by which ERK1/2, in turn, regulates MKP-3 (Fig. 7). Stimulation of both cell lines revealed a striking ERK1/2-dependent biphasic modulation of MKP-3. The initial downregulation of MKP-3 appears to involve ERK1/2-dependent protein destabilization. Our results are consistent with those of Marchetti et al., who used hamster fibroblast cells that overexpress exogenous MKP-3 to show that ERK1/2 phosphorylates MKP-3 and targets it for proteasomal degradation (Marchetti et al., 2005). The function of the initial ERK1/2-dependent downmodulation of MKP-3 is not yet clear. It has been suggested that MKP-3, which is localized to the cytoplasm, may regulate the subcellular localization of ERK1/2 (Karlsson et al., 2004). Further studies are required to determine if the initial loss of MKP-3 in stimulated H-*ras* MCF10A and DLD-1 cells liberates ERK1/2 from its cytoplasmic anchor so that this kinase can translocate to other subcellular compartments. Our studies indicate that transcription is required for MKP-3 protein levels to rebound following its initial downmodulation, and furthermore that ERK1/2 plays a major role in the regulation of both basal and stimulated MKP-3 gene expression. The rapid rebound in MKP-3 levels following its initial downmodulation suggests that cells that express oncogenic Ras are poised to maintain elevated MKP-3 levels, and thus limit ERK1/2 activity.

The regulation of MKP-3 gene expression varies widely depending on the system. In H-*ras* MCF10A cells the induction of MKP-3 is detected relatively rapidly (within 60 minutes). TPA and EGF induce MKP-3 gene expression in a similar manner in the parental MCF10A cells, indicating that the ability to stimulate MKP-3 gene expression in this cell line does not depend on the expression of oncogenic Ras (data not shown). In other systems, the induction of MKP-3 is only detected after prolonged (greater than 12 hours) exposure to stimuli (Woods and Johnson, 2006). Finally, MKP-3 has been reported to be mainly constitutively expressed and not strongly inducible in other systems, such as human skin fibroblasts (Groom et al., 1996; Dowd et al., 1998). Such differences may be due to cell type dependent differences in the expression of transcriptional machinery and the operation of signaling networks.

The data presented here generally show greater levels of phospho-ERK2 (pp42 ERK) than phospho-ERK1 (pp44) in both resting and simulated H-*ras* MCF10A and DLD-1 cells. This apparent preferential phosphorylation of ERK2 over ERK1 has been observed in several cell lines (Pelech, 2006; Vantaggiato et al., 2006). Interestingly, stimulation of the cells with EGF or TPA resulted in increases in both ERK1 and ERK2 phosphorylation, whereas knockdown of MKP-3 in serum-starved cells, in which oncogenic Ras is the primary stimulus, mainly resulted in an increase in ERK2 phosphorylation. The mechanism underlying such apparent preferential phosphorylation has not been established, but it is likely to be due, at least in part,

to the interactions between and subcellular localization and levels of several proteins, including ERK1, ERK2, MEK1, MEK2, protein scaffolds, and protein phosphatases (Pelech, 2006; Lefloch et al., 2008).

The observation that knockout of ERK2 in vivo is lethal, whereas knockout of ERK1 is not indicates that ERK1 and ERK2 have different functions (Pages et al., 1999; Yao et al., 2003). This suggests that differential dephosphorylation and inactivation of ERK1 and ERK2 could affect the cellular response to stimuli that activate the Ras/Raf/MEK/1/2/ERK1/2 pathway. Further research is required to determine whether the specificity of MKP-3 differs for ERK1 versus ERK2. Although knockdown of MKP-3 in serum-starved H-*ras* MCF10A and DLD-1 cells appears to primarily result in an increase in phospho-ERK2 levels, we have found that knockdown of MKP-3 in other cell lines results in a clear increase in both phospho-ERK1 and phospho-ERK2 (data not shown). Muda et al, reported that MKP-3 binds equally well to both ERK1 and ERK2, as indicated by the ability of a GST-MKP-3 fusion protein to precipitate ERK1 and ERK2 from cell lysates prepared from cells in which the different ERK isoforms were overexpressed (Muda et al., 1998). To our knowledge a direct comparison of the specificity of MKP-3 for dephosphorylating ERK1 versus ERK2 has not been published, however. The balance between the phosphorylated and desphosphorylated states of ERK1 and ERK2 is likely to depend on a complex set of interactions and conditions, as discussed above,

It might be expected that expression of oncogenic Ras would result in superactivation of ERK1/2. Instead, our studies suggest that cells may adapt to the expression of oncogenic Ras, and the chronic stimulation of the Ras/Raf/MEK1/2/ERK1/2 pathway, by upregulating MKP-3, which then suppresses ERK1/2. This hypothesis is consistent with the concept of MKP-3 as a tumor suppressor, and might help explain, at least in part, why activation of Ras alone is not sufficient to induce tumors. That is, cells respond to the expression of oncogenic Ras by upregulating MKP-3, which serves to rein in ERK1/2 activity; disruption of MKP-3, in turn, unleashes ERK1/2 activity and thus contributes another step along the pathway of carcinogenesis. **Previous results from our laboratory on the novel skin tumor promoter palytoxin support this hypothesis** (Warmka et al., 2002; Zeliadt et al., 2003; Warmka et al., 2004). **In the classic multi-stage mouse skin model of carcinogenesis, the first stage, known as initiation, typically involves activation of the oncogene Ras; subsequent repeated stimulation by tumor promoters results in tumor development** (Balmain and Pragnell, 1983; Yuspa, 1998). **We previously demonstrated that palytoxin, a non phorbol ester tumor promoter in the multi-stage mouse skin model of carcinogenesis, stimulates ERK1/2 activation by triggering the downmodulation of MKP-3 in mouse keratinocytes derived from initiated mouse skin that express oncogenic Ras** (Warmka et al., 2004). **Accordingly, we also showed that palytoxin modulates several targets that have been implicated in carcinogenesis, including c-Fos, AP-1, and matrix metalloprotease-13, through ERK1/2-dependent pathways in these cells** (Warmka et al., 2002). **Further research is required to determine whether knockdown of MKP-3 mimics palytoxin action and whether MKP-3 is a target for other carcinogenic agents.**

Interestingly, studies that implicate MKP-3 as a tumor suppressor in pancreatic cancer, which is characterized by a high frequency of activating Ras mutations, reported an association between elevated expression of MKP-3 at early stages of pancreatic cancer, but a loss of MKP-3 at advanced stages (Furukawa et al., 2003). **It has been estimated that oncogenic Ras is expressed in over 40% of colon tumors** (Bos et al., 1987; Downward, 2003). **In breast cancer the regulation of the Ras/Raf/MEK/1/2/ERK1/2 pathway is frequently undermined by the aberrant regulation of growth factor receptors** (Santen et al., 2002). **Limited information has been published concerning the expression of MKP-3 in these cancers** (Cui et al., 2006). **Further research is therefore required to determine how the expression**

of MKP-3 changes during colon and breast carcinogenesis and whether MKP-3 functions as a tumor suppressor in these types of cancers.

Given that activation of Ras frequently occurs in human cancers (Downward, 2003), there is an urgent need to understand the biochemical characteristics of cells that express activated Ras that make them particularly susceptible to agents and events that advance the process of carcinogenesis. The unstable nature of MKP-3 makes it a potentially vulnerable target in such cells. Altogether, the evidence that aberrant ERK1/2 activity plays a role in carcinogenesis together with the identification of MKP-3 as a potential tumor suppressor, underscores the importance of understanding the nature of the repartee between this centrally important protein kinase and its modulator.

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Fig. 1. MKP-3 knockdown via siRNA increases ERK1/2 phosphorylation in H-ras MCF10A and DLD-1 cells. (A) Whole cell lysates were prepared from serum starved MCF10A and H-ras MCF10A cells. MKP-3 (*top panel*) was detected by immunoblot analysis. The blot was stripped and reprobed for β-tubulin (*bottom panel*) as a loading control. **Whole cell lysates were prepared from (B) H-ras MCF10A cells or (C) DLD-1 cells that were either not transfected (lane 1), were transfected with duplex siRNA targeted against MKP-3 (lane 2), or were transfected with duplex scrambled siRNA (lane 3).** The following proteins were detected by immunoblot analysis: dually phosphorylated, active MEK1/2 (pMEK1/2); total MEK1; dually phosphorylated, active ERK1/2 (pERK1/2); total ERK2; or MKP-3. **The**

MKP-3 blot was stripped and reprobed for β -tubulin as a loading control. As a positive control for phospho-MEK1/2, nontransfected cells were incubated for 120 minutes with (B) 1.6 nM TPA (lane 4), or incubated for 30 minutes with (C) 3 nM EGF (lane 4). We and others have observed that MKP-3 typically runs as a doublet (Muda et al., 1996; Camps et al., 1998; Reffas and Schlegel, 2000; Warmka et al., 2004). The graphs represent average pERK1/2 densitometry relative to non-transfected controls for (B) 10 independent experiments and (C) 6 independent experiments; error bars represent SEM. Filled bars (nt), not transfected. Open bars (M), transfected with duplex siRNA targeted against MKP-3. Hatched bars (S), transfected with duplex scrambled siRNA. Treatment of H-ras MCF10A and DLD-1 cells with MKP3 siRNA resulted in an increase in pERK1/2 levels relative to nontransfected control or treatment with scrambled siRNA (H-ras MCF10A: $p=0.006$, $n=30$, 1-way ANOVA and Tukey's HSD, $\alpha=0.05$; DLD-1: $p=0.0008$, $n=18$, 1-way ANOVA and Tukey's HSD, $\alpha=0.05$). We did not detect a difference between non transfected cells or cells transfected with scrambled siRNA.

Fig. 2.

ERK1/2 is required to maintain elevated levels of MKP-3 RNA and protein. **(A) Whole cell lysates were prepared from H-ras MCF10A cells that were incubated for 60 minutes in the absence (–) or the presence (+) of 3 μ M U0126.** Dually phosphorylated, active ERK1/2 (pERK1/2, *top panel*) or total ERK2 (*bottom panel*) were detected by immunoblot analysis. **(B) Total RNA was extracted from H-ras MCF10A cells that were incubated for 60 minutes in the absence (–) or the presence (+) of 3 μ M U0126.** MKP-3 (*top panel*) and GAPDH (*bottom panel*) RNA levels were monitored by RT-PCR. **(C) H-ras MCF10A cells were incubated for 8 hours in the absence (–) or presence (+) of 3 μ M U0126.** MKP-3 protein levels (*top panel*) were monitored in whole cell lysates by immunoblot analysis, followed by reprobing for β -tubulin (*bottom panel*) as a loading control. The data shown are representative of at least three independent experiments.

Fig. 3.

TPA and EGF stimulate the loss and recovery of MKP-3 protein. Whole cell lysates were prepared from (A) H-ras MCF10A cells that were incubated for the indicated times with 1.6 nM TPA; (B) H-ras MCF10A cells; or (C) DLD-1 cells that were incubated for the indicated times with 3 nM EGF. **The following proteins were detected by immunoblot analysis: dually phosphorylated, active MEK1/2 (pMEK1/2); total MEK1; dually phosphorylated, active ERK1/2 (pERK1/2); total ERK2; or MKP-3. The MKP-3 blot was stripped and reprobed for β -tubulin as a loading control. The graphs represent average densitometry of pMEK1/2, pERK1/2, and MKP-3 for 3 independent experiments; error bars represent SEM. Filled bars, pMEK1/2. Open bars, pERK1/2. Hatched bars, MKP-3.**

H-ras MCF10A **Fig. 4.**

TPA stimulates a decrease in MKP-3 protein stability through an ERK1/2-dependent pathway in H-ras MCF10A cells. (A) H-ras MCF10A cells were incubated with 1.6 nM TPA for the indicated times. Total RNA was extracted and the levels of MKP-3 RNA (*top panel*) and GAPDH RNA (*bottom panel*) were monitored by RT-PCR. (B) The graph represents average densitometry of MKP-3 RNA relative to untreated control from three independent experiments; error bars represent SEM. (C) H-ras MCF10A cells were incubated for the indicated times with 36 μ M cycloheximide (CHX) alone or in the presence of 1.6 nM TPA (CHX + TPA). (D) H-ras MCF10A cells were incubated for the indicated times with cycloheximide in the presence of 3 μ M U0126 (CHX + U), or U0126 and TPA together (CHX + U + TPA). (C) and (D) The following proteins were detected by immunoblot analysis: dually phosphorylated, active ERK1/2 (pERK1/2); total ERK2; MKP-3. The MKP-3 blot was stripped and reprobed for β -tubulin as a loading control. (E) The graph represents average MKP-3 densitometry from three independent experiments; error bars represent

SEM. Cycloheximide alone (*open squares*). Cycloheximide plus TPA (*filled squares*). Cycloheximide plus U0126 (*open circles*). Cycloheximide plus U0126 and TPA (*filled circles*). **Incubation of the cells with TPA resulted in a more rapid decrease in MKP3 protein than that observed in control cells; there was no detectable difference among all other treatment groups (2-way ANOVA, n=48, main effect of both time and treatment significant ($p<0.0014$)).**

H-ras MCF10A **Fig. 5.**

TPA stimulates an ERK1/2-dependent increase in MKP-3 RNA in H-ras MCF10A cells. (A) H-ras MCF10A cells were incubated for the indicated times without (–) or with (+) 1.6 nM TPA and 6 μM actinomycin D. MKP-3 protein levels were monitored in whole cell lysates by immunoblot (*top panel*). The blot was stripped and reprobed for β-tubulin (*bottom panel*) as a loading control. (B) The graph represents average MKP-3 densitometry from three independent experiments; error bars represent SEM. Filled bars, actinomycin D alone. Open bars, TPA alone. Hatched bars, actinomycin D plus TPA. The zero time point represents nontreated cells. (C) H-ras MCF10A cells were incubated without (–) or with (+) 1.6 nM TPA and 3 μM U0126 for 120 minutes. The following proteins were detected by immunoblot analysis: dually phosphorylated, active ERK1/2 (pERK1/2); total ERK2; and MKP-3. The MKP-3 blot was stripped and reprobed for β-tubulin as a loading control. (D) H-ras MCF10A cells were incubated without (–) or with (+) 1.6 nM TPA and 3 μM U0126 for 90 minutes. Total RNA was extracted and the levels of MKP-3 RNA (*top panel*) and GAPDH RNA (*bottom panel*) were monitored by RT-PCR. The data shown are representative of at least three independent experiments.

DLD-1

Fig. 6. Knockdown of MKP-3 via siRNA prolongs EGF- and TNF- α -stimulated ERK1/2 phosphorylation in DLD-1 cells. **DLD-1 cells were either not transfected (-), or were transfected (+) with either duplex siRNA targeted against MKP-3 (MKP-3) or duplex scrambled siRNA (scrambled).** The cells were then incubated for the indicated times with **(A) 3 nM EGF or (C) 57 nM TNF- α .** Protein from whole cell lysates was analyzed by immunoblot for the following proteins: dually phosphorylated, active ERK1/2 (pERK1/2); MKP-3; total ERK2; or β -tubulin. Please note that the panels shown in (A) represent two different blots, but that the same lysate was used for the zero time point in both blots. **(B) The graph represents average pERK1/2 densitometry from three independent experiments; error bars represent SEM. Knockdown of MKP3 with siRNA prolonged the EGF-stimulated elevation of ERK1/2 phosphorylation; there was no detectable difference in the level of pERK1/2 in nontransfected and scrambled samples (2-way ANOVA, $n=45$, main effect of both treatment and time significant at $p<0.001$. Differences among treatment means were assessed using Tukey's HSD, $\alpha=0.05$). Filled bars, nontransfected. Open bars, transfected with duplex siRNA targeted against MKP-3. Hatched bars, transfected with duplex scrambled siRNA. (D) The graph represents average pERK1/2 densitometry relative to nontransfected controls from four independent experiments; error bars represent SEM. Knockdown of MKP3 with siRNA prolonged the TNF- α -**

stimulated elevation of ERK1/2 phosphorylation; there was no detectable difference in the level of pERK1/2 in nontransfected and scrambled samples (2-way ANOVA, $n=46$ main effect of treatment significant at $p<0.017$. Differences among treatment means were assessed using Tukey's HSD, $\alpha=0.05$). Filled bars, nontransfected. Filled bars, nontransfected. Open bars, transfected with duplex siRNA targeted against MKP-3. Hatched bars, transfected with duplex scrambled siRNA.

Fig. 7.

Reciprocal regulation of ERK1/2 and MKP-3. Phosphorylation and activation of ERK1/2 typically occurs through stimulation of the Ras/Raf/MEK1/2 protein kinase cascade. The GTPase Ras activates the protein kinase Raf, which phosphorylates and activates the protein kinases MEK1/2. MEK1/2 is highly specific for phosphorylating and activating ERK1/2. The magnitude and duration of ERK1/2 activity is determined by the balance between the activity of MEK1/2, the kinases that phosphorylate and activate ERK1/2, and the activity of phosphatases, such as MKP-3, which dephosphorylate and inactivate ERK1/2. The levels of MKP-3 protein available to inhibit ERK1/2 are regulated, at least in part, by ERK1/2. ERK1/2 can stimulate an increase in the expression of MKP-3 RNA. This results in an increase in MKP-3 protein, which can then feedback and inhibit ERK1/2 activity. ERK1/2 can also stimulate a decrease in MKP-3 protein stability, however, which can cause a loss of MKP-3 protein. Thus, the levels of MKP-3 protein are determined, at least in part, by the balance between the stimulation of new MKP-3 gene expression and the stability of the MKP-3 protein.