Mitogen-activated Protein Kinase Phosphatase-3 Is a Tumor Promoter Target in Initiated Cells That Express Oncogenic Ras*

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We have capitalized on the unique properties of the skin tumor promoter palytoxin, which does not activate protein kinase C, to investigate alternative mechanisms by which major signaling molecules can be modulated during carcinogenesis. We report here that palytoxin activates extracellular signal-regulated kinase (ERK) through a novel mechanism that involves inactivation of an ERK phosphatase in keratinocytes derived from initiated mouse skin (308 cells). Use of U0126 revealed that palytoxin requires the ERK kinase MEK to stimulate ERK activity, although palytoxin did not activate MEK. We found that 308 keratinocytes highly express mitogen-activated protein kinase phosphatase-3 (MKP-3), which selectively inactivates ERK. Palytoxin induced the loss of MKP-3 in a manner that corresponded to increased phosphorylation. Complementary studies showed that sustained expression of exogenous MKP-3 inhibited palytoxin-stimulated ERK activation. As is characteristic of initiated keratinocytes, 308 cells express activated H-Ras. To investigate whether expression of oncogenic Ras is key to palytoxin-stimulated ERK activation, we determined how palytoxin affected ERK and MKP-3 in MCF10A human breast epithelial cells and in H-ras MCF10A cells, which stably express activated H-Ras. Palytoxin did not affect ERK activity in MCF10A cells, which had no detectable MKP-3. Like 308 cells, H-ras MCF10A cells highly express MKP-3. Strikingly, palytoxin stimulated ERK activity and induced a corresponding loss of MKP-3 in H-ras MCF10A cells. These studies indicate that in initiated cells palytoxin unleashes ERK activity by down-regulating MKP-3, an ERK inhibitor, and further suggest that MKP-3 may be a vulnerable target in cells that express oncogenic Ras.

Carcinogenic agents classified as tumor promoters have proven to be powerful tools for investigating critical biochemical events involved in carcinogenesis (1). In the multistage mouse skin model of carcinogenesis the first rapid and irreversible stage, known as initiation, typically involves activation of the oncogene Ras (2). Tumor development requires the subsequent repeated stimulation by tumor promoters. The tumor promotion stage is prolonged and is reversible if tumor promoter treatment is ceased. Activation of Ras is a frequent early event in human cancers (3). Therefore, tumor promoters are excellent tools for identifying important molecular targets that are involved in carcinogenesis and for revealing how expression of oncogenic Ras makes cells susceptible to the subsequent action of tumor-promoting stimuli. The identification of protein kinase C as the receptor for the prototypical skin tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA)1 helped establish that tumor promotion involves the aberrant modulation of signaling cascades that govern cell fate and function (4). Palytoxin belongs to a class of non-TPA-type tumor promoters that do not act through protein kinase C (5). The novel properties of palytoxin make it a useful tool for investigating alternative mechanisms by which important targets in carcinogenesis can be modulated, which may be missed by the strong focus on TPA-stimulated signaling.

The initial signals stimulated by palytoxin differ significantly from those stimulated by TPA (5–7). Palytoxin is a large (M, 2,681), water-soluble polyalcohol isolated from zoanthids (genus Palythoa), which are closely related to sea anemones (8). Palytoxin was classified as a non-TPA-type tumor promoter because, in contrast to TPA, palytoxin does not activate protein kinase C in vitro, or induce ornithine decarboxylase in mouse skin (5). The putative palytoxin receptor is the Na+/K+-ATPase and stimulates ion flux by transforming the pump into an ion channel. Accordingly, stimulation of ion flux appears to be a critical component of palytoxin-stimulated signaling (7, 10, 11). Previous studies by this laboratory indicated that mitogen-activated protein kinases (MAPKs) are potentially important mediators of palytoxin-stimulated signals (10, 12, 13).

MAPKs are a family of serine/threonine kinases that coordinate the transmission of various types of signals to specific cellular targets (14, 15). For example, once activated, MAPKs can translocate from the cytoplasm to the nucleus, phosphorylate specific transcription factors, and thereby modulate gene expression (16). MAPKs regulate a wide variety of transcription factors that have been implicated in carcinogenesis, including activator protein-1 (AP-1) (1–16), which appears to be involved in tumor promotion (17). Extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 are three major MAPKs (14, 15). Mitogenic agents typically activate ERK. Stress-inducing agents typically activate JNK and p38. Our early work suggested that palytoxin and TPA activate different MAPK family members, which was not surprising considering that palytoxin and TPA stimulate distinctly differ-

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The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; AP-1, activator protein-1; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MKP, MAPK phosphatase; PP2A, protein phosphatase 2A; MEK, ERK kinase; GST, glutathione S-transferase.
ent initial signals. In COS7, HeLa, and Swiss 3T3 cells, palytoxin predominantly activates JNK and p38, but not ERK, whereas phorbol esters predominantly activate ERK, but not JNK or p38 (10, 11, 18). We also found that palytoxin-stimulated MAPK signaling can differ depending on the cell type (12). This finding led us to investigate the effects of palytoxin on MAPK activity in a cell culture model of initiated keratinocytes, which are the likely target cells of skin tumor promoters in vivo.

Surprisingly, we found that palytoxin activates ERK in a keratinocyte cell line derived from mouse skin initiated in vivo (308 cells), as does TPA (13, 19, 20). In 308 keratinocytes, palytoxin stimulates ERK-dependent gene expression and modulation of transcription factors involved in tumor promotion. These studies suggested that in the relevant target cells of tumor promoters the distinct signaling pathways stimulated by TPA and the non-TPA-type tumor promoter palytoxin converge to modulate ERK. ERK is a potentially critical common target of palytoxin and TPA because ERK modulates a constellation of downstream targets that are likely to be involved in carcinogenesis, including AP-1 (13, 17), and because aberrant regulation of ERK has been implicated as playing an important role in both the mouse skin model of carcinogenesis and in human carcinogenesis (21–26). These results also suggested that initiated keratinocytes express modulators of ERK activity that are particularly sensitive to palytoxin action. Thus, investigating how palytoxin activates ERK in initiated cells could reveal important alternative mechanisms by which ERK can be modulated and identify characteristics of these cells that make them susceptible to tumor promoter action.

In the studies presented here, we investigated the mechanisms by which palytoxin activates ERK in the 308 mouse keratinocyte cell line. 308 cells express an activated H-Ras oncogene, which is characteristic of initiated mouse skin (2, 19). ERK activity is regulated through the reversible phosphorylation of specific tyrosine and threonine residues (15, 27, 28). Phosphorylation and activation of ERK typically occurs through stimulation of the Ras/Raf/MEK/MAPK protein kinase cascade; the GTPase Ras activates the protein kinase Raf, which phosphorylates and activates the protein kinase MEK. MEK is highly specific for phosphorylating and activating ERK. MAPK phosphatases (MKPs) are a family of dual-specificity phosphatases that dephosphorylate both tyrosine and threonine residues, and specifically inactivate MKPs, including ERK (27, 28). The results presented here demonstrate that palytoxin does not activate ERK in 308 cells through direct stimulation of the Ras/Raf/MEK/ERK protein kinase cascade. Instead, we provide evidence that palytoxin increases ERK activity in 308 keratinocytes by inducing the loss of MKP-3, a phosphatase that is highly selective for dephosphorylating and inactivating ERK (29).

Furthermore, our results indicate that the ability of palytoxin to increase ERK activity by modulating MKP-3 is not unique to initiated keratinocytes, but instead may be characteristic of cells that express activated Ras oncogenes. Altogether, these studies suggest that MKP-3 may be a vulnerable target of tumor promoter action in cells that express oncogenic Ras.

**EXPERIMENTAL PROCEDURES**

**Materials**—Palytoxin was purchased from the Hawaii Biotechnology Group, Inc. (Aiea, HI). U0126 was purchased from Calbiochem (San Diego, CA). Antibodies were purchased from Sigma (St. Louis, MO). Palytoxin was purchased from Sigma (St. Louis, MO). Penicillin-streptomycin were purchased from Invitrogen Life Technologies (Carlsbad, CA). Cholera toxin, hydrocortisone, and epidermal growth factor were purchased from Sigma.

**Cell Culture**—308 cells were the generous gift of Dr. Stuart H. Yuspa (Laboratory of Cellular Carcinogenesis and Tumor Promotion, NCI, National Institutes of Health) and were grown in minimum essential medium supplemented with 8% fetal bovine serum (Interpen Company, Purchase, NY) at 37 °C with 7% CO2. MCF10A and H-ras MCF10A cells were the generous gift of Dr. Aree Moon (College of Pharmacy, Dukasung Women’s University, Seoul, Korea) and were grown in Dulbecco’s modified Eagle’s medium/F12 supplemented with 10% fetal bovine serum, 0.5 μg/ml hydrocortisone, 10 μg/ml insulin, 20 ng/ml epidermal growth factor, 0.1 μg/ml cholera toxin, 100 units/ml penicillin-streptomycin, 2 mM l-glutamine, and 0.5 μg/ml fungizone at 37 °C with 5% CO2. For experiments, the cells were grown until they were ~80% confluent and then switched to serum-free media overnight. All experiments were conducted in serum-free media.

**Plasmids and Transfections**—pGEX-GST-His-Elk1 (amino acids 308–428) was the generous gift of Dr. Robert A. Hippekind (Institute de Genetique Moleculaire CNRS, Montpellier, France). pEBG-ERK1 was the generous gift of Dr. Leonard Zon (Division of Hematologic Oncology, Howard Hughes Medical Institute, Children’s Hospital, Dana-Farber Cancer Institute, Harvard Medical School). pCDNA3.1-MKP-3 was the generous gift of Dr. Stefanie Dimmelmer (Department of Medicine, University of Frankfurt). pCMV4-hVH2-myc (MKP-2) and pCMV5-hVH1 (MKP-1) were the generous gifts of Dr. Kun-Liang Guan (Department of Biological Chemistry, University of Michigan). Cells were transfected with 0.25 μg of pEBG-ERK1 and 0.25 μg of either empty vector, pCMV4-MKP-3, or pCMV4-MKP-2, and pGEX-KH3, a plasmid expressing a GST fusion protein containing the enzyme catalytic domain of P32 phosphatase, was cotransfected with the plasmids to ensure that the plasmids were equally expressed in all transfections. The next day, cells were serum-starved. On day 3 cells were treated as described in figure legends and lysed for analysis of GST-ERK activity.

**Antibodies and Immunoblotting**—Proteins from whole cell lysates (20 to 40 μg) were resolved using SDS-PAGE minigels, and then transferred to Immobilon-P-PDVDF membrane (Millipore, Bedford, MA). Immunoblot analysis was conducted according to the protocol recommended by the manufacturer of the primary antibody. The signal for all immunoblots was detected using the ECL detection system purchased from Amersham Biosciences. The following antibodies were used: phospho-ERK, phospho-MAPK (Thr-202/Tyr-204) (E10) (mouse monoclonal), phospho-MEK-1 (Ser-290) (C-14) (rabbit polyclonal), MEK-1 (12-B) (rabbit polyclonal), MKP-1 (V-15) (rabbit polyclonal), MKP-2 (S-18) (rabbit polyclonal), MKP-3 (C-20) (goat polyclonal), and GST (B-14) (mouse monoclonal) 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 (Beverly, MA), and ERK 2 (C-14) (rabbit polyclonal), MEK-1 (12-B) (rabbit polyclonal), MKP-1 (V-15) (rabbit polyclonal), MKP-2 (S-18) (rabbit polyclonal), MKP-3 (C-20) (goat polyclonal), and GST (B-14) (mouse monoclonal) from Santa Cruz Biotechnology. Blots were stripped by incubating in diluted Re-Blot Plus Strong Antibody Stripping Solution (Chemicon International, Temecula, CA).

**Kinase Assays**—The assay for GST-ERK activity was conducted by transfecting cells as described above. After the treatments described in the figure legends, the cells were washed twice with ice-cold phosphate-buffered saline, and harvested in lysis buffer (20 mM Tris-Cl, pH 8.0, 2 mM EDTA, 1 mM Na2VO4, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 μM leupeptin). Cell lysates were centrifuged at 10,000 × g for 10 min, 4 °C for 10 min. The protein concentration of the supernatant was quantitated using the Bradford assay (Bio-Rad). 200 μg of protein was incubated with 2 μg of glutathione immobilized on agarose for 1 h at 4 °C. The glutathione-GST complexes were washed twice with lysis buffer, then lithium chloride buffer (0.5x LCl, 100 mM LiCl, 5% Triton X-100, 0.5 mM EGTA, 1 mM dithiothreitile, 0.03% Triton X-100, 10 mM MgCl2). The glutathione-GST complexes were then incubated in kinase buffer with 2 μg of GST-His-Elk1 and 15 μCi of [γ-14C]ATP for 30 min at 30 °C. Kinase reactions were terminated by the addition of Laemmli sample buffer. The proteins were resolved using 10% SDS-PAGE mini-gels. Phosphorylation was detected by autoradiography.

The assay for MEK activity was conducted as follows. After the treatments described in the figure legends, the cells were washed twice with ice-cold phosphate-buffered saline, and harvested in lysis buffer (10 mM Tris-Cl, pH 7.4, 50 mM NaCl, 2 mM EGTA, 0.1% Triton X-100, 1 mM dithiothreitol, 1 mM Na2VO4, 1 mM phenylmethylsulfonyl fluoride, 2 μM leupeptin). Cell lysates were centrifuged at 10,000 × g for 10 min, thawed at 4 °C, and then centrifuged at 10,000 × g for 4 °C for 10 min. The protein concentration of the supernatant was quantitated using the Bradford assay (Bio-Rad). 200 μg of cell lysate was incubated with 5 μl of MKP1 antibody and 25 μl of
palytoxin increases ERK activity in 308 cells. Whole cell lysates were prepared from 308 cells left untreated (C) or exposed to 30 pm palytoxin for the indicated times. Whole cell lysate (20 μg) was analyzed by immunoblot with an antibody that detects dually phosphorylated, active ERK (pERK) (top panel). The two bands represent ERK1 (p44) and ERK2 (p42). The pERK immunoblot was stripped and reprobed for total ERK (ERK) using an anti-ERK2 antibody (bottom panel). The results are representative of at least five independent experiments.

RESULTS

Palytoxin Stimulates ERK Activity in 308 Cells—In contrast to what we observed in several different cell lines (10, 11, 18), palytoxin stimulated ERK activation in 308 keratinocytes (Fig. 1). The activation of ERK by palytoxin was evident by 30 min and remained elevated for at least 8 h (data not shown). Palytoxin did not stimulate a detectable increase in ERK activity at time points measured prior to 30 min (data not shown). ERK activity was measured by immunoblot analysis and an antibody that specifically recognizes the dually phosphorylated, active form of ERK (Fig. 1, top panel). The phospho-ERK immunoblot was stripped and reprobed for total ERK (Fig. 1, middle panel). Palytoxin also stimulated the kinase activity of ERK (data not shown). These results show that palytoxin stimulates an increase in ERK activity, but not an increase in total ERK levels.

Palytoxin Does Not Stimulate MEK Activity—ERK is typically activated through stimulation of the Ras/Raf/MEK/ERK protein kinase cascade (15). Therefore, we first investigated the role of MEK in palytoxin-stimulated ERK activation. Precipitation of 308 cells with 3 μM U0126, a specific MEK inhibitor, effectively blocked both basal and palytoxin-stimulated ERK activity (Fig. 2A). These results indicate that MEK is required for palytoxin-stimulated ERK activation. Surprisingly, complementary experiments showed that palytoxin does not stimulate MEK activity (Fig. 2B). MEK activity was measured by immunoblot analysis with an antibody that recognizes the phosphorylated, active form of MEK (Fig. 2, top panel), and by immunoprecipitation of MEK followed by an in vitro kinase assay (Fig. 2B, middle panel). In contrast to palytoxin, TPA, as a positive control, stimulated an increase in MEK phosphorylation (Fig. 2B, top panel) and MEK activity, as indicated by phosphorylation of ERK2, the substrate used in the in vitro kinase assay (Fig. 2B, middle panel). We consistently observed lower levels of total MEK in lysates from TPA-stimulated cells (Fig. 2B, bottom panel). TPA may stimulate the redistribution of MEK to insoluble cellular compartments. Altogether, these results indicate that while basal MEK activity is required for palytoxin-triggered ERK activity, MEK activity is not increased in response to palytoxin.

Okadaic Acid Activate Both MEK and ERK—The observation that palytoxin stimulates prolonged ERK activity (Fig. 1), but does not stimulate MEK activity (Fig. 2) led us to investigate whether palytoxin inhibits an ERK phosphatase that is expressed in 308 cells. Two major classes of phosphatases can dephosphorylate and inactivate ERK: protein-tyrosine phosphatases and serine/threonine phosphatases (28, 30, 31). To help identify which class of phosphatase was the potential target of palytoxin we determined if sodium orthovanadate, a tyrosine phosphatase inhibitor, or okadaic acid, a serine/threonine phosphatase inhibitor, could mimic palytoxin action in 308 cells. Like palytoxin, sodium orthovanadate and okadaic acid stimulated ERK activity (Fig. 3, bottom panels). Unlike palytoxin, sodium orthovanadate and okadaic acid activated MEK (Fig. 3, top panels). Okadaic acid selectively inhibits the serine/threonine phosphatase protein phosphatase 2A (PP2A) at the concentration used in these studies (30, 32). Therefore, the observation that okadaic acid activates MEK indicates that palytoxin does not inhibit PP2A. Sodium orthovanadate is a general protein tyrosine phosphatase inhibitor (33, 34). Interestingly, at early time points, sodium orthovanadate stimulates a disproportionately high level of ERK phosphorylation relative to MEK phosphorylation. For example, at the 30 min time point, okadaic acid stimulates an ~2-fold increase in MEK phosphorylation and an ~4-fold increase in ERK phosphorylation (Fig. 3, right panels). By contrast, sodium orthovanadate stimulates an ~3-fold increase in MEK phosphorylation, but an ~36-fold increase in ERK phosphorylation (Fig. 3, left panels). One possible explanation for these results is that sodium orthovanadate increases ERK phosphorylation by inhibiting tyrosine phosphatases that act directly on ERK, as well as by inhibiting tyrosine phosphatases that act upstream of ERK. Altogether, these results indicate that palytoxin does not inhibit the total spectrum of phosphatases inhibited by sodium orthovanadate, and furthermore suggest that 308 cells express protein-tyrosine phosphatases that can directly dephosphorylate ERK.
308 Cells Express High Levels of MKP-2 and MKP-3—MKPs are a class of protein-tyrosine phosphatases that can directly dephosphorylate MAPks. MKP family members can differ with respect to several characteristics, including their selectivity for dephosphorylating ERK, JNK, and p38 and their tissue-specific expression (27, 28). We next analyzed 308 cells for the expression of three MKPs that can dephosphorylate ERK and are widely expressed: MKP-1, MKP-2, and MKP-3. Immunoblot analysis showed that 308 cells express very low basal levels of MKP-1 (Fig. 4, A). We detected MKP-1 in MCF10A cells transfected with MKP-1 as well as in 308 cells treated with TPA, which induces the expression of MKP-1 (35). This indicates we can detect endogenous MKP-1 under the conditions of our studies. In contrast to MKP-1, 308 cells expressed relatively high levels of both MKP-2 and MKP-3 (Fig. 4, B and C). MKP-3 typically runs as a doublet due to the presence of two translation start sites (36, 37). The relatively high expression of MKP-2 and MKP-3 suggested that these phosphatases were potential targets of palytoxin action.

Palytoxin Triggers the Loss of MKP-3—We next determined the effect of palytoxin on MKP-2 and MKP-3 protein levels. Incubating 308 cells with palytoxin for 30 min resulted in a substantial decrease in MKP-3 protein levels (Fig. 5A, middle panel). This decrease in MKP-3 coincided with a major increase in the levels of phosphorylated ERK detected in the same cell lysates (Fig. 5, A, top panel and B). MKP-3 levels remained suppressed through at least 4 h of palytoxin treatment. Accordingly, phosphorylated ERK levels remained elevated throughout this time course (Fig. 5A, top panel). In striking contrast, MKP-2 levels remained elevated throughout palytoxin treatment (Fig. 5A, bottom panel). These results show that MKP-3 is a target of palytoxin action and suggest that the palytoxin-induced loss of MKP-3 contributes to the increase in ERK phosphorylation and activation.

Sustained Expression of MKP-3 Blocks Palytoxin-stimulated ERK Activity—If the loss of MKP-3 is key to the activation of ERK by palytoxin, then we would expect that the sustained presence of MKP-3 would block the ability of palytoxin to activate ERK. To test this, 308 cells were co-transfected with a vector that expressed epitope-tagged ERK (GST-ERK) and either an empty vector or a vector that expressed MKP-3 (Fig. 6). As expected, sustaining MKP-3 levels, through expression of exogenous MKP-3, inhibited palytoxin-stimulated ERK activation. In cells that expressed GST-ERK alone, palytoxin stimulated the phosphorylation of GST-ERK—3-fold relative to non-treated cells, as detected by immunoblot analysis and an antibody that detects the phosphorylated, active form of ERK (Fig. 6A, compare lanes 1 and 2). Palytoxin stimulated a corresponding increase in GST-ERK activity, as detected by phosphorylation of the substrate Elk-1 (Fig. 6B, compare lanes 1 and 2). As expected, palytoxin also induced the almost complete loss of MKP-3 (Fig. 6D, lane 2). Expression of exogenous MKP-3 resulted in sustained elevated levels of MKP-3 even in the presence of palytoxin (Fig. 6D, compare lanes 2 and 4). GST-ERK phosphorylation and activity was lower in nontreated 308 cells that expressed both GST-ERK and exogenous MKP-3 than in nontreated cells that expressed GST-ERK alone (Fig. 6, A and B, compare lanes 1 and 3). Strikingly, expression of exogenous MKP-3 resulted in a dramatic attenuation of palytoxin-stimulated GST-ERK phosphorylation and activation (Fig. 6, A and B, compare lanes 2 and 4). In cells that expressed both GST-ERK and exogenous MKP-3, palytoxin only stimulated GST-ERK activity—1.5-fold relative to nontreated cells (Fig. 6, A and B, compare lanes 3 and 4). In a parallel set of experiments we found that expression of exogenous MKP-2 did not block palytoxin-stimulated ERK phosphorylation or activity (Fig. 7). Altogether, these results support an important role for
the down-regulation of MKP-3 in palytoxin-stimulated ERK activation.

Palytoxin Stimulates ERK Activity in Cells That Express Activated H-Ras—Because 308 cells express an activated H-Ras oncogene (19), we next wanted to investigate whether the ability of palytoxin to stimulate ERK activity is specific to keratinocytes or if it also occurs in other cell lines that express activated H-Ras. We compared the effects of palytoxin on ERK activity in MCF10A cells, transiently transfected with pEG-GST-MKP-3 and either empty vector (Vector) or pCMV4-kH2-myc (MKP-2). Whole cell lysates were prepared from transfected cells left untreated (Control) or exposed to 30 pm palytoxin (Paly) for 2 h. A, whole cell lysates (40 μg) were analyzed by immunoblot for dually phosphorylated, active ERK (pERK). B, GST-ERK was isolated from another portion of the whole cell lysates followed by an in vitro kinase assay. Phosphorylation of Elk-1 (pElk-1) indicates GST-ERK activity. Other portions of the same whole cell lysates (40 μg) were analyzed by immunoblot for the following: C, GST-ERK using an anti-GST antibody and D, MKP-3. E, the pERK blot was stripped and reprobed for MKP-2. ns indicates a nonspecific band. The results are representative of five independent experiments.

Discussion

The studies presented here reveal a novel mechanism by which carcinogenic agents can modulate ERK activity in cells that express oncogenic Ras. The activity of ERK, a major molecular switch that plays a central role in the regulation of cell proliferation, differentiation, and gene expression, depends on the balance between phosphorylation by kinases and dephosphorylation by phosphatases (27, 28, 38, 39). The duration and magnitude of ERK activation can dramatically affect the course of downstream cellular events (40–42). For example, in PC12 cells, transient ERK activation is associated with cell proliferation, whereas prolonged ERK activation is associated with differentiation (40). In mouse fibroblasts, prolonged ERK activation is associated with c-Fos stabilization and entry into S phase; transient ERK activation is not sufficient to induce these cellular events (42). Clearly, upsetting the balance between the phosphorylation and dephosphorylation of ERK can profoundly affect cell fate and function.

Growing evidence indicates that dysregulation of ERK plays an important role in carcinogenesis. For example, in the multistage mouse skin model, elevated ERK activity was detected in TPA-induced papillomas and squamous cell carcinomas (23). Elevated ERK activity has also been detected in several human cancers, including lung, colon, breast, melanoma, leukemia, and head and neck squamous carcinoma (26, 43–46). Moreover, inhibitors of MEK, the kinase that phosphorylates and activates ERK, blocked ERK activity and tumor growth in mice implanted with human colon or pancreatic tumors (24, 25). Significantly, we observed that in initiated cells, the prototypical tumor promoter TPA and the non-TPA-type tumor promoter palytoxin stimulate different signaling pathways that converge to modulate ERK and thereby stimulate common downstream targets that are likely to be involved in tumori-
times. Whole cell lysates (40 μg) from H9262 or H-ras MCF10A cells were analyzed by immunoblot for MKP-1 (top panel), MKP-2 (middle panel), or MKP-3 (bottom panel). A, H-ras MCF10A cells were left untreated (C) or exposed to 30 μM palytoxin for the indicated times. Whole cell lysates (40 μg) were analyzed by immunoblot for the following: dually phosphorylated, active ERK (pERK) (top panel); MKP-3 (middle panel); MKP-2 (bottom panel). ns indicates nonspecific bands. B, graphical representation of densitometry of the pERK and MKP-3 blots shown in B. Open bars represent pERK. Filled bars represent MKP-3. The results are representative of at least four independent experiments.

Fig. 9. H-ras MCF10A cells express elevated levels of MKP-2 and MKP-3 and palytoxin stimulates the down-regulation of MKP-3. A, whole cell lysates (40 μg) from MCF10A or H-ras MCF10A cells were analyzed by immunoblot for MKP-1 (top panel), MKP-2 (middle panel), or MKP-3 (bottom panel). B, H-ras MCF10A cells were left untreated (C) or exposed to 30 μM palytoxin for the indicated times. Whole cell lysates (40 μg) were analyzed by immunoblot for the following: dually phosphorylated, active ERK (pERK) (top panel); MKP-3 (middle panel); MKP-2 (bottom panel). ns indicates nonspecific bands. C, graphical representation of densitometry of the pERK and MKP-3 blots shown in B. Open bars represent pERK. Filled bars represent MKP-3. The results are representative of at least four independent experiments.

The observation that palytoxin stimulated ERK activity in H-ras MCF10A cells, but not the parental MCF10A cells, suggested that the mechanism by which palytoxin stimulates ERK activity is related to the expression of oncogenic Ras, as opposed to being a function specific to mouse keratinocytes. MKP-3, a dual specificity phosphatase that directly dephosphorylates and inactivates ERK (29), emerged as a likely target of palytoxin because it is highly expressed in cell lines in which palytoxin stimulates ERK activity (i.e. 308 and H-ras MCF10A), but was not detected in cells in which palytoxin does not affect ERK activity, including MCF10A, Swiss 3T3, and COS7 (data not shown). Furthermore, in 308 keratinocytes and H-ras MCF10A cells palytoxin stimulates the loss of MKP-3 in a manner that corresponds to the stimulation of ERK phosphorylation and activation. Finally, sustained expression of MKP-3 blocked the ability of palytoxin to activate ERK. Although other ERK phosphatases may also be targets of palytoxin action, the results presented here strongly support an important role for the down-modulation of MKP-3 in palytoxin-induced ERK activation.

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The studies presented here suggest that MKPs may be critical targets in tumor promotion. The fact that another class of phosphatases, serine/threonine phosphatases such as PP2A, are direct targets of the skin tumor promoter okadaic acid (50) further supports such a global and important role of phosphatases in carcinogenesis. As members of the protein tyrosine phosphatase superfamily, the MKPs are dual-specificity phosphatases that dephosphorylate both tyrosine and threonine residues, and specifically inactivate MAPKs (27, 28). MKP family members can differ with respect to several characteristics, including their selectivity for dephosphorylating ERK, JNK, and p38; subcellular location; tissue-specific expression; and inducibility by various types of signals. We initially focused our investigation on MKP-1, MKP-2, and MKP-3 based on reports that they can dephosphorylate ERK and are widely expressed. Interestingly, we did not detect elevated levels of MKP-1 in 308 cells or H-ras MCF10A cells. This is somewhat surprising considering that studies by others suggested that MKP-1 can be regulated by Ras-stimulated signaling cascades, and because MKP-1 is induced by many of the same signals that activate ERK (27, 35). Expression of MKP-1 could be induced in both 308 cells and H-ras MCF10A cells (data not shown), which indicates that MKP-1 gene expression is responsive to activation of signaling pathways in these cell lines. The
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presence of oncogenic Ras alone may not be sufficient to maintain the expression of MKP-1. We also show here that the up-regulation of MKP-2 and MKP-3 is not limited to initiated mouse keratinocytes. Instead, our results suggest that the up-regulation of MKP-2 and MKP-3 may be characteristic of cells that express oncogenic Ras. Accordingly, in pancreatic cancer cell lines that express oncogenic Ras, MKP-2, and MKP-3 also appear to be up-regulated (48, 49). We are currently investigating whether a broader constellation of MKPs is modulated in response to the expression of oncogenic Ras.

Although MKP-2 levels remained elevated throughout palytoxin treatment, we did observe that the MKP-2 band narrowed somewhat at 2 and 3 h. This could reflect an upshift in MKP-2 due to phosphorylation. Others have reported that phosphorylation increases the stability of MKP-1 (51). We are currently investigating whether palytoxin stimulates the phosphorylation of MKP-2 and whether such phosphorylation results in stabilization of this protein. Unlike MKP-3, which is highly specific for dephosphorylating ERK (29), MKP-2 can dephosphorylate both ERK and JNK (52). Interestingly, overexpression of MKP-2 did not block the ability of palytoxin to activate ERK. Furthermore, overexpression of MKP-2 did not decrease basal ERK activity and did not affect TPA-stimulated ERK activity in 308 cells (data not shown). These data suggest that ERK may not be the preferred substrate for MKP-2 in 308 mouse keratinocytes.

The observation that palytoxin stimulates the rapid loss of MKP-3 suggests that MKP-3 is a relatively unstable protein. This would make MKP-3 a particularly vulnerable target for agents that block the production of this phosphatase. We are currently investigating the mechanisms by which palytoxin stimulates the loss of MKP-3. We are focusing on how palytoxin affects MKP-3 translation because palytoxin does not appear to stimulate the loss of MKP-3. We are investigating whether palytoxin stimulates the loss of MKP-3 at the level of transcription or by stimulating MKP-3 redistribution to insoluble cellular compartments (data not shown).

Importantly, our studies indicate that cells may express a different constellation of MKPs in the presence of oncogenic Ras than they do in the presence of normal Ras alone. Such a cellular response suggests the enticing idea that MKPs, such as MKP-2 and MKP-3, may be part of a negative feedback loop that operates in cells that express oncogenic Ras. In essence, cells may compensate for chronic activation of the Ras-stimulated pathways by up-regulating phosphatases that constrain those protein kinase cascades. The idea that cells normally compensate for the expression of activated Ras by up-regulating negative regulators such as phosphatases may help explain, in part, the multistage nature of carcinogenesis. Perhaps one reason why activation of Ras alone is not sufficient to induce tumors is because cells normally compensate for the superactivation of Ras-stimulated signal transduction pathways, such as the Ras/Raf/MAPK/ERK cascade, by up-regulating phosphatases that constrain those pathways. Disrupting the compensatory mechanism by inactivating phosphatases would thus be an effective additional step, which would unleash such superactivated signaling cascades. The studies presented here suggest that MKP-3 may be a particularly vulnerable point in such a feedback mechanism due to its apparent instability. This idea is strongly supported by studies that suggest that MKP-3 is a tumor suppressor in human pancreatic cancer, where the frequency of Ras mutation approaches 90% (3, 49).

The relevance of tumor promotion to human cancer is illustrated by the long latency in the development of cancer, and by examples of reversibility, such as decreased lung cancer risk after smoking cessation (53). In addition, it has recently been suggested that the majority of chemicals released into the environment may not contribute to carcinogenesis by directly damaging DNA, but rather act by permitting the selective outgrowth of previously mutated cells (54). Given that activation of Ras frequently occurs in human cancers (3), 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. Our studies with palytoxin have revealed a characteristic of initiated cells that may make them vulnerable to the action of various types of tumor promoter action. The potentially important role of ERK in carcinogenesis, and the high frequency of Ras activation in human cancers, underscores the need to understand how the various mechanisms that regulate ERK can be subverted to stimulate carcinogenesis.

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Mitogen-activated Protein Kinase Phosphatase-3 Is a Tumor Promoter Target in Initiated Cells That Express Oncogenic Ras
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