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Extracellular signal regulated kinase 5 mediates signals triggered by the novel tumor promoter palytoxin

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ABSTRACT

Palytoxin is classified as a non-12-*O*-tetradecanoylphorbol-13-acetate (TPA)-type skin tumor because it does not bind to or activate protein kinase C. Palytoxin is thus a novel tool for investigating alternative signaling pathways that may affect carcinogenesis. We previously showed that palytoxin activates three major members of the mitogen activated protein kinase (MAPK) family, extracellular signal regulated kinase 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38. Here we report that palytoxin also activates another MAPK family member, called ERK5, in HeLa cells and in keratinocytes derived from initiated mouse skin (308 cells). By contrast, TPA does not activate ERK5 in these cell lines. The major cell surface receptor for palytoxin is the Na+,K+-ATPase. Accordingly, ouabain blocked the ability of palytoxin to activate ERK5. Ouabain alone did not activate ERK5. ERK5 thus represents a divergence in the signaling pathways activated by these two agents that bind to the Na+,K+-ATPase. Cycloheximide, okadaic acid, and sodium orthovanadate did not mimic the effect of palytoxin on ERK5. These results indicate that the stimulation of ERK5 by palytoxin is not simply due to inhibition of protein synthesis or inhibition of serine/threonine or tyrosine phosphatases. Therefore, the mechanism by which palytoxin activates ERK5 differs from that by which it activates ERK1/2, JNK, and p38. Finally, studies that used pharmacological inhibitors and shRNA to block ERK5 action indicate that ERK5 contributes to palytoxin-stimulated c-Fos gene expression. These results suggest that ERK5 can act as an alternative mediator for transmitting diverse tumor promoter-stimulated signals.

Introduction

Agents identified as tumor promoters in the classic multi-stage mouse skin model of carcinogenesis have helped reveal how the perturbation of signaling pathways contributes to the development of cancer (Yuspa, 1998). Initiation, the first stage of carcinogenesis in this model, typically involves one treatment with a genotoxic agent and is characterized by activation of the oncogene Ras (Balmain and Pragnell, 1983). Tumor promotion, the second stage, involves repeated stimulation over a prolonged period by agents that are typically non-genotoxic, and results in the development of tumors. Interestingly, whereas initiation is irreversible, tumor promotion is reversible if treatment is ceased. This suggests that investigating the action of tumor promoters may aid the development of strategies to block carcinogenesis. The identification of protein kinase C as the receptor for the prototypical skin tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA, also called PMA) helped establish that tumor promotion involves the subversion of signal transduction pathways (Nishizuka, 1984). The subsequent identification of non-TPA-type tumor promoters, which do not activate protein kinase C *in vitro* or require protein kinase C for signal transduction, indicated that protein kinase C-independent signaling pathways also play a role in carcinogenesis (Fujiki et al., 1986; Wattenberg et al., 1987). Accordingly, our laboratory has used the non-TPA-type tumor promoter palytoxin to investigate alternative signaling pathways that may play a role in carcinogenesis, but may have been missed by the historical focus on TPA-stimulated signaling.

The major cell surface receptor for palytoxin is the Na+,K+-ATPase (Habermann, 1989). Palytoxin, a large (M_r 2,681) water-soluble polyalcohol, which is isolated from zoanthids (genus *Palythoa*) (Moore, 1985), binds to the Na+,K+-ATPase and transforms the sodium pump into an ion channel. Palytoxin binding thus typically triggers sodium influx and potassium efflux. Our previous studies indicated that mitogen activated protein kinases (MAPKs) can mediate palytoxin-stimulated signaling (Kuroki et al., 1997; Li and Wattenberg, 1999; Warmka et al., 2002; Warmka et al., 2004).

MAPKs are a family of serine/threonine kinases that transmit a wide variety of signals to the cellular machinery that regulates gene expression, cell fate, and cell function (reviewed by Turjanski et al. (2007)). Several studies also indicate that aberrant regulation of MAPKs plays a role in carcinogenesis (Dhillon et al., 2007). The three groups of MAPKs that have been studied most extensively are the extracellular signal regulated kinases 1 and 2 (ERK1/2), the c-Jun N-terminal kinases (JNKs), and the p38s. In general ERK1/2 tends to be

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activated by mitogenic agents, whereas JNK and p38 are typically activated by stress (Turjanski et al., 2007). Our work indicates that MAPKs can mediate the convergence of the different signaling pathways stimulated by palytoxin and TPA, thus providing a mechanism by which these different types of tumor promoters can regulate common biochemical targets that play an important role in carcinogenesis (Warmka et al., 2002; Warmka et al., 2004). For example, we found that palytoxin and TPA both increase ERK1/2 activity, although by different mechanisms, in mouse keratinocytes that are derived from initiated mouse skin and express oncogenic Ras (308 cells); this results in the ability of palytoxin and TPA to regulate common downstream nuclear targets, including the transcription factor AP-1 (Warmka et al., 2002; Warmka et al., 2004; Zeliadt et al., 2004).

ERK5 (also called Big MAP kinase 1 or BMK1) is a MAPK family member that is likely to play a role in carcinogenesis, but has not been studied as extensively as ERK1/2, JNK, and p38 (reviewed by Wang and Tournier (2006)). Like other MAPKs, activation of ERK5 requires dual phosphorylation on specific threonine and tyrosine residues (Mody et al., 2003). MAPKs are typically activated by a protein kinase cascade, such that a MAPK kinase kinase (MAPKKK or MEKK) phosphorylates and activates a MAPK kinase (MAPKK or MEK), which phosphorylates and activates a MAPK (Wang and Tournier, 2006). For example, Raf is a MAPKKK that phosphorylates and activates MEK1/2, which phosphorylates and activates ERK1/2 (reviewed by Dhillon et al. (2007)). MEKK2 and MEKK3 have been identified as MAPKKKs, which phosphorylate and activate MEK5, the MAPKK that phosphorylates and activates ERK5 (Chao et al., 1999; Sun et al., 2001). A unique, large C-terminal non-kinase domain makes ERK5 approximately twice the size of ERK1/2 (Lee et al., 1995; Zhou et al., 1995). This C-terminal domain contains a nuclear localization signal (Hayashi and Lee, 2004). ERK5, like other MAPK family members, can translocate from the cytoplasm to the nucleus, where it can regulate transcription factors, including MEF2C (myocyte enhancer factor), Sap1a, and c-Fos (Kato et al., 1997; Kamakura et al., 1999; Terasawa et al., 2003). ERK5 is activated by mitogens, such as epidermal growth factor (EGF), and also by stress, such as osmotic shock and oxidative stress (Abe et al., 1996; Kato et al., 1998). Because palytoxin causes a type of osmotic stress by stimulating ion influx, we wanted to determine whether ERK5 mediates palytoxin-induced signals.

We used two cell culture models to determine whether ERK5 is involved in palytoxin signaling. We used 308 mouse keratinocytes, which were derived from mouse skin initiated *in vivo* with 7,12-dimethylbenz(a)anthracene and express endogenous oncogenic Ras (Strickland et al., 1988). This is thus an excellent model for studying tumor promoter action. We also used HeLa cells, a human cervical cancer cell line that has been used extensively to study MAPK signaling. HeLa cells, in contrast to keratinocytes, are relatively easy to transfect. This makes HeLa cells very useful for the study of signaling by methods that require the introduction of expression vectors. Our results indicate that ERK5 can mediate the transmission of palytoxin-stimulated signals from the Na+,K+-ATPase to the nucleus. In contrast to palytoxin, we did not detect activation of ERK5 by the prototypical phorbol ester tumor promoter TPA. Altogether, these studies indicate that ERK5 represents an alternative pathway through which diverse tumor promoters can transmit signals.

Materials and methods

Materials. Palytoxin was purchased from the Hawaii Biotechnology Group, Inc. (Aiea, HI). U0126 was purchased from Calbiochem (La Jolla, CA). High glucose Dulbecco's modified Eagle medium, minimum essential medium, and fetal bovine serum were purchased from Invitrogen Corporation (Carlsbad, CA). TPA, cycloheximide, EGF, protein G agarose beads, ouabain, sodium orthovanadate, okadaic acid, phenylmethylsulfonyl fluoride (PMSF), NaF, β-glycerophosphate, aprotinin, leupeptin, and sorbitol were purchased from Sigma (St. Louis, MO). Calf intestinal alkaline phosphatase was purchased from New England Biolabs (Ipswich, MA).

Cell culture. HeLa cells were the generous gift of Dr. Audrey Minden (Susan Lehman Cullman Laboratory for Cancer Research, Department of Chemical Biology, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey), and were grown as described in (Li and Wattenberg, 1998). 308 cells were the generous gift of Dr. Stuart H. Yuspa (Laboratory of Cellular Carcinogenesis and Tumor Promotion, National Cancer Institute), and were grown as described in (Warmka et al., 2004). For experiments, HeLa and 308 cells were plated at a density of approximately $2.4\times10^4/cm^2$ and $5.6\times10^4/cm^2$, respectively. The following day, the cells were switched to serum free media. The cells were incubated in serum free media for approximately 24 h before treatment, unless otherwise indicated.

Antibodies and immunoblotting. Cell lysates were prepared using the following buffer unless otherwise noted: 50 mM Tris–HCl, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 20 mM β-glycerophosphate, 1 mM Na_3VO_4, and 1 mM NaF. Lysates were cleared by centrifugation (16,000×*g*, 10 min, 4 °C). Protein (20–40 μg) was resolved using either 7.5% or 10% SDS–polyacrylamide minigels, and then transferred to Immobilon-P PVDF membrane (Millipore, Bedford, MA). After blocking in either a TBST/5% milk solution or TBST/3% BSA 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), phospho-ERK5 (Thr-218/Tyr-220) (rabbit polyclonal) (1:1000), ERK5 (rabbit polyclonal) (1:1000), phospho-p38 MAPK (Thr-180/Tyr-182) (rabbit polyclonal) (1:2000), and phospho-SAPK/JNK (Thr-183/Tyr-185) (G9) (mouse monoclonal) (1:2000) from Cell Signaling (Beverly, MA), and ERK2 (C-14) (rabbit polyclonal) (1:2000), JNK1 (FL) (rabbit polyclonal) (1:2000), and p38 (C-20) (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. Immunoblots were visualized using the Pierce SuperSignal West Pico substrate.

Densitometry. Protein bands from immunoblots were quantified using a Bio-Rad (Hercules, CA) Fluor-S MultiImager and Bio-Rad Quantity One software.

Quantitative real-time PCR analysis. Total RNA was harvested from cells using the RNeasy Plus Mini Kit from Qiagen (Valencia, CA). RNA quality was verified spectrophotometrically using the A260/A280 ratio. One microgram of RNA was reverse-transcribed using the SuperScript III from Invitrogen (Carlsbad, CA). Negative controls that lacked RNA or reverse transcriptase were included for each primer pair. cDNA was diluted 1:10, and 2 μl of diluted cDNA were used in 25 μl PCR reactions with 200 nM of each primer and SYBR GreenER qPCR SuperMix for iCycler (Invitrogen). PCR was performed on a Bio-Rad iCycler iQ5 using the following primers: human c-Fos (accession no. NM_005252), 5′-CGGGCTTCAACGCAGACTA-3′ (forward) and 5′-CTGGTCGAGATGGCAGTGA-3′ (reverse); human GAPDH (accession no. NM_002046), 5′-GGGAAGGTGAAGGTCGGAGT-3′ (forward) and 5′-GAGTTAAAAGCAGCCCTGGTGA-3′ (reverse); mouse c-Fos (accession no. NM_010234), 5′-GGGGCAAAGTAGAGCAGCTA-3′ (forward) and 5′-GGCTGCCAAAATAAACTCCA-3′ (reverse); and mouse GAPDH (accession no. NM_008084), 5′-ATTGTCAGCAATGCATCCTG-3′ (forward) and 5′-ATGGACTGTGGTCATGAGCC-3′ (reverse). The amplification program was 50 °C for 2 min, 95 °C for 8 min 30 s, and 40 cycles