Regulation of a c-Jun Amino-terminal Kinase/Stress-activated Protein Kinase Cascade by a Sodium-dependent Signal Transduction Pathway*

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Palytoxin is a novel skin tumor promoter that does not activate protein kinase C. Previous studies demonstrated that palytoxin stimulates a sodium-dependent signaling pathway that activates the c-Jun NH₂-terminal kinase/stress-activated protein kinase (JNK) in Swiss 3T3 fibroblasts. In this study we show that a JNK kinase known as the stress-activated protein kinase/extracellular signal-regulated kinase-1 (Sbf1) plays an important role in the regulation of JNK by palytoxin. We found that palytoxin stimulates the sustained activation of both JNK and SEK1 in COS7 and HeLa cells. Transiently expressed SEK1 isolated from palytoxin-treated cells can phosphorylate and activate JNK, which, in turn, can phosphorylate c-Jun. Furthermore, expression of a dominant negative mutant of SEK1 blocks activation of JNK by palytoxin. Sodium appears to play an important role in the regulation of JNK and SEK1 by palytoxin. Activation of JNK and SEK1 by palytoxin, but not anisomycin, requires extracellular sodium. Complementary studies showed that the sodium ionophore gramicidin can mimic palytoxin by regulating JNK and SEK1 through a sodium-dependent mechanism. Collectively, these results demonstrate that palytoxin stimulates a sodium-dependent signaling pathway that activates the SEK1/JNK/c-Jun protein kinase cascade.

The biochemical mechanism of action of the skin tumor promoter palytoxin differs significantly from that of the prototypical phorbol ester tumor promoters. Although palytoxin is as potent as phorbol esters in the two-stage mouse skin assay, this marine toxin does not activate protein kinase C and is therefore classified as a non-TPA¹-type tumor promoter (1). Indeed, the structure of palytoxin suggests that it would have a receptor type different from that of phorbol esters. In contrast to phorbol esters, which are lipophilic, palytoxin is a large (Mr 2,681), water-soluble polyalcohol (2). The putative receptor for palytoxin is Na⁺,K⁺-ATPase (3). Palytoxin appears to bind to Na⁺,K⁺-ATPase and either transform the pump into a sodium channel or form a sodium channel closely associated with this ion pump. Consequently, palytoxin stimulates sodium influx in every system where it has been tested (3). Presumably, the modulation of signal transduction pathways through activation of protein kinase C plays an important role in the carcinogenic effects of phorbol esters (4). This raises the question of whether palytoxin-stimulated sodium influx can also modulate signal transduction pathways that may be involved in carcinogenesis.

Recent studies demonstrated that palytoxin can stimulate the c-Jun NH₂-terminal kinase/stress-activated protein kinase (JNK) through a sodium-dependent pathway in Swiss 3T3 fibroblasts (5). JNK presents an important target for elucidating the signaling pathways that transduce the novel signals stimulated by palytoxin. JNK is a member of the mitogen-activated protein kinase (MAPK) family of serine/threonine kinases (6–8). One important function of MAPKs is to regulate gene expression. Upon activation by intracellular signals, MAPKs translocate to the nucleus where they phosphorylate transcription factors (9). For example, JNK is generally activated by agents that induce cellular stress, including UV light, tumor necrosis factor α (TNF-α), and hyperosmotic conditions (6–8). Once activated, JNK can translocate to the nucleus and phosphorylate c-Jun on Ser-63 and Ser-73 and thereby stimulate transcriptional activation (6). JNK may be a significant target for palytoxin action, providing a mechanism for transducing palytoxin-stimulated signals to the nucleus. In particular, the fact that c-Jun is a proto-oncogene product underscores the potential importance of aberrant regulation of JNK in carcinogenesis.

Another member of the MAPK family, the extracellular signal-regulated kinase (ERK), is generally activated by growth factors and other mitogenic agents (10). ERK also translocates to the nucleus upon activation and modulates gene expression through phosphorylation of transcription factors, including Elk-1 (11–13). Previous studies showed that phorbol esters, which are mitogenic, selectively activate ERK but not JNK in Swiss 3T3 fibroblasts, whereas the non-TPA-type tumor promoter palytoxin selectively activates JNK but not ERK in this cell type (5). These results indicate that both phorbol esters and palytoxin can activate MAPKs, although these distinct types of tumor promoters regulate different members of this kinase family.

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1 The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; JNK, c-Jun NH₂-terminal kinase or stress-activated protein kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; TNF-α, tumor necrosis factor α; SEK1, stress-activated protein kinase/extracellular signal-regulated kinase-1; DMEM, Dulbecco’s modified Eagle’s medium; PDBu, phorbol 12,13-dibutyrate; GST, glutathione S-transferase; HA, hemagglutinin; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; MKK and MEK, mitogen-activated protein kinase kinase; MEKK, MEK kinase; SPRK, src-homology 3 domain-containing proline-rich kinase.
Determining how palytoxin regulates JNK may help reveal the mechanisms by which the signals stimulated by palytoxin are transduced through the cell. JNK is activated by dual phosphorylation of Thr-183 and Tyr-185 (14). Stress-activated protein kinase/extracellular signal-regulated kinase-1 (SEK1, also called JNKK and MKK4) has been identified as a kinase that can directly phosphorylate and activate JNK (15–17). Therefore, to elucidate further the signal transduction pathways activated by palytoxin, we investigated the role of SEK1 in the regulation of JNK by palytoxin. The results from these studies support an important role for SEK1 in a protein kinase cascade that transduces the novel sodium-dependent signals stimulated by palytoxin.

**EXPERIMENTAL PROCEDURES**

**Materials—**Palytoxin was isolated from Hawaiian *Palythoa tuberculosa* according to published methods (18, 19). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, myelin basic protein, and LipofectAMINE were purchased from Life Technologies, Inc. Gramicidin, anisomycin, phosphor 12,13-dibutyrate (PDBu), protein G-agarose, horseradish peroxidase-conjugated goat anti-mouse polyclonal antibody, and glutathione (GSH)-agarose were purchased from Sigma. TNP-24 in propyl ether was purchased from R & D Systems (Minneapolis). (γ-32P)ATP was purchased from NEN Life Science Products. Sodium-free medium was made by reconstituting DMEM, replacing all of the sodium salts with the analogous potassium salts. Hyperosmotic medium was made by replacing the sodium chloride in DMEM with 0.5 M sorbitol.

**Plasmids—**The bacterial expression vector pGEX-2T encoding either glutathione S-transferase (GST) or the GST-c-Jun (1–232 amino acids) fusion protein was a gift of Dr. Daniel Mueller (Department of Medicine, University of Minnesota). pEBG-GST-SEK1, pEBG-GST-SEK1(K-R), and pEBG-GST-ERK1 were the gift of Dr. Leonard Zon (Children's Hospital, Howard Hughes Medical Institute, Department of Microbiology and Molecular Genetics, Harvard Medical School). pSirHA-JNK1 was described in (17). pGEX-KG-JNK1a was described in (15). GST-JNK, GST-JNK- and JNK were produced by transforming the HB101 strain of *Escherichia coli* with the appropriate GST fusion protein expression vector. Protein induction and purification were conducted according to standard procedures (20).

**JNK Assay—**JNK activation was assayed according to published methods (6). Briefly, COS7 or HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum in a gassed (5% CO2), humidified atmosphere. After the cultures became confluent, the medium was switched to serum-free medium. After 48 h, the cultures were incubated at 37 °C in DMEM, 0.1% fetal bovine serum in the presence or absence of the appropriate agent. Cultures were then washed with ice-cold phosphate-buffered saline and harvested in lysis buffer (25 mM HEPES, pH 7.7, 300 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, pH 8.0, 0.1% Triton X-100, 0.5 mM DTT, 20 mM β-glycerophosphate, 0.1 mM Na3VO4, 2 μg/ml leupeptin, and 100 μg/ml PMSF). Whole cell lysate containing 50 μg of protein was diluted to a final concentration of 20 mM HEPES, pH 7.7, 7.5 mM NaCl, 2.5 mM MgCl2, 0.1 mM EDTA, 0.05% Triton X-100, 0.5 mM DTT, 20 mM β-glycerophosphate, 0.1 mM Na3VO4, 2 μg/ml leupeptin, and 100 μg/ml PMSF and then mixed with GST-c-Jun bound to GSH-agarose beads for 3 h at 4 °C. The beads were pelleted, washed, and then resuspended in 30 μl of kinase buffer (20 mM HEPES, pH 7.6, 20 mM MgCl2, 20 mM β-glycerophosphate, 20 mM p-nitrophenyI phosphate, 0.1 mM Na3VO4, 2 mM DTT, 20 μM ATP, and 5 μCi of [γ-32P]ATP), and incubated for 15 min at 30 °C. The beads were washed, then the proteins were eluted in Laemmli sample buffer and resolved by SDS-PAGE using a 10% minigel. Phosphorylation was detected by autoradiography and quantified using either a Bio-Rad model GS-363 PhosphorImaging system or a Bio-Rad model GS-700 imaging densitometer, as noted in the figure legends.

**Coupled Kinase Assay—**GST-SEK1 was isolated as described above for the SEK1 assay. The GST-SEK1-GSH-agrose complex was washed and incubated for 20 min at 30 °C in the kinase buffer with 2 μg of GST-JNK1 and 15 μCi of [γ-32P]ATP followed by incubation for 15 min in the presence of 1 μg of GST-c-Jun and an additional 15 μCi of [γ-32P]ATP. The reaction was stopped by adding Laemmli sample buffer, and the proteins were resolved by SDS-PAGE using a 10% minigel. Phosphorylation was detected by autoradiography.

**ERK Assay—**The assay for ERK activation is essentially the same as the SEK1 assay except that cells were transiently transfected with pEBG-GST-ERK1, the GST-ERK1-GSH-agrose complex was incubated in a kinase buffer with 1 μg of myelin basic protein instead of JNK1, and proteins were resolved using a 12% minigel.

**Dominant Negative Inhibitor Assay—**Cultures of COS7 cells were transiently transfected with 0.5 μg of pSirHA-JNK1 and either 1.5 μg/plate empty pEBG-GST vector or 1.5 μg/plate pEBG-GST-SEK1(K-R)/35-mm plate using LipofectAMINE. Cells were serum starved and treated with the appropriate agent as described for the SEK1 assay. Cells were washed and incubated in a kinase buffer (20 mM HEPES, pH 7.5, 20 mM β-glycerophosphate, 0.2 mM leupeptin, and 1 μM Na3VO4) and then centrifuged at 4 °C for 10 min at 14,000 × g. HA-JNK1 was immunoprecipitated from the supernatant with an anti-HA antibody (Boehringer Mannheim) and protein-G agarose (Sigma). Immunocomplexes were washed and incubated in a kinase buffer (20 mM HEPES, pH 7.5, 20 mM β-glycerophosphate, 10 mM p-nitrophenyl phosphate, 10 mM MgCl2, 1 mM DTT, 50 μM Na3VO4, and 20 μM ATP) in the presence of 2 μg of GST-c-Jun and 5 μCi of [γ-32P]ATP for 20 min at 30 °C. Proteins were resolved by SDS-PAGE using a 10% minigel. Phosphorylation was detected by autoradiography.

**Western Blot Analysis—**Cells were plated and treated with the appropriate agent as described above for the JNK assay. Cultures were washed with ice-cold phosphate-buffered saline and lysed in 50 mM β-glycerophosphate, pH 7.4, 25 mM NaCl, 2 mM EDTA, 1 mM DTT, 40 mM p-nitrophenyl phosphate, 0.2 mM PMSF, 0.02 mM leupeptin, and 1 μM Na3VO4. Equal amounts of protein from cell lysates were resolved by SDS-PAGE on a 10% minigel and then transferred to nitrocellulose paper (Amer sham Corp.). The blots were blocked in a Tris-buff ered solution containing 5% non-fat dried milk and 0.1% Tween-20. The primary antibody used was an anti-phospho-specific SEK1/KMK4 polyclonal antibody (New England Biolabs, Beverly, MA). The secondary antibody was a horseradish peroxidase-conjugated goat anti-rabbit polyclonal antibody (Sigma). Western blot analysis was carried out according to the protocol recommended by Amersham. The signal was detected using the ECL detection system purchased from Amersham.

**RESULTS**

**Palytoxin Activates JNK and SEK1—**Previous studies demonstrated that treatment of Swiss 3T3 fibroblasts with palytoxin stimulates the activation of JNK (5). To begin elucidating the signaling cascade by which palytoxin regulates JNK, we determined if palytoxin activates the JNK kinase SEK1. Fig. 1 shows that treatment of either COS7 or HeLa cells with 300 pM palytoxin stimulates the activation of JNK (5). To begin elucidating the mechanisms by which the signals stimulated by palytoxin are transduced through the cell. JNK is activated by dual phosphorylation of Thr-183 and Tyr-185 (14). Stress-activated protein kinase/extracellular signal-regulated kinase-1 (SEK1, also called JNKK and MKK4) has been identified as a kinase that can directly phosphorylate and activate JNK (15–17). Therefore, to elucidate further the signal transduction pathways activated by palytoxin, we investigated the role of SEK1 in the regulation of JNK by palytoxin. The results from these studies support an important role for SEK1 in the protein kinase cascade that transduces the novel sodium-dependent signals stimulated by palytoxin.
FIG. 1. Palytoxin activates JNK and SEK1 in COS7 and HeLa cells. Panel A, COS7 cells were incubated in the absence (Control) or presence of 300 nM palytoxin, 190 nM anisomycin, or hypertonic medium for 30 min, treated with UV light as described below, or incubated with 590 nM TNF-α or 200 nM PDBu for 15 min and then lysed and assayed for JNK activity as described under “Experimental Procedures.” For UV light treatment, the medium was removed from the cultures, which were then exposed to 40 J/m² UV for 6 s. The medium was then replaced, and the cells were incubated for 30 min before lysis. JNK activity is indicated by the phosphorylation of GST-c-Jun.

Panel B, COS7 cells were transiently transfected with pEBG-GST-SEK1, treated as described in panel A, then lysed and assayed for SEK1 activity as described under “Experimental Procedures.” SEK1 activity is indicated by the phosphorylation of GST-c-Jun.

Panel C, HeLa cells were assayed for JNK activity as described in panel A after incubation in the absence (Control) or presence of 300 nM palytoxin for 15 min, 200 nM PDBu for 15 min, or 190 nM anisomycin for 30 min. Panel D, HeLa cells were transiently transfected with pEBG-GST-SEK1, treated as described in panel C, then lysed and assayed for SEK1 activity as described in panel B. Panel E, HeLa cells were treated as described in panel C and then lysed. 75 μg of protein from cell lysates was resolved by SDS-PAGE and analyzed by Western blot using a phospho-SEK1-specific antibody. A nonspecific band is indicated by ns.

Sodium-dependent Activation of SEK1

Two types of experiments indicate that palytoxin regulates SEK1 (Fig. 1, B, D, and E). First, we determined if palytoxin could activate SEK1 when it was transiently expressed as the fusion protein GST-SEK1 in COS7 and HeLa cells (Fig. 1, B and D). Treatment of either COS7 or HeLa cells with palytoxin caused a dramatic increase in SEK1 activity relative to controls, as monitored by the phosphorylation of recombinant JNK (Fig. 1, B and D). Consistent with the results describing JNK activation, palytoxin activated SEK1 to an extent similar to anisomycin, hypertonic medium, UV light, or TNF-α in COS7 cells (Fig. 1B). Palytoxin also stimulated a dramatic increase in SEK1 activity in HeLa cells (Fig. 1D). PDBu caused minimal activation of SEK1 in COS7 and HeLa cells, which corresponds to its minimal activation of JNK (Fig. 1, B and D). These results demonstrate that palytoxin can stimulate signals that activate SEK1.

Next, we investigated whether palytoxin treatment could regulate endogenous SEK1 (Fig. 1E). SEK1 is activated by phosphorylation of a serine residue and a threonine residue located in the kinase subdomain VIII (15, 16, 21). We monitored the effect of palytoxin on phosphorylation of endogenous SEK1 using Western blot analysis and a polyclonal antibody specific for the phosphothreonine located in subdomain VIII in human SEK1 (Fig. 1E). The phospho-SEK1-specific antibody reacted with a band (P-SEK1) whose molecular mass was similar to that of SEK1 (45 kDa) (22), which appeared in lanes containing protein from palytoxin-treated HeLa cells and HeLa cells treated with the established SEK1 activator anisomycin (15) (Fig. 1E). This band did not appear in the lanes containing protein from control cells or cells treated with PDBu (Fig. 1E). It is likely that Western blot analysis is not sensitive enough to detect the low level of SEK1 phosphorylation which would correspond to the lower level of SEK1 activity stimulated by PDBu. The nonspecific band that appears in the Western blot indicates that similar amounts of protein were loaded in each lane. We were not able to detect the phospho-SEK1 band in COS7 cells treated with either palytoxin or anisomycin using Western blot analysis. Two likely explanations, which require further investigation, are that 1) COS7 cells have a much lower level of SEK1 than HeLa cells or 2) this antibody does not cross-react with the SEK1 expressed in COS7 cells, which is a monkey cell line. In essence, the results from the Western blot correspond to the results obtained monitoring SEK1 activity in HeLa cells using the transient expression assay (Fig. 1D).

To investigate the relationship between the activation of SEK1 and JNK by palytoxin, we first compared the kinetics of activation of these two kinases in COS7 and HeLa cells treated with palytoxin (Fig. 2). The time courses of palytoxin-stimulated SEK1 and JNK activation were similar when measured in either COS7 cells (Fig. 2A) or HeLa cells (Fig. 2B). We detected an increase in SEK1 activation relative to background (the zero time point) within 5 min of palytoxin treatment in both COS7 and HeLa cells (Fig. 2). An increase in JNK activity was also detected within 5 min of palytoxin treatment in HeLa cells (Fig. 2B). COS7 cells have a relatively high basal level of JNK activity compared with HeLa cells (compare the Control lanes in Fig. 1, A and C). Therefore, it was difficult to detect a
were treated with 300 pM palytoxin for the indicated times and then lysed, and assayed for JNK activity as described in the "Experimental Procedures." Transiently expressed GST-SEK1 was isolated from cell lysates using GSH-agarose beads and then incubated for 20 min at 30 °C in a kinase buffer with [γ-32P]ATP in the absence (-) or presence (+) of GST-JNK. Proteins were separated by SDS-PAGE using a 10% minigel, and phosphorylation of GST-JNK was detected by autoradiography. For lanes 5–10, GST (lanes 5 and 6) or GST-SEK1 (lanes 7–10) was isolated as described above and incubated in the absence (-) or presence (+) of GST-JNK. GST-c-Jun and additional [γ-32P]ATP were then added, and this mixture was incubated for 15 more min. Proteins were resolved by SDS-PAGE as described above. Phosphorylation of GST-JNK indicates SEK1 activation. Phosphorylation of GST-c-Jun indicates JNK activation. The data shown are representative of two independent experiments.

Fig. 3. Palytoxin-stimulated SEK1 can phosphorylate and activate JNK. COS7 cells were transiently transfected with either pEBG-GST-SEK1 (+) or empty pEBG-GST vector (-). Cells were incubated for 30 min in the absence (-) or presence (+) of 300 pM palytoxin. The coupled kinase assay was conducted as described under "Experimental Procedures." Transiently transfected with pEBG-GST-SEK1 were treated in the absence (-) or presence (+) of 300 pM palytoxin. Lysates using GSH-agarose beads and then incubated for 20 min at 30 °C in a kinase buffer with [γ-32P]ATP in the absence (-) or presence (+) of GST-JNK. Proteins were separated by SDS-PAGE using a 10% minigel, and phosphorylation of GST-JNK was detected by autoradiography. For lanes 5–10, GST (lanes 5 and 6) or GST-SEK1 (lanes 7–10) was isolated as described above and incubated in the absence (-) or presence (+) of GST-JNK. GST-c-Jun and additional [γ-32P]ATP were then added, and this mixture was incubated for 15 more min. Proteins were resolved by SDS-PAGE as described above. Phosphorylation of GST-JNK indicates SEK1 activation. Phosphorylation of GST-c-Jun indicates JNK activation. The data shown are representative of two independent experiments.

Fig. 2. Kinetics of palytoxin-stimulated SEK1 and JNK activation. Panel A, time course of palytoxin-stimulated SEK1 (open squares, dashed lines) and JNK (closed circles, solid lines) activity in COS7 cells. Panel B, time course of palytoxin-stimulated SEK1 (open squares, dashed lines) and JNK (closed circles, solid lines) activity in HeLa cells. Cultures transiently transfected with pEBG-GST-SEK1 were treated with 300 pM palytoxin for the indicated times, lysed, and assayed for SEK1 activity as described under "Experimental Procedures." Confluent, serum-starved cultures were incubated with 300 pM palytoxin for the indicated times and then assayed for JNK activity as described under "Experimental Procedures." Phosphorylation was quantified using a Bio-Rad model GS-363 PhosphorImaging system. Fold kinase activity was determined by dividing the PhosphorImager units obtained for each time point by the PhosphorImager units obtained for the zero time point. The data points shown in the major graphs are the mean ± S.D. of results from either three independent experiments (SEK1) or four independent experiments (JNK). The data points shown in the inset are the mean ± S.D. of triplicate plates. Panel C, HeLa cells were treated with 300 pM palytoxin for the indicated times and then lysed. 65 µg of protein from cell lysates was resolved by SDS-PAGE and analyzed by Western blot using a phospho-SEK1-specific antibody. A non-specific band is indicated by ns. The Western blot shown is representative of two independent experiments.

small increase in JNK activity at early time points in COS7 cells. A more detailed temporal analysis revealed that palytoxin-stimulated SEK1 activation could be detected earlier than palytoxin-stimulated JNK activation in HeLa cells (Fig. 2B, inset). The SEK1 assay represented in the inset of Fig. 2B had extremely low background. Consequently, the fold stimulation of SEK1 activation is much greater than indicated by the other kinase assays shown. Nevertheless, when we plotted the SEK1 assay and JNK assay on the same graph, using different y axes, the time courses of SEK1 and JNK activation appeared to be parallel. SEK1 activation was detected at the 1-min time point (17 ± 2.7 fold over background) and increased slowly through 4 min. JNK activation was first detected at the 3-min time point (1.6 ± 0.2 fold over background) and increased slightly by 4 min. Both SEK1 and JNK activity increased dramatically after 4 min. In both COS7 and HeLa cells, JNK and SEK1 activity remained elevated through 60 min of palytoxin treatment (Fig. 2, A and B). Results from Western blot analysis using the phospho-SEK1-specific antibody corresponded to the results from the transient expression assay (Fig. 2C). We detected a faint band (P-SEK1) within 5 min of palytoxin treatment of HeLa cells. The intensity of this band increased dramatically within 10 min of palytoxin treatment and was sustained through 60 min of treatment. The kinetics of palytoxin-stimulated JNK and SEK1 activation are consistent with a role for SEK1 in the regulation of JNK by palytoxin.

The time course of palytoxin-stimulated SEK1 and JNK activation in COS7 cells appeared to lag behind that of HeLa cells (compare Fig. 2, A and B). For example, kinase activity appeared to plateau later in COS7 cells than in HeLa cells. A likely explanation for the more rapid response observed in HeLa cells is that this cell line is more sensitive to palytoxin than COS7 cells. We have shown previously that the time course of palytoxin action is dose-dependent (23). Consequently, the greater the dose of palytoxin, the more rapidly the response is detected and the more rapidly the response reaches a maximal point. HeLa cells may be more sensitive to palytoxin than COS7 cells because of a difference in affinity for palytoxin binding or a difference in concentration of effectors that are important for palytoxin action.

Palytoxin Activates the SEK1/JNK/c-Jun Protein Kinase Cascade—We used a coupled kinase assay to confirm that palytoxin-activated SEK1 can activate JNK (Fig. 3). The coupled kinase assay involves reconstituting the SEK1/JNK/c-Jun pathway in vitro. Transiently expressed GST-SEK1 was isolated from control cells (Fig. 3, odd lanes) or palytoxin-treated
COS7 cells (Fig. 3, even lanes). The GST-SEK1 was then incubated with a recombinant JNK fusion protein, GST-JNK, in the presence of [γ-32P]ATP. The GST-SEK1 isolated from palytoxin-treated cells phosphorylated GST-JNK to a far greater extent than the GST-SEK1 isolated from control cells (Fig. 3, compare lanes 1 and 2), indicating that palytoxin stimulates SEK1 activation. Palytoxin did not appear to stimulate the autophosphorylation of SEK1 (lanes 4 and 10). Although auto-phosphorylation of SEK1 has been reported (15), we and others do not detect it (22). This may be because of differences in assay conditions or specific SEK1 activators. Lanes 7 and 8 show that palytoxin-stimulated SEK1 can both phosphorylate and activate JNK. When GST-SEK1, isolated as described above, was incubated with [γ-32P]ATP in the presence of both GST-JNK and GST-c-Jun, phosphorylation of GST-JNK and GST-c-Jun was far greater in the mixture containing GST-SEK1 isolated from palytoxin-treated cells than GST-SEK1 isolated from control cells (Fig. 3, compare lanes 7 and 8). GST alone did not stimulate the phosphorylation and activation of GST-JNK (lanes 5 and 6). When GST-JNK was omitted from the mixture, no phosphorylation of GST-c-Jun was detected (Fig. 3, lanes 9 and 10), indicating that SEK1 does not directly phosphorylate GST-c-Jun. Altogether, these data indicate that palytoxin stimulates the activation of SEK1, which phosphorylates and activates JNK, which in turn phosphorylates c-Jun.

Palytoxin Activates JNK through an SEK1-dependent Pathway—Results from complementary experiments that involved transient expression of a dominant negative mutant of SEK1 support the conclusion that palytoxin can activate JNK through a protein kinase cascade that requires activation of SEK1 (Fig. 4). SEK1(K-R) is a kinase-inactive form of SEK1, in which Lys-129 in the ATP binding site of the kinase has been changed to an arginine (15). SEK1(K-R) presumably blocks SEK1 activation by sequestering cellular components that interact with this kinase (15). Palytoxin stimulated the activation of transiently expressed hemagglutinin-tagged JNK (HA-JNK) in COS7 cells, as did anisomycin (Fig. 4A). Coexpression of SEK1(K-R) effectively blocked activation of HA-JNK by palytoxin (Fig. 4A). Likewise, coexpression of SEK1(K-R) inhibited the activation of HA-JNK by anisomycin (Fig. 4A), which has been shown to regulate JNK through an SEK1-dependent pathway (15). Western blot analysis showed that expression of SEK1(K-R) does not cause a decrease in the expression of HA-JNK (Fig. 4A). In contrast to its efficient inhibition of JNK activation, SEK1(K-R) did not block activation of ERK by PDBu (Fig. 4B). PDBu stimulated robust activation of transiently expressed GST-ERK in COS7 cells regardless of the expression of SEK1(K-R). The observation that SEK1(K-R) does not block activation of ERK by PDBu in COS7 cells extends what is known from other studies, which showed that SEK1(K-R) does not block the activation of ERK by Abl in 293 cells (15). In contrast to PDBu, a 5-min treatment with 100 μM palytoxin did not activate ERK. Incubation of COS7 cells with 300 μM palytoxin for longer times also did not activate ERK. 2 Altogether, these data indicate that palytoxin can activate JNK through a signaling pathway that requires SEK1.

Palytoxin Activates SEK1 and JNK through a Sodium-dependent Pathway—Previously we had shown that palytoxin activates JNK through a sodium-dependent signal transduction pathway in Swiss 3T3 fibroblasts (5). Therefore, we wanted to investigate the role of sodium in the activation of SEK1 by palytoxin. Two lines of evidence indicate that sodium influx plays an important role in the regulation of SEK1 by palytoxin (Fig. 5). First, when HeLa cells were incubated in a sodium-free medium, palytoxin could no longer stimulate the activation of endogenous JNK (Fig. 5A) or the activation of transiently expressed GST-SEK1 (Fig. 5B). Likewise, Western blot analysis showed that the palytoxin-stimulated band that reacts with the phospho-SEK1-specific antibody (P-SEK1) does not appear when sodium is omitted from the medium (Fig. 5C). By contrast, anisomycin was able to activate JNK and SEK1 in both the presence and absence of extracellular sodium, indicating that removing sodium from the medium does not nonspecifically block activation of JNK and SEK1 (Fig. 5). Second, gramicidin, which is a pore-forming sodium ionophore, can mimic the effect of palytoxin on JNK and SEK1 (Fig. 5). Gramicidin stimulates the activation of JNK and transiently expressed GST-SEK1 (Fig. 5, A and B). Gramicidin also stimulates the appearance of a band that reacts with P-SEK1, which also appears with palytoxin treatment but does not appear in the control lane (Fig. 5C). Gramicidin appears to activate endogenous JNK and SEK1 more effectively than transiently expressed SEK1. The reason for this is not clear. One possibility is that transiently expressed SEK1 has a subcellular location that is different from that of endogenous SEK1. For example, the mechanism by which gramicidin interacts with the cell and activates SEK1 may require that the kinase occupy a specific subcellular location. Like palytoxin, the activation of JNK and SEK1 by gramicidin was inhibited in the absence of extracellular sodium (Fig. 5). These results indicate that palytoxin stimulates a sodium-dependent signal transduction pathway that can activate the SEK1/JNK/c-Jun protein kinase cascade.

DISCUSSION

The results presented here demonstrate that SEK1 is an important mediator of the sodium-dependent signal transduc-
palytoxin activates SEK1 suggests that the sodium-dependent signals stimulated by this tumor promoter might also activate p38. Two other kinases that directly phosphorylate and activate p38, MKK3 and MKK6, have also been identified (16, 29, 30). MKK3 and MKK6 do not appear to regulate JNK, however (16, 29, 30). Studies are in progress to investigate the regulation of p38 by palytoxin.

Biochemical studies suggest that SEK1 is not the only JNK kinase (22, 25, 26). Moriguchi et al. (22) fractionated cell lysates from rat fibroblast 3T3 cells treated with hyperosmotic medium and assayed column fractions for JNK kinase activity. In this cell type, hyperosmotic medium appears to activate at least two JNK kinases that do not elute with SEK1 or cross-react with antibodies that react with SEK1. By contrast, similar cell lysate fractionation studies by Meier et al. (25) indicated that in rat PC12 cells the only JNK kinase activated by hyperosmotic medium, UV light, anisomycin, and sodium arsenite was SEK1. Results from this same study indicated, however, that in human KB epidermal carcinoma cells, hyperosmotic medium and anisomycin can activate both SEK1 and another JNK kinase. This alternate JNK kinase does not elute with SEK1, is not immunoprecipitated by an antibody that reacts with SEK1, and is not activated by MEK kinase-1 (MEKK1) in vitro. Furthermore, interleukin-1 and UV light did not appear to activate SEK1 in KB cells, but they did appear to activate a JNK kinase that is potentially distinct from that activated by hyperosmotic medium and anisomycin in this cell type. Fractionation of lysates from macrophages treated with lipopolysaccharide revealed a major JNK kinase peak that coeluted with SEK1 and a minor peak that did not cross-react with antibodies that react with SEK1 (26). Collectively, these studies suggest that specific cell types may express different JNK kinases, which in turn are sensitive to different types of signals. We are not aware of studies that have investigated the possible presence of JNK kinases other than SEK1 in COS7 or HeLa cells. Therefore, although the results presented here clearly demonstrate that palytoxin regulates JNK through an SEK1-dependent pathway, it remains to be determined if palytoxin can also regulate JNK through an SEK1-independent protein kinase cascade.

The observation that palytoxin stimulates SEK1 activation to an extent similar to that of anisomycin, hyperosmotic medium, UV light, and TNF-α supports a central role for SEK1 in mediating a remarkably diverse range of signals. At least four different kinases have been identified which can phosphorylate and activate SEK1, suggesting that different types of signal transduction pathways may regulate SEK1 through modulation of specific upstream kinases. MEKK1, which was originally identified as a member of the ERK protein kinase cascade (31), was subsequently found to phosphorylate and activate SEK1 preferentially (21, 32). MEKK1 can be activated by Ras-dependent signaling pathways (32) and is also activated by osmotic shock (33). Another member of the MEKK family which preferentially activates the SEK1/JNK pathway, MEKK2, has recently been cloned (34). Another potential upstream activator of SEK1 is the mixed lineage kinase SPRK (35). The types of signals that activate SPRK remain to be identified. The serine/threonine kinase Tpl-2, which is a proto-oncogene product, can also phosphorylate and activate SEK1 (36). Tpl-2 can activate both the ERK and JNK pathways. The signals and upstream activators of Tpl-2 are not known. Based on the observation that palytoxin selectively activates JNK, but not ERK, Tpl-2 is not likely to mediate palytoxin-stimulated regulation of JNK. Another serine/threonine kinase called the apoptosis signal-regulating kinase-1 has recently been cloned and shown to activate SEK1 in vitro (37). Apoptosis...
Sodium-dependent Activation of SEK1

signal-regulating kinase-1 is activated by TNF-α and appears to play a role in TNF-α-induced apoptosis in Jurkat and 293 cells. The mechanism by which palytoxin regulates SEK1 is currently being investigated.

The mechanisms by which sodium influx activates the SEK1/JNK/c-Jun pathway are not yet clear. Although sodium influx may cause a type of osmotic stress, the response of the cell to this type of stress is likely to differ from the cellular response to incubation in hyperosmotic medium. For example, whereas sodium influx generally causes cell swelling, incubation of cells in hyperosmotic medium causes cells to shrink (38, 39). Therefore, the mechanisms by which the cell attempts to compensate for these distinct types of change in cell volume, for example through regulation of ion pumps, are likely to differ significantly for sodium influx and incubation in hyperosmotic medium. This suggests that the signals that trigger activation of the SEK1/JNK/c-Jun cascade by palytoxin differ from those stimulated by incubation of cells in hyperosmotic medium.

Support for this idea comes from the observation that hyperosmotic medium can activate both ERK and JNK (33), whereas palytoxin selectively activates JNK but not ERK (5). Preliminary work in our laboratory indicates that ouabain, which like palytoxin interacts with the Na⁺ /K⁺-ATPase, can activate JNK. We are currently investigating whether ouabain also regulates JNK through an SEK1-dependent pathway.

This study represents a significant step toward elucidating the mechanisms by which palytoxin-stimulated signals are transduced by the cell. For example, the results presented here suggest that blocking JNK activation through expression of the SEK1 dominant negative mutant may be a useful approach for studying the role of JNK in palytoxin action. This approach has been used to investigate the role of JNK in cell death induced by heat shock and by the cancer chemotherapy drug cis-platinum (40). The pivotal work that identified protein kinase C as the phorbol ester receptor (4) first suggested that tumor promoters may contribute to carcinogenesis by subverting the signaling pathways that regulate cell function. The observation that the non-TPA-type tumor promoter palytoxin activates the SEK1/JNK/c-Jun pathway suggests that aberrant regulation of this protein kinase C-independent signaling pathway may also be important in carcinogenesis.

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Regulation of a c-Jun Amino-terminal Kinase/Stress-activated Protein Kinase Cascade by a Sodium-dependent Signal Transduction Pathway
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