Stimulus-specific Requirements for MAP3 Kinases in Activating the JNK Pathway*

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Mitogen-activated protein kinases (MAPKs) are activated by numerous ligands typically through a protein kinase cascade minimally composed of the MAPK in series with a MAP2 kinase (MAP2K) and a MAP3K. This arrangement is thought to confer specificity and appropriate kinetic properties on the activation of MAPKs in response to physiological stimuli. Surprisingly, more than a dozen MAP3Ks have been identified that activate the c-Jun N-terminal kinases (JNKs) when overexpressed, but there is no clear understanding of which kinases actually mediate JNK activation by ligands. Here, we use double-stranded RNA-mediated interference of gene expression to reveal the explicit participation of discrete MAP3Ks in controlling JNK activity by multiple stimuli. Maximal activation of JNK by lipopolysaccharide requires the MAP3K TAK1. On the other hand, sorbitol requires expression of four MAP3Ks to cause maximal JNK activation. Thus, we demonstrate that specific stimuli use different mechanisms to recruit distinct MAP3Ks to regulate the JNK pathway.

An early model represented the events leading to stimulation of MAP kinases (MAPKs) as the sequential activation of a linear series of steps from ligand through receptor and signaling intermediates to MAPK (1, 2). This paradigm arose in part from studies of the pheromone mating pathway and other MAPK pathways in Saccharomyces cerevisiae, and the view was further substantiated by early work on regulation of ERK2 by growth factors. The paradigm was revised as it was recognized that diverse receptors may use different proximal signaling mechanisms to funnel into the three-protein kinase cascade, which became known as a MAPK module (3–5). In this three-tiered module, MAPKs are controlled by a MAP kinase kinase kinase (MAP3K) which activates a MAP kinase kinase (MAP2K) of a two-kinase module (14), and the apparently relaxed specificity of the MAP3Ks is an intrinsic property of the signaling, in vitro MAP3Ks display overlapping specificity in that they phosphorylate and activate MAP2Ks from different modules (9–13). These observations require a further re-evaluation of the model. Two possibilities, which are not mutually exclusive, might account for the diversity of the MAP3Ks. One possibility is that individual MAP3Ks may be coupled exclusively to activation by a specific type of ligand, i.e. a different MAP3K for each type of ligand. This would imply that a specific three-kinase module (containing the same MAP2K and MAPK) would be dedicated to mediate the functions of each ligand type. In this case, the apparently relaxed specificity of the MAP3Ks might be attributable to an artifact of overexpression.

To test these ideas, we examined the control of the MAPK c-Jun N-terminal kinase (JNK) in Drosophila S2 cells. We searched the Fly Data Base to find MAP3Ks and other proteins implicated upstream in regulating JNK in mammalian cells and the fly and asked if any of these molecules are involved in JNK activation in S2 cells using RNA interference (RNAi) (15). We found that the pro-inflammatory molecule lipopolysaccharide (LPS) uses a single MAP3K, whereas the osmotic stresses sorbitol and NaCl use multiple MAP3Ks to activate Drosophila JNK. These and other findings support both models above; LPS targets a discrete MAP3K module, whereas signals from osmotic stresses target multiple MAP3Ks to converge on a conserved MAP2K-MAPK module. Thus, distinct stimuli regulate JNK through disparate signal transduction mechanisms.

EXPERIMENTAL PROCEDURES

Cell Culture—Drosophila Schneider (S2) cells were cultured in 1 × Drosophila serum-free medium (DSFM, Invitrogen) supplemented with 20 mM 1-glutamine (Invitrogen) and 0.1% gentamicin (Sigma) in 100-mm dishes at room temperature.

Reverse Transcriptase (RT)-PCR—Total RNA was isolated from 10⁶ S2 cells using the High Pure RNA Isolation Kit (Roche Molecular Biochemicals). First-strand cDNA was synthesized from total RNA using the Superscript™ First-strand Synthesis System for RT-PCR.
Fig. 1. Activation of JNK in S2 cells. S2 cells were cultured for 3 days and then left untreated (control) or exposed to 0.4 M sorbitol for 30 min, 50 μg/ml LPS for 10 min, 10 μg/ml anisomycin for 30 min, UV for 80 J/m², 5 mM taxol for 30 min, 2 mM nocodazole for 30 min, 10 μg/ml insulin for 5 min, 1 mM phorbol 12-myristate 13-acetate (PMA) for 5 min, or 20 ng/ml tumor necrosis factor (TNF)-α for 15 min. Whole cell lysates were prepared and analyzed for activation of JNK. Top panel, immunoblot of lysates with JNK antibody, indicating that equal amounts of JNK were loaded. Middle panel, immunoblot of lysates with phospho-JNK antibody. Bottom panel, immunoprecipitated JNK assayed with glutathione S-transferase (GST)-c-Jun as substrate; far right lane, recombinant active JNK as a positive control.

(A) To choose candidates for RNAi, the BDGP-FlyBase (a data base of the Drosophila genome) was searched to identify fly homologs of the indicated proteins (Table I); full-length sequences of candidates were aligned with mammalian proteins to determine sequence similarities. The primers for RT-PCR are shown in Table I and include a 5′ T7 RNA polymerase-binding site (TAATACGACTCACTATAGGGAGA). The indicated primers were used both in amplifying DNA template for transcription of dsRNA and in RT-PCR for detecting the expression of genes. The following PCR program was used: an initial denaturation at 95 °C for 5 min followed by 30 cycles of amplification (95 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min) and an additional 10 min at 72 °C. With the exception of the small G proteins for which fragments were 500 bp, other fragments were ~700 bp.

Double-stranded RNA (dsRNA)—With PCR products as templates, the MEGASCRIPT T7 transcription kit (Ambion) was used to produce RNA according to manufacturer’s protocols. RNA products were precipitated with ethanol and resuspended in DEPC/water. dsRNA was annealed at 65 °C for 30 min following slow cooling to room temperature. RNA concentration was measured at A260 and in 1% agarose gels; dsRNAs were stored at −20 °C.

RNA Interference (RNAi) in Drosophila S2 Cell Culture—RNAi was conducted according to the Dixon laboratory protocol (16). 10° S2 cells were plated in 1 ml of DSFM per well of 6-well culture dishes. dsRNA was added at room temperature for 2 h, followed by 2 ml of fresh DSFM.

Cell Lysis and Immunoblotting—After 3 days of incubation, dsRNAs were lysed in 0.2 ml of RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 10 mM NaF, 0.1% SDS, 2 mM EDTA, 1 mM Na3VO4, 25 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride) on ice for 10 min. Clarified lysates were collected by microcentrifugation for 15 min at 4 °C. Protein concentration was measured by the Bradford assay using bovine serum albumin as standard. 30 μg of lyase protein was resolved by SDS-PAGE (10% gels) and transferred to nitrocellulose membranes. Antibodies that recognize JNK from multiple species (O977 (17)), doubly phosphorylated JNK (Promega), doubly phosphorylated p38 (New England Biolabs), and Ras (Oncogene Research) were used for immunoblotting as described (18).

Immunoprecipitation and Protein Kinase Assays—Endogenous JNK was immunoprecipitated from 300 μg of lysate protein with antibody O977 and 30 μl of protein A-Sepharose. Beads were washed three times with 1 ml of wash buffer (1 mM NaCl, 0.25% Tris-HCl, pH 7.4, 0.1% Triton X-100, 0.1% sodium deoxycholate), and one time with 1 ml of 10 mM HEPES, pH 8.0. Beads were resuspended in 20 mM HEPES, pH 7.8, 10 μM ATP (1 μCi of [γ-32P]ATP), 10 mM MgCl2, and 10 mM β-glycerophosphate, and incubated with 1 μg of glutathione S-transferase-c-Jun for 30 min at 30 °C.

RESULTS

Activation of JNK in Drosophila S2 Cells—In Drosophila the JNK pathway has been found to be essential not only in cell shape changes required for dorsal closure during embryonic development but also in cell polarity and immunity in the adult (19). Mutants have been generated with defects in dorsal closure, identifying molecules necessary for this process. Several of these molecules are likely to lie upstream in the JNK pathway; these include the following: MKK7 (hep), the MAP3Ks, TAK1 and MLK2 (slipper); misshapen (Msn), a MAP4K or-
MKK7 and MKK4 Are Both Required to Activate JNK in S2 Cells—Examination of the Drosophila genome sequence data base shows that there are two MAP2Ks, MKK7 (Hemipterous, Hep) and MKK4, that are expected to work on the JNK pathway. Published work (26–29) on these enzymes supports this idea. To examine the explicit contribution of these kinases to JNK activation, we inhibited their expression using RNAi (Table I). JNK was also knocked down as a control. Reducing the

expression of either MKK4 or MKK7 (Fig. 2A) only partially inhibited JNK activation in response to LPS or sorbitol (Fig. 2, B and C). If both MKK7 and MKK4 were suppressed in the same cells, JNK activation was inhibited below the limit of detection. These results indicate that MKK7 and MKK4 must both be present to elicit maximal activation of JNK in response to these stimuli (Fig. 2, B and C).

TAK1 Is the MAP3K in the LPS-stimulated JNK Pathway—Multiple mammalian MAP3Ks have been linked to JNK activation, based on in vitro reconstitution with MKK4s 4 and 7 or by overexpression in transfected cells. Analysis of the fly data base revealed six fly MAP3Ks, transforming growth factor-β-activated kinase (TAK1) (30), mixed lineage kinase (e.g. MLK2/MST) (31) and MLK3/PTK1/SPRK (32, 33)), dual leucine zipper kinase (DLK) (34), apoptosis signal-regulating kinases (e.g. ASK1/MAP3K5 (35, 36) and ASK2/MAP3K6 (37)), MAP/ERK kinase (MEKK) (4), and p38, that are homologous to mammalian MAP3Ks implicated in the JNK pathway. Expression of two of these, PKN and DLK, was not detected in S2 cells (data not shown). RNAi-mediated silencing of the other four MAP3Ks was confirmed by RT-PCR (Fig. 3A). Stimulation of JNK activity by LPS was unaffected by silencing of MLK2, ASK1, or MEKK1. On the other hand, no JNK activity was detected in lysates of cells in which expression of TAK1 was suppressed (Fig. 3B), indicating that TAK1, but none of the other MAP3Ks tested, is required for activation of JNK by LPS. The elimination of these MAP3Ks had no effect on the activity of the p38 MAPK (Fig. 3B).

Multiple MAP3Ks Transduce the Sorbitol Signal to JNK in S2 Cells—In contrast to signaling by LPS, no single MAP3K was essential for JNK activation by sorbitol (Fig. 3C). This suggested either that none of the four MAP3Ks tested were involved in the sorbitol signal to JNK or that multiple MAP3Ks work in this pathway. To evaluate the possibility that multiple MAP3Ks were involved in JNK activation, S2 cells were treated with the four dsRNAs in the indicated combinations (Fig. 4A). Suppression of the expression of all four of the MAP3Ks simultaneously blocked JNK activation by sorbitol (Fig. 4B). Treatment of cells with two or three of the four dsRNAs reduced but did not completely block stimulation of JNK by sorbitol (Fig. 4B and not shown). In experiments mentioned below, as many as six different dsRNAs were added to the cells at one time with no loss of phospho-JNK signal. Therefore, the loss of signal apparent in Fig. 4B is not due to a nonspecific effect on JNK.

Ras Is Required for Maximal Activation of the JNK Pathway by Sorbitol—Activation of the JNK pathway has been reported to involve the small GTPases Ras, Rac, Cdc42, and Rho in a cell-type and stimulus-dependent manner (39–45). To test whether small GTPases are involved in JNK activation in S2

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**Table I**

<table>
<thead>
<tr>
<th>Protein (name/orthology)</th>
<th>CG no.</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>DBSK/JNK</td>
<td>5680</td>
<td>GCCGGGAAAGGAACTGGG (272–289)</td>
<td>TCGACATCATACACAC (963–980)</td>
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<tr>
<td>DHEP/MKK7</td>
<td>4353</td>
<td>GTCGCAATCTTGGAGGCC (36–53)</td>
<td>ACATCCAGATCAACTGGG (720–737)</td>
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<tr>
<td>DMEKK4/MKK4</td>
<td>9728</td>
<td>AAGGCAGTATTGCAAGAA (1086–1103)</td>
<td>GCCGACGGTGGAGCTTG (1072–1090)</td>
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<tr>
<td>DTKAK1/TAK1</td>
<td>1388</td>
<td>GATGACCAAAACTTCCGCG (507–524)</td>
<td>GGGCGGAGGCCGACCTG (558–576)</td>
</tr>
<tr>
<td>DPK392B/ASK1</td>
<td>4720</td>
<td>GACATGCTCTGGCCTGTC (857–874)</td>
<td>AAAGGCTGAGGAGCTTG (1527–1543)</td>
</tr>
<tr>
<td>DMLK2/MKK2</td>
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<td>2849</td>
<td>AGCAGAAGGGCAGCAGCC (4–21)</td>
<td>CCGGAGTTAGGGAGG (458–475)</td>
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cells, seven small G proteins from the fly, counterparts of mammalian Ras, Ral, and Rho family members, were silenced by RNAi. Inhibiting the expression of the small G proteins, individually or in combinations, had no influence on JNK activation by LPS (data not shown); this finding is consistent with studies in mammalian cells that suggest that pro-inflammatory cytokines act through receptor-adaptor-MAP3K complexes to activate JNK (46).

In contrast, maximal stimulation of JNK activity by sorbitol required Ras (Fig. 5A). Inhibition of JNK was proportional to the amount of dsRNA directed at Ras incubated with the cells (not shown). No consistent effects of the other small G proteins were detected, either separately or in combination (data not shown). We probed the mechanism of JNK activation by the osmotic stress NaCl to determine whether there are common elements in signal transduction by osmotic perturbation. As was the case for sorbitol, no single MAP3K was essential to prevent JNK activation by NaCl (0.4 M, 10 min), but suppression of all four greatly reduced JNK activation by NaCl (Fig. 5B), indicating that NaCl, like sorbitol, uses multiple MAP3Ks to activate JNK. Suppression of Ras, however, had little or no effect on JNK activation by NaCl, indicating that NaCl, unlike sorbitol, does not activate JNK primarily through Ras (Fig. 5A).

**DISCUSSION**

JNKs are also known as stress-activated protein kinases because of their sensitivity to environmental stresses (e.g. hyperosmolarity and radiation), pro-inflammatory cytokines (e.g. tumor necrosis factor-α and interleukin-1), and biosynthetic inhibitors (e.g. anisomycin) (6, 47, 48). A variety of agents stimulate the JNK pathway, yet the intracellular signal transduction pathways that lead to JNK activation in response to any particular stimulus have not been determined. In mammalian cells, MKK4 and MKK7 are the two MAP2K family members that directly phosphorylate JNK based on extensive biochemical studies and gene disruption; they may have discrete biological functions or act simultaneously in JNK activation (49–54). In contrast, defining the relationships of MAP3Ks to the stress-sensitive MAPKs has been a daunting task, because of their broad specificities in vitro and when overexpressed. These MAP3Ks include MEKKs 1–4 (10, 12, 13, 38), MLK2 and MLK3 (31), DLK (34), MAP three kinase (MTK1) (10, 38, 55, 56), Tpl-2/Cot (57), TAK1 (30), and ASK1 and ASK2 (35–37).

Although we assume these MAP3Ks act in the JNK pathway as MAP2K kinases, they may also have other functions that contribute to pathway control (e.g. see Ref. 58).

Here, by using RNAi, we have been able to define the explicit
contributions of MAP3Ks and MAP2Ks to activation of the JNK cascade by three different agents in a cell culture system (Fig. 6). At the MAP2K level, both MKK7 and MKK4 are required for maximal JNK activation by either LPS or sorbitol, consistent with their having non-redundant contributions to JNK activation. In vitro experiments have suggested that MKK4 selectively catalyzes the phosphorylation of the tyrosine of JNK, whereas MKK7 catalyzes phosphorylation of the threonine of JNK (52); both residues must be phosphorylated to maximally activate it (39). Our results suggest that the presence of either of the MAP2Ks is sufficient to mediate phosphorylation of both JNK residues, although the amount of JNK activated is reduced by the absence of either.

In contrast to the MAP2K level, at the MAP3K level, stimuli use different MAP3Ks to mediate JNK activation. Remarkably, only TAK1 is required for LPS-stimulated JNK activity. Preliminary experiments in HeLa cells also suggest a significant role for TAK1 in regulating the JNK pathway.² The requirement for a single MAP3K fits the model in which individual signals are linked to single MAP3Ks. Interestingly, TAK1 was originally identified as an activator of p38 MAPK and has recently been shown to be a major IκB kinase, implicating it in control of NF-κB (30, 59). Our studies reveal that it has a prominent role in the JNK pathway, bolstering the idea that activation of TAK1 will impact multiple intracellular signaling systems.

On the other hand, four MAP3Ks contribute to JNK activation by sorbitol or NaCl. Although sorbitol and NaCl are widely studied as perturbations because of their significance as pathophysiologic agents, for example in diabetes (60), few studies explore how they influence signaling pathways (61). In this study we determined that both agents activated multiple MAP3Ks through different upstream mechanisms as discussed further below. Despite the fact that sorbitol has major effects on cells via osmotic stress, some sorbitol-stimulated signal transduction events may be mediated through effects on membrane receptors.³ Thus, the effects of sorbitol may be representative of the actions of other more conventional ligands. If this is so, the findings here also support the second model in which single ligands may employ multiple MAP3Ks. The required contribution of multiple MAP3Ks may be important to couple a more diverse group of signaling pathways to the regulatory events induced by sorbitol or may allow quantitatively different contributions from a largely overlapping set of pathways.

In mammalian cells, small GTPase proteins are important upstream components in the JNK signaling cascades (62–64). We found no evidence linking any small G proteins to activation of JNK by LPS. In contrast, the small G protein Ras made a demonstrable contribution to JNK activation by sorbitol. This linkage recapitulates the linkage between Ras and c-Jun in oncogenesis and the original identification of JNK as a Ras-sensitive regulator of c-Jun (65). We considered the possibility that the dependence on small G proteins such as Ras may be a general feature of agents that activate multiple MAP3Ks. To test this idea, we compared the requirements for signaling by sorbitol and NaCl to determine whether Ras is a common intermediate. JNK activation by both agents was mediated by multiple MAP3Ks; however, whereas the upstream signal from sorbitol is conveyed to the kinase cascade by Ras, NaCl activates JNK in a manner largely or entirely independent of Ras. Thus, these two osmotic stresses employ distinct mechanisms of action, and the ability to communicate to multiple MAP3Ks does not necessarily depend on the same small G proteins.

³ W. Chen and M. H. Cobb, unpublished data.

Many kinases acting at the MAP3K level have been identified, adding to the complexity of unraveling signaling mechanisms. There is no apparent similarity among these proteins outside of their kinase catalytic domains, which display only minimal identity. The relative contribution of each MAP3K to the activation of individual MAP2Ks, with the possible exception of Raf in the ERK1/2 module, is unclear. A specific MAP3K enzyme may regulate either a single or multiple MAP2Ks; this may depend upon the enzymatic specificity of the MAP3K, the cellular and subcellular distribution of the signaling components, and the formation of protein complexes. Some signals may utilize multiple MAP3Ks acting in concert. Consequently, significant differences in both the magnitude and kinetics of MAPK activation may occur in response to a given agent under different circumstances. The implication of our findings for JNK activation is that different MAP3Ks regulate JNK in response to different stimuli. In the ERK1/2 MAPK pathway, Raf isoforms appear to be the predominant MAP3Ks used by ligands to activate ERK1/2. In contrast, our findings suggest that MAP3Ks that are only modestly related may be required for JNK activation by different stimuli and that these enzymes may participate singly or in combination in stimulus-specific signal transduction pathways.

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