RESEARCH COMMUNICATION

Differential activation of stress-activated protein kinase kinases SKK4/MKK7 and SKK1/MKK4 by the mixed-lineage kinase-2 and mitogen-activated protein kinase kinase (MKK) kinase-1

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Overexpression of the protein kinases mixed-lineage kinase-2 (MLK2) or mitogen-activated protein kinase (MAPK) kinase kinase-1 (MEKK1) is known to trigger the activation of stress-activated protein kinase (SAPK1)/c-Jun N-terminal kinase (JNK). Here we demonstrate that MLK2 activates SAPK kinase-1 (SKK1)/MAPK kinase 4 (MKK4) and SKK4/MKK7, the two known direct activators of SAPK1/JNK (both in transfection studies and *in vitro*). In contrast, MEKK1 activates SKK1/MKK4 more efficiently than MLK2, but barely activates SKK4/MKK7. Since SKK4/MKK7 (but not SKK1/MKK4) is activated by interleukin-1 and tumour necrosis factor in several cells and tissues, we suggest that MEKK1 does not mediate the activation of SKK4/MKK7 and SAPK1/JNK induced by these pro-inflammatory cytokines. MLK2 and MEKK1 also activated SKK2/MKK3 and SKK3/MKK6, the direct upstream activators of SAPK2a/p38.

INTRODUCTION

At least ten mitogen-activated protein kinase (MAPK) family members have been identified in mammalian cells. Two of these, MAPK1/extracellular-signal-regulated kinase-1 (ERK1) and MAPK2/ERK2, are activated strongly by growth factors and by tumour-promoting phorbol esters, but much more weakly (in most cell contexts) by stress stimuli and pro-inflammatory cytokines. In contrast, the other MAPK family members are strongly activated by stress signals and pro-inflammatory cytokines, but only weakly (in most cell contexts) by growth factors and phorbol esters. For this reason, they are frequently referred to as stress-activated protein kinases (SAPKs).

All the SAPKs and MAPKs are activated by SAPK/MAPK kinases (SKKs/MKKs) that are themselves members of a single protein kinase subfamily. In the classical MAPK cascade, MAPK1/ERK1 and MAPK2/ERK2 are activated by MKK1 and MKK2, while in the SAPK pathways, the SAPK1/c-Jun N-terminal kinase (JNK) isoforms are activated by SKK1/MKK4 and SKK4/MKK7, the SAPK2/p38 isoforms by SKK2/MKK3 and SKK3/MKK6, SAPK3/ERK6 (also called p38γ) and SAPK4 (also called p38δ) by SKK3/MKK6, and SAPK5/ERK5 by SKK5/MKK5 (see [1] for a recent review).

In the MAPK/ERK cascade, MKK1 and MKK2 are themselves activated by Raf isoforms (A-Raf, B-Raf and c-Raf) or (in germ cells) by c-Mos. However, the identity of the protein kinases that lie upstream of the SKKs *in vivo* is unclear. For example, more than ten protein kinases have been shown to trigger activation of the SAPK/JNK pathway in co-transfection experiments. These include several MKK kinases (MEKKs) and 'mixed-lineage' kinases (MLKs), transforming growth factor β-activated protein kinase-1 (TAK1), the cellular homologue of the *Tpl2* oncogene (reviewed in [2]), apoptosis-signal-regulating kinase (ASK1) [3] and a mammalian homologue of SSK2/SSK22 (MTK1) [4]. In the case of the SAPK1/JNK pathway, the complexity of the problem is compounded because SKK1/MKK4 and SKK4/MKK7 are not always activated by the same signals. Thus although SKK1/MKK4 and SKK4/MKK7 are both activated in response to stress stimuli, such as osmotic shock, UV radiation and the protein synthesis inhibitor anisomycin, only SKK4/MKK7 is activated after stimulation by the pro-inflammatory cytokines interleukin-1 (IL-1) and tumour necrosis factor (TNF) in several cells and tissues [5–7]. This, in turn, implies that IL-1/TNF stimulation must trigger the activation of (a) protein kinase(s) that can switch on SKK4/MKK7, but not SKK1/MKK4.

Two MLKs (MLK2 and MLK3) have been reported to activate SAPK1/JNK in co-expression studies [8–12], suggesting that these enzymes may lie upstream of SKK1/MKK4 and/or SKK4/MKK7. In this paper, we show that MLK2 activates both SKK4/MKK7 and SKK1/MKK4, whereas MEKK1 activates SKK1/MKK4 preferentially and barely activates SKK4/MKK7. These findings suggest that MEKK1 cannot mediate the activation of SKK4/MKK7 by pro-inflammatory cytokines and raise the possibility that MLK2, or another MLK family member, plays a key role in this process. We also show that MLK2 and MEKK1 activate SKK2/MKK3 and SKK3/MKK6.

MATERIALS AND METHODS

Materials

Tissue culture reagents and microcystin-LR were purchased from Gibco Laboratories (Paisley, Renfrewshire, Scotland, U.K.), and Protein G–Sepharose from Pharmacia (Milton Keynes, U.K.). PKI, the specific peptide inhibitor of cAMP-dependent protein kinase (TTYADFIASGRTGRRNAIHD), was synthesized by Mr. F. B. Caudwell in the MRC Protein

Abbreviations used: ATF, activating transcription factor; ERK, extracellular-signal-regulated kinase; GST, glutathione S-transferase; HA, haemagglutinin; IL-1, interleukin-1; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEKK, MAPK or ERK kinase kinase; MLK2, mixed-lineage kinase-2; MKK, MAPK kinase; SAPK, stress-activated protein kinase; SKK, SAPK kinase; TNF, tumour necrosis factor.

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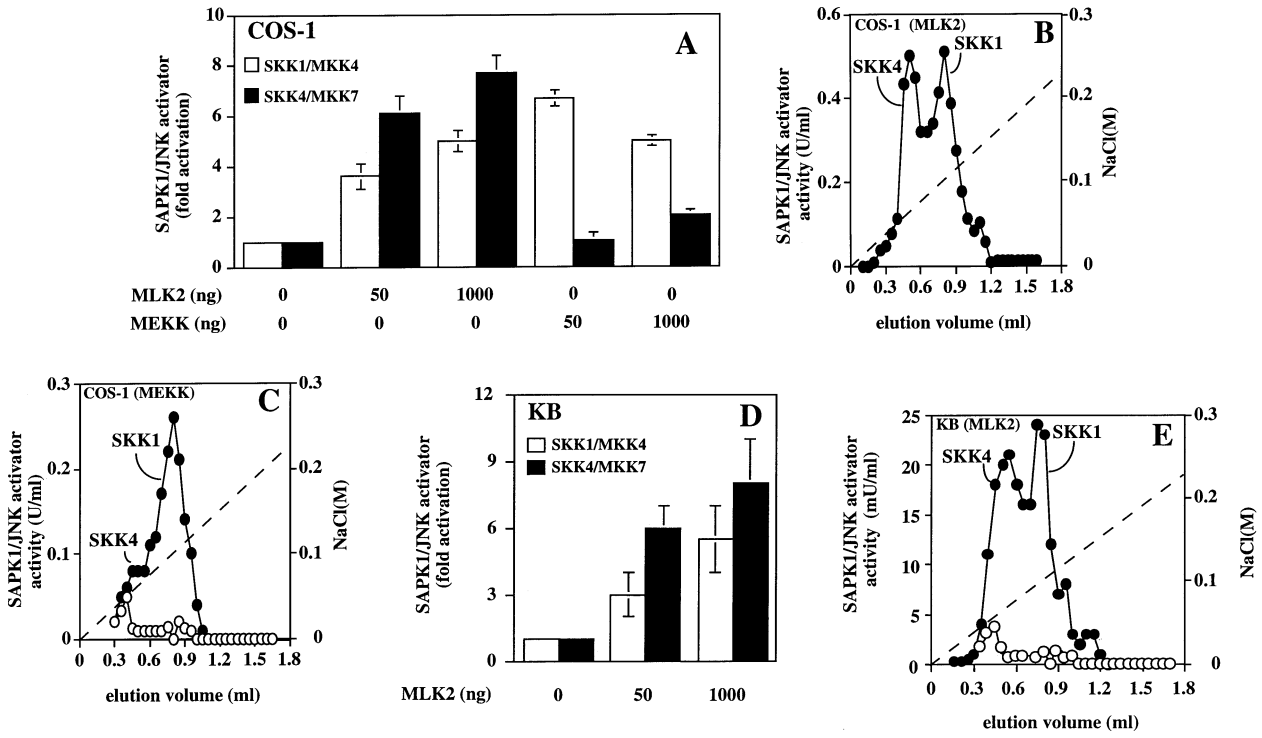

Figure 1 Activators of SAPK1/JNK induced by MLK2 and MEKK1 in COS-1 cells and by MLK2 in KB cells

(**A**) COS-1 cells or (**D**) KB cells were transfected with the indicated amounts of MLK2 DNA or MEKK1 DNA. The cells were lysed and SKK4/MKK7 and SKK1/MKK4 immunoprecipitated and assayed for their ability to activate SAPK1/JNK. The results are shown as averages (means ± S.E.M.) for three separate experiments. (**B**) and (**C**) Cell lysates (0.4 mg of protein) from COS-1 cells transfected transiently with 1 μg of DNA construct expressing either HA-MLK2 (**B**) or MEKK1 (**C**) were chromatographed on Mono S to resolve the different activators of SAPK1/JNK (●). (**E**) Lysates (0.4 mg of protein) from KB cells transfected transiently with 1 μg of HA-MLK2 DNA were chromatographed on Mono S to resolve SAPK1/JNK activators (●). Lysates from untransfected and unstimulated cells were chromatographed separately and assayed for activators SAPK1/JNK [parts (**C**) and (**E**); ○]. Similar results were obtained in at least five different experiments.

Phosphorylation Unit. 12CA5 and 9E10 monoclonal antobodies, which recognize the haemagglutinin (HA) epitope and Myc epitope respectively, were from Boehringer (Lewes, East Sussex, U.K.). Other reagents were of analytical grade or better and purchased from BDH Chemicals or Sigma Chemical Co. (Poole, U.K). *Escherichia coli* plasmids encoding glutathione S-transferase (GST)-fusion proteins were kindly provided by the investigators shown in parentheses, expressed in *E. coli* and purified by affinity chromatography on glutathione–agarose: GST–SKK1/MKK4 (Dr. J. Woodgett, Ontario Cancer Research Institute, Toronto, Canada), GST–ATF (activating transcription factor) 2(19–90) (Dr. N. Jones, ICRF, London) and GST–SKK4/MKK7 (Dr. S. Lawler, MRC Protein Phosphorylation Unit, Dundee). A plasmid encoding MalE fused to the *Xenopus* homologue of SAPK2a/p38 (MalE-Mpk2) was a gift from Dr. A. R. Nebreda (EMBL, Heidelberg, Germany), whereas a plasmid encoding the C-terminal 656 amino acids of MEKK1 [13,14] preceded by six histidine residues (His_6-MEKK1) was provided by Dr. G. Johnson (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO, U.S.A.) and Dr. A. R. Nebreda. A His_6-fusion protein expressing SAPK1/JNK was provided by Dr. M. Goedert (MRC Laboratory of Molecular Biology, Cambridge, U.K.) [15]. DNAs expressing Myc-tagged SAPK1/JNK and Myc-tagged SAPK2a/p38 were provided by Dr. S. Keyse (Biomedical Research Centre, Ninewells Hospital, Dundee). DNA expressing the C-terminal kinase domain of MEKK1 for transfection studies [16] was provided by Dr. A. Ashworth (Institute for Cancer Research, London, U.K.). A cDNA encoding full-length MLK2 [17] was cloned into the vector pKH3 [18], immediately 3′ to three copies of a sequence encoding the HA-tag peptide (YPYDVPDYA). The pKH3 vector was a kind gift of Dr. I. Macara (Department of Pathology, University of Vermont, VT, U.S.A.).

Cell culture and transient transfections

KB cells and COS-1 cells were cultured at 37 °C, in an atmosphere of 5 % CO_2 in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) foetal-calf serum. Transfection of cells was carried out using the lipofectamine method [19] using the amounts of DNA indicated.

Immunoprecipitation of SAPK1/JNK activators and SAPK2a/p38 activators

Cell lysates (300 μg of protein) or Mono S fractions (50 μl) were incubated for 2 h at 4 °C with 10 μg of affinity-purified anti-SKK1/MKK4 [13] or anti-SKK4/MKK7 [5] antibodies coupled to 5 μl of Protein G–Sepharose. The suspensions were centrifuged for 1 min at 13000 ***g*** and the pellets washed twice with 1 ml of lysis buffer [20 mM Tris/acetate (pH 7.0)/0.27 M sucrose/1 mM EGTA/1 mM EDTA/1 % (v/v) Triton X-100/10 mM sodium 2-glycerophosphate/25 mM NaF/2.5 mM sodium pyrophosphate/1 mM sodium orthovanadate/1 mM benzamidine/4 μg/ml leupeptin/2 μM microcystin-LR/0.1 % (v/v) 2-mercaptoethanol] containing 0.5 M NaCl, and twice with lysis buffer. The immunoprecipitates were then assayed for SAPK1/JNK activation [5]. Immunoprecipitation

of SKK2/MKK3 and SKK3/MKK6 from the Mono S fractions was carried out as described previously using 5 μg of the anti-SKK2/MKK3 (C-terminal) antibody and 2 μg of the anti-SKK3/MKK6 antibody respectively [20]. The two antibodies are available from UBI. The antibodies used to immunoprecipitate SAPK1/JNK activators and SAPK2a/p38 activators were specific and did not cross-react with any other SKKs/MKKs [5,13,20].

Partial purification and assay of SAPK activators

Cell lysates (400 μg of protein) were loaded on to a Mono S column (5 × 0.16 cm) attached to a SMART FPLC System (Pharmacia) [13]. The column was developed with a 4-ml linear salt gradient from 0 to 0.5 M NaCl and fractions of 50 μl were collected. SAPK2a/p38 activators (SKK2/MKK3 and SKK3/MKK6) were assayed by their ability to activate MalE-Mpk2 (the *Xenopus* homologue of SAPK2a/p38). SAPK2a/p38 activity was measured by the activation of bacterially expressed GST–MAPK-activated protein kinase-2 (46-400) [13]. SAPK1/JNK activators (SKK1/MKK4 and SKK4/MKK7) were assayed by their ability to activate His-SAPK1/JNK. Aliquots (7 μl) of each Mono S fraction were incubated for 3 min at 30 °C with 1 μl of 20 μM His-SAPK1/JNK in 50 mM Tris/HCl (pH 7.4)/0.1 mM EGTA/0.03 % (w/v) Brij-35/0.1 % (v/v) 2-mercaptoethanol/5 % (v/v) glycerol, and the reactions were initiated with 2 μl of 50 mM magnesium acetate/0.5 mM unlabelled ATP. After 30 min, an aliquot (10 μl) was assayed for SAPK1/JNK activity using GST–ATF2(19–90) and [γ-^{32}P]ATP [5]. One unit of SAPK1/JNK activator activity was defined as that amount of enzyme which increases the activity of His-SAPK1/JNK by 1 unit/min, whereas one unit of SAPK1/JNK was that amount which catalysed the phosphorylation of 1 nmol of ATF2 in 1 min.

Immunoprecipitation and assay of SAPK1/JNK and SAPK2a/p38

Lysates (50 μg of protein) from cells co-transfected with 1 μg of HA-tagged MLK2 or MEKK and 0.5 μg of Myc-SAPK1/JNK or Myc-SAPK2a/p38 were incubated for 2 h at 4 °C with 5 μl of Protein G–Sepharose coupled to 3 μg of 9E10 monoclonal antibody, which recognizes the Myc epitope. The immunoprecipitated Myc-SAPK1/JNK or Myc-SAPK2a/p38 was assayed, as indicated above.

Immunoprecipitation and assay of HA-tagged MLK2

Lysates prepared from cells transfected with HA-tagged MLK2 were centrifuged at 4 °C for 10 min at 13000 ***g***. Aliquots of the supernatant (50 μg of protein) were incubated for 2 h on a shaking platform with 5 μl of Protein G–Sepharose coupled to 1 μg of 12CA5 monoclonal antibody, which recognizes the HA epitope. The immunoprecipitated MLK2 was used to activate different SKKs/MKKs at 30 °C in the presence of 10 mM magnesium acetate and 0.1 mM ATP on a shaking platform, as described above.

RESULTS AND DISCUSSION

MEKK1 activates SKK1/MKK4 preferentially in COS-1 cells, whereas MLK2 activates SKK1/MKK4 and SKK4/MKK7

In order to examine whether SKK1/MKK4 and SKK4/MKK7 were activated differentially by some of the putative 'upstream' activators of these enzymes, we transfected COS-1 cells with two different amounts of MLK2 DNA or MEKK1 DNA (50 ng and 1 μg) and assayed SKK1/MKK4 and SKK4/MKK7 after their selective immunoprecipitation from cell lysates. MLK2 was active when overexpressed in transient transfections and its activity was not increased further by the cellular stresses used in this study (results not shown). Transfection with MLK2 results in activation of both SAPK1/JNK activators. MLK2 DNA (1 μg) causes an 8-fold increase in SKK4/MKK7 activity and a 5–6-fold activation of SKK1/MKK4 (Figure 1A), which were only reduced by 20 % when the amount of MLK2 DNA used was reduced to 50 ng. In contrast, transfection with 1 μg of MEKK1 DNA only activated SKK4/MKK7 weakly, and this activation disappeared when the amount of DNA was reduced to 50 ng, whereas transfection with 50 ng or 1 μg of MEKK1 DNA activated SKK1/MKK4 by 5–7-fold (Figure 1A).

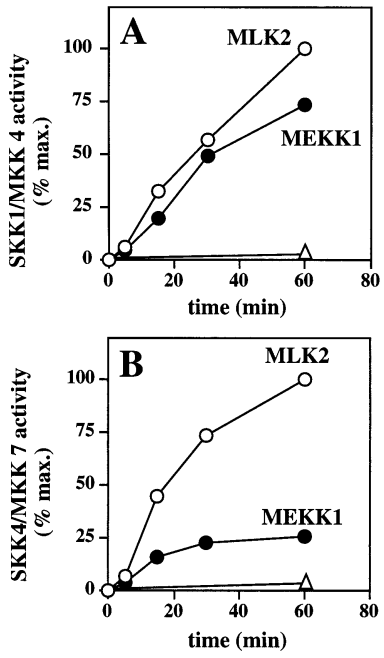

Figure 2 Activation of SKK4/MKK7 and SKK1/MKK4 by MLK2 and MEKK1 *in vitro*

HA-MLK2 was immunoprecipitated from the lysates (50 μg of protein) of COS-1 cells transfected with 1 μg of HA-tagged MLK2 DNA (○) and His_6-MEKK1 (●) was expressed in *E. coli* and purified on Ni^{2+}-nitrilotriacetic acid (NTA)-agarose. These enzymes were diluted appropriately to give the same rate of activation of 1 μM GST–SKK1/MKK4 (**A**) and were then used to activate 1 μM GST–SKK4/MKK7 (**B**) in the presence of 10 mM magnesium acetate/0.1 mM ATP. No activation of SKK4/MKK7 or SKK1/MKK4 occurred if HA-MLK2 or His_6-MEKK1 were omitted (△). Similar results were obtained in at least three different experiments.

Similar results were obtained when cell lysates were chromatographed on Mono S and the fractions assayed for activators of SAPK1/JNK (Figures 1B and 1C). Transfection with 1 μg of MLK2 DNA induced two activators of SAPK1/JNK, which were identified by immunoprecipitation with specific antibodies as SKK4/MKK7 and SKK1/MKK4 respectively (Figure 1B). In contrast, transfection with 1 μg of MEKK1 DNA induced a strong activation of SKK1/MKK4, but only a weak activation of SKK4/MKK7 (Figure 1C).

Co-transfection of Myc-tagged SAPK1/JNK with either 50 ng or 1 μg of MLK2 induced a 6-fold activation of SAPK1/JNK in COS-1 cells that was not increased further by exposure of the transfected cells to 10 μg/ml of the protein synthesis inhibitor anisomycin for 30 min or osmotic shock (0.5 M sorbitol for

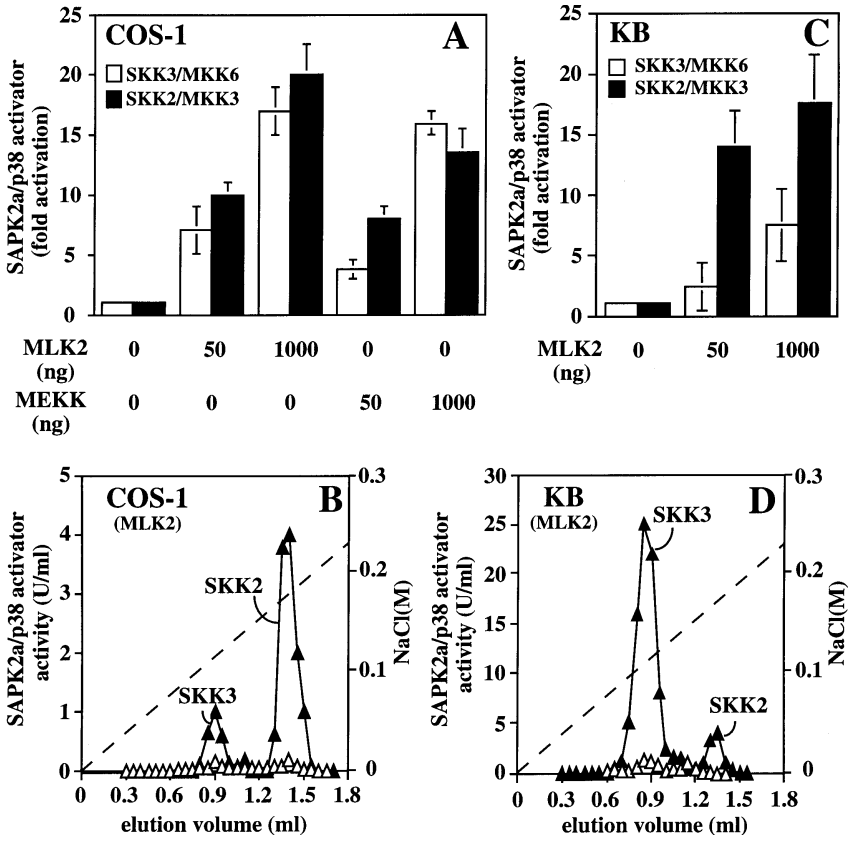

Figure 3 Activators of SAPK2a/p38 induced by MLK2 and MEKK1 in COS-1 cells and by MLK2 in KB cells

(**A**) COS-1 cells or (**C**) KB cells were transfected with the indicated amounts of MLK2 DNA or MEKK1 DNA. The cells were lysed and SKK3/MKK6 and SKK2/MKK3 were subsequently immunoprecipitated and assayed for their ability to activate SAPK2a/p38. The results are presented as averages (means ± S.E.M.) for three separate experiments. (**B**) and (**D**) Cell lysates (0.4 mg of protein) from COS-1 cells (**B**) or KB cells (**D**) transfected transiently with 1 μg of DNA construct expressing HA-MLK2 were chromatographed on Mono S to resolve the different activators of SAPK2a/p38 (Δ). Lysates from untransfected and unstimulated cells were chromatographed separately and assayed for activators SAPK2a/p38 [parts (**B**) and (**D**); ▲]. Similar results were obtained in at least five different experiments.

15 min) (results not shown). This indicates that SAPK1/JNK is maximally activated after transfection with MLK2. Co-transfection of SAPK1/JNK with 1 μg MEKK1 also induced a 6-fold activation of SAPK1/JNK.

MLK2 activates SKK4/MKK7 and SKK1/MKK4 in KB cells

In order to examine cell-to-cell variation in the response to MLK2, the experiments in COS-1 cells were repeated using the human epithelial KB cell line, in which the activation of SKKs and MKKs has been investigated in considerable detail [13,20]. Transfection with MLK2 DNA again activated both SKK1/MKK4 and SKK4/MKK7 (Figures 1D and 1E) and, as in COS-1 cells, the activation of SKK4/MKK7 was slightly greater than that of SKK1/MKK4. As in COS-1 cells, co-transfection of KB cells with 50 ng or 1 μg of MLK2 DNA causes a 3- or 5-fold Myc-SAPK1/JNK activation respectively.

The effects of MEKK1 could not be examined in KB cells, because it was very poorly expressed in this cell line.

Activation of SKK4/MKK7 and SKK1/MKK4 by MLK2 and MEKK1 *in vitro*

We next wanted to investigate whether the different effects of MLK2 and MEKK1 on SKK4/MKK7 and SKK1/MKK4 in transfection experiments (Figure 1) were a reflection of the different rates at which they activated these enzymes *in vitro*. HA-epitope-tagged MLK2 was transfected transiently in COS-1 cells and isolated from extracts by immunoprecipitation using the 12CA5 monoclonal antibody. The immunoprecipitated kinases HA-MLK2 and active His_6-MEKK1 were diluted appropriately so that they activated SKK1/MKK4 at similar rates *in vitro* (Figure 2A) and then their ability to activate SKK4/MKK7 under the same conditions was examined. As shown in Figure 2(B), MLK2 was much more effective in activating SKK4/MKK7 than MEKK1, consistent with the transfection experiments. The extent of activation correlated with the phosphorylation of SKK4/MKK7 and SKK1/MKK4; when determined in parallel incubations using [γ-^{32}P]ATP, the samples were electrophoresed on SDS/polyacrylamide gels and then autoradiographed. No phosphorylation was observed when MLK2 and MEKK1 were omitted (results not shown).

Activation of SKK2/MKK3 and SKK3/MKK6 by MLK2 and MEKK1 in COS-1 and KB cells

SAPK2a/p38 is also activated by stress stimuli and pro-inflammatory cytokines. It was therefore of interest to examine whether MLK2 and MEKK were capable of activating (in transfection experiments) the immediate 'upstream' activators of

SAPK2a/p38, namely SKK2/MKK3 and SKK3/MKK6. Transfection of COS-1 cells with MLK2 DNA or MEKK1 DNA was found to induce 5–20-fold activation of SKK2/MKK3 and SKK3/MKK6 (Figures 3A and 3B). The extent of activation of each enzyme was similar, but the absolute activity of SKK2/MKK3 generated was much higher than that of SKK3/MKK6 (Figure 3B). These findings raise the possibility that MLK2 and MEKK1 may activate SKK2/MKK3 and SKK3/MKK6 *in vivo*. Co-transfection with MLK2 or MEKK1 also induced a 2-fold activation of Myc-tagged SAPK2a/p38 (results not shown).

Transfection of KB cells with MLK2 induced a similar fold activation of SKK2/MKK3 to that observed in COS-1 cells (10–20-fold), but the activation of SKK3/MKK6 in KB cells was lower (3–7-fold). The absolute amount of SKK2/MKK3 activity generated by transfection with MLK2 DNA was similar in KB cells and COS-1 cells, but the amount of SKK3/MKK6 activity generated in KB cells was far higher (compare Figures 3C and 3D). The high level of SKK3/MKK6 activity in KB cells after stimulation with IL-1 or exposure to UV radiation, osmotic shock or anisomycin has been noted previously [13,20]. However, as in COS cells, co-transfection with MLK2 DNA only induced a 2-fold activation of Myc-tagged SAPK2a/p38 (results not shown).

Conclusions

SKK4/MKK7 and SKK1/MKK4 are both activated efficiently by MLK2 in either cell transfection studies (Figure 1) or *in vitro* (Figure 2). In contrast, MEKK1 activates SKK1/MKK4 even more efficiently than MLK2 (Figures 1A and 1C), but is a very poor activator of SKK4/MKK7 (Figures 1A and 1C). Since SKK4/MKK7 is activated by IL-1 and TNF in several cells and tissues, under conditions where SKK1/MKK4 is not activated [5–7], we suggest that MEKK1 may not mediate the activation of SKK4/MKK7 and SAPK1/JNK activation induced by pro-inflammatory cytokines. However, we cannot exclude the possibility that the poor activation of SKK4/MKK7 by the MEKK1 used in this study was the result of absence of necessary recognition sites at the N-terminal domain. MLK2 and MEKK1 also activate SKK2/MKK3 and SKK3/MKK6 efficiently, but this only activates SAPK2a/p38 weakly compared with the activation induced by stress stimuli or pro-inflammatory cytokines. The reason for this is unclear, but it could be explained if overexpressed MLK2 or MEKK1 exerted a dominant negative effect on the activation of SAPK2/p38 by sequestering SKK2/MKK3 and SKK3/MKK6.

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