Selective activation of JNK/SAPK by interleukin-1 in rabbit liver is mediated by MKK7

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1. Introduction

The mitogen-activated protein kinase (MAPK) cascades are intracellular signalling pathways which have been conserved from yeast to mammals. Activation of the MAPKs occurs by phosphorylation of threonyl and tyrosyl residues in a TXY motif by dual specificity MAPK kinases (MKKs). Three types of MAPKs are distinguished by the nature of the intervening amino acid. The first to be discovered, the p42 and p44 MAPKs, contain a TEY motif and are activated by MKK1 or MKK2, in response to mitogens and many other stimuli [1,2] including interleukin-1 (IL-1) in cultured connective tissue cells [3-5]. The c-jun N-terminal kinases (JNKs), also called p54 MAPKs or stress-activated protein kinases (SAPKs), contain a TPY motif and are activated in response to cellular stresses and the pro-inflammatory cytokines IL-1 and tumour necrosis factor (TNF) [6-8]. The p38 MAPKs contain a TGY motif and are usually activated by the same amino acid. The first to be discovered, the p42 and p44 MAPKs, contain a TEY motif and are activated by MKK1 or MKK2, in response to mitogens and many other stimuli [1,2] including interleukin-1 (IL-1) in cultured connective tissue cells [3-5]. The c-jun N-terminal kinases (JNKs), also called p54 MAPKs or stress-activated protein kinases (SAPKs), contain a TPY motif and are activated in response to cellular stresses and the pro-inflammatory cytokines IL-1 and tumour necrosis factor (TNF) [6-8]. The p38 MAPKs contain a TGY motif and are usually activated by the same amino acid.

Three activators of the stress-activated kinases have been cloned: MKK3 [14] and MKK6 [15-18], which are specific for p38 MAPK, and MKK4/SEK1 [14,19] which can activate both p38 MAPK and JNK/SAPK but when overexpressed in cell lines preferentially activates the latter. It is unclear which of the MKKs is responsible for activation of MAPKs in tissues.

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2.8. JNK/SAPK activator assay

To the protein A agarose beads from above was added 10 μl of substrate and 20 μl of assay buffer 1 (150 mM TRIS pH 7.4, 30 mM MgCl₂, 60 μM ATP, 0.4 μCi/μl [γ-32P]ATP): substrate for p42 MAPK was myelin basic protein (MBP) at 100 μg/ml; substrate for p38 MAPK was his-MAPKAPK-2 at 75 μg/ml and hsp27 at 50 μg/ml; substrate for JNK/SAPK was GST-jun (1-135) at 100 μg/ml. Samples were shaken for 20 min at 20°C, the assay was stopped and phosphorylated substrate was visualised by autoradiography of gels following SDS-PAGE.

2.6. Chromatography

Frozen livers were thawed and homogenised, using a Polytron (Kinematica, Switzerland), in lysis buffer (20 mM TRIS pH 7.4, 50 mM NaF, 0.2 mM Na₃VO₄, 1 mM EDTA, 1 mM EGTA, 2 mM DTT)+1 mM P-glycerophosphate, 0.2 mM Na₃, 0.5 mM EDTA, 0.5 mM EGTA, 2 mM DTT) at 4°C. Hundred μg of soluble protein was loaded to a column (20 μl Fast Flow S Sepharose) which was equilibrated in buffer C+0.3 M NaCl in buffer C was run. Two ml fractions were collected and assayed for activator of JNK/SAPK as described below.

2.7. JNK/SAPK activator purification

A 50 ml FFS Sepharose column (flow rate 60 cm/h) was equilibrated in buffer C (20 mM MES pH 6.0, 20 mM β-glycerophosphate, 10 mM NaF, 0.1 mM Na₃VO₄, 0.5 mM EDTA, 0.5 mM EGTA, 2 mM DTT) at 4°C. Hundred μg of soluble protein was loaded onto the column. A 40 ml gradient of 0-1.2 M NaCl in buffer C in buffer C was run. Two ml fractions were collected and assayed for activator of JNK/SAPK as described below.

2.8. JNK/SAPK activator assay

Fifteen μl of sample was incubated with 5 μl of GST-SAPKβ (100 μg/ml) and 10 μl of assay buffer 2 (150 mM TRIS pH 7.4, 30 mM MgCl₂, 60 μM ATP, 0.4 μCi/μl [γ-32P]ATP). Samples were mixed for 20 min at room temperature, the assay was stopped and phosphorylated GST-jun was visualised by autoradiography following SDS-PAGE.

2.9. Immunoprecipitation and assay for activator of JNK/SAPK

Fifty μl of sample was added to protein A agarose coated with 3 μl of antiserum. The samples were mixed at 4°C for 4 h. The agarose beads were washed 5×1 ml with wash buffer (as above). At the last wash, the sample was split into two, for assay with and without GST-SAPKβ. To each pellet was added 10 μl wash buffer (±0.5 μg GST-SAPKβ) and 10 μl assay buffer 4 (150 mM TRIS pH 7.4, 30 mM MgCl₂, 60 μM ATP). Samples were mixed for 1 h at room temperature, 10 μl of assay buffer 5 was added (100 μg/ml GST-jun, 0.4 μCi/μl [γ-32P]ATP), and the samples were shaken for another 20 min. The assay was stopped and phosphorylated GST-jun was visualised by autoradiography following SDS-PAGE.

3. Results

3.1. Activation of MAPKs in liver and in cultured cells by IL-1

The activation of all three types of MAPK in rabbit liver following injection of IL-1 was measured by immunoprecipitation of the enzymes and assay on appropriate substrates (Fig. 1A). JNK/SAPK was strongly activated, as expected, but p38 and p42 MAPKs were not, with the former showing constitutive activity. This was surprising in view of their activation in cultured cells (Fig. 1B). We feel that it is unlikely that the kinetics of activation of p38 and p42 MAPKs may be different to those of JNK/SAPK in liver, since they are not different in cultured cells.

3.2. IL-1 activates an activator of JNK/SAPK in liver

We next assayed liver cytosols of IL-1- or vehicle-injected rabbits for an activator of JNK/SAPK by use of a rat recombinant SAPKβ-GST fusion protein as substrate in a two-stage assay. Liver cytosol was incubated with the recombinant enzyme; the latter was then adsorbed to GSH Sepharose beads and its activity measured on GST-jun (1-135). Cytosol from the IL-1 treated animals activated the recombinant enzyme (Fig. 2A, lane 4), compared to cytosol from those treated with vehicle alone (Fig. 2A, lane 2). To show that hepatic JNK/SAPK is not carried over by the beads, GST-SAPKβ was omitted from the procedure (Fig. 2A, lanes 1 and 3). The untreated GST-SAPKβ did not phosphorylate the GST-jun substrate (Fig. 2A, lane 6).

To identify a putative activator on chromatography, cytosolic extracts were applied to a column of S Sepharose which was eluted with a salt gradient. The fractions were assayed for

Table 1

<table>
<thead>
<tr>
<th>Protein (mg)</th>
<th>Total activity (CPM)</th>
<th>Specific activity (CPM/mg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>640</td>
<td>0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cytosol</td>
<td>92</td>
<td>2.7</td>
<td>29</td>
<td>–</td>
</tr>
<tr>
<td>Fast Flow S Sepharose</td>
<td>24</td>
<td>0.15</td>
<td>6</td>
<td>–</td>
</tr>
<tr>
<td>Phenyl Sepharose</td>
<td>7.4</td>
<td>12</td>
<td>18</td>
<td>–</td>
</tr>
<tr>
<td>Resource Q</td>
<td>620</td>
<td>10</td>
<td>1.6</td>
<td>100</td>
</tr>
<tr>
<td>IL-1</td>
<td>10</td>
<td>50</td>
<td>50</td>
<td>60</td>
</tr>
</tbody>
</table>

1 mM benzimidine, 0.1% (v/v) β-ME, 0.2 mM PSMF and microfuged at 16000 × g, 4°C for 15 min. MAPKs were immunoprecipitated as above.
an activator of JNK/SAPK as described for Fig. 2A. Fractions from IL-1 treated animals showed increase in a peak of JNK/SAPK activator compared with controls. Activation in four experiments varied between two- and six-fold (Fig. 2B).

3.3. Purification of the IL-1 stimulated activator of JNK/SAPK from liver

Material from unstimulated and IL-1 stimulated rabbit livers was taken through three purification steps, so that the regulation by IL-1 could be confirmed. Table 1 shows that the overall recovery of activity after three steps of purification from IL-1 treated liver was 27%. Assay of equivalent fractions from control livers showed that IL-1 regulated material was being purified at each stage. The IL-1 regulated enzyme from Q Resource was next applied to a S Resource column and eluted by a salt gradient (Fig. 3A and Table 1). The enzyme did not elute sharply but over a wide conductivity range: there was little further increase in specific activity of the enzyme, which had been purified 200-fold. The single active peak was finally chromatographed on Superose 12: the activator eluted at a position corresponding to $M_r$ 40 kDa, but the protein was not homogeneous on SDS-PAGE (data not shown).

3.4. Identification of the activator as MKK7

The activator was tested for its ability to react with antibodies raised against known MKks. Antiserum to MKK3 and MKK4 did not immunoprecipitate or immunodeplete the activator, nor did they stain any co-eluting antigen by Western blot (data not shown). However, two antisera raised against the recently discovered MKK7, which is activated by anisomycin and hyperosmolar shock in NIH3T3 cells [21], immunoprecipitated the activator. Antiserum 2125 to a C-terminal synthetic peptide of MKK7 strongly precipitated activator from the fractions of S Resource chromatography, the fourth purification step (Fig. 3B). Antiserum 3936 to the C-terminal

![Fig. 1](image1.png)

Fig. 1. A: IL-1 activates JNK/SAPK, but not p42 or p38 MAPKs in rabbit liver. Immunoprecipitates from 1 mg of liver lysate of vehicle- or IL-1-injected rabbits were prepared with non-immune serum (NI) or with antiserum (I) against the relevant kinase (see Section 2). Immunoprecipitates were incubated with assay buffer containing [γ-32P]ATP and the appropriate substrate: MBP for p42 MAPK; GST-p38 for p38 MAPK and no activated jun kinase was detected if recombinant GST-SAPKβ (17 μg/ ml) in assay buffer containing ATP (50 μM) for 30 min at room temperature. GSH Sepharose beads were added for 15 min with mixing. Beads were spun down, washed twice and incubated with GST-jun (1-135) and assay buffer containing [γ-32P]ATP for 20 min at room temperature. Phosphorylated GST-jun (1-135) was detected by autoradiography, following SDS-PAGE. B: Reaction mixtures were separated by SDS-PAGE and phosphorylation of GST-jun (1-135) was measured by Cerenkov counting. See Section 2 for details. Error bars show standard error of the mean. 128 amino acids also precipitated the activator (Fig. 3C). Neither non-immune nor pre-immune sera precipitated activity and no activated jun kinase was detected if recombinant GST-SAPKβ was omitted. Both of these antisera recognised recombinant GST-MKK7 from E. coli upon Western blotting, but only faintly detected the putative hepatic MKK7 in fractions from Superose 12 gel filtration, presumably due to a low concentration of antigen (data not shown).

MKK7 was shown to activate JNK/SAPK preferentially, although it was able to activate p38 MAPK weakly in vitro. It did not activate p42 MAPK [20,21].

The hepatic JNK/SAPK activator did not phosphorylate p38 or p42 MAPKs, as judged by the inability of the peak fractions from S Resource chromatography (Fig. 3A) to phosphorylate GST-p38 or of the immunoprecipitates obtained with anti-MKK7 (Ab 3936) to phosphorylate GST-p38 or GST-p42 MAPKs (data not shown).

4. Discussion

Several laboratories, including our own, have reported that IL-1 activates all three types of MAPK in cultured cells. We felt it important to establish whether or not this also occurred
in a tissue in vivo. We chose to investigate liver, which is a physiological target for the cytokine in the acute phase response and in which we previously found striking activation of JNK2/SAPKα, rapidly following injection of IL-1 [6]. As before, IL-1 activated JNK/SAPK, but it did not significantly activate p42 MAPK or p38 MAPK. Generally, stressful stimuli and IL-1 or TNF have been found to activate both JNK/SAPK and p38 MAPK together. Indeed they are commonly referred to as the stress kinases. However, selective p38 MAPK activation has been reported in perfused heart subjected to ischaemia [23] and selective JNK/SAPK activation has been reported in mouse liver exposed to metabolic oxidative stress [24]. Our results suggest that IL-1 selectively activates JNK/SAPK in a differentiated tissue and that p38 MAPK may not be involved in certain physiological responses to IL-1. The JNK/SAPK activation may be occurring in hepatic parenchymal cells and/or vascular endothelial cells. Whether or not such selective JNK/SAPK activation occurs in other tissues remains to be seen. The contrast with cultured cells suggests that in dedifferentiated proliferating cells, such as fibroblasts, new connections may be established between IL-1 receptors and MAPK pathways, perhaps to enable a broader spectrum of responses.

We focussed on identifying the IL-1 stimulated activator of JNK/SAPK in rabbit liver, but were unable to purify it to homogeneity because it chromatographed broadly on cation exchange chromatography and did not bind to anion exchangers. As judged by (a) immunoprecipitation, (b) its substrate preference and (c) its size on gel filtration, it was provisionally identified as MKK7. However, without knowing its amino acid sequence, we cannot be sure that it is not an unidentified relative of MKK7. It did not correspond to MKK4/SEK1, the only other well characterised activator of JNK/SAPK. We found no activator corresponding to MKK4/SEK1, but if this were a minor activator in liver, it could have been overlooked. MKK4/SEK1 was found to be only a minor activator of JNK/SAPK in rat fibroblasts stressed by hyperosmolar shock [25].

MKK7 cDNA was originally isolated from a murine embryo cDNA library during a yeast two-hybrid screen with human MKK1 as bait. Subsequently the enzyme was shown to interact with JNK/SAPK, but not with p38 or p42.
MAPks. It activated JNK/SAPK strongly and p38 MAPK weakly in vitro. It did not activate p42 MAPK [21]. Our results suggest that MKK7 is the major activator mediating JNK/SAPK activation by IL-1 in liver. This may also be the case in other tissues since MKK7 is widely expressed [21].

Several MKK kinases (MKKKs) are candidates for the activation of MKK7 including MEKK1 [26], MAPKKK5 [27], ASK1 [28], TAK1 [29] and the mixed lineage kinases MLK3 [30] and DLK [31]. It is at present unclear how specific activation of MKK7 including MEKK1 [26], MAPKKK5, P7 [24], P5: [23], P4: [22], P3: [21], P2: [20], P1: [19], P0: [18], POT: [17], F1: [16], F2: [15], MAPK upon overexpression in cells. However, some of these observations may be artefacts due to enzyme overexpression. Which, if any, of these MKKKs mediates the effects of IL-1 remains to be established.

During the preparation of this manuscript, SKK4, the human homologue of MKK7, was cloned and shown to be activated by IL-1 in KB cells [32].

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References