FEBS Letters 418 (1997) 144–148 FEBS 19513

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

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Received 1 October 1997

Abstract Activation of jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) by interleukin-1 (IL-1) has been reported in many cells and in rabbit liver. Here we report selective activation of JNK/SAPK, without activation of p38 or p42 mitogen-activated protein kinases (MAPKs), by IL-1 in rabbit liver. We identified an IL-1 regulated JNK/SAPK activator present in rabbit liver using S Sepharose chromatography. It was purified and immunoprecipitated by two antisera to MAP kinase kinase 7 (MKK7). It was not recognised by an antibody to MKK4. We conclude that MKK7 is the activator of JNK/SAPK activated by IL-1 in liver and that JNK/SAPK is the only MAPK activated by IL-1 in liver.

Key words: Interleukin-1; Mitogen-activated protein kinase; Jun N-terminal kinase; Stress-activated protein kinase; Mitogen-activated protein kinase kinase 7; Liver

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 stimuli as JNK/SAPKs [9–13].

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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Abbreviations: JNK/SAPK, jun N-terminal kinase/stress-activated protein kinase; GST, glutathione *S*-transferase; IL-1, interleukin-1; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; MKKK, MAPKK kinase

IL-1 is able to activate all three types of MAPK in a variety of cells in culture [3,10,11] and we previously showed that it activates SAPKα/JNK2 in rabbit liver [6]. We have now investigated its ability to activate the other MAPKs in the same tissue. In this tissue, p38 MAPK is constitutively active and is not regulated by IL-1. Instead, the cytokine selectively activates JNK/SAPK and we have provisionally identified the activator involved as the recently discovered MKK7 [20,21].

2. Materials and methods

2.1. Materials

Human IL-1α was made as described [22]. [γ-^{32}P]ATP (Redivue) was from Amersham International (Slough, UK). Chromatography media and glutathione (GSH) Sepharose were from Pharmacia Biotech (St. Alban's, Herts, UK). cDNA for histidine-tagged MAPK-activated protein kinase-2 (MAPKAPK2) was a kind gift of M. Gaestel (Max Delbrueck Center for Molecular Medicine, Berlin, Germany). cDNAs for rat GST-SAPKβ [7] and human GST-jun (amino acids 1–135), were kind gifts of J.R. Woodgett (Ontario Cancer Institute, Toronto, Canada) and were expressed in *E. coli*. The proteins were purified by standard methods. Rabbit antiserum to p38 MAPK was to the synthetic peptide ISFVPPPLDQEEMES (amino acids 346–360). Rabbit antisera to JNK/SAPK were to the synthetic peptide DSSLDASTGPLEGCR (amino acids 409–423 of SAPKα), which was used for liver and to the synthetic peptide GVVKGQPSPSAQVQQ (amino acids 370–384 of SAPKβ) which was used for MRC-5. Rabbit antiserum to p42 MAPK was to the synthetic peptide EETARFQPGYRS and was a gift from C. Marshall (ICR, Chester Beatty Laboratories, Fulham Rd, London).

Rabbit antiserum 3936 was to GST fused with the C-terminal 128 amino acids of MKK7. Rabbit antiserum 2125 was to a synthetic C-terminal peptide of MKK7 (CRTSGVLSQHHLPFFR).

2.2. Stimulation

Female Dutch rabbits were sedated and injected via an ear vein with IL-1α (5 μg/kg) or vehicle. After 4 min a rapidly lethal anaesthetic was administered and the livers were removed and placed on ice: removal took about 4 min [6]. The livers were frozen in liquid nitrogen and stored at −70°C. Confluent MRC-5 fibroblasts (175 cm^2 flask per antiserum) were stimulated with 20 ng/ml IL-1α for 15 min.

2.3. Immunoprecipitation of MAPKs

250 mg frozen liver was thawed and homogenised using a Polytron (Kinematica, Switzerland) in buffer A (20 mM HEPES pH 7.5, 20 mM β-glycerophosphate, 10 mM NaF, 0.1 mM Na_3VO_4, 0.5 mM EDTA, 0.5 mM EGTA, 2 mM DTT)+0.2 M NaCl, 2 mM PMSF, 10 μM E64, 10 mg/ml aprotinin. Homogenates were microfuged at 16000×*g*, 4°C for 15 min. To 1 mg of cleared homogenate, made up to 500 μl was added 500 μl of 2×RIPA buffer (2×buffer A including 0.4 M NaCl 2% NP40, 1% DOC, 0.2% SDS) and 30 μl of protein A agarose coated with 5 μl of non-immune serum (NI) or 5 μl of one of the anti-MAPK antisera. Samples were shaken for 3 h at 4°C and the agarose beads were washed 4 times in RIPA buffer and twice in buffer A+10 mM $MgCl_2$ before assay.

MRC-5 fibroblasts were lysed in buffer B (20 mM Tris-acetate pH 7.0, 0.1 mM EDTA, 1 mM EGTA, 10 mM β-glycerophosphate, 50 mM NaF, 5 mM $Na_4P_2O_7$, 0.27 M saccharose, 1% TRITON X-100,

PII S0014-5793(97)01364-1

1 mM benzamidine, 0.1% (v/v) β-ME, 0.2 mM PMSF) and microfuged at 16000×g, 4°C for 15 min. MAPKs were immunoprecipitated as above.

2.4. Assay of MAPKs

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 $MgCl_2$, 60 μM ATP, 0.4 μCi/μl [γ-^{32}P]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.

2.5. Lysis for 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 Na_3VO_4, 1 mM EDTA, 1 mM EGTA, 2 mM DTT)+1 mM PMSF, 10 μg/ml aprotinin, 10 μM E64, 1 μM pepstatin. Five ml lysis buffer was used for each g of liver. Lysates were centrifuged at 20000×g, 4°C for 1 h, 1 M MES pH 6.0 was added to a final concentration of 20 mM and the sample was filtered (0.8 μM).

2.6. Chromatography

A 2 ml Fast Flow S Sepharose column was equilibrated in buffer C (20 mM MES pH 6.0, 20 mM β-glycerophosphate,10 mM NaF, 0.1 mM Na_3VO_4, 0.5 mM EDTA, 0.5 mM EGTA, 2 mM DTT) at 4°C. Hundred mg of soluble protein was loaded onto the column. A 40 ml gradient of 0–1.2 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 and approx. 5 g of liver cytosol protein was loaded. The column was washed in buffer C+0.3 M NaCl and the activity was eluted in buffer C+0.6 M NaCl. One M TRIS pH 8.5 was added to the eluate to a final concentration of 20 mM and pH 7.4. This was applied to a 10 ml phenyl Sepharose column (flow rate 60 cm/h) equilibrated in lysis buffer+0.6 M NaCl. The column was washed with lysis buffer and the active material was eluted in lysis buffer+1% CHAPS. One M TRIS pH 8.5 was added to 50 mM (final concentration) and the sample was passed through a 1 ml Q Resource column equilibrated in buffer D (20 mM TRIS pH 8.5, 10 mM NaF, 20 mM β-glycerophosphate, 0.2 mM Na_3VO_4, 0.5 mM EDTA, 0.5 mM EGTA, 2 mM DTT). The material passing through was dialysed overnight at 4°C into buffer E (20 mM HEPES pH 7.5, 10 mM NaF, 0.1 mM Na_3VO_4, 0.5 mM EDTA, 0.5 mM EGTA, 2 mM DTT, 0.05% BRIJ 35) and was loaded on a S Resource column equilibrated in buffer E at 4°C. This was eluted at 1 ml/min with a 20 ml gradient from 0 to 0.5 M NaCl in buffer E. One ml fractions were collected.

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 $MgCl_2$, 150 μM ATP) for 30 min at room temperature. Twenty μl of GSH Sepharose beads were added and the samples were shaken for 15 min. The beads were given 3×1 ml washes in wash buffer (buffer E+0.2 M NaCl). Ten μl of wash buffer was added to the beads, along with 10 μl of GST-jun (100 μg/ml) and 10 μl of assay buffer 1 (150 mM TRIS pH 7.4, 30 mM $MgCl_2$, 60 μM ATP, 0.4 μCi/μl [γ-^{32}P]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 $MgCl_2$, 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-^{32}P]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
Purification of IL-1 regulated activator of JNK/SAPK from rabbit liver

	Protein (mg)	Total activity (CPM)	Specific activity (CPM/mg)	Purification (fold)	Yield (%)
Control					
Cytosol	6460	0	–	–	–
Fast Flow S Sepharose	92	2.7	29	–	–
Phenyl Sepharose	24	0.15	6	–	–
Resource Q	7.4	0.13	18	–	–
IL-1					
Cytosol	6290	10	1.6	1	100
Fast Flow S Sepharose	75	6	80	50	60
Phenyl Sepharose	22	3.9	177	111	39
Resource Q	10	2.7	270	170	27
Resource S	5.15	1.6	311	200	16

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. Antisera 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

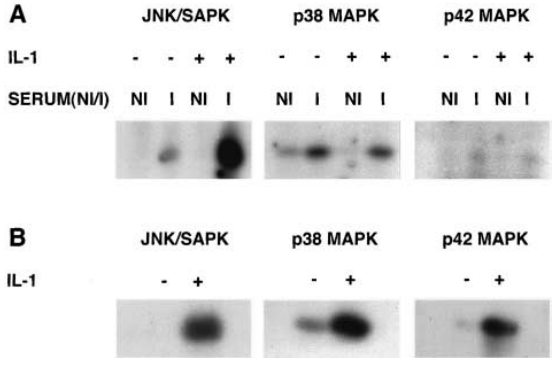

Fig. 1. A: IL-1 activates JNK/SAPK, but not p42 or p38 MAPKs in rabbit liver. Immunoprecipitates from 1 mg of liver lysate of vehicle- or IL-1-injected rabbits were prepared with non-immune serum (NI) or with antiserum (I) against the relevant kinase (see Section 2). Immunoprecipitates were incubated with assay buffer containing [γ-^{32}P]ATP and the appropriate substrate: MBP for p42 MAPK; GST-jun (1–135) for JNK/SAPK; his-MAPKAPK-2 and hsp27 for p38 MAPK; all three substrates for non-immune serum. Phosphorylated substrates were visualised by autoradiography following SDS-PAGE. B: IL-1 activates all three MAPK pathways in MRC-5 fibroblasts. Immunoprecipitates from unstimulated or IL-1 stimulated MRC-5 cells were prepared using antisera against the relevant kinase and were incubated with assay buffer and the relevant substrate as in A. Phosphorylated substrates were visualised by autoradiography following SDS-PAGE.

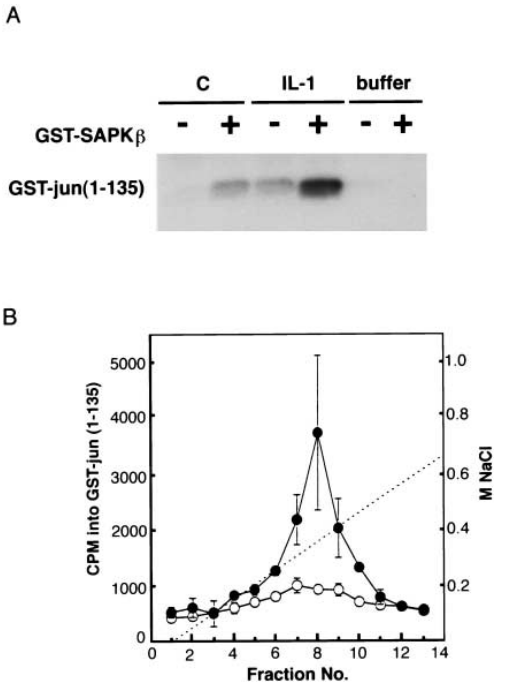

Fig. 2. A: IL-1 activates an activator of JNK/SAPK in rabbit liver. Cytosolic extracts from vehicle- or IL-1-injected rabbits, or buffer alone, were incubated with (+) or without (−) GST-SAPKβ (17 μg/ml) in assay buffer containing ATP (50 μM) for 30 min at room temperature. GSH Sepharose beads were added for 15 min with mixing. Beads were spun down, washed twice and incubated with GST-jun (1–135) and assay buffer containing [γ-^{32}P]ATP for 20 min at room temperature. Phosphorylated GST-jun (1–135) was detected by autoradiography, following SDS-PAGE. B: Cation exchange chromatography of hepatic activator of JNK/SAPK. Liver cytosol of control (open circle) or IL-1 stimulated (closed circle) rabbits was chromatographed on Fast Flow S Sepharose at pH 6.0 and eluted with a salt gradient. Fractions were assayed for activator of JNK/SAPK as for 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

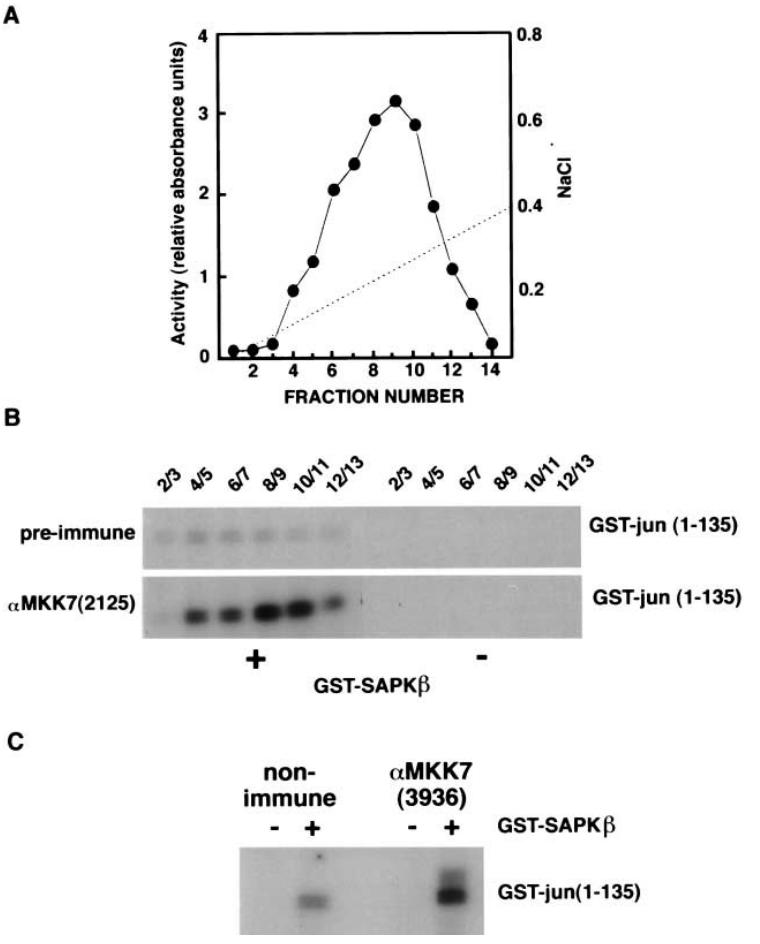

Fig. 3. A: Chromatography of JNK/SAPK activator on S Resource. Activator from Q Resource chromatography (Table 1) was applied to a S Resource column which was eluted with a salt gradient. Fractions were assayed for activator of JNK/SAPK as described for Fig. 1B. (See Section 2 for full details.) Absorbance units were obtained by densitometry of an autoradiograph. B: Antiserum 2125 to MKK7 immunoprecipitates the JNK/SAPK activator. Pairs of active fractions from A were treated with protein A agarose coated with antiserum 2125 or pre-immune serum of 2125 and the beads were incubated with (+) or without (−) GST-SAPKβ (25 μg/ml) in buffer containing ATP for 1 h at room temperature. GST-jun and [γ-^{32}P]ATP were then added for a further 20 min. Phosphorylation of GST-jun (1–135) was detected by autoradiography after SDS-PAGE. C: Antiserum 3936 to MKK7 precipitates JNK/SAPK activator from a pool of fractions 2–13 from S Resource (Fig. 2A).

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 these enzymes are for activation of JNK/SAPK. Many of them have also been shown to cause activation of p38 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].

Acknowledgements: We thank J.R. Woodgett and M. Gaestel for cDNAs, C.J. Marshall for the anti-ERK2 antibody and Lesley Rawlinson for help with immunoprecipitations. This work was supported by the Arthritis and Rheumatism Council and the Medical Research Council, UK. P.H. was supported by NSF, J.C. by NIH grant No. ROI CA54786.

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