Interleukin 1 α Activates Two Forms of p54α Mitogen-activated Protein Kinase in Rabbit Liver

By Michael Kracht,* Oanh Truong,§ Nick F. Totty,§ Masahiro Shiroo,‡ and Jeremy Saklatvala*

*From the *Cytokine Laboratory, Department of Development and Signalling, and the ‡Department of Immunology, Babraham Institute, Cambridge CB2 4AT, UK; and the §Protein Biochemistry Group, Ludwig Institute for Cancer Research, UCL Medical School, London W1P 8BT, UK*

Summary

We have identified in rabbits two hepatic forms of T669 peptide kinases that are very strongly activated after systemic injection with the inflammatory cytokine interleukin 1 (IL-1). The T669 peptide contains a major phosphorylation site of the epidermal growth factor receptor, threonine 699 and is a substrate for mitogen-activated protein (MAP) kinases. The kinases were purified to homogeneity and corresponded to 50- and 55-kD proteins on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Amino acid sequencing of 12 tryptic peptides of both kinases identified them as p54 MAP kinase α. This kinase belongs to the novel family of stress-activated protein kinases. This is the first evidence of IL-1 activating a specific protein kinase in vivo.

IL-1 is a major inflammatory hormone. It is produced at sites of injury or infection where it causes leukocyte accumulation, cytokine production, and resorption of connective tissue matrix. If it enters the circulation it induces the acute phase response and fever (1).

Its actions are mediated by the 80-kD (type I) receptor (2, 3), but the intracellular signaling pathways by which receptor triggering causes cellular responses are not yet clearly defined. There is rapid increase in phosphorylation of certain cellular proteins caused by induction of protein serine/threonine kinase activity. Some of the enzymes involved have been identified. For example, components of the growth factor–regulated pathway, the p42/p44 mitogen-activated protein (MAP)[1] kinases and their activator, MAP kinase kinase, are stimulated in some cells (4–6).

A second type of MAP kinase, p54, has been purified and recently cloned (7). It is activated by stressful stimuli (e.g., heat, protein synthesis inhibition, and UV light) and the cytokine TNF. Three very closely related cDNAs (α, β, γ) were identified that were ~50% homologous to p42/p44 MAP kinases. p54 and p42/p44 MAP kinases phosphorylate a similar range of substrates upon serine or threonine in the context of proline, and both depend for their activity upon phosphorylation of nearly adjacent threonine and tyrosine residues (8). The intervening glutamate residue found in p42/p44 MAP kinases is replaced by a proline residue in p54. A consequence of this is that p54 is not activated by MAP kinase kinase.

We recently purified an IL-1–activated kinase from KB cells that phosphorylated a MAP kinase substrate, a synthetic peptide (T669) containing the sequence around threonine 669 of the epidermal growth factor (EGF) receptor. Its properties suggested it could be a truncated form of p54 MAP kinase, but we were unable to obtain sufficient quantities for amino acid sequencing (9). We have now investigated the possibility that this or similar enzymes may be activated in a large organ such as the liver of a cytokine-injected animal. Such experiments provide direct evidence of which enzymes are activated in vivo (we have found cell lines to vary in the pattern of kinases activated by IL-1) and yield quantities sufficient for precise identification by amino acid sequencing.

Materials and Methods

Materials. Human recombinant IL-1 α and PP2A were prepared as previously (9). The cDNA of the truncated jun protein comprising amino acids 5–89 was a kind gift of Dr. J. R. Woodgett (The Ontario Cancer Institute, Toronto, Canada). It was expressed and purified from *Escherichia coli* as glutathione S-transferase (GST) fusion protein as described elsewhere (10). Microtubule associated protein 2 (MAP-2) was purified from porcine brain (11). Protein phosphotyrosine phosphatase (PTP) 1B was given by Dr. N. Tonks (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Antiphosphotyrosine mAb 4G10 and γ-ATP-Sepharose were from Upstate Biotechnology Inc. (Lake Placid, NY). Peroxidase-coupled rabbit anti–mouse IgG was from Dako (High Wycombe, Bucks,

[1] *Abbreviations used in this paper:* DTT, dithiothreitol; EGF, epidermal growth factor; GST, glutathione S-transferase; MAP, mitogen-activated protein; MAP-2, microtubule-associated protein 2; MBP, myelin basic protein; PTP, protein phosphotyrosine phosphatase.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/94/12/2017/09 $2.00
Volume 180 December 1994 2017–2025

UK). The T669 peptide (RRRELVEPLTPSGE) based on the sequence around T^{669} of the EGF receptor was synthesized at Babraham Institute. γ-[^{32}P]ATP (3,000 Ci/mmol) was from Amersham International (Amersham, Bucks, UK). Myelin basic protein (MBP), E64, aprotinin, pepstatin, PMSF, and all other chemicals were from Sigma (Poole, UK). Okadaic acid was from Calbiochem-Novabiochem Ltd. (Nottingham, UK) FFQ-, FFS-, and phenyl-Sepharoses were from Pharmacia (Milton Keynes, UK). The agarose coupled to blue dye no. 9 (mimetic blue 1 A6XL) was from Affinity Chromatography Ltd. (Isle of Man, UK). Hypnorm was from Janssen Pharmaceuticals Ltd. (Wantage, Oxon, UK) and Sagatal was from Rhone Merieux (Harlow, Essex, UK).

Procedures with Rabbits. Dutch rabbits (2–3 kg) were sedated by injection with 0.2 ml/kg s.c. Hypnorm (0.315 mg/ml Fentanyl, and 10 mg/ml Fluanisone). IL-1 (5 μg/kg) in PBS at a concentration of 50 μg/ml, or PBS alone, was injected intravenously and 8 min later, the rabbits were killed by injection of Sagatal (pentobarbitone sodium urethane, 100 mg/kg). Livers were rapidly removed and put on ice, before being homogenized or alternatively frozen in liquid nitrogen and stored at −80°C.

Buffers. We used the following buffers: A: 20 mM Tris, pH 8.5, 20 mM β-glycerophosphate, 50 mM NaF, 0.1 mM Na_3VO_4, 2 mM dithiothreitol (DTT), 0.5 mM EGTA, and 0.5 mM EDTA; B: 20 mM Tris, 0.1 mM EGTA, and 2 mM DTT; C: 20 mM MES (2-[*N*-Morpholino]ethane sulfonic acid), pH 5.9, 20 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 10 mM NaF, 2 mM DTT, 0.5 mM EGTA, and 0.5 mM EDTA; D: 20 mM Hepes, pH 7.0, 10 mM NaF, 0.1 mM Na_3VO_4, 2 mM DTT, and 0.5 mM EGTA; E: 20 mM Tris, pH 7.4, 0.1 mM EGTA, 2 mM DTT, 0.2 mM NaCl, and 0.05% Brij35; and F: 20 mM Tris, pH 7.4, 2 mM DTT, and 0.05% Brij35.

Preparation of Liver Extracts. 80–100 g of liver was homogenized in 500 ml of buffer A including 10 μM E64, 10 μg/ml aprotinin, 1 μM pepstatin, and 1 mM PMSF using a Politron® blender (Kinematica AG, Littau, Switzerland). The homogenate was centrifuged at 20,000 *g* for 1 h at 4°C and the supernatant filtered (8 μm).

Purification of T669 Kinases. 5–20 g of supernatant protein was loaded on to a 220-ml column of Fast Flow Q-Sepharose equilibrated in buffer A containing 0.1 M NaCl. Proteins were eluted with a linear gradient (250 ml/h) from 0.1–0.6 M NaCl in 2,400 ml of buffer A. Active fractions (made 1 M NaCl) were loaded onto a 40-ml phenyl-Sepharose column equilibrated in buffer B containing 1 M NaCl. The column was washed in that buffer and all the T669 kinase was eluted by buffer B including 50% ethylene glycol. This material was made 20 mM with MES, pH 5.9, and loaded onto a 10-ml S-Sepharose Fast Flow column equilibrated in buffer C. The T669 kinase flowed through and was made 20 mM Tris, adjusted to pH 8.5. It was loaded onto a MonoQ HR 10/10 column equilibrated in buffer A, and was eluted with a linear gradient from 0 to 0.5 M NaCl in 450 ml. Fractions (4 ml) were assayed for T669 kinase. The two peaks of activity obtained were pooled separately and dialysed overnight against buffer D at 4°C.

The later eluting kinase (peak II) was loaded onto a 1-ml column of blue agarose equilibrated in buffer D. This was eluted with a linear gradient from 0 to 0.35 M NaCl in 24 ml at a flow rate of 1 ml/min. Kinase-containing fractions were pooled and concentrated to 0.5–0.8 ml by ultrafiltration (Centricon 10, Amicon Ltd., Stonehouse, Gloucs, UK). The sample was than chromatographed on a Superose 12 column equilibrated in buffer E. The enzyme eluted in one peak and was diluted fivefold in buffer B and made 20 mM with $MgCl_2$. It was then loaded onto a 3.5-ml ATP-Sepharose column equilibrated in buffer B containing 20 mM $MgCl_2$. After washing the column, the kinase was eluted with 10 ml equilibration buffer containing 2 mM ATP. It was finally loaded onto a MonoQ HR 5/5 column equilibrated in buffer E. This was eluted with a linear gradient (55 ml at 1 ml/min) from 0 to 0.7 M NaCl.

The smaller peak of T669 kinase (peak I) eluted from MonoQ HR 10/10 was purified further using buffers and chromatography conditions identical to those described above except that material from four purification runs (e.g., four livers) that had been carried through the five initial steps was pooled, and the final sequence of chromatography steps was ATP-Sepharose, MonoQ HR 5/5, and Superose 12.

The first three purification steps were carried out at 4°C and reaction mixtures of the kinase assays separated on gels because of high background phosphorylation. Subsequent purification was at room temperature and reaction mixtures separated on P81 paper. Purified proteins were run on SDS-PAGE (12), and stained with silver or Coomassie Brilliant Blue, or Western blotted, stained for phosphotyrosine, and detected by chemiluminescence (9).

Total protein was measured by the method of Bradford (13). Concentration of pure proteins was estimated from stained SDS-PAGE. Purified enzymes were kept at 4°C.

Kinase Assays. The assays were done in a reaction mixture (15 μl) of a 5-μl sample, 5 μl T669 peptide (1.5 mM), or protein substrates, 5 μl 150 mM, Tris, pH 7.4, 30 mM $MgCl_2$, and 60 μM ATP (including 1–2 μCi γ-[^{32}P]ATP). Assays were started by adding ATP and then incubated at 20°C for 20 min. The reaction was terminated by adding 5 μl SDS electrophoresis sample buffer. The phosphorylated substrates were separated from the mixture by SDS-PAGE. Gels for protein substrates were 12.5% acrylamide for MBP and GST-jun, or 5% for MAP-2. They were stained, dried, and autoradiographed. T669 peptide reaction mixtures were separated on SDS gels containing 16% acrylamide and 6 M urea (14), which were autoradiographed wet. ^{32}P incorporated into substrates was measured by Cerenkov radiation of excised gel slices.

Alternatively, the reaction mixture containing T669 peptide was spotted onto 2 × 2-cm squares of anion exchange paper (model P81; Whatman Inc., Clifton, NJ). The squares were washed three times in 30% CH_3COOH and 0.5% H_3PO_4, once in EOH, dried, and Cerenkov counted. Background phosphorylation of reaction mixtures from which substrate was omitted was measured and subtracted.

Treatment of Kinases with Phosphatases. 5 μl of kinase was incubated with 2.5 μl PP2A (30 mU/ml) or 2.5 μl PTP1B (100 μg/ml) in 20 mM Tris, pH 7.4, 0.1 mM EGTA, 2 mM DTT, and 0.05% Brij35 at 30°C for 30 min. 2.5 μl of either okadaic acid (20 μM) to inhibit PP2A, or Na_3VO_4 (2 mM) to inhibit PTP1B was then added for an additional 10 min. Phosphorylation was then carried out on T669 peptide as described in the previous section. To show that inactivation was due to phosphatase, parallel assays were carried out in which 5 μl of T669 kinase was incubated either with 2.5 μl phosphatase that had been inactivated for 10 min with 2.5 μl inhibitor, or with buffer alone.

Protein Digestion, Peptide Separation, and Amino Acid Sequencing. After separation by SDS-PAGE, proteins were detected by staining with Coomassie Blue and excised. Gel pieces were digested with trypsin and applied directly to a tandem HPLC separation using 2.1 mm AX-300 anion exchange and OD-300 reverse phase columns on a Hewlett-Packard 1090M with diode array detection. Peak fractions were collected during elution with a linear acetonitrile gradient in 0.1% TFA and sequenced using fast cycle automated Edman chemistry on a protein sequencer (model 477A; Applied Biosystems Ltd., Warrington, UK) modified as described (15).

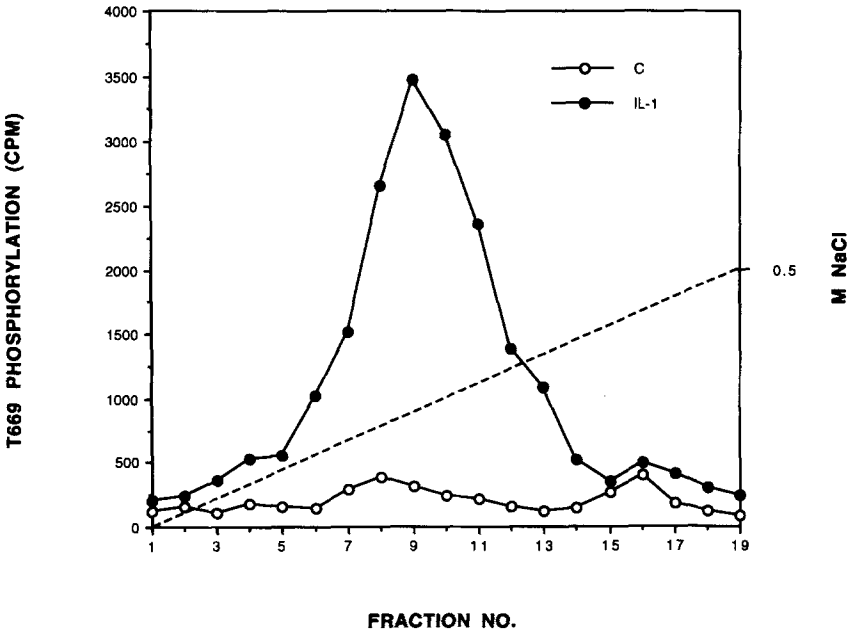

Figure 1. Hepatic T669 kinase activity in rabbits is increased after injection of IL-1. Liver extracts from two rabbits injected with IL-1 for 8 min or vehicle alone were prepared from 2 g of tissue, normalized for protein, and chromatographed on a 2-ml FFQ-Sepharose column as described in Materials and Methods. Proteins were eluted with a linear salt gradient from 0 to 0.5 M NaCl, and aliquots assayed for T669 kinase activity as described. Reaction mixtures were separated on SDS-PAGE. The gels were also autoradiographed and the T669 peptide phosphorylation quantitated as described.

Results

Hepatic T669 Kinase Activity Is Increased in Livers of Rabbits After Injection of IL-1. Cytosolic preparations were made from livers removed from rabbits 8 min after intravenous injection of IL-1 or vehicle, as described in Materials and Methods. Equal aliquots of these were chromatographed on a small FFQ-Sepharose column eluted with a salt gradient. Fig. 1 shows a result representative of three pairs of animals. The fractions from the IL-1–treated animal contained T669 kinase activity eluting as a broad peak between 100 and 250 mM NaCl. There was a 10-fold increase in active material in these fractions in comparison with those from an animal injected with vehicle alone.

Two T669 Kinases Can Be Purified from Livers of IL-1–treated Rabbits. T669 kinase was purified from extracts of livers of IL-1–treated animals by successive chromatography on FFQ-Sepharose, phenyl-Sepharose, and FFS-Sepharose as described in Materials and Methods. The active material was then applied to a MonoQ HR10/10 column and two forms of enzyme were separated by elution with a salt gradient. Peak I eluted at around 100 mM NaCl, peak II at around 150 mM NaCl (Fig. 2). Peak II contained about two thirds of the activity recovered. Both peaks were further purified by five additional chromatography steps. The purification of peak I enzyme is summarized in Table 1. About 5 μg of protein kinase

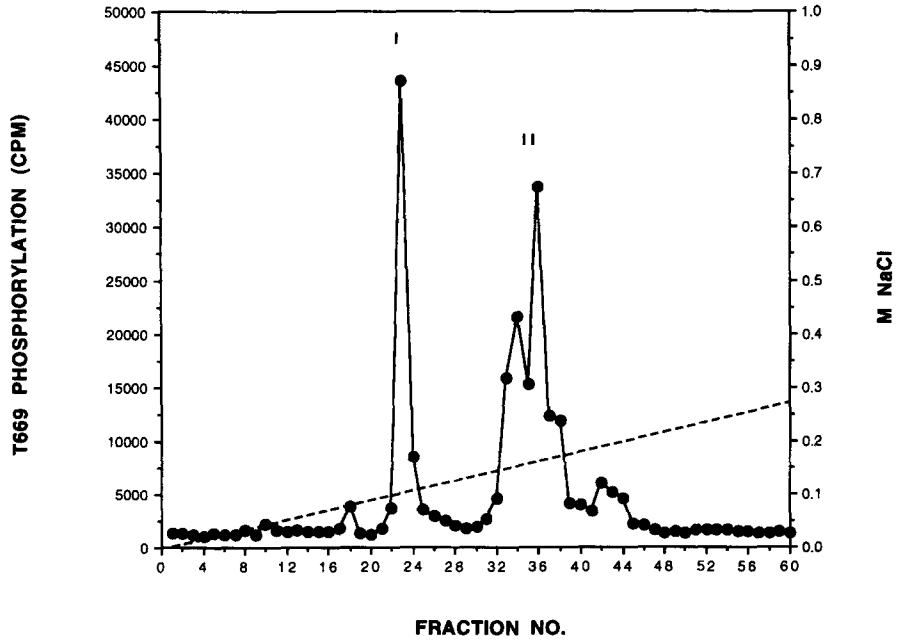

Figure 2. Livers of IL-1–treated animals contain two T669 kinases. T669 kinase purified on FFQ-Sepharose, phenyl-Sepharose, and FFS-Sepharose from one liver was chromatographed on a MonoQ HR10/10 column as described in Materials and Methods. Aliquots of the fractions were assayed for T669 kinase activity and reaction mixtures separated on P81 anion exchange paper and ^{32}P incorporation into the peptide quantitated as described.

Table 1. *Purification of Peak I of T669 Kinase*

	Volume	Protein	Activity	Sp act	Purification (-fold)	Yield
	ml	*mg*	*cpm* × 10^{-6}	(*cpm* ÷ *mg*) × 10^{-3}		%
Extract	1,460	34,398	–	–	–	–
Q-Sepharose	1,535	4,411	687	156	–	100
Phenyl-Sepharose	413	2,052	1,260	614	4	183
S-Sepharose	473	618	1,255	2,030	13	182
MonoQ						
HR 10/10	52	2.69	262	97,398	623	38
Blue-dye	12.5	1.2	59	49,167	315	9
ATP-Sepharose	17	0.1	73	730,000	4,677	11
MonoQ						
HR 5/5	7	0.02	26	1,300,000	8,329	4
Superose 12	2.5	0.005	20	4,000,000	25,628	3

T669 kinase activity from livers of IL-1–treated rabbits was purified over the first four steps. Peak I T669 kinase eluted from MonoQ HR 10/10 from four livers was pooled and purified as indicated through the subsequent steps. See Materials and Methods for details.

was obtained from four livers. The increase in specific activity over the material from the first anion exchange step was 25,000-fold. Peak II enzyme was purified in a similar manner (Table 2). About 3 μg of enzyme was obtained from one liver, representing about 30,000-fold purification from the first step.

In view of the likely relationship of these enzymes to MAP kinase they were treated with PP2A or PTP1B. Both were inactivated by either phosphatase (Fig. 3) and this was prevented by okadaic acid or vanadate, respectively. These findings suggested that the enzymes were activated by dual phosphorylation of serine/threonine and tyrosine. To identify the kinase of peak I aliquots of active fractions from the final Superose 12 column were separated on SDS-PAGE, transferred to nitrocellulose membrane, stained for protein, and probed with an antibody to phosphotyrosine. One phosphotyrosine-containing band (Fig. 4 *C*) corresponding to a 50-kD protein band (Fig. 4 *B*) coeluted with the enzyme activity (Fig. 4 *A*): this protein was therefore identified as the kinase. In the case of peak II, fractions from the final MonoQ column were separated on SDS-PAGE in two duplicate patterns: one half of the gel was silver stained whereas the other was blotted and probed with antibody to phosphotyrosine. The enzyme activity (Fig. 5 *A*) coeluted with a phosphotyrosine-containing 55-kD protein band (Fig. 5 *B* and *C*). This band was therefore identified as the peak II T669 kinase.

Table 2. *Purification of Peak II of T669 Kinase*

	Volume	Protein	Activity	Sp act	Purification (-fold)	Yield
	ml	*mg*	*cpm* × 10^{-6}	(*cpm* ÷ *mg*) × 10^{-3}		%
Extract	500	6,875	–	–	–	–
Q-Sepharose	485	752	152	202	–	100
Phenyl-Sepharose	73	234	168	718	4	111
S-Sepharose	93	98	237	2,418	13	156
MonoQ						
HR 10/10	28	6.4	92	14,375	77	61
Blue-dye	10	2.75	128	46,545	249	84
Superose 12	2	0.6	30	50,000	267	20
ATP-Sepharose	14	0.06	28	466,667	2,492	18
MonoQ						
HR 5/5	6	0.003	17	5,666,667	30,260	11

T669 kinase activity from the liver of an IL-1–treated rabbit was purified over four chromatography steps. Peak II from MonoQ HR 10/10 chromatography was further purified as indicated. See Materials and Methods for details.

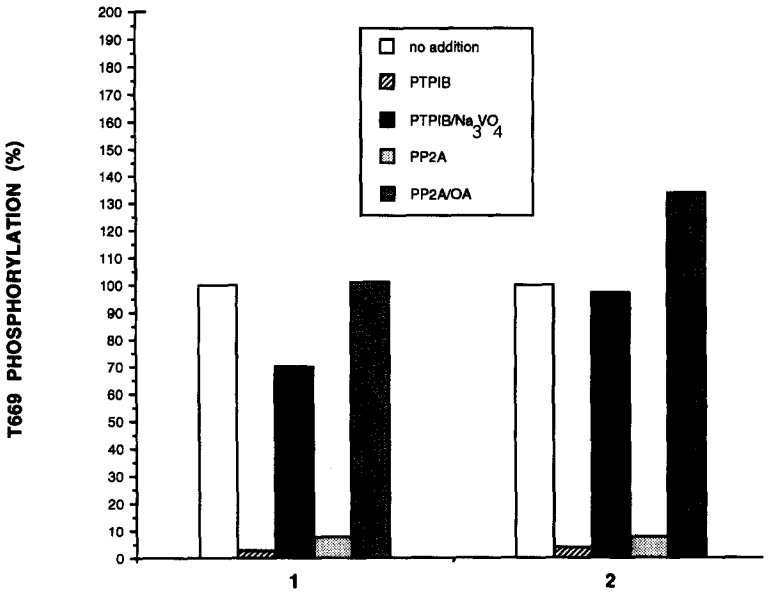

Figure 3. Both peak I and peak II T669 kinase are inactivated by phosphatases. Pure kinase of peak I (*1*) and peak II (*2*) were incubated with PP2A, or PTP1B, or phosphatase inactivated with okadaic acid or Na_3VO_4, respectively. After incubation, okadaic acid or Na_3VO_4 was added to the samples containing active phosphatase. Kinase activity was then assayed on T669 peptide. See Materials and Methods for details.

Substrate Specificity of p50 and p55 T669 Kinase. The ability of the enzymes to phosphorylate four MAP kinase substrates is shown in Fig. 6. Both phosphorylated T669 best and were least active against MBP. The enzymes phosphorylated MAP-2 or GST-c-*Jun* (amino acids 5-89) more rapidly than MBP. The ranking order for both enzymes was T669>MAP-2>MBP>GST-c-*Jun* (amino acids 5-89).

Amino Acid Sequencing of Peptides Identifies p50 and p55 T669 Kinase as Forms of p54α MAP Kinase. Purified enzymes were excised from SDS-PAGE and five tryptic peptides from p50 and seven from p55 were obtained and sequenced (Table 3). All were identical with the published sequence of the α form of p54 MAP kinase. Peptides, 3, 5, 6, and 7 were obtained from p50, peptides 1 and 4 were only recovered from p55. The sequences of some of these peptides show one or two differences in amino acids between the α and β and γ forms. Inspection of the sequences of peptides 2–6 show unequivocally that the small enzyme is a form of p54 α, rather than β or γ.

Discussion

The experiments provide the first direct evidence of IL-1 rapidly activating a protein kinase in a tissue in vivo. The two forms of enzyme that were purified accounted for most, and possibly all, of the T669 kinase induced by injecting IL-1. The amino acid sequences of the peptides, together with the biochemical properties of the enzymes, indicate that both are α forms of p54 MAP kinase, rather than p54 β or γ (7, 16).

T669 peptide is a substrate for p42/p44 MAP kinase, which IL-1 activates strongly in some cultured cells (4–6). We were surprised that essentially all the enzyme we detected could be accounted for by p50 and p55 and there was little evidence of any activated enzyme corresponding to p42/p44 MAP kinase. We also checked MonoQ chromatography fractions from liver extracts of unstimulated and IL-1–treated rabbits for their activity towards MBP, which is the substrate preferred by p42/p44 MAP kinase. However, no increase in MBP kinase activity was found in any fractions, including those containing p42/p44 MAP kinase as judged by immunostaining (Kracht, M., and A. Finch, unpublished observations). These fractions were also tested for MAP kinase kinase by their ability to activate a recombinant p42 MAP kinase. No increase in activity was found in the IL-1–stimulated material (Kracht, M., and A. Finch, unpublished observations).

The recently reported cDNAs of p54 MAP kinases code for full-length α, β, and γ proteins, and COOH-terminally truncated forms of β and γ. The truncated γ form is identical to another recently reported kinase, JNK1, which is activated in cells by UV irradiation and, much like p54, phosphorylates the NH_2-terminal activation domain of *c-jun*. JNK1 had a molecular mass on SDS-PAGE of 46 kD (17). Since we had purified a 45-kD T669 kinase from KB cells having properties similar to p54 we wondered if smaller forms would correspond to β or γ sequences. However, in the case of the liver, the smaller enzyme is clearly p54 α. The p54 family is not activated by MAP kinase kinase and has its own activator(s). They have been called stress-activated protein kinases since they are preferentially stimulated by stress (e.g., cycloheximide, heat, arsenite, and UV radiation), whereas mitogens and phorbol esters preferentially activate the p42/p44 MAP kinase path (7). IL-1 may also preferentially activate p54 in the liver, but further investigation of this response is needed since our observations on the absence of p42/p44 MAP kinase activation are restricted to a single time point after cytokine injection.

It will be important to establish which pathway is important for which cellular responses, and to find out how inflammatory cytokines such as IL-1 and TNF, which are themselves induced by stressful stimuli, activate the stress-kinase pathway. More needs to be known about the physiological significance of these signaling systems, but it is possible that they could be manipulated to interfere with cytokine action therapeutically.

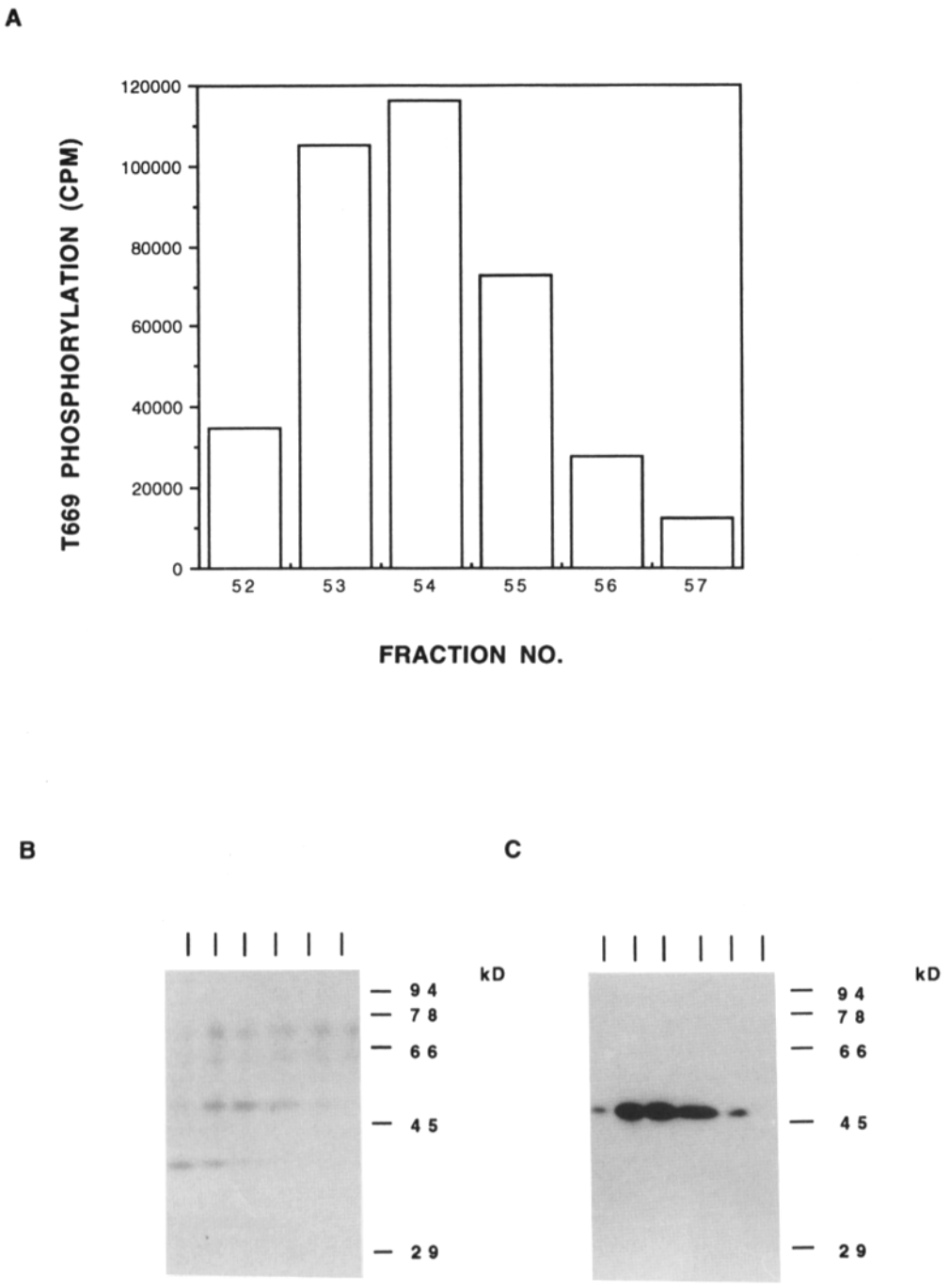

Figure 4. Identification of peak I T669 kinase. Aliquots of the most active fractions from Superose 12 chromatography of peak I T669 kinase (*A*) were separated on 10% SDS-PAGE and electrophoretically transferred to nitrocellulose. The blot was stained with Coomassie Brilliant Blue (*B*) and then probed for phosphotyrosine with antibody 4G10 (*C*).

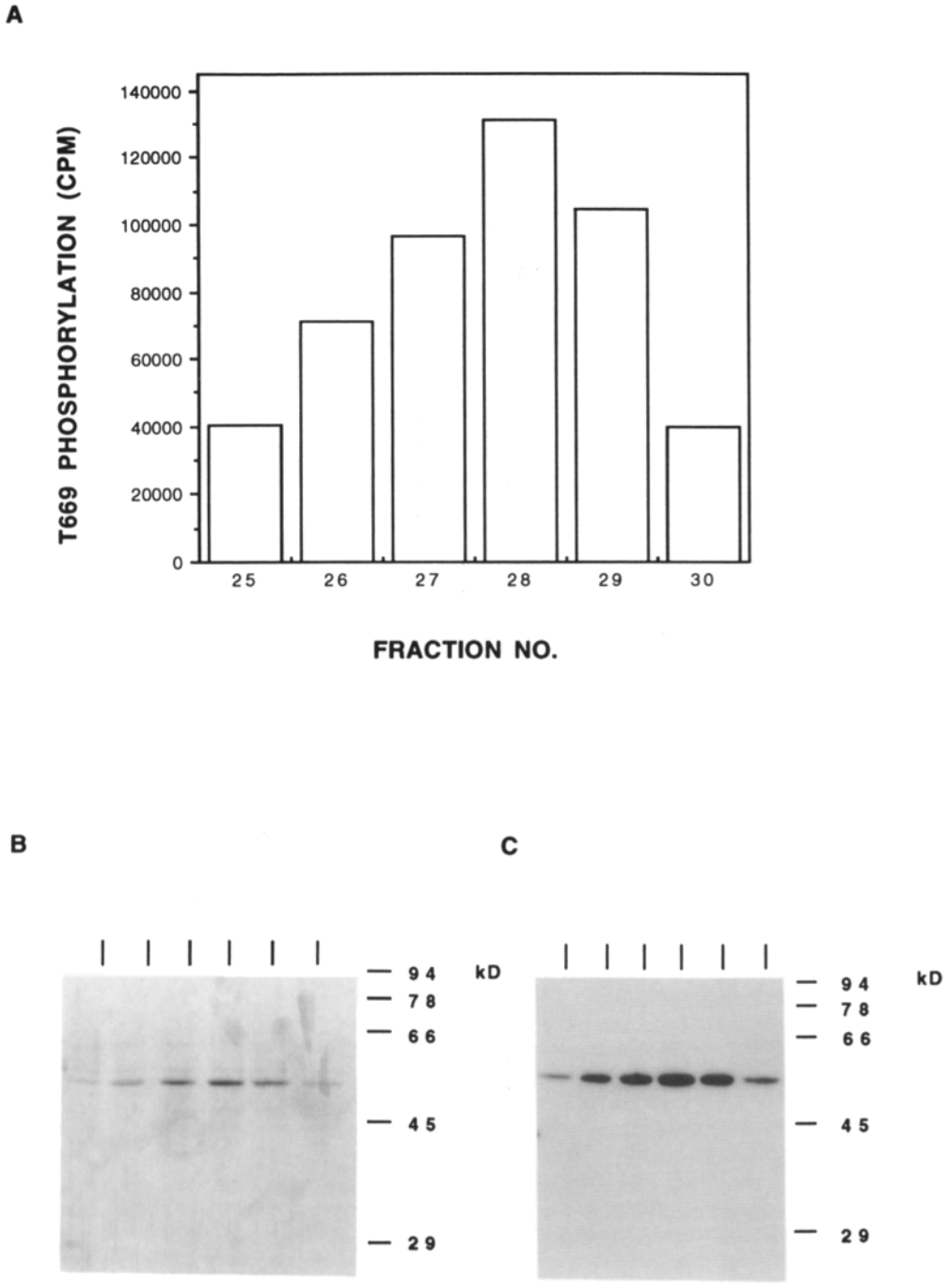

Figure 5. Identification of peak II T669 kinase. The active fractions of peak II purified on MonoQ HR5/5 (*A*) were concentrated six-fold. 80 μl of each fraction was run in two patterns on 10% SDS-PAGE. One half of the gel was stained with silver (*B*) and the other was blotted and probed for phosphotyrosine (*C*). Details are given in Materials and Methods.

Table 3. *Amino Acid Sequencing of Peptides Identifies p50 and p55 T669 Kinases as forms of p54α MAP Kinase*

Peptide	Amino acid	Sequence	Protein
1	73-78	ELVLLK	p55
		ELVLLK	α
		ELVL**M**K	β
		ELVL**M**K	γ
2	193-203	APEVILGMGYK	p50 and p55
		APEVILGMGYK	α
		APEVILGMGYK	β
		APEVILGMGYK	γ
3	237-250	VIEQLGTPSAEFMK	p50 and p55
		VIEQLGTPSAEFMK	α
		VIEQLGTP**C**PEFMK	β
		VIEQLGTP**C**PEFMK	γ
4	301-308	MLVIDPDK	p55
		MLVIDPDK	α
		MLVIDP**A**K	β
		MLVID**A**SK	γ
5	318-327	HPYITVWYDP	p50 and p55
		HPYITVWYDP	α
		HPYI**N**VWYDP	β
		HPYI**N**VWYDP	γ
6	346-353	EHAIEEWK	p50 and p55
		EHAIEEWK	α
		EH**T**IEEWK	β
		EH**T**IEEWK	γ
7	354-358	ELIYK	p50 and p55
		ELIYK	α
		ELIYK	β
		ELIYK	γ

Tryptic peptides from both enzymes were sequenced as described in Materials and Methods. All the peptides were obtained from p55, but only peptides 2,3,5,6, and 7 were from p50. The amino acids of the peptides are underlined and are aligned with the corresponding peptides in the published sequences of α, β, and γ rat p54 MAP kinases (7). The position of the peptides within the sequence is indicated by numbering the amino acids. Differences in amino acid residues between the α, β, and γ forms are shown in boldface type.

We thank Dr. N. Tonks for the gift of PTP1B, Lesley Rawlinson for making IL-1 and MAP-2; and Dr. François Guesdon for making PP2A.

We are grateful for the financial support from The Wellcome Trust, the Medical Research Council of the UK, and the Arthritis and Rheumatism Council of the UK.

Address Correspondence to Dr. Michael Kracht, Babraham Institute, Cytokine Laboratory, Department of Development and Signalling, Babraham Hall, Babraham, Cambridge CD2 4AT, UK.

Received for publication 25 July 1994.

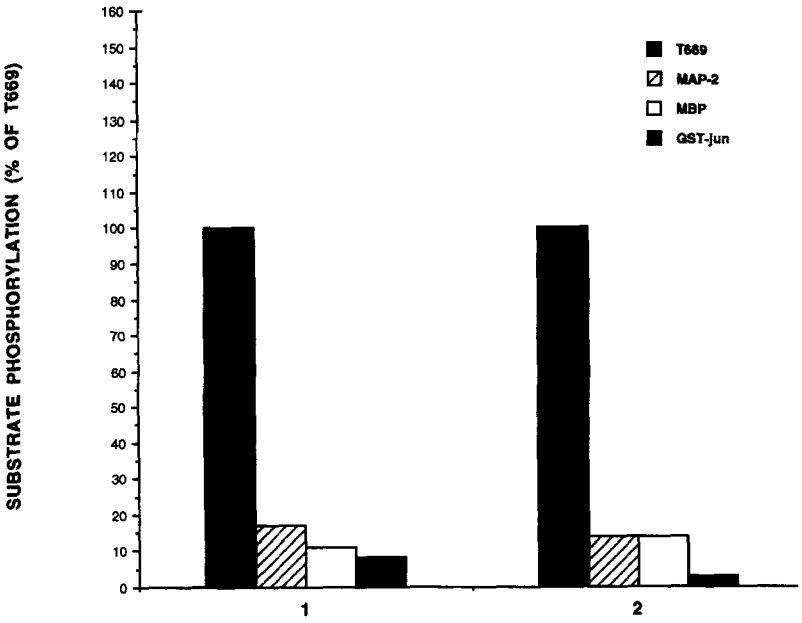

Figure 6. Substrate specificity of p50 (peak I) and p55 (peak II) T669 kinase. 5 μl of pure enzyme of peak I (*1*) and peak II (*2*) was assayed for 15 min at 20°C on MAP-2 (0.3 mg/ml), MBP (0.3 mg/ml), GST-jun (5-89) (0.17 mg/ml), and T669 peptide (0.5 mM). The ATP concentration was 50 μM. Reaction mixtures containing protein were separated on SDS-PAGE and for T669 peptide, on P81 anion exchange paper. ^{32}P incorporation into substrates was measured as described in Materials and Methods and expressed as a percentage of T669 peptide phosphorylation. Peak I kinase activity on T669 was 31,000 cpm/assay, peak II kinase activity on T669 was 16,000 cpm/assay.

References

1. Dinarello, C.A. 1991. Interleukin 1 and Interleukin 1 antagonism. *Blood.* 77:1629.
2. Sims, J.E., M.A. Gayle, J.L. Slack, M.R. Alderson, T.A. Bird, J.G. Giri, F. Colotta, F. Re, A. Mantovani, K. Shanebeck, et a. 1993. Interleukin signalling occurs exclusively via the type I receptor. *Proc. Natl. Acad. Sci. USA.* 90:6155.
3. Stylianou, E., L.A.J. O'Neill, L. Rawlinson, M.R. Edbrooke, P. Woo, and J. Saklatvala. 1992. Interleukin 1 induces NF-κB through its type I but not its type II receptor. *J. Biol. Chem.* 267:15836.
4. Saklatvala, J., L.M. Rawlinson, C.J. Marshall, and M. Kracht. 1993. Interleukin 1 and tumour necrosis factor activate the mitogen-activated (MAP) kinase kinase in cultured cells. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 334:189.
5. Guesdon, F., N. Freshney, R.J. Waller, L. Rawlinson, and J. Saklatvala. 1993. Interleukin 1 and tumor necrosis factor stimulate two novel protein kinases that phosphorylate the heat shock protein hsp27 and β casein. *J. Biol. Chem.* 268:14343.
6. Bird, T.A., P.R. Sleath, P.C. de Roos, S.K. Dower, and G.D. Virca. 1991. Interleukin 1 represents a new modality for the activation of extracellular signal-regulated kinases/microtubule-associated protein-2 kinases. *J. Biol. Chem.* 266:22661.
7. Kyriakis, J.M., P. Banerjee, E. Nikolakaki, T. Dai, E.A. Rubie, M.F. Ahmad, J. Avruch and J.R. Woodgett. 1994. The stress-activated protein kinase subfamily of c-Jun kinases. *Nature (Lond.).* 369:156–160.
8. Davis, R.J. 1993. The mitogen-activated protein kinase signal transduction pathway. *J. Biol. Chem.* 268:14553.
9. Kracht, M., M. Shiroo, C.J. Marshall, J.J. Hsuan, and J. Saklatvala. 1994. Interleukin 1 activates a novel protein kinase that phosphorylates the EGF receptor peptide T669. *Biochem. J.* 302:897.
10. Pulverer, B.J., J.M. Kyriakis, J. Avruch, E. Nikolakaki, and J. Woodgett. 1991. Phosphorylation of c-Jun mediated by MAP kinases. *Nature (Lond.).* 353:670.
11. Kim, H., L.I. Binder, and J.L. Rosenbaum. 1979. The periodic association of MAP_2 with brain microtubules in vitro. *J. Cell Biol.* 80:266.
12. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of head of bacteriophage T4. *Nature (Lond.).* 227:680.
13. Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 74:248.
14. Schapper, H., and G. von Jagow. 1987. Tricine-sodium dodecyl sulfate–polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kD. *Anal. Biochem.* 166:368.
15. Totty, N.F., M.D. Waterfield, and J.J. Hsuan. 1992. Accelerated high-sensitivity microsequencing of proteins and peptides using a miniature reaction cartridge. *Protein Science.* 1:1215.
16. Kyriakis, J.M., and J. Avruch. 1990. p54 microtubule-associated protein-2 kinase. A novel serine/threonine protein kinase regulated by phosphorylation and stimulated by poly-L-lysine. *J. Biol. Chem.* 265:17355.
17. Derijard, B., M. Hibi, I-Huan Wu, T. Barrett, B. Su, T. Deng, M. Karin, and R.J. Davis. 1994. JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-jun activation domain. *Cell.* 76:1025.