Interleukin-1 activates a novel protein kinase that phosphorylates the epidermal-growth-factor receptor peptide T669

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We have isolated from KB cells stimulated with interleukin-1 (IL-1) a protein kinase that phosphorylates a peptide (T669) based on the sequence around T^{669} of the epidermal growth factor (EGF) receptor. The enzyme, which had an apparent molecular mass of 45 kDa on gel-filtration chromatography, was purified 170000-fold from cytosolic extracts by sequential chromatography on Mono Q, Mono S, phenyl-Sepharose, Superose 12, ATP–Sepharose and Mono Q. The enzyme activity co-chromatographed at the last step with a 45 kDa protein band that stained for phosphotyrosine. This peak fraction also contained some actin and a 60 kDa protein that stained weakly for phosphotyrosine. The T669 peptide is a substrate for mitogen-activated protein (MAP) kinase. Amounts of IL-1-induced T669 kinase and activated recombinant p42 MAP kinase having equal activity on T669 peptide were compared on commonly used MAP kinase substrates. T669 kinase was two or three orders of magnitude less active on myelin basic protein or microtubule-associated protein-2 than was MAP kinase. The IL-1-induced T669 kinase did not react with antiserum to p42/p44 MAP kinase. It was inactivated by treatment with protein phosphatase 2A or protein phosphotyrosine phosphatase 1B, so it may be regulated by dual phosphorylation in similar fashion to MAP kinase. The dephosphorylated enzyme was not re-activated by MAP kinase kinase. This novel enzyme could lie on a kinase cascade induced by IL-1. It may be responsible for phosphorylating T669 of the EGF receptor.

INTRODUCTION

Interleukin-1 (IL-1) α and β are related 17 kDa cytokines with similar biological activity. They are major inflammatory hormones, produced mainly by activated mononuclear phagocytes. At local sites they cause leucocyte accumulation and activation. They also promote tissue resorption, and in the process may damage cartilage and bone. Systemically, they induce fever and the acute-phase response. If present in large amounts they cause hypotension and death. At a cellular level IL-1 induces many genes, but it is unclear how this is brought about (Dinarello, 1991).

There are two IL-1 receptors: the larger type I receptor (80 kDa), which appears to transduce the signal, and the smaller type II receptor (60 kDa), whose intracellular function (if any) is unknown (Sims et al., 1993). Both forms of IL-1 bind to these receptors with high affinity, but the mechanisms of signal transduction are obscure. A wide variety of post-receptor events have been reported: these include generation of second messengers such as cyclic AMP (Chedid et al., 1989), diacylglycerol (Rosoff et al., 1988) and ceramide (Mathias et al., 1993), and activation of G-proteins (O'Neill et al., 1990) and protein kinase C (Kracht et al., 1993). However, the significance of these changes for signalling by IL-1 is not established.

IL-1 rapidly increases phosphorylation of certain cellular proteins (Kaur and Saklatvala, 1988; Shiroo and Matsushima, 1990; Guy et al., 1991). Two which have been identified are the small heat-shock protein hsp27 (Kaur et al., 1989) and the epidermal growth factor (EGF) receptor (Bird and Saklatvala, 1989, 1990).

One approach to working out how IL-1 signals has been to identify the protein kinases responsible for such changes. Initial experiments showed that extracts of IL-1-stimulated fibroblasts contained at least three activated protein kinases that could be separated on chromatography. One was the mitogen-activated protein (MAP) kinase, and the others were unidentified enzymes that phosphorylated hsp27 and β-casein respectively (Guesdon et al., 1993). Such extracts were more recently shown also to contain activated MAP kinase kinase (Saklatvala et al., 1993).

MAP kinases are ubiquitous serine/threonine kinases whose activity depends on phosphorylation of nearly adjacent tyrosine and threonine residues. Mammalian cells contain two closely related proteins, p42 and p44. They are induced by a very wide range of stimuli, including insulin, growth factors, chemotactic agents, bacterial products and cytokines (Davis, 1993). This activation is carried out by MAP kinase kinases, which have dual specificity for tyrosine and threonine residues (Ahn et al., 1992).

MAP kinase is usually detected with myelin basic protein (MBP) substrate and phosphorylates threonine or serine residues in the motif P-X-T/S-P. This is found around T^{669} of the EGF receptor, and peptides based on this are substrates for MAP kinase (Davis, 1993). A study of IL-1-activated kinases in KB cells identified an enzyme that phosphorylated such a peptide, and concluded that it was MAP kinase (Bird et al., 1991).

When investigating the regulation of MAP kinase by IL-1, we used a peptide based on the sequence around T^{669} of the EGF receptor as a substrate, in addition to MBP. We found that these substrates detected different enzymes.

Abbreviations used: DTT, dithiothreitol; EGF, epidermal growth factor; MAP, mitogen-activated protein; MAP-2, microtubule-associated protein-2; MBP, myelin basic protein; IL-1, interleukin-1; PMA, phorbol 12-myristate 13-acetate; PP2A, protein phosphatase 2A; PTP1B, protein phosphotyrosine phosphatase 1B; T669, EGF receptor peptide.

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MATERIALS AND METHODS

Materials

Human recombinant IL-1α was purified from extracts of *Escherichia coli* expressing the protein (Bird and Saklatvala, 1990). Human recombinant p42 MAP kinase was expressed in *E. coli* and purified as described by Stokoe et al. (1992). Microtubule-associated protein 2 (MAP-2) was purified from pig brain (Kim et al., 1979). The catalytic subunit of protein phosphatase 2A (PP2A) was purified from rabbit muscle (Cohen et al., 1988): its specific activity was 3300 m-units/mg. Protein phosphotyrosine phosphatase 1B (PTP1B) was generously given by Dr. N. Tonks, Cold Spring Harbor Laboratory, NY, U.S.A. The rabbit antisera 124 and 122 were raised to C-terminal peptides of the sequence of p42 MAP kinase (Leevers and Marshall, 1992). Phosphotyrosine was detected by using the rat monoclonal antibody 2-53, which was raised against a mixture of phosphotyrosine, alanine and glycine coupled with keyhole-limpet haemocyanin (Kamps and Sefton, 1988) or the monoclonal antibody 4G10 from Upstate Biotechnology, Lake Placid, NY, U.S.A. For Western blotting, second antibodies were ^{125}I-labelled anti-rabbit IgG (from donkey) (Amersham International, U.K.), or a peroxidase-conjugated anti-rat Ig (from rabbit) or anti-rabbit IgG (from swine) from DAKO, U.K. Pansorbin was from Calbiochem (U.S.A.). The T669 peptide (RRRELVEPLTPSGE) based on the sequence around T^{669} of the EGF receptor was synthesized and purified in this Institute. [γ-^{32}P]ATP (3000 Ci/mmol) was from Amersham. Phorbol 12-myristate 13-acetate (PMA), β-glycerophosphate, E64 [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane], aprotinin, pepstatin, phenylmethanesulphonyl fluoride, MBP and phosphoamino acids were from Sigma. Okadaic acid was from Calbiochem–Novabiochem (U.K.). The ATP–Sepharose, in which the nucleotide was coupled through the γ-phosphate, was from Upstate Biotechnology. Phenyl-Sepharose was from Pharmacia (U.K.).

Cell culture

KB cells from the American Tissue Culture Collection were cultured in Dulbecco's modified Eagle's medium (GIBCO Bethesda Research Laboratories, Uxbridge, Middx., U.K.) which contained 10% heat-inactivated foetal-calf serum (Globepharm, Esher, U.K.).

Chromatography buffers

(A) 20 mM Tris, pH 8.5, 20 mM β-glycerophosphate, 50 mM NaF, 0.1 mM $NaVO_3$, 2 mM dithiothreitol (DTT), 0.5 mM EGTA, 0.5 mM EDTA; (B) 20 mM Mes, pH 5.8, 20 mM β-glycerophosphate, 0.1 mM $NaVO_3$, 10 mM NaF, 2 mM DTT, 0.5 mM EGTA, 0.5 mM EDTA; (C) 20 mM Tris, pH 7.4, 0.1 mM EGTA, 2 mM DTT.

Preparation of cell extracts

Confluent KB cells were stimulated with IL-1 (20 ng/ml) or PMA (100 ng/ml) for 15–20 min at 37 °C. The cells were then rapidly washed in ice-cold PBS/0.1 mM EGTA and resuspended by scraping them into buffer A (5 ml/175 cm^2 flask) containing proteinase inhibitors as indicated below. Cells were broken by passing them three times through a 26-gauge needle. Broken cells were centrifuged at 100000 *g* for 1 h at 4 °C. The supernatants (cytosolic extracts) were filtered through a 0.2 μm-pore filter before chromatography.

Anion-exchange chromatography

Equal amounts of cytosolic protein from untreated or stimulated cells were loaded on to a Mono Q HR 5/5 column run in a f.p.l.c. system (Pharmacia). The column was equilibrated in buffer A. Proteins were eluted by a linear 60 ml salt gradient (0–0.5 M NaCl in buffer A); 1 ml fractions were collected.

Purification of T669 kinase

For this, 50–60 175 cm^2 flasks of confluent KB cells were stimulated by replacing the culture medium with Dulbecco's modified Eagle's medium (DMEM) containing IL-1 (20 ng/ml) at 37 °C for 15 min. The flasks were then placed on ice, the DMEM was removed, and cells were rapidly scraped into 10 ml of ice-cold PBS. Cells were spun at 500 *g* at 4 °C for 15 min and resuspended in 150–180 ml of a hypotonic buffer (5 mM Tris, pH 7.0, 0.5 mM EGTA, 0.5 mM EDTA, 50 mM NaF, 0.2 mM $NaVO_3$, 20 mM β-glycerophosphate, 2 mM DTT, 10 μM E64, 10 μg/ml aprotinin, 1 μM pepstatin, 1 mM phenylmethanesulphonyl fluoride). After 15 min on ice, cells were broken by 30 strokes in a Dounce homogenizer, then centrifuged at 100000 *g* for 1 h at 4 °C. The pH of the supernatant was adjusted to 8.5 by adding 15 mM Tris. After being passed through a 0.2 μm filter, the cytosolic extract was loaded on to a Mono Q HR10/10 column equilibrated in buffer A. Proteins were eluted with a gradient of 0–0.5 M NaCl in 480 ml of buffer A at a flow rate of 4 ml/min. Fractions (4 ml) were collected and assayed for kinase activity. T669 kinase was eluted sharply around 0.18 M NaCl. The two peak fractions were pooled and dialysed overnight against buffer B at 4 °C, then filtered (0.2 μm) and loaded on to a Mono S HR 5/5 column equilibrated with buffer B, which was eluted with a gradient of 0–0.6 M NaCl in 15 ml of buffer B. The fraction running through was collected, and after NaCl was added to 1 M final concn. it was loaded on to a 0.5 ml phenyl-Sepharose column equilibrated in buffer C containing 1 M NaCl. After washing the column, the T669 kinase was eluted with 6 column vol. to buffer C containing 30% ethylene glycol. Brij 35 (to 0.05%) was added to this eluate, which was then concentrated by ultrafiltration to 220 μl. This was chromatographed on a Superose 12 HR 10/30 column equilibrated in buffer C, modified to include 0.2 M NaCl and 0.05% Brij 35; 0.25 ml fractions were collected at a flow rate of 0.3 ml/min. Fractions comprising the peak of T669 kinase were pooled and diluted 8–10-fold in buffer C containing 20 mM $MgCl_2$. This sample was loaded on to a 0.5 ml ATP–Sepharose column equilibrated in the modified buffer C. The column was washed with buffer C, then eluted with 6 column vol. each of buffer C containing firstly 0.5 mM ATP and then 1 mM ATP. Brij 35 was added to 0.05% and the eluate was loaded on to a Mono Q HR 5/5 column equilibrated in buffer C containing 0.05% Brij 35. Proteins were eluted with a gradient of 0–0.4 M NaCl in 30 ml of buffer C. The T669 kinase that was eluted was stored at −20 °C.

Electrophoresis

This was carried out in SDS/polyacrylamide (12.5% or 10% acrylamide) gels (Laemmli, 1970). Gels were stained with Coomassie Brilliant Blue or silver. Molecular-mass standards were: cytochrome *c* (12 kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa), BSA (68 kDa), transferrin (78 kDa) and phosphorylase *a* (94 kDa).

Kinase assays

The assays were carried out in a reaction mixture (15 μl) containing 5 μl of sample, 5 μl of substrate, 5 μl of 50 mM Tris,

pH 7.4, 10 mM $MgCl_2$ and 20 μM ATP (including 1–2 μCi of [γ-^{32}P]ATP). Final concentrations of each substrate were: 0.5 mM T669, 0.17 mg/ml MBP, 0.2 mg/ml MAP-2, 0.1 mg/ml p42 MAP kinase. Assays were started by adding the ATP and were then incubated at room temperature for 20 min. The phosphorylation reaction was terminated by adding 5 μl of electrophoresis sample buffer (8% SDS, 100 mM Tris, pH 6.8, 4% β-mercaptoethanol, 24% glycerol, 0.02% Bromophenol Blue). The phosphorylated substrates were separated from the mixture by SDS/PAGE. Gels for protein substrates were either 12.5% or 5% acrylamide for MBP or MAP-2 respectively. These were stained, destained, dried and autoradiographed against medical X-ray film. For T669 peptide, reaction mixtures were separated on SDS gels containing 16% acrylamide and 6 M urea (Schapper and von Jagow, 1987), which were autoradiographed wet. ^{32}P incorporated into substrates were measured by counting Čerenkov radiation of excised gel slices.

For monitoring the T669 kinase purification, 5 μl of the reaction mixture containing T669 peptide or MBP was spotted on to 1.5 cm × 1.5 cm squares of Whatman P81 anion-exchange paper. The squares were washed three times in 0.5% H_3PO_4/30% acetic acid, dried, and Čerenkov emission was measured. Background phosphorylation of reaction mixtures from which substrate was omitted was measured and subtracted. This method was less sensitive then the separation of reaction mixtures on SDS/PAGE, and was only used when background phosphorylation was less than 10%.

Kinase activity is given either as c.p.m./20 min (sp. radioactivity 2000–4000 c.p.m./pmol of ATP) or in units (1 pM ^{32}P/min) transferred to the substrates.

Western blotting

Proteins were separated on SDS/PAGE and electrophoretically transferred to nitrocellulose membranes. The membranes were blocked with 2% dried milk in Tris-buffered saline (TBS) for 30 min at 37 °C. Membranes were then incubated in milk/TBS with the first antibody for 4–24 h at 20 °C, washed in TBS, and incubated for a further 2–4 h in TBS with the second antibody. After washing in TBS, membranes were dried and either directly autoradiographed (if the second antibody was ^{125}I-labelled) or proteins were detected by using the Amersham enhanced chemiluminescence (ECL) system.

Immunoprecipitation

A 10 μl sample of partially purified T669 kinase or activated p42 MAP kinase was incubated with 2 μl of antibody 122 or 2 μl of pre-immune serum in buffer C containing 0.2 M NaCl and 0.05% Brij 35 (total volume 50 μl). The samples were rotated overnight at 4 °C, then 150 μl of Pansorbin was added and incubation was continued for 2 h at room temperature. The samples were centrifuged at 10000 *g* for 5 min, the supernatants collected and the pellets washed six times in buffer C containing 0.2 M NaCl and 0.05% Brij 35. Finally the pellets were resuspended in 50 μl of this buffer. Then 10 μl portions of supernatants or resuspended immunoprecipitates were assayed for kinase activity.

Treatment of T669 kinase with phosphatases

A 20 μl portion of T669 kinase purified as far as the Superose 12 step was incubated with 10 μl of PP2A (30 m-units/ml) or 10 μl of PTP1B (5 μg/ml) in 20 mM Tris (pH 7.4)/0.1 mM EGTA/4 mM DTT/0.1% Brij 35 at 30 °C. After the times indicated, 7.5 μl of that mixture was removed and incubated for a further 10 min at 30 °C either with 2.5 μl of okadaic acid (20 μM) to inhibit PP2A, or with 2.5 μl of $NaVO_3$ (2 mM) to inhibit PTP1B. Phosphorylation was then carried out on T669 peptide as described above. To show that inactivation was due to phosphatase, parallel assays were carried out in which 5 μl of T669 kinase was incubated with either 2.5 μl of phosphatase that had been inactivated for 10 min with 2.5 μl of inhibitor, or buffer alone, for 60 min.

Partial purification of MAP kinase kinase

Three flasks of confluent KB cells were stimulated with 100 ng/ml PMA for 15 min, and a cytosolic extract was prepared and chromatographed on a Mono Q HR 5/5 column as for T669 kinase. Fractions were then screened for MAP kinase kinase activity, with recombinant p42 MAP kinase as substrate. Active fractions were pooled, concentrated 10-fold by ultrafiltration to 100 m-units/μl, and stored in 50% glycerol at −20 °C.

Preparation of pure active recombinant p42 MAP kinase

A 50 μg portion of pure human recombinant MAP kinase was incubated with 100 μl of MAP kinase kinase prepared as described above in 50 mM Tris (pH 7.4)/10 mM $MgCl_2$/1 mM ATP in a total volume of 300 μl for 6 h at 20 °C. The sample was diluted 6-fold in buffer C and chromatographed on a Mono Q column equilibrated in buffer C. Elution was with a NaCl gradient (0–0.5 M in 30 ml of buffer C). Fractions were assayed for MBP-phosphorylating activity. The active MAP kinase was eluted around 0.2 M NaCl. Fractions containing this repurified MAP kinase were pooled, concentrated and stored at −20 °C in 50% glycerol. The specific activity was 3 units/μg.

Re-activation of dephosphorylated kinases with MAP kinase kinase

A 10 μl portion of either T669 kinase or active p42 MAP kinase was dephosphorylated with 2.5 μl of PP2A for 30 min at 30 °C. The phosphatase was then inactivated with 2.5 μl of okadaic acid for 10 min. Incubations were in buffer C. Then 5 μl of MAP kinase kinase and 10 μl of phosphorylation buffer (20 mM Tris, pH 7.4, 10 mM $MgCl_2$, 20 μM ATP, 2 μCi of [^{32}P]ATP) were added for 30 min at 30 °C to allow rephosphorylation. After that, 3 μl of MBP or T669 peptide was added and phosphorylation continued at room temperature for 20 min. In parallel, kinases were incubated with buffer alone, with PP2A inactivated with okadaic acid, or with okadaic acid alone at 30 °C for 70 min. After that time, 15 μl of phosphorylation mixture including MBP or T669 peptide was added and the samples were incubated for 20 min at room temperature. Reaction mixtures were then separated on SDS/PAGE as described above. Final concentrations were 3 m-units/ml PP2A, 20 μM okadaic acid, 0.2 μg/ml MBP and 0.5 mM T669 peptide. The activity of the MAP kinase kinase preparation on MBP was measured by incubating 5 μl with 10 μl of phosphorylation buffer including MBP. Under the same conditions no activity was found on T669 peptide.

Protein determination

Recovery of protein during purification up to the Superose 12 step was measured with a dye-binding assay (Bradford, 1976). From subsequent steps protein was estimated from stained SDS/PAGE.

Phosphoamino acid analysis

T669 peptide was phosphorylated by kinase-containing fractions in the presence of [γ-^{32}P]ATP. The peptide was separated by electrophoresis and eluted from the gel slices. It was hydrolysed in 5.7 M HCl at 110 °C for 1 h, then dried. The dried hydrolysates were mixed with phosphoamino acid standards and analysed by two-dimensional electrophoresis on cellulose plates, which were autoradiographed (Cooper et al., 1983). Standards were detected by ninhydrin staining.

Protein digestion, peptide separation and 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 tandem h.p.l.c. separation using 2.1 mm-diam. AX-300 anion-exchange and OD-300 reverse-phase columns on a Hewlett–Packard 1090M instrument with diode array detection. Peak fractions were collected during elution with a linear acetonitrile concentration gradient in 0.1% trifluoroacetic acid and sequenced by using fast-cycle automated Edman chemistry on an Applied Biosystems 477A instrument modified as described by Totty et al. (1992).

RESULTS

IL-1-stimulated KB cells contained a T669 kinase chromatographically distinct from p42/p44 MAP kinase

Extracts made from resting or stimulated cells were applied to a Mono Q chromatography column and eluted with a shallow salt gradient. Fractions from the IL-1-stimulated cells contained kinase activity which eluted as a single peak (0.18 M NaCl) that phosphorylated the T669 peptide (Figure 1a). The peak contained 5-fold more activity than did equivalent fractions from resting cells, and the enzyme phosphorylated the T669 peptide on threonine only (Figure 1a, inset). Because the T669 peptide may be a substrate for MAP kinase, the fractions were assayed for activity on MBP (Figure 1b). The peak fractions showed no corresponding activity towards MBP substrate (Figure 1b). In fact, no increased activity towards MBP was seen in any of the fractions from IL-1-stimulated cells. This was surprising, since we had found previously that extracts of IL-1-stimulated fibroblasts contained increased amounts of an MBP kinase which co-eluted with MAP kinase antigen (Guesdon et al., 1993). KB cells were therefore stimulated with PMA, in order to activate MAP kinase. The cytosolic extract of these cells was chromatographed on the Mono Q column, and the fractions were assayed on T669 peptide and MBP. They contained a minor and a major peak of activity on MBP (Figure 1b). The major one coincided with a PMA-inducible peak of T669 kinase activity (Figure 1a) and was eluted six or seven fractions later than the IL-1-induced enzyme. The fractions corresponding to the minor MBP kinase peak induced by PMA contained some T669 kinase activity, but this did not co-distribute with MBP kinase. If IL-1 and PMA were both activating MAP kinase, then the IL-1-induced T669 kinase should have shown corresponding activity towards MBP, and the T669 kinases induced by both agents should have been superimposable.

In order to find out the relationship of these enzymes to MAP kinase, samples of the fractions were immunoblotted either with an antiserum recognizing both p42 and p44 MAP kinases, or with antibodies to phosphotyrosine. Figure 1(c) shows that the main MAP kinase antigen detected was p42; it was present in fractions 21–27. Lesser amounts of a slower moving band,

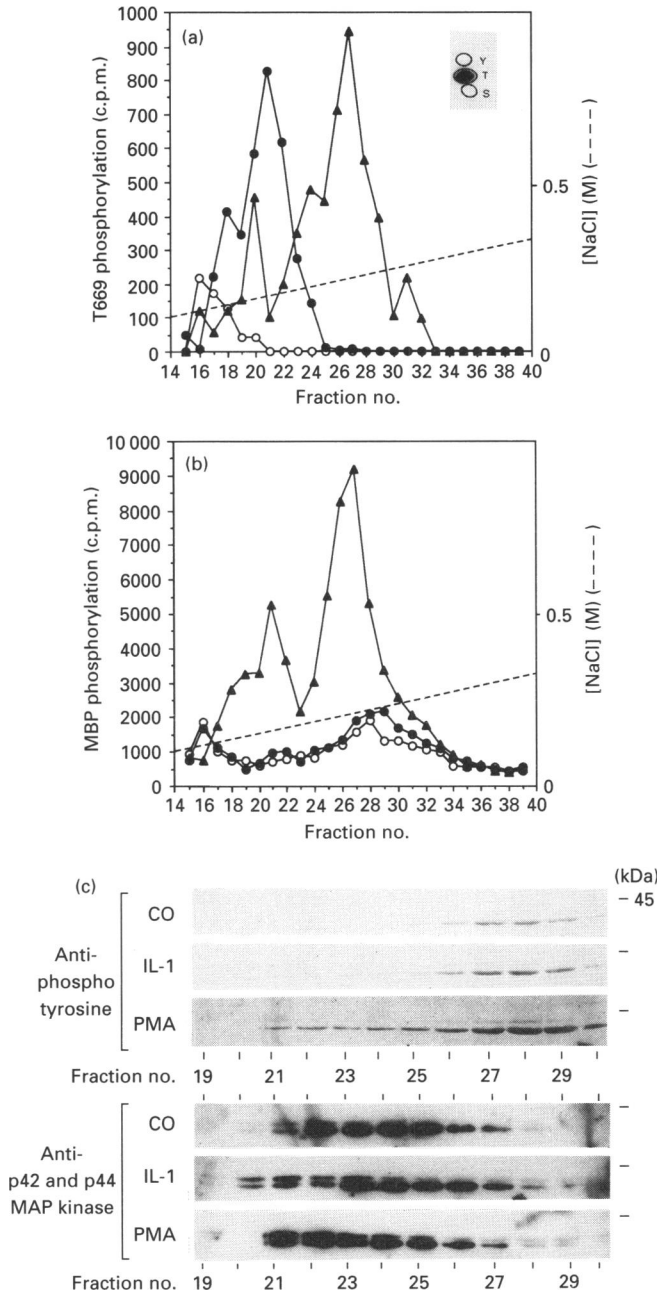

Figure 1 Distribution of T669 and MBP kinase activities and MAP kinase antigens in Mono Q chromatography fractions of resting and activated KB cells

Cytosolic extracts were prepared from resting KB cells (○) or from cells stimulated with IL-1 (●) or PMA (▲). A 10 mg portion of cytosolic protein was then chromatographed on a Mono Q column as described in the Materials and methods section. Samples of fractions were assayed for kinase activity on (**a**) T669 peptide and (**b**) MBP. Reaction mixtures were separated on SDS/PAGE, and substrate phosphorylation was measured as described in the Materials and methods section. Inset (**a**): Phospho amino acid analysis of T669 peptide phosphorylated by the IL-1-activated T669 kinase (fraction 21). Y, tyrosine; T, threonine; S, serine. (**c**) Portions (80 μl) of fractions 19–30 were separated on SDS/PAGE and Western blotted with anti-phosphotyrosine antibody 2-53 or anti-(MAP kinase) antibody 124. For full details see the Materials and methods section.

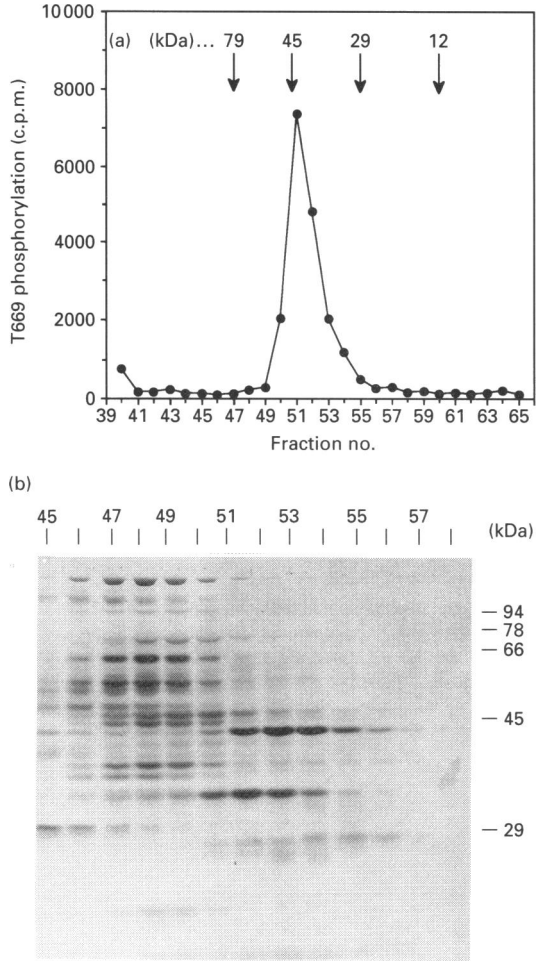

Figure 2 Chromatography of T669 kinase on Superose 12

(**a**) The fraction that ran through the Mono S column and contained T669 kinase was loaded on a phenyl-Sepharose column, eluted with 30% ethylene glycol and then concentrated and chromatographed on a Superose 12 HR10/30 column. Fractions were assayed for T669 kinase activity as described in the Materials and Methods section. Arrows indicate elution positions of standard proteins. (**b**) Portions (75 μl) of fractions 45–58 were run on SDS/PAGE and stained with silver.

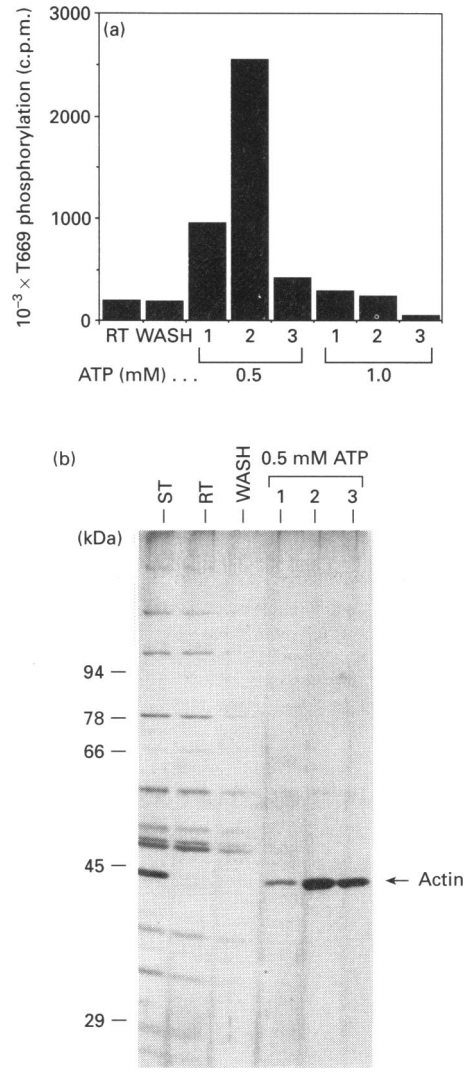

Figure 3 Chromatography of T669 kinase on ATP–Sepharose

T669 kinase-containing fractions (50–53) from Superose chromatography (Figure 2) were diluted and loaded on an ATP–Sepharose column as described in the Materials and methods section. Material running straight through (RT) was collected and the column was washed in buffer (WASH). Proteins were eluted stepwise with 0.5 mM and then 1 mM ATP. Portions of material applied to the column (ST) and fractions from it were assayed for T669 kinase activity (**a**) or run on SDS/PAGE and stained with silver (**b**).

presumably p44 MAP kinase, were detected in the earlier of these fractions. When the profiles of kinase activity are compared with the immunoblots, it can be seen that the maximum activity induced by PMA towards MBP was eluted about three fractions later than the maximum of p42 antigen. It was likely that the bulk of antigen was inactive MAP kinase, and that only a portion was activated by treating cells with PMA. When blots were stained for phosphotyrosine to identify active forms of MAP kinase (Figure 1c), a doublet phosphotyrosine-containing protein was seen corresponding in electrophoretic mobility to the p42 and p44 MAP kinases. The staining was significantly enhanced in the fractions from PMA-stimulated cells and was strongest in fractions 25–29, coinciding with the peak of MBP kinase (Figure 1b). These results were consistent with PMA activating a portion of p42 and p44 MAP kinases which eluted slightly later than the inactive enzymes. In contrast with PMA, IL-1 did not increase the phosphotyrosine content of any protein bands in the region where the MAP kinase antigen was eluted (Figure 1c).

The IL-1-activated T669 kinase was eluted before the bulk of the p42 and p44 MAP kinase antigens, and it did not appear to phosphorylate MBP. Because it differed from typical MAP kinases, we decided to purify it further.

Purification of T669 kinase

A cytosolic extract was made from 50 flasks of IL-1-stimulated KB cells and chromatographed on a large Mono Q column. The T669 kinase peak that was eluted in the expected position from Mono Q was pooled, dialysed and loaded on a Mono S column. Whereas the enzyme of interest passed through the column, the contaminating MBP kinase bound and was eluted as a single peak by a salt gradient (results not shown). Western blotting of these fractions showed that the p42 and p44 MAP kinase antigens were eluted at a similar salt concentration to the MBP kinase

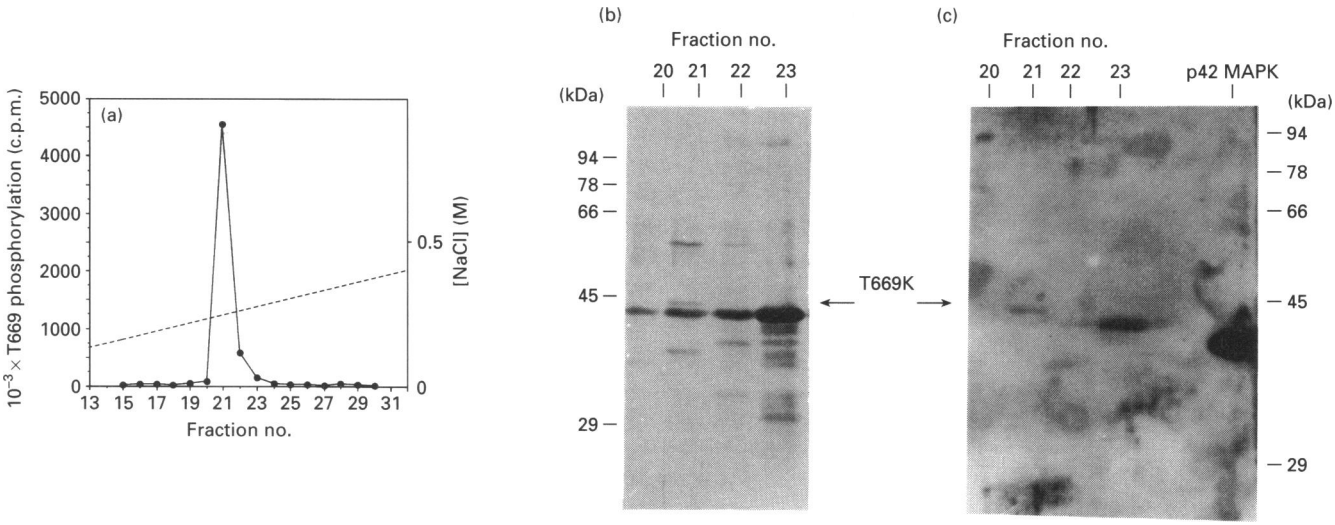

Figure 4 Final purification and identification of T669 kinase on Mono Q chromatography

T669 kinase purified through the chromatography steps (Mono Q, Mono S, phenyl-Sepharose, Superose 12) from 250 175 cm^2 flasks was pooled and further purified on ATP–Sepharose as shown in Figure 3. The fraction eluted by 0.5 mM ATP was then chromatographed on a Mono Q HR5/5 column eluted with a 0–0.4 M NaCl gradient (----). Fractions were assayed for T669 kinase activity (**a**). Fractions 20–23 were concentrated from 1 ml to about 150 μl by ultrafiltration. Half of each was run on SDS/PAGE and stained with silver (**b**), and the other half was run on the same gel but Western blotted for phosphotyrosine with antibody 4G10 (**c**) as described in the Materials and methods section. Activated recombinant p42 MAP kinase was included in one lane in (**c**). The arrows indicate the position of putative T669 kinase protein in fraction 21.

Table 1 Purification of T669 kinase from IL-1-activated KB cells

A total of 250 flasks of confluent KB cells were stimulated with IL-1 for 15 min. Cells were harvested, disrupted and centrifuged at 100000 ***g***. This cytosolic fraction was chromatographed through the indicated steps. At each stage the total protein amount and kinase activity on T669 substrate were measured.

	Protein (mg)	10^{-3} × Activity (c.p.m.)	10^{-3} × Specific activity (c.p.m./mg)	Purification (fold)	Yield (%)
Cytosol	873	13095	0.015	–	–
Mono Q HR10/10	16.48	99112	6.014	400	100
Mono S	3.02	109430	36.24	2400	110
Phenyl-Sepharose	0.701	34938	49.84	3300	35
Superose 12	0.1	17913	179	11852	18
ATP–Sepharose	0.03	18156	606	40125	18.4
Mono Q HR5/5	0.002	5150	2575	170500	5

(results not shown). The activity was eluted slightly earlier than the antigens on the cation exchanger, indicating that, as on Mono Q, most of the antigen probably represented unphosphorylated inactive MAP kinase. The difference in chromatographic behaviour on Mono S of the IL-1-activated T669 kinase compared with p42 and p44 MAP kinases suggested they were distinct enzymes.

The material that had run through the Mono S column was applied to a phenyl-Sepharose column. After washing of the column, T669 kinase was eluted stepwise with 30% ethylene glycol (results not shown). This material was then run on Superose 12. The T669 kinase was eluted as a single peak, corresponding to a molecular mass of about 45 kDa (Figure 2a). Silver staining of portions of the active fractions separated on SDS/PAGE revealed a prominent 44 kDa protein band that was co-eluted with the active material (Figure 2b). This band was excised, and two tryptic peptides were analysed for their primary structures. The two peptide sequences obtained were AVFPSIVGRPR and LDLAGRDLTDY, both of which are conserved in the major human actins, indicating that the protein was almost certainly actin. In order to achieve further purification of the enzyme, the active fractions were pooled and applied to a column of ATP linked to Sepharose via its γ-phosphate (Haystead et al., 1993). All the activity bound to the column and could be eluted with 0.5 mM ATP (Figure 3a). Silver staining of samples of the active fractions separated on SDS/PAGE showed that little protein bound to the column other than the 44 kDa protein which had been identified as actin (Figure 3b). We still could not detect an additional band which might be the kinase. Therefore material from five preparations of KB cells (i.e. from 250 flasks) that had been purified up to the gel-filtration step was pooled and separated on ATP–Sepharose as shown in Figure 3. T669 kinase activity eluted by ATP was then loaded on a Mono Q HR5/5 column, which was developed with a salt gradient. The T669 kinase was eluted sharply around 0.2–0.25 M NaCl (Figure 4a). Portions of the fractions of this region of the chromatogram were run on SDS/PAGE. Silver staining showed a 45 kDa protein band in fraction 21 co-eluting precisely with the T669

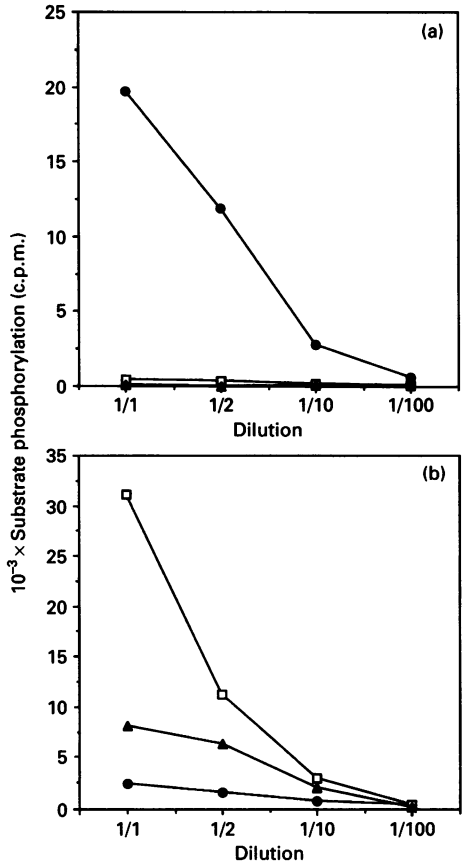

Figure 5 Substrate specificity of T669 kinase and p42 MAP kinase

(**a**) T669 kinase from Mono Q fraction 21 (Figure 4a) was assayed in increasing dilutions on T669 peptide (●), MBP (□) and MAP-2 (▲). Reaction mixtures were separated on SDS/PAGE and phosphorylation was measured as described in the Materials and methods section. (**b**) Increasing dilutions of recombinant p42 MAP kinase were assayed on the same substrates under the same conditions. The MAP kinase had been activated with MAP kinase kinase and repurified on a Mono Q column as described in the Materials and methods section.

kinase activity (Figures 4a and 4b). This band migrated slightly more slowly than actin, some of which was present in the fraction, although the great majority of it was eluted in fraction 23. Fainter bands of 60 kDa and 40 kDa were also seen.

Because the activity of T669 kinase was dependent on tyrosine phosphorylation (see below), samples of fractions 20–23 were Western blotted and stained for phosphotyrosine. As shown in Figure 4(c), the 45 kDa protein band in fraction 21 (Figure 4b) stained strongly for phosphotyrosine. The 60 kDa protein stained weakly, and the 40 kDa band did not stain. Most of the actin was eluted in fraction 23, and stained for phosphotyrosine: actin contains some phosphotyrosine (Machicao et al., 1983; Howard et al., 1993). In view of its molecular mass and co-distribution, the 45 kDa band was very likely to be the kinase, but the 60 kDa band could not be completely excluded. The putative kinase migrated more slowly than recombinant activated p42 MAP kinase (Figure 4c). We estimated that the total amount of kinase recovered in fraction 21 was less than 1 μg of protein. The purification is summarized in Table 1. Although a 170000-fold purification was achieved, it was not possible to generate sufficient protein for amino acid sequencing of either the 45 kDa or 60 kDa proteins.

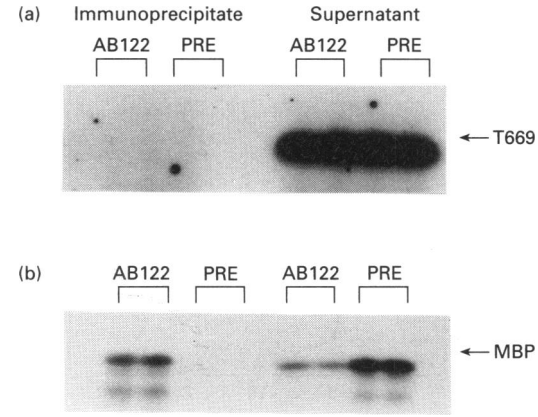

Figure 6 T669 kinase is not immunoprecipitated by antiserum to p42 MAP kinase

Duplicate samples of T669 kinase from the phenyl-Sepharose chromatography step (**a**), or repurified active p42 MAP kinase (prepared as described for Figure 5) (**b**), were incubated with a rabbit antiserum to p42 MAP kinase (AB 122) or with pre-immune serum (PRE). Immunoprecipitations were carried out as described in the Materials and methods section. Samples of the supernatants and the pellets were assayed for kinase activity on T669 (**a**) and MBP (**b**). Reaction mixtures were separated on SDS/PAGE and phosphorylated substrates detected by autoradiography. See the Materials and methods section for full details.

Substrate specificity of T669 kinase in comparison with p42 MAP kinase

Because T669 peptide serves as a substrate for MAP kinase, pure T669 kinase was tested in parallel with activated human recombinant p42 MAP kinase on the peptide, and on two other MAP kinase substrates, namely MBP and MAP-2. The T669 kinase phosphorylated the last two substrates poorly (Figure 5a). It phosphorylated 50 times more T669 than MBP, and it did not phosphorylate MAP-2 at the concentrations used. In contrast, p42 MAP kinase phosphorylated 13 times more MBP than T669. It also phosphorylated MAP-2, but it was less efficient on this than on MBP (Figure 5b). The T669 peptide was therefore a poor substrate for MAP kinase, whereas MBP was a poor substrate for the IL-1-activated T669 kinase.

T669 kinase is not immunoprecipitated by an anti-(p42 MAP kinase) antibody

An antibody to p42 MAP kinase was used to immunoprecipitate T669 kinase. The T669 kinase was not immunoprecipitated (Figure 6a), whereas active recombinant p42 MAP kinase was (Figure 6b).

Inactivation of T669 kinase by phosphatases

To investigate whether or not the T669 kinase was regulated by phosphorylation, it was treated with the protein serine/threonine phosphatase PP2A (Figure 7a) and the protein tyrosine phosphatase PTP1B (Figure 7b). Both phosphatases completely inactivated the enzyme, so its activity was apparently dependent on both tyrosine and serine or threonine phosphorylation.

Dephosphorylated T669 kinase is not re-activated by MAP kinase kinase

The need for phosphorylation of both tyrosine and serine/threonine for activity is reminiscent of the mechanism controlling

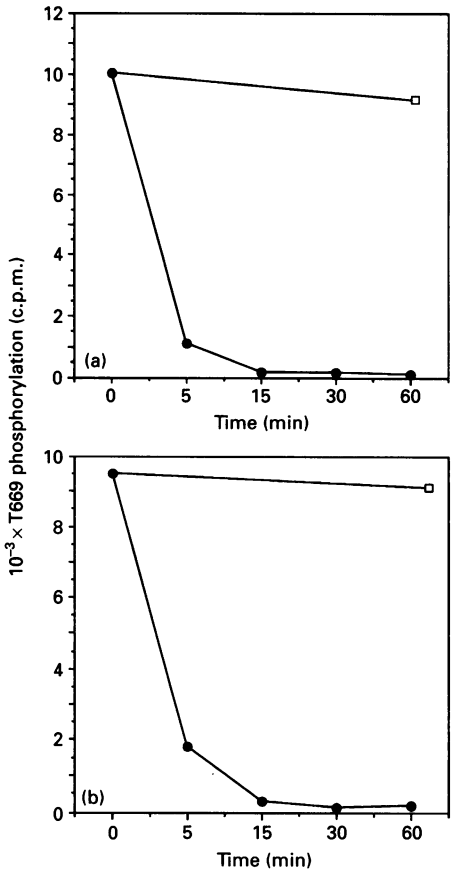

Figure 7 Inactivation of T669 kinase with phosphatases

(**a**) T669 kinase from the Superose 12 chromatography step was incubated for the times indicated with PP2A (●) or with PP2A inactivated with okadaic acid (□). At the end of the incubation, okadaic acid was added to samples containing active phosphatase. The kinase was then assayed on T669 peptide. (**b**) As for (**a**), except that T669 kinase was incubated with PTP1B (●) or PTP1B inactivated with $NaVO_3$ (□). At the end of the incubation $NaVO_3$ was added to the sample containing active phosphatase, and the kinase was assayed on T669 peptide. See the Materials and methods section for details.

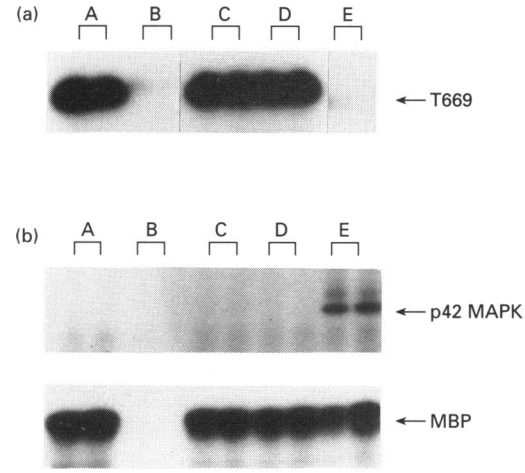

Figure 8 T669 kinase is not re-activated by MAP kinase kinase

(**a**) T669 kinase was incubated with PP2A (B, E), with PP2A that had been inactivated with okadaic acid (C), with okadaic acid alone (D) or with buffer alone (A). After 30 min, okadaic acid was added to the samples with active phosphatase (B, E). Samples were then incubated for a further 30 min with partially purified MAP kinase kinase and [^{32}P]ATP (E) or with buffer alone (A–D). Then T669 peptide was added to (E), or T669 peptide and [^{32}P]ATP to (A–D), and phosphorylation was allowed to continue for 20 min. Reaction mixtures were separated on SDS/PAGE and autoradiographed as described in the Materials and methods section. (**b**) An identical experiment was performed in which activated repurified p42 MAP kinase (MAPK) was inactivated by PP2A and then re-activated with the preparation of MAP kinase kinase (E). Kinase activity was measured on MBP substrate. Activity of T669 kinase was 6000 c.p.m./assay, and that of p42 MAP kinase was 12000 c.p.m./assay. Activity of MAP kinase kinase on MBP was 1609 c.p.m./assay.

MAP kinase. If the IL-1-activated enzyme were related to MAP kinase, then it might have been re-activated by MAP kinase kinase. However, T669 kinase dephosphorylated by PP2A was not re-activated by partially purified MAP kinase kinase (Figure 8a), whereas p42 MAP kinase was phosphorylated and re-activated in the same experiment (Figure 6b). The T669 kinase may therefore be regulated by kinase kinase(s) distinct from MAP kinase kinase.

DISCUSSION

We have substantially purified and characterized a protein kinase from cultured tumour cells that is activated by the major inflammatory cytokine IL-1. On gel-filtration chromatography its molecular mass was 45 kDa, and it was tentatively identified as a 45 kDa protein band on SDS/PAGE that could be stained for phosphotyrosine. Results of experiments with phosphatases suggested that, like MAP kinase, its activity was dependent on phosphorylation of both tyrosine and serine or threonine residues. The amino acids surrounding T^{669} represent the optimal sequence (P-X-S/T-P) for MAP kinase substrates (Davis, 1993). Therefore a major effort was made to establish the relationship of the IL-1-induced T669-peptide kinase to MAP kinase.

Although we purified the enzyme over six steps, it was not possible to isolate enough for amino acid sequencing: less than 1 μg was obtained from 250 flasks.

The specificity of the enzyme and recombinant p42 MAP kinase for commonly used substrates was investigated. When quantities causing equivalent T669 peptide phosphorylation were compared, the T669 peptide kinase had only 1/500 of the activity of p42 MAP kinase towards MBP, and no measurable activity towards MAP-2. Its lack of activity towards the latter substrate also contrasted it with p54 MAP kinase, a putative family member from rat liver, which is relatively lacking in activity towards MBP (Kyriakis and Avruch, 1990). Thus, although MBP is an excellent substrate for human recombinant p42 (as shown here) and for human recombinant p44 MAP kinase (Charest et al., 1993), it is a very poor substrate for the T669 kinase. By contrast, the T669 peptide is a poor substrate for p42 MAP kinase, but a good substrate for the IL-1-induced enzyme.

The phosphorylation of tyrosine and threonine that activates MAP kinase is carried out by MAP kinase kinases of dual specificity (Gomez and Cohen, 1991). The T669 peptide kinase which had been completely inactivated by dephosphorylation of serine/threonine was not re-activated by MAP kinase kinase. The results of the phosphatase treatment provided strong circumstantial evidence that activity depended on phosphorylation, but it remains to be established that T669 kinase contains a Y-X-T motif as found in MAP kinases. The enzyme is likely to be regulated by kinase kinase(s) different from the known MAP kinases.

At least four different cDNAs encoding MAP kinase sequences have been cloned from human tissues (Gonzalez et al., 1992), and

more have been purified or cloned from other species (Nishida and Gotoh, 1993; Pelech and Sanghera, 1992). The IL-1-induced enzyme could be a family member with a distinct substrate specificity, or it may be unrelated.

Another group of proline-seeking protein kinases are those dependent on cyclin (Draetta, 1990). These phosphorylate histone, but we were unable to detect any IL-1-regulated histone kinase in our chromatography fractions. The cytokine-regulated T669 kinase therefore did not possess the characteristics of this group.

Others have studied T669 peptide kinases in KB cells. One group identified an IL-1-activated T669 kinase as MAP kinase, although the enzyme was not extensively purified (Bird et al., 1991). In our initial experiments we fractionated cell extracts by high-resolution anion-exchange chromatography and were unable to detect MAP kinase activation in fractions from IL-1-stimulated cells. The T669 kinase induced was separable from MAP kinase. Upon further purification on cation-exchange chromatography, the T669 kinase was clearly separated from p42 and p44 MAP kinases as judged by activity and Western blotting. Furthermore, partially purified T669 kinase was not precipitated by an antiserum to p42 MAP kinase. The results of these early experiments led us to persevere in purifying the IL-1-stimulated enzyme.

Two kinases that phosphorylated a T669 peptide were isolated from KB cells stimulated with EGF (Northwood et al., 1991). One was identified as p42 MAP kinase; the other was later identified as p44 MAP kinase (Gonzalez et al., 1991).

It has been suggested that there is a T669 peptide kinase which is activated by ceramide formed in the cell membrane in response to IL-1 or tumour necrosis factor. This enzyme associates with the cell membrane upon cytokine stimulation, but has not been purified (Dressler et al., 1992; Mathias et al., 1993). We found some T669 kinase activity associated with the crude membrane fraction of KB cells, but the only enzyme clearly regulated by IL-1 was found in the cytosol (M. Kracht, unpublished work).

One conclusion from these experiments was that IL-1 did not activate MAP kinase in KB cells. This was in contrast with MRC5 fibroblasts, in which the cytokine did induce the enzyme, although less strongly than PMA (Guesdon et al., 1993). These findings are consistent with changes in MAP kinase kinase activity. In fibroblasts, IL-1 or PMA increased this enzyme's activity several-fold, but in KB cells IL-1 only doubled it, whereas PMA increased it 10-fold (Saklatvala et al., 1993). A doubling of MAP kinase kinase activity appears to be inadequate to activate MAP kinase significantly in KB cells.

Whether or not the MAP kinase cascade is important in IL-1 signalling remains to be seen; the fact that the system is not strongly activated in KB cells, which nevertheless respond to IL-1, suggests that it may not be a central or proximal part of the cytokine's signalling mechanism.

It is too early to assess the significance of the T669 kinase for IL-1 signalling: its substrates are likely to differ from those of MAP kinase, and it is therefore likely to have its own physiological role. It is a good candidate enzyme for phosphorylating T^{669} of the EGF receptor, and could partly account for the IL-1-induced phosphorylation of that molecule which is associated with a loss of affinity for the growth factor (Bird and Saklatvala, 1989, 1990).

The specificity of induction of T669 peptide kinase is unknown: it was not possible to be sure from the Mono Q chromatography profiles whether or not PMA was inducing the enzyme in addition to MAP kinase. Whether growth factors and other cytokines activate it remains to be investigated.

In conclusion, the T669 peptide kinase and its putative activator might be part of a novel cascade of phosphorylation induced by IL-1 which links the receptor to downstream events.

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