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Interleukin 1 and tumour necrosis factor activate the mitogen-activated protein (MAP) kinase kinase in cultured cells

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Interleukin 1 (IL1) activated mitogen-activated protein (MAP) kinase kinase in human gingival and foreskin fibroblasts and KB cells. Maximal activity was found in cytosolic extracts made after stimulating cells for 15 min. On anion-exchange chromatography two differently charged forms of MAP kinase kinase were identified, both phosphorylated a kinase-defective mutant MAP kinase, and activated recombinant wild type MAP kinase to phosphorylate MBP. Both were inhibited by an antiserum to recombinant MAP kinase kinase and the less acidic form was identified on Western blotting as an antigen of ~43 kDa. Indistinguishable forms were very much more strongly induced by phorbol myristate acetate (PMA). TNF had a similar effect to that of IL1.

Interleukin 1; Tumour necrosis factor; MAP kinase; MAP kinase kinase

1. INTRODUCTION

Interleukin1 (IL1) and tumour necrosis factor (TNF) induce mitogen-activated protein (MAP) kinase activity in some cell types [1–3]. Many agonists stimulate this enzyme, and most either activate protein kinase C or signal via tyrosine kinase receptors [4–6]. The signalling mechanisms of both IL1 and TNF are not understood. Their receptors are not tyrosine kinases, and several studies have shown they do not signal via PKC [1,7,8], although they do activate other serine and threonine kinases, including MAP kinase [1–3].

The two ubiquitous forms of MAP kinase, p42 and p44 (also known as extracellularly regulated kinases, ERK 2 and 1, respectively), are closely related proteins that are activated by phosphorylation of nearly adjacent tyrosine and threonine residues [4–6]. This is carried out by an upstream enzyme, MAP kinase kinase, which has been cloned and shown to have the dual specificity for phosphorylating both tyrosine and threonine [9–12]. The MAP kinase kinase is itself activated by phosphorylation and there is increasing evidence that the kinase Raf is its major activator [13–16].

How IL1 and TNF impinge on the MAP kinase system is unknown, so tracing this pathway upstream could cast light upon their signalling mechanisms. Here we show that they both induce two active forms of the MAP kinase kinase, and these are indistinguishable from those induced by the PKC activator, PMA.

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Abbreviations: ERK, extracellularly regulated kinase; IL, interleukin; MAP, mitogen-activated protein; MBP, myelin basic protein; PMA, phorbol myristate acetate.

2. MATERIALS AND METHODS

2.1. *Cell cultures*

Cells were cultured in Dulbecco's modified medium (DMEM) containing 10% foetal calf serum and maintained at 37°C in a humidified atmosphere of air with 5% CO_2. Human fibroblasts were made from tissue taken at surgery and were used at passages 7–12. KB cells were from the European Collection of Animal Cell Cultures at Porton Down, UK.

2.2. *Reagents*

Human recombinant IL1α and TNFα were purified from extracts of *E. coli* expressing the proteins [7]. Wild type and the kinase-defective mutant ($Lys^{52} \rightarrow Arg$)p42 MAP kinase (ERK 2) were recombinant proteins purified from bacterial lysates [17]. The rabbit antisera to MAP kinase and MAP kinase kinase were made against a C-terminal synthetic peptide [18] and rabbit muscle recombinant GST fusion protein [19] respectively. Phorbol myristate acetate (PMA) myelin basic protein (MBP), ATP, proteinase inhibitors and β-glycerophosphate were from Sigma. [γ-^{32}P]ATP (3200 Ci/mmol) and ^{125}I-labelled donkey anti-rabbit Ig antibody (500–2000 Ci/mmol) were from Amersham International plc (Aylesbury, UK). Other chemicals for electrophoresis were from BDH (Poole, UK) or Fisons (Loughborough, UK).

2.3. *Preparation and chromatography of cell extracts*

Fibroblasts grown in 175-cm^2 flasks were used 4 days after reaching confluence. They were stimulated by adding IL1 (20 ng/ml), TNF (100 ng/ml) or PMA (100 ng/ml) to the culture medium and incubating for 15 min, or other indicated times, at 37°C. Stimulated or unstimulated cells (6 flasks each) were washed 3 times in ice-cold phosphate-buffered saline, then scraped into ice cold buffer (2 ml/flask: 5 mM Tris-HCl, pH 8.5, 20 mM β-glycerophosphate, 10 mM NaF, 0.5 mM EGTA, 0.5 mM EDTA). After addition of proteinase inhibitors (final concentrations: 10 μM E64, 1 mM orthophenanthroline, 1 mM phenylmethylsulphonyl fluoride) the cells were homogenized by passage

through 23-gauge (twice) and 26-gauge 3/8 (five times) needles. The homogenates were centrifuged at 100,000 × *g* for 1 h at 4°C. The supernatants (cytosolic fractions) were kept on ice prior to chromatography.

Chromatography was done at room temperature on a 5/5 Mono Q column (Pharmacia Ltd., UK) equilibrated with a buffer containing 20 mM Tris-HCl, pH 8.5, 20 mM β-glycerophosphate, 10 mM NaF, 0.5 mM EDTA, 0.5 mM EGTA. For time course experiments extracts of cells were chromatographed by stepwise elution. Otherwise, elution was by a linear gradient of NaCl in the buffer (0–0.5 M over 30 ml). 1-ml fractions were collected and stored at 4°C.

2.4. *Assay for MAP kinase*

MAP kinase was detected by the use of MBP (0.1 mg/ml) as substrate. The assay mixture consisted of 5 μl of substrate in H_2O, 5 μl of sample and the reaction was started by adding 5 μl of buffer of 150 mM Tris-HCl pH 7.4, 20 mM $MgCl_2$, 60 μM ATP containing [γ^{32}P]ATP (specific activity 1.7 μCi/nmol). Mixtures were incubated for 20 min in 96-well microtitre plates at 30°C. The reactions were stopped by adding 5 μl of 4 × SDS sample buffer and the mixtures were loaded on to SDS-PAGE (12.5% acrylamide) and electrophoresed [20]. Gels were stained for protein, dried and autoradiographed. For quantitation, Cerenkov emission of excised gel slices was measured.

2.5. *Assay of MAP kinase kinase by phosphorylation of MAP kinase*

MAP kinase kinase was detected by the use of mutant MAP kinase (0.1 mg/ml) as substrate. This mutant is unable to bind ATP and undergoes no autophosphorylation which can complicate interpretation of assays for MAP kinase kinase. The assay was performed as for MBP.

2.6. *Assay of MAP kinase kinase by MAP kinase dependent phosphorylation of MBP*

Coupled assays were performed to show that MAP kinase was activated, rather than simply phosphorylated. For these assays wild type MAP kinase was used together with MBP as substrates. The assays were performed exactly as described for assay of MAP kinase except that the substrate solution contained 0.25 mg/ml wild type MAP kinase, and 0.25 mg/ml MBP.

2.7. *Western blotting*

Fractions from Mono Q chromatography were run on SDS-PAGE (12.5% acrylamide), then transferred to nitrocellulose membranes (Bio-Rad). The membranes were probed with antiserum to MAP kinase kinase (1:500 dilution), followed by ^{125}I-labelled second antibody. Antibody staining was visualized by autoradiography.

3. RESULTS

The onset of activation of kinases in IL1-stimulated cells has generally been found to be quite slow, and optimum activity is usually observed between 5 and 15 min after exposure to the cytokines [1,21,22]. In initial experiments the time course of activation of MAP kinase kinase was investigated in KB cells activated by either IL1 or PMA. Extracts were partially purified by stepwise elution from a Mono Q column. This was necessary because the effect of IL1 could not be seen above the background in unfractionated cytosol. Fig. 1 shows that the fractionated extracts of PMA-stimulated KB cells had very marked increased ability to phosphorylate the MAP kinase substrate. This reached a maximum of about 10-fold activation after 5 min of stimulation and declined slowly. By contrast, the effect of IL1

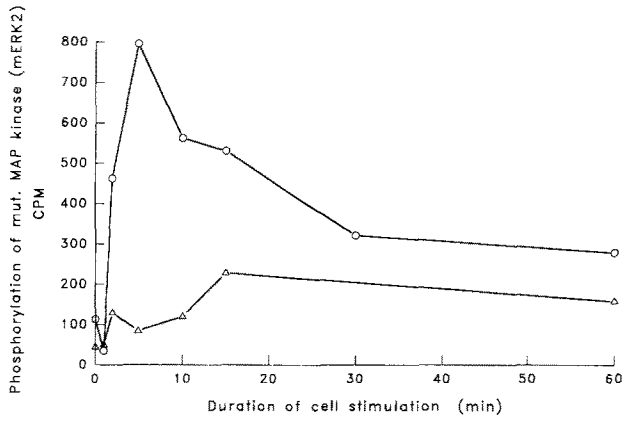

Fig. 1. Time course of MAP kinase kinase activation in KB cells. KB cells were stimulated for the indicated times with PMA (○) or IL1 (△). Cell extracts were chromatographed on a Mono Q column by stepwise elution and assayed for ability to phosphorylate the mutant MAP kinase. See section 2 for details.

was relatively weak: a 2- to 3-fold increase by 15 min. Similar but less detailed experiments carried out on human gingival fibroblasts with both stimulating agents gave comparable results. We therefore decided to analyse chromatographically and immunologically the MAP kinase kinase activity generated after 15 min of cell stimulation by either PMA or cytokine.

Cytosolic extracts were prepared from resting or stimulated human gingival fibroblasts and chromatographed on a Mono Q column which was eluted with a salt gradient. The fractions were assayed for MAP kinase activity on MBP substrate and Fig. 2A shows that the peak of MBP-kinase activity in the middle of the gradient was increased in stimulated cells. PMA caused a larger stimulation (about 6-fold) than IL1 (about 2.5-fold). Western blotting (data not shown) confirmed that p42 MAP kinase antigen was present in these fractions. This degree of stimulation was similar to that found previously in MRC5 fibroblasts [2].

The fractions were next assayed for MAP kinase kinase activity by mixing with unactivated recombinant wild type p42 MAP kinase, 20 μM ATP and MBP, and measuring the phosphorylation of MBP as described in the Methods section. Fig. 2B shows that the fractions from resting cells had some MAP kinase kinase activity in an early-eluting peak, while those from PMA-stimulated cells showed an increase in this, and the appearance of a second later eluting peak of activity. Both peaks eluted earlier than the MAP kinase itself (Fig. 2B) and it can also be seen that the fractions containing activator had little intrinsic MBP kinase activity (Fig. 2A). IL1 activated the same two components, but had a much weaker effect on the second one than PMA (Fig. 2B). The MAP kinase kinase was also detected directly by its ability to phosphorylate the inactive mutant MAP kinase substrate. Fig. 3A (a) shows that only fraction 9 from resting cells was able to phosphorylate the sub-

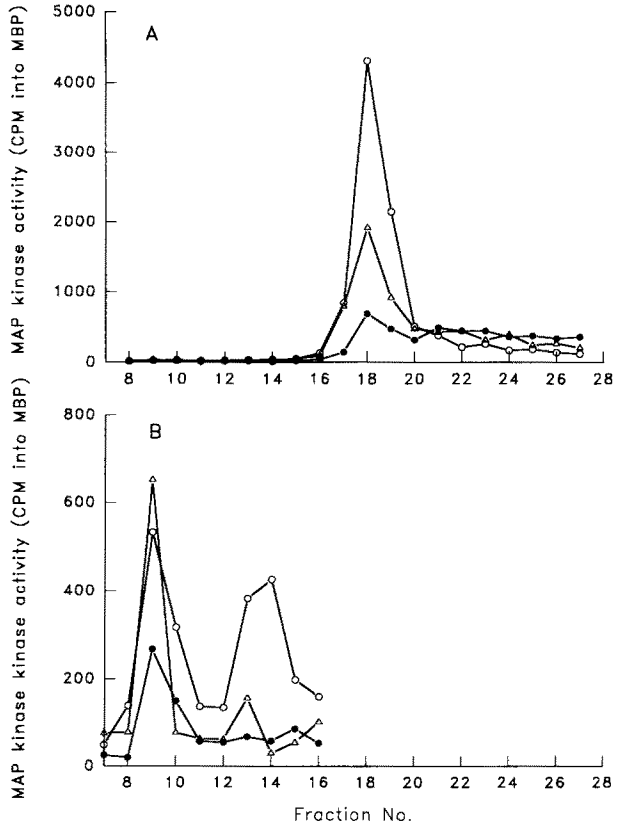

Fig. 2. MAP kinase and its activator (MAP kinase kinase) in Mono Q chromatography fractions of human gingival fibroblasts: effects of PMA and IL1. Confluent fibroblasts were treated for 15 min with vehicle (●), PMA (○), or IL1 (△). Cell extracts were chromatographed on a Mono Q column eluted with a salt gradient. Fractions were assayed (A) for MAP kinase activity on MBP substrate, and (B) for MAP kinase activator by adding recombinant wild type MAP kinase with MBP. The experiment is a representative one of three. See section 2 for details.

strate. This activity was increased in fractions from both the PMA and IL1-stimulated cells (Fig. 3A (c) and (b)). A later eluting component was also seen in fraction 13 particularly after PMA treatment (Fig. 3A (c)). This second component was only weakly activated by IL1. These two peaks of kinase kinase corresponded to those detected by activation of added MAP kinase shown in Fig. 2B.

The Mono Q fractions were next electrophoresed and immuno-stained with the antiserum to MAP kinase kinase (Fig. 3B). A 43 kDa band was detected in the fraction 9 of resting and stimulated cells. This corresponded to the first peak of kinase kinase (Fig. 3A). It was not possible to detect any similar antigen in the fractions of the second peak. Nevertheless, the antiserum was inhibitory to the activity of both peaks (Fig. 4), suggesting that the second component was immunologically related to the known MAP kinase kinase.

These experiments were extended by examining responses of another type of human fibroblast. Fig. 5 shows that IL1 and PMA induced the two forms of

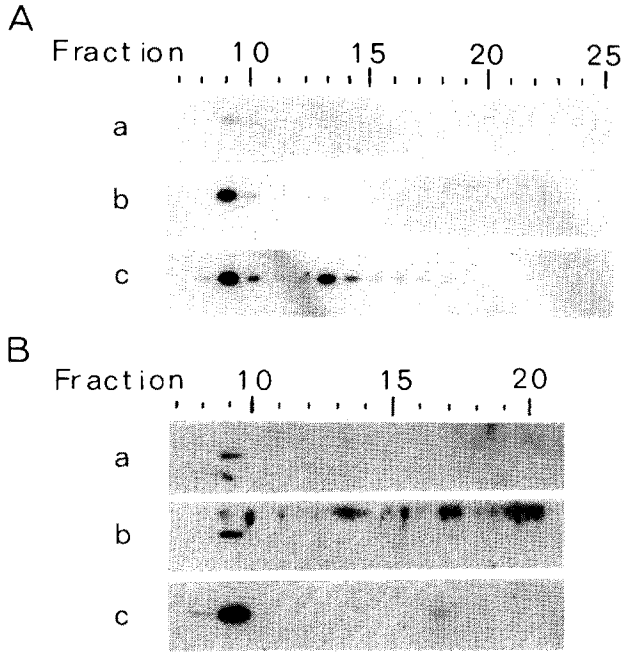

Fig. 3. MAP kinase kinase in Mono Q chromatography fractions of human gingival fibroblasts detected by (A) phosphorylation of mutant MAP kinase, or (B) Western blotting with antiserum. The Mono Q fractions were those shown in Fig. 2: (a) resting cells, (b) IL1, (c) PMA. (A) shows autoradiographs of mutant MAP kinase phosphorylated by the fractions, (B) shows autoradiographs of Western blots made with antiserum to MAP kinase kinase. See section 2 for details.

MAP kinase kinase in foreskin fibroblasts. We also tested the effect of TNF, which shares many biological actions with IL1, including activation of MAP kinase [3]. Fig. 5 shows that it too induced the same two forms of MAP kinase kinase as IL1, and to a similar extent. As in gingival fibroblasts and KB cells PMA had a significantly greater effect than the cytokines.

4. DISCUSSION

IL1, TNF or PMA all induced MAP kinase kinase

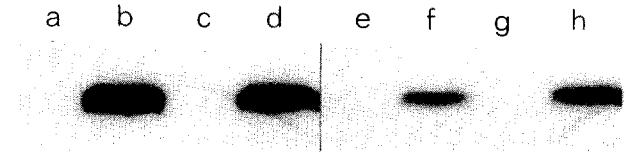

Fig. 4. Inhibition of MAP kinase kinase peaks from Mono Q chromatography by an antiserum. Peak fractions of MAP kinase kinase activity from Mono Q fractions of PMA- or IL1-stimulated human gingival fibroblasts as shown in Figs. 2 and 3 were preincubated with 1/20 dilution of rabbit antiserum to recombinant MAP kinase or preimmune serum, and then assayed for their ability to phosphorylate the mutant MAP kinase as described in section 2. A section of the autoradiograph showing phosphorylated substrate is shown. (a) and (b) are fraction 9 from PMA-stimulated cells; (c) and (d) fraction 9 from IL1-stimulated cells; (e) and (f) are fraction 13 from IL1-stimulated cells; (g) and (h) are fraction 13 from PMA-stimulated cells. (a), (c), (e) and (g) were treated with antiserum, (b), (d), (f) and (h) were treated with pre-immune serum.

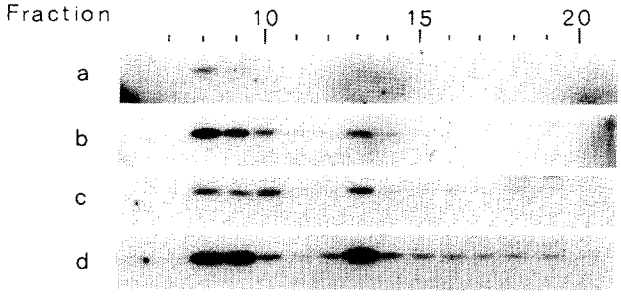

Fig. 5. MAP kinase kinase in Mono Q chromatography fractions of human foreskin fibroblasts detected by phosphorylation of mutant MAP kinase. The fibroblasts were stimulated with (a) vehicle, (b) IL1, (c) TNF, or (d) PMA for 15 min. Cytosolic extracts were chromatographed on a Mono Q Column extactly as described for Fig. 2. Fractions were assayed for ability to phosphorylate the mutant MAP kinase and autoradiographs of this are shown.

activity that comprised two differently charged forms. The less acidic co-eluted with a 43 kDa antigen detected by the antiserum to MAP kinase kinase. This antigen stained more strongly in fractions of activated cells. The reason for this is unexplained, since all the antigen of resting or stimulated cells appeared to have bound to the column. The first peak is likely to correspond to the cloned enzyme. The second peak was inhibited by the antiserum so it may represent a more active form of this which was not generated in sufficient quantity to be detectable by Western blotting. Being the more acidic form, it might represent a higher phosphorylation state. Some workers have found two [23,24] and others one [25–27] form of MAP kinase kinase. A second MAP kinase kinase 81% homologous to the first has been reported recently [28]. However, both enzymes were present in each of the two forms separated by Mono Q chromatography.

IL1 was a weaker inducer of MAP kinase kinase activity than PMA in both types of fibroblast, and in KB cells. This was consistent with the weaker induction of MAP kinase by IL1 than PMA found in the present work with gingival fibroblasts and previously with MRC5 fibroblasts [2]. In KB cells we have been unable to detect any significant activation of MAP kinase by IL1 as judged by increase in MBP kinase activity in Mono Q fractions.

IL1 and TNF therefore appear to be relatively weak activators of the MAP kinase pathway in cell lines. Whether the pathway is more strongly activated in physiological situations, and is important for generation of biological responses to the cytokines, remains to be seen.

Recent evidence indicates that a major activator of the kinase kinase is Raf [13–15], and that Raf can be directly activated by PKC [29]. Whether the cytokines are activating Raf, or a similar kinase, remains to be seen.

In conclusion, IL1 and TNF impinge on the MAP kinase pathway at a level above the MAP kinase kinase, but the relatively weak effects of the cytokines suggests that the MAP kinase system may not be central to their signalling mechanism, and could be being activated by cross talk from other pathways.

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