The chemotactic factor N-formylmethionyl-leucyl-phenylalanine activates microtubule-associated protein 2 (MAP) kinase and a MAP kinase kinase in polymorphonuclear leucocytes

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Incubation of human polymorphonuclear leucocytes (PMN) with either the chemotactic factor N-formylmethionyl-leucyl-phenylalanine (FMLP) or phorbol 12-myristate 13-acetate (PMA) activates a kinase with phosphorylating activity towards a known microtubule-associated protein-2 (MAP) kinase substrate, the epidermal growth factor receptor peptide (T669). Activation of this enzyme by FMLP was maximal at 1 min, decreasing by 10 min. Activation by PMA was slightly slower than that by FMLP, but more prolonged (maximal at 5 min, with no significant decrease by 20 min). The enzyme induced by either stimulant bound strongly to phenyl-Sepharose, had a molecular mass of 40 kDa on gel filtration and phosphorylated three MAP kinase substrates, i.e. MAP, myelin basic protein and the T669 peptide. By use of antibodies to MAP kinases and phosphotyrosine, the enzyme was identified as the 42 kDa MAP kinase (also known as extracellular-signal-regulated kinase 2, ERK2). Stimulation of PMN with FMLP or PMA was also found to induce a kinase kinase which phosphorylated human recombinant MAP kinase on threonine and tyrosine, with concomitant activation. These results suggest that MAP kinase and the kinase kinase are involved in the activation of PMN by chemotactic factors such as FMLP.

INTRODUCTION

Polymorphonuclear leucocytes (PMN) represent the first line of host defence against bacterial and fungal infections [1]. They are activated by a wide variety of stimuli, including chemotactic agents such as N-formylmethionyl-leucyl-phenylalanine (FMLP) and C5a [2–4]. These chemoattractants are thought to act via G-proteins to cause rapid increases in phosphoinositide turnover and intracellular free Ca^{2+}, together with translocation of protein kinase C (PKC) to the cell membrane [3–6]. PMN can also be activated by the tumour promotor phorbol 12-myristate 13-acetate (PMA), which directly activates PKC [3,7]. Much work has concentrated on showing that stimulants of PMN may work via activation of PKC, but very little is known about other protein kinases in these cells.

The microtubule-associated protein-2 (MAP) kinases are a widespread family of serine/threonine kinases that are activated by a variety of stimuli, including insulin, growth factors, T-cell receptor cross-linking, interleukin-1, colony-stimulating factor and PMA [8–17]. MAP kinases can phosphorylate a number of substrates besides MAP [9], such as myelin basic protein (MBP), epidermal growth factor (EGF) receptor, S6 kinase and c-jun [18–21]. The enzymes recognize sites containing threonine or serine in the context of proline, for instance the PXT/SP motif such as T669 in the EGF receptor [19]. The MAP kinases are activated by phosphorylation on threonine and tyrosine [22–24]. These two phosphorylations are carried out by an activator which appears to be a kinase with the ability to phosphorylate both threonine and tyrosine [25]. We report here the presence of a MAP kinase and a MAP kinase kinase which are rapidly activated by FMLP in PMN.

MATERIALS AND METHODS

Materials

The rabbit antiserum to human MAP kinase was raised against recombinant human 42 kDa MAP kinase (ERK2). It also recognizes the 44 kDa MAP kinase [26]. This antibody and recombinant human 42 kDa MAP kinase [27] were kindly supplied by Professor C. Marshall, Institute for Cancer Research, Chester Beatty Laboratories, London, U.K. The murine monoclonal antibody to the 42 kDa MAP kinase isoform was from Zymed (San Francisco, CA, U.S.A.). Rat 2-53 monoclonal antibody raised against phosphotyrosine, alanine and glycine mixture coupled with keyhole-limpet haemocyanin [28] was specific for phosphotyrosine. The peptide based on the sequence around T669 of the EGF receptor, RRRELVEPLTPSGE, contained three N-terminal arginine residues to confer basic charge and was synthesized in-house. MAP was purified from pig brain by a published method [29]. FMLP, PMA and MBP were from Sigma Chemical Co., Poole, Dorset, U.K. Lipopolysaccharide (LPS) was the international standard preparation 84/650 from *Escherichia coli* 0113:H10:K− and was given by Dr. S. Poole, National Institute of Biological Standards Control, South Mimms, Herts., U.K. Human recombinant tumour necrosis factor α and interleukin-1α were made and purified as described elsewhere [30]. [γ-^{32}P]ATP and ^{125}I-labelled donkey antibodies to rabbit immunoglobulin were from Amersham International, Aylesbury, Bucks., U.K. Ficoll-Paque was from Pharmacia, Milton Keynes, Bucks., U.K. P81 filter paper was from Whatman International, Maidstone, Kent, U.K.

Preparation and extraction of PMN

Venous blood from a healthy volunteer was mixed with 3.8% sodium citrate and 3% dextran in 0.9% NaCl. After sedimentation of erythrocytes, the granulocytes were purified by centrifugation on Ficoll-Paque. The remaining erythrocytes in the PMN-rich pellet were removed by hypotonic lysis with 0.2% NaCl. The cells were then counted and resuspended (1×10^7/ml) in RPMI containing 10% foetal-calf serum. The cells were then incubated with stimulants for the required time. After stimulation, the cells were rapidly centrifuged in a Microfuge and resuspended in buffer A (20 mM Tris/HCl, pH 7.4, 20 mM β-glycerophosphate, 100 μM EDTA, 100 μM EGTA, 100 μM sodium vanadate, 1 μM phenylmethanesulphonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin and 10 μg/ml pepstatin). They were then disrupted by two 15 s bursts of sonication, and the lysates were centrifuged for 10 min at 13000 g. The supernatants were tested for kinase activity.

Assay for MAP kinase activity

Assays were carried out with three substrates for the enzyme, namely the EGF receptor peptide T669, MBP or MAP. The assay mixture consisted of 5 μl of substrate in water and 5 μl of sample, and the reaction was started by adding 5 μl of buffer B containing 150 mM Tris/HCl, pH 7.4, 20 mM $MgCl_2$ and 40 μM [γ-^{32}P]ATP (sp. radioactivity 1.7 μCi/nmol). The mixtures were incubated for 15 min in 96-well microtitre plates at 25 °C. The reactions containing MBP or the T669 peptide were then stopped by addition of 3 μl of 90% formic acid. Samples (8 μl) from each of the wells were applied to P81 ion-exchange filter paper marked out in 1.27 cm $\times$ 1.27 cm squares. The paper was washed four times in 0.1% H_3PO_4. Alternatively, when MAP was used as substrate the reactions were stopped by adding 15 μl of 2 $\times$ SDS sample buffer. The mixtures were finally loaded onto 5%-acrylamide gels and subjected to SDS/PAGE. Both the filter papers and the gel slices were autoradiographed, and for quantification the individual samples were counted for radioactivity in a β-counter by using Čerenkov emission.

Chromatography of MAP kinase

Cell lysate in buffer A was ultracentifuged at 100000 g for 1 h at 4 °C. The lysate was then loaded on a Mono Q f.p.l.c. column previously equilibrated with buffer A. Elution was performed with a 30 ml gradient of increasing concentration of NaCl (0–500 mM) and 1 ml fractions were collected. Fractions containing MAP kinase activity were pooled and then loaded on a column (1 cm $\times$ 3 cm) of phenyl-Sepharose which had been equilibrated with buffer A containing 300 mM NaCl. Activity was eluted with a 20 ml gradient of decreasing concentration of NaCl (300 mM–0) and simultaneously increasing concentration of ethylene glycol (0–60%); 0.5 ml fractions were collected and assayed for kinase activity.

Gel filtration

Gel filtration was carried out on a column (1 cm $\times$ 56 cm) of Sephacryl S-200 equilibrated with buffer A containing 500 mM NaCl. Mono Q chromatography fractions containing MAP kinase activity were pooled and concentrated by centrifugation in a Centricon instrument (Amersham) to 250 μl before being loaded on to the S-200 column. Then 500 μl fractions were collected, and assayed for MAP kinase.

Immunoblotting with anti-MAP kinase or anti-phosphotyrosine antibodies

For immunological detection of MAP kinase or phosphotyrosine, PMN were stimulated and then lysed in 1% Triton X-100, containing 10 mM triethanolamine (pH 7.8), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2 mM Na_3VO_4 and protease inhibitors as for buffer A. The lysates, or fractions from Mono Q chromatography, were run on SDS/PAGE (7.5% gel) and transferred on to Immobilon-P membranes (Millipore). The membranes were then probed with antiserum to MAP kinases (1:10000 dilution) and ^{125}I-labelled donkey antibodies to rabbit Ig (1 μCi/5 ml). The MAP kinase was then detected by autoradiography. Phosphotyrosine was detected with the rat monoclonal antibody 2-53, which has been directly labelled with horseradish peroxidase. The phosphotyrosine-containing proteins were detected by using the enhanced-chemiluminescence system (Amersham).

Induction of MAP kinase activator

Fractions from Mono Q chromatography were assayed for MAP kinase kinase by incubation with both recombinant human MAP kinase and MBP in the presence of [γ-^{32}P]ATP. Inclusion of both MAP kinase and MBP in the reaction mixture made it possible to measure both the phosphorylation of MAP kinase and its activation state. The reaction mixture consisted of 5 μl of sample, 5 μl of substrate, containing both 0.5 mg/ml recombinant MAP kinase and 0.5 mg/ml MBP, and 5 μl of kinase buffer, containing 40 μM [γ-^{32}P]ATP (sp. radioactivity 1.7 μCi/nmol). The mixture was incubated for 15 min at 25 °C and the reaction was stopped by the addition of 15 μl of 2 $\times$ SDS/PAGE sample buffer. The samples were separated by SDS/PAGE on a 12.5% gel, stained for proteins and autoradiographed.

Phosphoamino acid analysis of MAP kinase

The recombinant MAP kinase that had been phosphorylated *in vitro* by its activator as described above was analysed for its phosphoamino acid content. The tryptic peptides were partially hydrolysed in 200 μl of 5.7 M HCl for 1 h at 110 °C [31]. The samples were mixed with 1 μl of a 0.6 mg/ml standard amino acid solution before being analysed by two-dimensional thin-layer electrophoresis [32]. The standards were detected by staining with ninhydrin, and the phosphoamino acids by autoradiography.

RESULTS

Phosphorylation of EGF receptor peptide by PMN extracts

Initial experiments to detect MAP kinase activity were carried out with the EGF receptor peptide, T669. Extracts from cells stimulated with FMLP or PMA phosphorylated the peptide more strongly than did those from unstimulated control cells (Figure 1). Extracts of cells treated with interleukin-1, tumour necrosis factor or LPS (results not shown) did not show any increase in activity. With FMLP the increase in kinase activity was found to be rapid and transient, being maximal within 1 min and decreasing by 10 min. In contrast, the response to PMA was less rapid but more sustained, being maximal at 5 min with no significant fall by 20 min. The amount of kinase activity was dependent on the concentration of FMLP or PMA when activity was measured at their maximal stimulation times (Figure 2).

Characterization of the kinase

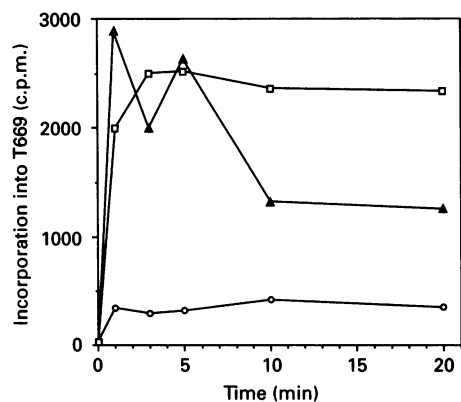The cell extracts were chromatographed on a Mono Q anion-

Figure 1 Phosphorylation of EGF receptor peptide (T669) by extracts from PMN stimulated by FMLP or PMA for various times

PMN were incubated with or without 0.1 μM FMLP or 100 ng/ml PMA for the times indicated. The unstimulated (O), FMLP (▲)- or PMA (□)-treated cells were then resuspended in buffer A (see the Materials and methods section) and disrupted by sonication. The lysates were assayed for phosphorylating activity against the EGF receptor peptide, T669. The data show one representative experiment of four.
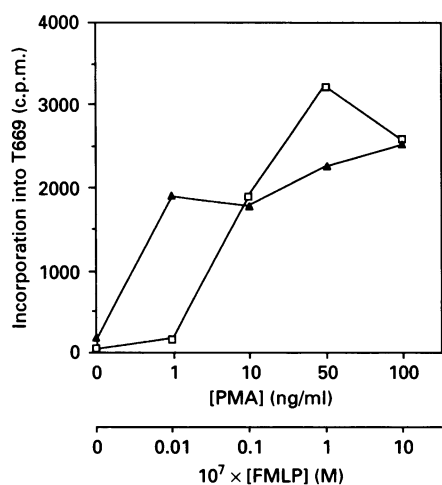

Figure 2 Effect of increasing dose of FMLP or PMA on phosphorylation of the EGF receptor peptide (T669) by PMN extracts

PMN were incubated with various concentrations of FMLP (▲) for 1 min or PMA (□) for 5 min. The cells were then disrupted and assayed for kinase activity as for Figure 1. The data show one representative experiment of four.

Figure 3 Anion-exchange chromatography of PMN extracts

PMN were incubated with or without 0.1 μM FMLP for 1 min or 50 ng/ml PMA for 5 min. Samples (0.5–1 mg of protein) from unstimulated (O), FMLP (▲)- or PMA (□)-stimulated extracts were applied to a Mono Q column equilibrated with buffer A. Proteins were eluted by a gradient of increasing concentration of NaCl (0–500 mM). The fractions were then assayed for kinase activity against T669, MBP or MAP.

exchange column, and fractions were assayed for kinase activity towards the EGF receptor peptide T669. A single sharp peak of activity was eluted at around 300 mM NaCl when cells had been stimulated with FMLP or PMA (Figure 3). The activity was contained in a single sharp peak, even when the column was eluted with a gradient of twice the volume (results not shown). No activity was detected in fractions of extracts of unstimulated cells (Figure 3). The fractions were also assayed for activity on two other MAP kinase substrates, namely MBP and MAP. Fractions from stimulated cells contained a peak of activity towards both substrates, which was found to co-distribute with that towards the T669 peptide (Figure 3). With MAP as substrate, an additional peak of inducible activity was eluted earlier on the gradient at 150 mM NaCl in the experiment shown in Figure 3.
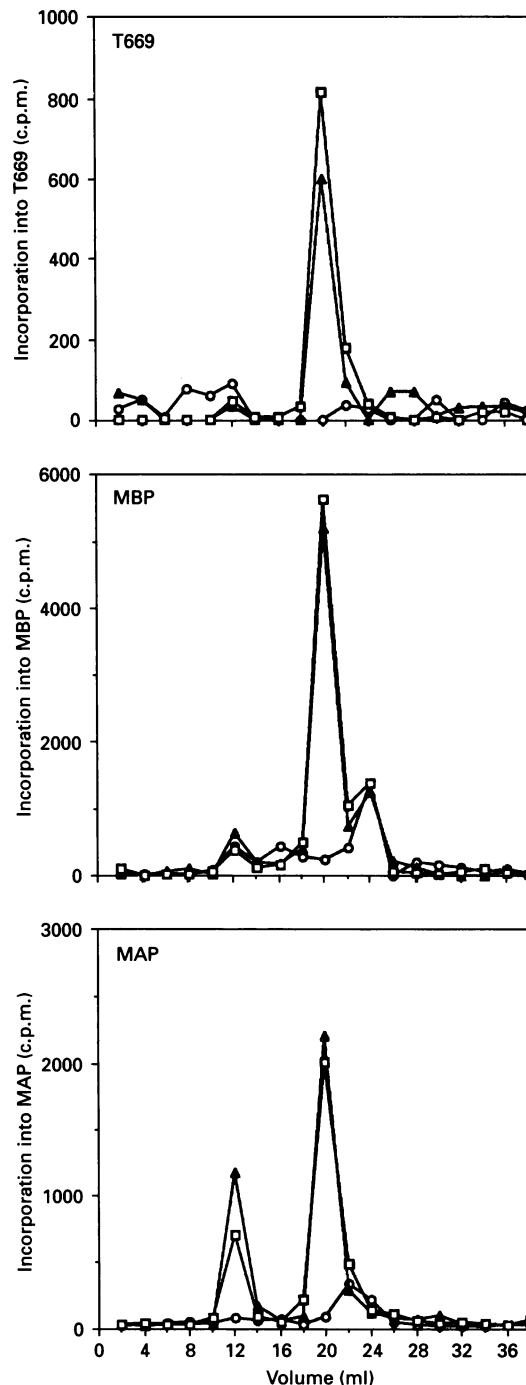
However, this enzyme was not activatable in cells from all donors (results not shown). One possibility was that it was being stimulated by contaminating endotoxin, LPS. To check this, cells from an individual in which the peak was not induced by PMA were incubated with LPS. Only a small amount of MAP kinase activity was detected in the earlier position, and there was no
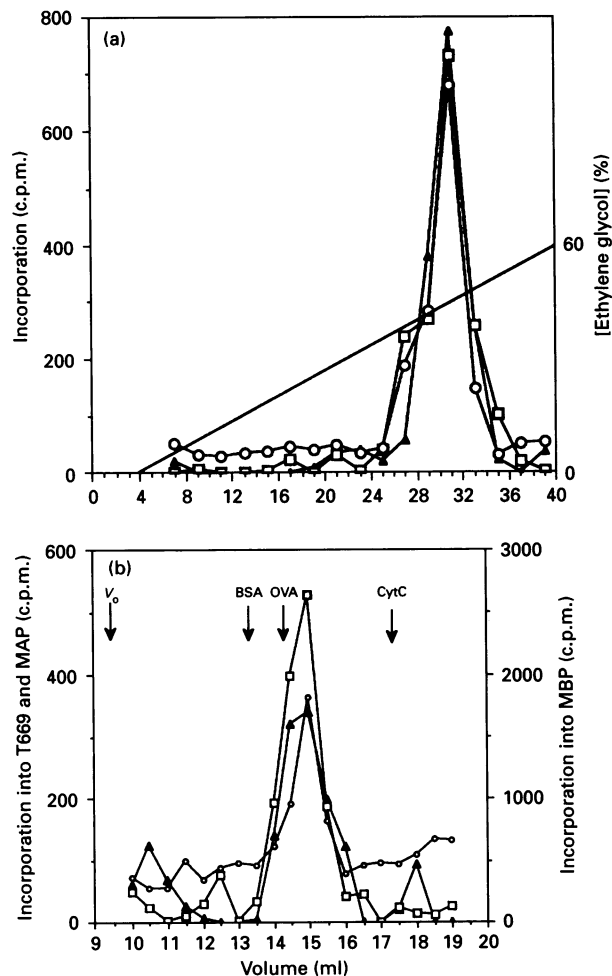
Figure 4   Phenyl-Sepharose (a) and Sephacryl S-200 (b) chromatography of PMA-treated cells

Chromatography was carried out as described in the Materials and methods section. (a) Co-elution of MAP kinase activity against T669 (▲), MBP (□) or MAP (○) from phenyl-Sepharose. (b) Elution profile of activity against the same substrates after chromatography on Sephacryl S-200. The elution position of standard proteins are indicated: V_0, Blue Dextran; BSA, 66 kDa; OVA, ovalbumin, 45 kDa; CytC, cytochrome c, 12.5 kDa.

difference between control, LPS- or PMA- stimulated cells (results not shown).

Further chromatography of the main peak of inducible enzyme was carried out. It bound strongly to phenyl-Sepharose, and was eluted at around 50 % ethylene glycol. Activity against the three substrates co-chromatographed (Figure 4a). Fractions containing the main peak of activity from the Mono Q column were also subjected to gel filtration, and the activity with all three substrates again co-eluted in a single peak that corresponded to a molecular mass of approx. 40 kDa (Figure 4b).

Western blotting with anti-MAP kinase and anti-phosphotyrosine antibody

To establish if the induced MAP kinase was a known MAP kinase, immunoblotting was performed. Immunoblotting of cell lysates with an antiserum which recognizes 42 kDa and 44 kDa MAP kinase (ERK1 and 2) revealed the presence of a single band
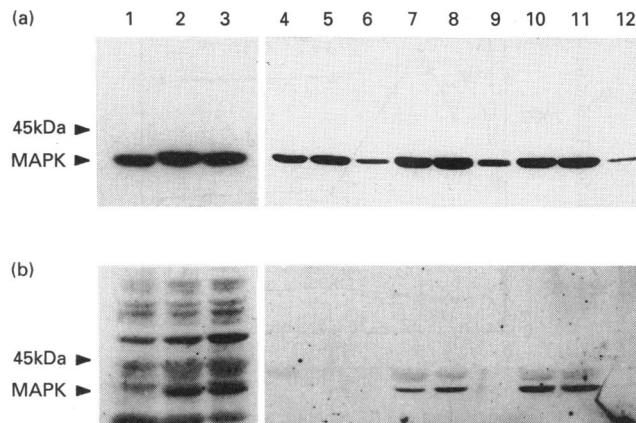

Figure 5   Immunoblot of PMN lysate and Mono Q fractions with antibodies to MAP kinase or phosphotyrosine

Lanes 1–3 are lysates of unstimulated (1), FMLP (2)- or PMA (3)-treated cells. Lanes 4–6 are Mono Q chromatography fractions 20, 22 and 24 of unstimulated cell extracts respectively. Lanes 7–9 are the equivalent fractions of FMLP-stimulated cell extracts, and lanes 10–12 the equivalent fractions of PMA-stimulated cells. (a) represents a membrane immunoblotted with a rabbit antiserum to MAP kinases (42 kDa and 44 kDa); (b) represents a membrane immunoblotted with a rat monoclonal antibody to phosphotyrosine. Abbreviation: MAPK, MAP kinase.

at 42 kDa in samples from resting or stimulated cells (Figure 5a, lanes 1–3). Its mobility was slightly slower in the lysates of stimulated cells than in unstimulated ones. This band was also detected in blots of Mono Q chromatography fractions (Figure 5a, lanes 4–12). However, with PMN stimulated with FMLP or PMA, the fractions containing the largest quantity of the 42 kDa band corresponded to fractions with the maximal kinase activity (Figure 5, lanes 7 and 8 for FMLP and 10 and 11 for PMA). Blotting with a monoclonal antibody which is specific for the 42 kDa MAP kinase also stained this band (results not shown).

Immunoblotting of cell lysates and Mono Q fractions with the antibody to phosphotyrosine showed that FMLP or PMA induced tyrosine phosphorylation of a 42 kDa protein (Figure 5b). The phosphotyrosine band detected on stimulated fractions (Figure 5b, lanes 7–12) corresponded exactly to the MAP kinase band (Figure 5a, lanes 7–12) and also to the distribution of MAP kinase activity in Mono Q chromatography (Figure 3). This phosphorylation was absent from the lysates of unstimulated cells (Figure 5b, lane 1).

Induction of the MAP kinase activator

We next decided to investigate the possible induction of a MAP kinase activator in PMN upon stimulation. Fractions from Mono Q chromatography were tested for their ability to phosphorylate the recombinant MAP kinase. Fractions from FMLP- or PMA-stimulated cells, eluted at around 150 mM NaCl, strongly phosphorylated the recombinant enzyme (Figure 6). To establish whether or not the phosphorylation state of the recombinant MAP kinase correlated with its kinase activity, MBP was included in the reaction mixture. As shown in Figure 6, an increased phosphorylation of recombinant MAP kinase was associated with increased phosphorylation of MBP. A small amount of MBP phosphorylation was observed in the control fractions ($<$ 10 % of label was incorporated into MBP in control compared with stimulated cells). This may be due to a low level of constitutively active MAP kinase kinase. Phosphorylation of the MBP was also seen in fractions 20–22, but this was due to
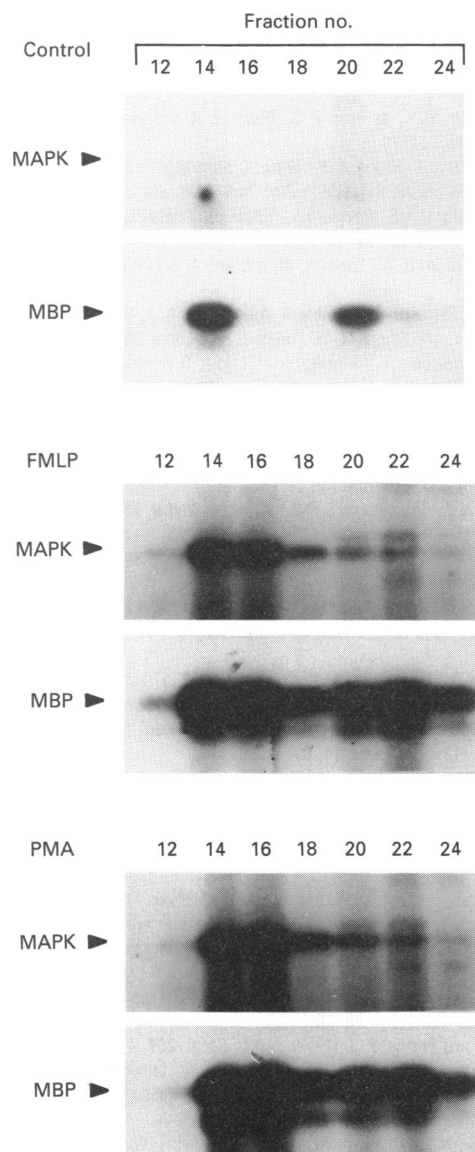
Figure 6   Activation of MAP kinase kinase in PMN by FMLP and PMA

Extracts from unstimulated control and FMLP- or PMA-stimulated cells were chromatographed on a Mono Q column as described in the Materials and methods section. Fractions were assayed for their ability to phosphorylate recombinant MAP kinase. The reaction mixture included recombinant MAP kinase (MAPK) and MBP, which were then separated by SDS/PAGE on a 12.5% gel. The panels show selected regions of the autoradiographs of Mono Q fractions showing phosphorylating activity towards MAPK or MBP.
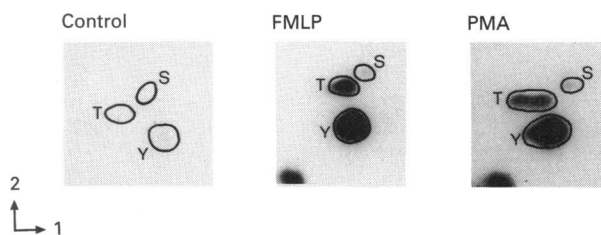

Figure 7   Phosphoamino acid analysis of recombinant MAP kinase

Recombinant MAP kinase was phosphorylated in vitro with fractions containing the MAP kinase kinase (fractions 14–16 of Figure 6) and the components separated by gel electrophoresis. The MAP kinase band was then excised, hydrolysed and electrophoresed on cellulose t.l.c. plates as described in the Materials and methods section. The first dimension was run at pH 1.9 and the second at pH 3.5. Abbreviations: S, serine; T, threonine; Y, tyrosine.

activated endogenous MAP kinase. Phosphoamino acid analysis of the recombinant MAP kinase which had been phosphorylated by the activator enzyme in fractions 14–16 (Figure 6) showed that it contained phosphothreonine and phosphotyrosine (Figure 7).

DISCUSSION

There are several members of the MAP kinase family. They are also known as Extracellular Signal-Regulated Kinases (ERK), and three related ERK cDNAs have been cloned [33]. The family includes the 42 kDa (ERK2) and 44 kDa (ERK1) enzymes, which are widely distributed, and the 54 kDa species, which is expressed in hepatocytes [8]. The 42 kDa and 44 kDa MAP kinases are often expressed in the same cells, and are more similar to each other than to the 54 kDa MAP kinase [8].

The present study demonstrates that FMLP stimulation of PMN results in the rapid and transient activation of a MAP kinase. The enzyme was also induced when the cells were stimulated with PMA, although the activation was slightly slower and more prolonged than with FMLP. Presumably the receptor-coupled response to FMLP is more rapid because the PMA has to diffuse into the cell. The enzyme bound strongly to phenyl-Sepharose, it had a molecular mass of about 40 kDa on gel filtration, and phosphorylated three MAP kinase substrates. It was recognized by antibodies to MAP kinase and became phosphorylated on tyrosine upon stimulation. Recent studies on PMN incubated with granulocyte–macrophage-colony-stimulating factor have shown the induction of tyrosine phosphorylation on a protein of molecular mass 40 kDa and have identified it as a MAP kinase [15–17]. We have confirmed these results and have found that, in contrast with FMLP, the induction of MAP kinase by granulocyte–macrophage-colony-stimulatory factor is slower and more prolonged (results not shown).

The 42 kDa enzyme that we have found corresponds closely to the 42 kDa MAP kinase of many cell types, including fibroblasts, pheochromocytoma cells and T lymphocytes [9,22,23,34]. However, these other cells also contain the 44 kDa MAP kinase [22,23,34–36]. The rabbit antiserum that we have used in this study detects both the 42 kDa and 44 kDa MAP kinases in human fibroblasts ([26]; H. L. Thompson, unpublished work), but in PMN only the 42 kDa enzyme could be detected. The reason for this preferential expression of one MAP kinase is not known, but it may lie in the MAP kinase system having different functions in PMN compared with other cells.

An additional kinase capable of phosphorylating MAP, but not MBP or T669 peptide, was detected in PMN. This enzyme was eluted from Mono Q earlier than 42 kDa MAP kinase. However, its inducibility by FMLP or PMA varied among donors. We wondered if this variability was due to the enzyme actually being induced by LPS, since small quantities of LPS could have been a contaminant of some experiments. However, incubation of PMN with LPS did not stimulate either this enzyme or, interestingly, the MAP kinase itself. The relationship of this protein to other MAP kinases is not known. In view of the large size of MAP, it is likely to serve as a substrate for many kinases in vitro.

Activation of MAP kinase depends on phosphorylation of threonine and tyrosine residues which are separated by a single isoleucine or leucine residue [22–24]. Dephosphorylation of either site inactivates the enzyme [37–39]. The activator of MAP kinase that has been characterized is itself a kinase and has specificity for both threonine and tyrosine [25,39]. MAP kinase may also undergo autophosphorylation, and it has been suggested that activators could work by increasing the rate at which this occurs

[40]. PMN stimulated by either FMLP or PMA contained a MAP kinase kinase activity which phosphorylated the recombinant 42 kDa substrate on threonine and tyrosine. These phosphorylations of MAP kinase were associated with activation, as judged by the enzyme's enhanced activity towards MBP. The activator induced by either FMLP or PMA showed similar chromatographic behaviour. A kinase kinase of similar activity has been found in PC-12 cells stimulated with nerve growth factor [25], in EL4 or U937 stimulated with phorbol esters [39,41], and in the muscle of insulin-treated rabbits [42]. Whether all the diverse stimuli which activate MAP kinase converge on a common activator, or work through different ones, remains to be established.

The activation of MAP kinase by a chemotactic factor represents a new category of stimulus acting on this kinase system. The enzyme's physiological function in PMN is open to speculation. MAP kinase is implicated in regulating cell division and protein synthesis [43], but PMN do not divide, and synthesize little, if any, protein. It could be involved in controlling cell movement, or other functions stimulated by chemotactic factors, such as the respiratory burst or degranulation. At present we are investigating whether or not MAP kinase is activated by all types of chemoattractants, by phagocytic stimuli and by priming agents such as colony-stimulating factors. Knowing the degree of MAP kinase activation caused by substances whose effects on PMN are extensively characterized should give some insight into the possible function of the enzyme in the physiology of phagocytic cells.

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