Direct stimulation by tyrosine phosphorylation of microtubule-associated protein (MAP) kinase activity by granulocyte–macrophage colony-stimulating factor in human neutrophils

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Human polymorphonuclear neutrophils exhibit a low level of the microtubule-associated protein kinase (MAPK) activity. This enzymic activity is enhanced up to 3-fold upon cell stimulation with the human haematopoietic hormone granulocyte–macrophage colony-stimulating factor (GM-CSF). This is demonstrated both in whole-cell lysates and in DEAE-anion-exchange semi-purified fractions prepared from GM-CSF-stimulated neutrophils, by assaying the kinase activity against either myelin basic protein or a phosphoacceptor peptide that bears the specific phosphorylation site of the MAPK natural substrate. Similarly, phosphorylation of MAPK in tyrosine residues, as found in immunoblots using anti-phosphotyrosine

antibodies, follows similar time- and dose-response curves as the kinase activation. Pretreatment of the cells with the tyrosine kinase inhibitor genistein abrogates the above-mentioned effect, whereas the phosphatase inhibitor okadaic acid enhances both the basal and the GM-CSF-stimulated kinase activities. Likewise, MAPK tyrosine phosphorylation is diminished in genistein-treated neutrophils, and enhanced in okadaic acid-treated cells. We conclude that MAPK activity is present in human neutrophils, and that it is stimulated by GM-CSF. This stimulation of the activity is most likely due to the phosphorylation of MAPK in tyrosine residues triggered upon binding of GM-CSF to its receptors.

INTRODUCTION

The microtubule-associated protein kinases (MAP-2 kinase, ERK-2 or p42^{MAPK}) are serine/threonine kinases that are activated upon stimulation of cells with several agents such as insulin, growth factors, proto-oncogene products and phorbol esters [1–3]. Several isoforms of MAPK have been described in different systems, although they are structurally related [4]. These enzymes phosphorylate other key kinases and proteins, and they play a central role in the signal-transduction mechanism that transduces mitogenic signals from an activated receptor kinase to the cell interior [3]. MAPKs themselves are regulated by phosphorylation. Activation of MAPKs requires the phosphorylation of both threonine and tyrosine residues [5]. The protein serine/threonine phosphatase-2A or the protein tyrosine phosphatase CD45 specifically inactivates MAPK activities [5,6]. A MAPK kinase has been described in nerve-growth-factor-stimulated pheochromocytoma (PC12) cells that phosphorylates MAPK on serine/threonine and tyrosine [7]. Another member of the MAPK family has been shown to be phosphorylated and activated by the tyrosyl kinase p56^{lck} that is related to *src*-protein tyrosine kinases [8]. Several MAPK substrates have been identified so far in several cell systems *in vitro* [9,10]. They include: MAP2, ribosomal-protein S6 kinase (S6K), myelin basic protein (MBP), *c-jun* proto-oncogene product and *c-Raf-1* proteins. One of these MAPK substrates, S6K, is another serine/threonine kinase that in turn phosphorylates S6, a major component of the large 40 S ribosomal unit [11]. The S6K is encoded by *rsk* in mouse and chick fibroblasts and also by the *raf* gene [12].

Granulocyte–macrophage colony-stimulating factor (GM-CSF) is a haematopoietic growth factor required for the pro-

liferation of myelomonocytic stem cells *in vivo* and *in vitro* [13,14]. GM-CSF also acts on mature neutrophils, potentiating their physiological functions. These include superoxide generation, platelet-activating factor and arachidonic acid release, phagocytosis and degranulation (reviewed in [15]). The mechanism of this priming process is still unknown. Recently it has been found [16–21] that incubation of human neutrophils with GM-CSF triggers a rapid phosphorylation of tyrosine residues of several proteins, including a protein of 41 kDa (p41).

The presence of tyrosine-specific protein kinase activities in human neutrophils has recently been demonstrated [22–24]. However, the roles of these kinases in neutrophil functions and the identities of their substrates remain largely unknown. Recently we have shown that the p41 protein, which is phosphorylated on tyrosine residues in neutrophils stimulated by various agonists, is a member of the MAPK family [25]. The present studies were undertaken to examine: (a) the presence of a MAPK activity in human neutrophils; (b) whether this activity is stimulated by the haematopoietic growth factor GM-CSF; and (c) whether a correlation exists between MAPK activation and its tyrosyl phosphorylation. Our data show that low MAPK activity can be measured in quiescent cells. This activity can be greatly enhanced in GM-CSF-stimulated neutrophils, and its stimulation correlates with the tyrosine phosphorylation of MAPK.

MATERIALS AND METHODS

Materials

GM-CSF was purchased from Amgen Biologicals (Thousand Oaks, CA, U.S.A.); Hepes, leupeptin, sodium orthovanadate, phenylmethanesulphonyl fluoride, cyclic-AMP-dependent pro-

Abbreviations used: MAPK, microtubule-associated protein kinase, mitogen-activated kinase or extracellular-signal-regulated kinase (ERK-1); GM-CSF, granulocyte–macrophage colony-stimulating factor; ECL, Enhanced Chemi-Luminescence; MBP, myelin basic protein; PKA, cyclic-AMP-dependent protein kinase; DFP, di-isopropyl fluorophosphate; PVDF, polyvinylidene difluoride.

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tein kinase (PKA) inhibitor (rabbit sequence TTYADFIAS-GRTGRRNAIHD) and MBP were obtained from Sigma (St. Louis, MO, U.S.A.); di-isopropyl fluorophosphate (DFP) was purchased from Aldrich (Milwaukee, WI, U.S.A.); Immobilon (polyvinylidene difluoride; PVDF) membranes were obtained from Millipore Corp. (Bedford, MA, U.S.A.); electrophoresis chemicals were supplied by Bio-Rad Laboratories (Richmond, CA, U.S.A.); [γ - 32 P]ATP (30 Ci/mmol) and the Enhanced Chemi-Luminescence (ECL) Western-blotting detection kit were purchased from Amersham Life Sciences (Arlington Heights, IL, U.S.A.); ion-exchange-chromatography cellulose phosphate paper was purchased from Whatman (Hillsboro, OR, U.S.A.). Okadaic acid was from LC Services Corp. (Woburn, MA, U.S.A.). Genistein and MAPK substrate peptide (sequence APRTPGRR, which includes amino acids 95–98 of bovine MBP) were purchased from Upstate Biotechnology Inc. (Lake Placid, NY, U.S.A.).

Antibodies

Protein A-purified monoclonal Ig2b_k anti-phosphotyrosine (clone 4G10) antibody (anti-PY) was purchased from Upstate Biotechnology Inc. and used at a 1:1000 dilution (1 μ g/ml final concn. in Western blots). MK12 monoclonal, IgG₁ antibody to MAP kinase (anti-MAPK) was purchased from Zymed Laboratories Inc. (South San Francisco, CA, U.S.A.) and used at a dilution of 1:4000 (0.4 μ g/ml final concn. in Western blots). MK12 is an anti-peptide antibody raised to the sequence TDEPVAEEPFTFDMELDDLK, corresponding to amino acids 325–345 near the C-terminus of ERK-1 [26].

Isolation of human neutrophils

Human neutrophils were isolated on Ficoll/Hypaque gradients by the method of English and Anderson [27]. The cells were resuspended in modified Hanks' Balanced Salt Solution containing 10 mM Hepes (pH 7.4), 124 mM NaCl, 4.9 mM KCl, 0.64 mM Na₂HPO₄, 0.66 mM KH₂PO₄, 15.2 mM NaHCO₃, 0.1 % dextrose and 0.1 % BSA.

Cell lysis

Neutrophils (1×10^7 cells/ml) were incubated with the appropriate agents and lysed by addition of 1 vol. of lysis buffer (10 μ M sodium orthovanadate, 10 mM *p*-nitrophenyl phosphate, 5.5 % Triton X-100, 1 mM DFP, 1 mM benzamidine, 2 μ g/ml aprotinin, 0.7 μ g/ml pepstatin A) to 10 vol. of the cell suspension for 10 min at 4 °C and then centrifuged for 15 min at 7000 *g* in a table-top centrifuge kept at 4 °C. These crude cell lysates were used to run SDS/PAGE gels or to perform the kinase assays. Lysates kept at –80 °C maintain activity for up to 4 weeks.

Anion-exchange chromatography

Neutrophils (2.5×10^7 cells/ml) were lysed as indicated above, and lysates were diluted 1:10 (v/v) with Buffer A (10 mM Hepes, pH 7.3, 1 mM EGTA, 0.1 mM phenylmethanesulphonyl fluoride, 1 mM DFP, 0.5 μ g/ml leupeptin, 50 mM β -glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM dithiothreitol, 10 μ M ammonium molybdate, 1 mM benzamidine, 2 μ g/ml aprotinin and 0.7 μ g/ml pepstatin A) and applied to an h.p.l.c. ion-exchange column (Bio-Gel SEC DEAE-5 PW; 75 mm $\times$ 7.5 mm; Bio-Rad) in a Bio-Rad h.p.l.c. system (400 HRLC series). The column was previously equilibrated with Buffer A. Elution was done with a linear gradient from 0 to 0.4 M NaCl in the same buffer at a flow rate of 1.0 ml/min.

Fractions were collected and assayed for MAPK activity or stored at –80 °C.

MAPK assay

The enzymic activity in whole lysates and in the purified DEAE-column fractions was measured as described [28,29] with some modifications. Briefly, the required substrate peptide was diluted in freshly prepared kinase buffer (13.4 mM Hepes, pH 7.3, 25 mM MgCl₂, 30 μ M sodium orthovanadate, 5 mM *p*-nitrophenyl phosphate, 2 mM EGTA, 0.021 mCi of [γ - 32 P]ATP/ml and 68 μ M unlabelled ATP) at the following concentrations: 16 μ M MBP or 1 mM MAPK substrate peptide. The kinase buffer also contains 2 μ M PKA inhibitor (Sigma) with an activity of 2000–6000 phosphorylation units of PKA/ μ g. The phosphotransferase reaction was initiated by addition of 20 μ l of kinase buffer to 20 μ l of the whole-cell lysates or the DEAE-column eluates. The reaction was carried out at room temperature for 30 min. The kinase reaction was terminated by blotting 20 μ l of the reaction mixture on to ion-exchange-chromatography cellulose phosphate paper [area 6.45 cm² (1 in²)]. Paper filters were washed twice, dried and counted for radioactivity in a liquid-scintillation counter (LKB-1217 Rack Beta).

Western blotting

This was performed as described previously [16–18]. Briefly, whole-cell lysates (60–100 μ l) from control and stimulated neutrophils were mixed with 50 μ l of Laemmli [30] stopping solution [9 % (w/v) SDS, 6 % (v/v) β -mercaptoethanol, 10 % (v/v) glycerol and a trace amount of Bromophenol Blue dye in 0.196 M Tris/HCl (pH 6.7)]. The mixture was electrophoresed through SDS/polyacrylamide gels (5–15 %). Pre-stained high-molecular-mass standards (Bethesda Research Laboratories) were run with each gel along with the samples. The proteins were then transferred to PVDF sheets (Millipore Corp.) and the blots were treated with the appropriate antibodies. Western blots were developed by the ECL method for detection. Briefly, blots were blocked in Tris-buffered saline/Tween-20 (TBS-T) (20 mM Tris base, pH 7.6, 137 mM NaCl, 0.1 % Tween-20) supplemented with 5 % BSA, followed by incubation with the primary antibody in TBS-T and finally with horseradish-peroxidase-conjugated anti-mouse IgG at 1:7000 dilution for 1 h at room temperature. Blots were autoradiographed for variable lengths of time (15 s–1 min) with Kodak X-Omat film. When needed, protein bands were quantified by laser scanning densitometry (LKB Ultrascan; Pharmacia).

Blot stripping and re-probing

When a Western-blotting procedure was completed and X-ray films were obtained, blots were stripped of the primary-antibody-secondary-antibody complex by incubating them in stripping buffer (100 mM β -mercaptoethanol, 2 % SDS, 62.5 mM Tris/HCl, pH 6.7) for 30 min at 50 °C. Blots were then subjected to autoradiography for confirmation that the antibody signal had been removed. After this procedure, the blots were blocked with buffer containing 5 % BSA, and re-probed with a different antibody, in accordance with the procedure indicated above.

RESULTS

Measurement of MAPK activity and tyrosine phosphorylation in neutrophil lysates

MAPK activity was measured in lysates isolated from control and GM-CSF-treated cells, by using as phosphoacceptor

Figure 1 Time course of MAPK activation and phosphorylation by GM-CSF in whole-cell lysates

Neutrophils (1×10^7 cells/ml) were incubated for the indicated times at 37 °C with 270 pM GM-CSF. After this time, cells were lysed and 20 μl portions of whole-cell lysates were used in a cell-free kinase assay with phosphocellulose paper as described in the Materials and methods section. Panel (a): ^{32}P incorporation into the phosphoacceptor substrate MAPK-substrate (MAPKS) peptide. ^{32}P incorporation in lysates from control cells (30 s before addition of GM-CSF) was taken arbitrarily as 1.0, and corresponds to 630 ± 40 c.p.m. Background incorporation of ^{32}P into phosphocellulose paper filters (peptide omitted from the kinase buffer) was 520 ± 70 c.p.m. Points represent means $\pm$ S.E.M. of three separate experiments. Panel (b): samples were subjected to SDS/PAGE, and proteins were transferred on to PVDF papers and immunoblotted with anti-MAPK antibody. The p41 band in X-ray films was quantified by laser densitometry. Optical density at 633 nm of controls was arbitrarily taken as 1.0.

substrate the MAPK substrate peptide (APRTPGGRR) that bears the Thr-97 phosphorylation site for MBP. As shown in Figure 1(a), the maximal MAPK activity is up to 3-fold higher in lysates prepared from GM-CSF-stimulated cells than in control lysates prepared from unstimulated cells. The increase in MAPK activity was seen after 3 min incubation with GM-CSF, reaching a maximum between 5 and 10 min and then decreasing at 15 min (Figure 1a). A similar time course was obtained when the phosphoacceptor substrate was MBP (results not shown). With this substrate, the maximum activation was 170% of control (at 5 min after addition of GM-CSF). This value is somewhat lower than that obtained with the MAPK substrate peptide. As shown in Figure 1(b), the time course of GM-CSF-induced MAPK activation is similar to that of tyrosine phosphorylation of MAPK (which we have previously shown to be p41, a phosphoprotein of 41 kDa [25]). Maximum phosphorylation levels can also be seen at 5–10 min. Both MAPK tyrosine phosphorylation and MAPK activity start to decline 15 min after GM-CSF addition (Figures 1a and 1b).

The stimulation of MAPK activity by GM-CSF is dose-dependent (Figure 2a). With the MAPK substrate peptide, a

Figure 2 Dose-response of MAPK activation by GM-CSF in whole-cell lysates

Neutrophils (1×10^7 cells/ml) were incubated with the indicated amounts of GM-CSF for 5 min at 37 °C. Samples were processed as indicated in Figure 1. ^{32}P incorporation into the phosphoacceptor substrates MAPK-substrate (MAPKS peptide) is indicated. Panel (a): ^{32}P incorporation in lysates from control cells (only buffer added to the cells) was taken arbitrarily as 1.0 and corresponds to 590 ± 45 c.p.m. Points represent means $\pm$ S.E.M. of three separate experiments. Panel (b): optical density (633 nm) of p41/MAPK (see Figure 1 legend).

Table 1 Effect of several neutrophil stimuli on MAPK activity and tyrosine phosphorylation

Cells were treated with the indicated stimuli for 3 min [*N*-formylmethionyl-leucyl-phenylalanine (fMet-Leu-Phe) and platelet-activating factor (PAF)] or 5 min [phorbol 12-myristate 13-acetate (PMA)]. Lysates were obtained and subjected to electrophoresis/immunoblotting or to the enzyme kinase assay. The Table was compiled from data (means $\pm$ S.E.M.) obtained in three independent experiments.

Condition	MAPK enzyme activity*	MAPK tyrosine phosphorylation†
Control	1.0 ± 0.08	1.0 ± 0.11
fMet-Leu-Phe (100 nM)	2.9 ± 0.21	2.6 ± 0.25
PAF (100 nM)	2.7 ± 0.18	2.5 ± 0.20
PMA (50 ng/ml)	4.1 ± 0.35	3.9 ± 0.40

* Data refer to incorporation of ^{32}P into the MAPK substrate peptide as indicated in the text. Control represents 510 ± 50 c.p.m.

† Data refer to optical density (OD) at 633 nm (measured in a LKB laser scanner) of the p41/MAPK protein of autoradiographs from immunoblots developed with anti-phosphotyrosine antibody. OD in control was arbitrarily taken as 1.0.

significant increase in the activity is obtained at concentrations > 50 pM, at values close to the K_d of GM-CSF for its receptor in human neutrophils [31]. Similarly, a maximum MAPK activity of 175% of control, at concentrations > 300 pM GM-CSF, was obtained with MBP in the kinase assay (results not shown). With

Figure 3 Effect of genistein and okadaic acid on MAPK activity

Neutrophils (1×10^7 cells/ml) were incubated for 1 h at 37°C with $10\text{ }\mu\text{g/ml}$ genistein (a, b) or $1\text{ }\mu\text{g/ml}$ okadaic acid (c, d) before being challenged with 270 pM GM-CSF for 5 min. After this time, cells were treated as indicated in the legend to Figure 1. ^{32}P incorporation into the phosphoacceptor substrates MAPK-substrate (MAPKS) peptide (a, c) or MBP (b, d) is indicated. Bars represent means $\pm$ S.E.M. of at least three separate experiments.

either of the aforementioned substrates, saturation of MAPK activity occurs at concentrations higher than $0.5\text{--}1\text{ nM}$ GM-CSF. The dose-response of GM-CSF-induced MAPK activation parallels that of MAPK tyrosine phosphorylation (Figure 2b).

In addition to GM-CSF, other known neutrophil stimulants are able to increase both the basal level of MAPK activity in the kinase assay *in vitro* and the phosphorylation of p41 in Western blots (Table 1). Those stimulants include the chemotactic peptide *N*-formylmethionyl-leucyl-phenylalanine platelet-activating factor and the protein kinase C activator phorbol 12-myristate 13-acetate. We have found that the potency of p41 phosphorylation correlates well with the degree of MAPK for each stimulus, PMA, at the indicated concentration, being the most potent (up to 4-fold increase in MAPK activity).

Effect of tyrosine kinase and phosphatase inhibitors on MAPK activity and tyrosine phosphorylation of MAPK protein in neutrophil lysates

Next we studied the effect of several known tyrosine kinase and protein phosphatase inhibitors on the MAPK activity. When cells were pretreated with the tyrosine kinase inhibitor genistein, the GM-CSF-enhanced MAPK activity in cell lysates was diminished (Figures 3a and b). The effect was dose-dependent, with $100\text{ }\mu\text{g/ml}$ genistein producing maximum inhibition (results not shown). We next examined the effect of the protein phos-

phatase inhibitor okadaic acid. This compound is a potent inhibitor of serine/threonine-specific protein phosphatases 1 and 2A (PP1 and PP2A) and of phosphatase 2B at higher concentrations [32]. Figures 3(c) and 3(d) show that okadaic acid, when added alone to the neutrophils, enhanced the MAPK activity.

Figures 4(b) and 4(d) show that the tyrosyl phosphorylation of p41, which we previously have shown to be MAPK, is increased in cell lysates obtained from GM-CSF-stimulated neutrophils. When cells were pretreated with the tyrosine kinase inhibitor genistein before adding GM-CSF, the level of tyrosine phosphorylation of p41 was diminished (Figure 4b). Okadaic acid alone also increased the level of tyrosine phosphorylation of p41 (Figure 4d). Autoradiographs showing the presence of the MAPK protein as determined by Western blots using anti-MAPK antibody are included (Figures 4a and 4c).

Measurement of MAPK activity in DEAE-anion-exchange-chromatographic fractions

Fractionation of cell lysates by DEAE-anion-exchange chromatography in a NaCl gradient allowed us to separate the different types of kinases that are present in the neutrophil. With the peptide APRTGGRR as a substrate, fractions prepared from resting cells contained little MAPK activity. On the other hand, a single and very sharp peak of MAPK activity was found

Figure 4 Tyrosine phosphorylation of MAPK in Western blots: effect of genistein and okadaic acid

Whole neutrophil lysates were prepared from 1×10^7 cells/ml; 100 μ l portions of those were mixed with Laemmli SDS-stopping buffer, subjected to SDS/PAGE and proteins were transferred to PVDF ('Immobilon'; Millipore Corp.). Blots were probed with monoclonal anti-MAPK MK12 antibody (**a, c**) as primary antibody and horseradish-peroxidase-conjugated anti-mouse IgG as secondary antibody. Proteins were made visible by the ECL method and exposed to X-ray films. The same blot was stripped (see the Materials and methods section) to remove the primary/secondary-antibody complex, and it was then re-probed with monoclonal anti-phosphotyrosine (anti-PY) antibody (**b, d**) and developed by ECL. The Figure depicts only the p41/MAPK protein band. Equal amounts of protein were loaded on to each lane of the gel.

Figure 5 MAPK activity in DEAE-column eluates of neutrophil lysates

Neutrophils (2.5×10^7 cells/ml) were incubated with 270 pM GM-CSF for 5 min, and cell lysates were obtained as indicated in the Materials and methods section. Lysates were diluted and loaded on to a DEAE-anion-exchange column in a Bio-Rad h.p.l.c. system. The column was washed with the equilibration buffer until protein readings returned to pre-loading basal levels (protein was monitored by measurement of A_{280} , and it is not shown here for sake of clarity). Elution was done with a linear gradient (---) from 0 to 0.4 M NaCl in the equilibration buffer at a flow rate of 1.0 ml/min; 0.5 ml fractions were collected every 30 s, and 20 μ l of each was used to assay MAPK activity with the MAPK substrate peptide (0.5 mM final concn. in the phosphotransferase reaction mixture); $\circ$, GM-CSF; $\bullet$, controls. Omission of peptide substrate from the kinase buffer yielded no peaks of activity throughout the gradient with fractions of either control or GM-CSF-stimulated cells. The Figure shows a representative experiment among three with near-identical results.

after 12.5 min of elution with the NaCl gradient in fractions prepared from GM-CSF-treated cells (Figure 5). Omission of the peptide substrate, from an otherwise complete reaction mixture, leads to a background incorporation of 32 P into phosphocellulose blots and consequently an absence of peaks of activity. Fractions

Figure 6 Immunoblotting of the DEAE-anion-exchange chromatography fractions with anti-MAPK and anti-phosphotyrosine (anti-PY) antibodies

Eluates (100 μ l) from the DEAE-anion-exchange chromatography were mixed with 60 μ l of Laemmli SDS-stopping buffer. Samples were processed as indicated in Figure 4. (**a**) Western blot probed with anti-MAPK monoclonal antibody MK12. (**b**) The same blot stripped and re-probed with monoclonal anti-PY antibody. Only relevant regions of the immunoblots spanning the range 28–60 kDa are represented. Numbers correspond to elution times (min) indicated in Figure 5. The Figure shows a representative experiment of three with near-identical results.

from unstimulated cells assayed with MBP contained some basal MAPK activity (results not shown). With this substrate, a couple of peaks were found at elution times of 10 min and 12.5 min in a NaCl gradient from 0 to 0.4 M. This is in agreement with published data in other systems [33] and corresponds to the elution time of MAPK. Only the second peak (eluted at approx. 200 mM NaCl) shows an increased activity in fractions from GM-CSF-stimulated cells. Interestingly, 32 P incorporation at the peak of maximum activity with GM-CSF is about 7 times (MAPK substrate peptide in the kinase assay) or 4 times (MBP) that found in the starting material (diluted lysates from GM-CSF-activated cells) loaded on to the column.

To confirm further the presence of the MAPK protein in our kinase assays, we loaded several eluate fractions from the DEAE column on to a SDS/PAGE gel; proteins were transferred to PVDF paper that was then probed with MK12 monoclonal antibody. As seen in Figure 6(a), only those fractions that correspond to the peak of maximum activity have MAPK-immunoreactive protein, and a clear phosphorylation increase can be seen in protein p41 in fractions from GM-CSF-treated neutrophils (Figure 6b).

DISCUSSION

The present data show that the activity of MAPK, as measured by the phosphorylation of myelin basic protein or the MAPK substrate peptide APRTGGRR, in lysates prepared from unstimulated human neutrophils is very low. This activity is

greatly increased in lysates prepared from GM-CSF-treated cells (Figure 1a). The stimulation is both time- and dose-dependent (Figures 1a and 2a). The activation of MAPK by GM-CSF described herein is in agreement with results reported, while this manuscript was in preparation, in the human MO7 cell line [34] and neutrophils and HL-60 cells [35] activated with haematopoietic growth factors, including GM-CSF. According to our data, both the time course and the dose-dependent curves of MAPK activation (Figures 1a and 2a) parallel those obtained from the GM-CSF-induced tyrosine phosphorylation of the 41 kDa protein (Figures 1b and 2b). Similarly, those neutrophil agonists that are able to elicit tyrosine phosphorylation of p41 are equally as capable of activating MAPK (the present paper). This p41 protein that becomes heavily phosphorylated on tyrosine upon cell stimulation has been shown to be MAPK [25,34–36]. This conclusion was based on several biochemical and immunological approaches [25], including molecular-mass determination, subcellular localization and immunoprecipitation-immunoblotting experiments. A protein of similar molecular mass is known to be covalently modified in systems other than the neutrophils, such as the nicotine-stimulated chromaffin cells and insulin-stimulated rat adipocytes [37,38]. In addition to being phosphorylated on tyrosine, this protein is also phosphorylated on threonine residues [39,40].

Genistein, a specific protein tyrosine kinase inhibitor [41], inhibited both the MAPK activity (Figures 3a and 3b) and tyrosine phosphorylation of p41/MAPK (Figure 4b). On the other hand, okadaic acid, a serine/threonine phosphatase inhibitor, stimulates MAPK activity (Figures 3c and 3d) and p41/MAPK tyrosine phosphorylation (Figure 4d). These results suggest that the inhibition of serine/threonine and tyrosine phosphatases is unmasking a large level of basal phosphorylation in a fashion similar to that described in other systems [42,43]. This could take place probably at the level of the MAPK kinase, the enzyme upstream of the activation cascade that activates MAPK by phosphorylation. Results obtained with tyrosine kinase and protein phosphatase inhibitors, taken together, strongly suggest the presence of a tyrosine kinase/phosphatase mechanism in neutrophils involved in the elevation of MAPK activity by GM-CSF. This is in agreement with the previously reported data that MAPK activity is regulated by phosphorylation on both serine/threonine and tyrosine [39,40].

Although measurement of MAPK activity in whole-cell lysates provides good information relative to the modulation of this enzyme, we wanted to carry out some experiments with a more enriched fraction of the enzyme. This was accomplished through DEAE-anion-exchange chromatography in a NaCl gradient. This method has been used successfully in our laboratory to assay the activities of other kinases such as Ca^{2+} /calmodulin kinase, protein kinase C and ribosomal-protein S6 kinase. With the inclusion of the PKA inhibitor in the kinase assay mixture (see the Materials and methods section), any contaminating PKA activity is eliminated [44]. PKA activity is present in neutrophils and could partially mask the results on MAPK substrate(s) phosphorylation in our assays.

Using lysates from neutrophils and DEAE-anion-exchange chromatography in a NaCl gradient, it was possible to localize the portion of the lysate that contains MAPK activity. A very sharp peak of MAPK activity was found after 12.5 min of elution with the NaCl gradient in fractions prepared from GM-CSF-treated cells (Figure 5). The presence of MAPK kinase was determined by Western blotting using the antibody MK12 against MAPK (Figure 6). As with the lysates, kinase assays were performed with two different substrates, MBP and the peptide APRTGGRR that includes amino acids 95–98 of bovine MBP,

bearing the specific phosphorylation site for MAPK. Nearly identical elution times were found with either the whole protein MBP or just the peptide, suggesting that both substrates are phosphorylated by the same enzyme. The profiles are slightly different, in the sense that it is possible to detect basal activity against MBP, but not against APRTGGRR, in resting cells. This is also observed in the kinase assays using whole-cell lysates. MBP could be a phosphoacceptor from kinases other than MAPK, although at a decreased specificity. It is worth pointing out that ^{32}P incorporation in MAPK substrates is much greater in the kinase assays performed with DEAE-column eluates than those performed in whole-cell lysates or even in the starting material that is loaded on to the DEAE column. An activity increase can be due either to protein concentration by the chromatographic column or to the elimination of an inhibitor present in the crude lysates.

The data presented here make several independent, though related, points. First, lysates prepared from human neutrophils contain MAPK activity. This activity is very low in unstimulated cells. Second, treatment of human neutrophils with GM-CSF greatly enhances MAPK activity both in total cell lysate and in a partially purified system. Third, this stimulation of the activity is most likely due to the generally observed phosphorylation of MAPK on tyrosine residues by GM-CSF. This conclusion is based on two lines of evidence. (i) The stimulation by GM-CSF of both the MAPK activity and the tyrosine phosphorylation of MAPK have similar time courses and dose-response curves. (ii) The enzymic activity of MAPK and the levels of tyrosine phosphorylation of MAPK are inhibited by genistein, a tyrosine kinase inhibitor, and enhanced by okadaic acid, a phosphatase inhibitor.

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