Effects of granulocyte–macrophage colony-stimulating factor and tumour necrosis factor-α on tyrosine phosphorylation and activation of mitogen-activated protein kinases in human neutrophils

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The present study was undertaken to determine the identities and characteristics of proteins with molecular masses between 40 and 44 kDa whose tyrosine phosphorylation increases in human neutrophils following stimulation of these cells with tumour necrosis factor α (TNF-α) and granulocyte–macrophage colony-stimulating factor (GM-CSF). Immunoblotting results demonstrate that addition of GM-CSF to human neutrophils increases the tyrosine phosphorylation of two proteins with molecular masses of 42 and 44 kDa. However, the addition of TNF-α to neutrophils induces a time- and dose-dependent increase in tyrosine phosphorylation of a 40 kDa protein. Immunoprecipitation using specific mitogen-activated protein kinase (MAPK) isoform antibodies and an antibody which recognizes phosphotyrosine-containing proteins demonstrated that the 42 and 44 kDa proteins are isoforms of MAPKs. Utilizing an *in situ* gel kinase activity assay, GM-CSF increases the kinase activity of the 42 and 44 kDa proteins. Moreover, using immunoprecipitated p42 and p44 MAPK isoforms in this gel assay revealed activity associated with the p42 and p44 MAPK isoforms. Using the same *in situ* assay, TNF-α induces an increase in kinase activity of a 40–42 kDa protein. However, the 40 kDa protein whose phosphorylation on tyrosine residues increased in human neutrophils following stimulation with TNF-α is not a member of the known MAPK family, demonstrating the divergences in pathways utilized by GM-CSF and TNF-α. This 40 kDa protein may be related to the recently identified protein that becomes phosphorylated on tyrosine residues upon stimulation of the human epidermal carcinoma cell line KB by interleukin-1. In these cells the p40 protein is part of a protein kinase cascade which results in the phosphorylation of the small heat shock protein, hsp27.

INTRODUCTION

Granulocyte–macrophage colony-stimulating factor (GM-CSF) and tumour necrosis factor α (TNF-α) are cytokines which can effect the growth and maturation of various cells, including myeloid cells, which differentiate into neutrophils [1]. In addition, certain functions of mature neutrophils such as adherence, superoxide (O_2^-) release and phagocytosis are recognized to be rapidly activated or potentiated by GM-CSF and TNF-α [1]. The mechanisms, however, by which these cytokines activate or prime mature neutrophils remain to be fully defined [2,3]. The intracellular transmission of cytokine signals is believed to be mediated by sequentially activated protein kinases integrated into a complex network. Phosphorylation and dephosphorylation of proteins is an important regulatory mechanism utilized by growth factors to mediate their actions. Although most protein phosphorylation occurs on serine and threonine residues, tyrosine phosphorylation has also been shown to play a crucial role in the control of cellular function. Protein kinase cascades emanating from cytokine receptors have recently been shown to involve activation of mitogen-activated protein kinase (MAPK) kinase which then activates MAPK [4].

MAPKs, or extracellular signal-regulated protein kinases (ERKs) as they are occasionally called, comprise a family of enzymes that are important intermediates in signal-transduction pathways initiated by many types of cell-surface receptors. These enzymes, which are serine/threonine kinases, become activated by phosphorylation on both a tyrosine and a threonine residue [5]. MAPKs that have been purified and analysed in depth include the $p42^{mapk}$, $p44^{erk1}$ and p54 isoforms. Recently, two other MAPKs have been identified using anti-MAPK peptide antibodies as probes: $p46^{erk4}$ and $p40^{hera}$. These proteins appear to be highly conserved during eukaryotic evolution and are particularly known for their possible roles in cell-cycle progression [6]. Their role, however, in terminally differentiated cells, such as the neutrophil, remains to be elucidated. The consensus recognition sequence for phosphorylation by the MAPKs, PX(Ser or Thr)P [7], potentially exists in many proteins and many of these are nuclear proteins [7,8]. Substrates that have been shown to be phosphorylated by MAPK include $p90^{rsk}$, c-Raf-1, c-Myc, c-Jun, c-Fos, the epidermal growth factor receptor and cytosolic phospholipase A_2 [9].

Recent studies have investigated the involvement of tyrosine kinases and MAPKs in the signal-transduction pathways of GM-CSF in various cell lines and in neutrophils [1,10–15]. GM-CSF has been shown to stimulate the rapid tyrosine phosphorylation of at least six unique and distinct proteins in neutrophils [10,14,15]. Recently, GM-CSF has been demonstrated to induce tyrosine phosphorylation and activation of MAPK or p42 MAPK in neutrophils [16–18]. The tyrosine

Abbreviations used: MAPK, mitogen-activated protein kinase; GM-CSF, granulocyte–macrophage colony-stimulating factor; TNF-α, tumour necrosis factor-α; ECL, enhanced chemiluminescence; MBP, myelin basic protein; PVDF, poly(vinylidene difluoride); PMSF, phenylmethanesulphonyl fluoride; HBSS, Hanks' balanced salt solution; α-PY, anti-phosphotyrosine antibody; α-p42 MAPK, anti-p42 MAPK antibody; α-p44 MAPK, anti-p44 MAPK antibody; α-cdc2, anti-cdc2 antibody.

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phosphorylation of other MAPKs in GM-CSF stimulation of human neutrophils remains largely unexplored.

TNF-α stimulation of many different types of cells leads to the activation of multiple signal-transduction pathways and to the induction of various cellular genes [19]. Protein kinases probably involved in TNF-α signal-transduction pathways include both protein kinase C and protein kinase A [20,21]. However, while TNF-α has been shown to induce activation and/or increased tyrosine phosphorylation of MAPKs in various human and murine cell lines [22–25], the involvement of tyrosine phosphorylation and activation of MAPKs in TNF-α stimulation of neutrophils remains to be defined. Although some investigators have demonstrated increased tyrosine phosphorylation of a 42-kDa protein following stimulation of human neutrophils with TNF-α [1], other investigators maintain that TNF-α-induced tyrosine phosphorylation is dependent upon adhesion of neutrophils to extracellular proteins [26].

The present studies were undertaken to address the following questions. First, does the addition of TNF-α to human neutrophils increase the tyrosine phosphorylation and/or activity of any of the MAPK isoforms? Secondly, does the addition of GM-CSF to human neutrophils increase the tyrosine phosphorylation and/or activities of other isoforms of MAPKs in addition to the p42 isoform? In addition, if these cytokines do increase the tyrosine phosphorylation and activities of MAPK isoforms, do the responses of the various isoforms differ from each other with respect to time course and dose response?

MATERIALS AND METHODS

Materials

GM-CSF and TNF-α were purchased from R&D Systems, Minneapolis, MN, U.S.A. The following antibodies were all obtained from Upstate Biotechnology Incorporated (UBI), Lake Placid, NY: anti-phosphotyrosine (α-PY), a monoclonal antibody derived from hybridoma 4G10; anti-MAPK R3 (α-p44 MAPK), a polyclonal antibody specific for the 44 kDa MAPK; anti-MAPK erk 2 (α-p42 MAPK), a monoclonal antibody specific for the 42 kDa MAPK; anti-cdc2 (α-cdc2), a polyclonal antibody which recognizes the $p40^{hera}$ MAPK isoform. Poly(vinylidine difluoride) (PVDF) protein transfer membrane, Immobilon-P, was from Millipore Corp., Bedford, MA, U.S.A.; electrophoresis reagents and molecular-mass markers were from Bio-Rad Laboratories, Melville, NY, U.S.A.; enhanced chemiluminescence (ECL) Western blotting reagents and [γ-^{32}P]ATP (3000 Ci/mmol) were purchased from Amersham, Arlington Heights, IL, U.S.A. All other reagents were from Sigma, St. Louis, MO, U.S.A.

Isolation of human neutrophils

Neutrophils were isolated from normal human donors utilizing a Ficoll/Hypaque gradient according to the method of English and Andersen [27]. Cells were resuspended in modified Hanks' balanced salt solution (HBSS) containing 0.1% (w/v) BSA and 10 mM Hepes, pH 7.35.

Immunoblotting

Immunoblotting was performed as described previously [17]. Briefly, cells (1×10^7/ml) were stimulated with either buffer (HBSS), GM-CSF or TNF-α at 37 °C and the reaction was stopped by rapid centrifugation (5 s using a tabletop Eppendorf 3200 centrifuge). Pellets were resuspended in 40 μl of ice-cold HBSS containing 2 mM sodium orthovanadate, 10 μM sodium pyrophosphate, 1 mM phenylmethanesulphonyl fluoride (PMSF), 1 mM EGTA, 10 mM NaF, 10 μg/ml leupeptin and 10 μg/ml aprotinin; mixed 1:1 with Laemmli sample buffer (8% SDS, 10% 2-mercaptoethanol, 10% glycerol, 66 mM Tris/HCl, pH 6.8, 0.05% Bromophenol Blue), heated at 100 °C for 10 min and loaded on to either 8% or 12% SDS/polyacrylamide gel. After electrophoresis, proteins were electrophoretically transferred from the gel to PVDF membranes in transfer buffer (20 mM Tris base, 150 mM glycine, 20% methanol, pH 8.9) at 4 mA/cm² for 3 h at 4 °C. Residual binding sites on the membrane were blocked by incubating the membrane in Tris-buffered saline/Tween (20 mM Tris base, 137 mM NaCl, pH 7.6, 0.1% Tween 20) containing 5% (w/v) BSA for either 1 h at room temperature or overnight at 4 °C. Blots were washed in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and then incubated with the appropriate antibody. Incubation time was 2 h at room temperature for the α-PY antibody and the α-p44 MAPK antibody and 1 h at room temperature for the α-p42 MAPK antibody. Blots were again washed with TBS-T and then incubated with the appropriate secondary antibody [either horseradish peroxidase-conjugated anti-(mouse IgG) or anti-(rabbit IgG)] for 20 min at room temperature. The ECL method was utilized for detection. Where appropriate, blots were stripped of the primary antibody–secondary antibody complex by incubation in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris/HCl, pH 6.7) for 30 min at 50 °C and reprobed with a different antibody according to the procedure above.

Immunoprecipitation

Immunoprecipitation was carried out according to the method of Campos-Gonzalez and Glenney [28] with slight modifications. Briefly, neutrophils (5×10^7/ml) were treated with buffer (HBSS), GM-CSF, or TNF-α. Reactions were terminated by rapid centrifugation; 100 μl of boiling 1% SDS was added to the pellets and samples were immediately boiled for 15 min. Aliquots (400 μl) of ice-cold water and (500 μl) of double-strength lysis buffer (20 mM Tris/HCl, 300 mM NaCl, 2.0% Triton X-100, 1.0% Nonidet P-40, 2 mM EDTA, 2mM EGTA, 2 mM PMSF, 4 mM sodium orthovanadate, 20 μg/ml leupeptin, 20 μg/ml aprotinin) were then added to the samples. After centrifugation at 15000 *g* for 10 min, supernatants were transferred to tubes containing anti-(mouse IgG) or anti-(rabbit IgG) agarose beads to which the appropriate antibody had been conjugated. Briefly, conjugation consisted of the following procedure: anti-(mouse IgG) or anti-(rabbit IgG) agarose beads were rinsed with 1 × lysis buffer and then incubated with 500 μl of 1 × lysis buffer containing 10 mg/ml BSA and either α-PY, α-p42 MAPK, or α-p44 MAPK. Following overnight incubation at 4 °C, beads were washed with 1 × lysis buffer and resuspended in 100 μl of 1 × lysis buffer. Lysate–bead–antibody complexes were incubated for 2 h at 4 °C for α-p42 MAPK antibody or 4 h at 4 °C for α-PY antibody or α-p44 MAPK antibody. After incubation, complexes were centrifuged at 15000 *g* for 5 min and the supernantants were removed. The immune complexes were then washed with buffer (25 mM Tris/HCl, 150 mM NaCl, 2 mM EDTA, pH 7.4), dissociated by addition of 50 μl of Laemmli sample buffer, boiled for 5–10 min, and loaded on to an 8% SDS/polyacrylamide gel. Proteins were transferred and analysed by immunoblotting as described above.

Kinase assay

Kinase activity was determined as previously described [29] with minor modifications. Briefly, neutrophils were treated with buffer (HBSS), GM-CSF, or TNF-α; reactions were terminated by

rapid centrifugation. Cell pellets were lysed in lysis buffer (50 mM Hepes, 150 mM NaCl, 10% glycerol, 10 mM EDTA, 1 mM $MgCl_2$, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 mM PMSF, 10 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1% Nonidet P-40) and equivalent amounts of lysate were electrophoresed utilizing 8% SDS/PAGE containing 0.5 mg/ml myelin basic protein (MBP). After electrophoresis, the gel was washed for 1 h at room temperature with three changes of Buffer 1 (50 mM Tris, pH 8.0, 20% isopropanol). The gel was then treated for 1 h with Buffer 2 (50 mM Tris, pH 8.0, 6 M guanidine hydrochloride, 5 mM 2-mercaptoethanol). Following this step, the gel was incubated in Buffer 3 (50 mM Tris, pH 8.0, 0.04% Tween 40, 5 mM 2-mercaptoethanol) for 16 h at 4 °C changing the buffer several times. The gel was then incubated with kinase buffer (40 mM Hepes, 2 mM DTT, 0.1 mM EGTA, 5 mM magnesium acetate, pH 8.0) for 30 min at room temperature and subsequently incubated in kinase buffer containing 20 μM ATP and 50 μCi [γ-^{32}P]ATP for 1 h at room temperature. The gel was washed for approximately 2–3 h with 5% trichloroacetic acid containing 1% sodium pyrophosphate, dried, and then autoradiographed at −70 °C using Kodak X-ray film and an intensifying screen.

RESULTS

Tyrosine phosphorylation of proteins with molecular masses between 40 and 44 kDa in human neutrophils stimulated with GM-CSF and TNF-α

Human neutrophils were stimulated with GM-CSF and TNF-α at 37 °C and tyrosine phosphorylated proteins were evaluated by immunoblotting with UBI α-PY. As shown in Figure 1(a), GM-CSF induced a time-dependent increase in tyrosine phosphorylation of two proteins with molecular masses of approximately 42 kDa and 44 kDa. Phosphorylation was evident as early as 2 min after stimulation with GM-CSF. Phosphorylation of both bands peaked at approx. 5 min and subsequently decreased to a sustained level which lasted for approx. 45 min. GM-CSF also induced a dose-dependent increase in tyrosine phosphorylation of two proteins with molecular masses of approx. 42 kDa and 44 kDa (results not shown). TNF-α also induced a time- and dose-dependent increase in tyrosine phosphorylation (Figures 1b and 1c), but of only one significant and consistant band of approx. 40 kDa. Phosphorylation was detected at concentrations as low as 2 ng/ml and within 2 min of stimulation with TNF-α. Phosphorylation peaked at approx. 5 min and decreased rapidly by 30 min. A comparison of the 40-kDa band tyrosine phosphorylated by TNF-α with the 42 and 44 kDa bands tyrosine phosphorylated by GM-CSF suggests that the band phosphorylated by TNF-α does not co-migrate with either the 42 or 44 kDa bands phosphorylated by GM-CSF. This is demonstrated in Figure 1(d). Utilizing 12% SDS/PAGE, three tyrosine phosphorylated bands of approximate molecular mass 40–44 kDa are induced by GM-CSF stimulation of neutrophils (lane 1), but only one tyrosine phosphorylated band in this molecular-mass range is induced by TNF-α (lane 3; arrowheads indicate bands). Stripping and reprobing of this immunoblot with the specific MAPK isoform antibodies demonstrated that the upper two tyrosine phosphorylated bands induced by GM-CSF co-migrate with the upper bands of the p42 and p44 MAPKs respectively, while the TNF-α-induced tyrosine phosphorylated band did not co-migrate with either of these MAPK isoforms. These results also demonstrate that GM-CSF induces the tyrosine phosphorylation of a 40-kDa band in human neutrophils.

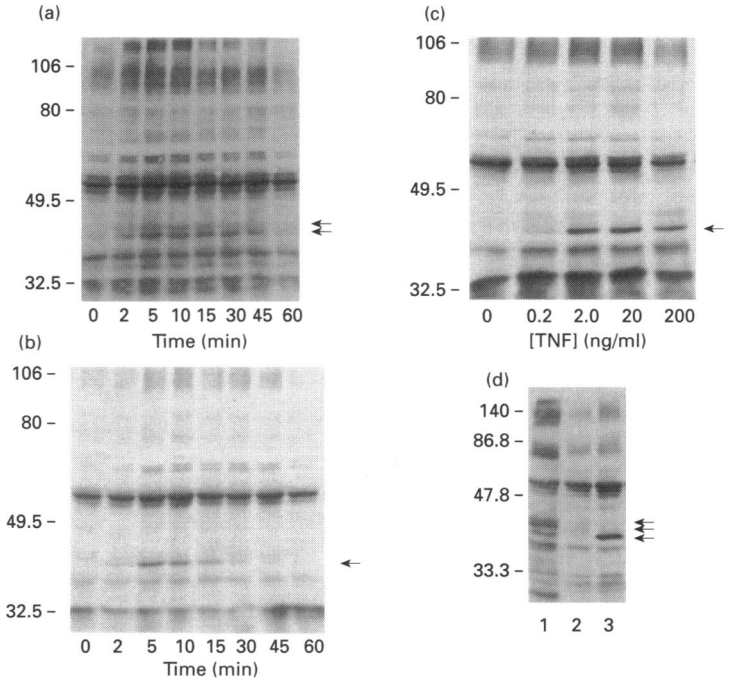

Figure 1 GM-CSF- and TNF-α-induced tyrosine phosphorylation in human neutrophils

(**a**) Neutrophils were stimulated with 500 pM GM-CSF for the indicated times. (**b**) Neutrophils were stimulated with 200 ng/ml TNF-α for the indicated times. (**c**) Neutrophils were stimulated with the indicated concentrations of TNF-α for 5 min. (**d**) Neutrophils were treated with 500 pM GM-CSF (lane 1), HBSS buffer (lane 2), or 20 ng of TNF-α (lane 3) for 5 min. In Figures (**a**, **b** and **c**) 8% SDS/PAGE was utilized, whereas in (**d**) 12% SDS/PAGE was used. Following SDS/PAGE, proteins were transferred to a Millipore membrane and immunoblotted with UBI α-PY (a monoclonal antibody directed against phosphotyrosine proteins). Detection was determined by the Amersham ECL system. Molecular-mass standards are given in kDa. Arrowheads indicate the positions of the respective 40, 42, and 44 kDa bands. Results are representative of experiments.

Characterization of the tyrosine phosphorylated proteins

In order to characterize these proteins further, immunoblots were stripped and reprobed with antibodies specific for the p42 MAPK and the p44 MAPK. As shown in Figures 2(a) and 2(b), GM-CSF induced time-dependent formation of p42 MAPK doublet and p44 MAPK doublet, respectively. Doublet formation of both the p42 and p44 MAPK isoforms was also dose-dependent (results not shown). The upper bands of both the p42 and p44 MAPKs were most prominent at 5 min following stimulation with GM-CSF and were sustained for at least 45 min. This pattern correlates with that observed for tyrosine phosphorylation and the upper bands of both the p42 and p44 proteins appear to co-migrate with the tyrosine phosphorylated bands. The appearance of the upper band is characteristic for MAPK activation and is thought to be associated with its enhanced phosphorylation [30].

MAPK doublet formation was also investigated in TNF-α-stimulated neutrophils by stripping immunoblots and reprobing with the antibodies specific for the p42 and p44 MAPKs. As shown in Figure 2(c), TNF-α seems to induce a time-dependent formation of the p42 MAPK doublet. The upper band of the p42 MAPK is evident by 5 min, peaks at 10 min and returns to a decreased level which is maintained for approx. 45 min following

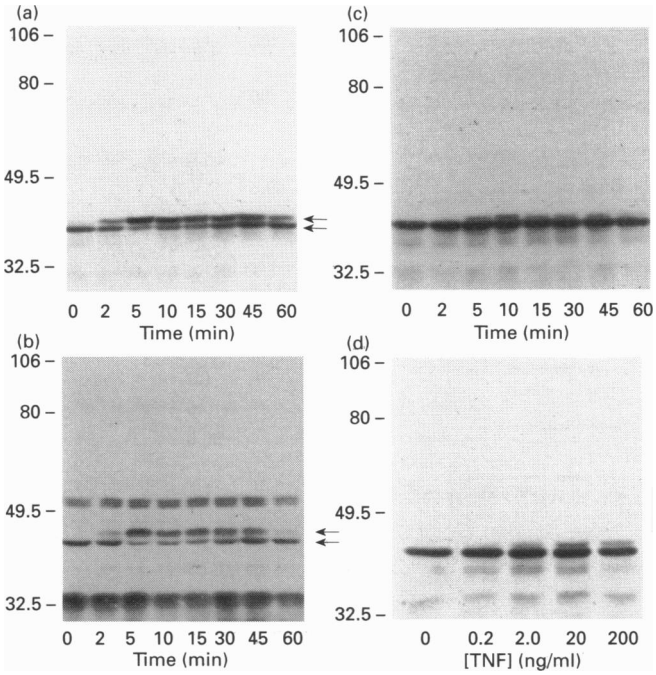

Figure 2 GM-CSF- and TNF-α-induced MAPK doublet formation in human neutrophils

(**a, b**) Neutrophils were stimulated with 500 pM GM-CSF for the indicated times. Proteins were transferred as described and the membrane was probed first with UBI α-p42 MAPK, a monoclonal antibody specific for the 42 kDa MAPK isoform (**a**). The blot was then stripped and reprobed with UBI α-p44 MAPK, a polyclonal antibody specific for the 44 kDa MAPK (**b**). (**c**) Neutrophils were stimulated with 200 ng/ml TNF-α for the indicated times. Following transfer of proteins, the blot was probed with the α-p42 MAPK antibody. (**d**) Neutrophils were stimulated with the indicated concentrations of TNF-α for 5 min. Proteins were transferred and the blot was probed with the α-p42 MAPK antibody. Molecular-mass standards are given in kDa. Results are representative of experiments.

TNF-α treatment. As shown in Figure 2(d), p42 MAPK doublet formation appears to be dose-dependent with a concentration of 2.0 ng/ml TNF-α sufficient to induce doublet formation. TNF-α does not induce doublet formation of p44 MAPK (results not shown), suggesting that TNF-α does not induce phosphorylation or activation of this MAPK isoform. However, a comparison of the 40 kDa band tyrosine phosphorylated by TNF-α with the p42 MAPK doublet induced by TNF-α reveals that the tyrosine phosphorylated band does not co-migrate with the upper p42 band as with GM-CSF-stimulated neutrophils, suggesting that the tyrosine phosphorylated band induced by TNF-α may not be the p42 MAPK.

Identity of the tyrosine phosphorylated proteins induced by GM-CSF and TNF-α stimulation of human neutrophils

To determine the identity of the tyrosine phosphorylated proteins induced by GM-CSF and TNF-α, immunoprecipitation was performed. The antibody used to immunoprecipitate the p42 MAPK isoform was UBI anti-MAPK erk 2 (α-p42 MAPK), a monoclonal antibody specific for the 42 kDa MAPK. Neutrophils were treated with buffer (HBSS), 500 pM GM-CSF, or 200 ng/ml TNF-α for 5 min. Reactions were terminated by rapid centrifugation and p42 MAPK was immunoprecipitated utilizing a denaturing protocol as described in the Materials and methods section. Following SDS/PAGE, proteins were transferred to PVDF membranes and blots were probed with either α-PY or α-p42 MAPK antibodies. As shown in Figure 3(a), the p42 MAPK protein is tyrosine phosphorylated only in neutrophils stimulated by GM-CSF (lane 2) and not in neutrophils stimulated by TNF-α (lane 3). To demonstrate that the p42 MAPK was actually immunoprecipitated, the blot was stripped and reprobed with α-p42 MAPK antibody (Figure 3b). Note that in Figure 3(b) the p42 MAPK doublet is induced by GM-CSF, but not by TNF-α, stimulation. While this correlates with previous whole-cell immunoblot studies of GM-CSF-stimulated neutrophils, it differs somewhat from whole-cell immunoblot studies of TNF-α-stimulated neutrophils. Absence of the p42 MAPK doublet in immunoprecipitates of TNF-α-induced neutrophils was puzzling. Repetitive experiments, however, yielded the same consistent results. While the exact explanation is not clear at present, one possible explanation may be antibody cross-reactivity. If the 40 kDa band is a protein closely related to the p42 MAPK, α-p42 MAPK antibody may recognize the 40 kDa band in addition to the p42 MAPK in whole-cell immunoblots. However, while α-p42 MAPK antibody may recognize the 40 kDa band sufficiently in whole-cell immunoblots, it may not possess the affinity necessary to immunoprecipitate this protein. Immunoprecipitation, therefore, will result in isolating only the p42 MAPK, which would show up as a single band.

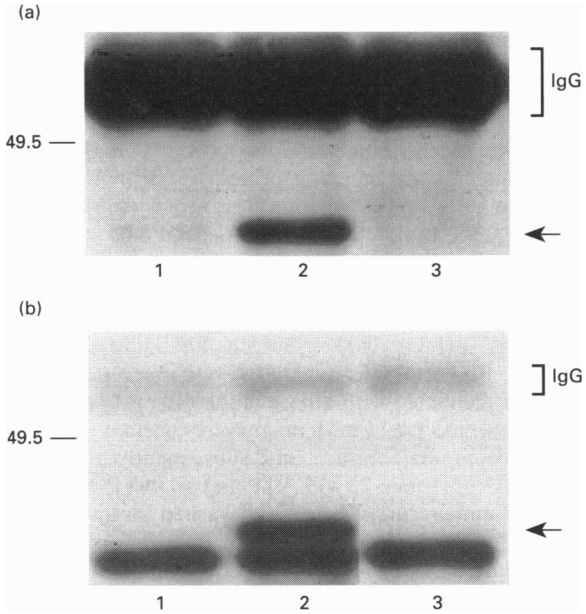

Figure 3 Immunoprecipitation of the p42 MAPK

(**a**) Neutrophils were treated with HBSS buffer (lane 1), 500 pM GM-CSF (lane 2), or 200 ng/ml TNF-α (lane 3) for 5 min. Reactions were terminated by rapid centrifugation and p42 MAPK was isolated with α-p42 MAPK antibody utilizing a denaturing immunoprecipitation protocol as described in the Materials and methods section. Following SDS/PAGE, proteins were transferred as described and immunoblotted with the α-PY antibody. A molecular-mass standard is given in kDa. An arrowhead indicates that the p42 MAPK protein is tyrosine phosphorylated only in neutrophils stimulated by GM-CSF (lane 2). (**b**) The blot was stripped and reprobed with the α-p42 MAPK antibody. Results are representative of experiments.

In order to determine the identity of the 44 kDa protein whose tyrosine phosphorylation is increased in neutrophils stimulated with GM-CSF, immunoprecipitation with the α-p44 MAPK antibody was done. Following protein transfer, the immunoblot was probed with the α-PY antibody. However, blots were difficult to interpret due to the interference of heavy and light IgG chains as maintained by the antibody's manufacturer, UBI.

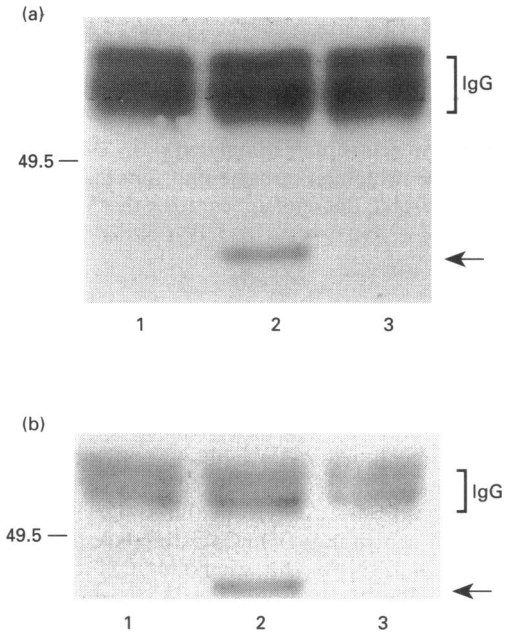

Figure 4 Immunoprecipitation of tyrosine phosphorylated proteins

(**a**) Neutrophils were treated with HBSS buffer (lane 1), 500 pM GM-CSF (lane 2), or 200 ng/ml TNF-α (lane 3) for 5 min. Reactions were terminated and tyrosine phosphorylated proteins were isolated with α-PY antibody utilizing a denaturing immunoprecipitation as described in the Materials and methods section. Following SDS/PAGE, proteins were transferred as described and immunoblotted with the α-p42 MAPK antibody. A molecular-mass standard is given in kDa. An arrowhead indicates the p42 MAPK protein is only present in immunoprecipitates from GM-CSF-stimulated neutrophils. (**b**) The blot was stripped and reprobed with the α-p44 MAPK antibody. An arrowhead indicates the p44 MAPK protein is only present in immunoprecipitates from GM-CSF-stimulated neutrophils. Results are representative of experiments.

To substantiate further the identity of the tyrosine phosphorylated proteins induced by GM-CSF and TNF-α, immunoprecipitation with UBI α-PY was performed. Neutrophils were stimulated with buffer (HBSS), 500 pM GM-CSF, or 200 ng/ml TNF-α for 5 min. Reactions were terminated and tyrosine phosphorylated proteins were immunoprecipitated utilizing a denaturing protocol as described. After SDS/PAGE and transfer of proteins to PVDF membranes, the blot was probed with α-p42 MAPK antibody (Figure 4a). The blot was then stripped and reprobed with α-p44 MAPK antibody (Figure 4b). As shown in Figure 4(a), p42 MAPK is immunoprecipitated by α-PY antibody in neutrophils stimulated by GM-CSF (lane 2), but not in neutrophils stimulated by TNF-α (lane 3). Figure 4(b) demonstrates that p44 MAPK is also immunoprecipitated by α-PY antibody in neutrophils stimulated by GM-CSF (lane 2), and not in neutrophils stimulated by TNF-α (lane 3). These results confirm that the p42 MAPK and p44 MAPK isoforms are both tyrosine phosphorylated in neutrophils stimulated by GM-CSF. However, the protein(s) tyrosine phosphorylated by TNF-α stimulation of neutrophils does not appear to be either the p42 or p44 MAPKs.

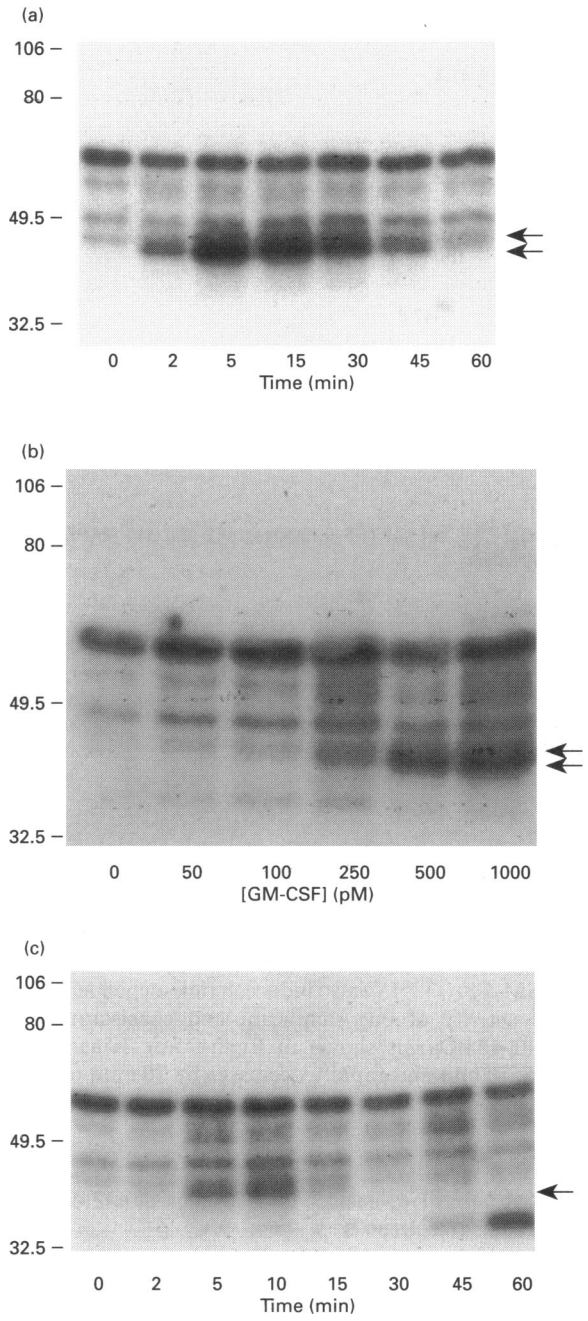

Figure 5 GM-CSF and TNF-α induce kinase activation in human neutrophils

(**a**) Neutrophils were stimulated with 500 pM GM-CSF for the indicated times and (**b**) neutrophils were stimulated with indicated concentrations of GM-CSF for 5 min. (**c**) Neutrophils were stimulated with 200 ng/ml TNF-α for the indicated times. Reactions were terminated by rapid centrifugation and equivalent amounts of protein were electrophoresed using 8% SDS/PAGE containing 0.5 mg/ml MBP. The proteins were then renatured and submitted to an in-gel kinase assay as described in the Materials and methods section. Gels were dried and autoradiographed at −70 °C. Molecular-mass standards are indicated in kDa. Arrowhead(s) indicate kinase activities. Results are representative of experiments.

GM-CSF and TNF-α induce kinase activation

In order to show that tyrosine phosphorylation increases MAPK activation, an *in situ* gel kinase activity assay was utilized. Neutrophils (1×10^7) were treated with buffer (HBSS), GM-CSF, or TNF-α; following reaction termination by rapid centrifugation, cell pellets were lysed and equivalent amounts of lysate were electrophoresed utilizing 8% SDS/PAGE containing 0.5 mg/ml MBP. After electrophoresis, gels were washed and subjected to a kinase activity assay as described in the Materials and methods section. As shown in Figures 5(a) and 5(b) respectively, GM-CSF induces a time- and dose-dependent

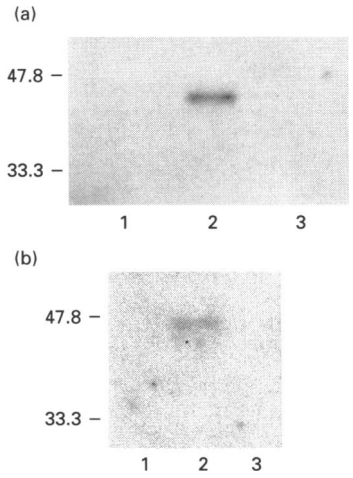

Figure 6 GM-CSF, but not TNF-α, induces p42 and p44 MAPK activity in human neutrophils

(**a** and **b**) Neutrophils were treated with HBSS buffer (lane 1), 500 pM GM-CSF (lane 2), or 200 ng/ml TNF-α (lane 3) for 5 min. The p42 MAPK (**a**) and the p44 MAPK (**b**) were isolated by immunoprecipitation as described. Immunoprecipitates were then submitted to the in-gel kinase assay according to the protocol described in the Materials and methods section. p42 MAPK and p44 MAPK activity are induced only in neutrophils stimulated with GM-CSF, not in neutrophils stimulated with TNF-α. Molecular-mass standards are indicated in kDa. Results are representative of experiments.

increase in kinase activity of two bands, of approx. 42 and 44 kDa. Kinase activity is evident by 2 min, peaks at 5 min, and is sustained for approx. 45 min (Figure 5a). This time-dependence characteristic of the kinase activity correlates with that of tyrosine phosphorylation and MAPK doublet formation in cells stimulated by GM-CSF. TNF-α also induces a time-dependent increase in kinase activity of one significant and consistant band of approx. 40–42 kDa, as shown in Figure 5(c). Kinase activity peaks at 5–10 min and rapidly decreases by 30 min, correlating with the characteristic time course of tyrosine phosphorylation induced in neutrophils stimulated by TNF-α.

GM-CSF, but not TNF-α, induces the activation of p42 and p44 MAPKs in human neutrophils

Since the substrate used in the *in situ* gel kinase activity assay, MBP, can be utilized by kinases other than MAPKs, these results suggest, but do not confirm, the activation of MAPKs in neutrophils stimulated by GM-CSF and TNF-α. Therefore, cells were treated with buffer (HBSS), GM-CSF, or TNF-α and p42 MAPK and p44 MAPK were isolated by immunoprecipitation with the respective antibodies. Immunoprecipitates were then subjected to the *in situ* gel kinase activity assay. Results (Figure 6a) demonstrate that p42 MAPK is activated only in neutrophils stimulated by GM-CSF (lane 2) and not in neutrophils stimulated by TNF-α (lane 3). Similarly, p44 MAPK is activated only in neutrophils stimulated by GM-CSF (Figure 6b, lane 2) but not in neutrophils stimulated by TNF-α (Figure 6b, lane 3).

The kinase activity of a band of approx. 40 kDa molecular mass induced by TNF-α may be due to the activation of another MAPK isoform. This would correlate with the observed tyrosine phosphorylation induced by TNF-α stimulation of neutrophils. The possibility that this isoform may be the p40^{hera} was investigated by immunoprecipitating GM-CSF- and TNF-α-stimulated neutrophils with the α-PY antibody and probing the immunoblot with α-cdc2 antibody, a polyclonal antibody which also recognizes p40^{hera}. Cdc2 kinases and MAPKs are highly related in their primary structure [31] and therefore antibodies directed against one may recognize the other. Results, not shown, demonstrated that the p40^{hera} MAPK is not tyrosine phosphorylated in neutrophils stimulated with GM-CSF or TNF-α. Therefore, the protein(s) tyrosine phosphorylated in TNF-α-stimulated neutrophils does not appear to be this MAPK isoform.

DISCUSSION

Tyrosine phosphorylation and activation of MAPKs are thought to play a key role in signalling processes initiated by various cytokines in many types of cells [32]. Although receptors for GM-CSF and TNF-α are structurally different, these cytokines seem to share some functional redundancy in their effects on the neutrophil. We therefore sought to determine which of the various MAPK isoforms are tyrosine phosphorylated and activated in human neutrophils following stimulation of these cells with GM-CSF and TNF-α. Although GM-CSF has previously been shown to induce tyrosine phosphorylation and activation of the p42 MAPK in human neutrophils [17,18], phosphorylation and activation of other MAPK isoforms has not been investigated. While in some cells, both the p42 and p44 kinases are co-activated by a variety of stimuli [33–35], it has recently been demonstrated that only the p42 MAPK is phosphorylated and activated in thrombin-stimulated platelets [36].

The data presented here show very clearly that GM-CSF increases the tyrosine phosphorylation and activity of the p42 and p44 MAPK isoforms. Recently, it has been demonstrated that certain MAPKs, such as the p44^{erk1} and the newly discovered p40^{hera}, are physically associated with certain growth factor receptors [31,37] in various cell lines. It will be interesting to see if this phenomenon also exists in a differentiated cell such as the neutrophil.

The data presented in this study show clearly that neither the p42 nor the p44 MAPK is tyrosine phosphorylated or activated in human neutrophils stimulated with TNF-α. This is unlike the situation in proliferating cells, in which it has been shown that the addition of TNF-α to fibroblasts or HL-60 cells increases the tyrosine phosphorylation of the p42 MAPK [23,24]. This difference in the patterns of tyrosine phosphorylation of the two MAPK isoforms between GM-CSF and TNF-α stimulation of human neutrophils provides evidence for distinction in tyrosine kinase and/or phosphatase regulation of the actions of these two cytokines. While TNF-α does not induce tyrosine phosphorylation or activation of p42 MAPK, it does increase the activity of a kinase of approximate molecular mass 40–42 kDa, as evidenced by the observed increase in the *in situ* phosphorylation of MBP (Figure 5c). The identity of this protein or proteins is not known. It must be kept in mind that MBP is a substrate for kinases other than MAPKs [38].

More interestingly, while TNF-α does not increase the tyrosine phosphorylation of either of the two MAPK isoforms in human neutrophils, it increases the tyrosine phosphorylation of a protein of approximate molecular mass of 40 kDa (Figures 1b and 1c). This response is time- and dose-dependent. This result demonstrates that TNF-α does induce tyrosine phosphorylation in suspended human neutrophils as is maintained by some investigators [1]. While the identity of the 40 kDa protein whose phosphorylation is increased in human neutrophils following stimulation by TNF-α is not known, it is not related to the newly discovered MAPK, p40^{hera}. This conclusion is based on the finding that the phosphorylated protein is not recognized by the

antibody against cdc2. This antibody has been shown to cross-react with $p40^{hera}$ [31].

The data presented here make three distinct yet related points. First, although GM-CSF and TNF-α both potentiate certain functions of mature neutrophils, such as adherence, superoxide generation, arachidonic acid release and phagocytosis, these results demonstrate that distinctive signalling pathways are utilized by these cytokines. Secondly, since neutrophils are non-proliferative cells, the signal-transduction pathway that involves p42 and p44 MAPKs cannot lead to a mitogenic signal and instead may regulate secretory or metabolic changes during neutrophil activation. Substrates that are phosphorylated and activated by MAPKs include cytosolic phospholipase A_2 ($cPLA_2$) and $p90^{rsk}$ [9]. Whereas $p90^{rsk}$, a ribosomal S6 kinase, is thought to participate in regulating gene expression [36], activation of $cPLA_2$ leads to the production of arachidonic acid and its metabolites, prostaglandins and leukotrienes. Since GM-CSF can directly augment the synthesis of leukotriene B4 and platelet-activating factor in neutrophils [39,40], these substances themselves may play a role in signal transduction [2]. Recently, arachidonic acid has also been shown to be involved in the signal-transduction pathways utilized by TNF-α stimulation of HL-60 cells, a promyelocytic cell line. In this system, TNF-α stimulated a rapid release of arachidonic acid which preceded TNF-α-stimulated sphingomyelin hydrolysis [41]. Therefore, although distinct signalling pathways seem to be utilized in GM-CSF and TNF-α stimulation of neutrophils, lipid molecules such as arachidonic acid may act as central mediators involved in the activation of the neutrophil. Thirdly, determining the identity and the distribution of the 40 kDa protein, whose phosphorylation on tyrosine is increased following stimulation of human neutrophils with TNF-α, and understanding the role of this protein in mediating TNF-α-induced action are important in elucidating the signalling mechanism of TNF-α. Determination of the identity, distribution and function of this protein are the subject of future studies.

While this paper has been under review, another member of the MAPK family has been identified. An MAPK, p38, was isolated from a murine cell line and demonstrated to be tyrosine phosphorylated in response to endotoxin and hyperosmolarity [42]. The likely human equivalent of this protein has recently been identified in the human epidermal carcinoma cell line KB. This protein, p40, becomes tyrosine phosphorylated and activated in response to interleukin-1 and is part of a protein kinase cascade which results in the phosphorylation of the small heat shock protein, hsp 27 [43]. Tyrosine phosphorylation of p40 was demonstrated by phosphoamino acid analysis, protein tyrosine phosphatase treatment, and immunoblotting. The 40 kDa band tyrosine phosphorylated in human neutrophils stimulated by TNF-α may be related to this recently identified protein.

This work is supported by National Institutes of Health Grants HL-53786-06 and AI-28810-03.

REFERENCES

1 Yuo, A., Kitagawa, S., Azuma, E., Natori, Y., Togawa, A., Saito, M. and Takaku, F. (1993) Biochim. Biophys. Acta **1156**, 197–203

2 Rapoport, A. P., Abboud, C. N. and DiPersio, J. F. (1992) Blood Rev. **6**, 43–57

3 Aggarwal, B. B. and Vilcek, J. (1992) Immunology Series. **56**, Dekker Inc, New York

4 Roberts, T. M. (1992) Nature (London) **360**, 534–535

5 Levin, D. E. and Errede, B. (1993) J. NIH Res. **5**, 49–52

6 Pelech, S. L. and Sanghera, J. S. (1992) Trends Biochem. Sci. **17**, 233–238

7 Clark-Lewis, I., Sanghera, J. S. and Pelech, S. L. (1991) J. Biol. Chem. **266**, 15180–15184

8 Gonzalez, F. A., Raden, D. L. and Davis, R. J. (1991) J. Biol. Chem. **266**, 22159–22163

9 Davis, R. J. (1993) J. Biol. Chem. **268**, 14553–14556

10 McColl, S. R., DiPersio, J. F., Caon, A. C., Ho, P. and Naccache, P. H. (1991) Blood **78**, 1842–1852

11 Miyazawa, K., Hendrie, P. C., Mantel, C., Wood, K., Ashman, L. K. and Broxmeyer, H. E. (1991) Exp. Hematol. **19**, 1110–1123

12 Hanazono, Y., Chiba, S., Sasaki, K., Mano, H., Miyajima, A., Arai, K., Yazaki, Y. and Hirai, H. (1993) EMBO J. **12**, 1641–1646

13 Corey, S., Eguinos, A., Puyana-Theall, K., Bolen, J. B., Cantley, L., Mollinedo, F., Jackson, T. R., Hawkins, P. T. and Stephens, L. R. (1993) EMBO J. **12**, 2681–2690

14 Gomez-Cambronero, J., Yamazaki, M., Metwally, F., Molski, T. F. P., Bonak, V., Huang, C.-K., Becker, E. L. and Sha'afi, R. I. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 3569–3573

15 Kanakura, Y., Druker, B., Cannistra, S. A., Furukawa, Y., Torimoto, Y. and Griffin, J. D. (1990) Blood **76**, 706–715

16 Raines, M. A., Golde, D. W., Daeipur, M. and Nel, A. E. (1992) Blood **79**, 3350–3354

17 Gomez-Cambronero, J., Huang, C., Gomez-Cambronero, T. M., Waterman, W. H., Becker, E. L. and Sha'afi, R. I. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 7551–7555

18 Gomez-Cambronero, J., Colasanto, J. M., Huang, C. and Sha'afi, R. I. (1993) Biochem. J. **291**, 211–217

19 Vilcek, J. and Lee, T. H. (1991) J. Biol. Chem. **266**, 7313–7316

20 Brenner, D. A., O'Hara, M., Angel, P., Chojkier, M. and Karin, M. (1989) Nature (London) **337**, 661–663

21 Zhang, Y., Lin, J.-X., Yip, Y. K. and Vilcek, J. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 6802–6805

22 Van Lint, J., Agostinis, P., Vandevoorde, V., Haegeman, G., Fiers, W., Merlevede, W. and Vandenheede, J. R. (1992) J. Biol. Chem. **267**, 25916–25921

23 Vietor, I., Schwenger, P., Wei, L., Schlessinger, J. and Vilcek, J. (1993) J. Biol. Chem. **268**, 18994–18999

24 Raines, M. A., Kolesnick, R. N. and Golde, D. W. (1993) J. Biol. Chem. **268**, 14572–14575

25 Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E. A., Ahmad, M. F., Avruch, J. and Woodgett, J. R. (1994) Nature (London) **369**, 156–160

26 Fuortes, M., Wen-Wen, J. and Nathan, C. (1993) J. Cell Biol. **120**, 777–784

27 English, D. and Andersen, B. R. (1974) J. Immunol. Methods **7**, 2267–2275

28 Campos-Gonzalez, R. and Glenney, J. R., Jr. (1991) Cell Regul. **2**, 663–673

29 Kameshita, I. and Fujisawa, H. (1989) Anal. Biochem. **183**, 139–143

30 Leevers, S. J. and Marshall, C. J. (1992) EMBO J. **11**, 569–574

31 Williams, R., Sanghera, J., Wu, F., Carbonaro-Hall, D., Campbell, D., Warburton, D., Pelech, S. and Hall, F. (1993) J. Biol. Chem. **268**, 18213–18217

32 Nishida, E. and Gotoh, Y. (1993) Trends Biol. Sci. **18**, 128–131

33 Blenis, J. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 5889–5892

34 Cobb, N. H., Boulton, T. G. and Robbins, D. J. (1991) Cell Regul. **2**, 965–978

35 Pelech, S. L. and Sanghera, J. S. (1992) Science **257**, 1355–1356

36 Papkoff, J., Chen, R., Blenis, J. and Forsman, J. (1994) Mol. Cell. Biol. **14**, 463–472

37 Loeb, D. M., Tsao, H., Cobb, M. H. and Greene, L. A. (1992) Neuron **9**, 1053–1065

38 Chao, T.-S. O., Byron, K. L., Lee, K.-M., Villereal, M. and Rosner, M. R. (1992) J. Biol. Chem. **267**, 19876–19883

39 De Nichilo, M. O., Stewart, A. G., Vadas, M. A. and Lopez, A. F. (1991) J. Biol. Chem. **266**, 4896–4902

40 DiPersio, J. F., Billing, P., Williams, R. and Gasson, J. C. (1988) J. Immunol. **140**, 4315–4322

41 Jayadev, S., Linardic, C. M. and Hannun, Y. A. (1994) J. Biol. Chem. **269**, 5757–5763

42 Han, J., Lee, J.-D., Bibbs, L. and Ulevitch, R. J. (1994) Science **265**, 808–811

43 Freshney, N. W., Rawlinson, L., Guesdon, F., Jones, E., Cowley, S., Hsuan, J. and Saklatvala, J. (1994) Cell **78**, 1039–1049

Received 1 August 1994/28 October 1994; accepted 21 November 1994