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Granulocyte–macrophage colony-stimulating factor-induced protein tyrosine phosphorylation of microtubule-associated protein kinase in human neutrophils

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ABSTRACT **Granulocyte–macrophage colony-stimulating factor (GM-CSF), formylmethionylleucylphenylalanine, tumor necrosis factor α, platelet-activating factor, phorbol ester (phorbol 12-myristate 13-acetate), and calcium ionophore A23187 are able to increase the level of tyrosine phosphorylation of different protein substrates, as demonstrated by Western blotting with anti-phosphotyrosine antibody (anti-PY). A protein of 41 kDa (p41) consistently showed more intense reactivity to anti-PY than controls. Blots treated with anti-PY, stripped of the antibody, and reblotted with microtubule-associated protein kinase (MAPK, p42^{MAPK}) antibody show only one band. The molecular mass of that band exactly matches that of p41. MAPK-reactive protein is present in control and stimulated cells, although the intensity of the band is greater in the latter. GM-CSF-stimulated phosphorylation of p41 is time- and dose-dependent. Anti-MAPK antibody detects a single band of 41 kDa, whose intensity increases with time of incubation and concentration of the agonist. Thus, the anti-MAPK antibody appears to react better to the phosphorylated form of p41 from GM-CSF-stimulated cells than to the dephosphorylated form. The p41 and MAPK proteins are localized in the cytosol. Finally, MAPK immunoprecipitates were probed with anti-PY in Western blots and a band of 41 kDa was found. In summary, these results suggest that this 41-kDa protein in neutrophils that is tyrosine phosphorylated in response to GM-CSF and other stimuli is MAPK. Its phosphorylation may represent an early and crucial signal associated with the GM-CSF neutrophil stimulation cascade.**

The presence of tyrosine-specific protein kinase activities in human neutrophils has been demonstrated (1–3). The roles of these kinases in neutrophil functions and the identities of their substrates are totally unknown. Microtubule-associated protein kinase (MAPK or p42^{MAPK}) is a serine/threonine kinase originally described in 3T3 L1 cells that is activated upon stimulation of cells with insulin, growth factors, protooncogene products, and phorbol esters (4–6). Activation of MAPK requires threonine and tyrosine residue phosphorylation (7). A MAPK has been recently described in nerve growth factor-stimulated pheochromocytoma (PC12) cells that phosphorylates MAPK in serine/threonine and tyrosine (8). On the other hand, protein serine/threonine phosphatase 2A or the protein tyrosine phosphatase CD45 completely and specifically inactivates MAPK (7, 9). Several proteins have been identified to date as substrates of MAPK. They are MAP2, ribosomal S6 kinase II, myelin basic protein, c-*jun* protooncogene product, and Raf-1 (10, 11). For its ability to phosphorylate other key kinases and proteins, MAPK might be crucial in the control of cell growth (6).

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Granulocyte–macrophage colony-stimulating factor (GM-CSF) is a hemopoietic growth factor required for the *in vivo* and *in vitro* proliferation of myelomonocytic stem cells (12, 13). GM-CSF also acts on mature cells such as the neutrophil, cells in which it primes or enhances their physiological functions. These include superoxide generation, platelet-activating factor (PAF) and arachidonic acid release, phagocytosis, and degranulation (14). The mechanism of this priming process is, however, unknown. Recently, it has been found (15–17, 35, 36) that incubation of human neutrophils with GM-CSF triggers a rapid phosphorylation of tyrosine residues of several proteins, among them one with a molecular mass of 41 kDa (p41).

The present studies were undertaken to examine the characteristics of phosphorylation on tyrosine residues of this 41-kDa protein and to determine its identity and location. On the basis of an identical relative mobility in SDS/polyacrylamide gels of the anti-phosphotyrosine antibody (anti-PY)- and the anti-MAPK-reactive protein, its kinetics and dose dependency, calcium dependence, subcellular localization, and immunoprecipitation/immunoblotting characteristic, we conclude that the neutrophil p41 protein is a member of the MAPK family.

MATERIALS AND METHODS

Isolation of Human Neutrophils. Human neutrophils were isolated on a Ficoll/Hypaque gradient according to the method of English and Andersen (18). 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 Na_2HPO_4, 0.66 mM KH_2PO_4, 15.2 mM $NaHCO_3$, 0.1% dextrose, and 0.1% bovine serum albumin.

Cell Fractionation. Neutrophils were suspended at 10^7 cells per ml in buffered sucrose solution (10 mM Hepes, pH 7.5/0.25 M sucrose, 1 mM EGTA/1 mM diisopropyl fluorophosphate/1 mM phenylmethylsulfonyl fluoride/50 μg of leupeptin per ml). Cells were disrupted by nitrogen cavitation at 250 psi (1 psi = 6.89 kPa) for 15 min at 4°C as indicated (19). The homogenate was centrifuged at 450 × *g* and the pellet was discarded. This supernatant was centrifuged at 8000 × *g* for 30 min and the resulting supernatant was then centrifuged at 104,000 × *g* for 1 hr at 4°C. The pellet (membrane fraction) and the supernatant (cytosol fraction) were saved.

Abbreviations: MAPK, microtubule-associated protein kinase, mitogen-activated kinase, or extracellular signal-regulated kinase (ERK-1); MK12, monoclonal antibody to MAPK; PY, phosphotyrosine; anti-PY, polyclonal antibody to phosphotyrosine; GM-CSF, granulocyte–macrophage colony-stimulating factor; fMet-Leu-Phe, formylmethionylleucylphenylalanine; ECL, enhanced chemiluminescence; PAF, platelet-activating factor; PVDF, poly(vinylidene difluoride); PMA, phorbol 12-myristate 13-acetate.

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Western Blotting. Western blotting was performed as described (15–17). Briefly, cells were stimulated with the agent (GM-CSF, unless otherwise indicated) for a specific time and the reaction was stopped by quick centrifugation (15 s; 7000 × *g* using a tabletop Eppendorf 3200 centrifuge). The pellets were then resuspended in 100 μl of buffer and mixed with 50 μl of Laemmli stopping solution [9% (wt/vol) SDS, 6% (vol/vol) 2-mercaptoethanol, 10% (vol/vol) 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%). Prestained high molecular mass standards (Bethesda Research Laboratories) were run with each gel along with the samples. The proteins were then transferred to poly(vinylidene difluoride) (PVDF) sheets (Millipore) and the blots were treated with the appropriate antibodies. Western blots were developed by incubation with ^{125}I-labeled staphylococcal protein A when the primary antibody was polyclonal anti-PY (14–16). In the case of monoclonal MK12 (MAPK antibody) we chose the enhanced chemiluminescence (ECL) method for detection. In ECL, blots were blocked in Tris-buffered saline/Tween 20 (TBST) (20 mM Tris base, pH 7.6/137 mM NaCl/0.1% Tween 20) supplemented with 5% bovine serum albumin; this was followed by incubation with the primary antibody in TBST and finally with horseradish peroxidase-conjugated anti-mouse IgG at 1:7000 dilution for 1 hr at room temperature. Membranes were exposed for 1 min with Kodak X-Omat film.

Blot Stripping and Reprobing. When a Western blotting procedure was completed and x-ray films were obtained, blots were stripped of the primary antibody–secondary antibody (or protein A) complex by incubating them in the stripping buffer (100 mM 2-mercaptoethanol/2% SDS/62.5 mM Tris·HCl, pH 6.7) for 30 min at 50°C. Blots were subjected to autoradiography for confirmation that the antibody signal had been removed. After this procedure, the blots were blocked with buffer containing 5% bovine serum albumin and reprobed with a different antibody according to the procedure indicated above.

Immunoprecipitation. Immunoprecipitation was carried out based on the method of Campos-Gonzalez and Glenney (20) with slight modifications. Briefly, neutrophils (2×10^7 cells per ml) were lysed in lysis buffer (10 mM Tris·HCl, pH 7.4/150 mM NaCl/1.0% Triton X-100/0.5% Nonidet P-40/1 mM EDTA/1 mM EGTA/0.5 mM Na_3VO_4/1 mM diisopropyl fluorophosphate/0.3 mM phenylmethylsulfonyl fluoride/50 μg of leupeptin per ml). The MK12 or anti-PY (in separate experiments) was used at a concentration of 3 μg/ml. PY20 monoclonal anti-PY (ICN) was used for immunoprecipitation purposes, since it yielded better results than the rabbit anti-PY. After a 2-hr incubation period at 4°C with the corresponding antibody, the immunocomplex was recovered by incubation with 50 μl of 10% protein A (Zysorbin, Zymed Laboratories). Pellets were washed three times with lysis buffer, boiled, and loaded onto 5–15% SDS/polyacrylamide gels. After electrotransference of proteins to PVDF sheets, Western blotting was performed as indicated above and detected with the ECL system (Amersham). In control experiments, the immunoprecipitation efficiency of MK12 and PY20 was monitored by Western blotting of the immunoprecipitates probed with the same kind of antibody used in the immunoprecipitation step.

Antibodies. Polyclonal anti-PY was prepared in New Zealand White rabbits using phosphotyrosine coupled to gamma globulin. Antiserum was affinity purified and used at the dilution of 1:100 (vol/vol). Specificity has been published elsewhere (21). Saturating amounts of phosphoserine or phosphothreonine did not inhibit the antibody signal. PY20 monoclonal anti-PY was purchased from ICN. Monoclonal MK12 to MAPK (anti-MAPK) was purchased from Zymed Laboratories and used at a dilution of 1:4000. MK12 is an anti-peptide antibody raised to the sequence TDEPVAEEPFTFDMELDDLPK corresponding to amino acids 325–345 near the C end of ERK-1 (22).

Materials. GM-CSF was from Amgen Biologicals; formylmethionylleucylphenylalanine (fMet-Leu-Phe), phorbol 12-myristate 13-acetate (PMA), Hepes, leupeptin, and phenylmethylsulfonyl fluoride were obtained from Sigma; diisopropyl fluorophosphate was purchased from Aldrich; Immobilon PVDF membranes were obtained from Millipore; electrophoresis chemicals were supplied by Bio-Rad; ^{125}I-labeled protein A (1 μCi/ml; 1 Ci = 37 GBq) was purchased from New England Nuclear; ECL Western blotting detection kit was purchased from Amersham; Zysorbin was purchased from Zymed Laboratories.

RESULTS

Characterization of p41 Tyrosine Phosphorylation. To characterize the phosphorylation of p41, we first investigated the effects of various neutrophil agonists on the levels of tyrosine phosphorylation of this protein using Western blotting with anti-PY. As seen in Fig. 1*A*, GM-CSF, fMet-Leu-Phe, and PMA increased the levels of tyrosine phosphorylation of several human neutrophil proteins, including that with the molecular mass of 41 kDa (p41). Other stimuli that were able to induce phosphorylation of the same protein were tumor necrosis factor α (10–1000 pM), PAF (1–100 nM), and calcium ionophore A23187 (0.1–1 μM, only when added in the presence of 1 mM $CaCl_2$). Phosphorylation of p41 was partially prevented by preincubation with several protein kinase inhibitors. Inhibitory concentration (IC_{50}) values were 10 μg/ml for the cyanamide derivative ST 638 (15) and 12 μg/ml for the epidermal growth factor receptor kinase tyrphostin inhibitors [compounds 8 and 11 (23)]. Staurosporine at 100 nM completely blocked phosphorylation of p41. This concentration of staurosporine inhibits tyrosine kinase(s) in addition to protein kinase C (24).

To determine that p41 is MAPK, we used several biochemical and immunological approaches—specifically, molecular mass determination, time course and dose–response curves,

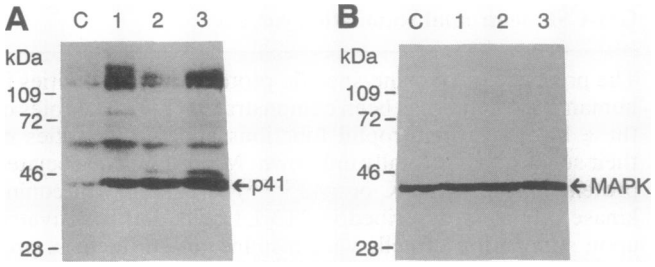

FIG. 1. Phosphorylation on tyrosine of a 41-kDa protein in response to stimulation of neutrophils by GM-CSF, PMA, and fMet-Leu-Phe and detection of MAPK. Neutrophils (10×10^6 cells per ml) were incubated with the indicated agonists at the following concentrations and length of time: 200 pM GM-CSF (lanes 1), 5 min; 20 ng of PMA per ml (lanes 2), 3 min; and 100 nM fMet-Leu-Phe (lanes 3), 3 min. The reactions were stopped by centrifugation, the cellular pellets were resuspended in SDS-stopping buffer and electrophoresed, and proteins were transferred onto PVDF Immobilon (Millipore). C, control. (*A*) Blots were probed with affinity-purified polyclonal anti-PY, decorated with ^{125}I-labeled protein A, and autoradiographed. (*B*) The same blot was stripped to remove the antibody and radioactive protein A; it was then reprobed with monoclonal anti-MAPK MK12. The blot was developed by ECL. Molecular mass markers are indicated in kDa. The protein of 41 kDa exhibiting tyrosine phosphorylation (*A*) or immunoreactive MAPK (*B*) is indicated. Equal amounts of protein (assayed by Bradford method) were loaded onto each lane of the gel.

subcellular localization, and immunoprecipitation/immunoblotting experiments.

MAPK Detection and Molecular Mass Comparison. Blots were initially treated with anti-PY followed by ^{125}I-labeled protein A (see *Materials and Methods*). A film was obtained in which p41 was clearly located (Fig. 1*A*). Then the blot was stripped to remove the noncovalent binding of the complex Ag–Ab until no bands were seen in exposed x-ray films. The blot was then reprobed with MK12. Proteins reactive to the antibody were visualized by the ECL method (see *Materials and Methods*). Fig. 1*B* shows that a band lines up with that in the blot originally treated with anti-PY. The same result was found if the blots were first treated with MK12, developed by ECL, stripped off of antibody, reincubated with anti-PY, decorated with ^{125}I-labeled protein A, and developed by autoradiography (data not shown). This reported identity in apparent size was not evident when blots were probed with other antibodies able to recognize proteins in the 37- to 44-kDa region of SDS/polyacrylamide gels, such as lipocortin I, lipocortin II, and $G_{i\alpha_2}$ (data not shown).

Time Course and Dose–Response. The time of maximum phosphorylation depended on the agonist used. Thus, fMet-Leu-Phe- and PAF-induced phosphorylation on tyrosine of the p41 detected by immunoblotting was rapid, peaking at 1–2 min. Conversely, GM-CSF needed longer incubation times to maximize phosphorylation of the 41-kDa protein. In the following studies we concentrated on the use of the human cytokine GM-CSF as a neutrophil stimulant. Fig. 2*A* shows the time course of tyrosine phosphorylation with GM-CSF. Maximum phosphorylation levels can be seen at 5–10 min. Anti-PY-treated blots stripped and reprobed with MK12 produced a single band when developed by ECL. When autoradiographs from both blots (anti-PY-treated and anti-MAPK-treated) were superimposed, the proteins aligned exactly. The intensity of MAPK-reactive protein increased with the time of incubation (Fig. 2*B*) almost paralleling that of tyrosine phosphorylation (Fig. 2*A*).

Fig. 3*A* shows that the phosphorylation of p41 is dependent on the dose of GM-CSF. Phosphorylation is seen at concentrations of >50 pM, at values close to the K_d for its receptor (25). The range of concentrations used correlates with that needed to exert biological effects, such as the expression of plasma membrane proteins CD11b (26) and CR1 and CR3 (27) and priming of the respiratory burst (26), arachidonic acid (28), and PAF release (29, 30). Saturation of the response is achieved at concentrations above 1 nM. As seen in Fig. 3, the intensity of MAPK-reactive protein produced in anti-PY-treated blots stripped and reprobed with MK12 increased with the dose of GM-CSF (Fig. 3*B*), almost paralleling that of p41 (Fig. 3*A*).

Subcellular Localization. We next investigated the subcellular localization of p41. Cells were disrupted by nitrogen cavitation and sequential centrifugation as described in *Materials and Methods* and two fractions were prepared: post-104,000 × *g* pellet (membranes) and post-104,000 × *g* supernatant (cytosol). Membrane fractions, whether obtained from control or from stimulated human neutrophils, contained no protein in the 41-kDa region that could be identified by anti-PY, although other proteins are readily phosphorylated (Fig. 4). Conversely, cytosolic fractions did contain the p41 (Fig. 4). It can also be seen that in cytosol prepared from GM-CSF-treated neutrophils, the phosphorylated form of 41 kDa gives a stronger signal to the anti-PY than it does in controls. In parallel experiments, we observed that membrane preparations contained no immunoreactive MAPK. On the other hand, MAPK was present in cytosol to as great an extent as p41. Our positive controls (not shown) included a membrane preparation that was immunoblotted with AS7, an antibody to $G_{i\alpha_2}$ (courtesy of Allen Spiegel, National Institutes of Health, Bethesda). This preparation showed a single strong band of about 39–40 kDa in x-ray films.

Immunoprecipitation of MAPK and Immunoblotting. To further prove the homology between p41 and MAPK, we carried out the following set of experiments. Cells incubated with or without GM-CSF for 5 min were lysed and MAPK was immunoprecipitated before being subjected to Western blotting for phosphotyrosine substrate. Fig. 5*A* shows that a protein immunoprecipitated with anti-MAPK in cell lysates was indeed recognized by anti-PY in the blots. Furthermore, immunoprecipitates obtained from cells stimulated with GM-CSF clearly exhibit more reactivity to the latter antibodies, thus further proving that GM-CSF increases the level of tyrosine phosphorylation of the neutrophil MAPK. Similar results were obtained if immunoprecipitation was carried out with monoclonal anti-PY20 and immunoblots of the resulting immunoprecipitates were probed with anti-MAPK antibodies

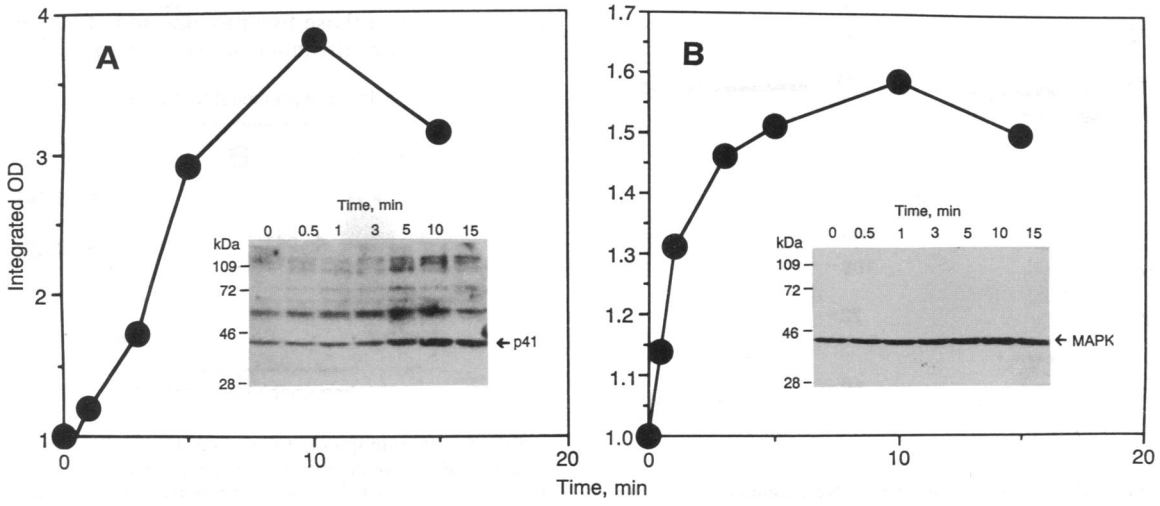

FIG. 2. Time course of p41 phosphorylation on tyrosine upon neutrophil stimulation with GM-CSF and MAPK detection. (*A*) Neutrophils were incubated with 200 pM GM-CSF at the indicated length of time. Samples were processed as indicated in the legend of Fig. 1. p41 of the resulting autoradiographs was quantified by laser scanning densitometry (LKB Ultroscan, Pharmacia). The optical density (program GelScan version 1.21, Pharmacia) in the absence of GM-CSF (only solvent vehicle added) was arbitrarily taken as 1.0. (*Inset*) X-ray film from a Western blot treated with anti-PY. (*B*) The same blot stripped and reprobed with anti-MAPK as indicated in legend of Fig. 1. Only a single band was found to immunoreact with the antibody (shown in *Inset*). The scanning densitometry was performed on that band as indicated above. The *Insets* show a representative experiment among three.

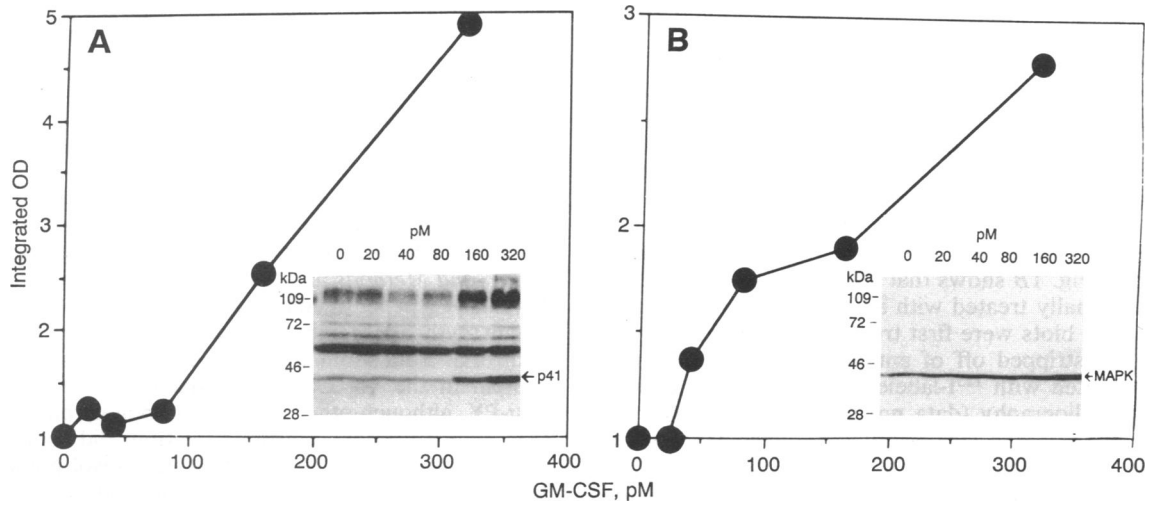

FIG. 3. Dose–response of p41 phosphorylation on tyrosine upon neutrophil stimulation with GM-CSF and MAPK detection. Neutrophils were incubated with the indicated amounts of GM-CSF for 5 min at 37°C. Samples were processed as indicated in the legend to Fig. 1. (*A*) Western blot probed with anti-PY (*Inset*) and densitometric scanning of p41 band. (*B*) The same blot stripped and reprobed with anti-MAPK (*Inset*) and densitometric scanning of the only band visible in the x-ray film. The *Insets* show a representative experiment among three.

(Fig. 5*B*). In a similar fashion as seen in the first set of experiments, MK12 antibodies reacted more with PY immunoprecipitates obtained from GM-CSF-activated cells than they did from untreated cells (Fig. 5*B*).

DISCUSSION

In this paper we report the following findings: (*i*) human polymorphonuclear neutrophils contain a protein of 41 kDa (p41) that undergoes major phosphorylation on tyrosine residues upon receptor activation by several well-known neutrophil agonists, (*ii*) these cells contain the MAPK, (*iii*) these two proteins (p41 and MAPK) are similar, if not identical, which implies that the kinase is phosphorylated in tyrosine residues upon cell activation, (*iv*) MAPK and p41 are located in the cytosol, (*v*) the immunoreactivity of the MAPK to anti-MAPK antibody increases during cell activation, almost paralleling the pattern of tyrosine phosphorylation.

We suggested earlier the possibility that p41, a major phosphoprotein in the neutrophil system, could be MAPK (17, 31). The anti-MAPK antibody used in this study reacted strongly with a single protein in Western blots. In our hands, this antibody has proven to be a useful reagent for the identification of MAPK in neutrophils. This enzyme [referred to as $p42^{MAPK}$ in the literature (6, 32)] has been shown to be phosphorylated in both threonine and tyrosine residues (Thr-183, Tyr-185) upon cell stimulation in other systems (32, 33). According to our results, MAPK and p41 share the following features: they have identical relative mobilities in SDS/polyacrylamide gels, they are located in the cytosol and are absent in membranes, and there is a close parallelism in reactivity to anti-MAPK and anti-PY antibodies with respect to time and dose of the agonist. Finally, the combination of immunoprecipitation and immunoblotting provided results (Fig. 5) compatible with the suggestion of similarity or identity between those two proteins and of a covalent modification after agonist-induced cell activation.

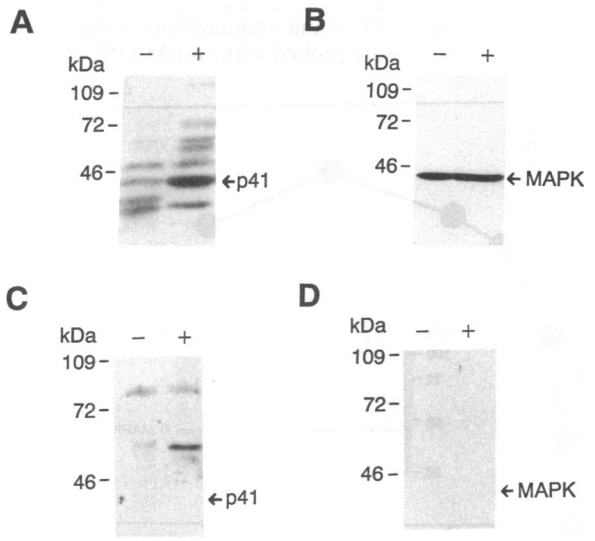

FIG. 4. Subcellular localization of p41. Neutrophils were stimulated with 200 pM GM-CSF (+) or buffer (−) for 5 min at 37°C. Cells were then disrupted by nitrogen cavitation and sequential centrifugation and post-104,000 × *g* pellet (*C* and *D*) and post-104,000 × *g* supernatant (*A* and *B*) fractions were saved. Protein content was measured by Bradford analysis (Bio-Rad) and the same amount of protein was loaded in each lane of SDS/polyacrylamide gels. Samples were electrophoresed, transferred to PVDF sheets, and probed with antibodies (either anti-PY or anti-MAPK as indicated).

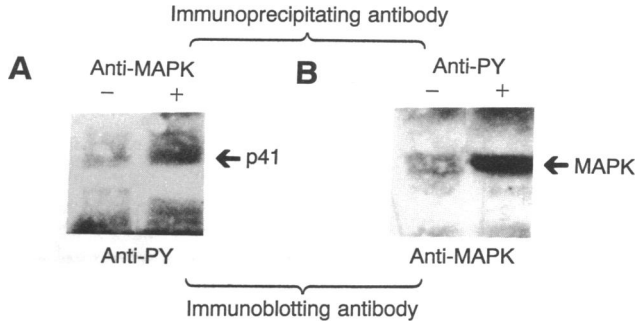

FIG. 5. Immunoprecipitation and immunoblotting of neutrophil lysates. Cells were incubated with 200 pM GM-CSF (+) or buffer (−) for 5 min at 37°C. (*A*) Lysates corresponding to equal number of cells were immunoprecipitated with anti-MAPK. The immunoprecipitates were recovered with protein A, subjected to SDS/PAGE, and transferred to PVDF sheets, and the blots were probed with anti-PY. (*B*) Whole-cell lysates immunoprecipitates with anti-PY and subsequent Western blot developed with anti-MAPK antibody. One representative experiment among three with nearly identical results is shown. Only relevant regions of the blots spanning a range of 28–50 kDa are shown.

Also, MAPK activity (assayed against myelin basic protein) is higher in whole cell lysates prepared from GM-CSF-stimulated neutrophils than in controls. The enhancement in kinase activity parallels that of tyrosine phosphorylation (J.G.-C., J. Colasanto, C.-K.H., and R.I.S., unpublished data).

The finding that the immunoreactivity of MAPK to anti-MAPK antibody varies with the time or dose of the cell stimulant was surprising. The explanation of this phenomenon could be that either new protein is formed in the course of cell stimulation or that the covalent modification of the protein by the insertion of phosphate groups alters the antigenicity of the protein. The former explanation is unlikely, since we have observed changes in MAPK bands in immunoblots from fMet-Leu-Phe-stimulated cells (Fig. 1*A*) or PAF at very early times (<3 min). For the second possible explanation to be valid, the anti-MAPK antibody would have to recognize an epitope that is in close spatial proximity to the phosphorylation site(s).

The phosphorylation and MAPK reactivity in Western blots do not parallel exactly (Fig. 2), at least at early stages. The small discrepancies in the curves are within the margins of experimental error and might be related to the densitometry measurements and/or the greater sensitivity of the ECL detection system for MAPK. It also could be argued that phosphorylation of a site(s) other than tyrosine, as reported (7), could be taking place and, again, that would be enough to alter the protein antigenicity. Tyrosine phosphorylation, in the case of that induced by the cytokine GM-CSF, could occur slightly later. In this regard, we have found that PMA also promotes phosphorylation in tyrosine of p41 (34). This is in agreement with the findings of Gomez and Cohen (8), who suggested that PMA may stimulate Ser/Thr phosphorylation of MAPK as an early event in the phosphorylation cascade. Calcium may also be involved at some point in this cascade of kinase activation. Calcium ionophore in the presence of Ca^{2+} triggers tyrosine phosphorylation (this paper; also ref. 17).

With the finding of MAPK in nondifferentiating cells such as the neutrophil, the role considered for MAPK (until now related to cell growth) has to be broadened to include other unrelated mechanisms. MAPK, with its particular upstream localization in the signal transduction cascade, might be important during the priming process observed with GM-CSF that is related to physiological functions of the neutrophil, such as phagocytosis, degranulation, and oxygen radical release.

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