

FcγR-dependent Mitogen-activated Protein Kinase Activation in Leukocytes: A Common Signal Transduction Event Necessary for Expression of TNF-α and Early Activation Genes

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Summary

Cross-linking the receptors for the Fc domain of IgG (FcγR) on leukocytes induces activation of protein tyrosine kinases. The intermediary molecules that transduce to the nucleus the signals leading to induction of the diverse biological responses mediated by these receptors are not clearly identified. We have investigated whether mitogen-activated protein kinases (MAPK) are involved in transmembrane signaling via the three FcγR present on monocytic, polymorphonuclear, and natural killer (NK) cells. Our results indicate that occupancy of FcγRI and FcγRII on the monocytic cell line THP-1 and on polymorphonuclear leukocytes (PMN) induces, transiently and with fast kinetics, MAPK phosphorylation, as indicated by decreased electrophoretic mobility in sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and increased amounts of the proteins in antiphosphotyrosine antibody immunoprecipitates. This, associated with increased enzymatic activity, also occurs upon stimulation of the transmembrane isoform of CD16 (FcγRIIIA) in NK cells and in a T cell line expressing transfected FcγRIIIA α ligand-binding chain in association with ζ, but not upon stimulation of the glycosyl-phosphatidylinositol-anchored FcγRIIIB on PMN. Using the specific MAP kinase kinase inhibitor PD 098059, we show that activation of MAPK is necessary for the FcγR-dependent induction of *c-fos* and tumor necrosis factor α mRNA expression in monocytes and NK cells. These results underscore the role of MAPK as signal-transducing molecules controlling the expression of different genes relevant to leukocyte biology upon FcγR stimulation.

Mitogen-activated protein kinases (MAPK)¹, also known as extracellular signal-regulated kinases (ERK), transduce signals elicited via several receptors that either have intrinsic tyrosine kinase activity or are associated with non-receptor tyrosine kinases (1), including hematopoietic receptors like FcεRI, TCR, and B cell receptor (BCR) (2, 3). MAPK are serine-threonine kinases activated by MAPK kinases (MEK), that phosphorylate the TEY domain on tyrosine and threonine residues (4). Stimuli that activate MAPK induce a signaling cascade involving transient formation of *ras*-GTP and activation of *raf* kinase at the membrane, followed by sequential activation of MEK and

MAPK (5). Several transcription factors have been identified in eukaryotic cells as targets of MAPK (6). The *ras*-MAPK pathway mediates *c-fos* induction, via phosphorylation of the transcription factor TCF/*Elk1* (ternary complex factor) (7). MAPK also activate cytoplasmic enzymes (e.g., phospholipase A2) or other proteins regulating protein synthesis (8, 9), and have been implicated in proliferative responses and differentiation (10).

Receptors for the Fc domain of IgG (FcγR) link humoral and cellular immune responses and mediate different functions upon ligand binding (11). Three different classes of FcγR have been identified, FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16), that differ in relative affinity for IgG, cellular distribution, and molecular composition (11). Cross-linking FcγRs on macrophages, PMN, and NK cells induces signals leading to the activation of diverse biological responses such as phagocytosis, Ab-dependent cellular cytotoxicity, and transcription of genes encoding cytokines and surface molecules relevant to leukocyte functions (12, 13).

Initiation of signal transduction after occupancy of FcγR

¹Abbreviations used in this paper: BCR, B cell receptor; [Ca²⁺]_i, intracellular Ca²⁺ concentration; EA, IgG-sensitized E; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; FcγR, receptor for the Fc fragment of IgG; GαMlg, goat anti-mouse Ig; GPI, glycosyl-phosphatidylinositol; ITAM, immune receptor tyrosine-based activation motif; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MEK, MAP kinase kinase; PI-3K, phosphatidylinositol-3 kinase; PLC, phospholipase C.

as well as other immune receptors (TCR, BCR, and FcεRI) shares common molecular mechanisms involving sequential activation of tyrosine kinases of the *src* and Syk families, depending on induced phosphorylation of immune receptor tyrosine-based activation motifs (ITAM) (14–16). In the IFN-γ-inducible high affinity FcγRI and in the low affinity FcγRIIA, ITAM is present in the ζ family members (γ only or γ and ζ chains in myelomonocytic and in NK cells, respectively) linked as dimers to the highly homologous ligand-binding α chains (17, 18). The ITAM motif in the α chain's cytoplasmic domain of the low affinity FcγRIIA in monocytes/macrophages and PMN confers signal transduction capability to the α chain itself (19). It has been demonstrated that this chain is expressed and functional at the cell membrane independently from association with additional molecules (19). Whether or not the glycosyl-phosphatidylinositol (GPI)-linked FcγRIIB on PMN transduce signals is still controversial, but the recent observation that *hck* is activated after FcγRIIB cross-linking supports a signal-transducing capability of this receptor (20). Activation of protein tyrosine kinases results in tyrosine phosphorylation and activation of cellular substrates (21, 22), among which phospholipase C (PLC) γ1 and γ2, followed by increased intracellular Ca²⁺ concentration ([Ca²⁺]_i) (23, 24). Activation of phosphatidylinositol-3 kinase (PI-3K) also occurs upon tyrosine kinase activation (25, 26).

A major event in the activation of myelomonocytic and NK cells induced by FcγR-generated signals is the induced expression of genes encoding cytokines (12, 13). Recently, p21^{ras} activation has been observed upon FcγRIIA occupancy in NK cells (27). In T cells, TCR-mediated p21^{ras} activation is necessary for ERK2 activation and TCR-induced transcription of IL-2 mRNA (28, 29). However, no formal proof exists that ERK2 links p21^{ras} activation with IL-2 gene induction.

Here, we analyzed the involvement of MAPK in the signal transduction pathways elicited upon FcγR cross-linking. Our data show that occupancy of each of the transmembrane FcγR forms, but not of the GPI-linked FcγRIIB, induces MAPK activation. A role for this kinase(s) in the FcγR-mediated induction of *c-fos* and TNF-α mRNA is demonstrated using the MEK inhibitor PD 098059 to inhibit, in leukocytes, expression of these genes. These results identify MAPK as common intermediate molecules in FcγR-induced activation of several genes relevant to leukocyte's functions.

Materials and Methods

Cell Lines and Primary Cell Preparations. The human monocytic THP-1 and B lymphoblastoid RPMI-8866, and the murine mAb-producing hybrid B cell lines were maintained in culture in RPMI-1640 medium (BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma Chemical Co., St. Louis, MO) and 100 μg/ml glutamine. FcγRIIA-transfected CD3⁻/CD2⁻ J32.65.3.1 Jurkat T cells, expressing FcγRIIAα chain in association with endogenous ζ chain, was maintained in culture in RPMI-1640 supplemented

with 10% heat-inactivated, protein G-adsorbed FBS and geneticin (G418; GIBCO BRL, Gaithersburg, MD) (23). PBMC, separated on Ficoll-Hypaque (F/H) (Histopaque-1077, Sigma Chemical Co.) density gradient from heparin anticoagulated venous peripheral blood of healthy donors, were depleted of the majority of monocytes after adherence to plastics (1 h, 37°C). PMN were obtained from the F/H pellet of the upper two thirds of leukocyte-rich plasma after sedimentation (30 min, 37°C) of total peripheral blood with Dextran T500 (Pharmacia Biotech, Uppsala, Sweden; 3% Dextran in PBS/blood, vol/vol) (30). Alternatively, PMN were collected from the pellet of F/H density gradients of total blood, resuspended in autologous plasma and, after E sedimentation as above, they were recovered in the pellet of a second F/H gradient performed to deplete PBL and monocyte contaminants. If needed, contaminating E were eliminated after lysis with 0.15 M NH₄Cl, 0.01 M KHCO₃, and 1 mM EDTA, pH 7.3. PMN represented >99% of the cell preparations, as determined by morphological examination and surface phenotyping in indirect immunofluorescence (flow cytometry) using anti-CD15 mAb B40.9 (31). Monocyte (CD14⁺ cells) contamination was undetectable. When indicated, PMN were used after 16 h of incubation (5 × 10⁶ cells/ml RPMI-10% FBS) with 10³ U/ml rIFN-γ (Genentech Inc., South San Francisco, CA; 7 × 10⁷ antiviral U/mg on HeLa cells) as previously described (30). IFN-γ-treated PMN were >99% FcγRI⁺, as detected by immunofluorescence using murine IgG2a or anti-FcγRI mAb 32.2 (32).

Homogeneous NK cell preparations were obtained from 10-d cocultures of PBL with 30 Gy-irradiated RPMI-8866 cells after negative selection using a mixture of anti-CD14, anti-CD3, and anti-CD5 mAb and indirect antiglobulin rosetting (33). The purity of each preparation (>98% CD16⁺/CD56⁺/CD3⁻ and <1% CD3⁺ cells) was confirmed by indirect immunofluorescence (flow cytometry) using a panel of mAb.

Abs. mAb B52.1 (anti-CD14, IgM), B36.1 (anti-CD5, IgG2b), OKT3 (anti-CD3, IgG2a), 3G8 (anti-CD16, IgG1), B159.5 (anti-CD56, IgG1), IV.3 (anti-CD32, IgG2b), B137.17 (IgG2a), 32.2 (anti-CD64, IgG2a), and B40.9 (anti-CD15, IgM) have been previously described (33–35). IgG were purified on protein G-Sepharose columns (Pierce, Rockford, IL). Fab and F(ab')₂ fragments were prepared following standard protocols (36). Undigested IgG and Fc fragments were depleted after adsorption to protein G-Sepharose, and the Ab purity was confirmed in SDS-PAGE. Labeling with *N*-hydroxysuccinimidobiotin (Pierce) was according to standard procedures (37). The goat anti-mouse Ig (GαMIg) was produced in our laboratory, adsorbed on human Ig, and affinity purified on a mouse IgG-Sepharose 4B column (Pharmacia Biotech). Rabbit anti-mouse Ig serum (RαMIg) was obtained from Cappel (Durham, NC). The polyclonal rabbit serum ERK2 (anti-MAPK ERK2), and the antiphosphotyrosine murine mAb PY20 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The polyclonal rabbit serum R2 (anti-MAPK ERK1 and ERK2), and the murine antiphosphotyrosine mAb 4G10 were purchased from Upstate Biotechnology, Inc. ([UBI] Lake Placid, NY).

Cell Stimulation. Cells were incubated (5–10 × 10⁶/ml) for the indicated times at 37°C in serum-free medium containing 10 μg/ml FcγR-specific or irrelevant control mAb and 50 μg/ml streptavidin (Sigma Chemical Co.) or 20 μg/ml GαMIg, as appropriate. When indicated, the cells were first incubated with the biotin-labeled mAb (10 min on ice), washed, and resuspended in medium containing streptavidin; results were identical. Cells stimulated with 50 ng/ml PMA (Sigma Chemical Co.) were used as positive control. Immune complexes (rabbit IgG-sensitized bo-

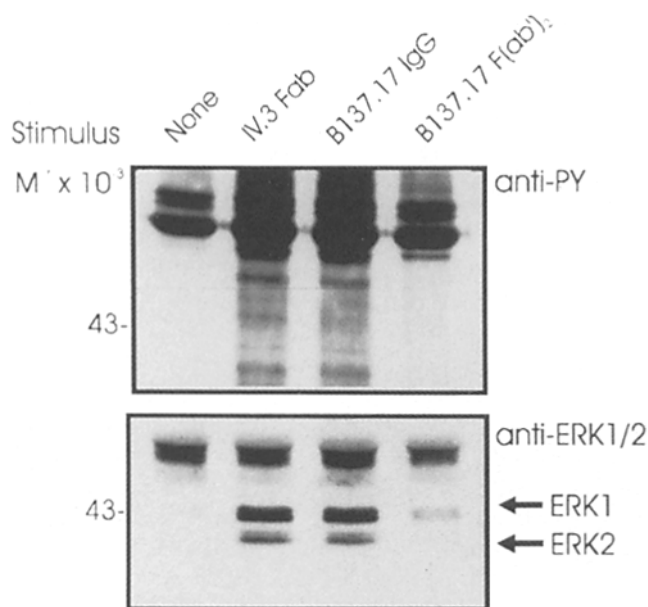


Figure 1. Presence of ERK kinases in the antiphosphotyrosine immunoprecipitates from THP-1 cells. THP-1 cells (15×10^6 /sample) were incubated (2 min, 37°C) in serum-free medium without (*None*) or with reagents indicated (biotin labeled) and streptavidin. Phosphotyrosine proteins were precipitated with mAb PY20 from NP-40 lysates of the cells and resolved in 7.5% SDS-PAGE. Western blot analysis was performed, sequentially on the same filter, using antiphosphotyrosine (PY, *top*) and anti-MAPK ERK1 and ERK2 Ab (*bottom*) 4G10 and R2, respectively. Experiment representative of three performed with identical results.

vine erythrocytes [EA]) were prepared as described (13), nonsensitized bovine E were used as control. When indicated, cells were pretreated (30 min, 37°C) with the indicated concentrations of MEK inhibitor PD 098059 (38; kindly provided by Dr. A. Saltiel, Parke-Davis Pharmaceutical Research/Warner-Lambert Co., Ann Arbor, MI).

Immunoprecipitation and Western Blotting. These were performed as previously described (25); lysis buffer was 10 mM Hepes, pH 7.5, 0.15 M NaCl, 10% glycerol, 10 $\mu\text{g}/\text{ml}$ each aprotinin and leupeptin, 1 mM PMSF, 1 mM Na_3VO_4 , and 1% NP-40. Abs coupled to protein A-Sepharose (Pharmacia Biotech) via R α Mlg or not, as appropriate, were used for immunoprecipitation (2 or 18 h, 4°C) after preclearing with the same beads not coupled with Ab. Proteins were separated in SDS-PAGE under reducing conditions. Western blot analysis was performed as previously reported (25), and Ab-reactive proteins were detected with horseradish peroxidase-labeled donkey anti-rabbit or G α M Ig and enhanced chemiluminescence (ECL; Amersham Corp., Arlington Heights, IL). When indicated, different Abs were used sequentially on the same filter, after stripping (30 min, room temperature, 0.1 M glycine/HCl, pH 2.6).

MAPK Assay. Immunocomplex MAPK assays were performed as described by Thomas et al. (39). Briefly, cells (5×10^6 /sample) were lysed with 100 μl 1% Triton X-100, 50 mM NaCl, 10 mM Tris, pH 7.5, 50 mM NaF, 1 mM PMSF, 1 mM Na_3VO_4 , 5 mM benzamidine, 1 mM EGTA, and 10 $\mu\text{g}/\text{ml}$ each aprotinin and leupeptin. MAPK were precipitated using the polyclonal ERK2 Ab (0.5–1 μg Ab/sample) and protein A-Sepharose. The beads were washed four times in the same buffer and twice in 10

mM Tris, pH 7.5, 0.05 M NaCl supplemented with 1 mM Na_3VO_4 , and 5 mM benzamidine. After adding 30 μl reaction buffer (30 mM Hepes, 10 mM MgCl_2 , 1 mM dithiothreitol, 20 μM ATP, 10 μCi γ - ^{32}P ATP {4,000 Ci/mmol, sp act; ICN Pharmaceuticals, Inc., Costa Mesa, CA}, 5 mM benzamidine, 5 μg myelin basic protein [MBP; Sigma Chemical Co. or UBI]), the reaction was performed for 30 min at 30°C . Eluted products were analyzed on 13% SDS-PAGE and transferred to nitrocellulose filters. Western blot with ERK2 Ab was performed on the filter to verify the amounts of MAPK precipitated from each sample. In vitro phosphorylation of MBP was visualized after exposure of the filters or dried gels to X-AR films (Eastman Kodak Co., Rochester, NY). Levels of phosphorylation were quantitated by optical densitometry (Personal Densitometer SI, Molecular Dynamics Inc., Sunnyvale, CA).

Northern Blot Analysis. Total RNA was extracted as previously described using RNazol (Biotecx Laboratories, Houston, TX). RNA was size fractionated in 1% agarose-formaldehyde gels, transferred by capillary onto Hybond-nylon membranes (Amersham Corp.), and hybridized to cDNA probes specific for human TNF- α , *c-fos*, and β_2 -microglobulin, as described (40). RNA was visualized in ethidium bromide-stained gels, and the amount of RNA loaded was determined after hybridization to β_2 -microglobulin cDNA. cDNA probes were labeled with α - ^{32}P dCTP (sp act 3,000 Ci/mmol; NEN Dupont, Wilmington, DE) by random priming or nick translation (Boehringer Mannheim Corp., Indianapolis, IN). Filters were exposed to X-AR films for autoradiography. Levels of expression of each mRNA species were quantitated as above.

Results

MAPK Phosphorylation and Activation after Fc γ RI and Fc γ RII Stimulation. Among the proteins phosphorylated on tyrosine residues after Fc γ RI-ligand (IgG2a) binding or Fc γ RII cross-linking with mAb IV.3 Fab and streptavidin in THP-1 cells is a set of proteins with an apparent molecular mass of ~ 40 kD, similar to that of MAPK (Fig. 1, *top*). ERK1 and ERK2 were detectable at significant levels by Western immunoblotting in the PY20 antiphosphotyrosine immunoprecipitates from IgG2a- or anti-Fc γ RII-treated cells (Fig. 1, *bottom*). Minimal levels of ERK1 and ERK2 were present in the same immunoprecipitates from control nonstimulated or IgG2a F(ab') $_2$ -stimulated cells. To determine whether MAPK were phosphorylated as a result of Fc γ R occupancy, Western blot analysis was performed with R2 antiserum on lysates of THP-1 cells treated with the Fc γ RI-ligand (IgG2a) or anti-Fc γ RII mAb IV.3 Fab and streptavidin (Fig. 2, *top*). ERK1 and ERK2 were detected in both lysates as bands migrating slower than those detected in controls. ERK2 appeared as a doublet in which the faster migrating band migrated as in controls. Decreased electrophoretic mobility indicates phosphorylation of both kinase isoforms after Fc γ R stimulation. The results of MAPK assays performed, with MBP as exogenous substrate, on the ERK2 immunoprecipitates from the same cell samples (Fig. 2, *bottom*) revealed that MBP phosphorylation, detectable at minimal levels in the immunoprecipitates from control IgG2a F(ab') $_2$ -treated cells, was significantly increased (5 ± 2 - and 4 ± 1 -fold increase, respectively; $n = 3$) in those

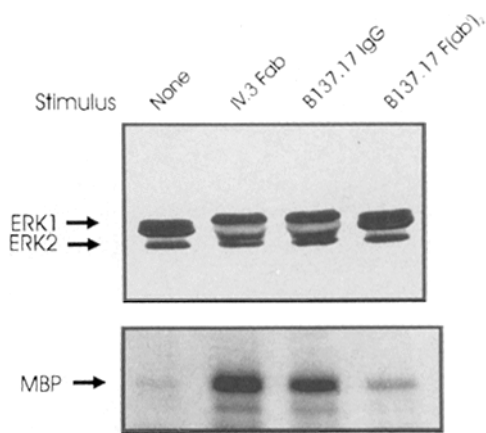


Figure 2. ERK1 and ERK2 phosphorylation and activation of MAPK upon Fc γ R stimulation in THP-1 cells. THP-1 cells were treated as in Fig. 1 and ERK2 was immunoprecipitated from 5×10^6 cell lysate equivalent; aliquots equivalent to 10^6 cells were left untreated. (Top) Western blot analysis (7.5% SDS-PAGE) was performed, with R2 Ab. (Bottom) Kinase assay with MBP as exogenous substrate on the ERK2 immunoprecipitates. Phosphorylation products were resolved in 13% SDS-PAGE. Experiment representative of three performed with identical results.

from Fc γ R1- or Fc γ R2-stimulated cells, proving activation of MAPK enzymatic activity. The same analysis was performed in PMN, to confirm the generality of these findings, and to determine whether the GPI-anchored form of Fc γ R3 mediates similar activation. Electrophoretic mobility shift of ERK2 was detected in freshly separated PMN after stimulation with IV.3 Fab or PMA (Fig. 3, top left), and significant MBP phosphorylation was present in the ERK2 precipitates from the same cells (middle left), but undetectable in those from cells stimulated with either IgG2a or anti-CD16 mAb F(ab')₂. In IFN- γ -treated PMN (Fig. 3, right) ERK2 and MBP phosphorylation were over basal levels in the lysates and in the ERK2 immunoprecipitates both from Fc γ R2- and Fc γ R1-stimu-

lated cells. Western blot analysis with anti-ERK2 Ab confirmed the presence of equal amounts of ERK2 in each precipitate used (Fig. 3, bottom).

Kinetics of MAPK Phosphorylation. Western blot analysis with anti-ERK2 Ab was performed on NP-40 lysates prepared from IFN- γ -treated PMN at different times after Fc γ R1 and Fc γ R2 stimulation (Fig. 4). In both cases, significant levels of ERK2 phosphorylation were detected within 0.5-min stimulation and declined to basal levels within 32 min. Similar results were obtained in THP-1 cells (data not shown).

MAPK Phosphorylation and Activation upon Fc γ R3A Stimulation. To determine whether all transmembrane Fc γ R induce activation of MAPK, similar experiments were performed in Fc γ R3A⁺ NK cells. Untreated or PMA-treated NK cells were used as negative and positive controls, respectively. In lysates from Fc γ R3A- or PMA-stimulated cells, the electrophoretic mobility of MAPK ERK2 was decreased (Fig. 5, top) and MBP phosphorylation was observed in immune complex kinase assays (Fig. 5, middle); CD56 stimulation was ineffective (not shown). Western blot analysis with anti-ERK2 Ab confirmed equal amounts of ERK2 in all precipitates (Fig. 5, bottom). MBP phosphorylation in ERK2 immunoprecipitates from Fc γ R3A-stimulated cells was, on average, twofold that in precipitates from untreated or control CD56-treated cells, as determined by densitometry. Similar results were obtained in the Jurkat T cell line expressing transfected Fc γ R3A α chain in association with ζ chain (data not shown).

Role of MAPK in Fc γ R-induced Gene Expression. Transcription of the immediate early gene *c-fos*, a component of AP-1 transcriptional activator (41), is rapidly induced upon MAPK activation. To determine whether *c-fos* is induced upon Fc γ R occupancy, and whether MAPK are involved in its induction, expression of *c-fos* mRNA was analyzed in THP-1 and NK cells, pretreated or not with the MEK inhibitor PD 098059 (38), and stimulated for 20 min with

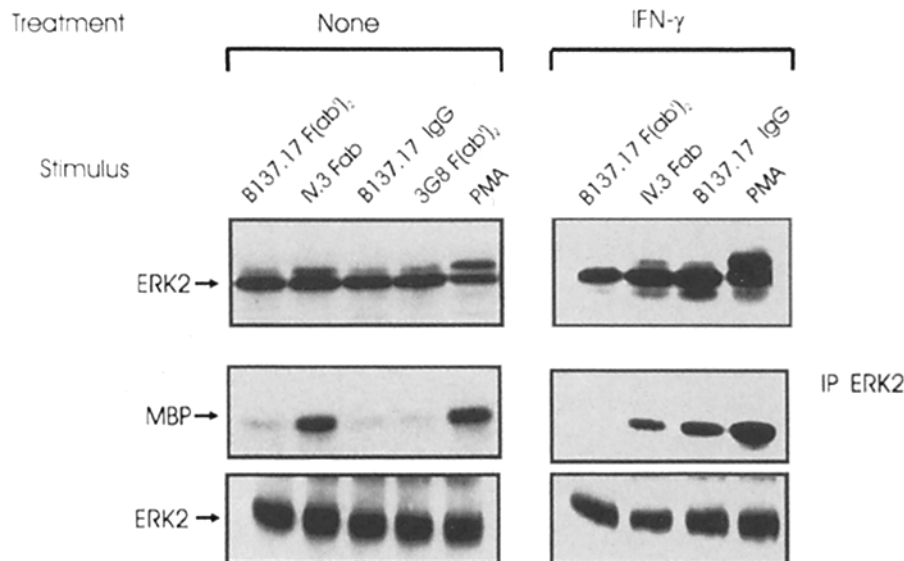


Figure 3. Phosphorylation and activation of ERK2 upon Fc γ R stimulation in PMN. PMN were incubated (5×10^6 /ml, 16 h, 37°C) in medium without (None) or with IFN- γ (500 U/ml), as indicated, washed, and treated (2 min, 37°C) with the indicated biotin-labeled reagents (10 μ g/ml) and streptavidin (50 μ g/ml) or PMA (50 ng/ml). NP-40 lysates were prepared and divided in two aliquots. ERK2 was immunoprecipitated from 5×10^6 cell equivalent lysates; an aliquot corresponding to 10^6 cells was left untreated. (Top) Western blot analysis (7.5% SDS-PAGE) on total lysates with anti-ERK2 Ab. (Middle) MAPK assays on the ERK2 immunoprecipitates as in Fig. 2. (Bottom) Western blot analysis (13% SDS-PAGE) was performed on the same ERK2 immunoprecipitates with anti-ERK2 Ab. Experiment representative of nine performed with identical results.

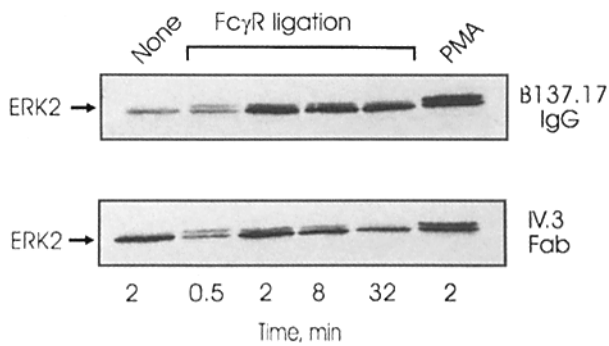


Figure 4. Kinetics of MAPK phosphorylation after Fc γ R I and II stimulation in IFN- γ -treated PMN. IFN- γ -treated PMN were incubated in medium without (*None*) or with biotin-labeled B137.17 IgG2a (*top*), IV.3 Fab, and streptavidin (*bottom*), or PMA (50 ng/ml), as in Fig. 3. Identical aliquots of cells were collected at the indicated times and lysed with 1% NP-40. Western blot analysis was performed as in Fig. 3 on the lysates with ERK2 Ab. Experiment representative of four performed with identical results.

immune complexes (EA). *c-fos* mRNA was induced in both cell types after Fc γ R cross-linking, and its induction was almost completely inhibited in cells pretreated with 30 μ M PD 098059 (Fig. 6, *top*). At this concentration, the inhibitor prevented the induced phosphorylation and enzymatic activity of ERK2 kinase without affecting tyrosine phosphorylation induced upon Fc γ R stimulation (not shown). *c-fos* mRNA expression upon Fc γ R stimulation was inhibited by $83.2 \pm 8.0\%$ ($n = 3$) and by $77.5 \pm 3.5\%$ ($n = 2$) in PD 098059-pretreated THP-1 and NK cells, respectively, as detected by densitometric analysis.

Cross-linking of Fc γ R induces synthesis of cytokines, including IFN- γ , GM-CSF, and TNF- α in NK cells (13), and IL-1, IL-6, and TNF- α in monocytes (12). The effect of the MEK inhibitor on the induction of TNF- α mRNA after Fc γ R occupancy by immune complexes was analyzed. Significant TNF- α mRNA levels were detectable in both THP-1 and NK cells stimulated with EA for 2 or 1.5 h, respectively (Fig. 7, *top*), but not in control E-stimulated cells. The induction was almost completely inhibited in cells pretreated with PD 098059 (Fig. 7, *top*), as determined by densitometric analysis ($92.7 \pm 8.9\%$ and $83.4 \pm 8.1\%$ inhibition, $n = 3$).

Discussion

Fc γ R-dependent signaling in monocytes, PMN, and NK cells is initiated by receptor ligand occupancy and results in the activation of *src* (42, 43) and zeta-associated protein (ZAP)-70-family related kinases (44–46), which associate with the ITAM-containing receptor complex subunits. Subsequent events include activation of PLC- γ 1, - γ 2 (23), and PI-3K (25, 26), increases in $[Ca^{2+}]_i$, and transcription of genes encoding cytokines (13). Protein tyrosine kinase inhibitors block these events (21, 23). Here, we show that activation of the MAPK ERK2 is also induced upon Fc γ R stimulation and is indispensable for the induced transcrip-

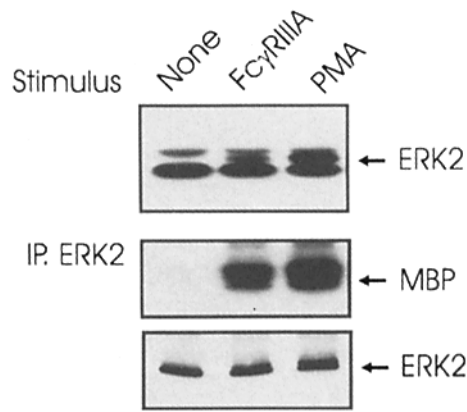


Figure 5. ERK2 phosphorylation and activation upon Fc γ R IIIA stimulation. NK cells (5×10^6 /ml) were incubated in serum-free medium without (*None*) or with 3G8 (anti-Fc γ R IIIA) (10 μ g/ml) and G α Mlg (20 μ g/ml), or PMA (50 ng/ml) for 1 min. NP-40 lysates were prepared and Western blot (*top*, 7.5% SDS-PAGE), and MAPK assay with MBP (*middle*) were performed on ERK2 immunoprecipitates as in Fig. 2. Western blot analysis (*bottom*, 13% SDS-PAGE) was performed on the same ERK2 immunoprecipitates with anti-ERK2 Ab. Experiment representative of four performed with identical results.

tion of early activation genes such as *c-fos*, and cytokines, such as TNF- α .

Stimulation of Fc γ R I and Fc γ R II in the monocytic cell line THP-1 and U937 (not shown) induces tyrosine phosphorylation of several proteins with similar apparent molecular weights. The presence of proteins with electrophoretic mobility similar to that of MAPK in the total lysates (not shown) and in antiphosphotyrosine immunoprecipitates of Fc γ R-stimulated cells, together with the notion that MAPK regulate gene induction (6), prompted us to examine whether these kinases were present in the antiphosphotyrosine precipitates of stimulated cells. Both ERK1 and ERK2, the best characterized MAPK isoforms in mammalian cells, are associated with antiphosphotyrosine precipitates, indicating that MAPK are activated, likely after phosphorylation on threonine and tyrosine residues (4), and suggesting that they may play a role in Fc γ R-mediated functions. Induced phosphorylation of ERK2 was confirmed, based on its decreased electrophoretic mobility in SDS-PAGE and increased enzymatic activity. Although we did not analyze the enzymatic activity of ERK1, the induction of its phosphorylation in the same conditions supports the hypothesis that this isoform is also activated. Our studies with PMN confirm the results obtained in THP-1 cells and demonstrate that Fc γ R I and Fc γ R II occupancy induces MAPK activation in primary cells. The specificity of the reagents used, Fab, F(ab')₂, and avidin, assures that in each condition a single Fc γ R is stimulated; the ability of each receptor to induce MAPK activation was confirmed in both resting (Fc γ R I⁻/Fc γ R II⁺) and IFN- γ -treated (Fc γ R I⁺/Fc γ R II⁺) PMN. ERK2 phosphorylation is a transient event upon Fc γ R cross-linking, possibly because a phosphatase is induced, upon Fc γ R cross-linking, that inactivates MAPK by dephosphorylation, in

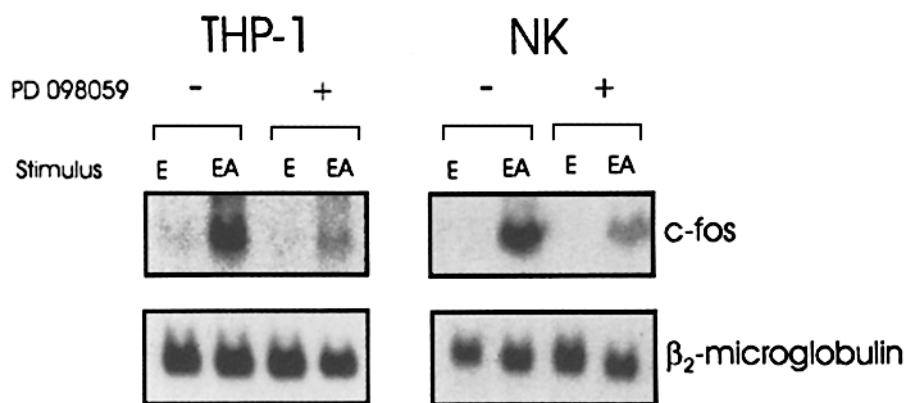


Figure 6. *c-fos* mRNA induction upon FcγR stimulation. THP-1 (left) and NK cells (right) were incubated in serum-free medium for 30 min with 0.3% DMSO (vehicle) (-) or 30 μM PD 098059 (+). After stimulation with E or EA (20 min, 37°C), total RNA was extracted. Northern blot analysis was performed using *c-fos* cDNA (top). The blots were then stripped and hybridized to β₂-microglobulin cDNA (bottom). Experiment representative of two (NK) and three (THP-1) cells performed with identical results.

keeping with the observation that MAPK are inactivated by the dual specificity phosphatase PAC1, specific for the tyrosine and threonine residues in the TEY motif of activated MAPK (47).

ERK2 kinase activation in FcγRIIIA-mediated signaling was evaluated in NK and Jurkat cells constitutively expressing FcγRIIIA (data not shown). As expected, FcγRIIIA crosslinking also induced ERK2 phosphorylation in these cells, thus demonstrating that activation of this kinase represents a common event in the signal transduction mediated by each of the three transmembrane FcγR. These receptors share common ITAM motifs, present either in the cytoplasmic domain of the α chain (FcγRII) or in the γ and ζ α chain-associated molecules (48). Although FcγRIIIA has been reported to be expressed in association with γ chain in monocytes (49), our attempts to reproduce those data were negative and our data indicate no association of FcγRII with γ chain (expressed both in monocytes and PMN; 18, and data not shown) even after receptor occupancy (Trotta, R. and B. Perussia, unpublished results). The experimental protocol of Masuda and Roos (49) was complicated by the use of adherent cell populations, previously demonstrated to contain NK cells (50) and of reagents that may have contained contaminating Fc capable of binding FcγRIII and FcγRI; thus, FcγRII-γ association may have been artifactual. Therefore, we favor the hypothesis that FcγRII is not associated to other signal transduc-

tion chains as previously suggested (18), and that the ITAM sequence in the FcγRIIIA α chain is sufficient to transduce the early events mediated via this receptor (19, 51). MAPK transduce signals from other hematopoietic receptors that contain ITAM (FcεRI, BCR, and TCR) and share with FcγR most signaling events (14–16). No detectable MAPK activation, instead, follows occupancy of the membrane GPI-anchor FcγRIIIB, and the mechanisms of signal transduction via FcγRIIIB (20), if any, remain unclear.

Several reports indicate the involvement of p21^{ras} in MAPK activation (for a review see reference 1), and in FcγR-mediated signaling (27). Galandrini et al. (27) demonstrated that FcγRIIIA induces p21^{ras} activation in NK cells, suggesting that this is one of the mechanisms of MAPK activation via FcγRIIIA in NK cells. Additional data demonstrate that FcγRIIIA-dependent MAPK activation in NK cells occurs via a PI-3 kinase-dependent mechanism (Kanakaraj, P., R. Trotta, and B. Perussia, manuscript in preparation). Stimulation of macrophages with CSF-1 and bacterial LPS induces MAPK activation via both p21^{ras}-dependent and -independent mechanisms (52), and activation of MAPK, upon IL-2 stimulation in T cells, involves PI-3 kinase-dependent and -independent pathways (53). The possibility that p21^{ras} and PI-3 kinase are involved in inducing MAPK activation in FcγRI- and FcγRII-stimulated monocytes and PMN needs to be investigated.

Transcription of the protooncogene *c-fos* is rapidly and

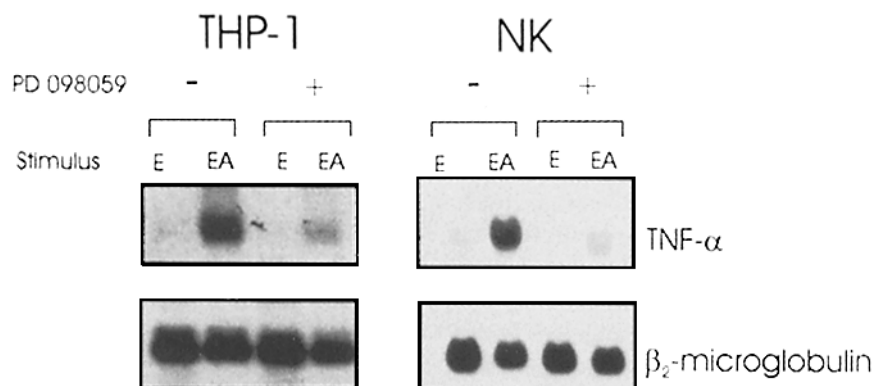


Figure 7. Effect of PD 098059 on FcγR-induced TNF-α mRNA accumulation. THP-1 (left) and NK cells (right) were incubated (30 min, 37°C) with DMSO (vehicle) (-) or 30 μM PD 098059 (+), and for 2 or 1.5 additional h, respectively, with EA or E, as described in Fig. 6. Total RNA was extracted and subjected to Northern blot analysis using TNF-α (top) and β₂-microglobulin cDNA (bottom). Experiment representative of three performed with identical results.

transiently induced in several cell types upon stimulation of hematopoietic receptors (e.g., TCR, IL-2R) (15, 54) that activate MAPK, depending on induced phosphorylation of the transcription factor Elk-1 by these kinases (7). *c-fos* associates in the nucleus with preexisting *c-jun* proteins to form AP-1 transcription factor (41). Our data show that *c-fos* mRNA expression is rapidly induced in NK and THP-1 monocytic cells upon binding of immune complexes to FcγR and that MAPK activation is necessary for its induction upon FcγR occupancy, as demonstrated using the MEK-specific inhibitor PD 098059 (38), which prevents FcγR-dependent ERK2 activation (not shown). We previously reported that FcγRIIIA stimulation induces increased AP-1 activity in NK cells (40). Here we present additional and direct evidence that MAPK are signal-transducing molecules necessary for the transmission of FcγR-originated signals to the nucleus, mediating in this way the expression of early genes (i.e., *c-fos*) involved in transcriptional gene regulation.

Our previous data indicated that cytokine gene transcription upon FcγRIIIA stimulation in NK cells occurs in a Ca²⁺ and cyclosporin A-dependent fashion (40, 55). In THP-1 cells, inhibition of FcγR-induced TNF-α mRNA accumulation was observed using the tyrosine kinase inhibitor herbimycin A (21), thus supporting that early signaling

events, such as tyrosine kinase activation and [Ca²⁺]_i increases, are essential to induce TNF-α mRNA expression via FcγR in monocytes and NK cells. The present data demonstrate that MAPK are indispensable to allow FcγR-induced TNF-α mRNA accumulation, based on the demonstration that induced TNF-α mRNA accumulation after FcγR-immune complex interaction is almost completely inhibited upon pretreatment of the cells with the MEK inhibitor PD 098059.

The signaling molecules, targets of MAPK, involved in TNF-α induction have not been analyzed. However, the presence of an active AP-1-binding site in the TNF-α promoter (56), and the notion that MAPK regulate AP-1 activity (41), strongly suggest that AP-1 represents a MAPK-inducible transcription factor necessary for TNF-α expression upon FcγR stimulation. Preliminary data in NK cells also indicate that IFN-γ expression after FcγRIIIA stimulation is MAPK dependent (Kanakaraj et al., manuscript in preparation). All together, our findings are consistent with the conclusion that MAPK play an essential role in allowing cytokine production in each leukocyte type upon stimulation with immune complexes. It remains to be investigated whether other FcγR-mediated functions, e.g., phagocytosis and cytotoxicity, are similarly regulated in a MAPK-dependent way.

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