Fcγ Receptor-mediated Mitogen-activated Protein Kinase Activation in Monocytes Is Independent of Ras*

(Received for publication, July 9, 1998)

Gabriela Sánchez-Mejorada and Carlos Rosales;
From the Immunology Department, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Mexico City 04510, Mexico

Receptors for the Fc portion of immunoglobulin molecules (FcR) present on leukocyte cell membranes mediate a large number of cellular responses that are very important in host defense, including phagocytosis, cell cytotoxicity, production and secretion of inflammatory mediators, and modulation of the immune response. Cross-linking of FcR with immune complexes leads, first to activation of protein-tyrosine kinases. The molecular events that follow and that transduce signals from these receptors to the nucleus are still poorly defined. We have investigated the signal transduction pathway from Fc receptors that leads to gene activation and production of cytokines in monocytes. Cross-linking of FcR, on the THP-1 monocytic cell line, by immune complexes resulted in both activation of the transcription factor NF-κB and interleukin 1 production. These responses were completely blocked by tyrosine kinase inhibitors. In contrast, expression of dominant negative mutants of Ras and Raf-1, in these cells, did not have any effect on FcR-mediated nuclear factor activation, suggesting that the mitogen-activated protein kinase (MAPK) signaling pathway was not used by these receptors. However, MAPK activation was easily detected by in vitro kinase assays, after FcR cross-linking with immune complexes. Using the specific MAPK/extracellular signal-regulated kinase (MAPK kinase) inhibitor PD98059, we found that MAPK activation is necessary for FcR-dependent activation of the nuclear factor NF-κB. These results strongly suggest that the signaling pathway from Fc receptors leading to expression of different genes important to leukocyte biology, initiates with tyrosine kinases and requires MAPK activation; but in contrast to other tyrosine kinase receptors, FcR-mediated MAPK activation does not involve Ras and Raf.

Antibodies (immunoglobulins) present two main functions in host defense: the binding to antigen via their antigen-combining sites and the mobilization of cellular defense mechanisms via their carboxyl-terminal Fc portion. Cross-linking of receptors for the Fc portion of immunoglobulin G molecules (FcγR)1 on many cells of the immune system triggers various functions such as phagocytosis, antibody-dependent cell-mediated cytotoxicity, generation of the respiratory burst, and production of inflammatory mediators and cytokines (1–3).

Three classes of FcR have been identified, FcyRI (CD64), FcyRII (CD32), and FcyRIII (CD16). They are coded for by different genes and differ in their relative avidity for IgG, molecular structure, and cellular distribution (4). Activation of FcγR as well as other immunoreceptors (such as TCR, BCR, and FcεRI) results in common molecular events involving activation of Src family kinases followed by activation of Syk family kinases (5–7). The particular kinases involved depend on the particular immunoreceptor tyrosine-based activation motif (ITAM) present on the cytoplasmic portion of each receptor (8, 9).

After FcγR aggregation and activation of protein-tyrosine kinases (10), several substrates are phosphorylated and other enzymes are also activated. Among them, phospholipase C γ1 and γ2 (11–14), phosphatidylinositol 3-kinase (15, 16), and paxillin (17), a cytoskeletal protein, have all been reported.

One of the major cellular responses initiated by FcγR cross-linking, specially in myelomonocytic and natural killer (NK) cells, is the activation of genes encoding cytokines important in inflammation, such as interleukin 1 (IL-1), IL-8, and tumor necrosis factor (TNF) (2, 18, 19). The signaling pathway from FcγR to the nucleus is not known, but it probably shares elements with the biochemical cascade used by other receptors known to activate gene transcription. In particular, receptors with intrinsic tyrosine kinase activity have been shown to induce transcription of genes via activation of the Ras signaling pathway (20), which turns on sequentially Ras, Raf-1, MEK, and mitogen-activated protein kinase (MAPK) (21, 22). MAPK, also known as extracellular signal-regulated kinase (ERK) (23) phosphorelays and activates several transcription factors (24, 25).

Due to the fact that recent reports indicate that MAPK is activated after FcγR cross-linking in various cell types (26–32), it has been assumed that the classical Ras pathway is activated upon FcγR signaling. However, no direct proof that Ras is used in FcγR signaling has been provided, except for a single report on NK cells (33).

Because activation of the transcription factor NF-κB is required for IL-1 gene induction (34–36), we decided to investigate directly if FcγR cross-linking on mononuclear cells resulted in activation of this nuclear factor, and then we used this

---

1 The abbreviations used are: FcγR, receptor(s) for the Fc portion of immunoglobulin G molecules; ERK, extracellular signal-regulated kinase; HA-MAPK, MAPK containing the influenza hemagglutinin epitope tag; IL-1, interleukin 1; IL-8, interleukin 8; MAPK, mitogen-activated protein kinase; MAP, mitogen-activated protein; MEK, MAPK/ERK kinase; TNF, tumor necrosis factor; MBF, myelin basic protein; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; GFP, green fluorescent protein; HA, hemagglutinin; ELISA, enzyme-linked immunosorbent assay; NK, natural killer; NF-κB, nuclear factor κB; IIC, insoluble immune complex.

* This work was supported by Grant 2356P/N from Consejo Nacional de Ciencia y Tecnología and Grant IN201797 from Dirección General de Asuntos del Personal Académico, Universidad Nacional Autónoma de México. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Immunology, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Apto. Postal 70228, Cd. Universitaria, México D.F. 04510, Mexico. Tel.: 52-5-822-3369; Fax: 52-5-822-3369; E-mail: carosa@servidor.unam.mx.

§ The abbreviations used are: FcγR, receptor(s) for the Fc portion of immunoglobulin G molecules; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; MEK, MAPK/ERK kinase; TNF, tumor necrosis factor; MBF, myelin basic protein; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; GFP, green fluorescent protein; HA, hemagglutinin; ELISA, enzyme-linked immunosorbent assay; NK, natural killer; NF-κB, nuclear factor κB; IIC, insoluble immune complex.

27610 This paper is available on line at http://www.jbc.org
response as a final read-out to examine the involvement of the several elements of the Ras pathway in FcγR signaling, leading to gene activation and cytokine production.

We found that stimulation of the THP-1 monocytic cell line with insoluble immune complexes results in production of IL-1, and also in activation of the nuclear factor NF-κB. Moreover, activation of this nuclear factor is mediated by MAPK but activation of this kinase does not seem to involve the classical pathway that is independent of Ras and Raf.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Reagents**—The following antibodies were used: Anti-PH anti-monoclonal antibody (catalog no. E171120, Transduction Laboratories, Lexington, KY), horseradish peroxidase-conjugated F(ab′)2 goat anti-mouse IgG (Cappel, Aurora, OH) The specific MEK (MAP kinase) inhibitor PD98059 was from New England Biolabs, Inc. (Beverly, MA). The plasmids HIV-luc and E18pal-luc were a generous gift from Dr. John Westwick and Dr. David A. Brenner of the University of North Carolina, Chapel Hill, NC. HIV-luc contains NF-κB-responsive elements within the human immunodeficiency virus long terminal repeat promoter placed upstream of the luciferase (luc) gene and directs the expression of luciferase in response to activation of the nuclear factor NF-κB. E18pal-luc activates luciferase transcription in response to the nuclear factor Ets. The plasmid encoding HA-MAPK was a gift from Mike Weber from the University of Virginia, Charlottesville, VA. E18pal-luc was selected by ELISA. Positive hybridomas secreted antibodies binding to the phosphorylated substrate bands were analyzed by autoradiography. To evaluate the amount of protein immunoprecipitated, an aliquot of the sample was separated and Western blotted with anti-MAPK antibody at 0.1 mg/ml in blocking buffer, for 1 h at room temperature. Membranes were washed with PBS six times for 5 min each and incubated with horseradish peroxidase-conjugated F(ab′)2 goat anti-mouse IgG (Cappel, Aurora, OH), for 1 h at room temperature. After washing, membranes were exposed to an X-ray film for 1 day at −70 °C.

**Cell Lysates**—Cells were lysed in buffer (150 mM NaCl, 5 mM EDTA, 10% FBS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 10 mM 2-mercaptoethanol, pH 7.5) containing 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 250 μM aprotinin, 25 μg/ml leupeptin, and 25 μg/ml pepstatin, for 15 min at 4 °C. Cell lysates were then cleared by centrifugation at 20,000 × g for 5 min and kept cold on ice.

**Western Blot**—Total cell lysates or MAP kinase immunoprecipitates were resolved on 12% SDS-PAGE. Proteins were then electrotransferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). Membranes were incubated in blocking buffer (1% bovine serum albumin, 5% nonfat dry milk (Carnation; Nestle Food Co., Glendale, CA) and 0.1% Tween 20 in PBS) overnight at room temperature. Membranes were subsequently probed with anti-pan ERK monoclonal antibody at 0.1 μg/ml in blocking buffer, for 1 h at room temperature. Membranes were washed with PBS six times for 5 min each and incubated with horseradish peroxidase-conjugated Fab′2 goat anti-mouse IgG (Cappel, Aurora, OH), for 1 h at room temperature. After washing, membranes were exposed to an X-ray film for 1 day at −70 °C.

**Cell Culture**—The human monocytic THP-1 cell line was maintained in RPMI 1640 medium (Life Technologies, Inc.), supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc., Grand Island, NY), 20 μM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin.

**IL-1 Measurement**—THP-1 cells (1 × 10⁶) were stimulated with 40 μM insoluble immune complexes in 0.5 ml of RPMI 1640 complete medium for various times (0–48 h) at 37 °C. At the end of the incubation time, cells were centrifuged at 20,000 × g and the supernatant collected and immediately frozen at −80 °C. Interleukin 1 was measured in the supernatants with an ELISA kit (Amersham, Buckinghamshire, United Kingdom) according to the manufacturer’s instructions. In some experiments, 30 μM PD98059 or 10 μM herbimycin A (Life Technologies, Inc.) was added 1 h before stimulation.

**Transfections**—THP-1 monocytic cells were transiently transfected with a DEAE-dextran method as described previously (41). Briefly, 1 × 10⁶ cells were transfected with 0.5 ml of serum-free RPMI 1640 medium, supplemented with 5 μg of plasmid DNA by mixing cells with 20 μl of DEAE-dextran (Pharmacia Biotech, Uppsala Sweden) for 20 min and after one wash, with 0.1 mM chloroquine for another hour at 37 °C. Twenty-four hours after transfection, cells were resuspended in 4 ml of serum-free RPMI 1640 medium and stimulated with 40 μM of insoluble immune complexes. Cells were collected after a 5-h incubation at 37 °C and lysed with a detergent lysis buffer (0.2% Triton X-100, 0.5 mM dithiothreitol, 2 mM EDTA). To evaluate transfection efficiencies in selected experiments, cells were transfected with the plasmid pGL3 control (Promega, Madison, WI), which constitutively expresses luciferase from the SV40 promoter. Cells were also transfected with the plasmid pEGFP-N1 (CLONTECH) containing the cDNA for the green fluorescent protein (GFP) under control of the cytomegalovirus promoter. Efficiency was estimated from the number of cells presenting green fluorescence at 24 h after transfection.

**Luciferase Activity**—Luciferase enzymatic activity was determined in cell lysates using a luminesimeter (Monolight 2010 Luminesimeter, Ann Arbor, MI). Briefly, 50 μl of cell lysate were mixed with 100 μl of buffer (30 mM tris-glycine, pH 7.8, 3 mM ATP, 15 mM MgSO₄, 10 mM dithiothreitol, 100 μM β-luciferin, pH 6.5). Light production was measured during 20 s.

**Cell Lysates**—Cells were lysed in buffer (150 mM NaCl, 5 mM EDTA, 50 mM HEPES, 0.5% sodium deoxycholate, 1% Nonidet P-40, 10 mM 2-mercaptoethanol, pH 7.5) containing 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 250 μg/ml aprotinin, 25 μg/ml leupeptin, and 25 μg/ml pepstatin, for 15 min at 4 °C. Cell lysates were then cleared by centrifugation at 20,000 × g for 5 min and kept cold on ice.

**Western Blot**—Total cell lysates or MAP kinase immunoprecipitates were resolved on 12% SDS-PAGE. Proteins were then electrotransferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). Membranes were incubated in blocking buffer (1% bovine serum albumin, 5% nonfat dry milk (Carnation; Nestle Food Co., Glendale, CA) and 0.1% Tween 20 in PBS) overnight at room temperature. Membranes were subsequently probed with anti-pan ERK monoclonal antibody at 0.1 μg/ml in blocking buffer, for 1 h at room temperature. Membranes were washed with PBS six times for 5 min each and incubated with horseradish peroxidase-conjugated Fab′2 goat anti-mouse IgG (Cappel, Aurora, OH), for 1 h at room temperature. After washing, membranes were exposed to an X-ray film for 1 day at −70 °C.
overnight culture to allow for cell recovery, the THP-1 cells were stimulated in various forms. HA-MAPK was immunoprecipitated from THP-1 cell lysates (1.5 × 10^6 cell equivalent) with 14 μg/ml of anti-HA monoclonal antibody 12CA5 (Boehringer Mannheim). The antibody was first incubated with cell lysates for 2 h and then 20 μl of protein A-Sepharose (Pharmacia Biotech, Uppsala, Sweden) were added and the mixture incubated for another 4 h at 4 °C. Sepharose beads were then washed once with cold RIPA buffer and four more times with cold washing buffer (0.25 M Tris-HCl, pH 7.5, 0.1 M NaCl). Immunoprecipitates were subjected to a kinase assay just as described above.

RESULTS

Fcγ Receptor Stimulation Induces Interleukin 1 Production—THP-1 monocytic cells were stimulated with insoluble immune complexes for various periods of time, and IL-1 produced and secreted in the supernatant was measured with a commercial ELISA kit. FcγR cross-linking by immune complexes induced a rapid and strong production of IL-1 by these cells (Fig. 1). IL-1 production reached a maximum (around 400 pg/ml) at about 24 h of stimulation. Previous treatment of cells with the selective tyrosine kinase inhibitor herbimycin A (44) abolished cytokine production, indicating that FcγR-mediated production of IL-1 requires protein-tyrosine kinase activity.

Fcγ Receptor Stimulation Induces Tyrosine Phosphorylation—Because treatment of cells with the selective tyrosine kinase inhibitor herbimycin A (44) abolished cytokine production, we decided to look more directly at the effect of this drug on the FcγR response. Stimulation of THP-1 cells with insoluble immune complexes caused rapid phosphorylation on tyrosine of several proteins. Prominent phosphotyrosine bands are increased at 1 min of stimulation at around 30, 35, 40, 44, and 70 kDa (Fig. 2). Treatment of cells with herbimycin A prior to stimulation completely abolished tyrosine phosphorylation of these proteins. Moreover, several other bands that were tyrosine-phosphorylated in the resting state also showed a significant reduction in presence of herbimycin A (Fig. 2). This result is in agreement with previous data indicating that Fc receptors recruit tyrosine kinases for their signaling (3, 10). It also shows that herbimycin A is working well and it is blocking the tyrosine phosphorylation needed for FcγR-mediated IL-1 production.

Fcγ Receptor Stimulation Induces Activation of the Nuclear Factor NF-κB—Because the IL-1 gene, as well as other early immediate genes (such as those for IL-8 and TNF), requires activation of the nuclear factor NF-κB for transcriptional activation (34, 45), we evaluated NF-κB activation in response to FcγR cross-linking in monocytes. THP-1 cells were transiently transfected with the NF-κB reporter plasmid, HIV-luc, and luciferase activity was measured in cell lysates. Stimulation of transfected THP-1 cells by immune complexes resulted in a strong activation of the nuclear factor NF-κB, as indicated by an increase (around 4-fold) in luciferase activity (Fig. 3A). Pretreatment of THP-1 cells with herbimycin A also completely blocked NF-κB activation (Fig. 3A). Specificity of this response was tested by transfecting cells with the plasmid E18pal-luc that activates luciferase transcription in response to activation of the nuclear factor Ets. Stimulation of THP-1 cells with insoluble immune complexes did not induce luciferase activity from this plasmid (Fig. 3B). Treatment of the cells with 15 μg/ml lipopolysaccharide induced luciferase activity from this plasmid (Fig. 3B), indicating that the response observed after immune complex stimulation is not due to general cell activation.

To confirm that only immune complexes were stimulating the cells via Fc receptors, transfected THP-1 cells were treated with various preparations of antibody and immune complexes (Fig. 4). None of the following stimuli caused activation of NF-κB as indicated by an increase in luciferase activity: ferritin, the protein used to form the immune complexes, F(ab9)2 fragments of the anti-ferritin antibodies, and immune complexes formed with these F(ab9)2 fragments and ferritin (Fig. 4). The complete IgG molecule of anti-ferritin antibodies caused only a small activation, while the insoluble immune complexes gave the optimal response previously observed (Fig. 4). These data collectively indicate that FcγR aggregation is responsible for nuclear factor activation and production of IL-1, and that both events require protein-tyrosine kinase activity to take place.

FcγR-dependent NF-κB Activation Is Independent of Ras—There is evidence that cross-linking of several immunoreceptors in leukocytes activates various elements of the Ras con-
mediated NF-κB reporter plasmid. HIV-luc, were placed in 4 ml of serum-free medium and left untreated or stimulated by 40 μl of IIC or by anti-flt3 receptor IgG, by F(ab)2 fragments of this anti-flt3 receptor antibody (III), or by immune complexes formed with flt3 receptor and the F(ab')2 fragments of this anti-flt3 receptor antibody (III). Five hours later, cells were collected and cell lysates prepared as described under "Experimental Procedures." Luciferase activity, representing NF-κB activation, was determined in a luminometer. Data are means ± S.E. of three different determinations.

sensus signaling pathway (26–32). To explore the possibility that FcR signaling leading to nuclear factor activation in monocytic cells also involved elements of the Ras pathway, THP-1 cells were co-transfected with the NF-κB reporter plasmid and an expression plasmid directing the synthesis of a mutant form of Ras or Raf. Transfected THP-1 cells were then stimulated with insoluble immune complexes and left untreated or stimulated by 40 μl of IIC or by 15 μg/ml lipopolysaccharide (III). Five hours later, luciferase activity was determined as described. Data are means ± S.E. of three different determinations.

**Fig. 3.** FcR receptor stimulation induces activation of the nuclear factor NF-κB. A, 3 × 10^6 THP-1 cells transiently transfected with the NF-κB reporter plasmid, HIV-luc, were placed in 4 ml of serum-free medium and left untreated or stimulated by 40 μl of IIC (III). Some cultures were treated with 10 μg/ml herbimycin A (III) for 60 min before IIC stimulation. Five hours later, cells were collected and cell lysates prepared as described under "Experimental Procedures." Luciferase activity, representing NF-κB activation, was determined in a luminometer. B, 3 × 10^6 THP-1 cells transiently transfected with the E18 pal-luc reporter plasmid, were placed in 4 ml of serum-free medium and left untreated or stimulated by 40 μl of IIC (III) or by 15 μg/ml lipopolysaccharide (III). Five hours later, luciferase activity was determined as described. Data are means ± S.E. of three different determinations.

**Fig. 4.** Immune complex stimulation is mediated by FcR receptors. 3 × 10^6 THP-1 cells, transiently transfected with the NF-κB reporter plasmid, HIV-luc, were placed in 4 ml of serum-free medium and left untreated or stimulated by 40 μl of IIC (III), by anti-ferritin IgG (III), by F(ab)2 fragments of this anti-ferritin antibody (III), or by immune complexes formed with ferritin and the F(ab')2 fragments of this anti-ferritin antibody (III). Five hours later, cells were collected and cell lysates prepared as described under "Experimental Procedures." Luciferase activity, representing NF-κB activation, was determined in a luminometer. Data are means ± S.E. of three different determinations.**

confirmed that, in these cells, the mutant forms of Ras were affecting Ras signaling activity as expected.

After Ras activation, the serine-threonine kinase Raf is the next element in the Ras signaling pathway (20, 46). To explore if this kinase was involved in FcR signaling in monocytes, THP-1 cells were also co-transfected with the NF-κB reporter plasmid and the dominant negative mutant form of Raf-1, Raf

FcR-dependent MAPK Activation Is Independent of Ras

**Fig. 4.** Immune complex stimulation is mediated by FcR receptors. 3 × 10^6 THP-1 cells, transiently transfected with the NF-κB reporter plasmid, HIV-luc, were placed in 4 ml of serum-free medium and left untreated or stimulated by 40 μl of IIC (III), by anti-ferritin IgG (III), by F(ab)2 fragments of this anti-ferritin antibody (III), or by immune complexes formed with ferritin and the F(ab')2 fragments of this anti-ferritin antibody (III). Five hours later, cells were collected and cell lysates prepared as described under "Experimental Procedures." Luciferase activity, representing NF-κB activation, was determined in a luminometer. Data are means ± S.E. of three different determinations.
stimulation with insoluble immune complexes resulted in complete inhibition of FcR-mediated NF-κB activation (Fig. 8). This result clearly showed the participation of MEK in the signal transduction pathway from Fc receptors leading to activation of this nuclear factor. Also, PD98059 affected the production of IL-1 by these cells after IIC stimulation. However, a concentration of PD98059 that completely blocked NF-κB activation (Fig. 8) inhibited IL-1 production only about 40% (Fig. 9). Increasing concentrations of the MEK inhibitor did not further block IL-1 production.

**FcγR Stimulation by Insoluble Immune Complexes Results in MAPK Activation**—Our results, described above, indicated that FcγR signaling in monocytes did not involve Ras and Raf, but clearly activated MEK and the nuclear factor NF-κB. To determine if FcγR was connecting MEK and NF-κB, we decided to look directly at MAPK activation by immune complex kinase assays. Stimulation of THP-1 cells with IIC resulted in a clear and strong stimulation of MAPK activity (Fig. 10). The kinetics of FcγR-mediated activation of MAPK showed maximal activity by 1 min of IIC stimulation. This activity had returned to basal levels around 3 min (Fig. 10). This result was in agreement with previous reports that FcγR cross-linking results in MAPK activation. Moreover, treatment of THP-1 cells with PD98059 for 1 h before IIC stimulation demonstrated that FcγR-mediated MAPK activation is completely blocked when MEK activation is inhibited (Fig. 11). These data suggested that, in FcγR signaling, MEK activation is an upstream event of MAP kinase activation, which then leads to nuclear factor NF-κB activation.

**FcγR-dependent MAPK Activation Is Independent of Ras**—Data presented above indicated that MAPK is clearly activated by insoluble immune complexes and that the Ras and Raf dominant negative constructs did not inhibit NF-κB activation. It was then important to determine directly if these dominant negative mutants did not block FcγR-dependent MAPK activation. It is not easy to see the effect of these mutants on MAPK directly because the efficiency of transfection of monocytes is rather low, approximately 5%, as estimated by transfections with a plasmid that expresses the GFP (data not shown). Therefore, the effect of the dominant negative constructs is only on those cells that were successfully transfected.

To test for the effect of the negative mutants on MAPK activity of only transfected cells, THP-1 cells were co-transfected with a plasmid that expresses the GFP (data not shown). To this end, THP-1 cells were co-transfected with the vector pZIP or with the dominant negative mutant form of Ras N17 cloned into pZIP. Luciferase activity, representing NF-κB activation, was determined 5 h later. Data are means ± S.E. of five different determinations. B, THP-1 cells co-transfected with the Ras-reporter system Gal-Elk/5XGal-luc, and pZIP, Ras N17, or the activated oncogenic form of Ras L61. After transfection cells were serum-starved for 48 h and then left untreated or stimulated with 10% serum. Luciferase activity, indicating Ras pathway activation, was determined 5 h later. Data are means ± S.E. of three different determinations.

**FcγR-dependent MAPK Activation Is Independent of Ras**—Data presented above indicated that MAPK is clearly activated by insoluble immune complexes and that the Ras and Raf dominant negative constructs did not inhibit NF-κB activation. It was then important to determine directly if these dominant negative mutants did not block FcγR-dependent MAPK activation. It is not easy to see the effect of these mutants on MAPK directly because the efficiency of transfection of monocytes is rather low, approximately 5%, as estimated by transfections with a plasmid that expresses the GFP (data not shown). Therefore, the effect of the dominant negative constructs is only on those cells that were successfully transfected.

To test for the effect of the negative mutants on MAPK activity of only transfected cells, THP-1 cells were co-transfected with a plasmid that has the HA epitope tag and the corresponding Ras N17 or Raf 23–284 dominant negative mutants. After transfection cells were stimulated with insoluble immune complexes and cell lysates prepared. HA-MAPK was immunoprecipitated from these lysates with the HA-specific
monoclonal antibody 12CA5, and its activity tested by in vitro kinase assays. The expression of Ras N17 did not inhibit the kinase activity stimulated by FcγR cross-linking with immune complexes (Fig. 12A). Control experiments using serum stimulation of transfected THP-1 cells indicated that Ras N17 could block MAPK activation induced by a different stimulus (Fig. 12C).

In a similar fashion we tested the effect of the Raf 23–284 dominant negative mutant on HA-MAPK activation after transfected THP-1 cells were treated with insoluble immune complexes. Expression of this mutant did not inhibit FcγR-dependent activation of MAPK (Fig. 13A). To confirm the efficacy of this negative constructs, transfected THP-1 cells were stimulated with serum and HA-MAPK activity measured in an in vitro kinase assay. Similarly to previous results, Raf 23–284 inhibited activation of MAPK induced by serum (Fig. 13C). These results all together support the idea that FcγR signaling does not use Ras or Raf to activate MEK and MAPK in the pathway that leads to nuclear factor NF-κB activation.

**DISCUSSION**

Membrane receptors for the Fc portion of immunoglobulin G class antibodies (FcγR) are expressed on almost every type of hematopoietic cells. Cross-linking of these receptors by aggregated IgG, in the form of antigen-antibody complexes, triggers a very wide array of responses important for host defense and for modulation of the immune response (3). There is a great deal of interest in understanding the signaling mechanisms that lead to the various cell responses. One of the most important functions activated by immune complexes on myelomono
cytic and NK cells is the production of inflammatory cytokines such as IL-1, IL-8, and TNF. This means that FcγR cross-linking induces transcription of the genes encoding these response (2, 18). To have initiation of transcription of these genes, activation of diverse nuclear factors has to take place. Very little is known about the signal transduction pathway from FcγR to nuclear factors in the cell nucleus.

It has been observed that the 5’ regulatory sequences of the cytokine genes induced by FcγR cross-linking (IL-1, IL-8, TNF), all contain sites for the nuclear factor NF-κB (34–36). We, therefore reasoned that NF-κB activation would be an ideal
immune complexes indeed caused NF-κB. In addition, the IL-1 and activation of the nuclear factor NF-κB on THP-1 monocytic cells also resulted in production of cytokines, leading to activation of nuclear factors and cytokine production.

Receptor cross-linking and activation of Src family (48, 49) induces receptor cross-linking and activation of Src family kinases, including Syk/ZAP-70 family related kinases (50–52), which associate with phosphorylation sites important for signal transduction. Polyvalent ligands interact with the phosphorylated ITAM in the cytoplasmic tail of the FcγR, and also the antigen receptors on T lymphocytes and B lymphocytes, present a common feature that is important for signaling by all these immunoreceptors (5). They all contain a conserved motif, known as ITAM for immunoreceptor tyrosine-based activation motif (7, 9), which contains phosphorylation sites important for signal transduction. Polyvalent ligands induce receptor cross-linking and activation of Src family (48, 49) and Syk/ZAP-70 family related kinases (50–52), which associate with the phosphorylated ITAM in the cytoplasmic tail of the receptor. After FcγR aggregation, these activated kinases catalyze the phosphorylation of cellular substrates on tyrosine residues (Fig. 2) (10).

Activation of tyrosine kinases leading finally to nuclear factor activation resembles the signal transduction pathway defined for receptor tyrosine kinases that induces mitogenic signals in response to growth factors such as epidermal growth factor and platelet-derived growth factor. This signaling pathway is also known as the Ras pathway (20, 37). Receptor tyrosine kinase activity induces transient formation of Ras-GTP and activation of Raf kinase at the membrane, followed by sequential activation of MEK and MAPK (22). MAPK is then responsible for activation of several transcription factors (25).

Moreover, recent reports indicate that MAPK is activated after FcγR cross-linking in various cell types (26–32), supporting the idea that the Ras pathway is used by FcγR to induce gene transcription. However, no direct evidence of Ras involvement has been provided in these reports. A recent publication indicates that an increase in Ras-GTP was observed after cross-linking of FcγRIIIA on NK cells (33), further supporting the idea for FcγR using the Ras signaling pathway. Because MAPK activation does not necessarily mean that the Ras pathway is being utilized, and because diverse signaling pathways may be used in different cell types, a direct evaluation for the involvement of the various elements of the Ras pathway in different cell types becomes important.

In this report, we investigated the participation of the Ras signaling pathway in the activation of cytokine genes upon FcγR cross-linking on monocytes, probing for the different elements involved in this signaling cascade.

The involvement of Ras signal pathway elements in the FcγR signal transduction pathway was investigated by measuring...
NF-κB activation in the presence of specific inhibitors. Expression of either wild-type Ras or the dominant negative mutant Ras N17, in THP-1 cells did not have any effect on FcγR-mediated NF-κB activation. Similarly, expression of the dominant negative mutant form of Raf-1, Raf 23–284, did not prevent NF-κB activation by insoluble immune complexes. These mutants have been shown to efficiently block signal transduction via the classical Ras pathway (38, 39) (see also Figs. 5B and 6B). These results were obtained in the monocytic cell line THP-1. In order to confirm that this was a more general behavior of monocytes, the experiments were repeated in a different monocytic cell line. Data on U937 cells (Fig. 7) also showed that dominant negative mutant forms of Ras and Raf did not have any effect on activation of NF-κB by immune complexes. Thus, in monocytic cells, Ras and Raf are not directly involved in the FcγR-mediated signal transduction pathway that leads to activation of NF-κB. Although Ras and Raf did not seem to be involved in activation of the nuclear factor NF-κB, many reports have indicated that MAPK is activated after cross-linking of FcyR, so downstream elements in the Ras pathway may still be used for FcyR signaling.

MEK is a cytoplasmic serine-threonine kinase that directly activates MAPK (47) and it is found downstream of Raf in the Ras signaling cascade activated by receptor tyrosine kinases (20). The selective MEK inhibitor, PD98059, completely blocked NF-κB activation, indicating that MEK participated in FcyR-mediated signal transduction. Immune complex stimulation of THP-1 cells also resulted in activation of MAPK, as indicated by in vitro kinase assays using MBP as substrate for the kinase (Fig. 10). In addition, PD98059 was able to block MAPK activation back to basal levels (Fig. 11). This established a link between FcyR and the pathway MEK, MAPK, and NF-κB. Moreover, evaluating directly MAPK activity only in transfected cells by using the HA-MAPK, it was found that the dominant negative mutants of Ras and Raf did not affect FcyR-dependent activation of this kinase (Figs. 12 and 13). These data further supported the idea that FcyR cross-linking activates a MAPK pathway without using the proteins Ras and Raf.

Treatment of THP-1 cells with a concentration of PD98059 that completely blocked NF-κB activation resulted only in partial inhibition of IL-1 production. To initiate transcription of the IL-1 gene, more than one nuclear factor is required. The promoter region of this and many other genes contains multiple and different sites for nuclear factor binding (36, 53, 54). NF-κB is one of the nuclear factors identified to bind at the 5’ regulatory region of the IL-1 gene (34–36). Thus, blockage of MEK and MAPK activity by the inhibitor PD98059 resulted in failure to activate NF-κB, and therefore reduced FcyR-mediated IL-1 production in monocytes. Full transcriptional activation of the IL-1 gene needs NF-κB and cooperation from other transcription factors (36, 53, 54). This cooperation effect has been demonstrated in the THP-1 cells for the IL-8 gene (41).

Stimulation of monocytic cells was done with immune complexes. These interact and stimulate all types of Fc receptors. However, it is known that the various types of Fc receptors activate different cellular responses (1, 3, 55), so it would be very interesting to know what type of receptor is responsible for the MAPK and nuclear factor activations that are connected to the induction of cytokine production by monocytes. We are now investigating this by stimulating cells with the monoclonal antibodies IV.3 and 3G8, which are specific for FcγRII and FcγRIII, respectively. Our preliminary results indicate that both receptors FcγRII and FcγRIII, expressed on monocytic cells, are capable of inducing NF-κB activation, although they seem to do it at lower levels compared with immune complex stimulation.

How MEK is activated without Raf participation is not clear, but there is evidence for diverse ways to activate this kinase (56). MEK is phosphorylated and activated by an upstream kinase, which in the case of many serum growth factor receptors is the proto-oncogene Raf. Direct action of Raf over MEK is clearly established (57, 58). Another MEK kinase, also called MEKK, has been described upstream of MEK in the signaling pathway from a different type of receptors (56). MEKK is the mammalian counterpart of the yeast protein kinases Byr2 (from Schizosaccharomyces pombe) and Ste11 (from Saccharomyces cerevisiae), which in turn activate the protein kinases Byr1 and Ste7, respectively. Byr1 and Ste7 have considerably sequence homology to MEK and function in the pheromone-induced signaling pathway that leads to mating (56). Some pheromone receptors have a seven-membrane-spanning ser-
FIG. 14. Model for FcR-mediated signal transduction in monocytic THP-1 cells. FcR are aggregated by immune complexes formed by IgG bound to polyvalent antigens. Tyrosine kinases, such as Syk, are activated and bound to the tyrosine-phosphorylated receptor. Direct substrates for these tyrosine kinases are not clearly defined. The signaling pathway leads to activation of MEK, which in turn phosphorylates and activates MAPK. These enzymes are then responsible for activation of the nuclear factor NF-xB. There is no involvement of Ras and Raf proteins upstream of MEK.

pentine structure coupled to G proteins. Similarly, in mammalian cells, some serpine receptors coupled to heterotrimeric Gt2 proteins can stimulate DNA synthesis via MAPK activation (59). For example, Gt2-coupled acetylcholine muscarinic M2 receptors have been reported to activate MEK and MAPK independently of Raf. The kinase responsible for this effect is MEKK (59). Moreover, in mouse (NIH3T3) and rat (Rat1a) fibroblasts, MEK and MAPK are activated in response to epidermal growth factor (recognized by a receptor tyrosine kinase) and also in response to thrombin (recognized by a serpine G protein-coupled receptor). Raf is activated by epidermal growth factor but not thrombin (60). It seems, then, that MEKK is a conserved kinase for the regulation of G protein-coupled signal pathways in yeast and vertebrates and Raf represents a divergence in vertebrates from the yeast rho-like protein/tyrosine protein kinase system (59, 61). Whether G protein subunits activate MEKK directly or through an unknown intermediary molecule remains to be determined (61).

FcR are not associated with G proteins. The kinase responsible for MEK activation after immune complexes stimulation remains unknown. Because G proteins may activate MEKK indirectly, it may be possible that this kinase is also used by FcR to activate MEK. Another possibility is that FcR may activate MEK via a different and yet undescribed kinase that has MEKK kinase properties. It will be interesting to determine the involvement of MEK in FcR signaling to the nucleus to activate gene transcription.

FcR signaling initiates with tyrosine phosphorylation (10). Herbimycin A, a selective inhibitor of tyrosine kinases, completely blocked both IL-1 production and activation of the NF-xB-driven reporter plasmid in transient transfection assays, confirming that FcR aggregation triggers activation of tyrosine kinases (see Fig. 2) as an early event in the signal transduction pathway from Fc receptors to gene activation and production of cytokines in monocytes. Syk kinase (72 kDa) has been implicated in FcR signaling in several cell types. Syk belongs to the ZAP-70 kinase family. These enzymes are not myristoylated and therefore are exclusively cytoplasmic. Syk is present in all hematopoietic cells, whereas ZAP-70 is expressed in T cells and NK cells (62). In mast cells (RBL-2H3), MAPK activation has been clearly shown to be dependent of Syk, probably through the GTP/GDP exchange factor Vav (63). The link between FcER and MAPK may also be through Shc, which is phosphorylated by Syk and then binds to Grb2. This adaptor protein is known to associate with Sos to activate Ras upstream of MAPK (64), although in mast cells the FcER for IgE seems to connect Syk to MAPK via Ras. In monocytes, we did not find evidence for Ras or Raf involvement in MEK and MAPK activation after FcER cross-linking. Syk is the most likely tyrosine kinase involved in FcER signal transduction in THP-1 monocytic cells. In this report we did not look directly at Syk, but it will be interesting to confirm that Syk activation is required for MEK and MAPK activation in THP-1 cells. The mechanism that Syk may use to activate this downstream kinases bypassing Ras and Raf is unknown, but as discussed above, it may be through activation of MEKK. As of this report, there are no studies directly looking for a functional interaction between Syk and MEKK in any cell type.

Taken together, data presented in this work strongly suggest that the monocyte signaling pathway from Fc receptors leading to expression of different genes important to leukocyte biology (Fig. 14), initiates with tyrosine kinases and requires MAPK activation, but in contrast to other tyrosine kinase receptors, FcR-mediated MAPK activation does not involve Ras and Raf.

Acknowledgments—we thank Dr. John Westwick and Dr. David A. Brenner (University of North Carolina, Chapel Hill, NC) for generously donating the HIV-luc and the E18pal-luc plasmids, and Dr. Mike Weber (University of Virginia, Charlottesville, VA) for the HA-MAPK. We especially thank Dr. Channing Der (University of North Carolina, Chapel Hill, NC) for donating all normal or mutant forms of Ras and Raf. We also thank Michelle Soriano Leish for purifying the anti-phosphotyrosine monoclonal antibody AFT8, and Nancy Mora Perez for excellent technical assistance.

REFERENCES

1. Unkeless, J. C., Boros, P., and Fein, M. (1992) in Inflammation: Basic Principles and Clinical Correlates (Galili, J. L., Goldstein, I. M., and Snyderman, R., eds) 2nd Ed., pp. 497–510, Raven Press, Ltd., New York
2. Van de Winkel, J. G. J., and Capel, P. J. (1993) Immunol. Today 14, 215–221
3. Sánchez-Mejorada, G., and Rosales, C. (1996) J. Leukocyte Biol. 63, 521–533
4. Ravetch, J. V., and Kinet, J. P. (1991) Annu. Rev. Immunol. 9, 457–492
5. Keegan, A. D., and Paul, W. E. (1992) Immunol. Today 13, 63–68
6. Weiss, A., and Littman, D. R. (1994) Cell 76, 263–274
7. Lamb, C. J. (1995) J. Immunol. 155, 5281–5285
8. Lamb, C. J., and Johnson, S. A. (1995) Immunol. Lett. 44, 77–80
9. Isakov, N. (1997) J. Leukocyte Biol. 61, 6–16
10. Santiana, C., Noris, G., Espinosa, B., and Ortega, E. (1996) J. Leukocyte Biol. 60, 433–440
11. Azzoni, L., Kamoun, M., Saleedo, T. W., Kanakaraj, P., and Perussia, B. (1992) J. Exp. Med. 176, 1745–1759
12. Ting, A.-T., Karnitz, L. M., Schoon, H. A., Abraham, R. T., and Leibson, P. J. (1992) J. Exp. Med. 176, 1751–1755
13. Liao, F., Sing, H. S., and Rhee, S. G. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 3699–3703
14. Shen, Z., Lin, C.-T., and Unkeless, J. C. (1994) J. Immunol. 152, 3017–3023
15. Ninoyima, N., Hazeki, K., Fukui, Y., Seya, T., Okada, T., Hazeki, O., and Ui, M. (1994) J. Biol. Chem. 269, 22732–22737
16. Kanakaraj, P., Duckworth, B., Azzoni, L., Kamoun, M., Cantley, L. C., and Perussia, B. (1994) J. Exp. Med. 179, 551–558
17. Greenberg, S., Chang, P., and Silverstein, S. C. (1994) J. Biol. Chem. 269, 3997–3902
18. Anegon, I., Cuturi, M. C., Trinchieri, G., and Perussia, B. (1988) J. Exp. Med. 167, 452–472
19. Polat, G. L., Lauder, J., Fabian, I., and Passwell, J. H. (1993) Immunology 88, 287–292
20. Egan, S. E., and Weinberg, R. A. (1993) Nature 365, 781–783
21. Macdonald, S. O., Crews, C. M., Wu, L., Driller, J., Clark, R., Erikson, R. L., and McCormick, F. (1993) Mol. Cell. Biol. 13, 6615–6620
22. Marshall, C. J. (1994) Curr. Opin. Genet. Dev. 4, 82–89
23. Benezet, J. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5889–5892
24. Gille, H., Sharaoka, A., and Perisic, O. (1996) Nature 385, 141–147
25. Hill, C. S., and Treisman, R. (1995) Cell 80, 199–211
26. Durden, D. L., Kim, H. M., Calore, B., and Liu, Y. (1995) J. Immunol. 154, 4039–4047
27. Hiraseawa, N., Santini, F., and Bevan, M. A. (1995) J. Immunol. 154, 5391–5402
28. Izquierdo, M., Levers, S., Marshall, C. J., and Cantrell, D. A. (1993) J. Exp. Med. 179, 1199–1208
29. Casillas, A., Hanekom, C., Williams, K., Katz, R., and Nel, A. E. (1991) J. Biol. Chem. 266, 19088–19094
30. Trotta, R., Kanakaraj, P., and Perussia, B. (1996) J. Exp. Med. 184, 1027–1035
31. Milella, M., Gismondi, A., Roncaisi, P., Bisogno, L., Palmieri, G., Frati, L., Cifone, M. G., and Santoni, A. (1997) J. Immunol. 158, 3148–3154
32. Karimi, K., and Lennartz, M. R. (1998) Inflammation 22, 67–82
33. Galandrini, R., Palmieri, G., Piccoli, M., Frati, L., and Santoni, A. (1996) J. Exp. Med. 183, 179–186
34. Haskell, L., Johnson, C., Eierman, D., Becker, S., and Warren, K. (1988) J. Immunol. 140, 1690–1694
35. Cogswell, J. P., Godlevski, M. M., Wisely, G. B., Clay, W. C., Leesnitzer, L. M., Ways, J. P., and Gray, J. G. (1994) J. Immunol. 153, 712–723
36. Haskill, S., Johnson, C., Eierman, D., Becker, S., and Warren, K. (1988) J. Immunol. 140, 1690–1694
37. Cogswell, J. P., Godlevski, M. M., Wisely, G. B., Clay, W. C., Leesnitzer, L. M., Ways, J. P., and Gray, J. G. (1994) J. Immunol. 153, 712–723
38. Hiscott, J., Marois, J., Garoufalis, J., D’Addario, M., Roulston, A., Kwan, I., Pepin, N., Lacoste, J., Nguyen, H., Bensi, G., and Fenton, M. (1993) Mol. Cell. Biol. 13, 6231–6240
39. Khosravi-far, R., and Channing, D. J. (1994) Cancer Metast. Rev. 13, 67–89
40. Chen, S. Y., Huff, S. Y., Lai, C. C., Der, C. J., and Powers, S. (1994) Oncogene 9, 2691–2698
41. Hauser, C. A., Westwick, J. K., and Quilliam, L. A. (1995) Methods Enzymol. 255, 412–426
42. Rosales, C., and Juliano, R. (1996) Cancer Res. 56, 2302–2305
43. Schook, L. B. (1987) Monoclonal Antibody Production: Techniques and Applications, Marcel Dekker, New York
44. Zola, H. (1987) Monoclonal Antibodies: A Manual of Techniques, CRC Press, Boca Raton, Fl.
45. Uehara, Y., and Fukazawa, H. (1994) Methods Enzymol. 210, 370–379
46. Sporn, S. A., Eierman, D. F., Johnson, C. E., Morris, J., Martin, G., Ladner, M., and Haskell, S. (1990) J. Immunol. 144, 4434–4441
47. Vojtek, A. B., Hollenberg, S. M., and Cooper, J. A. (1993) Cell 74, 205–214
48. Payne, D. M., Roissamando, A. J., Martino, P., Erickson, A. K., Her, J.-H., Shabazi, A., and Hunt, D. F. (1998) Immunol. Rev. 165, 133–142
49. Wang, A. V. T., Scholl, P. R., and Geha, R. S. (1994) J. Exp. Med. 180, 1165–1170
50. Viver, E., da Silva, A. J., Ackerley, M., Levine, H., Rudd, C. E., and Anderson, P. (1993) Eur. J. Immunol. 23, 1872–1876
51. Kiener, P. A., Rankin, B. M., Burkhardt, A. L., Schieven, G. L., Gilliland, L. K., Rowley, R. B., Bolen, J. B., and Ledbetter, J. A. (1993) J. Biol. Chem. 268, 24442–24448
52. Darby, C., Geahlen, R. L., and Schreiber, A. D. (1994) J. Immunol. 152, 5429–5437
53. Gray, J. G., Chandra, G., Clay, W. C., Stinnett, S. W., Haneline, S. A., Lorenz, J. J., Patel, I. R., Wisely, G. B., Furdon, P. J., Taylor, J. D., and Kost, T. A. (1993) Mol. Cell. Biol. 13, 6678–6689
54. Scharenberg, A., Faito, K., Bonagura, C. A., Galson, D. L., Fenton, M. J., Webb, A. C., and Auren, P. E. (1993) Mol. Cell. Biol. 13, 1322–1324
55. Erbe, D. V., Pfefferkorn, E. R., and Fanger, M. W. (1991) J. Immunol. 146, 3145–3151
56. Lange-Carter, C. A., Pleiman, C. M., Gardner, A. M., Blumer, K. J., and Johnson, G. L. (1993) Science 260, 315–319
57. Pyriakis, J. M., App, H., Zhang, X.-P., Banerjee, P., Brautigan, D. L., Rapp, U. R., and Avruch, J. (1992) Nature 358, 417–421
58. Howe, L. R., Leevers, S. J., Gimenez, N., Nakielny, S., Cohen, P., and Marshall, C. J. (1992) Cell 71, 335–342
59. Johnson, G. L., Gardner, A. M., Lange-Carter, C., Qian, N. X., Russell, M., and Winz, S. (1994) J. Biol. Chem. 269, 17896–17901
60. Gardner, A. M., Vaillancourt, R. R., and Johnson, G. L. (1993) J. Biol. Chem. 268, 17896–17901
61. Crews, C. M., and Erickson, R. L. (1993) Cell 74, 215–217
62. Agarwal, A., Saleem, P., and Robbins, K. C. (1993) J. Biol. Chem. 268, 15900–15905
63. Hiraiwa, N., Scharenberg, A., Yamamura, H., Beaven, M. A., and Kinet, J. P. (1995) J. Biol. Chem. 270, 10960–10967
64. Jabril-Cuenod, B., Zhang, C., Scharenberg, A. M., Paolini, R., Numerof, R., Beaven, M. A., and Kinet, J. P. (1996) J. Biol. Chem. 271, 16268–16272