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Signaling by the Mpl Receptor Involves IKK and NF-κB

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Abstract Binding of tumor necrosis factor-α (TNF-α) to its receptor activates IKK complex, which leads to inducement of NF-κB activity. Here we report that activation of Mpl ligand is also linked to IKK and NF-κB activity. Mpl ligand, also known as thrombopoietin (TPO) or megakaryocyte growth and development factor (MGDF), induces megakaryocyte differentiation and inhibition of mitotic proliferation, followed by induction of polyploidization and fragmentation into platelets. The latter process is often observed in megakaryocytes undergoing apoptosis. Treatment of a Mpl ligand-responding megakaryocytic cell line with this cytokine led to an immediate, transient increase in IKK activity followed by a profound decrease in this kinase activity over time. This decrease was not due to an effect on the levels of the IKK regulatory components IKKα and IKKβ. Proliferating megakaryocytes displayed a constitutive DNA-binding activity of NF-κB p50 homodimers and of NF-κB p50–p65 heterodimers. As expected, reduced IKK activity in Mpl ligand-treated cells was associated with a significant reduction in NF-κB DNA binding activity and in the activity of a NF-κB-dependent promoter. Our study is thus the first to identify a constitutive NF-κB activity in proliferating megakaryocytes as well as to describe a link between Mpl receptor signaling and IKK and NF-κB activities. Since a variety of proliferation-promoting genes and anti-apoptotic mechanisms are activated by NF-κB, retaining its low levels would be one potential mechanism by which inhibition of mitotic proliferation is maintained and apoptosis is promoted during late megakaryopoiesis. J. Cell. Biochem. 85: 523–535, 2002.

Key words: megakaryocyte; Mpl receptor; IKK; NF-κB

Platelets arise from a precursor cell, the megakaryocyte, which undergoes polyploidization. Many facets of megakaryocyte differentiation, such as the mechanism that leads to commitment, signal transduction that triggers polyploidization, and the interplay of gene expression that initiates cytoplasmic maturation have been gradually dissected in recent years. Although much of the detailed mechanism concerning megakaryopoiesis is still unclear, it is known that c-Mpl ligand (also referred to as thrombopoietin, TPO, or megakaryocyte growth and development factor, MGDF) is the primary cytokine that regulates megakaryopoiesis [Kaushansky, 1995]. This cytokine promotes expansion of cells in this lineage via mitotic proliferation, induces expression of differentiation markers, and induces an endomitotic cell cycle resulting in polyploidization as well as cellular maturation.

To elucidate its effect on megakaryocyte polyploidization and differentiation, intense research has been done on Mpl ligand-mediated signal transduction. Signaling pathways that involve Ras, Grb2, Shc, PKC, Jak2, Stat, and ERKs have been implicated. Among these, the Jak2-Stat pathway appears to constitute an essential element for programming megakaryocyte differentiation, since hematopoietic liver progenitors of Jak2-deficient mice failed to produce megakaryocytes in response to Mpl ligand [Parganas et al., 1998]. Ras [Matsumura et al., 1998] or an ERK activating kinase, MEK1 [Rouyez et al., 1997], were implicated in Mpl ligand signaling [Hong et al., 1998]. Recent studies also described the involvement of phos-

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phatidylinositol 3-kinase as well as of a MEK-phosphorylating activity of protein kinase Czeta in Mpl-mediated signaling [Geddis et al., 2001; Rojnuckarin et al., 2001]. As described above, Mpl ligand promotes within the same lineage different cell cycles (mitotic and endomitotic). It is then reasonable to suggest that it exerts its effects on these cell cycles via different signaling pathways and/or via opposite effects of the same signaling route, depending on the developmental state of the megakaryocyte.

Numerous studies on nuclear factor kappa B (NF-κB) have placed this transcription factor in the center of biological processes such as the inflammatory response [Baeuerle and Henkel, 1994], proliferation [Bellas et al., 1995], survival, and anti-apoptosis [Liu et al., 1996; Wang et al., 1998]. Nuclear localization and activation of NF-κB involves the removal of its cognate cytoplasmic inhibitor, IκB [Baeuerle and Baltimore, 1988]. IκB is targeted for degradation by phosphorylation in response to environmental cues [Beg et al., 1993; Henkel et al., 1993; Brown et al., 1995; Chen et al., 1995; Traenckner et al., 1995]. Consequently, the activity of IκB kinase, IKK, must be regulated in a similar fashion [Ling et al., 1998]. Several signaling pathways have been linked to IKK activation, including the phosphatidylinositol 3-kinase pathway [reviewed in Penninger and Woodgett, 2001]. In this study, we report a link between the IKK/NF-κB signaling pathway and activation of the Mpl receptor in megakaryocytes. We found that these cells, at a proliferation mode, display a constitutive DNA-binding activity of NF-κB p50 homodimers and of NF-κB p50–p65 heterodimers. Mpl ligand induces an immediate, transient increase in IKK activity followed by a significant reduction in IKK and NF-κB activities over time. The latter coincides with a decrease in mitotic proliferation and an augmentation in the fraction of apoptotic cells.

MATERIALS AND METHODS

Cell Culture and Ploidy Analysis

Rat bone marrow cultures were prepared as previously described [Ravid et al., 1991; Wang et al., 1995]. Y10 mouse megakaryocytes [Zhang et al., 1998], derived from L8057 megakaryocytes [Ishida et al., 1993], were maintained in F12 nutrient mixture plus 10% fetal bovine serum (FBS) (Gibco/BRL, Gaithersburg, MD). Cells were induced to differentiate by growing in Iscove's modified Dulbecco's medium (IMDM) (Gibco/BRL) containing 10% FBS in the presence of 25 ng/ml of an Mpl ligand, PEG-rHu-MGDF (MGDF) (generously supplied by Amgen, Thousand Oaks, CA) for indicated times. This compound is reported to have the biological features of native Mpl ligand, such as effects on megakaryopoiesis, ploidy, and platelet level [Kabaya et al., 1996; Nichol, 1996]. When indicated, murine tumor necrosis factor-α (TNF-α) was used at a concentration of 20 ng/ml (R & D Systems, Minneapolis, MN). Y10 cells, treated or not treated with MGDF, were subjected to ploidy analysis as we described before [Zhang et al., 1998].

Western Blot Analysis and Immunoprecipitation

For Western blotting, cells were collected, washed with phosphate buffered saline (PBS), pH 7.4, and the pelleted cells were treated in a buffer containing 250 mM NaCl, 5 mM EDTA, 0.5% NP-40, complete protease inhibitor (Boehringer Mannhem, Indianapolis, IN). Samples were incubated on ice for 15 min, followed by centrifugation in a microcentrifuge at top speed for 10 min at 4°C. Supernatants were recovered, and protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Proteins (15 or 75 μg) were analyzed by SDS–PAGE and electrophoretically transferred from the gel onto a nitrocellulose membrane (Schleicher & Schull, Keene, NH) in a buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol. Proteins on the membrane were visualized by Ponceau S staining (0.5% ponceau S (Sigma, St. Louis, MO), 5% acetic acid) to confirm equal loading. After destaining with water, the membrane was washed in TBS (10 mM Tris, pH 8.0/150 mM NaCl) and blocked for 1 h with TBST (TBS with 0.1% Tween-20) containing 5% dry milk. The blot was incubated for 1 hr at room temperature in 10 ml TBST supplemented with the appropriate antibody. The blot was washed four times, 10 min each, and incubated for 1 h with HRP-conjugated anti-rabbit antibody (Santa Cruz Biotechnology, Inc. Santa Cruz, CA), at 1:1,500 dilution in TBST. The blot was then washed four times, each for 10 min, with TBST and the Enhanced Chemiluminescence system (Amersham, Arlington Heights, IL) was used for detection of immunologically reacting proteins, as instructed by the manufacturer. The primary antibodies used were as follows: 7 μg/

10 ml of our anti-IKKβ, 1:500 dilution of anti-phosphorylated IκB (New England Biolab, Beverly, MA), 5 μg/10 ml of anti-IκB, 7 μg/10 ml of anti-IKKα (both from Santa Cruz Biotechnology) and 5 μg/10 ml of phospho-specific p44/42 MAP kinase (Thr202/Tyr204), and anti MAP kinase (from B (New England Biolab)).

For immunoprecipitation or kinase assays, cells were collected, washed with PBS, and then incubated on ice in 100μl of lysis buffer containing 20 mM HEPES pH 7.9, 1 mM EDTA, 0.4 M NaCl, 0.1% Tween 20, 1 mM DTT, 10% glycerol, complete protease inhibitor (Boehringer Mannhem), 1 mM sodium orthovanadate, 1 mM sodium fluoride, 20 mM β-glycerophosphate. Samples were sonicated twice for 15 s. Debris were removed by centrifuging at a top speed for 10 min at 4°C in a microcentrifuge. Bio-Rad protein assay was used to determine protein concentration of the lysates. For each sample, 300 μg protein (100 μg proteins for Hela cells) was used for immunoprecipitation in 250 μl of lysis buffer with NaCl concentration adjusted to 150 mM. A 1.5 μg antibody was added and the samples were agitated on a rotator for 2 h at 4°C. To collect immunoreactive proteins, 30 μl of protein A/G beads (Santa Cruz biotechnology, Inc.) were added to the mixture and rotated overnight at 4°C. The antibodies used for immunoprecipitation were: anti-IKKα (Santa Cruz Biotechnology, Inc.) and anti-FLAG (Sigma). Beads were spun down and washed three times with Wash buffer (same as the lysis buffer, except that NaCl concentration is 150 mM).

Kinase Assay

For the kinase assay, beads with immunoprecipitated material, as described above were washed twice more with kinase buffer containing 20 mM HEPES pH 7.4, 1mM EDTA, 1 mM DTT, 10% glycerol. The beads were then suspended in 15 μl of kinase buffer containing 2.5 μg of kinase substrate, 10 mM $MgCl_2$, 20 μM ATP, and 5 μCi γ-^{32}P-ATP (New England Nuclear, 3,000 Ci/mmol). After 30 min incubation at 30°C, each tube was quickly spun and 15 μl each of the reaction mixture was transferred to a fresh tube containing 10 μl of 2.5 × SDS loading buffer. A 20 μl of 1 × SDS loading buffer were added to the tube with the remaining beads for Western blot analysis. All samples were boiled for 5 min and loaded onto a SDS–polyacrylamide gel. The gel for kinase assay was fixed in 10% acetic acid, and 10% methanol for 15 min and dried before autoradiography. The gel for Western blotting was transferred onto a nitrocellulose membrane and processed as described above. Kinase substrates, IκB55 and IκB55-AA, were generated by subcloning IκBα 1-55 or mutated IκBα (32A/35A) 1-55 (generous gifts from Dr. Thomas Maniatis of Harvard University, Cambridge, MA) into the pET tag-fusion bacteria expression system (Novagen, Madison, WI). The reading frame of constructs was verified by DNA sequencing. The recombinant fusion proteins produced were purified to greater than 90% homogeniety as instructed by manufacturer. Protein concentration was determined with a Bio-Rad protein assay kit (Bio-Rad).

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear proteins for EMSAs were extracted according to a method described before [Ritzenthaler et al., 1991]. To this end, packed nuclei from Y10 cells were isolated and extracted with an equal volume of nuclear binding protein extraction buffer (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM dithiothreitol (DTT), 1.0 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 μg/ml of leupeptin, 2.0 μg/ml of aprotinin, and 0.7 μg/ml of pepstatin). The nuclear extracts were gently stirred, using a sterile pipette tip with a cut end to disrupt the nuclear membranes and were incubated on ice for 20 min, followed by centrifugation at 14,000 rpm (18,000*g*) for 30 min at 4°C. Supernatants were recovered and nuclear protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad). The extracts were aliquoted (20 μl) and stored at −80°C until used. These were used for mobility shift assays along with the following oligonucleotides: NF-κB (AGTTGAGGGGATTTCCCAGGC) [Sen and Baltimore, 1986]; mNF-κB (AGTTGAGGCC-ACTTTCCCAGGC); URE (GATCCAAGTCC-GGGTTTCCCCAACC) [Duyao et al., 1990; Lawrence et al., 1994]. To prepare probes, equimolar aliquots of complementary oligonucleotide strands were combined and annealed by heating in 10 mM Tris, pH 8.0 and 10 mM $MgCl_2$ to 95°C for 5 min then slowly cooled to room temperature. The probes were end labeled with [γ-^{32}P]-ATP, using T4 polynucleotide kinase (New England Biolabs), and then purified by a ProbeQuant G-50 Micro column (Pharmacia, Piscataway, NJ). Nuclear protein (2–10 μg) was mixed with 2 μg of poly (dI-dC) (Pharmacia) in

30 μl of binding buffer (20% glycerol, 20 mM Hepes, pH 7.9, 4 mM Tris, pH 7.9, 100 mM NaCl, 1 mM EDTA, and 1 mM DTT) along with approximately 40,000–100,000 cpm of end-labeled probe at room temperature for 30 min. In reactions using competitor DNA or oligonucleotides, a 50 M excess of the indicated unlabeled competitor DNA was used. In antibody supershift experiments, 1 μg of anti-NF-κB was preincubated with the nuclear extract for 30 min on ice before adding the ^{32}P-labeled probe. The reactions were then loaded on a 4.5% non-denaturing PAGE and analyzed by autoradiograhy. The antibodies were from Santa Cruz Biotech, Inc., except for one efficiently reacting batch of anti-p65 (in supershift assay), which was a generous gift of Nancy Rice (National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD).

Transient Transfections and CAT Assay

Y10 cells were plated in 2.5 ml of IMDM, 10% FCS, at a density of 5×10^5 cells/ml and transfected with 2 μg of appropriate DNA (NFκB-TK-CAT or mut-NFκB-TK-CAT, generous gifts of Dr. Gail Sonenshein, Boston University School of Medicine) using 6 μl of FuGENE6 reagent based on manufacturer's protocol (Boeringer Mannheim, Indianapolis, IN). After 3 h of incubation, cells transfected with the same plasmid were pooled to assure consistent transfection efficiency between wells and re-allocated to new 6-well plates prior to the addition of MGDF (to 25 ng/ml), or the vehicle as a control. After 2 days in culture, cells were collected and washed with first PBS and then STE buffer (0.1 M NaCl, 10mM Tris, pH 8.0, 1 mM EDTA). Whole cell soluble protein extracts were prepared by four cycles of freeze-thaw in 100 μl, 0.25 M Tris, pH 7.4 and spun at top speed in a microcentrifugor for 5 min to pellet debris. Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad). Extracts were stored at −20°C until use. Aliquots were prepared from each sample such that 75 μg of protein was diluted with 0.25 M Tris to a total volume of 100 μl. Endogenous enzymatic activity was inactivated by heating the samples to 60°C for 10 min. Each sample was transferred to a scintillation vial, to which was added a cocktail containing 1 μl ^{3}H-acetyl CoA, 2.4 μl 100 μM chloramphenicol, 0.163 μl unlabeled 40 mM acetyl CoA, and 27.4 μl water. The mixture was incubated at 37°C for 1 h, overlaid with 3 ml toluene-based scintillation fluid, and counted in a scintillation counter. Total counts were determined by using aqueous scintillation fluid in place of the toluene-based fluid in one control sample. HeLa or Y10 cells were also subjected to transient transfection with the FuGENE6 reagent, using 2 μg pCDNA3-rIKKβ (in which the cDNA was tagged with a FLAG sequence) or pCMVβ-Galactosidase. It appeared that approximately 50% of the HeLa cells and 1–5% of the Y10 cells were transfected, as indicated by in situ staining for β-galactosidase [Ravid et al., 1991]. The efficiency of transfection of Y10 cells was not improved by employing other available methods of transfection (data not shown). Thus only transfected HeLa cells were used for IKβ kinase assay using the anti FLAG antibody, as described above.

RESULTS

NF-κB DNA Binding Activity Is Downregulated by MGDF

Since the population of bone marrow cells is heterogeneous, and since megakaryocytes are rare in the marrow, we resorted to a megakaryocytic cell line that we characterized before in order to examine a potential link between Mpl ligand signaling and NF-κB DNA binding activity. As we reported earlier, the expression of megakaryocyte-specific markers, such as acethylcholine esterase [Zhang et al., 1998] and platelet factor four (Fig. 1A), was detected within few hours of treatment of the Y10 megakaryocytic cell line (derived from L8057 cells as described under Materials and Methods) with the Mpl ligand, MGDF. As also described for primary megakaryocytes [Rouyez et al., 1997], treatment with Mpl ligand activated in Y10 cells the mitogen activated protein (MAP) kinase pathway (Fig. 1B). While all the above characteristics were consistently observed in Y10 cells of all batches and passages, the ploidy response to MGDF was often lost upon prolonged passaging in culture, thus necessitating re-cloning of the cells. In the following studies we used a batch of cloned Y10 cells that also polyploidized in response to MGDF. Polyploidy became somewhat apparent only by one day of MGDF treatment (Fig. 1D). At 3 days of MGDF treatment, the number of polyploid cells ($>$ 4N) increased, as well as the fraction of $<$ 2N cells (from 16 to 25%), as indicated by flow

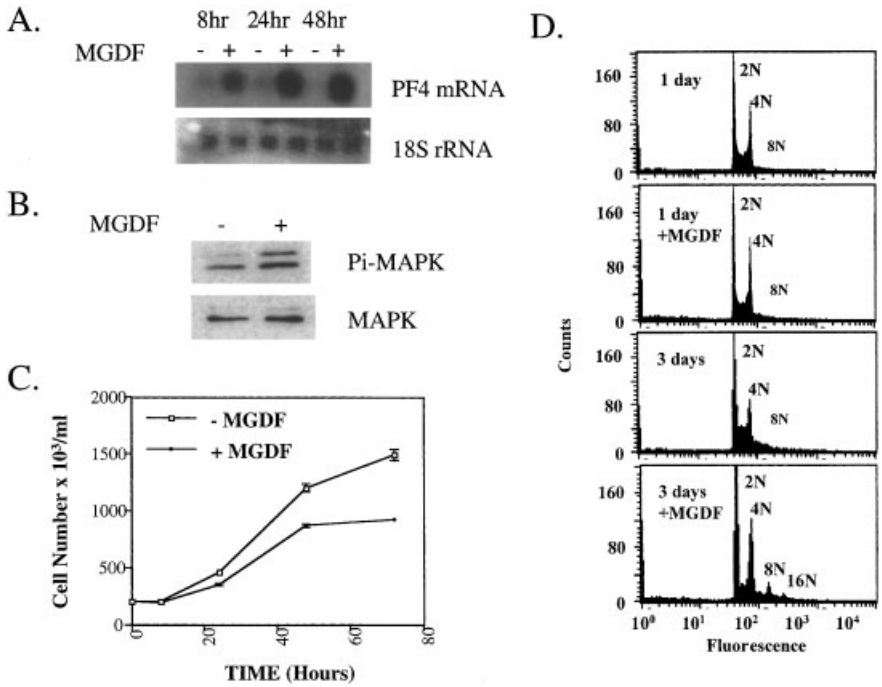

Fig. 1. Effects of MGDF on expression of a megakaryocytic marker, on cell number and ploidy level. **A**: Y10 cells were cultured without (−) or with (+) 25 ng/ml MGDF for the time indicated. Total RNA was subjected to Northern blotting (15 μg/lane), using the rat platelet factor four (PF4) cDNA as a probe. Equal loading of RNA was confirmed by probing the blot with the cDNA hybridizing to the 18S rRNA. The data shown are representative of four experiments. **B**: Western blot analysis of Y10 cells treated or not treated with MGDF for 24 h, using phosphor-specific p44/42 MAP kinase antibody (Pi-MAPK) or an antibody to MAPK. The data presented are representative of two performed. **C**: Y10 cells were subjected to trypan blue exclusion and counting of viable cells by a hemocytometer following incubation with or without 25 ng/ml MGDF for different time periods. Results are averages of four experiments ± SD. **D**: Y10 cells were cultured without (−) or with (+) 25 ng/ml MGDF for the time indicated prior to subjection to ploidy analysis, as detailed under Materials and Methods.

cytometry analysis of propidium iodide-stained cells [Zhang et al., 1998] (Fig. 1D, representative of three experiments). The < 2N fraction represents apoptotic cells, indicating that as polyploidization takes place, the percentage of apoptotic cells increases. It should be pointed out that in cultures of primary megakaryocytes too, expression of a variety of differentiation markers precedes the process of polyploidization, which is followed by fragmentation associated with apoptosis [Zauli et al., 1997]. Y10 megakaryocytes, however, are not capable of fragmenting into platelets [Ishida et al., 1993]. In accordance with cessation of cellular proliferation in Y10 cells, the cell number decreased in MGDF-treated cultures, as compared to non-treated megakaryocytes (Fig. 1C). We examined in this cell system whether MGDF affected NF-κB binding activity, a major inducer of cellular proliferation. To this end, an oligonucleotide containing a consensus NF-κB binding site (NF-κB) was used as a probe in gel mobility shift assay. Two specific DNA-protein complexes were observed when this probe was incubated with nuclear extracts prepared from Y10 cells cultured in the absence or presence of MGDF (Fig. 2A). NF-κB binding activity was reduced by approximately three-fold in response to MGDF (Fig. 2A). When the NF-κB binding element from the myc promoter, URE [Duyao et al., 1990], was used as a probe, MGDF reduced the specific binding activity similarly. When an oligonucleotide containing a consensus Sp1 site was used as a probe in gel mobility shift assay, the level of protein bound to the oligomer was higher in the MGDF-treated cells as compared to control (Fig. 2C), as we reported before [Wang et al., 1999]. This control was used to exclude the possibility that the reduction seen in NF-κB binding ability in MGDF-treated cells is a result of a reduced amount of active proteins in the cytokine-treated sample. In order to establish the identity of the protein(s) that were present in the specific protein-DNA complex, several antibodies that react with distinct NF-κB family members were used in supershift

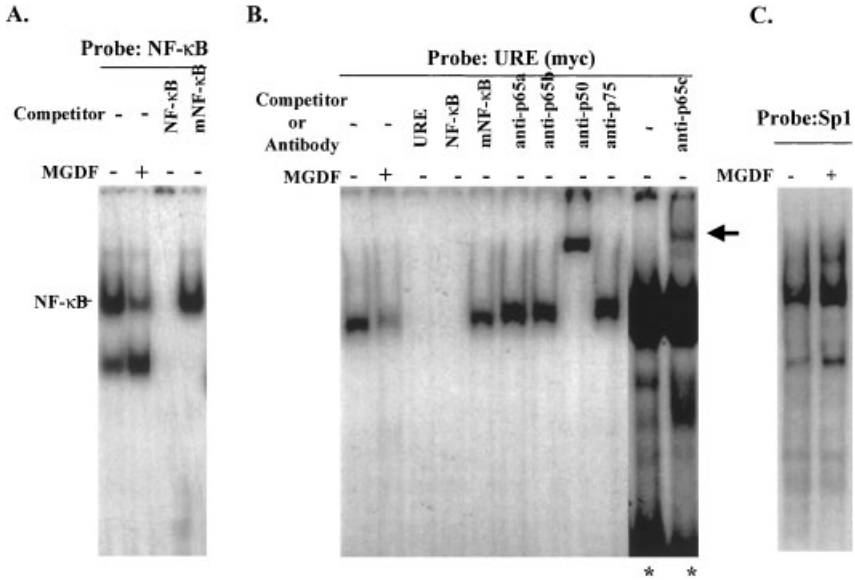

Fig. 2. NF-κB DNA binding activity is downregulated in differentiated megakaryocytes. EMSAs were performed using nuclear extracts from Y10 cells cultured in the absence or presence 25 ng/ml MGDF for 2 days. **A**: Consensus NF-κB oligonucleotide containing the B site of the κ light chain (NF-κB) was used as a probe. A 50-fold molar excess of the unlabeled NF-κB oligonucleotides or unlabeled oligonucleotides in which NF-κB site was mutated (mNF-κB), were added as competitors. **B**: An oligonucleotide containing the upstream regulatory element (URE) of c-myc was used as a probe. A 50-fold molar excess of the following unlabeled oligonucleotide competitors or 1 μg antibody were added as indicated: URE, NF-κB, and mNF-κB, as well as antibodies a, b, c against the NF-κB p65 subunit (anti-p65), the NF-κB p50 subunit (anti-p50) or c-rel (anti-p75). It appears that among the antibodies to p65, only anti-p65c (see Materials and Methods) was able to supershift a complex in WEHI 231 murine B cells nuclear extracts (data not shown) and in our Y10 cells (indicated by an arrow), except that the EMSA film of the experiment with Y10 cells needed an extended exposure in order to visualize well the supershifted band. Lanes indicated by a star (*) were exposed 24 h longer than other lanes. Extended exposure of the film containing supershifts with anti-p50 (not shown) indicated that all the complex was supershifted. The data shown are representative of three experiments performed. **C**: As a control for the binding activity of each nuclear extract used, an oligonucleotide containing the Sp1 consensus site (ATTCGATGGGGCGGGGCGAGC) was used as a probe in EMSAs, using nuclear extracts from Y10 cells cultured in the absence or presence 25 ng/ml MGDF for 3 days. Similar results were obtained with cells treated with MGDF for 1 or 2 days (data not shown).

assays. Anti-p50 NF-κB (but not anti-p75) was able to supershift all the NF-κB complex (Fig. 2B). Since anti-p65 NF-κB supershifted a very small portion of the complex (Fig. 2B), we conclude that Y10 megakaryocytes contain mainly p50 homodimers as well as some p50–p65 heterodimers. It should be pointed out that antibodies-induced supershifts were also observed in EMSA of WEHI 231 murine B cells nuclear extracts (generous gift of Gail Sonenshein), used as a control to confirm the activity of the antibodies used.

NF-κB Transcription Activation Activity Is Present in Megakaryocytic Cells and is Downregulated by MGDF

In order to assess the transactivating potential of NF-κB in response to MGDF, Y10 cells were transiently transfected with a CAT reporter construct that contains two consecutive NF-κB sites (NFκB-CAT). A pool of transfected cells was cultured in the absence or presence of MGDF. CAT assays were performed as described under Materials and Methods. As can be seen from Figure 3, the NF-κB promoter construct was active in Y10 megakaryocytes. Extracts from MGDF-treated cells showed a remarkable decrease in CAT activity, indicating that the expression of genes, which depend on NF-κB binding for expression, are likely to be reduced. A mutated form of this promoter that lacks the ability to bind NF-κB (mut-NFκB-CAT) [La Rosa et al., 1994] was used as a control, to indicate that the activity measured was indeed dependent on NF-κB binding.

IKK Activity Is Downregulated in Polyploidizing Megakaryocytes

IKKβ and IKKα are major components of the IKK complex which in turn regulates NF-κB activity. As shown in Figure 4, while the levels of intracellular IKKβ or of IKKα remained

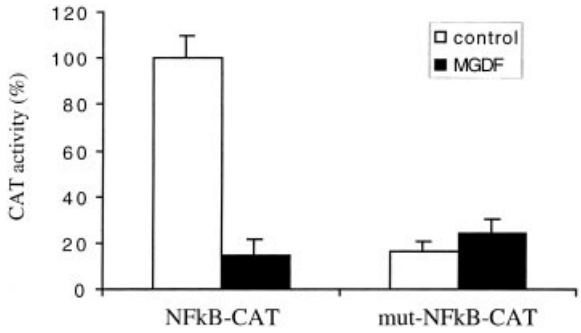

Fig. 3. The activity of an NF-κB-dependent promoter is downregulated by MGDF. Y10 megakaryocytes were transiently transfected with constructs containing CAT driven either by two NF-κB sites within a TK promoter (NFκB-CAT) or a form of this promoter containing point mutations that disrupt NF-κB binding (mut-NFκB-CAT). Transfected cells were treated with or without 25 ng/ml MGDF for 2 days. Cells were then collected, and whole-cell lysates were subjected to CAT assay, as described under Materials and Methods. Baseline values obtained in mock-transfected cells were subtracted from the values obtained in cells transfected with the indicated constructs. Data are averages ± SD derived from three determinations.

unaltered in MGDF-treated cells, the level of the phosphorylated form of IκB, the substrate for IKK, was transiently reduced, as compared to non-treated cells. The extent of reduction was greater at day one than day three. We next investigated whether IKK activity was altered in megakaryocytes in response to MGDF treatment. Anti IKKβ, while reacting well in Western blot analysis, failed to precipitate the IKK complex from cell extracts. Also, since the efficiency of transient transfection of Y10 cells was very low (see Materials and Methods), the FLAG antibody did not immunoprecipitate a sufficient amount of the FLAG-tagged IKKβ, transiently expressed in cells transfected with pCDNA3-rIKKβ. Consequently, we resorted to determining the endogenous IKK activity in Y10 cells in response to MGDF by using a commercial antibody against IKKα, which also co-precipitated IKKβ in the immune complex kinase assay (Fig. 5C). This assay was successfully employed for HeLa cells (Fig. 5A) before

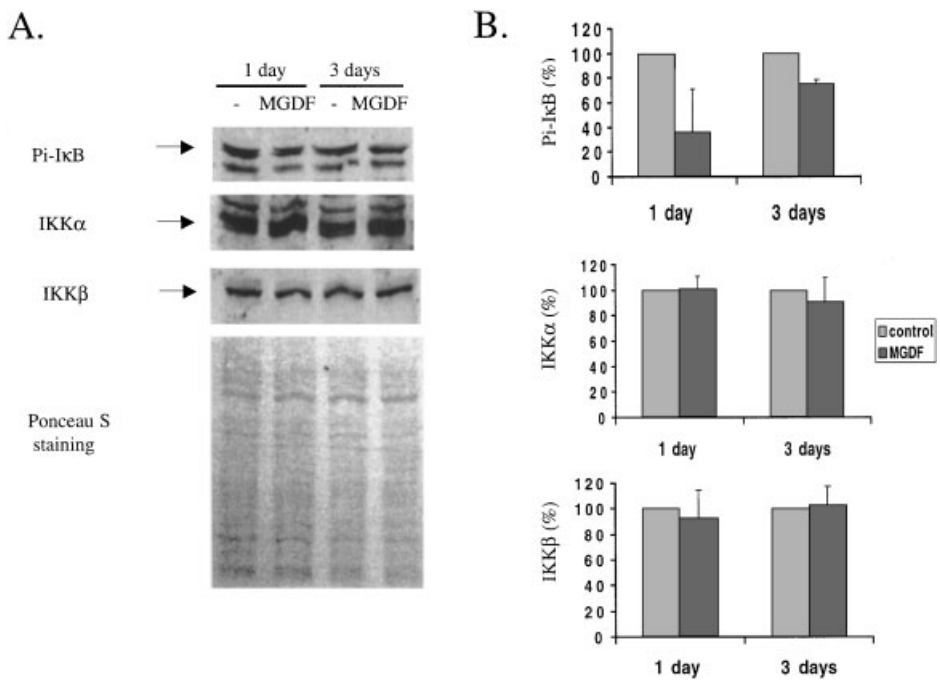

Fig. 4. Effects of MGDF on the levels of IKKβ, IKKα, and phosphorylated IkB. **A**: Y10 cells were cultured without (−) or with 25 ng/ml MGDF for the indicated times. Cells were subjected to Western blotting, using antibodies to the proteins indicated on the margin by an arrow. Equal loading of protein was confirmed by staining the blot with Ponceau S. Some of the antibodies used react with two proteins or more, as also indicated by the manufacturer. The arrows point to the expected molecular weights of the indicated proteins (as compared to the Benchmark prestained protein ladder, Gibco BRL) (not shown) as follows: 43 kDa Pi-IkB (phosphorylated IkB), which migrates slower than the non-phosphorylated form (IkB); 74 kDa IKKα; and 87 kDa IKKβ. **B**: The intensities of the Pi-IkB, or IKKα, or IKKβ bands obtained under each condition was quantitated by using the Electrophoresis Documentation and Analysis System (Kodak). The difference in the levels of Pi-IkB in non-treated and MGDF-treated cells was expressed as averages ± SD. Application of *t*-test indicated a difference in the level of Pi-IkB at 1 day ($P < 0.01$) and 3 days ($P < 0.05$) of incubation with MGDF as compared to control.

proceeding to assay IKK activity in megakaryocytes. Y10 megakaryocytes were treated with MGDF for short or prolonged times. As indicated under Materials and Methods, addition of the cytokine was routinely initiated upon shifting the cells from F12 medium to the differentiation-promoting medium IMDM [Ishida et al., 1993]. Cells cultured for short time periods with MGDF or TNF-α displayed a transient increase in IKK activity of up to 2.2-fold or 1.6-fold, respectively, within 10 min (Fig. 5B) (averages derived from three determinations using the Electrophoresis Documentation and Analysis System). We also noted that in cells cultured with IMDM alone, there was some increase in IKK activity over 30 min of incubation, but to a lesser extent than the induction by MGDF at 10 min incubation (Fig. 5B). Extracts prepared from Y10 cells treated for 1 day with MGDF showed a sustained, significant decrease in IKK activity (Fig. 5C). Since it has been shown that IKKβ is essential for IKK activation [Li et al., 1997; Delhase et al., 1999; Hu et al., 1999; Takeda et al., 1999], we normalized IKK activity to the level of IKKβ in each immunoprecipitated complex (Fig. 5C). The level of IKKβ in the complex tested for kinase activity was not diminished in response to MGDF (Fig. 5C), further confirming that the inhibitory effect of this ligand on IKK activity was not due to changes in IKKβ levels in the immunoprecipitated material. Since the steady state levels of the phosphorylated form of IκB reflect the activity of IKK and that of a phosphatase, it would be possible to obtain a decrease in IKK activity (Fig. 5) that is larger than the decrease in the level of phosphorylated IκB (Fig. 4). In this regard it should be pointed out that as of yet, it is not certain by which upstream kinase IKKβ is predominately activated in vivo and which phosphatases are involved in the process. Nevertheless, our data point to a signaling from the Mpl receptor to IKK activity, which in turn results in a decrease in NF-κB activity.

DISCUSSION

Mpl ligand, the primary cytokine that drives megakaryopoiesis [Kaushansky, 1995], promotes differentiation, cessation of mitotic proliferation and polyploidization of megakaryocytes [Borge et al., 1996; Rasko et al., 1997; Yoshida et al., 1997]. These polyploid cells fragment into platelets in association with programmed cell death [Zauli et al., 1997]. In view of the diverse effects of this cytokine on the megakaryocytic cell cycle, it is reasonable to assume that it affects different signaling pathways, in a transient or sustained manner, as well as it may induce opposite effects on the same signaling route, depending on the developmental state of the cell. In relation to these effects of the Mpl ligand, examination of NF-κB activation seems to be particularly pertinent, since in many systems NF-κB α activation has been associated with increased proliferation and protection from apoptosis [e.g., Bellas et al., 1995; Liu et al., 1996; Wang et al., 1998].

Signal transduction leading to IκB phosphorylation has been intensively studied [reviewed in Maniatis, 1997], especially where inflammatory cytokines TNF-α and IL-1 were involved. A cytokine-challenged receptor recruits members of the adapter signal transducer TNF receptor-associated factor 2 (TRAF2), in the case of TNF-α [Rothe et al., 1995], or TRAF6 in

Fig. 5. IKK activity during megakaryopoiesis. **A**:. Autoradiography of in vitro kinase activity from extracts prepared from HeLa cells, using anti-IKKα for immunoprecipitation. When indicated, cells were treated with 20 ng/ml murine TNFα for 10 min. Assays were carried out with either wild type IκBα 1-55 (IκB) or mutated IκBα 1-55 (IκB-AA) as substrates, as indicated. The data shown are representative of two separate experiments. **B**, **C**: Autoradiography of kinase activities. Kinase assays were carried out with cell lysates (300 μg protein) prepared from Y10 cells cultured without (−) or with 25 ng/ml MGDF, or 20 ng/ml murine TNFα for the time indicated (in the same experiment). At zero time, the cells were shifted from F12 to IMDM medium to allow differentiation to proceed . This shift in medium causes an increase in IKK activity within 30 min incubation, which is not further augmented by MGDF. Wild type IκBα 1–55 (IκB) was used as a substrate in all assays, unless otherwise indicated. The mutate substrate IkB-AA was used as a control to evaluate the specificity of the immunoprecipitated kinase towards its substrate. Anti-IKKα was used for immunoprecipitation, while anti IgG was used as a control (IgG lane in C). The level of IKKβ in the complex immunoprecipitated was determined by Western blotting (12% SDS–PAGE blotted and probed with anti IKKβ) of the same samples subjected to kinase assay (panel B). **D**: For calculations of percent kinase activity, the intensities of the bands obtained under each condition were quantitated by using the Electrophoresis Documentation and Analysis System (Kodak). IKK activity immunoprecipitated by anti IKKα was subtracted from the value obtained when anti IgG was used. Each value was normalized to the amount of IKKβ in the immunoprecipitated complex. Data are averages of three experiments ± SD. The statistical differences between groups were derived by *t*-test. The difference in activity between the MGDF-treated cells and the control is significant ($P < 0.01$ and $P = 0.05$ for 1 day and 3 days incubation, respectively).

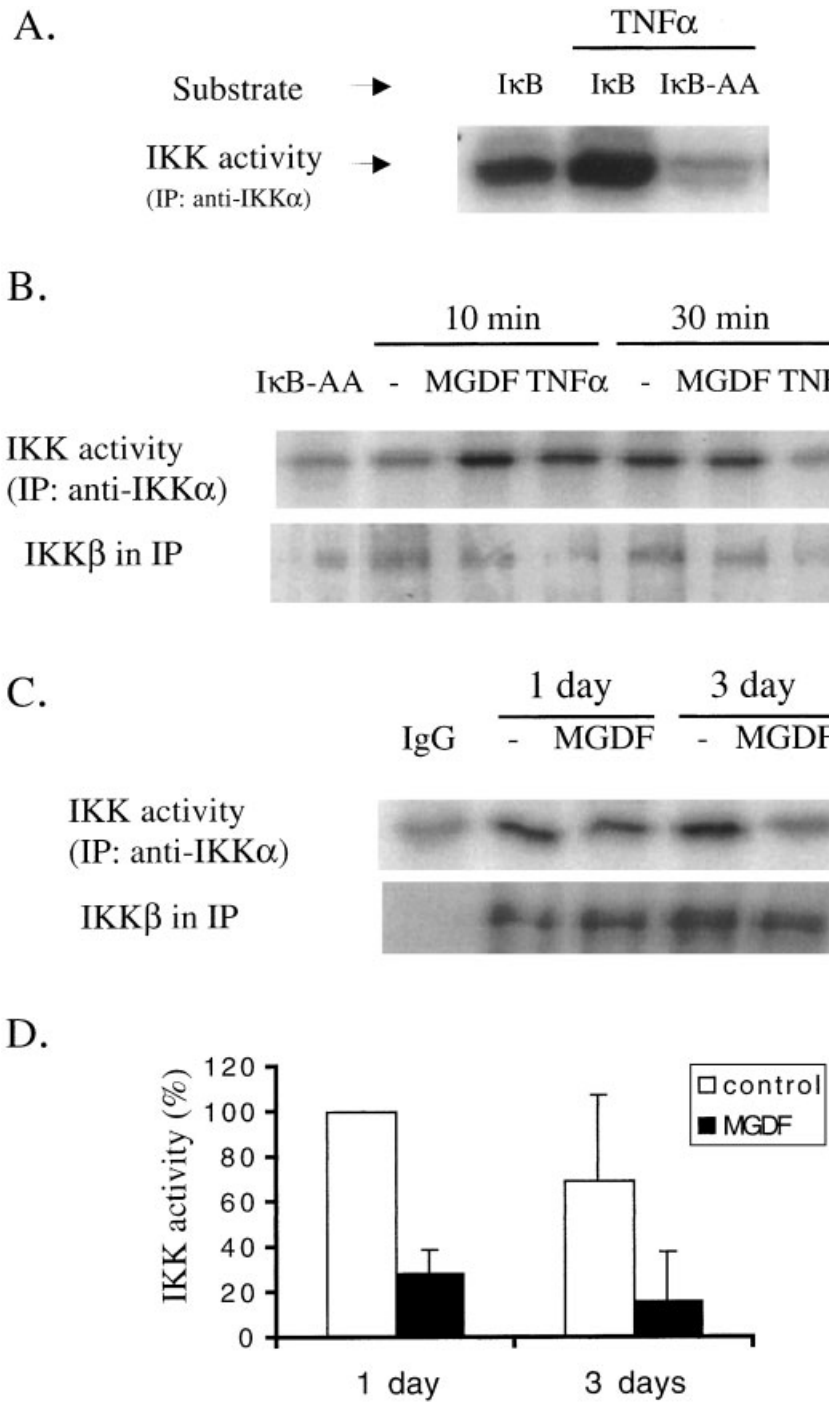

Fig. 5.

the case of IL-1 [Cao et al., 1996] through their interaction with the receptor-associated adapter protein TRADD [Hsu et al., 1996]. Downstream the receptor complex, a member of the MAP kinase kinase family, the NF-κB-inducing kinase (NIK), is activated through its interaction with the TRAF family members [Malinin et al., 1997]. Downstream of NIK lies the IκB kinase IKK [Ling et al., 1998], which phosphorylates IκB to target its ubiquitination and subsequent degradation. The long-sought IKK was finally identified in recent years. It was isolated as a large complex of 700–900 kDa. Within this complex, two kinases (IKKβ and IKKα) were identified and characterized [DiDonato et al., 1997; Mercurio et al., 1997; Regnier et al., 1997;

Woronicz et al., 1997; Zandi et al., 1997]. The activity of IKK is stimulated by cytokines such as, TNFα and IL-1, which are known to activate NF-κB. To date, at least three accessory molecules that could modulate the activity of IKKs have been identified [Cohen et al., 1998; Rothwarf et al., 1998; Yamaoka et al., 1998]. Recent studies indicate, that among these components IKKβ is essential for IKK activation and for cytokine-induced activation of NF-κB [Li et al., 1997; Delhase et al., 1999; Hu et al., 1999; Takeda et al., 1999]. It is not certain yet, however, by which kinase IKKβ is predominantly activated in vivo. Severe abnormalities in multiple morphogenic events are observed in mice lacking IKKα [Hu et al., 1999; Takeda et al., 1999], while severe liver degeneration and death of embryos are observed in mice lacking IKKβ [Li et al., 1999].

The NF-κB-mediated transactivation of a NF-κB-dependent promoter decreased in response to prolonged exposure of Y10 megakaryocytes to the recombinant Mpl ligand, MGDF. At least part of this decrease was due to an attenuated DNA-binding activity of NF-κB. Reduced NF-κB activation in our system was, as expected, associated with a decrease in IKK activity and in the levels of phosphorylated IκB. The levels of IKKβ, or of IKKα did not change during the course of MGDF stimulation of Y10 cells. As indicated above, however, proteins other than IKKβ or IKKα have been demonstrated to contribute to substrate recognition and/or modulation of kinase activity [Chen et al., 1996; DiDonato et al., 1997]. Among these are NEMO [Yamaoka et al., 1998], IKAP [Cohen et al., 1998], and IKKγ [Rothwarf et al., 1998]. Furthermore, since NIK was identified either as a TRAF-interacting kinase [Maniatis, 1997] or as a component of the large IKK complex [Cohen et al., 1998], formation of a super-complex between a cytokine receptor complex and the IKK complex has been implied [Maniatis, 1997]. Although NIK is undoubtedly an IKK-activating kinase [Ling et al., 1998], other kinases have been reported to function either as IKK-activating kinases or as IκB-kinases. These include MEKK1 [Lee et al., 1998; Nakano et al., 1998; Nemoto et al., 1998], TAK1 [Sakurai et al., 1998], PKC [Steffan et al., 1995; Lindholm et al., 1996], and tyrosine kinase $p56^{lck}$ [Imbert et al., 1996; Briant et al., 1998]. IKK is also activated by PKB, which in turn is activated by high levels of 3′-phosphorylated phosphatidylinositol (PIP_3) [reviewed in Penninger and Woodgett, 2001]. The mechanisms by which MGDF induces a transient increase in IKK activity followed by a sustained decrease in this activity are not clear yet. Since a Mpl ligand was reported to signal via PIP_3 [Rojnuckarin et al., 2001], it is possible that the transient increase in IKK activity involves activation of phosphoinositide 3-kinase. It is also possible that the PIP_3 phosphatase, called PTEN [Penninger and Woodgett, 2001], is activated upon prolonged incubation with Mpl ligand, thus preventing the flow of PIP_3 signaling and potentially IKK activation. However, since the activity of the heterodimeric IKK probably accounts for most of the activity observed in vitro [Mercurio et al., 1997; Woronicz et al., 1997; Zandi et al., 1997], full activation of IKK may require integration of more than one signal transduction pathway. Consequently, the reduction in IKK activity we observed may be due to the absence of potential regulatory molecules in the immunocomplex or due to a modulation of a complementary signal transduction pathway. In this regard, a recent study indicated that while the phosphorylation by upstream kinases of two sites in the activation loop of IKKβ is essential for IKK activation, IKK can also be inactivated due to an autophosphorylation of a COOH-terminal serine cluster in IKKβ [Delhase et al., 1999]. Future studies would aim to characterize those upstream kinases/phosphatases and their potential link to Mpl receptor activation.

Our study is the first to demonstrate an active NF-κB in proliferating megakaryocytes and the modulation of IKK and consequently NF-κB DNA-binding activity in response to an Mpl ligand. In our system, as also recently described in bone marrow stem cells [Pyatt et al., 1999], the majority of the DNA binding activity detected is of NF-κB p50 homodimers. Mpl ligand treatment decreased the DNA binding activity of p50 homodimers as well as of p50–p65 heterodimers. Although, p50 homodimers only weakly bind IkB and are generally not considered as transcriptional activators [Rice and Ernst, 1993], they have been implied as important in altering gene expression and response to stimuli, for example, the tolerance to lipopolysacharide involves mobilization of NF-κB with predominant p50 homodimers [Zeigler-Heitbrock et al., 1994]; the regulation of transcription of the interleukin-2 gene alpha depends on p50 homodimers that can impair SRF binding to the promoter of this gene and

its activation [Algarte et al., 1995]. It is then possible that Mpl ligand allows upregulation of some genes via a decrease in the level of p50 homodimers as well as downregulation of genes via a decrease in p50–p65 heterodimers. Since the expression of a variety of genes such as, c-myc [La Rosa et al., 1994], nitric oxide synthase, [Marks-Konczalik et al., 1998], IL-8 [Breton and Chabot-Fletcher, 1997], IL-12 [Yoshimoto et al., 1997], IL-15 [Washizu et al., 1998], and p53 [Hellin et al., 1998] is affected by NF-κB activation, it is likely that these genes are highly regulated during megakaryopoiesis. In addition, several studies led to the conclusion that NF-κB acts as a survival and anti-apoptosis factor [Liu et al., 1996;Wang et al., 1998]. Of the many possible genes regulated by NF-kB, c-myc is of most interest because Mpl ligand induces a decrease in c-myc mRNA [our unpublished data; Dorn et al., 1994], and because our studies indicated that high levels of c-myc induce megakaryocyte proliferation and hinder the process of megakaryocyte polyploidization [Thompson et al., 1996]. Also of interest is the fact that as polyploidization progresses in Y10 megakaryocytes, a larger fraction of the cells undergo apoptosis. Apoptosis, on the other hand, has been speculated to be an important part of fragmentation of bone marrow megakaryocytes into platelets [Zauli et al., 1997]. Since Y10 cells are not capable of fragmenting into platelets [Ishida et al., 1993], programmed cell death may be essential, but not sufficient for fragmentation. Future exploration of other proliferation-promoting and anti-apoptotic genes potentially suppressed by reduced activity of NF-kB in megakaryocytes will be of interest.

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