Cytokine Receptor Common β Subunit-mediated STAT5 Activation Confers NF-κB Activation in Murine proB Cell Line Ba/F3 Cells*

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The cytokine receptor common β subunit (βc) transmits intracellular signals upon binding ligand such as granulocyte-macrophage colony-stimulating factor or interleukin-3 (IL-3); however, transcriptional regulation under the control of signaling events downstream of the βc is not fully understood. Using murine Ba/F3 cells, here we demonstrate that the βc-mediated signals stimulate NF-κB-driven gene expression of not only the reporter construct but also endogenous target genes such as IL-6. Analyzing the effects of several inhibitors or mutant receptors revealed that this NF-κB activation is mediated neither by MEK/ERK/MAPK nor by the phosphatidylinositol 3-kinase pathway but by STAT5. Overexpression experiments of the wild-type or constitutive active form of STAT5 further confirmed this notion. In addition, STAT5-dependent NF-κB activation is mediated not through an inducible nuclear translocation but via up-regulation of both DNA binding activity and transactivation potential of NF-κB. Furthermore, we also show that as yet undefined humoral factor(s) may be involved in this NF-κB activation process. Taken together, we may propose that cytokine receptor-mediated STAT5 activation and expression of its target genes culminates in a unique mode of NF-κB activation and gene expression.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) play important roles in regulating multiple cellular functions such as proliferation, differentiation, and survival in various hematopoietic cell lineages and their precursors (1). These cytokines bind to their cognate receptors and trigger a cascade of signaling events leading to various biological responses. The receptors for GM-CSF (GMR) and IL-3 (IL-3R) are composed of two subunits, the cytokine-specific α and the common β subunit (βc), the latter of which is also shared by the receptor for IL-5 (2). Both subunits belong to the type I cytokine receptor superfamily, and the βc, having a relatively large cytoplasmic domain, plays a pivotal role in downstream signal transduction (2). These receptors lack intrinsic kinase activity but interact with and activate Janus kinase 2 (JAK2) in response to binding ligand (3). Subsequently, the cytoplasmic domain of the βc becomes tyrosine-phosphorylated and then cue multiple signal transduction pathways (4, 5) including the mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3-kinase (PI3K) pathway (6, 7). In addition, at the transcriptional level, not only expression of some immediate early genes such as c-fos, c-jun, c-myc, egr-1, cip, and pim-1 (6, 8, 9) but also the functional regulation of transcription factors such as STAT5, AP-1, and CREB (9–12) is known to be achieved by these cytokine receptors.

The STAT proteins are activated upon various cytokine stimulations and play a central role in following the transcriptional regulation of gene expression (13, 14). Among seven known members of STAT family, both STAT5A and STAT5B, closely related isoforms, are the most predominant members activated by the βc (10, 11). Upon cytokine stimulation, JAK2 phosphorylates STAT5 on a tyrosine residue near the C-terminal transactivation domain in the cytoplasm (15). The phosphorylated STAT5 proteins dimerize and translocate into the nucleus, where they bind to specific DNA elements and thereby activate target gene expression (10, 11). Using murine IL-3-dependent Ba/F3 cells, we have reported previously that STAT5 participates in growth-promoting signals downstream of the βc (16–18). Moreover, we have shown that STAT5 is a key regulator of not only proliferation but also differentiation or apoptosis in these cells (19). In particular, pim-1, JAB/SOCS-1/SSI-1, and p21/WAF1/Cip1, all of which are transcriptionally regulated by STAT5, are suggested to be involved in a variety of biological outcomes induced by STAT5 (19). Our knowledge of the gene expression profile regulated by STAT5, however, has been extremely limited. A recent study using microarray analysis revealed that more than 300 genes are regulated at the transcriptional level by IL-3 signaling in murine pro-B cells (20), raising the possibility that more complicated mechanisms than described previously are operated in these cytokine- and STAT5-dependent biological responses.

It was recently reported that several growth factors, including GM-CSF (21) and IL-3 (22), could induce the activation of another transcription factor NF-κB in cells of hematopoietic cell lineages (23, 24). This observation raised the possibility that STAT5 might also regulate NF-κB activation in various cellular contexts. Here we demonstrate that STAT5 activates transcription of the NF-κB-dependent gene promoters under the control of signaling events downstream of the βc. Moreover, we show that several cytokine receptors converge on the STAT5 signaling pathway. In particular, we confirm that cytokines such as IL-3, GM-CSF, GMR, G-CSF, and IL-6 signal through STAT5, and we identify the MAPK (ERK1/2 and p38) and PI3K pathways as parallel signals downstream of STAT5. Our analysis of STAT5 activation by various cytokines also reveals that STAT5 is a key regulator of cytokine-induced NF-κB activation in hematopoietic cell lineages.
lineages, although underlying mechanisms have remained unclear. NF-κB was originally described as a regulator of immune and inflammatory responses. NF-κB consists of a dimer from five related proteins, most typically a heterodimer composed of p65/RelA and p50 subunits (23–25). The regulation of NF-κB is achieved through interaction with a family of inhibitory protein known as IkB that binds to NF-κB and sequesters it in the cytoplasm (26). Once cells are stimulated with inducers such as tumor necrosis factor-α (TNFα) and IL-1, two serine residues of the IkB protein are phosphorylated by IkB kinases (27, 28). Phosphorylation of IkB targets it for ubiquitination and subsequent degradation by the 26S proteasome and renders the nuclear localization signal of NF-κB unmasked (29, 30). Then NF-κB translocates from the cytoplasm into the nucleus and regulates the transcription of target genes. In addition to this “classical” milieu, recent reports have suggested that alternative pathways lead to NF-κB activation through several nuclear mechanisms (31–35). As well as its well established role in activating the transcription of genes involved in immunological responses, it is indicated that NF-κB is one of the central mediators of hemopoiesis (36). Therefore, it appears to be of importance to elucidate the linkage between the GM-CSF or IL-3 signaling and NF-κB activation for a better understanding of biological responses under the control of these cytokines.

In the present study, we demonstrated that in Ba/F3 cells, GM-CSF as well as IL-3 activates NF-κB-dependent transactivation, and STAT5 is essential for this process. Of note, STAT5 affects neither IkB degradation nor nuclear translocation of NF-κB. In contrast, we indicated that STAT5 increases not only the DNA binding activity but also the transactivation potential of NF-κB, possibly through a mechanism involving STAT5-dependent synthesis of unknown humoral factor(s).

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—A murine (m) IL-3-dependent proB cell line, Ba/F3 (37), was maintained in RPMI 1640 (Sigma) medium containing 10% fetal calf serum (Medical & Biological Laboratories, Nagoya, Japan), 4 ng/ml IL-3 (Pepro Tech EC, London, England), 100 units/ml penicillin, and 100 μg/ml streptomycin. Various Ba/F3 clones stably expressing human (h) GM-CSF together with hGMRFβ (Ba/F3-wild) or hGMRFβ mutant γ6 or FαI (Ba/F3-γ6 or Ba/F3-FαI, respectively) have been described before (16). These engineered cells were grown in medium in the presence of 500 μg/ml G418 (Sigma). Another clone of Ba/F3 cells in which STAT5A1*6 is stably transduced (Ba/F3-STAT5A1*6) was a murine cell line myelomonocytic cell line WEHI 3B cells, was maintained with the same medium but without IL-3 (tentatively termed as depletion medium). In all experiments, cells were extensively washed with the depletion medium three times and stimulated with either 4 ng/ml IL-3 or 5 ng/ml hGM-CSF (kindly provided from Kirin Brewery, Tokyo, Japan), or indicated concentrations of mIL-3, mIL-6 (Sigma), m-oncostatinM (OSM, Sigma), or mIL-1β (Pepro Tech EC) or left unstimulated. Nonstimulated medium was prepared by culturing either parental Ba/F3 or Ba/F3-STAT5A1*6 cells at a density of 1 × 10^6 cells/ml for 24 h in the depletion medium. The culture supernatant was subsequently collected, cleared by centrifugation (1,000 × g for 20 min), and freshly used as a conditioned medium for stimulating cells.

**Plasmids**—The reporter plasmids NF-κB-Luc (formerly pNFxBHL) and AP-1-Luc (formerly pAP-1HL) in which expression of the luciferase gene is under the control of transcription factors NF-κB and AP-1, respectively, were described elsewhere (38). A β-casein promoter-inducible luciferase reporter construct, β-casein-Luc (pZzI) (39), and an expression plasmid, pXM-STAT5A1*6/Bsu36 encoding a fusion protein, were described in detail described in Ref. 40 as indicated. The DNA probe corresponding to nucleotides 16–761 of the m IL-6 cDNA was generated by reverse transcription–PCR from an RNA sample of parental Ba/F3 cells cultured under usual conditions. The probe was labeled with [α-32P]dCTP by random priming using RediPrime II (Amersham Biosciences, Inc.) according to the manufacturer’s instructions. Hybridization was carried out under ULTRAhyb conditions (Ambion Inc., Austin, TX) at 42 °C overnight.

**Preparation of Cell Extracts**—For preparation of whole cell extracts, 1 × 10^6 cells were washed with phosphate-buffered saline twice and incubated on ice for 15 min in lysis buffer (1% Nonidet P-40, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 1 μM pepstatin, 50 mM NaF, 1 mM Na3VO4). After centrifugation at 12,000 × g for 20 min in a microcentrifuge, the supernatant was used as whole cell extracts. Cy...
toxol and nuclear extracts were prepared as described by Dignam et al. (45) with minor modifications. In brief, after two cycles of washing in ice-cold phosphate-buffered saline, 2 × 10^7 cells were harvested and centrifuged at 1,000 × g for 5 min in a microcentrifuge. The cell pellet was resuspended in 80 μl of buffer A (10 mM Tris-HCl, pH 7.5, 1.5 mM MgCl2, 10 mM dithiothreitol, 0.4% Nonidet P-40, 1 mM PMSF, 10 μg/ml aprotinin, 1 μg pepstatin, 50 mM NaCl, and 1 mM Na3VO4) and incubated on ice for 5 min. Cell lysates were centrifuged, and the supernatant was used as cytosol. The nuclear pellet was then resuspended in 75 μl of buffer C (20 mM Tris-HCl, pH 7.3, 1.5 mM MgCl2, 454 mM KCl, 1 mM dithiothreitol, 0.2 mM EDTA, 25% glycerol, 1 mM PMSF, 10 μg/ml aprotinin, 1 μg pepstatin, 50 mM NaCl, and 1 mM Na3VO4) and incubated for 30 min at 4°C. Nuclear debris were pelleted with centrifugation at 12,000 × g for 15 min, and the supernatant was used as nuclear extracts.

Protein concentration was determined using protein assay reagent (Pierce).

Electrophoretic Mobility Shift Assay (EMSA)—Ten μg of protein was subjected to EMSA. The double-stranded oligonucleotide probes harboring NF-κB (top strand, 5′-AAAACTTGGAGCTTTCGGCTC-3′; bottom strand, 3′-GGTTACCCTGAAGGCGCAGCCGG-5′) and STAT5 (top strand, 5′-GATCCGAATTTCCGAGATC-3′; bottom strand, 3′-GCTTAAGGTCTTAAGTCTAG-5′) binding sequences were annealed and filled in by BcaBEST DNA polymerase (TaKaRa, Kyoto, Japan) using [α−32p]dCTP and unlabeled dNTPs. Proteins were incubated with 0.7 ng of the radiolabeled probe in 20 μl of the reaction mixture containing 20 mM HEPES, pH 7.9, 4 mM dithiothreitol, 0.2 mM EDTA, 12% glycerol, and 100 mM KCl in the presence of 2 μg of sheared salmon sperm DNA (Wako Pure Chemical Industries, Osaka, Japan) for NF-κB or 2 μg of poly(dI-dC)/dI-dC) (Amersham Biosciences, Inc.) for STAT5. For competition experiments, a 2-, 5-, or 20-fold excess of unlabeled double-stranded oligonucleotides of NF-κB or STAT5 or 20-fold excess of non-specific oligonucleotide containing glucocorticoid responsive element (43) was added prior to the oligonucleotide probe. In supershift assays, 2 μg of antibodies against either p65 (sc-372), p50 (sc-1190), p52 (sc-298), RelB (sc-226), c-Rel (sc-70), Bcl-3 (sc-185), or STAT5 (sc-836) (purchased from Santa Cruz Biotechnology, Santa Cruz, CA) was added to the nuclear extracts for 1 h prior to the addition of oligonucleotide probe. Samples were then incubated for 15 min at room temperature and electrophoresed on a 4% non-denaturing polyacrylamide gel in 0.5 TBE (10 mM Tris borate, 89 mM boric acid, and 2 mM EDTA) buffer. Gels were run at 300 V for 2 h at 4°C, dried, and autoradiographed.

Immunoblotting—Ten μg of protein was separated in 10% SDS-polyacrylamide gels and then transferred to polyvinylidene difluoride membranes. The membranes were blocked in Tris-buffered saline (TBS, pH 7.4, 0.1% Tween 20) with 5% non-fat dried skim milk. The membranes were probed with either anti-p65 (sc-372), anti-p50 (sc-1190), or anti-IκBα (sc-945, Santa Cruz Biotechnology) antibodies at 1:1000 dilution followed by incubation with a secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology). For analysis of IκBα protein, the membranes were first probed with anti-phospho-IκBα (Ser-32) antibody (New England Biolabs, Beverly, MA) at 1:1000 dilution followed by incubation with a secondary antibody conjugated to horseradish peroxidase. Subsequently, after detection of proteins, the same membranes were stripped, reprobed for anti-IκBα antibody (sc-371, Santa Cruz Biotechnology) at 1:500 dilution, and probed with a secondary antibody. In all experiments, proteins were visualized with ECL detection systems (Amersham Biosciences, Inc.) according to the manufacturer’s instructions.

Immunocytochemical Analysis—For immunocytochemical analysis, Ba/F3-wild cells were collected and washed once with phosphate-buffered saline, and then 2 × 10^6 cells were cytosinized to a silane-coated glass slide. Cells were fixed with 3.7% paraformaldehyde in TBS for 20 min at room temperature, permeabilized for 30 min using TBS containing 0.1% Triton X-100 and 3% bovine serum albumin, and washed twice with TBS. Slides were then treated with 0.1 mg/ml RNase per 1 h at 37°C. Incubation with 1:1000 dilution of anti-p65 antibody was performed for 1 h at 37°C, followed by washing two times with TBS, and then incubation was carried out for 1 h with anti-rabbit IgG conjugated to fluorescein (Santa Cruz Biotechnology) at 1:200 dilution. The slides were rinsed and exposed to propidium iodide for 2 h at room temperature, and coverslips were mounted for viewing by a laser scanning confocal microscopy (Fluoview FV500, OLYMPUS, Tokyo, Japan).

RESULTS

GM-CSF as Well as IL-3 Induces NF-κB Activation in Ba/F3 Cells through Distinct Mechanisms from MEK/ERK/MAPK or PI3K Pathway—To analyze signaling events downstream of the βc, we used an engineered subline of m IL-3-dependent Ba/F3 cells stably expressing both wild-type α and βc subunits...
of the h GMR (Ba/F3-wild cells, Fig. 1C) (16) because signals evoked by the hGMR in these cells are able to substitute for those of endogenous IL-3R. To test whether NF-κB-dependent gene expression is induced by IL-3 or GM-CSF, both Ba/F3-wild and parental Ba/F3 cells were transiently transfectected with an NF-κB-inducible reporter plasmid NF-κB-Luc and cultured in the absence or the presence of these cytokines, and then cellular lysates were assayed for luciferase activities. As shown in Fig. 1A, treatment with IL-3 induced nearly 3-fold induction of reporter gene expression in both of these cells. In contrast, treatment with GM-CSF resulted in 5-fold increase of reporter gene expression only in Ba/F3-wild cells. These results suggested that not only the endogenous IL-3R but also transfectected hGMR could activate NF-κB-dependent gene expression in Ba/F3 cells and that cells expressing hGMR provide a suitable tool for analyzing this βc-dependent NF-κB activation.

As described previously, the βc, upon ligand binding, activates multiple distinct signals such as the MEK/ERK/MAPK cascade, the PI3K pathway, and STAT5 (6, 7, 11). Because it has been revealed that both the MEK/ERK/MAPK cascade and PI3K pathway activate NF-κB in certain conditions (31, 46–52), the involvement of these pathways in NF-κB activation in our systems was tested using specific inhibitors for each pathway. As shown in Fig. 1B, neither the MEK1 inhibitor PD98059 nor PI3K inhibitor wortmannin did affect GM-CSF-dependent induction of NF-κB-responsive reporter gene expression. The bioavailability of PD98059 was verified by its 50 to 60% of inhibitory effect on AP-1-inducible reporter gene activation in response to GM-CSF (data not shown). Similarly, both PI3K activity and phosphorylation of Akt, a downstream target of the PI3K pathway, or STAT5 (16) (Fig. 1C). As shown in Fig. 1D, treatment with IL-3 activated expression of the NF-κB-responsive reporter gene not only in Ba/F3-wild but in Ba/F3-Y6 and -Fall cells as well. In contrast, treatment with GM-CSF stimulated reporter gene activation in Ba/F3-wild and -Y6 cells but not in Ba/F3-Fall cells (Fig. 1D). These results suggest that a certain signal involving neither MAPK nor PI3K pathway but involving phosphorylation of at least Tyr-750 on the βc is essential for the βc-dependent NF-κB activation. Together with the potent ability of Ba/F3-Y6 cells but not Ba/F3-Fall cells to activate STAT5 (16), the involvement of STAT5 in this cytokine-dependent NF-κB activation was strongly suggested.

We next asked whether induction of NF-κB activity may contribute to expression of endogenous target genes in Ba/F3 cells. For this purpose, we examined mRNA expression of proinflammatory cytokine IL-6, gene expression of which is regulated by NF-κB in various cell types and known to be induced by IL-3 in Ba/F3 cells (54). Moreover, analysis of the IL-6 gene seems appropriate for monitoring NF-κB-driven gene expression in the present study because the promoter region of the IL-6 gene is lacking STAT5 response element (55). As shown in Fig. 1E, the mRNA level of IL-6 was strongly induced within 3 h in response to IL-3 treatment in parental Ba/F3 cells. Furthermore, similar induction of IL-6 mRNA expression was observed after a 3-h stimulation of GM-CSF in Ba/F3-wild and -Y6 cells. In contrast, treatment with GM-CSF did not induce IL-6 mRNA expression in Ba/F3-Fall cells, reflecting the incapability of this Fall mutant receptor to induce the activation of NF-κB. Taken together, these results clearly indicate that the βc-dependent NF-κB activation leads to expression of endogenous target genes including IL-6.

STAT5 Activates NF-κB in Ba/F3 Cells—Next, we explored the possibility that STAT5 activation is involved in cytokine-mediated NF-κB activation. For this purpose, we cotransfected into Ba/F3-wild cells an expression plasmid for WT STAT5A together with the reporter plasmids for either NF-κB or STAT5. As shown in Fig. 2A, WT STAT5A amplified GM-CSF-dependent induction of NF-κB activity, suggesting the primary role of STAT5 in NF-κB activation in these cells (left). The
cotransfection efficiency of these plasmids was confirmed by the enhancement of β-casein promoter activity induced by the presence of WT STAT5A in a dose-dependent fashion (Fig. 2A, right). The effect of STAT5B, an isoform of STAT5A, was also examined, and the obtained results were quite similar to that of STAT5A (data not shown).

To bypass the effects of the other signaling cascades triggered by the β rather than STAT5 activation, we also analyzed the effect of a constitutive active mutant of STAT5, STAT5A1*6, on NF-κB activation in the absence of cytokine treatment. STAT5A1*6 has two amino acid substitutions in the WT STAT5A backbone, one in the DNA binding domain (H298R) and the other in the transactivation domain (S710F) (18). This mutant is constitutively phosphorylated on its tyrosine residue, localizes in the nucleus, and is transcriptionally active in the absence of growth factor stimulation (18). Efficient introduction of this strong transcriptional activator was verified by the fact that β-casein promoter activity was induced in a dose-dependent manner of STAT5A1*6 even in the absence of cytokine stimulation (Fig. 2B, top right). Note, overexpression of STAT5A1*6 significantly induced NF-κB activation despite the absence of cytokine stimulation (Fig. 2B, top left). We also investigated another subline of Ba/F3 cells stably expressing STAT5A1*6 (Ba/F3-STAT5A1*6), which are able to survive and proliferate without upstream cytokine signals (18). As expected, Ba/F3-STAT5A1*6 cells exhibited strong NF-κB-dependent transcription in parallel with the activation of STAT5-dependent reporter gene expression (Fig. 2B, top). Analysis of AP-1-driven reporter gene expression revealed that transcriptional activity of AP-1 was induced by GM-CSF stimulation but not by STAT5A1*6 (Fig. 2B, bottom). Taken together, we may conclude that STAT5 is essential for the β-mediated NF-κB activation in Ba/F3 cells.

**STAT5 Enhances the DNA Binding Activity of NF-κB without Influencing Its Subcellular Localization**—To investigate the DNA binding activity of NF-κB and STAT5 in Ba/F3 cells, EMSA was performed. As shown in Fig. 3A, two complexes were observed between NF-κB oligonucleotide probe and the nuclear extracts prepared from GM-CSF-treated Ba/F3-wild cells (lane 1). These complexes were sequence-specific because not nonspecific (lane 5) but specific competitor DNA (lanes 2–4) diminished the complex formation. Supershift assays revealed that anti-p65 antibody shifted the upper band (lane 6) and anti-p50 antibody shifted both upper and lower bands (lane 7). Antibodies against other Rel family proteins or Bcl-3, a member of the IκB family proteins, did not affect these complex formations (lanes 8–11), indicating that the upper complex is a heterodimer of p65/p50 and the lower complex is a p50/p50 homodimer. We obtained similar results when using the nuclear extracts from Ba/F3 cells after IL-3 stimulation (Fig. 3B, lanes 5, 10, and 15; and data not shown). Note that the anti-STAT5 antibody, which recognizes both STAT5A and STAT5B, could not supershift either of these NF-κB complexes (Fig. 3A, lane 12). We also demonstrated the formation of protein-DNA complex between STAT5 oligonucleotide probe and the nuclear extracts from GM-CSF-treated Ba/F3-wild cells (Fig. 3A, lanes 14–20).

Based on these experimental settings, we examined the time-dependent alteration of NF-κB DNA binding in response to GM-CSF stimulation in Ba/F3-wild, -Y6, and -Fall cells. As shown in Fig. 4B, the DNA binding activity of STAT5 was hardly detectable at the time point of stimulation in any of these cells (top panels, lanes 1, 6, and 11), indicating the rapid attenuation of STAT5 DNA binding activity after the removal of IL-3. When stimulated with GM-CSF, the DNA binding activity of STAT5 reached the maximum level within 15 min and was sustained for at least 3 h in both Ba/F3-wild (lanes 1–4) and -Y6 (lanes 6–9) cells, whereas induction of STAT5 DNA binding was hardly observed in Ba/F3-Fall cells (lanes 11–14). When assayed for NF-κB, all of these cells showed a substantial amount of NF-κB DNA binding activity in the nuclear extracts even after a 5-h deprivation of IL-3 (bottom panels, lanes 1, 6, and 11). After GM-CSF stimulation, the DNA binding activity of NF-κB was induced 2–2.5-fold at 1 and 3 h after stimulation in Ba/F3-wild (bottom panel, lanes 1–4) and -Y6 cells (lanes 6–9). In Ba/F3-Fall cells, however, the DNA binding activity of NF-κB seemed weaker at 3 h than at that the time point of stimulation (lanes 11–14). Treatment with IL-3 resulted in an increase in DNA binding of NF-κB in any of these cells (lanes 5, 10, and 15). These observations indicate that cytokine receptor-mediated STAT5 activation enhances the DNA binding activity of NF-κB.

Regulation of NF-κB activity in most cell types involves the targeted phosphorylation and degradation of IκB proteins, allowing subsequent nuclear translocation of NF-κB (see the Introduction). To test this in Ba/F3 cells, Ba/F3-wild cells were deprived of IL-3 for 5 h, stimulated with GM-CSF, and processed for immunocytochemical staining of p65 subunit of NF-κB. As a control experiment, cells were treated with TNFα and examined in parallel. In TNFα-treated cells, drastic nuclear translocation of p65 was observed after 15–30 min of stimula-
Fig. 4. Nuclear translocation of NF-κB is not involved in GM-CSF-dependent NF-κB activation. Ba/F3-wild cells were stimulated with 5 ng/ml GM-CSF (left) or 20 ng/ml TNFα (right) after 5 h of cytokine deprivation (A). After the indicated time periods, cells were fixed and processed for immunocytochemical analysis. Subcellular localization of p65 was analyzed by indirect immunocytochemical staining using anti-p65 antibody as described under “Experimental Procedures.” FITC, subcellular localization of p65 with fluorescein isothiocyanate; PI, nuclear staining with propidium iodide; Merged, the merged image of both. Ba/F3-wild cells were stimulated with GM-CSF (left) or TNFα (right) as described above (B). After the indicated time periods, cells were harvested, and both cytosol and nuclear extracts were isolated. Ten μg of each was separated on a 10% SDS-polyacrylamide gel and immunoblotted with anti-p65 antibody. Ba/F3-wild cells were stimulated with GM-CSF (left) or TNFα (right) and harvested at the indicated time points, and whole cell extracts were prepared (C). Ten μg of each was separated on a 10% SDS-polyacrylamide gel and immunoblotted with anti-phosphorylated IκBα (p-IκBα) antibody, and the protein blot was reprobed with anti-IκBα antibody. As a control, whole cell extracts from cells treated with TNFα for 5 min were loaded on the same gel (lane 8). Immunoblotting with anti-IκBβ antibody was also performed. N.S., non-specific.

A

B

C

STAT5 Activates NF-κB

that both inducible but undetectable phosphorylation and rapid resynthesis of IκB proteins may occur, promoting the nuclear translocation of NF-κB, whereas the gross protein amount of IκBs remains constant. To test this, we also analyzed the time-dependent alteration of IκBα and IκBβ in the presence of the protein synthesis inhibitor cycloheximide in IL-3- or GM-CSF-treated Ba/F3-wild cells; however, the elimination of interference by newly synthesized IκB proteins did not explore any degradative process of these proteins (data not shown).

These somewhat curious findings that the β-dependent NF-κB activation involves enhancement of DNA binding activity without affecting its subcellular localization prompted us to precisely investigate the effects of STAT5 on NF-κB DNA binding activity. Previous reports described that when Ba/F3 cells are deprived of cytokine, the DNA binding activity of NF-κB slowly declines (22). Thus, we also tested whether the decrease in NF-κB DNA binding following the cytokine deprivation was affected by STAT5 activation. As shown in Fig. 5A, both STAT5 and NF-κB are recovered as DNA-bound complexes in both Ba/F3-wild and -pFall cells cultured in the presence of IL-3 (top part, lanes 1 and 5). The DNA binding activity of NF-κB was hardly discernible after a 9-h deprivation of IL-3 (lanes 4 and 8). Longer deprivation resulted in total loss of the DNA binding, although cell viability was seriously impaired (data not shown). In the case of STAT5, deprivation of IL-3 rapidly decreased DNA binding in both of these cells (lanes 4 and 8 and data not shown). Interestingly, once these cells were deprived of IL-3 but complemented with GM-CSF just after IL-3 de-
moval, the decline of DNA binding of STAT5 and NF-κB was inhibited in Ba/F3-wild cells (lane 3). This preservation of NF-κB DNA binding was of course observed after complementation with IL-3 (lanes 2 and 6). In clear contrast, although GM-CSF was complemented, preformed NF-κB DNA complexes in the absence of IL-3 were not sustained in Ba/F3-Fall cells (lane 7), suggesting the involvement of STAT5 in the preservation of the β-dependent interaction between NF-κB and DNA. Because protein levels of p65 and p50 appeared to be constant during the experiments (Fig. 5A, bottom part), it was suggested that this alteration in NF-κB DNA binding activity is not due to a decrease of p65/p50 proteins. Moreover, subcellular localization of p65 in either of these cells was unaffected after a 9-h deprivation of IL-3 as assessed by Western blot analysis (Fig. 5B), indicating that STAT5-dependent signals downstream of the hGMR positively modulate the DNA binding activity of NF-κB without influencing either the subcellular sequestration or protein amounts of p65/p50 complexes. To examine the effect of STAT5 more directly, we analyzed the effect of STAT5A1*6 on the DNA binding activity of NF-κB. When the nuclear extract was prepared just after electroporation (Fig. 5C, bottom panel), Relative loss of DNA binding of STAT5 in the same nuclear extract is reasonable because the cells had to stay cytokine-free for nearly 1 h during the procedures of cytokine removal before electroporation (top panel, lane 1). The DNA binding of NF-κB then gradually decreased to the minimum level through 9 h of culture in the depletion medium (lanes 1–4). In contrast, when STAT5A1*6 was overexpressed, not only STAT5 but also NF-κB DNA binding was observed until 9 h after electroporation even in the absence of IL-3 (top and bottom panels, lanes 5–8). Thus, we may conclude that STAT5 is able to preserve the DNA binding activity of NF-κB within the nucleus. We next tested the dose effect of STAT5 on the DNA binding activity of NF-κB. For this purpose, vector alone or indicated amounts of the expression plasmid for WT STAT5A were transfected into Ba/F3-wild cells. Cells were cultured in the presence or the absence of GM-CSF for 9 h, and then EMSA was performed. The top panel of Fig. 5D represents that the DNA binding activity of STAT5 increased in proportion to increasing amounts of the expression plasmids for WT STAT5A. In contrast, the DNA binding activity of NF-κB did not show a correspondent increase with the amounts of transfected plasmids (bottom panel). Note that these doses of the expression plasmids enhanced expression of NF-κB-responsive reporter gene in a dose-dependent manner (Fig. 2A). These results, therefore, may argue the presence of an additional mechanism that enhances the transcriptional function of nuclear NF-κB after DNA binding.

**STAT5 Up-regulates Transactivational Function of p65**—To test this hypothesis, we performed one hybrid assay to evaluate whether the transactivational function of p65 is also affected by STAT5. We constructed the indicated plasmids expressing GAL4 fusion proteins with either the N- or C-terminal half of p65 (Fig. 6A). We then transfected each of these plasmids, the GAL4-reporter plasmid, and either expression plasmid for WT STAT5A or STAT5AΔ49 into Ba/F3-wild cells, and cells were treated with GM-CSF as indicated (Fig. 6B). As in the case of GAL4 alone, the N-terminal half of p65 did not transactivate reporter gene expression in the presence of either GM-CSF or WT STAT5A (Fig. 6B). However, reporter gene expression in
the presence of the C-terminal half of p65 was significantly increased when WT STAT5A was overexpressed (Fig. 6B), indicating that the transactivation function of NF-κB p65 is augmented by STAT5A. Moreover, this effect of STAT5 was supposed to be ascribed to the C-terminal transactivation domain because coexpression of STAT5AΔ749 exhibited no detectable enhancement of reporter gene expression (Fig. 6B).

Both DNA Binding Activity and Transactivation Functions of STAT5 Are Required for NF-κB Activation—To further elucidate the mechanism for STAT5-dependent NF-κB activation, we used several mutants of STAT5 and performed cotransfection experiments. STAT5AVVV mutant, in which amino acid residues Val-Val-Val (amino acids 466–468) within the DNA binding domain of STAT5A are replaced by Ala-Ala-Ala, lacks DNA binding activity and is transcriptionally inactive (42). STAT5AΔ3749, in which the C-terminal 44 amino acids containing the transactivation domain are deleted, exerts dominant-negative effects on wild-type STAT5-induced transcription despite retaining its DNA binding ability (56). As shown in Fig. 7, neither of these mutants was able to augment NF-κB activation in response to GM-CSF, suggesting that NF-κB activation requires both STAT5 DNA binding and transactivation domains. These data raise the possibility that STAT5 induces transcription of a certain gene, the product of which in turn enhances NF-κB function. We tried to confirm this possibility by using a protein synthesis inhibitor cycloheximide on GM-CSF/H9260 cells. The indicated sequences of murine p65 were fused to the DNA binding domain (DBD) of Gal4 (amino acids 1–147), which is indicated as gray boxes. B, Ba/F3-wild cells transiently transfected with 3 μg of Gal4-inducible luciferase reporter plasmid in combination with 1 μg of expression vectors encoding various Gal4 fusion proteins. The indicated amounts of expression vectors encoding WT STAT5A or STAT5AΔ749 were also co transfected. Cells were cultured in the absence or presence of 5 ng/ml GM-CSF for 12 h and subjected to luciferase assays. Luciferase activities were normalized to RLU per μg of protein and indicated as fold activation as compared with those of cells transfected with Gal4 alone and left nonstimulated. Results are the mean ± S.D. of 3 independent experiments.

Fig. 6. STAT5 up-regulates transactivation potential of NF-κB p65. A, schematic drawing of Gal4 fusion constructs. The indicated sequences of murine p65 were fused to the DNA binding domain (DBD) of Gal4 (amino acids 1–147), which is indicated as gray boxes. B, Ba/F3-wild cells transiently transfected with 3 μg of Gal4-inducible luciferase reporter plasmid in combination with 1 μg of expression vectors encoding various Gal4 fusion proteins. The indicated amounts of expression vectors encoding WT STAT5A or STAT5AΔ749 were also co transfected. Cells were cultured for 12 h in the absence or presence of 5 ng/ml GM-CSF. Luciferase activities were assayed and indicated as fold activation as compared with those of cells transfected with Gal4 alone and left nonstimulated. Results are the mean ± S.D. of 3 independent experiments.
tal Ba/F3 cells (top). This biological activity of CM STAT5A1*6 is supposed to be mediated through a protein factor(s), because heat inactivation of CM STAT5A1*6 at 65°C for 1 h nearly abolished the activity. Similar properties of inducing NF-κB-dependent transcription were also observed in the CM of several different clones of Ba/F3-STAT5A1*6 or Ba/F3 cells in which another form of constitutive active mutant STAT5A-N642H (58) is stably transfected (data not shown). An autocrine mode of NF-κB activation through secretion of IL-3 or GM-CSF was not likely because CM STAT5A1*6 induced neither STAT5- nor AP-1-dependent transcription as determined by reporter gene expressions (Fig. 8A, middle and bottom panels). We next tested the possibilities of several other humoral protein factors to be involved in this NF-κB activation. As shown in Fig. 8B, however, neither IL-6 nor OSM could induce NF-κB activation. In addition, IL-1β, widely known as a potent inducer of NF-κB in multiple cell types, also had no effect (top). TNFα could stimulate NF-κB-dependent reporter activities to a

**FIG. 8.** STAT5-inducible humoral factor activates NF-κB in Ba/F3 cells. Parental Ba/F3 cells were transiently transfected with either 3 μg of NF-κB-Luc (top), 3 μg of β-casein-Luc (middle), or 10 μg of AP-1-Luc (bottom) reporter plasmids (A). Cells were then cultured for 12 h in either depletion medium alone (MED), depletion medium plus 4 ng/ml IL-3 (MED + IL-3), conditioned medium obtained from parental Ba/F3 cells (CM parent Ba/F3), or conditioned medium from Ba/F3 cells stably expressing STAT5A1*6 (CM Ba/F3-STAT5A1*6). Heat inactivation of CM Ba/F3-STAT5A1*6 was performed at 65°C for 1 h. Luciferase activities were assayed and indicated as fold activation as compared with those of cells cultured in the depletion medium. Parental Ba/F3 cells were transiently transfected with 3 μg of NF-κB-Luc and cultured in the indicated conditions for 12 h (B). For cell stimulation, 4 ng/ml IL-3 or various doses (5, 20, or 80 ng/ml) of either IL-6, OSM, IL-1β, or TNFα was added to the depletion medium, or cells were cultured in the CM Ba/F3-STAT5A1*6 (top). The effects of anti-mouse TNFα antibodies (Ab) were examined by adding the indicated amounts of neutralizing antibodies or control IgG (asterisks) 2 h prior to cellular stimulation with 20 ng/ml TNFα or CM Ba/F3 STAT5A1*6 (bottom). Luciferase activities were assayed and indicated as fold activation as compared with those of cells cultured in the depletion medium. Parental Ba/F3 cells were deprived of IL-3 for 5 h, collected by centrifugation, and then resuspended in CM Ba/F3-STAT5A1*6 (C and D). Total RNA and whole cell extract were prepared after the indicated time periods, and Northern blot analysis of IL-6 mRNA (B) and immunoblot analysis of IκBα proteins (D) were performed. N.S., non-specific.
level comparable with that induced by IL-3 or CM STAT5A1*6 (top); however, involvement of this cytokine in STAT5-dependent NF-κB activation seemed unlikely because the mechanism of NF-κB activation induced by TNFα was distinct from that induced by the β3-mediated signals in Ba/F3 cells (Fig. 5). In agreement with this, CM STAT5A1*6-dependent NF-κB activation was not blocked by the addition of neutralizing antibodies against TNFα (Fig. 8, bottom), and furthermore, enzyme-linked immunosorbent assay revealed no detectable amount of TNFα in the CM STAT5A1*6 (data not shown). Strikingly, we found that this putative protein factor(s) in CM STAT5A1*6 could activate not only reporter gene expression but also transcription of endogenous NF-κB target genes such as IL-6 (Fig. 8C). It should be emphasized that the time-dependent increase of IL-6 mRNA in response to CM STAT5A1*6 was earlier than that observed in the case of IL-3 or GM-CSF stimulation (compare Fig. 8C with Fig. 1E). These results strongly support our hypothesis that the β3-mediated NF-κB activation is in some extent dependent on a secondary mechanism involving STAT5-dependent transcription and de novo protein synthesis. In Western blot analysis, we again found that phosphorylation and subsequent degradation of IkB proteins are not affected in this soluble factor-dependent NF-κB activation process (Fig. 8D). We, therefore, may conclude that NF-κB activation downstream of the β3 is mediated at least in part through STAT5-dependent transcription and the production of a(n) unknown humoral factor(s) that in turn evokes a quite unique mode of NF-κB activation.

Humoral Factor(s) Produced by Ba/F3-STAT5A1*6 Could Activate NF-κB-dependent Transcription in WEHI 3B Cells—Finally, we examined whether STAT5 may induce NF-κB activation in different cell types other than Ba/F3 cells. When myelomonocytic leukemic WEHI 3B cells were transiently transfected with an expression vector encoding STAT5A1*6 along with reporter plasmid β-casein-Luc, significant induction of reporter gene expression was observed (Fig. 9A, left). In contrast, transfection of the same amount of STAT5A1*6 expression plasmid did not augment but rather suppressed NF-κB-dependent reporter gene activation (right). These results indicate that the mechanisms by which STAT5 up-regulates NF-κB activation in Ba/F3 cells may not be generally conserved in a variety of cell types. Indeed, it was recently reported that prolactin-activated STAT5 inhibits NF-κB-mediated signaling through a mechanism of competition for limiting transcriptional coactivators such as p300 (59). However, when WEHI 3B cells were stimulated with CM Ba/F3-STAT5A1*6 and assayed for reporter gene activity, an ~4-fold induction of NF-κB-driven reporter gene expression was observed (Fig. 9B). Thus, it is strongly proposed that this as yet undetermined soluble factor(s) secreted from certain cell types such as Ba/F3 cells may serve as an inducer(s) of NF-κB-dependent gene expression in not only Ba/F3 cells but also other cells including WEHI 3B cells.

DISCUSSION

Here we show that signals downstream of the β3 lead to the activation of the transcription factor NF-κB. To date, it is well characterized that the β3, upon ligand binding, activates JAK2 and then provokes several distinct and experimentally separable signals such as the MEK/ERK/MAPK cascade, the PI3K pathway, and STAT5 (6, 7, 11). Therefore, we first attempted to determine the responsible signaling cascade that links the β3 to the NF-κB activation pathway because several previous studies revealed that both ERK/MAPK and PI3K pathways functionally up-regulate the activity of NF-κB in various conditions (31, 46–52). In addition, one recent report showed that erythropoietin receptor-mediated JAK2 activation leads to NF-κB activation in neuronal cells (60). In our case with Ba/F3 cells, however, involvement of either the MEK/ERK/MAPK cascade or the PI3K pathway seemed unlikely because specific inhibitors for each pathway had no effect on NF-κB activation. In addition, our observation that Ba/F3-Y6 cells did induce NF-κB activation further supports this because this mutant receptor was previously shown to lack the potential to elicit MAPK or PI3K activation in the presence of GM-CSF (16). The possibility that JAK2 directly affect the NF-κB signaling was also excluded by the observation that the Fall mutant receptor could not activate NF-κB despite its retaining the potential to activate JAK2 (16). Instead, our results strongly suggested the involvement of STAT5, which was further supported by the observations that this NF-κB activation is augmented by overexpression of wild-type STAT5. Furthermore, our findings that the constitutively active mutant STAT5A1*6 induced NF-κB activation even in the absence of cytokines lead us to conclude the essential role of STAT5 in activating NF-κB. Thus, we are able to indicate that whether signaling events such as JAK2, MEK/ERK/MAPK, or PI3K activation interact with NF-κB activation pathway may vary depending on the cell type or the type of upstream stimulus.

Recent studies have revealed that STAT5 functionally interacts with other transcription factors. These include several distinct mechanisms by which STAT5 suppresses the function of other transcription factors such as glucocorticoid receptor (GR) (61) and peroxisome proliferator-activated receptor-α (62, 63); the direct protein-protein interaction-mediated mechanism for the former and the yet unidentified indirect mechanism for the latter. In addition, it has also been revealed, in contrast to our present study, that prolactin-activated STAT5

**Fig. 9.** CM Ba/F3-STAT5A1*6 activates NF-κB in WEHI 3B cells. WEHI 3B cells were transiently transfected with either 10 μg of NF-κB-Luc (left) or β-casein-Luc (right), cultured in the depletion medium for 18 h, and subjected to assays for reporter activities (A). Luciferase activities were normalized to RLU per μg of protein. WEHI 3B cells were transiently transfected with 10 μg of NF-κB-Luc and cultured in the indicated conditions for 18 h (B). Cytokine stimulation was performed by adding 20 ng/ml TNFα, IL-1β, OSM, or IL-6 to the depletion medium, and stimulation with the CM parent Ba/F3 or CM Ba/F3-STAT5A1*6 was performed. Luciferase activities were normalized to RLU per μg of protein, and the results are the mean ± S.D. of 3 independent experiments.
inhibits NF-κB-mediated signaling through a mechanism of competition for limiting transcriptional coactivators such as p300 (59). In the present study, we were unable to detect a physical association between STAT5 and NF-κB, as assessed by EMSA supershift analysis and pull-down assays using *in vitro* translation products (Fig. 3A and data not shown), suggesting a mechanism distinct from the previously described STAT5-GR association. In contrast, our data strongly indicate an indirect mechanism involving as yet unidentified humoral factor(s) in STAT5-dependent NF-κB activation. At this moment, therefore, it remains unclear whether the reported STAT5-dependent NF-κB suppression mechanism via, for example, the squelching of transcriptional coactivators may be present in Ba/F3 cells. Because we reproduced the suppressive effects of STAT5A1*6* on NF-κB-dependent reporter gene expression in WEHI 3B cells (Fig. 9), competition for limiting coactivators between STAT5 and NF-κB may play a role in STAT5-mediated inhibition of NF-κB-mediated signaling. Alternatively, enhancement of NF-κB function in our systems may be considered as the net effect of the opposite influences of STAT5 on NF-κB activation pathway: the inhibitory effect caused by protein-protein interaction between coactivators and these transcription factors and the promoting effect possibility mediated through intervening secondary humoral factor(s). In this regard, identification of this factor(s) responsible for activating NF-κB in Ba/F3 cells would provide insight into the mechanism of cross-talk between STAT5 and NF-κB signal transduction pathways in various cells.

In this report, we also addressed which step of the NF-κB activation pathway is influenced by the β*−*-mediated STAT5 activation. Ba/F3 cells exhibit a substantial amount of nuclear NF-κB composed of p65/p50 under usual culture conditions as seen in other cells of B cell lineage (64–66) or certain malignant cells (67). We showed that STAT5 activation leads to enhancement of the DNA binding activity of NF-κB without its nuclear translocation. In addition, our results also suggested that STAT5 up-regulates the transactivation potential of p65 even after the DNA binding of NF-κB is saturated. This mode of NF-κB regulation within the nucleus displays a striking contrast with that observed when Ba/F3 cells are stimulated with TNFα, which induces the rapid phosphorylation and subsequent degradation of IkB proteins. Consistently, a growing body of evidence has suggested that NF-κB-dependent gene expression is regulated not only via this well characterized IkB-dependent mechanism but also at several regulatory steps within the nucleus (31–35, 68). One possible mechanism of these nuclear regulations is modulatory phosphorylation of NF-κB p65 because both DNA binding (69) and the transactivation potential of NF-κB (33, 51, 70, 71) are positively regulated by phosphorylation of p65 by such kinases as cAMP-dependent protein kinase (33), CK2 (72), protein kinase Cζ (71), and IkB kinases (73). Thus, it is suggested that STAT5-dependent signals may consequently target the catalytic activity of these kinases. The second possible mechanism is that STAT5-dependent signals may up-regulate NF-κB via a redox-dependent mechanism because it has been shown that DNA binding activity of NF-κB is modulated by oxidation-reduction *in vitro* (74). Furthermore, we demonstrated that a redox-related factor, Ref-1, could enhance the DNA binding activity of NF-κB without affecting the degradation of IkB, nuclear translocation of NF-κB, or phosphorylation status of NF-κB proteins (35, 38). Thirdly, STAT5-dependent signals may regulate the function of transcriptional coactivators because NF-κB-dependent gene expression involves a growing number of such coactivators (34, 75, 76), and recent investigations have revealed that not only recruitment but also inducible activation of coactivators plays an important role in the efficient induction of transcription (77–79). Although the precise mechanisms of our described nuclear regulation of NF-κB remain undetermined, it should be emphasized that the activation of NF-κB is regulated through different modes of signaling pathways within the same cell. In this regard, it would be essential to identify and characterize the putative soluble factor for a better understanding of not only a new biological function exerted by STAT5 but also a unique mode of the NF-κB activation pathway distinctively utilized from the classical IkB-dependent pathway depending on the type of extracellular stimuli *in vivo*.

In summary, we demonstrated that signals downstream of the β*−* induce NF-κB activation in murine proB cell line Ba/F3 cells, which is mediated neither by MEK/ERK/MAPK nor by the PI3K pathway but instead exclusively mediated by STAT5. This NF-κB activation is suggested to be induced in part through as yet unidentified humoral factor(s) that is expressed depending on the activation of STAT5. Furthermore, STAT5-dependent signals confer a unique mode of NF-κB activation that is distinct from well characterized mechanisms.

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Cytokine Receptor Common \( \beta \) Subunit-mediated STAT5 Activation Confers NF-\( \kappa \)B Activation in Murine proB Cell Line Ba/F3 Cells

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