

PIAS3 Suppresses NF- κ B-mediated Transcription by Interacting with the p65/RelA Subunit*

Received for publication, December 1, 2003, and in revised form, March 16, 2004
Published, JBC Papers in Press, March 26, 2004, DOI 10.1074/jbc.M313018200

Hyun Duk Jang, Kwiyeom Yoon, Young Joo Shin, Jaesang Kim, and Soo Young Lee‡

From the Division of Molecular Life Sciences and Center for Cell Signaling Research, Ewha Womans University, Seoul 120-750, Korea

Nuclear factor- κ B (NF- κ B) is a transcription factor critical for key cellular processes, including immune response, apoptosis, and cell cycle progression. A yeast two-hybrid screening, using the Rel homology domain (RHD) of the p65 subunit (RelA) of NF- κ B as bait, led to the isolation of PIAS3, previously identified as a specific inhibitor of STAT3. We show that PIAS3 can directly associate with p65 using an *in vitro* pull-down and *in vivo* coimmunoprecipitation assays. When overexpressed, PIAS3 inhibits NF- κ B-dependent transcription induced by treatment with tumor necrosis factor α (TNF- α) or interleukin-1 β or by overexpression of TNF family receptors such as RANK, TNFR1, and CD30 or signal transducers of TNF receptor-associated factors (TRAFs), including TRAF2, TRAF5, and TRAF6. Down-regulation of PIAS3 by RNA interference reverses its effect on TNF- α -mediated NF- κ B activation. We found that an N-terminal region of PIAS3 is necessary for both the interaction with p65 and the transcriptional suppression activity. In addition, we found that an LXXLL coregulator signature motif located within the N-terminal region of PIAS3 is the minimal requirement for the interaction with p65. Furthermore, we demonstrate that PIAS3 interferes with p65 binding to the CBP coactivator, thereby resulting in a decreased NF- κ B-dependent transcription. Taken together, these data suggest that PIAS3 may function *in vivo* as a modulator in suppressing the transcriptional activity of p65.

NF- κ B¹ is an inducible cellular transcription factor that plays a critical role in the expression of a variety of genes involved in immune and inflammatory responses and cell survival (1–3). There are five known members of the mammalian NF- κ B/Rel family: p65 (RelA), c-Rel, RelB, p50 (NF- κ B1), and

p52 (NF- κ B2). These proteins share a conserved 300-amino acid region known as the Rel homology domain (RHD), which is responsible for DNA binding, dimerization, and nuclear translocation of NF- κ B (4–6). In most cells, Rel family members form hetero- and homodimers with distinct specificities in various combinations. p65, RelB, and c-Rel are transcriptionally active members of the NF- κ B family, whereas p50 and p52 primarily serve as the DNA binding subunits (4–6). The most widely studied and most abundant form of NF- κ B is the heterodimer of p50 and p65.

A common feature of the regulation of NF- κ B is the sequestration in the cytoplasm as an inactive complex physically associated with a class of inhibitory molecules known as I κ Bs (7). Treatment of cells with a variety of inducers such as phorbol esters, interleukin-1 (IL-1), and tumor necrosis factor- α (TNF- α) results in phosphorylation, ubiquitination, and degradation of the I κ B proteins (2, 8, 9). The degradation of I κ B proteins exposes the nuclear localization sequence in the remaining NF- κ B dimers, which in turn leads to a rapid translocation of NF- κ B to nucleus where it activates target genes by binding to cognate DNA regulatory elements (4–6).

A recent work has shown that NF- κ B-dependent transcription requires the function of transcriptional coactivator proteins (10–12). The CBP and its homologue, p300, are coactivators that interact with the p65 subunit of NF- κ B to enhance its ability to activate transcription. Inducible phosphorylation of p65 by the protein kinase A catalytic subunit stimulates NF- κ B-dependent gene expression by enhancing the interaction of p65 with CBP (13, 14). Two other coactivators are also known to be involved in regulating the transactivation function of NF- κ B. The HAT function of the p/CAF coactivator was shown to be required for the activation of NF- κ B-dependent transcription (15), and the SRC-1 coactivator protein was shown to interact with the p50 subunit of NF- κ B to potentiate NF- κ B-mediated transactivation (16). Thus, interaction with transcriptional coactivators is important in mediating the transactivation potential of NF- κ B. Moreover, the differential association of NF- κ B with coactivator proteins and possibly with corepressor proteins in unstimulated cells as well as in cells in which NF- κ B has been activated is likely to determine the level of activation or repression of NF- κ B-regulated genes (17–19).

Although the requirement of coactivators for transcriptional activation by NF- κ B is well established, little work has been done to determine the importance of repressors in regulating NF- κ B transactivation. To gain more insights into negative regulation of NF- κ B-activity, we performed a yeast two-hybrid screen and found PIAS3 as a potential binding partner for p65. We provide evidence that PIAS3 exerts a strong inhibitory effect on NF- κ B-dependent transactivation through binding to the Rel domain of p65. Additionally, we found that the interaction between p65 and PIAS3 requires the LXXLL motif of

* This work was supported by Korea Research Foundation Grant (KRF-2000-015-FS0006) (to S. Y. L.), by Molecular Medical Science Research Grant (M1-0106-00-0025) (to S. Y. L.), and by the Ewha Womans University Research Grant of 2000. 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: Division of Molecular Life Sciences and Center for Cell Signaling Research, Ewha Womans University, Seoul 120-750, Korea. Tel.: 82-2-3277-3770; Fax: 82-2-3277-3760; E-mail: leesy@ewha.ac.kr.

¹ The abbreviations used are: NF- κ B, nuclear factor- κ B; RHD, Rel homology domain; STAT3, signal transducers and activators of transcription 3; IL-1, interleukin-1; TNF- α , tumor necrosis factor α ; TNFR1, TNF receptor 1; aa, amino acid(s); GST, glutathione S-transferase; wt, wild type; CMV, cytomegalovirus; MEKK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; TRAF, TNF receptor-associated factor; Ab, antibody; siRNA, small interference RNA; RANK, receptor activator of nuclear factor κ B; PIAS3, protein inhibitor of activated STAT3.

N-terminal PIAS3. Moreover, we show that PIAS3 negatively regulates NF- κ B-dependent transcription by interfering with the ability of the CBP coactivator to interact with p65.

EXPERIMENTAL PROCEDURES

Plasmids and Antibodies—pSos-p65-(1–313) containing the N-terminal RHD of p65, the bait plasmid for the Cytotrap yeast two-hybrid screen, was generated by inserting the p65 N-terminal region corresponding to amino acids 1–313 into pSos (Stratagene). To generate bacterial expression vectors for GST-p65N (aa 1–313) and GST-p65C (aa 314–551), the corresponding p65 cDNA fragments were generated by PCR and cloned in-frame into pGEX-5x-1 (Amersham Biosciences). A full-length murine PIAS3 cDNA was obtained by PCR from a cDNA library of T cell hybridoma KMLs-8.3.5 (20). FLAG epitope-tagged PIAS3 cDNAs (PIAS3 wt (aa 1–584), PIAS3-N (aa 1–391), and PIAS3-C (aa 391–584)) were generated by PCR amplifying corresponding fragment from the full-length PIAS3 cDNA and subsequently cloning into pFLAG-CMV2 (Sigma). FLAG epitope-tagged PIAS3 LL-AA mutant was generated by site-directed mutagenesis using the QuikChange kit (Stratagene). To generate eukaryotic expression vectors for the GST-CBP-(1–450) mutant, CBP cDNA fragment encoding the corresponding N-terminal region was amplified by PCR and cloned in-frame into pEBG vectors as described previously (21). The nucleotide sequences of all constructs obtained by PCR were confirmed by DNA sequencing. The NF- κ B reporter vector (κ B)₃-interferon-luciferase and pCMV- β -gal plasmids have been described previously (22). The c-Jun (pFA-Jun) pathway-specific transactivators, the GAL4 UAS-containing luciferase reporter (pFR-Luc) plasmid, and the MEKK (pFC-MEKK) expression vectors were purchased from Stratagene (La Jolla, CA). Expression constructs encoding RANK, TNFR1, CD30, TRAF2, TRAF5, and TRAF6 have been described previously (21, 23, 24). CBP expression vector was kindly provided by Dr. Choe J (25). The antibody (Ab) specific for phosphorylated I κ B- α was from New England Biolabs; anti-I κ B α (C-21), anti-p65 (C-20), anti-PIAS3 (H-169), and anti-CBP (A-22) Abs were from Santa Cruz Biotechnology (Santa Cruz, CA); and the anti-FLAG epitope Ab (M2) was from Sigma.

Yeast Two-hybrid Assay—The CytoTrap™ (Stratagene) yeast screening was performed with murine thymus cDNA library (Stratagene) as prey and pSos-p65-(1–313) as the bait according to the manufacturer's instructions. *Saccharomyces cerevisiae* strain cdc25H was transformed sequentially with pSos-p65-(1–313) and murine thymus cDNA library fused to the pMyr plasmid containing the myristylation sequence of v-Src. Because the cdc25H yeast cell contains a temperature-sensitive mutant of the yeast homologue (*cdc25H*) of human hSos, it cannot grow at 37 °C. Positive clones were selected by the ability of cdc25H cells to grow on galactose plate at 37 °C. The hSos-p65-(1–313) protein is recruited to the plasma membrane because of the interaction with the myristoylated protein encoded by a pMyr clone selected from the target library, thereby complementing the *cdc25* defect and allowing the growth of the cdc25H yeast clone at 37 °C due to activation of the Ras-signaling pathway. The pMyr plasmids were rescued from positive colonies and identified by nucleotide sequencing.

Cell Culture, Transfections, and Luciferase Assays—293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml streptomycin, and 100 units/ml penicillin (Invitrogen). The cells were seeded at a density of 5×10^5 cells/well in a 6-well dish 1 day prior to transfection by calcium phosphate method as previously described (21). Each transfection was done using an equal amount of total DNA by adding appropriate amount of the control vector, pcDNA3.1 (Invitrogen). At 36-h post-transfection, the cells were harvested, and the whole cell extracts were prepared for the luciferase assay. The luciferase activity was measured by the Luciferase Assay System (Promega) and normalized relative to β -galactosidase activity as described previously (21). The data are obtained from three independent transfections and presented as the -fold increase in luciferase activities (means \pm S.D.) relative to the control. Recombinant human TNF- α and IL-1 β were purchased from R&D Systems.

Immunoprecipitations and Western Blot Analysis—To examine the protein-protein interaction in cultured 293T cells, subconfluent plates were transfected with 2–7 μ g of the indicated combinations of expression vectors by calcium phosphate method. At 36 h post-transfection, cells were lysed in 0.5% Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, and protease inhibitors). Cell lysates were incubated with anti-FLAG Ab, anti-p65 Ab, or anti-CBP Ab for 3 h at 4 °C, and immune complexes were collected by incubation (1 h, 4 °C) with protein G-agarose (Roche Ap-

plied Science). After extensive washing, immunoprecipitated proteins were resolved by 6–10% SDS-PAGE and analyzed by Western blotting with anti-FLAG, anti-p65, or anti-CBP Abs. Membranes were developed with enhanced chemiluminescence (Amersham Biosciences). For endogenous immunoprecipitations, mouse T cell hybridoma KMLs-8.3.5 cells (20) were lysed in 0.5% Nonidet P-40 lysis buffer and incubated with polyclonal anti-p65 Ab or IgG as a control. Western blotting for detection of endogenous PIAS3 and endogenous p65 was performed with polyclonal anti-PIAS3 and anti-p65 Abs, respectively.

Preparation of GST Fusion Proteins and GST Binding Assay—GST-p65 fusion proteins were expressed in *Escherichia coli* BL21(DE) (Novagen) and purified with glutathione-Sepharose 4B beads (Amersham Biosciences) as specified by the manufacturer. Equal amounts of GST fusion proteins (2 μ g) immobilized on the beads were used for binding assay. Cell lysates transfected with FLAG epitope-tagged PIAS3 DNA were incubated with either GST or GST-p65 fusion proteins for 3 h at 4 °C in the 0.5% Nonidet P-40 lysis buffer. Samples were washed four times with the lysis buffer, and the bound proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and detected by Western analysis using enhanced chemiluminescence.

RNA Interference—Custom SMARTpool plus small interfering RNA (siRNA) to target human PIAS3 (catalog no. M-004164-00-05) was designed and synthesized by Dharmacon (Lafayette, CO). siRNA (5 nmol) was transfected into MCF-7 cells using Oligofectamine (Invitrogen) according to the manufacturer's protocol. 48 h after transfection, the cells were treated with TNF- α for 6 h and lysed for NF- κ B-luciferase reporter assay. A nonspecific RNA duplex (Dharmacon, catalog no. D-001130-01-05) was used in control experiments. The relative expression of endogenous PIAS3 was monitored by Western blotting of cell extracts isolated from control or siRNA-treated cells, using a polyclonal antibody against PIAS3 (Santa Cruz Biotechnology).

RESULTS

Identification of PIAS3 as a Novel p65-interacting Partner Using Yeast Two-hybrid System—We used the yeast two-hybrid system to identify proteins that interact with NF- κ B p65. The cDNA fragment encoding amino acids 1–313 of p65, containing the Rel homology domain, was used as bait. We screened \sim 1.5 million transformants from the mouse thymus cDNA library. One of the isolated clones encoded the C-terminal region of the PIAS3 starting from amino acid 298 (data not shown). We subsequently isolated a full-length mouse PIAS3 cDNA clone from the cDNA library derived from a mouse T cell hybridoma cell line KMLs-8.3.5 (20) for further study.

To confirm the interaction between p65 and PIAS3 *in vitro*, we performed a GST pull-down assay. GST fusion proteins with N-terminal or C-terminal portions of p65 (Fig. 1A) were synthesized in *E. coli*, immobilized on glutathione-Sepharose beads, and incubated with the cell lysates containing PIAS3. As shown in Fig. 1B, PIAS3 was retained by GST-p65N-(1–313) (lane 2), but not by GST alone or GST-p65C-(314–551) (lanes 1 and 3). To demonstrate the interaction between p65 and PIAS3 in mammalian cells, we performed a coimmunoprecipitation assay. FLAG-tagged full-length PIAS3 was coexpressed with p65, and cell lysates were immunoprecipitated with either control IgG or anti-FLAG monoclonal Ab. The coimmunoprecipitated protein was examined for the presence of p65 by immunoblot assay using anti-p65 Ab. In Fig. 1C, it can be seen that p65 can be coprecipitated by antibody against the FLAG epitope but not by control mouse IgG Ab. To further confirm this finding, the same cell lysates were immunoprecipitated with anti-p65 polyclonal Ab, and then bound PIAS3 was detected by immunoblotting with anti-FLAG Ab (Fig. 1D). These data demonstrate that p65 interacts with PIAS3 in mammalian cells.

To test whether endogenous PIAS3 and p65 can interact *in vivo* in response to treatment with TNF- α , we prepared total cell lysates from T cell hybridoma KMLs-8.3.5 where both proteins are naturally expressed. With these extracts, we performed immunoprecipitations with either polyclonal anti-p65 Ab or with IgG as a control. As shown in Fig. 1E, endogenous PIAS3 was coimmunoprecipitated from the KMLs-8.3.5 cell ly-

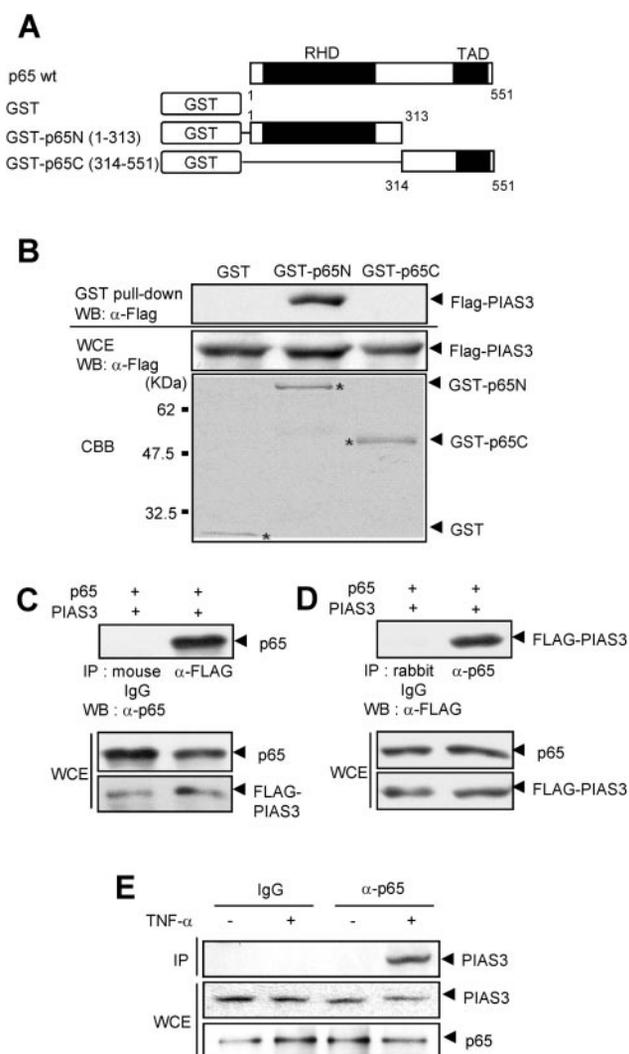


FIG. 1. PIAS3 interacts with p65 *in vitro* and *in vivo*. *A*, schematic illustration of p65 and its mutants. Full-length p65 wild type (*wt*)-(1–551) and two fragments of GST-fused p65, GST-p65N-(1–313) and GST-p65C-(314–551). *RHD*, Rel homology domain; *TAD*, transactivation domain; *GST*, glutathione *S*-transferase. Amino acid positions of p65 are indicated. *B*, 293T cells were transfected with FLAG-tagged PIAS3 plasmid. After 36 h of transfection, cell lysates were incubated with GST, GST-p65N-(1–313), or GST-p65C-(314–551) fusion proteins (2 μ g of each) produced in *E. coli* and immobilized on glutathione-Sepharose beads. The beads were washed, and half of the bound proteins was resolved on SDS-PAGE and stained with Coomassie Brilliant Blue (CBB) to confirm their expression (*lower panel*). The bands corresponding to GST, GST-p65N, or GST-p65C are labeled with asterisks. The other half was resolved on SDS-PAGE and detected by Western blotting (WB) using anti-FLAG Ab (*upper panel*). Whole cell extracts (WCE) were also subjected directly to Western blot analysis with the same Ab to show that equal amounts of PIAS3 were expressed (*middle panel*). *C*, 293T cells were cotransfected with pcDNA-p65 (0.5 μ g) and pFLAG-PIAS3 (1 μ g) as indicated. After 36 h of transfection, cell lysates were immunoprecipitated (IP) with either control mouse IgG or anti-FLAG monoclonal Ab. The bound proteins were detected by Western blotting with anti-p65 polyclonal Ab. The presence of both p65 and FLAG-PIAS3 proteins in the whole cell lysates was verified by Western blotting with either anti-FLAG or anti-p65 Ab. *D*, reciprocally, cell lysates used in *C* were immunoprecipitated with either rabbit IgG or p65 polyclonal Ab, and the bound proteins were detected by Western blotting with Abs as indicated. *E*, lysates from KMI5-8.3.5 cells treated and untreated with TNF- α (20 ng/ml) were immunoprecipitated with either control IgG or polyclonal p65 Ab. The immunoprecipitates were resolved on SDS-PAGE and were probed with anti-PIAS3 Ab (*upper panel*). The *middle* and *lower panels* show equal amounts of PIAS3 and p65 present in whole cell lysates.

sates treated with TNF- α by the p65 Ab (*lane 4*) but not with the control IgG (*lane 2*). In contrast, when untreated with TNF- α , PIAS3 was not detected in the immunoprecipitates of

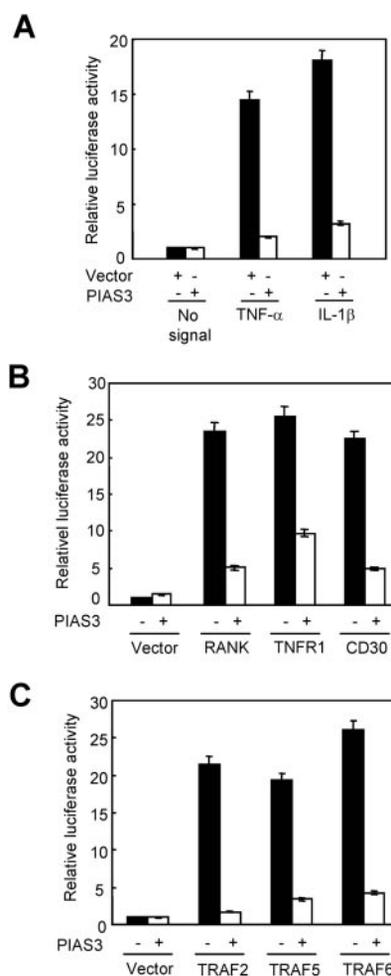


FIG. 2. PIAS3 inhibits NF- κ B activation induced by TNF- α , IL-1 β , TNF family receptors or various signal transducers involved in the NF- κ B signaling. *A*, PIAS3 inhibits TNF- α - and IL-1 β -induced NF- κ B activation. 293T cells were cotransfected with 50 ng of (κ B)₃-interferon-luciferase reporter plasmid, 25 ng of CMV- β -galactosidase plasmid together with 1 μ g of pFLAG empty vector or pFLAG-PIAS3 as indicated. At 36 h after transfection, cells were treated with TNF- α (20 ng/ml) or IL-1 β (10 ng/ml) for 6 h or left untreated. Luciferase activity was then measured as described under “Experimental Procedures.” Relative luciferase activity was normalized to β -galactosidase activity to correct for variability in transfection efficiency. *B*, 293T cells were cotransfected with reporter plasmids and with either plasmids encoding TNF superfamily receptors (RANK, 250 ng; TNFR1, 250 ng; CD30; 500 ng) alone or PIAS3 plasmid (1 μ g) as indicated. NF- κ B reporter gene activity was determined as described in Fig. 2*A*. *C*, as in *B*, except that various TRAFs (TRAF2, 250 ng; TRAF5, 500 ng; TRAF6, 250 ng) expression plasmids were tested for NF- κ B activity toward PIAS3 effect. The results shown in *A*, *B*, and *C* represent the mean \pm S.D. of triplicate experiments.

p65 Ab (*lane 3*). Taken together, these results demonstrate that p65 interacts with PIAS3 *in vivo* upon TNF- α stimulation.

PIAS3 Inhibits Transcriptional Activation of NF- κ B—Because PIAS3 was shown to bind p65, we asked whether PIAS3 had any effect on the NF- κ B-dependent transcription through diverse NF- κ B activating signals. 293T cells were cotransfected with NF- κ B-luciferase reporter plasmids either with empty vectors or with PIAS3 expression plasmids. At 36 h post-transfection, cells were either left untreated or treated with human TNF- α (20 ng/ml) or IL-1 β (10 ng/ml) for 6 h, and then NF- κ B activity was analyzed. As shown in Fig. 2*A*, endogenous NF- κ B activity was maintained at a basal level, and this activity was not affected by PIAS3 overexpression. When overexpressed, PIAS3 inhibited TNF- α - and IL-1 β -mediated NF- κ B activations. The similar effects of PIAS3 were observed when NF- κ B

was activated by overexpressing TNF family receptors (Fig. 2B) or various TRAF molecules (Fig. 2C), including RANK, TNFR1, CD30, TRAF2, TRAF5, and TRAF6, all of which are well known to mediate NF- κ B activation. These results suggest that PIAS3 might function at a point where divergent signals merge in the NF- κ B activation pathways. Because divergent NF- κ B activation signals converge on I κ B- α phosphorylation and degradation, we investigated the effect of PIAS3 on I κ B- α . We did not find any differences between PIAS3-overexpressed and normal cells in the extent of the TNF- α -induced I κ B- α phosphorylation and degradation (Fig. 3A), suggesting PIAS3 might act downstream of I κ B- α .

To investigate whether PIAS3 suppresses the activity of NF- κ B through acting on the p65, we examined the effect of PIAS3 on NF- κ B-dependent transcriptional activity when p65 is overexpressed. As shown in Fig. 3B, PIAS3 was able to suppress p65-mediated NF- κ B activity without affecting p65 expression levels. Therefore, the ability of PIAS3 to inhibit NF- κ B activity most likely results from direct interaction between p65 and PIAS3 rather than from its activity on upstream signaling events involving TRAFs and I κ B- α . Next, we asked whether PIAS3 could affect the transactivation function of any other transcription factor that can be activated by TNF- α . To investigate this, we examined the effect of PIAS3 on the c-Jun transcriptional activity using reporter constructs expressing c-Jun-dependent luciferase. In contrast to the results seen with NF- κ B reporter, overexpression of PIAS3 in 293T cells did not repress a c-Jun-dependent reporter but, rather, enhanced it to a certain extent (Fig. 3C). Therefore, PIAS3 suppression of NF- κ B activity did not result from a general suppression of transcription.

Because our data indicated that PIAS3 overexpression could inhibit TNF- α -mediated NF- κ B activation (Fig. 2A), it seemed likely that inhibition of PIAS3 expression would result in increased TNF- α -mediated NF- κ B activity. MCF-7 cells were transfected with Oligofectamine alone or siRNA directed against PIAS3 or cyclophilin as a control. After 48 h of siRNA transfection, an NF- κ B luciferase reporter construct was co-transfected along with a β -galactosidase expression vector. After an additional 24 h, the cells were stimulated with TNF- α for 6 h. TNF- α stimulation increased the NF- κ B activity in both Oligofectamine and control siRNA-transfected cells in a dose-dependent manner, and there was a further increase in TNF- α -mediated NF- κ B activity in PIAS3 siRNA-transfected cells (Fig. 4A), suggesting that the reduction of PIAS3 leads to increased TNF- α -mediated NF- κ B activity. Western blot analysis demonstrated that PIAS3 siRNA, but no RNA interference or cyclophilin siRNA, specifically reduced the expression of PIAS3 but not β -actin (Fig. 4B).

The LXXLL Motif in PIAS3 Is Exclusively Required for the Interaction with p65—The LXXLL motif has been previously demonstrated to be a protein interaction module that can mediate coactivator-nuclear receptor (NR) interactions or coactivator-coactivator interactions (26–30). Because PIAS3 contains the LXXLL motif starting at the amino acids residue 19, it was of interest to examine whether this putative LXXLL motif is required for the interaction with p65. We generated FLAG-tagged N-terminal (aa 1–391) or C-terminal (aa 391–584) deletion constructs of PIAS3 and a mutant PIAS3 (PIAS3 LL-AA) with point mutations in the LXXLL motif of PIAS3 (LQVLL to LQVAA) (Fig. 5A) and cotransfected these PIAS3 mutants with p65 DNAs into 293T cells. Coimmunoprecipitation data showed that p65 interacted with the C-terminal deletion mutant of PIAS3 expressing N-terminal domain (P3N) but failed to interact with the N-terminal deletion mutant (P3C) (Fig. 5B, lane 4 and 5). Interestingly, the PIAS3 LL-AA

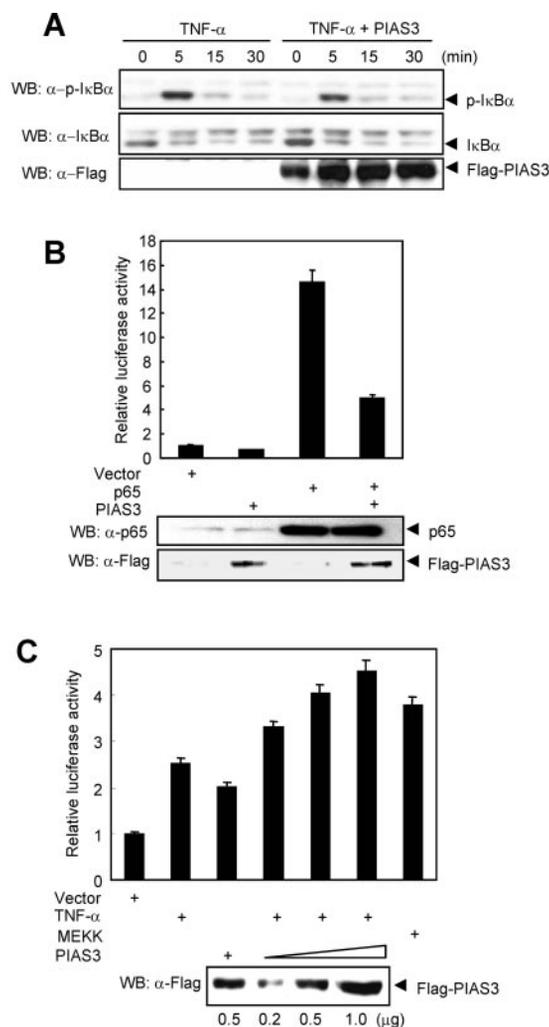


FIG. 3. Effects of PIAS3 on the p65-mediated NF- κ B-transactivation. A, PIAS3 does not interfere with TNF- α -induced I κ B α phosphorylation and degradation. 293T cells were transfected with either 1 μ g of pFLAG-PIAS3 or same amount of empty vector. After 36 h of transfection, cells were stimulated with 20 ng/ml TNF- α for indicated minutes and lysed. Western blotting was performed on the lysates with specific antibody for I κ B α and phospho-I κ B α (upper and middle panels). The lower panel shows that equal amounts of PIAS3 were expressed in each transfection. B, PIAS3 represses p65-mediated transcriptional activity. 293T cells were transfected with (κ B) $_3$ -interferon-luciferase reporter plasmid and with either pcDNA-p65 (0.5 μ g) or pFLAG-PIAS3 (1 μ g) expression plasmids as indicated. Reporter assays were performed as described in Fig. 2A. Controls for the expression of p65 and FLAG-tagged PIAS3 are shown in the bottom panel. The results shown represent the mean \pm S.D. of triplicate experiments. C, effects of PIAS3 on the c-Jun transcriptional activity monitored with a pathway-specific trans-activator plasmid encoding a GAL4 DNA-binding domain fused to c-Jun activator domains and a GAL4 UAS/luciferase reporter construct. 293T cells were transfected with 100 ng of pFR-luc and 50 ng of pFA-c-Jun together with the indicated amounts of PIAS3 expression plasmids. As positive controls, either mock transfected cells were treated with TNF- α (20 ng/ml) for 6 h or cells or 293T cells were transfected with 0.2 μ g of an expression vector containing the MEKK. The results shown represent the mean \pm S.D. of triplicate experiments. Controls for the expression of FLAG-tagged PIAS3 are shown in the bottom panel.

mutant was unable to interact with p65 (lane 3), even though all PIAS3 derivatives were produced in comparable levels (middle panel). These results indicated that the LXXLL motif of PIAS3 is minimally required for the interaction with p65.

To determine the functional significance of the LXXLL motif-mediated interaction between PIAS3 and p65 in terms of NF- κ B activity, 293T cells were transfected with expression vectors encoding the wild type PIAS3 or the various PIAS3

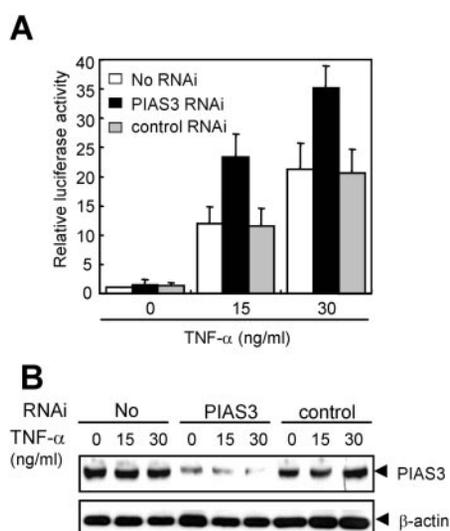


FIG. 4. PIAS3 siRNA increases TNF- α -induced NF- κ B activity. *A*, MCF-7 cells were transfected with either Oligofectamine alone (lanes 1, 4, and 7) or Oligofectamine containing 5 nM siRNA directed against PIAS3 (lanes 2, 5, and 8) or cyclophilin (lanes 3, 6, and 9). After 48-h siRNA transfection, the 50 ng of (κ B)₃-interferon-luciferase reporter plasmid and 25 ng of CMV- β -galactosidase plasmid were cotransfected. Following an additional 24 h, the cells were treated with TNF- α for 6 h, and luciferase activity was determined and normalized for transfection efficiency. The results shown represent the mean \pm S.D. of triplicate experiments. *B*, Western blot analysis using PIAS3 antibody was performed to determine PIAS3 protein levels following siRNA transfection.

mutants together with p65 and NF- κ B reporter. Consistent with the *in vivo* coimmunoprecipitation assays, PIAS3-C lacking N-terminal portions (1–391) containing the LXXLL motif and the PIAS3 LL-AA mutant, did not inhibit the transcriptional activity of NF- κ B. However, PIAS3-N, which has the LXXLL motif and is able to bind to p65, inhibited the NF- κ B activity comparable to the wild type PIAS3. These results suggest that the N-terminal region of PIAS3 and most likely the LXXLL motif are important in interacting with p65 and negatively regulating p65-mediated NF- κ B activity.

PIAS3 Interferes with the Interaction of p65 with CBP—Because the CBP and its homologue, p300 coactivator, interact with the p65 subunit of NF- κ B, thereby enhancing its ability to activate transcription (12, 13), we sought next to determine whether PIAS3 could also suppress the NF- κ B activity enhanced by coexpression of p65 and CBP. Whereas overexpression of p65 stimulated transcription from the reporter gene by nearly a factor of 10 and CBP alone stimulated transcription by 2.5-fold, the combination of p65 plus CBP led to a 20-fold increase in transcriptional activity (Fig. 6A). Importantly, cotransfection of PIAS3 with p65 plus CBP resulted in significantly less activation of NF- κ B. In contrast, expression of PIAS3 mutant with point mutations in the LXXLL motif had no effect on the NF- κ B activity. We next asked whether overexpression of CBP could overcome the inhibition effects of PIAS3 on NF- κ B activity. Interestingly, the inhibitory effects of wild type PIAS3 were relieved when CBP was expressed at a high dose (Fig. 6B). Together, these results suggest that PIAS3 can

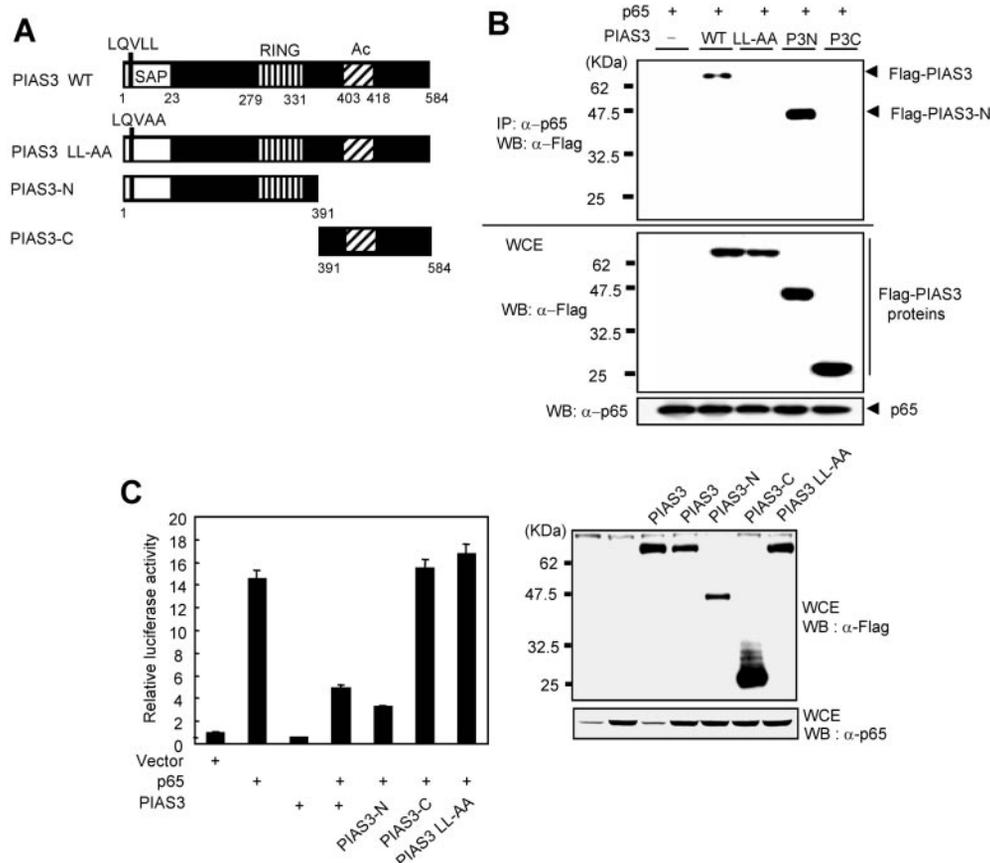


FIG. 5. The LXXLL motif of PIAS3 is required for the interaction with p65. *A*, schematic illustration of wild type PIAS3 and its mutants. LQVLL, LQVLL coregulator signature motif present at the NH₂ terminus of PIAS3; SAP, SAF-A, Acinus, PIAS module; RING, a putative DNA-binding domain; Ac, an acidic region. *B*, the PIAS3-p65 interaction requires the LXXLL motif of PIAS3. 293T cells were transfected with the indicated combinations of expression plasmids. After 36 h of transfection, cell lysates were immunoprecipitated with anti-p65 polyclonal Ab. The bound proteins were detected by Western blotting with anti-FLAG Ab (upper panel). Controls for the expression of FLAG-PIAS3 and p65 are shown (middle and bottom panels). *C*, NF- κ B reporter assays were performed as in Fig. 3B except that FLAG-tagged PIAS3 mutants were tested for NF- κ B activity (left panel). The right panel shows the relative levels of PIAS3 and p65 expressed in each transfection.

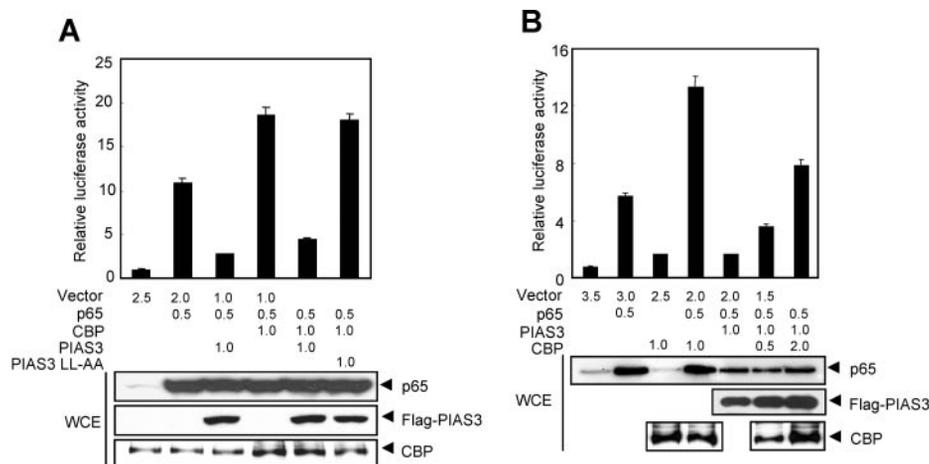


FIG. 6. Effect of PIAS3 on the enhancement of the NF- κ B activity by CBP. A, 293T cells were cotransfected with the reporter plasmids together with the indicated amounts (in micrograms) of expression plasmids, including p65, PIAS3, PIAS3 LL-AA mutant, and CBP. After 36 h of transfection, NF- κ B reporter assays were performed as in Fig. 3B. To control for the expression of FLAG-tagged PIAS3, PIAS3 LL-AA mutant, p65, and CBP, whole cell extracts were analyzed by Western blotting (*bottom panel*). B, PIAS3 effects on NF- κ B activity can be reversed by CBP overexpression. NF- κ B reporter assays were performed as in Fig. 6A except that the indicated amounts of CBP were transfected in the presence of p65 and PIAS3. To control for the expression of FLAG-tagged PIAS3, p65 and CBP, whole cell extracts were analyzed by Western blotting (*bottom panel*). The results shown in A and B represent the mean \pm S.D. of triplicate experiments.

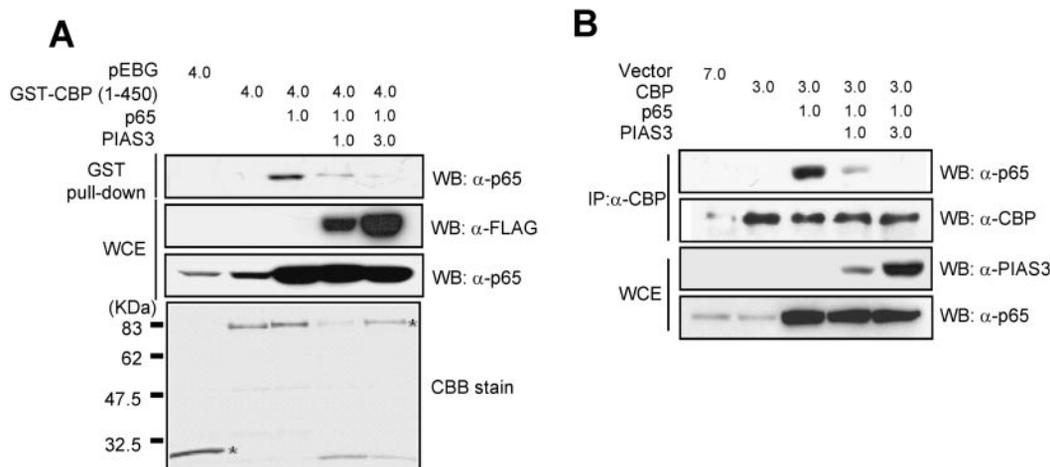


FIG. 7. PIAS3 inhibits the interaction between p65 and CBP. A, 293T cells were transfected with the indicated amounts (in micrograms) of pEBG-CBP(1-450), p65, and PIAS3 expression vectors. After 36 h of transfection, aliquots of cell lysates were subjected for purification with glutathione-Sepharose beads as described under "Experimental Procedures." Proteins coprecipitated with GST beads were analyzed by Western blotting with anti-p65 Ab. Whole cell extracts were analyzed by Western analysis with anti-FLAG and anti-p65 Ab to monitor the expression of transfected DNAs. Coomassie Brilliant Blue stain (*CBB stain*) shows the expression of GST or GST-CBP(1-450). The bands corresponding to GST or GST-CBP(1-450) are labeled with *asterisks*. B, Western blot analyses were performed as in Fig. 6A except that full-length CBP expression vectors were transfected with the indicated combinations together with p65 and PIAS3 expression vectors.

suppress the transcriptional enhancement of NF- κ B activity by CBP.

Previous studies have shown that the N-terminal region of CBP (aa 1-450) is sufficient for the interaction with p65 (10, 12). Moreover, the N-terminal 450 amino acids of CBP are required to mediate NF- κ B-induced transcriptional enhancement (31). Thus, it was conceivable that PIAS3 blocks coactivation by CBP by inhibiting the formation of the p65-CBP complex. To test this possibility, 293T cells were cotransfected with an expression plasmid encoding the GST-fused N-terminal region of CBP (aa 1-450) together with p65 and increasing amounts of PIAS3 expression vectors. As shown in Fig. 7A, p65 was detected in lysates precipitated with GST beads containing the N-terminal region of CBP (*lane 3*). Importantly, when GST-CBP and p65 were expressed together with PIAS3, the amounts of p65 found in GST beads precipitates were severely decreased in a dose-dependent manner (Fig. 7A, *lane 4 and 5*). Similar results were obtained when a full-length CBP was used to transfect and to immunoprecipitate the proteins (Fig. 7B,

lanes 3-5). Taken together, these results suggest that transcriptional suppression by PIAS3 is dependent on its ability to compete with CBP for binding to p65.

DISCUSSION

In this study, we present data demonstrating the interaction of the p65/RelA subunit of NF- κ B with PIAS3. By both *in vitro* binding assay and coimmunoprecipitation analysis, we demonstrate that the Rel homology domain of the p65, but not the C-terminal domain, which contains the transactivation domain, interacts with the N-terminal region of PIAS3. Further mapping of the PIAS3 interaction revealed that an LXXLL coregulator signature motif of PIAS3 is responsible for the functional interaction with p65. When PIAS3 was overexpressed, the transcriptional activity of p65 was significantly suppressed. Interestingly, overexpression of PIAS3 interfered with the ability of the CBP coactivator to interact with p65. This suggests that PIAS3 exerts its effects through interfering with the interaction between p65 and CBP. Based on these

results, we conclude that PIAS3 is a novel negative regulator of NF- κ B p65.

Biochemical studies indicate that PIAS proteins interact directly with different transcription factors, including STATs, steroid receptors, and p53, and can regulate their transcriptional activities both positively or negatively (32–35). In particular, PIAS3 was initially isolated as a specific inhibitor of STAT3 signaling and shown to inhibit the DNA-binding activity of STAT3 (36). Recently, however, additional roles of PIAS3 have been reported in different transcriptional regulations through its physical interaction with other proteins, including a zinc finger protein Gfi-1 (37), microphthalmia transcription factor (38), and nuclear receptor coactivator TIF2 (39). In addition to these different classes of signal-activated transcriptional factors or coactivators, our findings put NF- κ B p65 to the list of PIAS3-interacting proteins. It is likely that the transcriptional effects of PIAS3 might be quite diverse, depending on their interaction partners, activation signals, and cell types.

PIAS3 expression did not block upstream events in the NF- κ B activation cascades, including phosphorylation and degradation of I κ B- α or nuclear translocation of p65 (data not shown). Recent data indicated that PIAS3 predominantly exists in the nucleus (37). When stimulated with TNF- α , which causes the translocation of NF- κ B, the activated NF- κ B might interact with PIAS3 already present in the nucleus. Consistently, we detected TNF- α -dependent interaction of p65 with PIAS3. Similar to this, STAT3-PIAS3 interaction, in which their interaction depends on IL-6 stimulation, is also found in the nucleus (36). Our *in vitro* gel shift assays did not reveal any effect of PIAS3 on the DNA-binding activity of NF- κ B (data not shown). This finding is consistent with a previous report (36), in which GST-PIAS3 had no effect on the TNF- α -induced DNA-binding activity of NF- κ B. It is therefore likely that the mechanism by which PIAS3 inhibits p65-mediated transcriptional activity is different from those involving STAT3 or microphthalmia transcription factor, whose DNA-binding activity is inhibited by PIAS3.

One attractive hypothesis is that PIAS3 regulates p65-mediated transcriptional activity through promoting p65 sumoylation, because a putative RING domain of the PIAS family has recently been shown to be involved in sumoylation and in fact has been suggested to function as a sumo-E3 ligase (40, 41). Unlike ubiquitination, sumoylation does not appear to promote protein degradation but rather was shown to be involved in protein-protein interactions, subcellular compartmentalization, and protein stability (42, 43). In this context, we tested whether sumoylation regulates p65-mediated transcriptional activity. However, SUMO conjugation of p65 is not detected in the presence of PIAS3 and overexpression SUMO and p65 together with κ B reporter gene did not repress p65-mediated NF- κ B activity (data not shown). Furthermore, p65 lacks the consensus sequences (ψ KXE; ψ , hydrophobic amino acid), required for SUMO-1 conjugation (44).

The LXXLL signature motif was originally identified in several nuclear receptor coactivators and corepressors. The α -helical LXXLL signature motif is a protein interaction module that has been demonstrated to mediate coregulator-NR interactions or coregulator-coregulator interactions (27–29). In addition, the LXXLL motif in PIASy is required for suppression of androgen receptor (AR) transactivation (45). Recently, Liu *et al.* (30) observed that the LXXLL motif of PIASy is not involved in PIASy-STAT1 interaction but is absolutely required for the repression activity of PIASy. Thus, it seems that the LXXLL motif can act either as a protein interaction module or as a functional repressor module depending on the binding partners. In our experiments with PIAS3, mutating the LXXLL

motif to LXXAA resulted in the loss of interaction with p65, suggesting the motif is an interaction module of p65.

Recent work has shown that NF- κ B-dependent gene expression requires the function of transcriptional coactivators, including CBP, SRC-1/NcoA-1, TIF-2/GRIP-1/NcoA2, and p/CAF (17). Among them, CBP plays a critical role as a bridge factor between sequence-specific transcriptional activator and basal transcriptional machinery. In this context, interaction of CBP with p65 may facilitate recruitment of other transcriptional coactivators. Because the level of CBP is limiting in comparison to that of p65 (46), it is possible that competition for p65 between CBP and PIAS3 may regulate p65 transactivation. In accordance with this scenario, increasing amounts of CBP could counteract PIAS3-mediated repression on NF- κ B activity. Conversely, increasing amounts of PIAS3 interfere with CBP-p65 interaction.

Taken together, we propose a model whereby PIAS3 functions as a negative regulator of NF- κ B. In the absence of PIAS3 for a given cell type, the activated NF- κ B interacts with various factors, including general transcription factors and coactivators, to activate the transcription of NF- κ B target genes. In contrast, when PIAS3 exists in the nucleus, PIAS3 physically interacts with p65, thereby affecting NF- κ B activity depending on the relative levels of NF- κ B, coactivators such as CBP, and PIAS3.

Acknowledgment—We thank Dr. Jaesang Kim for critical reading for the manuscript.

REFERENCES

- Baldwin, A. S., Jr. (1996) *Annu. Rev. Immunol.* **14**, 649–683
- Karin, M., and Ben-Neriah, Y. (2000) *Annu. Rev. Immunol.* **18**, 621–663
- Ghosh, S., and Karin, M. (2002) *Cell* **109**, (suppl.) 81–96
- May, M. J., and Ghosh, S. (1997) *Semin. Cancer Biol.* **8**, 63–73
- Perkins, N. D. (2000) *Trends Biochem. Sci.* **25**, 434–440
- Karin, M., Cao, Y., Greten, F. R., and Li, Z. W. (2002) *Nat. Rev. Cancer* **2**, 301–310
- Foo, S. Y., and Nolan, G. P. (1999) *Trends Genet.* **15**, 229–235
- Bours, V., Bonizzi, G., Bentires-Alj, M., Bureau, F., Piette, J., Lekeux, P., and Merville, M. (2000) *Toxicology* **153**, 27–38
- Mercurio, F., and Manning, A. M. (1999) *Curr. Opin. Cell Biol.* **11**, 226–232
- Gerritsen, M. E., Williams, A. J., Neish, A. S., Moore, S., Shi, Y., and Collins, T. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 2927–2932
- Perkins, N. D., Felzien, L. K., Betts, J. C., Leung, K., Beach, D. H., and Nabel, G. J. (1997) *Science* **275**, 523–527
- Zhong, H., Voll, R. E., and Ghosh, S. (1998) *Mol. Cell* **1**, 661–671
- Zhong, H., SuYang, H., Erdjument-Bromage, H., Tempst, P., and Ghosh, S. (1997) *Cell* **89**, 413–424
- Zhong, H., May, M. J., Jimi, E., and Ghosh, S. (2002) *Mol. Cell* **9**, 625–636
- Sheppard, K. A., Rose, D. W., Haque, Z. K., Kurokawa, R., McInerney, E., Westin, S., Thanos, D., Rosenfeld, M. G., Glass, C. K., and Collins, T. (1999) *Mol. Cell Biol.* **19**, 6367–6378
- Na, S. Y., Lee, S. K., Han, S. J., Choi, H. S., Im, S. Y., and Lee, J. W. (1998) *J. Biol. Chem.* **273**, 10831–10834
- Goodman, R. H., and Smolik, S. (2000) *Genes Dev.* **14**, 1553–1577
- Ashburner, B., Westerheide, S. D., and Baldwin, A. S., Jr. (2001) *Mol. Cell Biol.* **21**, 7065–7077
- benezra, M., Chevallier, N., Morison, D., MacLachlan, T. K., El-Deiry, W. S., and Licht, J. D. (2003) *J. Biol. Chem.* **278**, 26333–26342
- Lee, S. Y., Park, C. G., and Choi, Y. (1996) *J. Exp. Med.* **183**, 669–674
- Lee, S. Y., Lee, S. Y., and Choi, Y. (1997) *J. Exp. Med.* **185**, 1275–1285
- Lee, S. Y., Lee, S. Y., Kandala, G., Liou, M.-L., Liou, H. C., and Choi, Y. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 9699–9703
- Wong, B. R., Josien, R., Lee, S. Y., Vologodskaja, M., Steinman, R. M., and Choi, Y. (1998) *J. Biol. Chem.* **273**, 28355–28359
- Chung, Y. M., Park, K. J., Choi, S. Y., Hwang, S. B., and Lee, S. Y. (2001) *Biochem. Biophys. Res. Commun.* **284**, 15–19
- Hwang, S., Gwack, Y., Byun, H., Lim, C., and Choe, J. (2001) *J. Virol.* **75**, 9509–9516
- Glass, C. K., and Rosenfeld, M. G. (2000) *Genes Dev.* **14**, 121–141
- Darimont, B. D., Wagner, R. L., Apriletti, J. W., Stallcup, M. R., Kushner, P. J., Baxter, J. D., Fletterick, R. J., and Yamamoto, K. R. (1998) *Genes Dev.* **12**, 3343–3356
- Neear, A. M., Beaurang, P. A., Zhou, S., Abraham, S., Solomon, W., and Tjian, R. (1999) *Nature (Lond.)* **398**, 828–832
- Rachez, C., Lemon, B. D., Suldan, Z., Bromleigh, V., Gamble, M., Neear, A. M., Erdjument-Bromage, H., Tempst, P., and Freedman, L. P. (1999) *Nature (Lond.)* **398**, 824–828
- Liu, B., Gross, M., Hoeve, J., and Shuai, K. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 3203–3207
- Hiroi, M., and Ohmori, Y. (2003) *J. Biol. Chem.* **278**, 651–660
- Liu, B., Liao, J., Rao, X., Kushner, S. A., Chung, C. D., Chang, D. D., and Shuai, K. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 10626–10631

33. Kotaja, N., Aittomaki, S., Silvennoinen, O., Palvimo, J. J., and Janne, O. A. (2000) *Mol. Endocrinol.* **14**, 1986–2000
34. Gross, M., Liu, B., Tan, J., French, F. S., Carey, M., and Shuai, K. (2001) *Oncogene* **20**, 3880–3887
35. Nelson, V., Davis, V., and Maxwell, S. A. (2001) *Apoptosis* **6**, 221–234
36. Chung, C. D., Liao, J., Liu, B., Rao, X., Jay, P., Berta, P., and Shuai, K. (1997) *Science* **278**, 1803–1805
37. Rodel, B., Tavassoli, K., Karsunky, H., Schmidt, T., Bac, P., and Möröy, T. (2000) *EMBO J.* **19**, 5845–5855
38. Levy, C., Nechushtan, H., and Razin, E. (2002) *J. Biol. Chem.* **277**, 1962–1966
39. Jiménez-Lara, A. M., Heine, M. J. S., and Gronemeyer, H. (2002) *FEBS Lett.* **526**, 142–146
40. Johnson, E. S., and Gupta, A. A. (2001) *Cell* **106**, 735–744
41. Kahyo, T., Nishida, T., and Yasuda, H. (2001) *Mol. Cell* **8**, 713–718
42. Schdev, S., Bruhn, L., Sieber, H., Pichler, A., Melchior, F., and Grosschedl, R. (2001) *Genes Dev.* **15**, 3088–3103
43. Kentsis, A., Gordon, R. E., and Borden, K. L. B. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 667–672
44. Rodriguez, M. S., Dargemont, C., and Hay, R. T. (2001) *J. Biol. Chem.* **276**, 12654–12659
45. Alen, P., Claessens, F., Verhoeven, G., Rombauts, W., and Peeters, B. (1999) *Mol. Cell. Biol.* **19**, 6085–6097
46. Hottigen, M. O., Felzien, L. K., and Nabel, G. J. (1998) *EMBO J.* **17**, 3124–3134

PIAS3 Suppresses NF- κ B-mediated Transcription by Interacting with the p65/RelA Subunit

Hyun Duk Jang, Kwiyeom Yoon, Young Joo Shin, Jaesang Kim and Soo Young Lee

J. Biol. Chem. 2004, 279:24873-24880.

doi: 10.1074/jbc.M313018200 originally published online March 26, 2004

Access the most updated version of this article at doi: [10.1074/jbc.M313018200](https://doi.org/10.1074/jbc.M313018200)

Alerts:

- [When this article is cited](#)
- [When a correction for this article is posted](#)

[Click here](#) to choose from all of JBC's e-mail alerts

This article cites 46 references, 24 of which can be accessed free at <http://www.jbc.org/content/279/23/24873.full.html#ref-list-1>