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Ribosomal Protein S3: A KH Domain Subunit in NF-κB Complexes that Mediates Selective Gene Regulation

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SUMMARY

NF-κB is a DNA-binding protein complex that transduces a variety of activating signals from the cytoplasm to specific sets of target genes. To understand the preferential recruitment of NF-κB to specific gene regulatory sites, we used NF-κB p65 in a tandem affinity purification and mass spectrometry proteomic screen. We identified ribosomal protein S3 (RPS3), a KH domain protein, as a non-Rel subunit of p65 homodimer and p65-p50 heterodimer DNA-binding complexes that synergistically enhances DNA binding. RPS3 knockdown impaired NF-κB-mediated transcription of selected p65 target genes but not nuclear shuttling or global protein translation. Rather, lymphocyte-activating stimuli caused nuclear translocation of RPS3, parallel to p65, to form part of NF-κB bound to specific regulatory sites in chromatin. Thus, RPS3 is an essential but previously unknown subunit of NF-κB involved in the regulation of key genes in rapid cellular activation responses. Our observations provide insight into how NF-κB selectively controls gene expression.

INTRODUCTION

NF-κB was originally detected as a site-specific DNA-binding protein complex (Sen and Baltimore, 1986b) required for the activity of the immunoglobulin (Ig) light-chain gene intronic enhancer (Lenardo et al., 1987). The mammalian NF-κB family includes five proteins: p65 (RelA), c-Rel, RelB, p50, and p52, which function as homo- or heterodimers to control gene transcription (Ghosh and Karin, 2002). Individual dimers exhibit varying binding affinities for specific recognition sites and differ in their regulatory function (Kang et al., 1992; Natoli et al., 2005). The most abundant form of NF-κB contains a p65-p50 heterodimer that regulates many important immunity, inflammation, and antiapoptosis genes (Lenardo and Baltimore, 1989; Sen, 2006). Although NF-κB activation has been studied extensively, how NF-κB recognizes specific DNA-binding sites and governs selected gene expression in response to distinct inducers in different cell types remains an important unresolved question.

NF-κB exerts its fundamental role as a transcription factor by binding to variations of κB sites (Chen et al., 1998). The binding of Rel protein dimers to these sites in target genes is minimally required, but questions remain about whether this is sufficient for NF-κB regulation. Crosslinking and structural studies of NF-κB binding reveal that p50 binds to the relatively invariant 5′ half of κB sites and p65 binds to the more variable 3′ half (Chen et al., 1998; Urban et al., 1991). The complexity of p65 binding was highlighted by a chromatin immunoprecipitation (ChIP)-microarray assay revealing 209 regions on chromosome 22, of which only 60% contain canonical or related κB sites (Martone et al., 2003). High-affinity NF-κB binding cannot be explained solely by the number or character of heterodimer:DNA contacts (Chen et al., 1998). Interestingly, the original size estimate of natural NF-κB found in nuclear extracts was >200 kDa, but purified p50 and p65 proteins form a 115 kDa complex, the size of a simple heterodimer (Urban et al., 1991). Furthermore, reconstituted heterodimers from purified proteins have an > 100-fold lower affinity than native NF-κB, at least for binding to the Ig κB motif (Phelps et al., 2000). These findings hint that natural NF-κB may contain more than just Rel protein dimers.

We hypothesized that other proteins are integral parts of NF-κB and used NF-κB p65 as a bait protein in a proteomic screen. We identified the KH domain protein ribosomal protein S3 (RPS3) as a p65-binding component of NF-κB complexes that was essential for NF-κB binding

to specific regulatory sites and for inducible expression of a variety of genes. This surprising extraribosomal function for RPS3 provides insight into how NF-κB selectively controls gene expression.

RESULTS

p65 Interacts with RPS3

To search for novel NF-κB proteins, we fused p65 with tandem affinity peptides (TAP), overexpressed this "bait" protein in 293T cells, and copurified p65-binding proteins through two consecutive affinity steps. Mass spectrometry identified ribosomal protein S3 (RPS3) as a major coprecipitant (Figures 1A and S1, see Supplemental Data available with this article online). No other ribosomal proteins were detected, ruling out contamination with whole ribosomes. RPS3 consists of 243 amino acids with a nuclear localization signal (NLS) domain and a KH domain (Figure 1A). RPS3 is a component of the 40S ribosomal subunit, but it also has extraribosomal functions in DNA repair (Hegde et al., 2004).

In a GST pull-down assay, we demonstrated that RPS3 binds to p65 (Figure 1B). Immunoprecipitation of endogenous RPS3 revealed a strong association with endogenous p65 in Jurkat cells (Figure 1C). We also detected p50 and IκBα in this immunoprecipitate (Figure 1C). Additional experiments showed no direct interaction of RPS3 with p50, RelB, c-Rel, or IκBα (data not shown), suggesting that the association of p50 and IκBα with RPS3 was indirect and occurred through an inhibitory p50:p65:IκBα complex. Conversely, endogenous RPS3 was precipitated with antibodies against endogenous p65 and p50 (Figure 1D). Hence, there is a strong physical interaction between RPS3 and p65.

RPS3 Knockdown Impairs NF-κB Signaling

We next measured the effect of RNAi-mediated knockdown of RPS3 on the expression of an NF-κB-driven luciferase reporter construct. Four different siRNA duplexes decreased the total amount of RPS3 protein by ~50% compared to a nonspecific (NS) control siRNA (Figure 2A). As expected, T cell receptor (TCR) and TNFα stimulation induced NF-κB-driven luciferase activity in NS siRNA-treated cells, but this was strongly and equivalently suppressed by RPS3 knockdown or p65 knockdown (Figures 2A and S2). NF-κB activity was restored by an RPS3 expression construct containing a mutated nucleotide sequence that could not be targeted by the RPS3 siRNA (mut1), confirming that the loss of NF-κB transcription was not due to off-target effects (Figure 2B). Knockdown of RPS16, a different 40S ribosomal protein, had no effect on NF-κB reporter activity (Figure 2C). Taken together, these data specifically implicate RPS3 in NF-κB-induced reporter gene expression during T cell stimulation. Conversely, reduction of RPS3 did not impair phorbol myristate acetate (PMA)-induced AP-1 signaling (Figure 2D). Therefore, silencing RPS3 affected NF-κB signaling, but not all TCR-induced signal transduction.

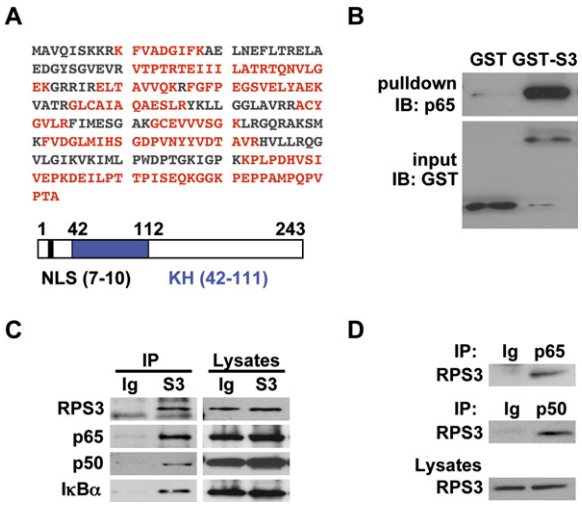

Figure 1. The Interaction between p65 and RPS3

(A) The amino acid sequence and a diagram of RPS3; the residues detected by mass spectrometry are shown in red. NLS, nuclear localization signal; KH, K homology.

(B) Pull-down with GST-S3 or GST proteins with nuclear extracts from TCR-stimulated Jurkat cells, followed by immunoblotting for p65 or GST.

(C) Whole-cell lysates from Jurkat cells were immunoblotted directly or after immunoprecipitation with RPS3 antiserum (S3) or preimmune serum (Ig) for the indicated proteins.

(D) Lysates as in (C) were immunoblotted for RPS3 directly or after immunoprecipitation with p65, p50, or isotype control (iso) antibodies.

Ribosomal and Cytosolic Pools of RPS3

As a component of the ribosomal 40S subunit, RPS3 plays a key role in protein translation. However, protein synthesis in cells transfected with NS versus RPS3 siRNA was equivalent, showing that the abrogation of NF-κB signaling by silencing RPS3 is not due to a general protein translation defect (Figure S3). These data also suggest that the RPS3 siRNA had a disproportionate effect on p65-associated RPS3. Given the known stability of ribosomes, we hypothesized that there was a distinct pool of RPS3 sequestered from ribosome-associated RPS3 that was more labile and therefore more susceptible to RNAi. We subjected cell lysates to ultracentrifugation to separate the soluble, ribosome-free cytosol (S100) from a particulate pellet containing ribosomes (P100). We detected RPS3 in both the S100 and P100 fractions, in proportions of ~40% and 60% of total cellular RPS3 (Figure 2E). We also found that the cytosolic RPS3 was clearly reduced after RNAi knockdown, whereas the ribosomal RPS3 was largely unaffected (Figure 2E). Hence, it is likely that the cytosolic pool of RPS3 plays the major role in NF-κB signaling, marked by a striking inhibition of NF-κB-driven transcription when it is eliminated by RPS3 siRNA.

RPS3 Translocates to the Nucleus

We next investigated whether RPS3 affected p65 liberation from IκBα or nuclear translocation. We found no

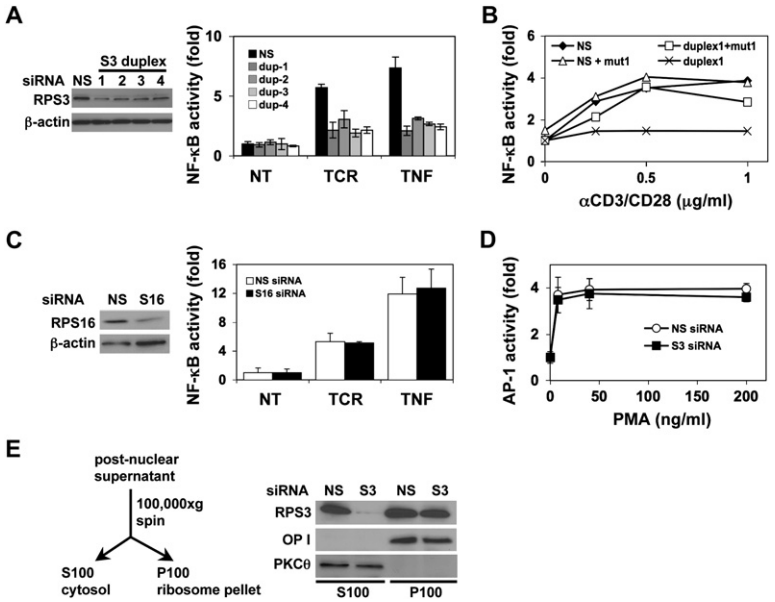

Figure 2. RPS3 Depletion Impairs NF-κB Signaling

(A) Jurkat cells were transfected with p-κB-Luc reporter and pTKRL plasmids plus NS or four different RPS3 siRNAs (S3 duplex 1–4). After 48 hr, the cells were stimulated with αCD3/CD28 (TCR) or TNFα (TNF), or were left untreated (NT), and were analyzed. Shown are relative NF-κB-driven luciferase activity (fold increase). In this and all other figures, error bars represent ± SD. RPS3 knockdown is shown at the left by immunoblotting, with a β-actin control.

(B) Jurkat cells were cotransfected with siRNA, luciferase reporter plasmids, and either vector plasmid or a plasmid encoding RPS3 with mutations in the 21 bp site recognized by the siRNA that did not alter the amino acid sequence (mut1). A total of 48 hr later, the cells were stimulated and analyzed as in (A).

(C) The effect of reducing ribosomal protein S16 (S16) by RNAi on TCR- and TNFα-stimulated NF-κB signaling determined as in (A).

(D) Jurkat cells were analyzed as described above by using p-AP-1-Luc reporter plasmid and PMA stimulation. Shown are means ± SD of relative AP-1-driven luciferase activity (fold increase).

(E) Subcellular fractions were prepared from Jurkat cells transfected with NS or RPS3 (S3) siRNA. Equal amounts of S100 (cytosol) and P100 (crude ribosome pellet) fractions were immunoblotted with antibodies for RPS3, oxidative phosphorylation complex I (OP I, P100 marker), and PKCθ (S100 marker).

such effect; rather, RPS3 itself translocated from the cytoplasm to the nucleus when assessed by immunofluorescence staining after stimulation with αCD3/CD28 or TNFα, but not geldanamycin (GA), an NF-κB-independent stimulus (Figure 3A). Immunoblots revealed that, after stimulation, the cytoplasmic levels of p65 and RPS3 decreased and nuclear levels increased with similar kinetics, whereas RPS16 remained unchanged (Figure 3B). We also found that nuclear translocation of p65 was identical after TCR stimulation in NS- and RPS3-silenced cells, as judged by western blot and immunofluorescence (Figures 3B and 3C). Furthermore, IκBα degradation occurred normally in response to activating stimuli despite RPS3 knockdown (data not shown). Thus, RPS3 does not govern p65 nuclear accumulation. Conversely, p65 knockdown has no influence on TCR-induced RPS3 nuclear accumulation (Figures 3B and 3D). Each protein contains an NLS that may facilitate independent nuclear translocation. The surprising observation that RPS3 translocates into the nucleus after cellular activating stimuli underscores its novel extraribosomal function in NF-κB signaling since the primary translation function of RPS3 operates in the cytoplasm.

RPS3 Contributes to NF-κB Signaling Depending on Relative p65 Expression

A variety of coregulators are known to influence NF-κB signaling, and most of these can physically associate with NF-κB-family proteins (Gao et al., 2005). We therefore tested whether RPS3 serves as a coactivator for p65, but we found that RPS3 overexpression failed to boost NF-κB signaling either before or after TCR stimulation (Figure S4A). Using a standard coactivation assay, we found that RPS3 possesses little, if any, intrinsic transcriptional activating ability and therefore does not resemble a conventional coactivator (Figures S4B and S4C, detailed results in Supplemental Data). We also tested whether p65 overexpression could bypass the requirement for RPS3 in NF-κB signaling. In cells that received NS siRNA, p65 overexpression significantly increased NF-κB signaling (Figure 4A). By contrast, RPS3 knockdown prevented p65 overexpression from increasing NF-κB activity, indicating that excess p65 cannot override the requirement for RPS3 (Figure 4A). A similar result was obtained by overexpressing the inhibitor of κB kinase β (IKKβ), an upstream activator for p65 (Figure 4B). Thus, RPS3 affects a later step in NF-κB signaling, either downstream or in conjunction with p65. Interestingly, coexpression of the two proteins successfully augmented NF-κB signaling, in a RPS3 dose-dependent manner, compared to the signal induced by p65 overexpression alone (Figure 4C). This suggests that RPS3 plays a critical role in p65-mediated NF-κB signaling (Figures 4A and 4B), most likely in stoichiometric relation to the cellular concentration of p65. A mutual transcriptional function involving binding of RPS3 and p65 was also supported by enhanced transcription of pG5-Luc involving coexpression of GAL4-RPS3 and p65 compared with p65 alone (Figure S4D).

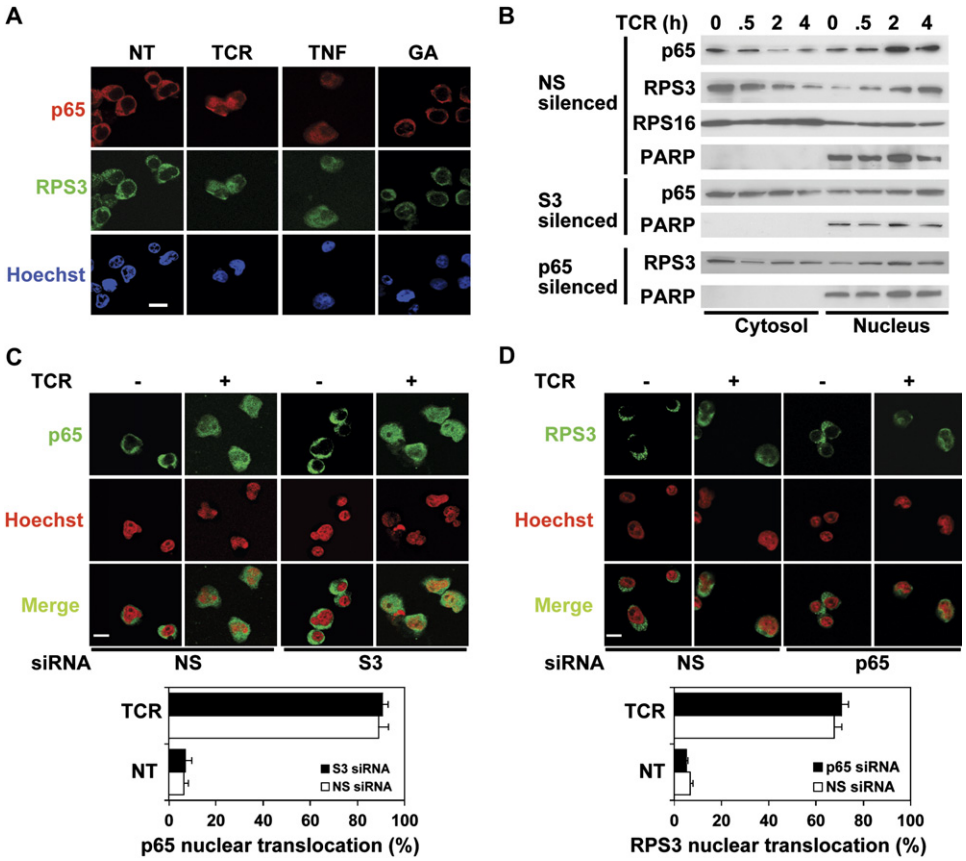

Figure 3. Independent Nuclear Translocation of p65 and RPS3

(A) Confocal micrographs of Jurkat cells stimulated with no treatment (NT), αCD3/CD28 (TCR), TNFα (TNF), or Geldanamycin (GA). The fixed cells were stained with FITC-anti-RPS3 (green), Alexa Fluor 594-anti-p65 (red), and the nuclear dye Hoechst 33342 (blue).

(B) Immunoblots with the indicated antibodies of Jurkat cells that were transfected with NS, RPS3 (S3), and p65 siRNAs, TCR-stimulated 72 hr after transfection, and then fractionated into cytosolic and nuclear extracts. PARP was a nuclear fraction control.

(C) Confocal micrographs of Jurkat cells treated as described above, fixed, and stained with FITC-anti-p65 (green) and Hoechst (red). Percentages of cells with nuclear p65 after TCR stimulation are quantified below.

(D) Jurkat cells treated as in (C) and stained with FITC-anti-RPS3 (green) and Hoechst (red). Percentages of cells with nuclear RPS3 after TCR stimulation are quantified below. The scale bars are 10 μm.

RPS3 Is a Subunit of the NF-κB DNA- and Chromatin-Binding Complexes

To understand the association of RPS3 with p65, we carried out a structure-function study with deletions of both proteins (Figures 4D–4G). We found that the N-terminal portion (NTD, aa 21–186) of the Rel homology domain (RHD) of p65 was necessary for binding to RPS3, whereas the C-terminal transcriptional activation domain (TAD) was not involved (Figure 4E). A similar analysis of RPS3 revealed that the N-terminal region (aa 1–111), particularly the KH domain (aa 41–111), was needed for p65 binding (Figure 4G). The p65 NTD and the RPS3 KH domain both have nucleic acid-binding capacities, suggesting that RPS3 might regulate the DNA binding of NF-κB.

We first tested whether RPS3 could be recruited to promoters in NF-κB target genes in vivo by using a ChIP assay. We found that precipitation of either p65 or RPS3 gave the same signal for the 200–300 bp region containing κB sites of two well-studied NF-κB target genes, IκBα and IL-8 (Figures 5A and S5). Therefore, RPS3 is a component of the NF-κB transcriptional complex formed at relevant promoter sites. To determine whether RPS3 is incorporated into the NF-κB DNA-binding complex, we employed electrophoretic mobility shift assays (EMSAs) with nuclear extracts prepared from 293T cells transfected with or without FLAG-tagged RPS3. TNFα stimulation strongly increased two NF-κB bands (Figure 5B, compare lanes 2 and 11 to lanes 1 and 10). The specificity of these bands was confirmed by cold oligonucleotide competition: wild-type κB oligonucleotides competed away both induced bands as well as nonspecific bands (Figure 5B, lane 7), whereas mutant κB and unrelated OCT1 oligonucleotides only diminished the nonspecific bands (Figure 5B, lanes 8 and 9). The addition of p65 (Figure 5B, lane 5) and p50

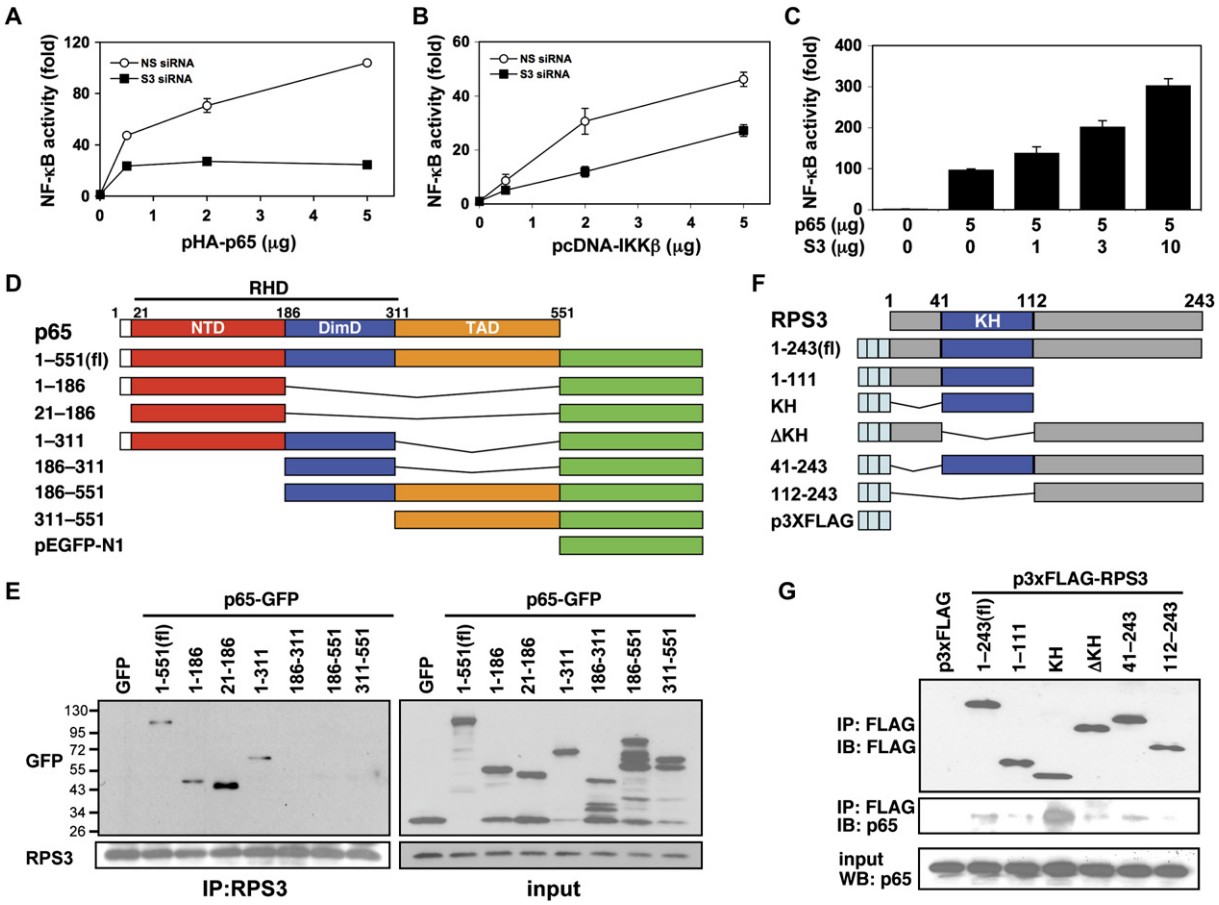

Figure 4. RPS3 Augments NF-κB Signaling through Association with p65

(A) Jurkat cells were cotransfected as in Figure 2A with the indicated siRNA and the amount of pHA-p65 plasmid shown and, 48 hr later, were analyzed by luciferase assay.

(B) Jurkat cells treated and analyzed as in (A) with the indicated amount of pcDNA-IKKβ plasmid.

(C) Jurkat cells were transfected with pHA-p65 (p65) plus 3FLAG-RPS3 (S3) plasmids as shown and were analyzed as in (A).

(D) Diagram of the truncation mutants of p65 fused to EGFP. RHD, Rel homology domain; NTD, N-terminal domain; DimD, dimerization domain; TAD, transcriptional activation domain.

(E) 293T cells transfected with constructs expressing full-length or truncated p65-GFP fusion proteins, lysed after 24 hr, and immunoblotted for GFP directly (input, right panel) or after immunoprecipitation with RPS3 antiserum (immunoprecipitation, RPS3, left panel). The blots at the bottom show RPS3 in each sample.

(F) Diagram of RPS3 and FLAG-tagged truncation mutants. KH, K homology.

(G) 293T cells transfected with constructs expressing full-length or truncated FLAG-tagged RPS3 proteins, lysed after 24 hr, and immunoblotted (IB) for FLAG-RPS3 or p65 after immunoprecipitation with FLAG antibody or for p65 in lysates (Input WB: p65).

(Figure 5B, lane 6) antibodies indicated that the upper and lower bands contained p65 homodimers and p65-p50 heterodimers, respectively, consistent with previous reports (Sathe et al., 2004). Most importantly, FLAG antibody supershifted both NF-κB bands (Figure 5B, lane 14), similar to p65 antibody (Figure 5B, lane 13), in cells overexpressing FLAG-tagged RPS3. Thus, NF-κB gel-shift complexes, widely believed to contain only Rel proteins such as p65 and p50, also contain the non-Rel protein RPS3. To validate this result, we preincubated the nuclear extracts with an antibody that specifically recognizes native RPS3 and found that it too blocks the formation of both p65-containing complexes, verifying that endogenous RPS3 is an essential component for both NF-κB complexes that form with the Ig κB oligonucleotide (Figure 5C, lanes 19–21). These complexes were unaltered by isotype control antibody (Figure 5B, lanes 3 and 12; Figure 5C, lane 18), whereas the RPS3 antibody did not modify OCT1 or AP-1 EMSA complexes (Figure 5D), indicating that the effect on NF-κB binding was specific. A similar result was also obtained in nuclear extracts from TCR-stimulated Jurkat cells (Figure S6) and LPS-stimulated 70Z/3 cells (data not shown). These results, combined with ChIP data, strongly suggest that RPS3 and p65 are bound to each other and

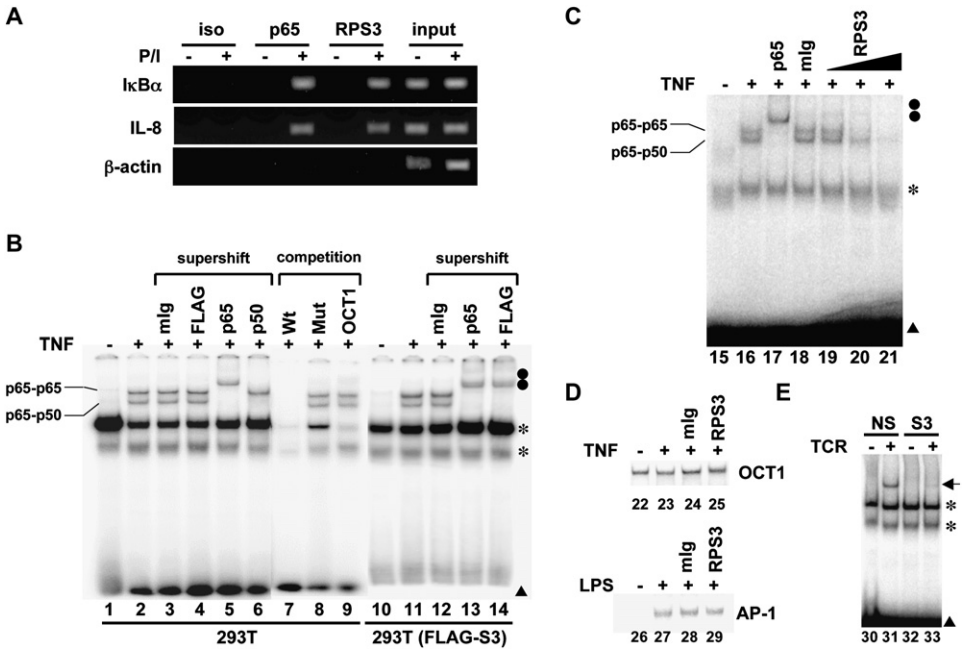

Figure 5. RPS3 Is Involved in NF-κB Gene Regulation

(A) Jurkat cells treated with or without PMA plus ionomycin (P/I) were used for chromatin immunoprecipitation (ChIP) assays with p65 antibody (p65), RPS3 antiserum (RPS3), or control antiserum (iso). The κB sites in the IκBα promoter, IL-8 promoter, or β-actin promoter were detected by PCR.

(B) Nuclear extracts of 293T cells (lanes 1–9) and 293T cells overexpressing 3FLAG-RPS3 (lanes 10–14) treated with (+) or without (–) TNFα were analyzed by EMSA with a ^{32}P-labeled, double-stranded Ig κB probe; in some cases, cells were analyzed with 100-fold unlabeled wild-type (WT) Ig κB, mutant (Mut) Ig κB, or nonspecific OCT1 oligonucleotide competitors. Supershift analysis with the indicated antibodies is shown in lanes 3–6 and 12–14. p65-p50 and p65-p65 complexes are designated, and the supershifted bands, nonspecific bands, and free oligonucleotides are labeled with dots, asterisks, and triangles, respectively.

(C) Nuclear extracts of 293T cells stimulated as in (B) were analyzed by EMSA. Supershift analysis was conducted with p65, an isotype control (mIg), or different doses of purified RPS3 antibodies (lanes 19–21). The NF-κB complexes, supershifted bands, nonspecific bands, and free oligonucleotides are labeled as in (B).

(D) The nuclear extracts are as in (C), and those from 70Z/3 cells stimulated with or without LPS were analyzed by EMSA and super shift analysis with ^{32}P-labeled OCT1 (upper panel) and AP-1 (bottom panel) probes, respectively.

(E) Jurkat cells transfected with NS or S3 siRNAs were stimulated with or without αCD3/CD28, and nuclear extracts were analyzed by EMSA with a ^{32}P-labeled Ig κB probe. The labels are the same as in (C), and NF-κB complex is labeled with an arrow.

are jointly recruited, in some cases along with p50, as an NF-κB complex to certain cognate DNA-binding sites in target genes.

Having established that RPS3 can be part of NF-κB complexes, we next investigated whether endogenous RPS3 is necessary for NF-κB binding to target genes. We performed a ChIP analysis of endogenous p65 recruitment to κB sites in chromatin by using cells either with or without RPS3 knockdown. As expected, p65 was recruited to IκBα and IL-8 promoters after stimulation of cells transfected with NS siRNA (Figure 6A). By contrast, p65 recruitment was severely diminished when RPS3 was depleted (Figure 6A). We also observed that RPS3 recruitment to those promoter sites was severely diminished in cells transfected with p65, but not NS siRNA (Figure 6A). Hence, RPS3 and p65 bind in a mutually dependent fashion to κB sites in gene control sequences. Lack of binding to these promoter sites correlated with the endogenous function of IL-8 and IκBα genes since TCR-induced mRNA expression from both genes was decreased by RPS3 knockdown (Figure 6B).

RPS3 Causes Synergistic Binding of p65 to κB Sites

Given the in vivo effects of RPS3 on NF-κB binding and function, we next explored how RPS3 influences NF-κB complexes in vitro by using purified recombinant p65, p50, and RPS3 (as a GST-RPS3 fusion) proteins in EMSAs. Recombinant p65 alone bound weakly to an Ig κB DNA site with only one detectable band (Figure 6C, lane 1), which likely represents a homodimer (Urban et al., 1991). However, preincubation of RPS3 with recombinant p65 increased the magnitude of p65 gel-shift complex nearly 100-fold in a concentration-dependent manner (Figure 6C, lanes 2–5). RPS3 presumably incorporated into the p65 complex because the complex migrated more slowly in proportion to the amount of RPS3 added (Figure 6C, lanes 2′–5′). We then formed complexes with

recombinant p50 and p65 proteins and observed two weak bands that ostensibly represent homodimers and heterodimers (Figure 6E, lane 9). Again, the addition of RPS3 dramatically increased binding and slowed migration in the gel as a single complex (Figure 6E). The NF-κB complex was confirmed by cold oligonucleotide competition and supershift analysis (Figure 6E and data not shown). Addition of RPS3 had no effect on DNA binding or the migration of gel-shift complexes formed by the p50 protein alone, which RPS3 does not bind (Figure S7B). Under the same conditions, no band was apparent with GST or GST-RPS3 alone (Figure S7C), suggesting that RPS3 does not bind the Ig κB DNA site itself, but rather enhances p65 NF-κB DNA-binding activity by direct association with p65. Binding site occupancy measurements showed a 70-fold and a greater than 30-fold effect on p65 homodimer and p65-p50 heterodimer complexes, respectively (Figures 6D and 6F). Since the number of binding sites was limiting in these analyses, the increase in site occupancy is likely an underestimate of the true effect on DNA-binding affinity. Thus, RPS3 exerts a dramatic synergistic effect on the binding of p65-containing NF-κB complexes to DNA. The synergy in binding of RPS3 and p65 to DNA was independently verified by an ELISA assay (TransAM assay) (Figure S7, detailed results in Supplemental Data). These data suggest that, in vitro, RPS3 formed a selective complex with p65 that dramatically increases binding to Ig κB site-containing DNA.

We next examined whether RPS3 was required for the formation of native NF-κB complexes with nuclear extracts from the cells transfected with NS or RPS3 siRNAs. In nuclear extracts from nonsilenced Jurkat cells, TCR stimulation induced a strong NF-κB gel-shift complex (Figure 5E). By contrast, essentially no endogenous NF-κB complex could be detected in nuclear extracts from similarly treated RPS3 knockdown Jurkat cells (Figure 5E, compare lane 31 to lane 33). Cold oligonucleotide competition and supershift assays confirmed the specificity of this complex (data not shown). We also found that RPS3 knockdown significantly decreased p65 DNA-binding activity in a TransAm assay even though it did not impair p65 nuclear translocation (Figure S7E). Thus, knockdown of endogenous RPS3 abrogated native NF-κB DNA-binding complexes, indicating that RPS3 is necessary and sufficient for the ability of p65 and p50 to form strong NF-κB-binding complexes with the Ig κB site.

RPS3 Is Required for Efficient Expression of NF-κB-Dependent Genes

To assess the NF-κB transcriptome after the activation of T cells with RPS3 knockdown, we compared p65- and RPS3-silenced versus nonspecific siRNA (NS)-treated samples on the Lymphochip microarray (Shaffer et al., 2000). We found that TCR stimulation induced 57 p65-dependent and 88 RPS3-dependent genes, as judged by their impaired expression after p65 and RPS3 knockdown, respectively (Figure 7A). Of these, 21 genes (37% and 24% in the p65 and RPS3 knockdown groups, respectively) were reduced by both knockdowns, and most were found to be NF-κB target genes (Figures 7A and S8A). A selection of these genes was confirmed by real-time PCR (Figure 6B and data not shown). The overlap between the p65- and RPS3-dependent genes was highly significant (χ^2 test, $p < 0.001$), suggesting parallel in vivo function of RPS3 and p65 at the transcriptional level for this subset of NF-κB-dependent genes. Conversely, a panel of TCR-induced genes known to be NF-κB independent were unaffected by either p65 or RPS3 knockdown (Figure S8B). Thus, RPS3 is required for the expression of a subset of NF-κB target genes driven by p65.

To assess whether the requirement for RPS3 is due to the assembly of binding complexes on κB sites, we carried out ChIP assays. We tested the promoter sites for IκBα and IL-2, both of which are RPS3-dependent genes, and we found that immunoprecipitation with antibodies against both p65 and RPS3 gave equivalent signals. However, when testing the κB sites for CD25 and CD69, whose expression is RPS3 independent, we obtained a strong signal with p65 immunoprecipitation, but essentially no signal for RPS3 immunoprecipitation (Figure 7B). This indicates that the endogenous κB sites in the promoters of the IκBα and IL-2 genes are occupied by both proteins, but those in the CD25 and CD69 genes are occupied by p65, but not RPS3. We then tested the association of endogenous p65 with promoter κB sites in cells with intact or knocked down RPS3. We found that the p65 occupancy of κB sites in the promoters of IκBα, IL-8, and IL-2 was markedly reduced in RPS3-knockdown cells (Figure 6A, left panel, and Figure 7C). By contrast, p65 occupancy of κB sites in the RPS3-independent genes, CD25 and CD69, was unaffected in RPS3-knockdown cells (Figure 7C). Moreover, EMSA analysis showed that RPS3 knockdown failed to inhibit the TCR-induced NF-κB complex that formed with the κB DNA site from CD25, which contrasts with the dramatic abolition of the NF-κB complex formed with an Ig κB DNA site (Figures 5E and S9). Therefore, the selective gene expression revealed by the microarray analysis can be explained by differing requirements of specific promoter sites for the RPS3 subunit in NF-κB (Figure 7A). Furthermore, these differences were manifested in the physiological function of these genes in T cells: IL-2 synthesis was impaired by RPS3 knockdown (Figure 7D), whereas upregulation of cell-surface CD25 (necessary for the high-affinity IL-2 receptor) occurred normally (Figure 7E). However, the net result was that TCR-induced proliferation of human T lymphocytes (that depends on IL-2) was blocked by RPS3 knockdown (Figure S10). Thus, the synergistic effect of RPS3 in forming NF-κB complexes on the endogenous chromatin sites paralleled the selective expression of RPS3-dependent genes.

RPS3 Knockdown Decreases Ig κ Light-Chain Expression

To further examine the dependence of NF-κB on RPS3 in a key physiological context, we revisited one of the original cell systems in which the NF-κB transcription factor

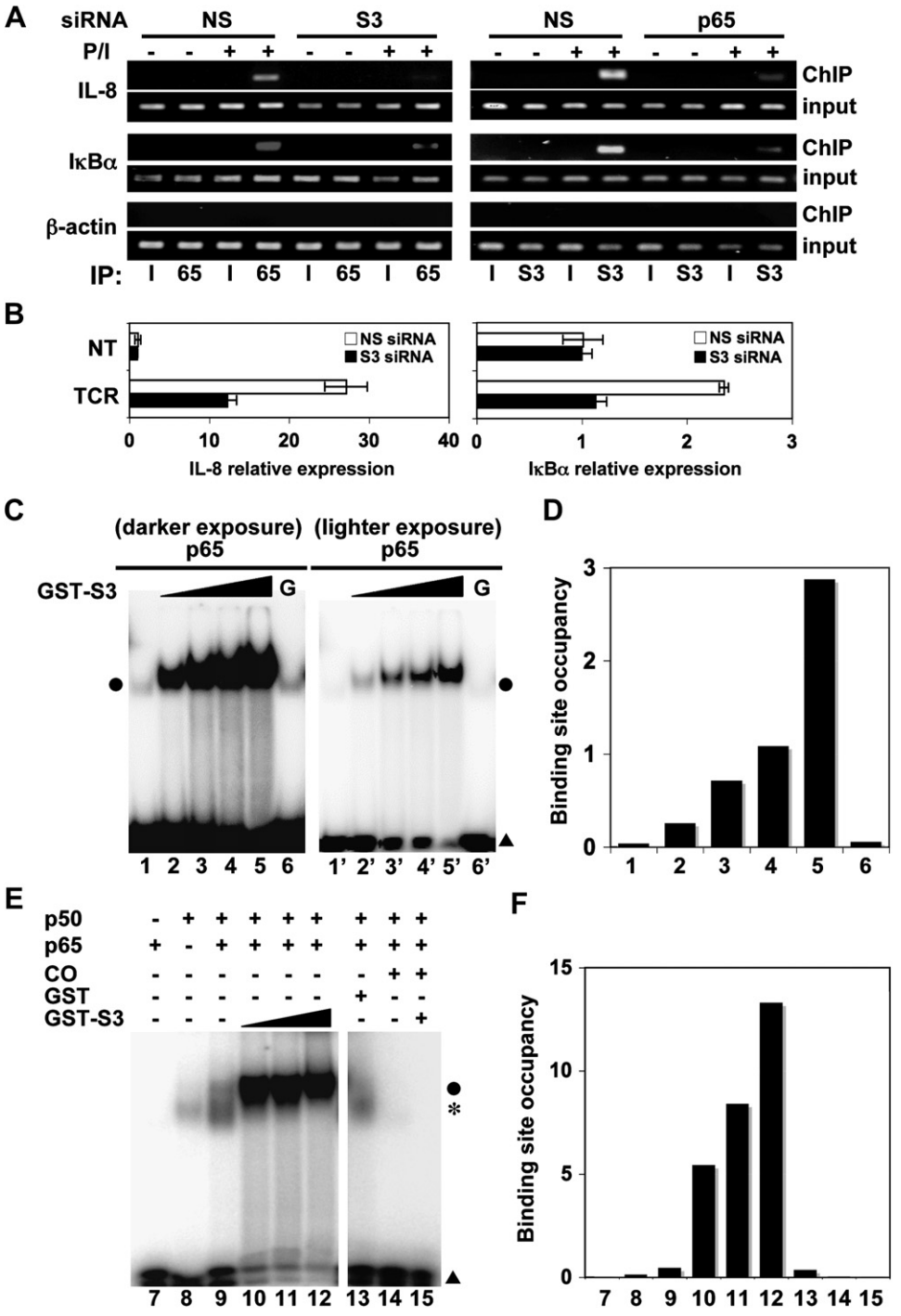

Figure 6. RPS3 Facilitates p65 Binding to κB Sites

(A) Jurkat T cells silenced with nonspecific (NS) or RPS3 (S3) siRNA (left panel) or p65 siRNA (right panel) were treated as in Figure 5A for ChIP analysis with isotype (I), p65 (65), or RPS3 antibodies and PCR evaluation of the κB sites in the IL-8, IκBα, or β-actin promoters.

(B) Real-time PCR quantitation of mRNA levels of IL-8 and IκBα normalized to GAPDH in Jurkat cells silenced with NS or RPS3 (S3) siRNAs and stimulated with no treatment (NT) or αCD3/CD28 (TCR).

(C) Recombinant p65 (200 ng/sample) was incubated with increasing amounts of GST-RPS3 protein (0, 100, 200, 400, and 800 ng in lanes 1–5, respectively) or GST protein (800 ng, lane 6) at 25°C for 30 min, followed by an EMSA with a ^{32}P-labeled Ig κB probe. The p65 homodimer and free oligonucleotide were labeled by dots and a triangle, respectively. Darker and lighter autoradiographic exposures are shown.

(D) The binding site occupancy of the complex shown in (C), calculated as a ratio of the intensity of the complex band to the free oligonucleotide band.

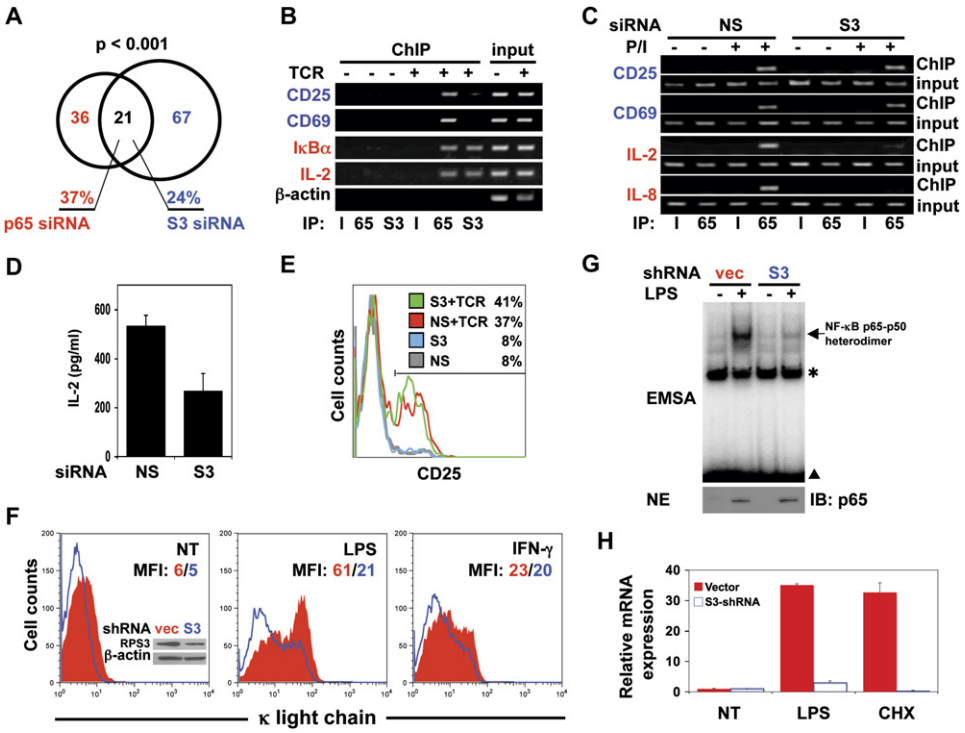

Figure 7. RPS3 Regulates a Subset of Physiologically Important NF-κB Target Genes

(A) The Venn diagram shows genes that are downregulated after p65 siRNA (red), RPS3 siRNA (blue), or both in TCR-stimulated versus untreated Jurkat T cells determined by the Lymphochip microarray. Of the 3035 genes with multiple spots available, 88 (2.9%) were decreased (≥1.4-fold) in the S3 sample, and 57 (1.9%) were decreased in the p65 sample. A total of 21 (37%) were downregulated in both samples (≥2 repeats in both samples), which is greater than expected by random chance alone (χ^2 test, $p < 0.001$).

(B) Jurkat cells treated with or without αCD3/CD28 (TCR) were analyzed by ChIP with p65 antibody (65), RPS3 antiserum (S3), or isotype control serum (iso). PCR was used to detect the promoter κB sites for CD25, CD69, IκBα, or IL-2 and the control β-actin DNA.

(C) Jurkat cells were transfected with NS or S3 siRNAs, stimulated as in Figure 6A, and ChIP was performed with isotype (I) or p65 (65) antibodies as shown. PCR was used to detect the promoter κB sites for CD25, CD69, IL-2, or IL-8.

(D) Human peripheral blood lymphocytes (PBLs) were transfected with NS or RPS3 (S3) siRNAs and were stimulated with αCD3/CD28 for 36 hr. IL-2 in supernatants was measured by ELISA.

(E) PBLs were transfected and stimulated as in (D) only for 12 hr, and they were analyzed by flow cytometry after cell-surface staining for CD25. The percentage of CD25-positive cells is indicated.

(F) 70Z/3 cells were transduced with pNUTS (vec, red) or pNUTS-RPS3-shRNA (S3, blue) lentiviruses and were stimulated with LPS or IFN-γ, or were left not treated (NT), and they were analyzed by flow cytometry with live gating on GFP^+, i.e., lentivirally transduced, cells and staining for Ig κ light chain. RPS3 knockdown is shown by immunoblotting, with a β-actin control (inset). The mean fluorescence intensity (MFI) in relative units of cell-surface κ light-chain expression in GFP^+ cells is illustrated.

(G) Identical amounts of nuclear extracts (NE) prepared from 70Z/3 cells transduced as in (F) and stimulated with or without LPS were analyzed by EMSA with a ^{32}P-labeled Ig κB probe. The NF-κB band is indicated, and a nonspecific band and free oligonucleotide are labeled with an asterisk and triangle, respectively. The lower panel shows an immunoblot for p65 in the NE samples.

(H) Total RNA was isolated from 70Z/3 cells transduced as in (F) and stimulated with LPS (1 μg/ml) or cycloheximide (CHX, 20 μM) for 8 hr, or not treated (NT). The mRNA levels of κ light chain were measured by quantitative real-time PCR and were normalized to levels of GAPDH; the mRNA level from the cells transfected with pNUTS alone and mock treated was set as 1.

was first identified by measuring LPS-induced Ig κ light-chain expression in murine pre-B 70Z/3 cells, in which the p65-p50 heterodimer plays a central role (Miyamoto et al., 1994; Sen and Baltimore, 1986a). We stably silenced RPS3 by inserting an RPS3 short hairpin RNA (shRNA) into a GFP-expressing lentiviral vector that was transduced into 70Z/3 cells (Furumoto et al., 2006). The transduction efficiency ranged from 90% to 94% in both RPS3-downregulated and control (empty vector) 70Z/3 cells (Figure S11). We found that silencing RPS3 with shRNA slightly diminished basal expression, but substantially inhibited LPS-induced κ light-chain expression on

(E) Recombinant p65 (100 ng/sample), p50 (100 ng/sample), GST-RPS3 (GST-S3) (0, 100, 200, and 400 ng in lanes 9–12, respectively), or GST protein (400 ng, lane 6) as indicated were incubated and analyzed by EMSA as in (C), in some cases with 100-fold unlabeled Ig κB competitor (CO, lanes 14 and 15). The p65-p50 heterodimer, p50 homodimer, and free oligonucleotide were labeled with a dot, asterisk, and triangle, respectively.

(F) The binding site occupancy of the major complex in (E), quantitated as in (D).

the cell surface (Figures 7F and S12). By contrast, κ light-chain expression induced by IFNγ, which modestly upregulates the κ light chain in 70Z/3 cells via an NF-κB-independent mechanism (Briskin et al., 1988), was essentially the same whether RPS3 was silenced or not (Figures 7F and S12). To confirm that the inhibition of Ig κ expression was due to effects on NF-κB, we examined nuclear extracts from the 70Z/3 cells by EMSA. We found that LPS induced a strong NF-κB complex in the vector control cells, which was almost completely eliminated in the RPS3-knockdown cells (Figure 7G, upper). Cold competition and supershift analyses revealed it to be a p65-p50-containing NF-κB complex (data not shown). Despite the failure of this NF-κB complex to form with the Ig κ-binding motif, p65 had translocated normally into the nucleus (Figure 7G, bottom). Furthermore, real-time PCR measurements showed that decreasing RPS3 impaired the induction of κ light-chain mRNA (Figure 7H). Finally, we used a protein synthesis inhibitor, cycloheximide (CHX) (Figure S13), which blocks new synthesis of IκB and allows this labile inhibitor to turn over and release NF-κB to the nucleus, to test the effect of RPS3 knockdown on the induction of NF-κB and Ig κ light-chain mRNA expression in 70Z/3 cells (Sen and Baltimore, 1986a), on its own in the context of RPS3 knockdown. We found that κ light-chain mRNA induction by CHX was eliminated by RPS3 knockdown in 70Z/3 cells (Figure 7H), definitively excluding the possibility that this effect was associated with defective translation. Taken together, these data suggest that the ability of NF-κB to exert its full physiological regulatory function on Ig gene expression depends on RPS3 acting at the transcriptional level. Taken together with the effects on cytokine production and lymphocyte proliferation, our data demonstrate a prominent role for RPS3 in mediating important physiological cellular responses involving NF-κB.

DISCUSSION

The NF-κB complex was identified in B cell extracts by using a probe from κ light-chain gene intronic enhancer (Sen and Baltimore, 1986b), and it was established to have transcriptional function important for κ light-chain expression (Lenardo et al., 1987). After purification of subunits and cloning of their genes, NF-κB became synonymous with the p65-p50 heterodimer, in the first instance, and later with various homo- and heterodimers of the five Rel-homology proteins. We now show that this model is oversimplified, and that RPS3, which has no Rel homology, is a functional subunit of specific NF-κB DNA-binding complexes. When RPS3 expression is reduced, NF-κB fails to bind to selected regulatory sites, and a variety of p65-regulated genes are not expressed normally. The identification of RPS3 indicates that selective recruitment of p65 to certain promoters involves DNA-binding complexes containing additional essential components, and that natural NF-κB is more than a Rel heterodimer.

RPS3 is a 26 kDa protein that shuttles between the cytoplasm and the nucleus and functions in both compartments. Notably, the protein lacks any obvious TAD, Rel homology, or the conserved LXXLL consensus sequence present in most coactivator proteins (Heery et al., 1997). Our evidence shows that RPS3 does not function as a transcriptional activator or coactivator. However, RPS3 contains an NLS that, in combination with ERK1/2 phosphorylation, causes nuclear localization in DNA-damage responses (Yadavilli et al., 2007). We now show that RPS3 can specifically translocate to the nucleus in response to TCR and TNF stimulation, which fits with the notion that it is a subunit of nuclear NF-κB complexes. It will be interesting to determine how specific activating signals can dissociate RPS3 from its cytoplasmic berth, which is essential for its cooperative function with p65.

A prominent feature of RPS3 is the presence of a KH domain. This structural motif, rare among ribosomal proteins, can bind single-stranded RNA and DNA with some sequence specificity (Siomi et al., 1993). Whereas the KH domain was found to be essential for p65 association, our data show that RPS3 alone exhibits no measurable binding to the Ig κB motif. Originally defined in hnRNP K protein, KH domains are found in a diversity of proteins with important roles in RNA synthesis and metabolism (Ostareck-Lederer and Ostareck, 2004). The full regulatory role of these proteins, which are highly conserved throughout phylogeny, continues to be elucidated. For example, although hnRNP K was originally detected in association with RNA in RNP particles, later evidence revealed that it bound to DNA recognition motifs and had transcriptional-promoting effects (Michelotti et al., 1996; Tomonaga and Levens, 1995, 1996). More recently, hnRNP K was found to be essential for p53-mediated gene expression during DNA-damage responses (Moumen et al., 2005). Our results with RPS3 and NF-κB share many of the same hallmarks as hnRNP K and p53—coregulation by inducing stimuli, coordinated DNA binding, and interdependence for physiologically important transcriptional effects. These findings may prefigure a new regulatory paradigm in which KH domain proteins serve as essential functional components of other sequence-specific DNA-binding transcription complexes.

Our findings illustrate how RPS3 influences NF-κB function through two major effects. First, RPS3 is a DNA-binding exponent that dramatically stabilizes the association of Rel subunits with certain cognate sites. It provides a selective and inducible mechanism by which to generate a high-affinity binding complex. This could explain the extremely high DNA-binding capacity of semi-purified natural NF-κB, which is not manifested by complexes formed solely of purified p50 and p65 subunits. Based on previous studies, it has been assumed that NF-κB heterodimers and homodimers alone have high affinity in the absence of RPS3. In fact, careful review of this literature would suggest the opposite. Studies measuring a high affinity of NF-κB for its cognate DNA motif were carried out with crude nuclear extracts in which the complex likely contained RPS3 (Thanos and Maniatis, 1992). By contrast, Phelps et al. (2000), found that the affinity of heterodimers

of the purified p50 and p65 RHD proteins was approximately 100- to 1000-fold lower than that of crude extracts. This discrepancy has not been previously explained. Remarkably, this difference is nearly equivalent to the synergistic increase in binding that we have observed on gel shift by adding RPS3 to purified p65-p50 subunits in EMSAs by using the Ig κB site, although more precise measurements are needed under equilibrium conditions. Thus, the high affinity of natural NF-κB in nuclear extracts may be accounted for by the presence of the RPS3 subunit. Moreover, the binding of KH domain proteins to single-stranded DNA/RNA suggests a potentially interesting twist to this function. For example, during repeated rounds of transcription of the *Igk* gene, melting of the double helix at the intronic κB site would occur. RPS3 could stabilize NF-κB association with the separated strands or serve as a "bookmark" for NF-κB on the active gene.

Our data also imply that RPS3 may be a "specifier" that selects particular genomic κB sites to be activated under certain conditions. Our microarray data indicate that only a subset of p65-dependent genes activated by TCR stimulation are contingent upon RPS3. ChIP experiments investigating endogenous κB sites revealed that p65 binding to chromatin depends on the presence of RPS3 at the chromatin regulatory sites in genes whose expression relies upon RPS3. By contrast, the κB sites in the promoters of other genes, whose expression is unaffected by RPS3 knockdown, appeared to recruit p65 without requiring RPS3. With these differences in mind, we envision that selectivity could be conferred by a cooperative binding specificity involving RPS3 contacting DNA itself, or more complex conformations of the DNA:protein holocomplex.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents

Jurkat A3, 70Z/3, and HEK 293T cells (ATCC) were cultured in RPMI 1640 medium containing 10% fetal calf serum, 2 μM glutamine, and 100 U/ml each of penicillin and streptomycin (Cambrex). Human peripheral blood lymphocytes were isolated from heathy donor blood by Ficoll-Hypaque gradient centrifugation. Antibodies used were p65, p50, c-Rel, RelB, IκBα, and PKCθ (Santa Cruz); β-actin, GST, and FLAG (Sigma); PARP, CD3, CD28, and mouse Ig κ light chain (BD); RPS16 (Aviva) and GFP (Roche). Rabbit antibodies against RPS3 were custom prepared by Proteintech Group, Inc. (Hegde et al., 2007) and by PrimmBiotech, Inc. (Cambridge, MA, USA). Recombinant human TNFα was purchased from R&D, and p65 and p50 proteins were purchased from Active Motif, Inc.

RNAi and Transfection

The siRNA sequences for human RPS3, p65, and RPS16 are provided in Supplemental Data. Transient transfection of siRNA and DNA constructs into Jurkat cells was described previously (Su et al., 2005). 293T cells were transfected by using ExGen500 (Fermentas) according to the manufacturer's protocol.

TAP Purification and Mass Spectrometry

293T cells were transfected with pCTAP-p65 or pCTAP vector as described above. A total of 36 hr later, cells were lysed after stimulation (30 ng/ml TNFα, 30 min) and TAP purified by using the InterPlay TAP purification kit (Stratagene) per the manufacturer's protocol. Eluted proteins were separated on a 4%–20% Tris/glycine-SDS PAGE gel and stained with Colloidal Blue (Invitrogen). Mass spectrometry and a peptide database search were carried out as described in Supplemental Data.

In Vitro Binding Assay with GST Fusion Protein

A total of 1 μg purified GST or GST-RPS3 proteins was applied to glutathione Sepharose 4B resins (Amersham Pharmacia) and incubated for 1 hr at 4°C. The resins were washed and mixed with 0.2 mg nuclear extract prepared from stimulated Jurkat cells (αCD3/CD28, 1 μg/ml each, 3 hr) and were incubated for 2 hr at 4°C. After washing, bound proteins were eluted and subjected to SDS-PAGE, followed by immunoblotting.

Confocal Microscopy

Cells were fixed and stained with FITC-conjugated rabbit anti-p65 antibodies (Santa Cruz) or with Alexa Fluor 594-conjugated rat anti-mouse Ig κ light-chain antibodies (BD) as described previously (Su et al., 2005).

Luciferase Reporter Gene Assays

Cells were cotransfected at a ratio of 10:1 with firefly luciferase constructs driven by consensus κB, AP-1, or GAL4 sites, together with the renilla luciferase pTKRL plasmid (Promega), cultured for 1–2 days, and then stimulated as described above in triplicate for 4–6 hr before harvest. Lysates were analyzed by using the Dual-Luciferase Kit (Promega) with firefly fluorescence units (FU) normalized to renilla FU. Fold induction was calculated as (relative FU of stimulated)/(relative FU of unstimulated) samples.

Chromatin Immunoprecipitation

Jurkat cells (5–10 × 10^7) were either untreated or treated with 50 ng/ml phorbol myristate acetate (PMA) (Sigma) plus 1.5 μM ionomycin (Sigma) for 3 hr. The chromatin immunoprecipitation (ChIP) assay was executed as described (Ainbinder et al., 2002) with 2 μg anti-FLAG (M2) (Sigma), anti-RPS3 (PrimmBiotech), anti-p65 (Santa Cruz), or control antibody. The eluates were precipitated and resuspended in 30 μl TE. A 2 μl sample was used for PCR (30–35 cycles) with a PCR Master Mix Kit (Qiagen) with primers for the promoters of the human IL-8, IκBα, β-actin, IL-2, CD25, and CD69 genes (sequences detailed in Supplemental Data). The PCR products were separated in E-Gel 1.2% agarose (Invitrogen).

Preparation of Subcellular Protein Fractions and Electrophoretic Mobility Shift Assays

Cytosolic and nuclear protein extracts were prepared as previously described (Lenardo et al., 1989) from Jurkat cells stimulated as described above, 293T cells, or FLAG-tagged 293T cells activated with 30 ng/ml TNFα for 1 hr, and their protein concentration was determined with the BCA kit (Pierce). Cytosolic fractions (S100) and crude ribosome pellets (P100) from Jurkat cells transfected with NS or RPS3 siRNAs were prepared as previously described (Chen and Lin, 2004). Electrophoretic mobility shift assays (EMSAs) were carried out with the EMSA kit (Promega) with modifications as previously described (Lenardo et al., 1989) by using Ig κB (WT), Ig κB (Mut), OCT1, AP-1, and CD25 double-stranded oligonucleotide probes (sequences detailed in Supplemental Data). For supershift analyses, nuclear extracts were preincubated with 1 μl antibodies against mIg (isotype control, Jackson Laboratory), FLAG (M2, Sigma), p65 (F-6, Santa Cruz), p50 (E-10, Santa Cruz), and RPS3 (PrimmBiotech) for 20 min on ice prior to the addition of the ^{32}P-labeled probe. For competition binding analyses, 100-fold unlabeled Ig κB (WT), Ig κB (Mut), or the OCT1 oligonucleotide probe was added. For the EMSA with recombinant proteins, p65 or p50 (200 ng/sample) was incubated with the indicated purified GST-RPS3 or GST protein at 25°C for 30 min, followed by an additional incubation with labeled Ig κB oligonucleotide or supershift antibody as described above. Samples were resolved on a 6% DNA retardation gel

(Invitrogen) in 0.25 × TBE buffer. Autoradiography was carried out on dried gels.

Gene Expression Profiling and Data Analysis

DNA microarray analysis was performed as described (Lam et al., 2005). For each sample, 50 μg total RNA, prepared by using the Trizol reagent (Invitrogen), was used for fluorescent probe preparation. The raw gene expression data from each DNA microarray hybridization were normalized as described (Shaffer et al., 2000). For the microarray experiments, mRNA from Jurkat cells transfected with nonspecific siRNA was labeled with the Cy3 dye, and that from Jurkat cells transfected with p65 or RPS3 siRNA was labeled with the Cy5 dye. Data were selected such that the Cy3 signal intensity was > 200 relative FU. Genes were considered to be affected if either p65 or RPS3 siRNA-transfected cells exhibited decreased expression by >1.4-fold at ≥1 time points in both time courses. The microarray data set was subjected to statistical analysis by the chi-square test and was deposited in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo) under the series submission codes GSE7231 and GSM174472–GSM174477.

RT-PCR and ELISA

Total RNA was isolated as described above and treated with the TURBO DNA-free kit (Ambion) to remove residual genomic DNA. cDNA was prepared from 1 μg RNA by using the Superscript First Strand System (Invitrogen) with oligo (dT). Real-time PCR reactions were performed in triplicate with primers (sequences are detailed in Supplemental Data) by using the SYBR Green PCR Master Mix (Qiagen) in a 7900HT sequence-detection system (Applied Biosystems). The relative transcription level was calculated by using the ΔΔCt method. The IL-2 in lymphocyte supernatants was quantified by using the Human IL-2 ELISA Ready-SET-Go kit (eBioscience) as instructed by the manufacturer.

Lentivirus shRNA Vector Construction and Gene Transduction

Mouse RPS3 shRNA (Supplemental Data) was ligated into the pNUTS vector via Apa I/Bst XI sites, and packaging of the RPS3 shRNA or control pNUTS vectors was carried out as previously described (Furumoto et al., 2006). 70Z/3 cells (1×10^6) were transduced by resuspension in 3 ml virus supernatant. A total of 24 hr after infection, the medium was changed, and the transduction efficiency (percentage of GFP-positive cells) was measured by flow cytometry. A total of 5–6 days later, RPS3 knockdown was assessed by western blot, and cells were stimulated with Lipopolysaccharide (LPS) (Sigma) or IFNγ (R&D). Cell-surface Ig κ light chain was assessed by flow cytometry.

Supplemental Data

Supplemental Data contain Supplemental Experimental Procedures, Supplemental Results, Supplemental References, thirteen figures and are available with this article online at http://www.cell.org/cgi/content/full/131/5/927/DC1/.

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