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RKIP inhibits NF-κB in cancer cells by regulating upstream signaling components of the IκB kinase complex

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ABSTRACT

RKIP was first identified as an inhibitor of the Raf-MEK-ERK signaling pathway. RKIP was also found to play an important role in the NF-κB pathway. Genetic and biochemical studies demonstrated that RKIP functioned as a scaffold protein facilitating the phosphorylation of IκB by upstream kinases. However, contrary to what one would expect of a scaffold protein, our results show that RKIP has an overall inhibitory effect on the NF-κB transcriptional activities. Since NF-κB target gene expression is subject to negative regulation involving the optimal induction of negative regulators, our data support a hypothesis that RKIP inhibits NF-κB activity via the auto-regulatory feedback loop by rapidly inducing the expression and synthesis of inhibitors of NF-κB activation.

Structured summary:
MINT-7386121: *TRAF6* (uniprotkb:Q9Y4K3) *physically interacts* (MI:0915) with *RKIP* (uniprotkb:P30086) by *anti bait co-immunoprecipitation* (MI:0006)

1. Introduction

NF-κB is a transcriptional activator that is required for the up-regulation of a large number of genes encoding proteins that are important for immunity, cell proliferation and survival [1,2]. NF-κB consists of homo- and hetero-dimeric complexes of the REL family polypeptides. In the absence of stimulating signals, the NF-κB dimeric complex is retained in the cytoplasm in an inactive form bound to IκB. In response to stimulatory signals IκB is rapidly phosphorylated by the IKK complex, which contains two kinase subunits, IKKα and IKKβ and a regulatory subunit called IKKγ/NEMO. The phosphorylated IκB will subsequently be ubiquitinated and degraded releasing the NF-κB dimer for nuclear import [3].

One of the potent activators of the NF-κB is the pro-inflammatory cytokine IL1-β [4]. Genetic studies have further shown that TAK1 is the critical direct upstream activating kinase of IKK [5]. The activation of NF-κB in response to stimulation by IL1-β has been extensively studied, however, the mechanisms that modulate and eventually limit these responses are still poorly understood.

RKIP was first identified as an interacting partner of Raf-1, and shown to function as a negative regulator of the MAPK cascade initiated by Raf-1 [7]. The Raf-1 initiated pathway is comprised of three sequentially acting protein kinases: a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK), and a MAPK; this basic relationship has now been found to be conserved in several protein kinase pathways including the one required for the phosphorylation of IκB. In the Raf-1 pathway the MAPK is ERK1/2, the MAPKK is MEK1/2, and the MAPKKK is Raf-1 itself. Functional studies using both gain-of-function and loss-of-function approaches demonstrated that RKIP disrupts the interaction between Raf-1 and MEK1/2 resulting in a downregulation of MEK1/2 activation phosphorylation [6]. Previously we showed that RKIP interacted with TAK1 and IKKα/β of the NF-κB core kinase cascade. Here we show that RKIP also interacts with TRAF6, the upstream activator of IKK kinase complex. The interaction is ligand-dependent and precedes the degradation of IκB. We show that the effect of RKIP was concentration-dependent. The presence of RKIP favored the optimal ubiquitination of IRAK, TRAF6, and TAK1 as well as the assembly of the IKK complex. We show that RKIP facilitated the phosphorylation of IκB by IKKs. Our results therefore are consistent with the notion that RKIP is a scaffold protein of the IKK complex.

2. Methods

2.1. Cell cultures

Cos-1, and 293mycIL1R cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine

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serum and 1% penicillin/streptomycin. A549 cells were grown in Ham'12 medium supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 0.15% $NaHCO_3$.

2.2. Antibodies

Antibodies to p-IkBα were purchased from Upstate Biotechnology. Antibodies to ERK, p-ERK, p38, p-p38, JNK, p-JNK, IκBα, p65, IKKα, and phospho-IKKα were purchased from Cell Signaling. Anti-HA peroxidase was purchased from Roche. 12CA5 and 9E10 antibodies were purified from hybridoma supernatants with protein G-Agarose (Upstate).

2.3. Real time PCR and PCR array analysis

Total cellular RNA was extracted with the Trizol reagent (Invitrogen) and reverse transcribed using random hexamer primers (Applied Biosystems). The resulting cDNAs were used for PCR using SYBR-Green Master PCR mix (Applied Biosystems) in triplicates. PCR and data collection were performed on ABI7500 (Applied Biosystems). β-Actin were used as an internal standard.

A detailed description of materials and methods is given in Supplementary data.

3. Results and discussion

3.1. Both ectopic expression and knockdown of RKIP expression impairs the phosphorylation of IκBα in response to IL1-β stimulation

Previously we showed that over expression of RKIP inhibited IκB degradation following treatment with pro-inflammatory cytokines [8]. Mechanistically RKIP may impair by either inhibiting the phosphorylation of IκB by IKK or acting after the phosphorylation of IκB. To evaluate these alternative possibilities, we examined the effect of RKIP on IκB phosphorylation in DU145 cells treated with proteasome inhibitor ALLN. The prostate cancer cells DU145 have an elevated level of IKK activity and IκB is continuously being phosphorylated and degraded. In the presence of ALLN phosphorylated IκB is stabilized and will not be degraded. We monitored directly the effect of RKIP on the phosphorylation of IκBα at $Ser^{32/36}$ by phosphospecific Ab. Ectopic expression of RKIP reduced the amount of phospho-IκB without affecting the expression levels of IκB (Fig. 1a). Our results therefore show that ectopic expression of RKIP inhibits the activation of NF-κB at least partly by preventing the phosphorylation of IκB.

We reasoned that if RKIP is a repressor of IκB degradation we should expect an opposite effect when RKIP is inactivated. Unexpectedly, we observed the same effect when the expression of RKIP was down regulated by specific siRNA. We observed changes both in the kinetics and magnitude of the IκB degradation in RKIP knockdown cells (Fig. 1b). To evaluate the effect of RKIP knockdown on IκB phosphorylation we constructed an IκB variant IκBKR, which contained mutations in two of its major ubiquitination sites and hence could be phosphorylated but not degraded. Consistent with results with ectopic RKIP expression we observed that the effect of RKIP knockdown was also at the level of IκB phosphorylation (Fig. 1c). Our results therefore suggest that RKIP facilitates the IκB phosphorylation/degradation upon IL1-β stimulation. The effect of RKIP on the NF-κB signaling is specific, as knocking down of RKIP expression has little effect on the IL1-β-mediated phosphorylation activation of p38 and JNK (Fig. 1d).

The effect of the siRNA was specific, as the control siRNA (siLUC) had no effect on the IL1-β-induced IκB degradation when compared with normal cell control (data not shown). Furthermore the effect of RKIP siRNA could be reversed by expression of a version of RKIP gene that was not recognized by the RKIP specific siRNA (Fig. 1e). To ensure the effect is not cell type specific we also examined the effect of RKIP expression in human lung epithelial cells A549 that are highly responsive to IL1β stimulation. We observed a similar effect of RKIP on IL1-β-mediated IκB degradation/phosphorylation in A541 cells (Fig. 1f).

3.2. RKIP functions as a scaffold in NF-κB pathway

Since both RKIP gain-of-function and loss-of-function have the same effect on the phosphorylation of IκB, our results therefore raise the possibility that RKIP may function as a scaffold protein. Several essential attributes of a scaffold protein have been described. A scaffold protein has the ability to interact with many components of the signaling pathway and thereby facilitates the assembly of a multi-component protein complex. The function of a scaffold protein is concentration-dependent.

We have previously shown that RKIP associates with NIK, IKKα/β, and TAK1 of the NF-κB pathways [8]. To investigate whether RKIP also interacts with upstream activators of the IKK complex we studied the interaction of RKIP with TRAF6 by co-immunoprecipitation. We observed that RKIP interacted with TRAF6 when overexpressed (Fig. 2a). The association is physiologically relevant as the endogenous RKIP was also found associated with TRAF6 and the association was ligand-dependent (Fig. 2b). The interaction with TRAF6 was specific, as no detectable RKIP was found in co-immunoprecipitates when other closely related TRAFs (TRAF1, 2, 3, and TRAF5) or IRAK were used (Fig. 2a and data not shown). It has been shown previously that the association of TRAF6 with IRAK, TAK1, and IKK is essential for the IL1-β-induced phosphorylation and degradation of IκB. Consistent with the notion that RKIP is a scaffold protein, the association of TRAF6 with IRAK, IKKβ or TAK1 is greatly reduced in the absence of RKIP (Fig. 2c and d).

Next we examined whether RKIP exists as a single complex with components of the NF-κB pathway. We began by examining whether RKIP interacts with TAK1 and TRAF6 simultaneously by co-immunoprecipitation after the proteins were crosslinked in vivo by DTBP. Consistent with the notion that RKIP, TAK1, and TRAF6 coexist in the same complex we detected RKIP and TAK1 in complexes immunoprecipitated by Abs that recognized TRAF6 (Fig. 2e). To ensure that the whole complex but not individual sub-complexes were co-immunoprecipitated, the complexes were eluted from the 1st Ab and immunoprecipitated with an Ab that recognized another component in the complex, TAK1. The results are shown in (Fig. 2e, *right panel*).

Lastly we examined whether the effect of RKIP on NF-κB signaling pathway is concentration-dependent. We co-transfected 293IL1R RKIP knockdown cells with flag-tagged IκB and different amounts of RKIP and monitored the degradation of the IκB by Western blotting. With expected function as a scaffold protein, RKIP exhibited a biphasic effect on the IL1-β mediated degradation of IκB (Fig. 2f).

3.3. Down regulation of RKIP decreases the ubiquitination of IRAK, TRAF6, and TAK1

The activation of IKK results from a sequential ubiquitination and phosphorylation of several upstream modulators and kinases including IRAK, the E3 ubiquitin ligase TRAF6 and TAK1. Conceptually RKIP loss-of-function may directly affect the phosphorylation of IκB by IKK. Alternatively, the effect of RKIP loss-of-function mutation may be upstream of IKK. To evaluate these alternative possibilities, we examined the effect of RKIP knockdown on the phosphorylation and/or ubiquitination of IRAK, TRAF6, TAK1, and IKK.

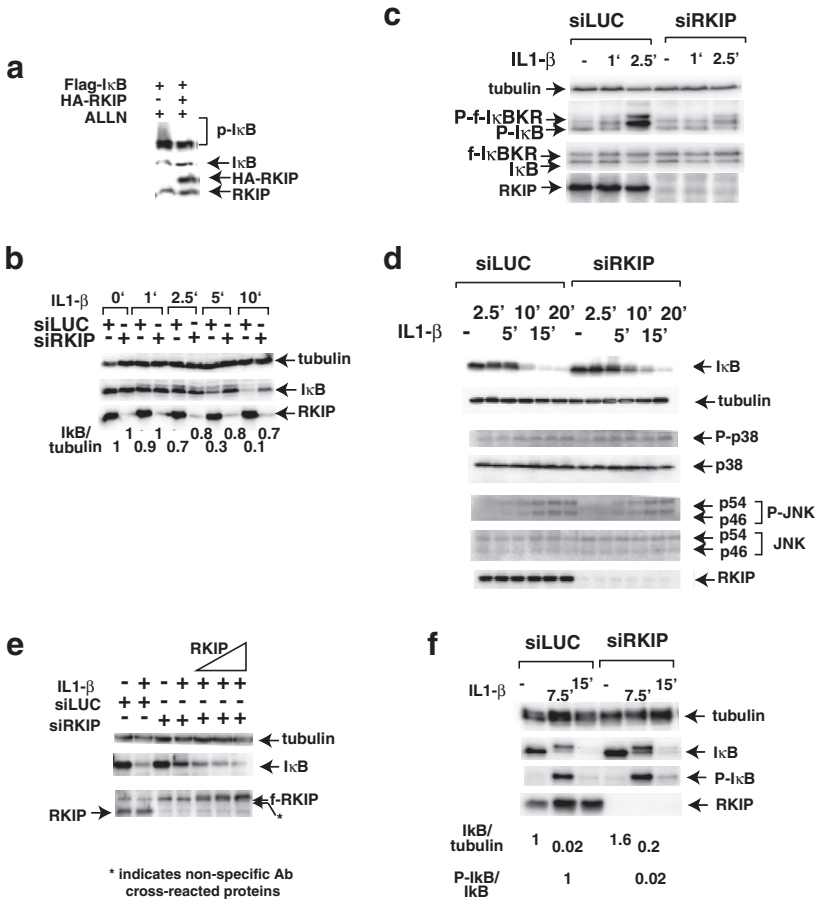

Fig. 1. (a) DU145 cells were infected with the indicated expression plasmid DNAs. Transfected cells were treated with ALLN or carrier control for 3 h before harvesting for immunoblotting with specific Abs as indicated. (b) Serum starved RKIP or control knockdown 293ILR cells were stimulated with IL1-β. Lysates were immunoblotted with the indicated specific antibodies. Relative levels of IκB/tubulin are indicated. (c) Same as (b), except the cells were transfected with flag-tagged IκB (f-IκBKR). (d) 293ILR cells were stimulated and analyzed as described in (b). (e) Same as (b), except the cells were transfected with the indicated expression plasmid DNAs. (f) Serum starved RKIP or control knockdown A549 cells were stimulated and analyzed as described in (b). The experiments were repeated at least three times with similar results.

In agreement with the effect of RKIP knockdown on IκB phosphorylation and degradation, we observed decreases in IRAK, TRAF6, and TAK1 ubiquitination as well as phosphorylation of TAK1 and IKK kinases (Fig. 3a–c). As expected from decreases in the activation of the upstream activators of IκB phosphorylation and degradation, we also observed reduced nuclear translocation of the p65 and p50 subunits of the NF-κB and its subsequent binding to promoters of target genes (Fig. 3d and e). In short our data suggest that RKIP is a scaffold protein that facilitates the assembly of the IKK complex leading to the degradation of IκB releasing the NF-κB for nuclear translocation (see Fig. 3f for summary).

3.4. Down regulation of RKIP increases the basal expression of a selected group of NF-κB target genes

To investigate the biological consequences of the scaffold function of RKIP in the NF-κB pathway we decided to monitor the expression profile of a representative group of inflammatory cytokines and receptors genes by quantitative RT-PCR array analysis. We focused on 84 key genes of chemokines, cytokines, and interleukins as well as their receptors. This group of genes was chosen because it contained verified targets of the NF-κB pathway initiated by the pro-inflammatory cytokine IL1-β. Normalized mRNA levels ranging from 300 to −2 relative to the non-stimulated control were observed in A549 control or RKIP knockdown cells after stimulation for 6 h with IL1-β. As expected a group of NF-κB regulated genes including CXCL1-3, CXCL5, CXCL10-11, CCL2, CCL4-5, CCL20, IL8, TNFα, and LTβ were highly activated (~10–300-fold) (Fig. 4a, the last six columns) when cells were stimulated with IL1-β for 6 h. Unexpectedly, the expression of this same group of genes was also elevated in quiescent RKIP knockdown cells when compared with the control GFP knockdown A549 cells (Fig. 4a, first two columns). Consequently the differences in the overall increase in IL1-β stimulated gene expression due to the specific RKIP knockdown are insignificant. Although NF-κB is essential for the expression of this group of genes, other signaling pathways including JNK, ERK, and p38 are also involved. To ensure the effect of RKIP is NF-κB specific we examined the expression an artificial NF-κB reporter in A549 cells when RKIP expression was knocked down by specific siRNA. Consistent with the quantitative RT-PCR arrays analysis results, we also observed elevated activities of an artificial NF-κB reporter in quiescent A549 cells when RKIP expression was knocked down by specific siRNA and these activities were further induced after IL1-β stimulation (Fig. 4b).

3.5. RKIP is required for the auto-regulation of NF-κB targeted genes expression

Previously we showed that neutralization of RKIP by Ab increased NF-κB responsive reporter activities in quiescent cells [9]. Consistent with our previous results, here we show that

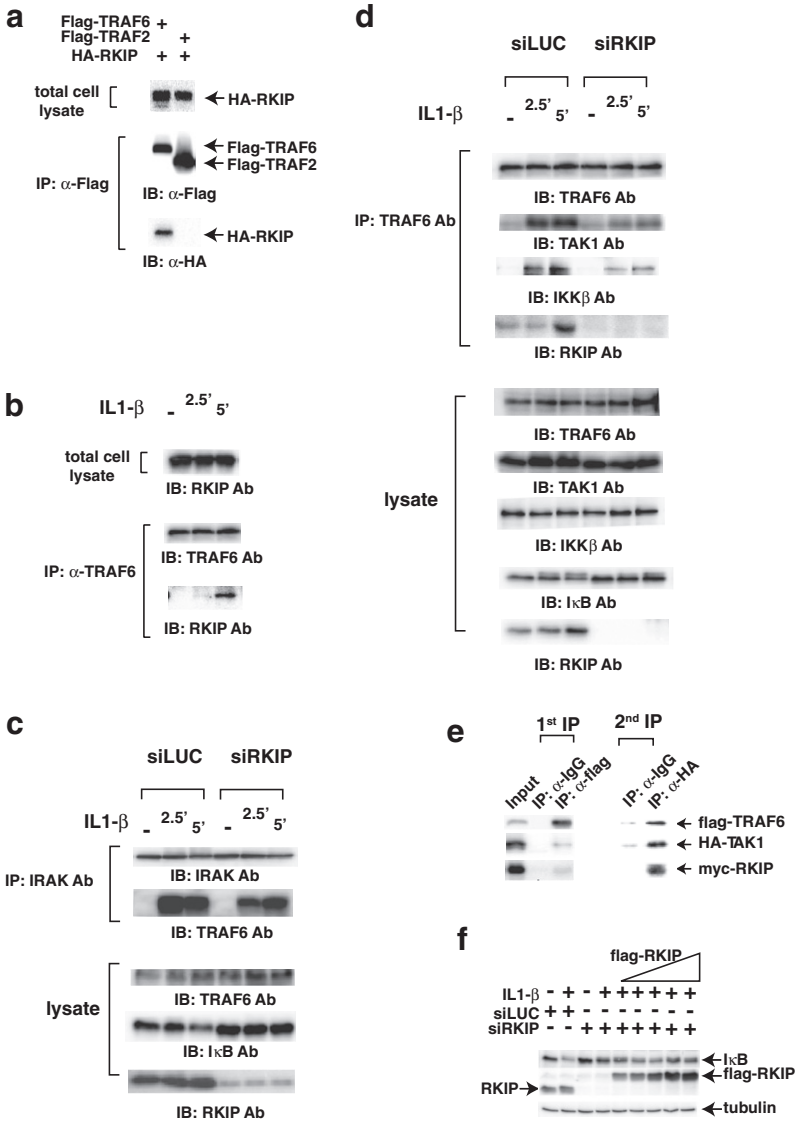

Fig. 2. (a) Cos-1 cells were transfected with the indicated expression plasmid DNAs. Cell lysates were immunoprecipitated with the indicated Ab and subsequently immunoblotted with specific Abs as indicated. (b) Total cell extracts prepared from IL1-β stimulated 293ILR cells were immunoprecipitated with the indicated Abs and immunoblotted with specific Abs. (c and d) Serum starved RKIP or control knockdown 293ILR cells lysates were immunoprecipitated and analyzed as described in (b). (e) Cos cells were transfected with the indicated expression plasmids and crosslinked before harvest. Cell lysates were subjected to a sequential co-IP assay as described [18]. (f) Same as described in Fig. 1c. The experiments were repeated at least three times with similar results.

downregulation of RKIP expression by siRNA increases the basal expression of NF-κB target genes (Fig. 4a and b). Our results therefore seem at odds with the observation that the signaling leading to degradation of the IκB was reduced in knockdown cells. However, since the regulation of NF-κB target genes expression is subjected to negative regulation involving the optimal induction of negative regulators, our results could result from the impairment of the negative feedback loop imposed by the induction of negative regulators. Indeed we observed a decrease in *IκB* gene expression in RKIP knockdown A549 cells at earlier times after stimulation with IL1-β (Fig. 4c) and reduction in the re-synthesis of IκB protein after its degradation (Fig. 4d). Beside *IκB* we also observed reductions in the expression of two other NF-κB target genes, *A20* and *cyld*, that encode negative regulators of the NF-κB pathway (Fig. 4e). Our data therefore are consistent with the model that RKIP inhibits the NF-κB pathway by enhancing the negative regulatory loop.

Here we show that RKIP interacts with multiple components of the IL1-β stimulated NF-κB pathway. Both overexpression and downregulation of RKIP expression impaired the degradation of IκB upon IL1-β stimulation. Studies of IKK/TAK1 activating phosphorylation, and IRAK/TRAF6/TAK1 ubiquitination suggested that RKIP acted downstream of or parallel to IRAK and upstream of IκB. Downregulation of RKIP decreased the phosphorylation of IκB, IKKα/β, and TAK1 and diminished the ubiquitination of TAK1, TRAF6, and IRAK. Our results therefore suggest that RKIP functions as a scaffold protein facilitating the activation of IKK complex by the E3 ubiquitinase TRAF6. Scaffold proteins can bind concurrently with several signaling molecules in the kinase cascade. We showed that RKIP associated with TRAF6, TAK1, or IKKα/β. Although we also demonstrated that RKIP existed in a ternary complex with TRAF6, and TAK1, it remains to be determined whether RKIP also can bind simultaneously with the rest of the IKK complex. It will also be of interest to determine regions in RKIP that interact with different components of the NF-κB pathway.

The involvement of scaffolding proteins in signal transduction is evolutionarily conserved. The functions of scaffold proteins in signaling pathways requiring MAPKs are well studied [10–14]. On the

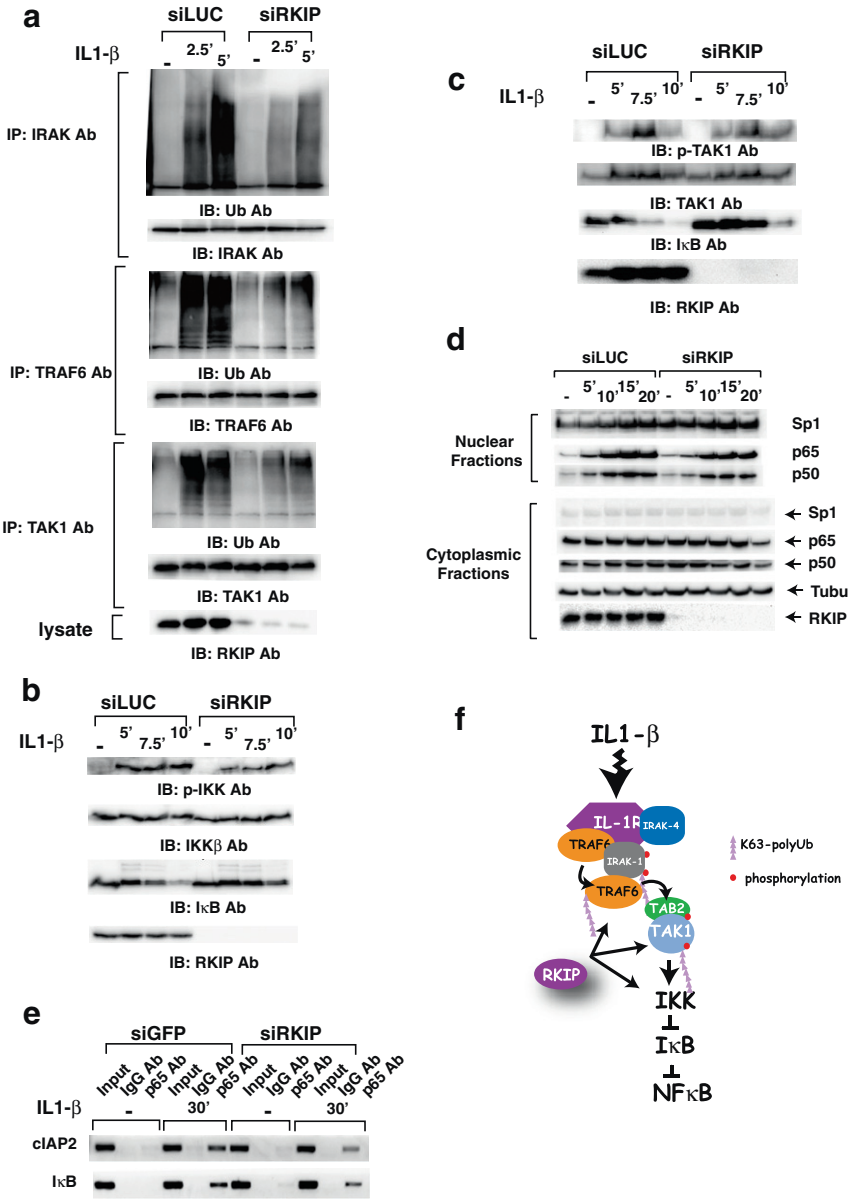

Fig. 3. (a–c) Same as described in Fig. 2d. (d) RKIP or control knockdown IL1R cells were stimulated with IL1-β. At indicated times cells were harvested and subjected to cellular sub-fractionation. Nuclear and cytoplasmic fractions were immunoblotted with specific Abs as indicated. (e) ChIP assays performed with anti-p65 (NF-κB) on RKIP or control knockdown HeLa cells. DNA that was immunoprecipitated with antibodies was amplified by PCR with specific primer pairs as indicated. The experiments were repeated at least three times with similar results. (f) A schematic summary of the IL1-mediated signaling pathway illustrates the possible interactions of RKIP with various gene products in the presence of IL1-β.

contrary, a scaffold protein that is directly involved in the IκB phosphorylation by upstream kinases has not been conclusively demonstrated. Hence, RKIP is the first example of a scaffold protein that directly stimulates the association of the IKK complex with the TRAF6 E3 ligase. RKIP is a member of the PEBP protein family. Recently it was shown that another PEBP family member, PEBP4, was a scaffolding protein of the Raf-MEK-ERK signaling pathway [15]. Unlike PEBP4, we have previously demonstrated that RKIP does not function as a scaffold in the Raf-MEK-ERK pathway but functions as a competitive inhibitor of the activation phosphorylation of MEK by Raf1 [6]. Our results therefore showed that RKIP employs different mechanisms to regulate different signaling pathways. The effect of PEBP4 on NF-κB pathway is currently unknown.

RKIP is a small protein with one identified ligand-binding pocket. How can this small protein bind concurrently with multiple proteins? One possibility is that additional protein(s) may be involved. It is possible that the scaffolding function of RKIP requires the presence of a novel RKIP yet-to-be-identified interacting protein. Together they provide a platform for other components of NF-κB pathway to interact. One precedent example of such a scaffold complex is the MP1/p14 complex [16,17].

In agreement with our earlier knockdown study with RKIP neutralization Ab, we observed an overall stimulatory effect on the basal transcription activities of NF-κB target genes by reporter assay and PCR array analysis. Increase in NF-κB transcriptional activities in spite of a decrease in IκB degradation is largely unexpected. Studies on expression of NF-κB target genes raise the possibility that the inhibitory effect of RKIP results from increased transcription of negative regulators of NF-κB signaling. In fact we observed a decrease in the expression of NF-κB early gene targets that encode negative regulators as IκB, A20, and Cyld in IL1-β stimulated RKIP knockdown A549 cells. Future work involving global monitoring of NF-κB target genes expression kinetics is required to further investigate this possibility.

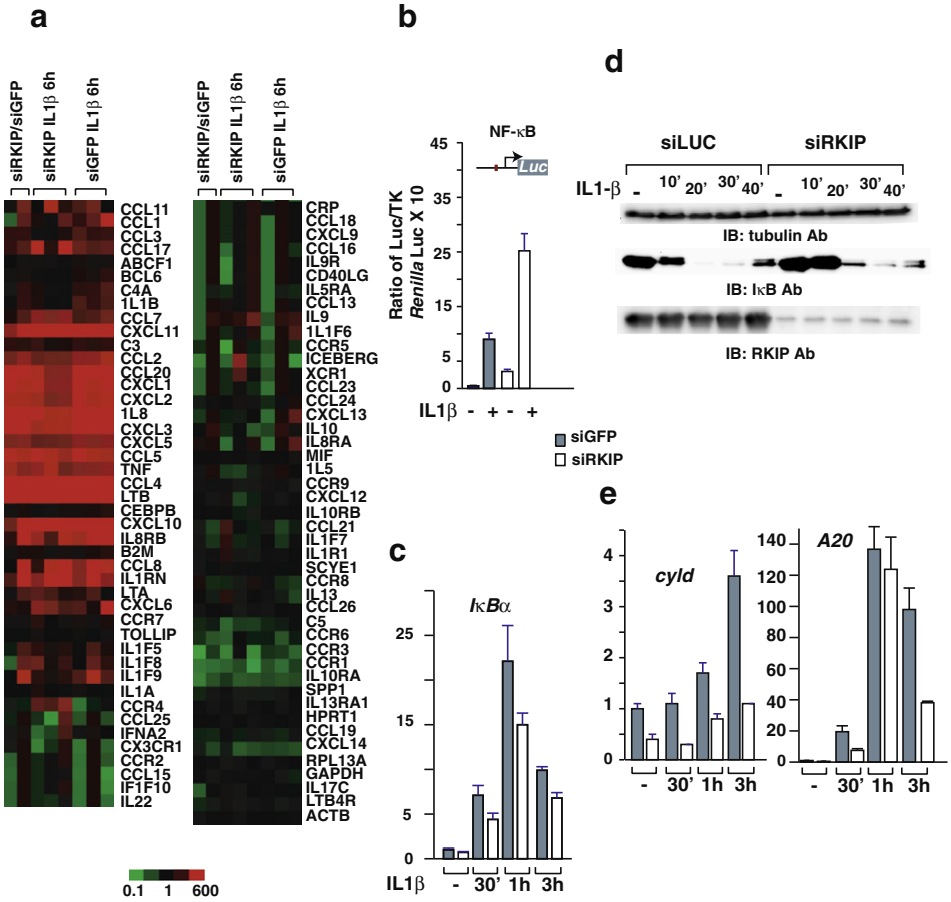

Fig. 4. (a) Heat map shows the relative expression of 84 inflammatory and cytokines genes in RKIP and control knockdown A549 cells in quiescent (first two lanes) or IL1-β stimulated (the last six lanes) conditions. mRNA expression levels are normalized to β-2-microglobulin, hypoxanthine phosphoribosyltransferase 1, ribosomal protein L13a, GAPDH, and β-actin expressions and displayed with genes as rows and samples as columns and color coded to indicated high (red) or low (green) expression levels. (b) RKIP or control knockdown A549 cells were transfected with a NF-κB luciferase reporter (NF-κB×3). Thirty hours after transfection, cells were either left untreated or were stimulated for 6 h with IL-1β and extracts were prepared and analyzed for luciferase activity. Activity of reporters in control knockdown cells not treated with IL1-β were set to 1. The means and standard deviations of three independent experiments are shown. (c) RKIP or control knockdown A549 cells were stimulated with IL1-β. At indicated times cells were harvested and the mRNA expression levels of the indicated genes were quantified by qRT-PCR and normalized to the level of GAPDH ($n \geqslant 3$). (d) Same as described in Fig. 2. (e) Same as described in (c).

RKIP was first identified as an endogenous negative regulator of the Raf/MEK/ERK signaling pathway [7]. Here we show that RKIP acts like a scaffold facilitating the phosphorylation of IκB by upstream kinases. The kinetics of RKIP interaction with NF-κB pathway components also differs significantly from its interaction with Raf-1. While RKIP associates with Raf-1 in quiescent cells, the association of RKIP with TRAF6 or TAK1 is IL1-β-dependent. Therefore, depending on how RKIP interacts with its targets, disparate mechanisms are used by RKIP to control different signaling pathways. Our studies therefore suggest that RKIP represents a novel regulator of multiple signal transduction pathways.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.12.051.

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