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The tumor suppressor Sef is a scaffold for the classical NF-κB/RELA:P50 signaling module

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ABSTRACT

The classical NF-κB transcription factor (RelA:p50) and the tumor suppressor Sef axis constitute a negative regulatory loop in which Sef, a target of NF-κB/RelA:p50, fine-tunes NF-κB/RelA:p50 transcriptional-activation in response to inflammatory stimuli trough binding to p50. Similar to the inhibitor IκBα, Sef sequesters NF-κB/RelA:p50 in the cytoplasm of unstimulated cells. Despite its key roles in regulating multiple cellular processes and its potential role as mediator between inflammation and cancer, Sef structural domains required to fulfill its tasks are poorly characterized, and how Sef specificity towards RelA:p50 is achieved is unknown. *In-vitro* binding assays using bacterially expressed Sef and Co-IP experiments, revealed that in addition to p50, Sef directly interacts with IκBα, and the IKKβ subunit of the IKK complex which mediates RelA:p50 induction by inflammatory stimuli. These interactions are ligand-independent and do not require Sef post-translational modifications. Deletion mutagenesis mapped binding site to IKKβ in a 74- residue segment juxtaposing Sef transmembrane domain, whereas several Sef regions seem to interact with IκBα. Moreover, we identified two new sites which together with the previously identified conserved tyrosine constitute three discontinuous Sef regions each indispensable for Sef binding to RelA:p50 and inhibiting its cytokine induced transcriptional activation. Contrary to IκBα, endogenous Sef is not degraded upon cytokine-stimulation, and its targeting in different cell types markedly enhances cytokine-induced NF-κB nuclear translocation. These results reveal Sef as the first scaffold that brings together the components of NF-κB/RelA:p50 signaling-module. Sef scaffolding function explains the basis for Sef specificity towards inhibiting inflammatory cytokine-induction of NF-κB/RelA:p50.

1. Introduction

Nuclear Factor-kappa B (NF-κB) family of dimeric transcription factors (TFs) play central roles in diverse physiological processes, while excessive and prolonged NF-κB activity is implicated in various pathologies including cancer [1–3]. The NF-κB/RelA:p50 heterodimer is the primary mediator of NF-κB activities. It is well accepted that in unstimulated cells, RelA:p50 exists predominantly as a cytoplasmic complex bound to its inhibitor, IκBα. Ligand stimulation by a wide range of stimuli induces phosphorylation of IκBα by the IκB kinase (IKK) complex which destines IκBα for rapid degradation, thus enabling NF-κB nuclear translocation where it activates the expression of its target genes, including its own inhibitor, IκBα. The newly synthesized IκBα exports NF-κB back to the cytosol [2,4]. IKK, IκBα, and NF-κB constitute a molecular relay switch mechanism known as the NF-κB signaling module [5]. NF-κB target genes are involved in numerous biological responses such as cell survival and proliferation, cell migration, tissue remodeling and inflammatory responses [1,2,6]. Thus, understanding how the NF-κB response is modulated is essential for developing useful therapeutic strategies for many human diseases [7].

Recently we described a previously unrecognized negative regulatory mechanism for the inhibition of inflammatory-cytokine induced NF-κB/RelA:p50 activation by the tumor suppressor SEF [similar expression to FGF, also known as IL17RD, [8–10]]. SEF is a known inhibitor of receptor tyrosine kinase (RTK) signaling, and our findings expand Sef function as a feedback antagonist for major pro-inflammatory cytokines, interleukin-1 (IL-1) and tumor necrosis factor alpha (TNFα). We showed that Sef is a target of NF-κB(p50:p65) and AP-1 but Sef specifically inhibits NF-κB(p50:p65). Sef interacts with NF-κB/RelA:p50 in unstimulated cells *via* binding to the p50 subunit, and constrains NF-κB nuclear entry upon IL-1/TNFα stimulation even though IκBα is degraded [9]. This newly identified negative feedback

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mechanism mediated by Sef likely represents a buffer constraining NF-κB activity. Subsequently, it was reported that Sef also inhibits IL-17 and pathogen induced NF-κB activation [11,12] pointing to Sef as a general inhibitor of canonical NF-κB.

Sef regulates key biological processes including cell proliferation, migration, apoptosis, bone homeostasis, and genomic stability [reviewed in [8,10]]. Sef is downregulated in a variety of human carcinomas in a manner correlating with tumor aggressiveness and poor prognosis, whereas restoring Sef expression in pre-established tumors suppresses tumor growth and tumor angiogenesis [13–18]. Additionally, Sef is down-regulated in rheumatoid arthritis [19], and inherited mutations in Sef are strongly linked to hearing loss in Kallmann's syndrome where fibroblast growth factor (FGF) signaling is involved [10,20]. Sef multi-functional nature points to its importance both for biological and pharmaceutical implications and provides a strong impetus for investigating how its multiple roles are carried out at the structural level. Surprisingly, however, Sef functional domains are poorly characterized.

Sef is encoded by a single locus, but several isoforms are generated *via* an alternative splicing mechanism of which prototypical Sef is mostly studied [8]. Human prototypical Sef (hSef-a) encodes a receptor-like protein, with intracellular domain (ICD) of 419 residues. Sef ICD contains a conserved tyrosine residue (Y330 in hSef-a) and a unique structural motif termed SEFIR domain that shares sequence similarity with IL17R SEFIR domain and with TOLL/IL-1R (TIR) domains, known as protein-protein interaction domains in TLRs/IL-1R signaling [8,21]. Thus far, it has been shown that 75 residues juxtaposing the transmembrane domain in Sef ICD are sufficient for binding of Sef to MEK (MAPKK) and inhibiting FGF-dependent biological activity [22]. The conserved tyrosine residue resides within this segment and is dispensable for Sef inhibition of RTK signaling but essential for Sef inhibition of inflammatory cytokines induced NF-κB(p50:p65) activation [9]. Additionally, box-3 of the Sef SEFIR domain was shown to interact with the adaptor protein MyD88 in a SEFIR/TIR-dependent fashion in TLR signaling. However, its deletion had only mild effect on Sef biological activity [12]. Thus, the role of Sef SEFIR domain in mediating Sef inhibitory activity remains elusive.

The goal of the present study was to delineate Sef functional subdomains in the context of pro-inflammatory cytokine signaling, and to gain better understanding of the mechanism by which Sef inhibits NF-κB(p50:p65). By combining mutagenesis with *in vivo* and *in vitro* binding assays, we found that the majority of Sef-ICD is dedicated for inhibiting cytokine induced NF-κB activation. We provide strong evidence that Sef interaction with NF-κB requires not only the conserved tyrosine but also the SEFIR domain and Sef C-terminal tail. Each of these sites is essential for efficient interaction with NF-κB and for inhibiting cytokine induced NF-κB activation. Moreover, we show that Sef physically interacts not only with p50 but also with IκBα and the IKKβ subunit of the IKK complex thus revealing Sef scaffolding function for the classical NF-κB signaling module.

2. Materials and methods

2.1. Enzymes, growth factors, reagents and chemicals

Restriction enzymes were from NEB (Ipswich, USA), and Thermo Scientific (Waltham, USA). IL-1α and TNFα were from Peprotech (Rocky Hill, USA). Recombinant FGF-2 was produced in bacteria and purified as previously described [23]. Fetal calf serum and media were from Gibco laboratories (Gaithersburg, USA) and Biological Industries (Beit Haemek, Israel). Generation of antibodies directed against Sef ICD was previously described [24]. Antibodies directed against Actin (sc-1615), Myc-tag (9E10, sc-40), IκBα (sc-371), p50 (sc-7178) and HA-tag (sc-805) were from Santa Cruz Biotechnology (Dallas, USA). Human Sef antibodies were from R&D systems (Minneapolis, USA), and αFlag antibody (F3165) from Sigma-Aldrich (Saint Louis, USA). HisPur NiNTA resin was from Thermo Scientific (Waltham, USA), and Glutathione resin was from Genscript (Piscataway, USA). ^{35}S-Methionine was from Perkin Elmer (Waltham, USA) (Specific Activity: > 1000 Ci/mMole).

2.2. Plasmids and generation of Sef mutants

Generation of Myc and Flag tagged Sef, as well as the SefY330F mutant was previously described [24–26]. To generate prokaryotic expression vector for the production of Sef ICD in bacteria (residues 321–739), the amino terminus of Sef ICD was adapted for cloning 3′ to the GST gene in the pGEX4T-1 vector to generate a fusion product in which GST is N-terminally fused to Sef-ICD (designated pGEX-SEF-ICD). This construct was used as a platform for the generation of the various Sef mutants. Internal deletions (rSefΔSEFIR; rSefΔ561-660) were created by PCR, and C-terminal truncations of Sef-ICD (rSefΔ395, rSefΔ534 and rSefΔ560, rSefΔ713) were generated using linker ligation methodology. To generate the same mutations in native human Sef, WT fragments were replaced by mutated fragments in pcDNA3.1/Sef. We also cloned GST-Sef ICD into pcDNA3.1. All Sef proteins were also fused to 6-His and myc-epitope tags at the carboxy-terminal end. Plasmids encoding p50-HA and IκBα-HA, IKKα-HA and IKKβ-flag, were kindly provided by Yinon Ben-Neriah (The Hebrew University, Israel), and Aaron Ciechanover (Technion, Israel), respectively. Plasmids for *in-vitro* translation of p50 and IKKγ, and IKKβ were kindly provided by Amir Orian (Technion, Israel), and Allan Israel (Pasteur Institute, France), respectively. We thank Eisuke Nishida for providing us with Sef SefΔ443 mutant (Kyoto University, Japan).

2.3. Cell culture, transfection and luciferase assays

HEK 293 cells stably expressing IL-1R (HEK/IL-1R) were previously described [27]. Cells were grown in DMEM containing 5% FCS. Transient transfections were performed with Polyethylenimine (PEI, from Polysciences, Warrington, USA) according to manufacturer's protocol. Sef knockout in HEK 293 was performed by utilizing CRISPR-Cas9 technology. Sef knock-down in HaCaT cells was performed by stable RNA interference with validated shRNAs as previously described [13].

2.4. CRISPR/Cas9 mediated Sef knockout

The *SEF* CRISPR construct was generated based on a previously described protocol [28]. Briefly, two pairs of gRNAs for a CRISPR-Cas9n construct were designed (http://crispr.mit.edu/) to target the second common exon in all human Sef splice variants (gRNA A: 5′- GTCACC ATTCTTTGGTCCCC -3′ and gRNA B: 5′- ACTGGCTGATGGTGATATTC -3′) and were cloned into pSpCas9n (BB)-2A-GFP (PX461) plasmid [a generous gift from Feng Zhang; Addgene plasmid #48140 [28]]. Co-transfection with each PX461 gRNA plasmid (1.2 μg each) was performed using PEI reagent according to manufacturer's instructions, after which eGFP-positive clones were enriched by FACS and seeded at various densities. Isolated clones were screened for *SEF* knockout (KO) by immunoblotting with antibody directed against human Sef as described [9], and Sef genomic region was sequenced to assess the extent of deletion.

2.5. Dual luciferase assay

Assays were performed essentially as described using pFIRE and NF-κB luciferase-reporter plasmids [9]. Sixteen hours post-transfection, cells were left untreated or treated with the indicated ligands for 6 h. Luciferase activity in cell lysates was measured by using the dual luciferase assay system (Promega, Madison, USA) in a GLOMAX™ 20/20 luminometer. Reporter activity was normalized to the activity of co-expressed Renilla.

2.6. Generation and purification of recombinant Sef proteins

Recombinant Sef and Sef mutant proteins were produced in BL21 strain of *E. coli*, and purified from bacterial supernatants using two consecutive affinity purification steps. First, bacterial soluble fraction was loaded onto NiNTA column in buffer containing 40 mM imidazole, and elution was performed following extensive washes as previously described [29]. Aliquots from eluted fractions were analyzed by SDS-PAGE and Coomassie Brilliant Blue staining as well as western blotting. Selected fractions were combined and subjected to Glutathione Agarose affinity chromatography. Concentrations of purified Sef proteins were determined by Bradford assay and further verified by SDS-PAGE and Coomassie Brilliant Blue staining relative to BSA standard.

2.7. Protein detection methods and binding assays

Co-immunoprecipitation and protein immunodetection by Western-blotting were performed as previously described [9,24]. Immunofluorescence (IF) for testing NF-κB(p65) nuclear translocation was performed essentially as described [9]. Nuclear staining, when indicated, was performed using 10 μM DRAQ5 (Biostatus, Shepshed Leicestershire, UK), and images were examined by using standard fluorescence microscopy. For cell free binding assays, p50, IKKα/β/γ and IκBα proteins were translated *in vitro* using the TNT quick-coupled transcription-translation kit (Promega, Madison, USA) in the presence of ^{35}S-methionine according to manufacturer's protocol. Pull-down assays with GST-fused Sef proteins were carried out as described [9]. Densitometry analysis was performed using Quantity One software (Bio-Rad, Hercules, USA).

3. Results

3.1. The majority of Sef intracellular domain is dedicated to inhibiting pro-inflammatory cytokine signaling

To identify new functional motifs within Sef ICD, we generated a panel of Sef mutants including C-terminal truncations (SefΔ395, SefΔ443, SefΔ534 and SefΔ560) as well as deleted residues 356–512 that encompass the SEFIR domain [SefΔSEFIR, (illustrated in Fig. 1A)]. Biological activity of the Mutants was assessed in HEK/IL-1R cells by luciferase reporter assay using pFIRE and NF-κB reporters for FGF and cytokine signaling, respectively. Mutants functionality was compared to that of the SefY330F mutant previously shown by us to retain inhibitory capacity towards FGF but not proinflammatory cytokine signaling [9]. All mutants, including SefΔ443, SefΔ534 and SefΔ560 that have been expressed at lower levels relative to full length Sef and to the SefΔSEFIR mutant (Fig. 1B), inhibited FGF reporter activity (Fig. 1*C*) in agreement with Torii et al., [22]. SefY330F, as expected, lost inhibitory capacity in the context of cytokine signaling and even enhanced NF-κB transcriptional activation due to its previously demonstrated strong dominant-negative (DN) effect on endogenous Sef [9]. Similar to SefY330F, all the other mutants failed to inhibit IL-1-induced NF-κB activation (Fig. 1D), indicating that the majority of Sef ICD is dedicated to inhibition of cytokine induced NF-κB activation.

3.2. Sef regions downstream to the conserved tyrosine are also essential for interaction with NF-κB

The findings that any alterations C-terminal to residue 395 in Sef ICD abrogated Sef capacity to inhibit IL-1 induced NF-κB activation, led us to hypothesize that the conserved tyrosine, although essential for Sef inhibitory activity in the context of pro-inflammatory cytokine signaling, is not enough for this Sef function. To examine this hypothesis, we initially focused on investigating the role of the SEFIR domain. First, we examined the outcome of SEFIR deletion on the interaction of Sef with p50, which mediates the association of Sef with NF-κB/RelA:p50. Co-immunoprecipitation (Co-IP) assays were performed on HEK/IL-1R extracts co-expressing myc-tagged Sef or SefΔSEFIR constructs with or without p50. WT-Sef, but not SefΔSEFIR, co-precipitated with p50 albeit its expression level was similar to WT-Sef (Fig. 2*A*).

Since Sef homodimers interact with NF-κB/relA:p50 [9] and SEFIR alone can form homodimers when expressed in cells (Supplementary Fig. 1), one plausible explanation for the failure of SefΔSEFIR to bind p50 could be its inability to dimerize. To test this, HEK/IL-1R cells were co-transfected with Flag-tagged WT-Sef, along with a 3-fold excess of either myc-tagged WT-Sef or SefΔSEFIR and co-immunoprecipitated with anti-Flag antibodies to exclude homomeric complexes of myc-tagged proteins from being precipitated. As seen in Fig. 2B, deletion of Sef SEFIR domain did not abolish mutant ability to heterodimerize with WT-Sef. Furthermore, when expressed alone, the mutant protein was capable of forming dimers/oligomers as efficiently as WT Sef, which were readily observed in gel-electrophoresis under semi-denaturing conditions (Fig. 2C). To further substantiate that the SefΔSEFIR mutant retained the ability to dimerize, we examined whether it exerts a dominant negative effect when co-expressed with WT-Sef. Indeed, when co-expressed with WT-Sef, the SefΔSEFIR mutant reversed WT-Sef inhibition of IL-1-induced NF-κB activation in a dose dependent manner (Fig. 2D), consistent with its ability to form heterodimers with WT-Sef. Collectively, these results strongly indicate that the SEFIR domain is essential for the interaction of Sef with p50 but not for Sef homotypic interactions. Similar to the SefΔSEFIR mutant, SefΔ560 also failed to associate with p50 (Fig. 2E). However, as SefΔ560 was expressed at relatively low levels compared to parental Sef, we could not rule out the possibility that this might be the reason for the lack of a detectable interaction. Nevertheless, these results clearly point to the involvement of the SEFIR domain in Sef interaction with NF-κB and subsequent inhibition of its transcriptional activation.

3.3. Sef mutants binding to upstream components of the NF-κB cascade

Previously, we showed that Sef associates with IKKβ and IκBα in Co-IP experiments in the absence of ligand stimulation [9]. Since these two proteins are found in a large complex containing NF-κB in resting cells [30], it was not possible to exclude that Sef interaction with IKKβ and IκBα is indirect through its binding to NF-κB [9]. The availability of Sef mutants with reduced/null binding to p50 provided the opportunity to resolve this problem as binding of these mutants to IKKβ and IκBα would indicate that their binding to Sef was independent of NF-κB. We initially performed Co-IP experiments with either IKKβ or IκBα with a panel of Sef mutants (SefY330F, SefΔSEFIR, SefΔ443, SefΔ534 and SefΔ560) and found that all the tested mutants co-precipitated both with IKKβ and IκBα to a degree that correlate with the expression levels of each mutant protein (Fig. 2F, and 2G). Taken together, the above findings prove that Sef interacts with IKKβ and IκBα independent of NF-κB and point to Sef as a scaffold for the NF-κB/RelA:p50 signaling module.

3.4. Establishing a cell-free system for studying Sef interaction with the components of the NF-κB signaling module

To further substantiate the function of Sef as a scaffold for the NF-κB signaling module we established a cell-free binding assay with bacterially expressed Sef. Such an assay would also allow us to better quantify interaction potencies. To this end, we generated a construct in which Sef ICD was N-terminally fused to the glutathione S-transferase (GST) gene and C-terminally to His/Myc tags (Fig. 3A). This construct was then cloned into a prokaryotic and eukaryotic expression vector. In transient transfections into HEK/IL-1R cells, Sef-ICD inhibited IL-1-induced NF-κB reporter activity in a dose dependent manner and as effectively as full-length Sef (Fig. 3B), indicating that the extracellular and transmembrane domains of Sef are not essential for its inhibitory function in the context of pro-inflammatory cytokine signaling.

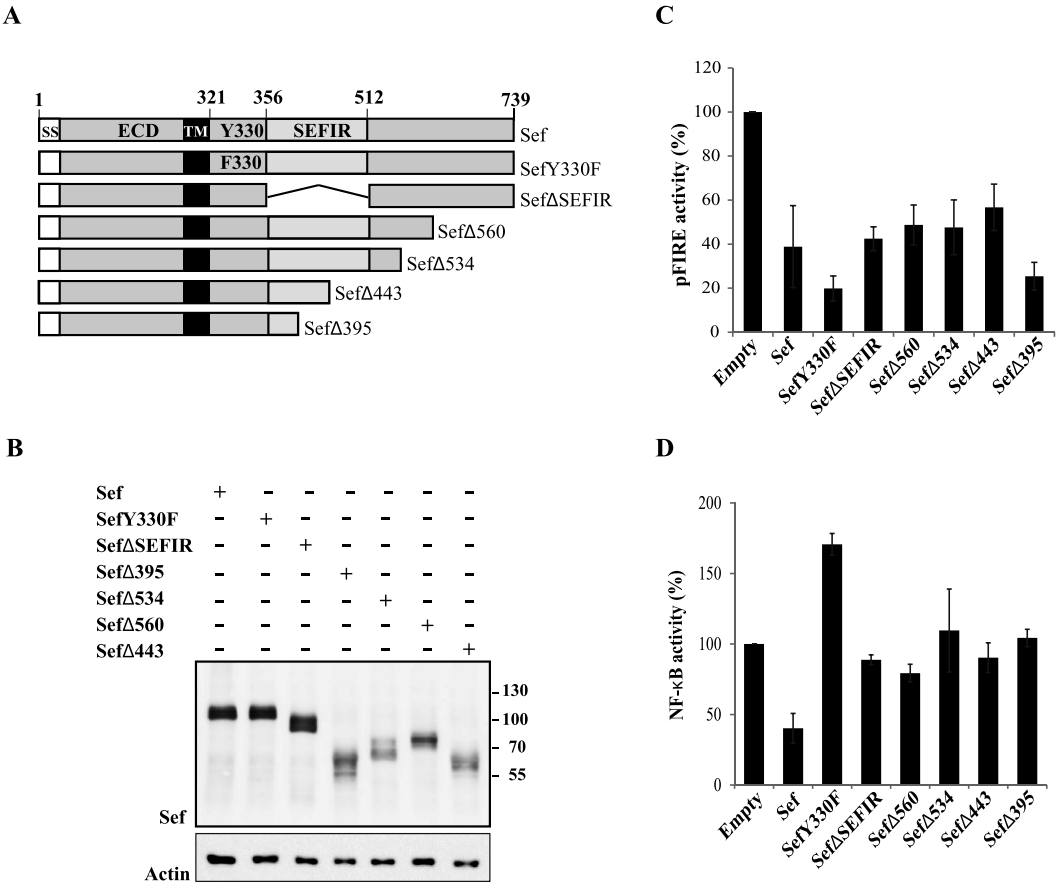

Fig. 1. The majority of Sef ICD is dedicated to inhibiting NF-κB. (A) Schematic diagram of Sef mutants. SS – signal sequence; ECD – extracellular domain; TM – transmembrane domain. The conserved tyrosine Y330 and SEFIR domain are indicated. (B) Expression level of Sef mutant proteins. HEK/IL-1R cells were transiently transfected with the indicated plasmids and whole cell extracts were subjected to Western blot analysis using antibodies directed against human Sef-ECD or anti-actin antibody, the latter serving to monitor relative protein loading. (C, D) Sef ICD mutants efficiently inhibit GF signaling, but not inflammatory-cytokine induced NF-κB activation. HEK/IL-1R cells were transfected with FGF (C) or NF-κB (D) luciferase reporter plasmid alone or with a plasmid encoding hSef or the indicated hSef mutants. Stimulation was with 40 ng/ml FGF2 (C) or 10 ng/ml IL-1 (D) for 6 h. Error bars indicate SD ($n = 2$).

Next, we expressed the GST-Sef protein (designated rSef) in bacteria and purified it as described under Materials and Methods using two consecutive affinity purification steps (Fig. 3C). To test whether rSef is biologically active, we initially examined its binding to *in-vitro* translated (IVT) p50 in a GST pull-down assay using 0.1 and 0.2 μg/ml rSef. As shown in Fig. 3D, rSef efficiently interacted with p50 and binding could be already observed at the lowest rSef concentrations, while no detectable signal was obtained in a binding assay with 0.5 μg/ml purified GST protein.

Having established a cell-free binding system with bacterially expressed Sef, we next tested its dose-dependent binding (0.1–2 μg/ml) to each individual component of the IKK complex and to IκBα, compared to p50. rSef efficiently interacted with p50, the catalytic subunit IKKβ of the IKK complex, and IκBα in a dose-dependent manner. Both for p50 and IKKβ a specific signal could be observed already at 0.1 μg/ml of Sef, while in case of IκBα, a positive signal could be detected starting from 0.2 μg/ml of rSef most likely due to differences in their specific activities (Fig. 3E, left panel). By contrast, rSef did not interact with IKKα catalytic subunit or the regulatory subunit IKKγ (also known as NF-κB essential modifier/IκB kinase gamma, NEMO) even at Sef concentrations as high as 1.5 μg/ml (Fig. 3E, right panel). These findings show unequivocally that Sef physically interacts with three components of the NF-κB signaling cascade (p50, IKKβ and IκBα) and with a similar high efficiency.

3.5. *In vitro binding properties of Sef mutants*

The established cell free binding assay provided the opportunity to compare binding potencies of Sef mutants under similar concentrations, thus overcoming the problem of low expression levels of C-terminally truncated mutants in eukaryotic cells as compared to WT Sef. Initially, we expressed mutants rSefY330F, rSefΔSEFIR, rSefΔ534, rSefΔ560 and rSefΔ395 in bacteria (See illustration in Fig. 4*A*). Then, increasing concentrations of purified mutant proteins were bound to IVT- p50, IKKβ and IκBα in a GST pull-down assay. Similar to pull-down assays performed with full length SefY330F mutant expressed in eukaryotic cells [9], the bacterially expressed rSefY330F mutant displayed a significantly reduced binding affinity to p50 (~10-fold decrease as compared to rSef, see Fig. 4B and 4C). None of the remaining mutant proteins bound to p50, even at concentration as high as 2 μg/ml (Fig. 4B). Staining of the gel following binding indicated that similar WT and mutant proteins levels were loaded (data not shown). By contrast, all the mutants bound to IVT-IKKβ with a parental binding potency (Fig. 4D). The mutants also retained binding capacity to IκBα but displayed a reduced affinity as compared to rSef, with rSefΔSEFIR, rSefΔ534 and rSefΔ560 having the lowest binding potency (~50% reduction, Fig. 4E).

To further narrow down the region within Sef residues 560–739 required for binding to p50, we generated two additional mutants in which 100 residues carboxy-terminal to the SEFIR domain (rSefΔ561–660) and the last 23 residues (rSefΔ713) were deleted. The

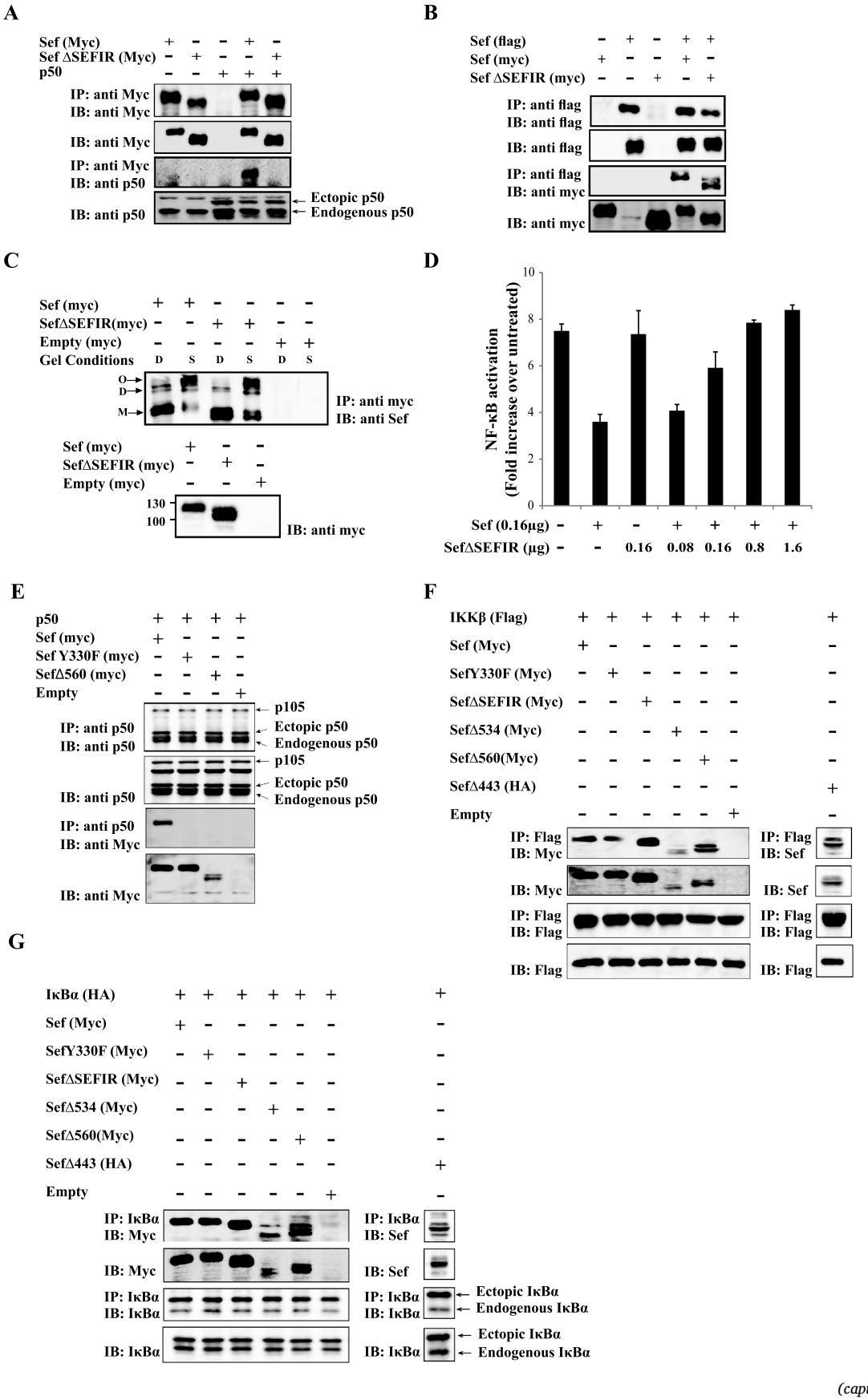

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Fig. 2. Sef SEFIR domain and its carboxy-terminal tail are essential for interaction with NF-κB. (A) SefΔSEFIR does not co-precipitate with p50. For the Co-IP, HEK/IL-1R cells were transfected with the indicated combinations of constructs, and whole cell lysates were either immunoblotted (IB), or immunoprecipitated (IP) with the indicated antibodies. (B, C) Deletion of Sef SEFIR domain does not abrogate Sef homotypic interaction. (B) SefΔSEFIR heteromerizes with full length Sef in Co-IP assay. (C) Whole cell extracts of HEK/IL-1R cells overexpressing Sef or SefΔSEFIR were immunoprecipitated with anti Myc-tag antibody, separated in gel electrophoresis under denaturing (D) or semi-denaturing (S) conditions, and immunoblotted with anti-Sef antibodies. Different oligomerization states of Sef/SefΔSEFIR proteins are indicated with arrows (M-monomer; D-dimer, O-oligomer). Expression levels of Sef/SefΔSEFIR proteins in whole cell lysates is shown in the bottom panel. (D) SefΔSEFIR abrogates WT Sef inhibition of NF-κB. Reporter assays were performed in HEK/IL-1R cells as indicated in the legend to Fig. 1D. (E) Sef region C-terminal to the SEFIR is also required for Sef interaction with p50. Co-IP assay was performed as described in panel A. (F, G) All Sef mutant proteinss co-precipitate with IKKβ and IκBα. HEK/IL-1R cells were co-transfected with IKKβ (F) or IκBα (G) along with WT Sef or the indicated Sef mutants. Co-IP assays were performed as described in panel A.

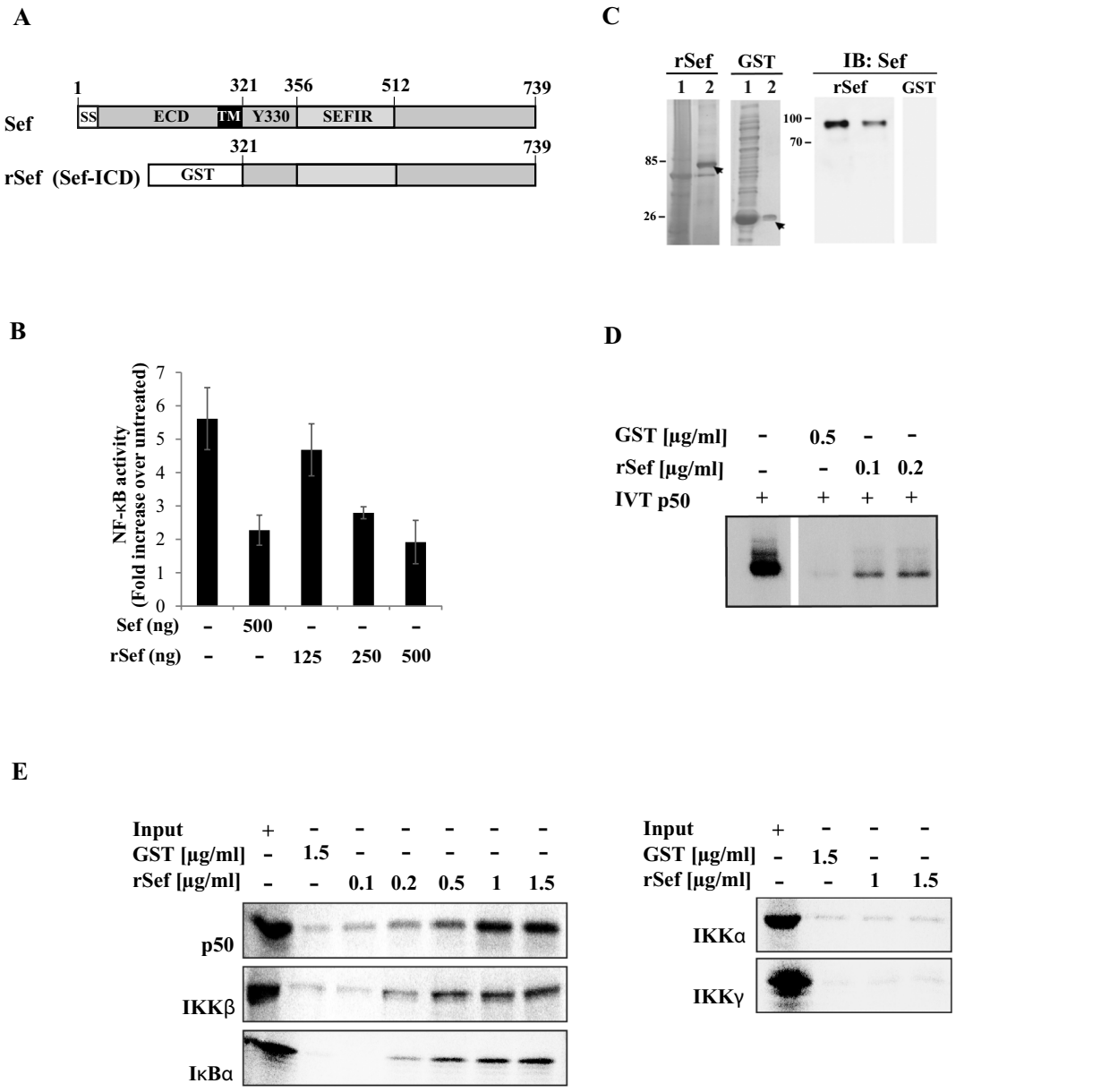

Fig. 3. Establishment of cell-free system for evaluating Sef physical interaction with the components of NF-κB/RelA:p50 signaling module (A) Schematic representation of human Sef coding sequence cloned into pGEX4T- for bacterial expression (rSef). (B) Biological activity of Sef-ICD. Sef ICD was cloned into pCDNA3.1 eukaryotic expression vector and NF-κB reporter activity was assessed as described in Fig. 1D legend. Error bars indicate SD ($n = 2$). (C) Purification of bacterially expressed rSef and GST. rSef and recombinant GST were each expressed in BL21 *E.coli* and purified from bacteria soluble fraction as described under Material and Methods. SDS-PAGE and coomassie blue staining of bacterial soluble fraction (lane 1, left and middle panels) and purified proteins in a selected fraction (lane 2, left and middle panel) from the Glutathione agarose affinity-chromatography. Presence of rSef protein in the bacteria lysate and in combined fractions following purification was verified by immunoblotting with antibody directed against Sef-ICD (right panel). (D) rSef efficiently interacts with IVT-p50. rSef immobilized on Glutathione agarose resin was subjected to *in vitro* binding assay with ^{35}S-labeled p50, as described under Materials and Methods. GST served as control for background binding. (E) Dose dependent binding of rSef to all components of the NF-κB signaling module. Each of the indicated proteins was *in-vitro* translated and subjected to cell free binding assays with increasing concentrations of rSef, and the indicated concentration of purified GST. 5% input is shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

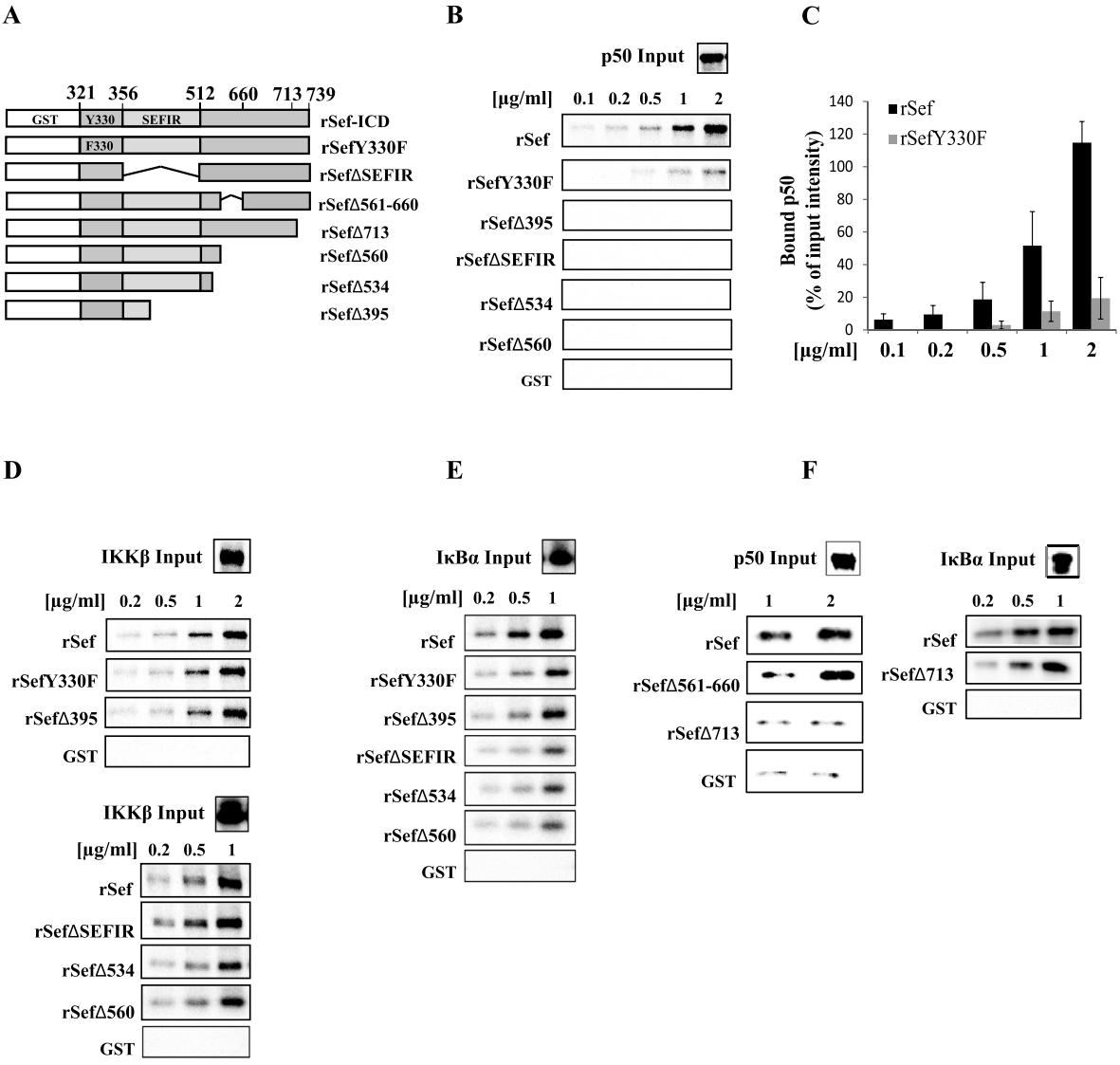

Fig. 4. Mapping Sef domains required for Sef interaction with p50/IKKβ/IκBα in a cell-free system. (A) Schematic diagram of rSef mutants expressed in bacteria for *in vitro* binding assays. (B) *In vitro* binding of rSef and its mutants to IVT-p50. (C) Densitometry analysis of rSef/rSefY330F binding intensity to p50, presented as percentage of input intensity. Error bars indicate SD (n = 2). (D, E) *In vitro* binding of rSef and its mutants to IVT- IKKβ (D) and IκBα (E). (F) *In vitro* binding of rSef and the indicated mutants to IVT-p50 and IVT- IκBα. 4% input is shown.

binding of bacterially expressed mutants to IVT-p50 was compared to that of rSef. Mutant rSefΔ561–660 displayed nearly parental binding affinity whereas rSefΔ713 exhibited background binding level to p50 (Fig. 4F, left panel). By contrast, rSefΔ713 interacted with IVT-IκBα as efficiently as rSef (Fig. 4F, right panel). These findings locate a third region within Sef ICD that is indispensable for Sef interaction with p50 but not for interaction with IKKβ and IκBα.

3.6. The effect of SEF mutations on cytokine induced nuclear translocation

Since the outcome of Sef interaction with NF-κB is inhibition of stimulus dependent nuclear translocation of the TF, we assessed the outcome of the hSef mutations on this process. To avoid complication that may arise from heterodimerization of mutants with endogenous Sef, we performed the assay in HEK 293 null for hSef expression (HEK/*SEF*$^{-/-}$). These cells, predominantly express the hSef-a isoform used in this study (Fig. 5A), and Sef knock-out was achieved using CRISPR technology (representative results are shown in Fig. 5B). Initially, we transfected various hSef-a plasmid concentrations (0.16, 0.32, and 0.64 μg) into HEK/*SEF*$^{-/-}$ cells in order to choose an effective inhibitory concentration. hSef-a, efficiently inhibited TNFα induced NF-κB nuclear translocation at all the examined concentrations (data not shown). Next, we compared the capacity of various hSef-mutants to inhibit TNFα dependent NF-κB nuclear translocation to that of WT Sef following transfection with 0.32 μg of each construct, and also with a 2-fold higher concentration for mutants SefΔ534 and SefΔ560 that exhibit low expression levels under steady-state conditions. In cells transfected with empty plasmid, p65 nuclear translocation was observed in over 98% of the cells 20 min post- TNFα stimulation, and hSef suppressed nuclear translocation of p65 in over 90% of the transfected cells. Similar results were obtained with mutant SefΔ561–660 that retained the capacity to interact with NF-κB(p50) in the above described binding assays. By contrast, SefΔSEFIR, SefΔ534, SefΔ560, and SefΔ713 failed to inhibited p65 nuclear translocation (Fig. 5C and 5D). Moreover, mutants SefΔ534, SefΔ560 also lacked an inhibitory capacity when expressed at the higher concentration (data not shown). Collectively, these results fully corroborate the results of the binding assays and clearly indicate that lack of biological activity of the Sef mutants in the context of cytokine signaling results from their inability to interact with NF-κB and consequently to inhibit its nuclear translocation.

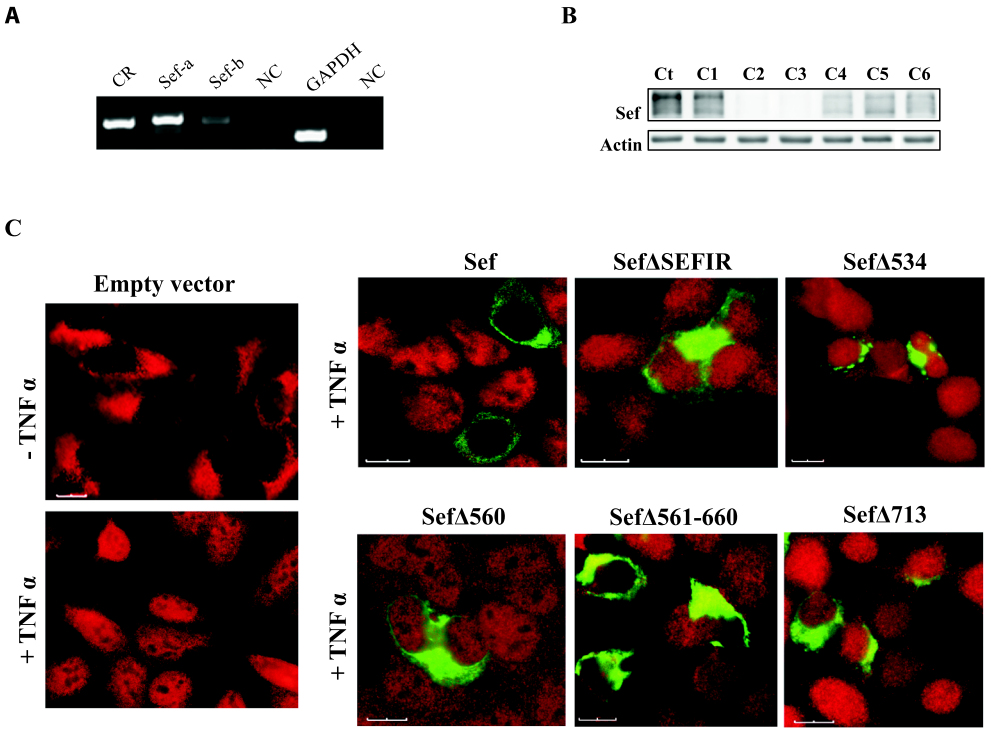

Fig. 5. The effect of mutations in hSef on cytokine dependent NF-κB nuclear translocation. (A) RT-PCR analysis of Sef isoforms in HEK 293 cells. PCR was performed with primers from Sef common region (CR) and isoform (hSef-a and hSef-b) specific primers as previously described (24). NC denotes negative control where PCR was performed without template. (B) Detection of Sef protein in control (Ct) and targeted HEK 293 clones (C1-C6). Whole cell lysates from the various clones were immunoblotted with anti-hSef and anti-actin antibodies where the latter served to monitor relative loading. (C, D) HEK/*SEF*$^{-/-}$ cells were transfected with 0.32 μg of the indicated constructs. Cells were stimulated with 7.5 ng/ml TNFα for 20 min or left unstimulated, and then analyzed by indirect immunofluorescence (IF) with myc epitope tag (green) and p65 (red) antibodies. Quantification of the extent of inhibition is shown in panel D Over 200 cells were counted in two individual experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.7. The outcome of targeting SEF on cytokine induced NF-κB nuclear translocation

Previously we showed that knock-down of endogenous Sef in a cervical carcinoma cell-line enhances IL-1 induced NF-κB nuclear translocation and transcriptional activity [9]. The availability of hSef null cells provided the opportunity for assessing whether this is a general phenomenon. For this aim, we compared the extent of NF-κB nuclear translocation in control and HEK/*SEF* $^{-/-}$ cells stimulated with TNFα for 20 min, a time point at which IκBα protein is fully degraded (Fig. 6E). In control cultures stimulated with TNFα (2.5 ng/ml), NF-κB remained cytoplasmic in 82% of the cells. Complete and partial nuclear localization was observed in 4% and 14% of cells, respectively. By contrast, the number of Sef$^{-/-}$cells displaying complete or partial nuclear localization was 8 and 3-fold higher, respectively (Fig. 6A and 6B). Although the extent of NF-κB nuclear localization was increased by 15% and 32.6% (predominant and partial, respectively) in control cells stimulated with 5 ng/ml ligand, Sef null cells still displayed 3-fold (46%) and 1.6-fold (52%) higher predominant and partial nuclear localization, respectively, as compared to the stimulated control cells (Fig. 6A and 6B). Similar results were obtained in human keratinocytes (HaCaT cells) wherein Sef expression was targeted by stable RNA interference using previously validated shRNAs [[9,22,26], Fig. 6C and 6D]. These findings indicate that Sef sequestration of NF-κB in the cytoplasm of cytokine stimulated cells is a general phenomenon and that a rather large proportion of NF-κB is prevented from entering the nucleus due to the interaction with Sef. Furthermore, follow up of the fate of Sef and IκBα proteins in TNFα stimulated HEK 293 cells for up to 120 min, clearly showed that while IκBα protein is rapidly lost within 15 min of TNFα stimulation, Sef levels remain unchanged. As expected, IκBα protein levels were fully restored 60 min post stimulation (Fig. 6E). Collectively, these findings point to a principal difference between regulation of IκBα and Sef proteins in stimulated cells and explain why Sef is able to constrain NF-κB nuclear translocation in spite of IκBα

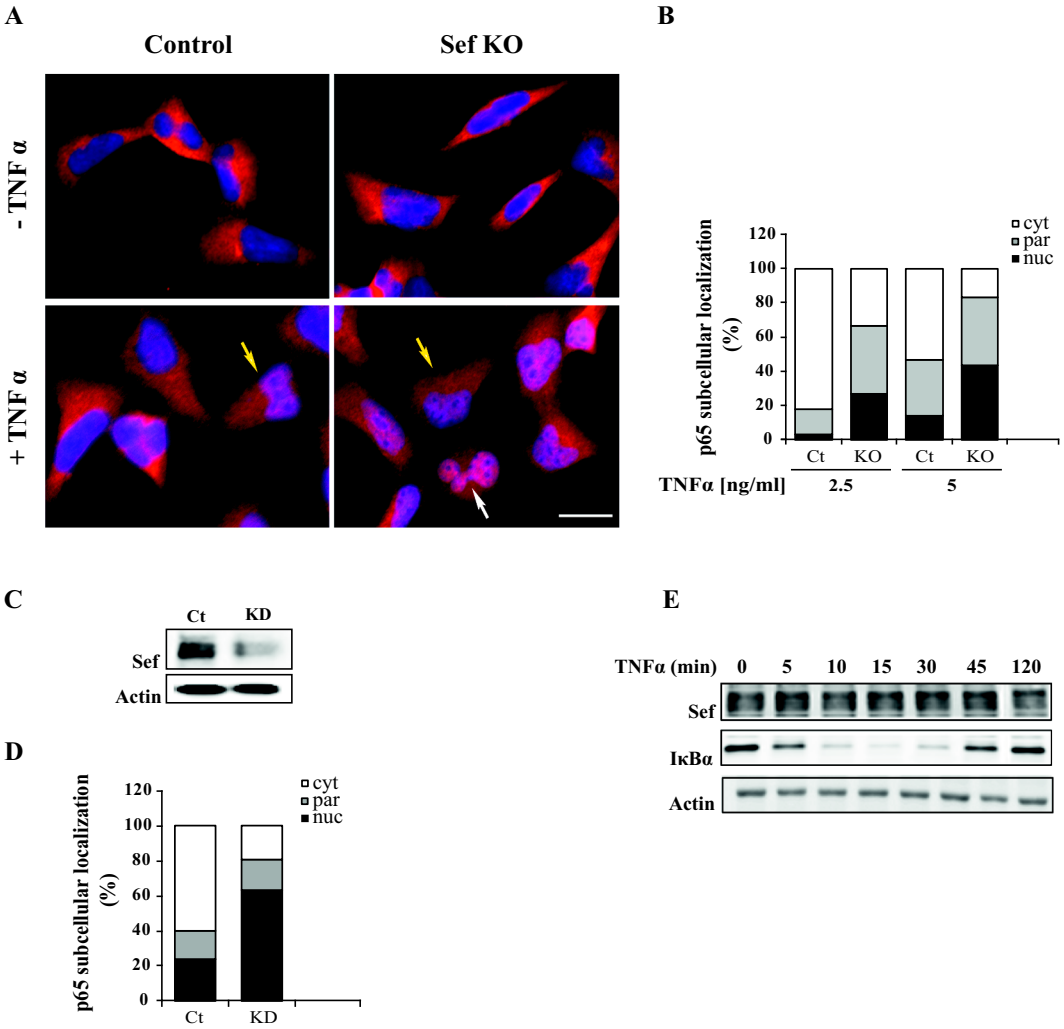

Fig. 6. Sef knock-out (KO) in HEK 293 cells significantly enhances NF-κB nuclear translocation. A, Control and HEK/*SEF*$^{-/-}$ cells were stimulated with 2.5 and 5 ng/ml of TNFα for 20 min, then fixed and immunostained with RelA(p65) antibody. Arrows point to complete (white) or partial (yellow) nuclear localization of RelA(p65). (B) Quantification of the data described in panel A. > 200 cells were counted in 2 independent experiments (cyt, par and nuc denote cytoplasmic, partial nuclear and nuclear localization, respectively). (C, D) The effect of targeting Sef in human keratinocytes (HaCaT cells) on ligand induced NF-κB nuclear translocation. (C) Immunodetection of Sef in control (Ct) and targeted (KD) keratinocytes. Immunodetection was performed as described in the legend to Fig. 5. (D) For assessing RelA(p65) nuclear translocation, cells were stimulated with 5 ng/ml TNFα for 20 min, fixed and immunostained for RelA(p65). Nuclei were stained with Hoechst reagent (blue staining). (E) The fate of Sef and IκBα in cytokine stimulated cells. HEK 293 cells were treated with TNFα (10 ng/ml) for the indicated time points or left untreated. Whole cell lysates were immunoblotted with anti-hSef, anti IκBα, or anti-Actin antibodies. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

degradation. These results also indicate that Sef acts as a universal inhibitor of major inflammatory stimuli induced NF- κB activation.

4. Discussion

Sef capacity to negatively modulate NF-κB/RelA:p50 by distinct stimuli, its ubiquitous expression in human tissues [8] combined with its downregulation in the most common types of human carcinomas point to Sef as an important link between inflammation and cancer and as a central modulator of the multitude of responses triggered by NF-kB. In spite of this, little is known about how Sef specificity towards NF-κB/RelA:p50 is governed, and Sef structural motifs required for Sef modulation of NF-κB signaling are poorly characterized. By using cultured cells and by developing a cell-free binding system with bacterially expressed Sef, we show here that most of Sef ICD is dedicated to inhibiting NF-κB/RelA:p50. Most importantly, our work reveals that in addition to directly binding to NF-κB(p50), Sef physically/independently interacts with IκBα and the IKKβ subunit of the IKK complex. Scaffold proteins that link receptor signaling to the IKK complex [*e.g.* [31]], and other proteins (*e.g.*, Notch-1) that interact with NF-κB and individual components of the IKK complex have been reported [32–34]. To the best of our knowledge, hSef, is the first known protein capable of bringing together the three components of the canonical NF-κB signaling module.

The efficient binding of bacterially expressed Sef to p50, IκBα and IKKβ *in-vitro*, suggests that post-translational modifications (PTMs) in Sef are not essential for the interaction. This conclusion is consistent with reported observations from others and us that Sef interaction with NF-κB is signal independent [9,11]. Most importantly, the current study revealed two previously unknown Sef regions that are essential for interaction with NF-κB. One resides within the SEFIR domain (residues 356–512), and the other within Sef carboxy-terminal end (residues 713–739), the latter providing an explanation to as why any C-terminal truncation abolished Sef binding to p50 and its inhibitory activity in the context of cytokine signaling. Each of the newly identified regions, similar to the previously published binding site [hSefa^{Y330}, [9]], is

equally essential for Sef modulation of NF-κB/RelA:p50 as deletion of each region abrogated Sef binding to p50 and Sef inhibition of cytokine-dependent nuclear translocation. Since these three sites are discontinuously dispersed on hSef primary structure, they are likely to form a binding pocket in the folded protein.

In IL-17R signaling, the SEFIR domain can participate in homotypic and heterotypic interactions, and can also mediate IL-17RA signaling independent of classic TIR adaptors such as MyD88 [35–37]. In our hands, when expressed alone in cells, the SEFIR domain of Sef was also capable of homodimerization (Supplementary Fig. S1). In view of this finding and Yang et al., report that deletion of Sef ICD impaired Sef oligomerization [25], it was rather surprising that SEFIR domain deletion was without an effect on Sef homotypic interaction (see Fig. 2). One plausible explanation may be that Sef ICD harbors more than one homodimerization domain, and that the SEFIR domain plays a secondary role in Sef homotypic interactions but primary role in Sef heterotypic interactions [current study, and [12]]. In the context of GF signaling, Torii et al., found that truncation of residues 356–739, which removes the entire SEFIR domain, abolishes Sef inhibition of FGF whereas truncation that spares box 1 of Sef SEFIR (aa 395–739) retained inhibitory activity [22]. Contrary to this report, we found that Sef mutant lacking the entire SEFIR domain (aa 356–512) efficiently inhibits FGF signaling (Fig. 1C). This discrepancy may be due to effect of C-terminal truncation of residues 356–739 on Sef stability. This notion is supported by the current findings that large C-terminal truncations within Sef ICD result in lower expression levels of the mutant proteins in cells as compared to those of WT Sef and SefΔSEFIR (see Fig. 1B, 2E, and 2F).

Unlike p50, IκBα bound all hSef mutants and except for the Y330F mutant, all displayed slight but a similarly reduced affinity to Sef suggesting that IκBα/Sef share an extended binding interface. IκBα possesses six ankyrin repeats, which participate in binding to p50/p65 in such a way that the interacting residues are discontinuously dispersed on the protein [38,39]. A similar situation could exist for IκBα interaction with hSef and may explain the similar reduction in binding to IκBα observed for the Sef mutants. Resolution of the 3D-structure of Sef and the components of the NF-κB signaling module is likely to add valuable information in this direction. Our ability to successfully express large amounts of biologically active hSef in bacteria should facilitate this task.

The panel of mutants we generated, with no exception, retained parental binding affinity to IKKβ. These findings locate Sef binding site to IKKβ within residues 321–395 in Sef ICD. Interaction site with IKKβ, however, does not overlap with the binding site to p50 as the SefY330F mutant displayed WT affinity towards IKKβ. Importantly, these findings support the conclusion that lack of binding to p50 is not due to improper folding of the mutant proteins. The IKK complex exists as a hetero-trimeric complex containing two catalytic subunits (IKKα; IKKβ) and a regulatory subunit (NEMO/IKKγ), but genetic evidence also suggest that a complex containing either kinase is functional [40]. While IL-1 can activate NF-κB cells lacking either IKKα or IKKβ, the latter is indispensable for TNF-induced NF-κB activation [40]. Sef binding to IKKβ explains why Sef can inhibit both IL-1 and TNFα induced NF-κB activation. Since IKKβ activity depends on interaction with IKKγ [7,41], it appears that Sef can recruit either the hetero-trimeric IKK complex (IKKαβγ) or the IKK$\beta_2\gamma$ complex through its direct binding to IKKβ. In support with the first possibility is our previous finding that Sef inhibits NF-κB/RelA:p50 activation in HeLa cells expressing constitutively active IKKα [9]. Apparently, interaction with Sef does not interfere with the IKK complex activity as Sef has no effect on IκBα degradation in response to either IL-1 or TNFα [current data, and [9]]. Furthermore, co-expression assays combined with Co-IP experiments indicate that IKKβ and Sef associate in both unstimulated as well as IL-1 stimulated cells [current data, and [9]]. Existence of additional scaffold proteins which share with Sef the capacity to interact with IKK but recognize different IκB/NF-κB combinations might be plausible and may explain the findings by Hielker et al., who co-purified various IκB isoforms, Rel family members, and the precursor forms of p50/p52 along with IKK [30].

Scaffold proteins organize subsets of proteins in space and time, thus determining the specificity of information flow in signaling networks [42]. Binding of hSef to three components of the NF-κB signaling module could fulfill several roles. As IKK preferentially phosphorylates NF-κB–bound IκB proteins [43], one role could be to expedite IκBα phosphorylation and subsequent NF-κB nuclear translocation. At the same time, hSef provides a braking system that fine-tunes the NF-κB response. This idea is supported by the fact that Sef targeting in different cell types results in a substantial increase in NF-κB nuclear translocation and hyper activation of NF-κB transcriptional activity [current data, and [9,19]] as well as low to mild inflammation in many organs of Sef null mice (http://www.mmrrc.org/strains/32387/032387.html). Since prototypical hSef is known to localize to early endosomes [9,22,44], another potential role of hSef scaffolding function might be to bring the components of the NF-κB signaling module in close proximity to Toll-like receptors (TLR), some of which are known to signal from this subcellular compartment [45]. This should assist in efficient TLR-mediated activation of NF-κB and enable an efficient attenuation of the signal by a negative feedback mechanism. Such a functional role for hSef could be relevant for IL-1R signaling as well, as recent studies have shown that IL-1R endocytosis upregulates NF-κB activity [46,47]. However, Sef action is not restricted to the endosomal compartment as the soluble cytoplasmic hSef isoform (hSef-b) also inhibits IL-1 and TNFα induced NF-κB/RelA:p50 activation in prostate carcinoma cells [18], and as shown here, soluble Sef ICD also inhibited IL-1- induced NF-κB activation. Another attractive role for hSef could be determining pathway specificity, a known role for many scaffold proteins [42]. For example, hSef binding to IKKβ/IκBα/NF-κB could serve as a mechanism to preclude hSef from binding to p50 homodimers as hSef interacts with NF-κB through p50 [9]. Binding of hSef to p50 homodimers would convert hSef from an inhibitor to a stimulator of the NF-κB response because p50 homodimers generally act as repressors [7]. NF-κB/RelA:p50 can also bind to NF-κB_2 (p100), that functions as an IκB-like protein, and be activated by non-canonical stimuli [48]. Since this activation requires IKKα with which Sef does not interact, it is unlikely that Sef can modulate RelA:p50 by non-canonical stimuli. However, hSef could potentially act to minimize interaction of NF-κB/RelA:p50 with p100 in order to prevent inappropriate expression of inflammatory genes by non-canonical stimuli. A model explaining the basis for Sef specificity towards inhibiting inflammatory cytokine-induction of NF-κB/RelA:p50 is depicted in Fig. 7.

In summary, this study provides new insights into structure-function relation of Sef protein and the molecular mechanism by which Sef serves to limit classical NF-κB transcriptional activity. Our results further highlight the growing complexity of NF-κB signaling and its intricate regulation. The availability of biologically active bacterially expressed Sef should greatly facilitate future resolution of Sef 3D-structure in complex with its binding partners. We hope this will allow to further study the role of Sef in the cross-talk between inflammation and cancer, bearing future opportunities for developing therapy for these diseases.

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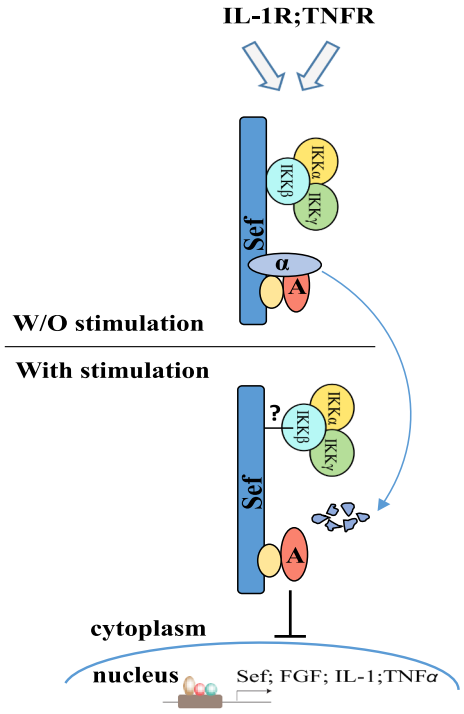

Fig. 7. A revised model for Sef interaction with the NF-κB/RelA:p50 signaling module.
According to this model Sef homodimer either in a soluble cytoplasmic form or membrane bound form sequesters the components of the classical NF-κB signaling module *via* direct binding to the IKKβ subunit, IκBα (α), and p50 subunit (orange ellipse). Sef can recruit either of the trimeric complexes: IKKα/β/γ or IKKβ$_2$/γ through its direct binding to IKKβ. Sef recruitment of the signaling module is signal independent and does not require PTMs. Sef interaction with these components determines its specificity towards inhibiting NF-κB/RelA:p50 in response to inflammatory stimuli and in addition assists in keeping the IκBα/p50/p65 complex predominantly in the cytoplasm. Upon stimulation, Sef protein level remains unchanged and, therefore, it attenuates the NF-κB response in spite of stimulus-induced IκBα degradation. The binding of Sef to the IKK complex serves likely to expedite IKK-induced IκBα phosphorylation and subsequent degradation on the one hand, and on the other hand allows Sef to fine-tune NF-κB transcriptional activation. (A) denotes RelA.

Declarations of interest

None

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2019.01.009.

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