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Biochimica et Biophysica Acta 1783 (2008) 941 – 952
BBA Biochimica et Biophysica Acta
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IEX-1 directly interferes with RelA/p65 dependent transactivation and regulation of apoptosis

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Received 8 September 2007; received in revised form 29 November 2007; accepted 12 December 2007
Available online 23 December 2007

Abstract

The early response gene IEX-1 plays a complex role in the regulation of apoptosis. Depending on the cellular context and the apoptotic stimulus, IEX-1 is capable to either enhance or suppress apoptosis. To further dissect the molecular mechanisms involved in the modulation of apoptosis by IEX-1, we analysed the molecular crosstalk between IEX-1 and the NF-κB pathway. Using GST-pulldown assays, a direct interaction of IEX-1 with the C-terminal region of the subunit RelA/p65 harbouring the transactivation domain of the NF-κB transcription factor was shown. This interaction negatively regulates RelA/p65 dependent transactivation as shown by GAL4-and luciferase assay and was confirmed for the endogenous proteins by co-immunoprecipitation experiments. Using deletion constructs, we were able to map the C-terminal region of IEX-1 as the critical determinant of the interaction with RelA/p65. We could further show, that IEX-1 mediated NF-κB inhibition accounts for the reduced expression of the anti-apoptotic NF-κB target genes Bcl-2, Bcl-xL, cIAP1 and cIAP2, thereby sensitizing cells for apoptotic stimuli. Finally, ChIP-assays revealed that IEX-1 associates with the promoter of these genes. Altogether, our findings suggest a critical role of IEX-1 in the NF-κB dependent regulation of apoptotic responses.
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Keywords: NF-κB; apoptosis; Signal transduction; IEX-1; IAP; Chromatin

1. Introduction

The nuclear factor-κB (NF-κB) family of transcription factors serves as an important master switch in the regulation of cell death and differentiation [1]. The members of the Rel-protein family share a sequence motif of approximately 300 amino acids within their N-termini, termed the Rel homology domain (RHD) [2,3]. This domain mediates hetero- or homo-dimerization, DNA binding and interaction with the IκB inhibitory proteins. In contrast to the p50 and p52 isoforms, RelA, RelB and c-Rel contain a transactivation domain (TAD) within their C-termini. Distinct combinations of the subunits and dimer exchange at the promoters of target genes determine the specificity and mode of transcriptional activation [4]. Persistent NF-κB activation has been identified as a pivotal factor in etiopathogenesis of inflammation-induced malignancies. The primary anti-apoptotic mechanism, which is held to be involved in NF-κB-induced oncogenesis is the transcription of anti-apoptotic target genes, including the inhibitor of apoptosis proteins cIAP1 and cIAP2 and Bcl family members [5,6]. NF-κB activation has also been shown to contribute to the development of chemoresistance of several types of tumors. However, the differential influence of basal or inducible NF-κB activity on chemoresistance is still poorly defined [7–10]. Although NF-κB dependent cellular programs are preferentially associated with suppression of apoptosis, under certain circumstances NF-κB signaling may exert pro-apoptotic functions as well [11–14].

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doi:10.1016/j.bbamcr.2007.12.010

In unstimulated cells, NF-κB is retained in the cytoplasm by a group of inhibitory proteins designated as inhibitor of κB (IκB) and thus prevented from activating transcription. Upon stimulation by a wide range of stimuli, including death receptor ligands and chemotherapeutic drugs [15,16], the IκB proteins are subject of rapid phosphorylation by the IκB kinase complex (IKK) followed by their polyubiquitination and subsequent degradation by the 26S-proteasome. The dissociation of the IκB proteins leads to an unmasking of a nuclear localization signal, which allows the NF-κB dimers to translocate to the nucleus and regulate the transcription of target genes.

In addition to these regulatory steps, several other post-translational modifications, primarily of the RelA subunit, are able to enhance or to suppress NF-κB dependent gene expression [2]. These modifications include reversible acetylation [17] and phosphorylation [18]. NF-κB-dependent gene expression is also modulated through interaction with histone deacetylase (HDAC) enzymes [17,19,20]. These HDAC proteins function to negatively regulate NF-κB transcriptional activity by deacetylating the N-terminal tails of the core histones as well as by deacetylating the p50 and p65 subunits of NF-κB. Other proteins, like the DEK proto-oncogene [21] and the COMMD family of proteins [22], have been shown to inhibit NF-κB dependent signalling by a physical interaction with RelA/p65 and thereby affecting the association of NF-κB with chromatin. These data indicate that regulation of NF-κB activity through interaction with transcriptional corepressor proteins plays an important role in the overall control of NF-κB activity and apoptosis.

One of the NF-κB target genes involved in the regulation of apoptosis is the immediate early gene X-1 (IEX-1). This gene-also referred to as IER3, p22/PRG1, DIF2- is induced by various pro-apoptotic stimuli and its promoter is controlled by transcription factors such as NF-κB, p53 and retinoic acid [23,24]. Several reports indicated that IEX-1 is involved in the regulation of apoptosis but with contradicting results regarding the pro-apoptotic [24–32] or anti-apoptotic [11,12,23,29,33–37] properties of IEX-1. A cell type and stimulus dependency for the differential outcome of IEX-1 expression has been proposed but the exact cellular mechanisms involved still remain unclear. From recent studies, evidence accumulated that the pro-apoptotic effect of IEX-1 depends on a functional nuclear localisation signal (NLS) [26,29], and mutants lacking this domain are unable to promote apoptosis in HeLa cells under etoposide and UV treatment [26].

In the present study, we further delineate the mechanisms involved in the effects of IEX-1 on NF-κB activation and cell death. We show that the C-terminal regions of IEX-1 and RelA/p65 directly interact with each other. Whilst this interaction seems to be dispensible of the nuclear localization of IEX-1, the capacity of IEX-1 to block NF-κB activation and hence to augment apoptosis by interfering with the expression of anti-apoptotic target genes – i.e. Bcl-2, Bcl-xL, cIAP1 and cIAP2 – requires its translocation into the nucleus. Since IEX-1 binds to the promoters of these genes, IEX-1 might function as a transcriptional corepressor by interacting with RelA/p65 on the level of chromatin. These findings indicate that IEX-1 is involved in the shaping of NF-κB apoptotic responses in the nucleus.

2. Material and methods

2.1. Materials

Cell culture medium was purchased from PAA-Labs (Linz, Austria), fetal bovine serum from Seromed (Berlin, Germany), Etoposide from Bristol Myers Squibb (München, Germany), killer-TRAIL from Axxora/Alexis (Grünberg, Germany), recombinant TNF-α from Sigma. IEX-1 antibody, monoclonal and polyclonal RelA/p65, RelB and c-Rel antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Generation of rabbit polyclonal IEX-1 antibody was previously described [11]. Mouse monoclonal M2 antibody was fom Stratagene. cIAP1 and cIAP2 antibody were from R&D Systems (Wiesbaden, Germany). Bcl-2 antibody was from Cell Signaling (Danvers, USA) and Bcl-xL antibody was from Calbiochem (Darmstadt, Germany).

2.2. Cell culture

HeLa and PT45-P1 cells were cultured in RPMI 1640 supplemented with 1% (w/v) glutamine, 10% (w/v) fetal bovine serum, and gentamicin (0.1 mg/ml). HEK293 cells were cultered in DMEM low glucose medium supplemented with 1% (w/v) glutamine and 10% (v/v) fetal calf serum. Cells were split twice a week and incubated at 37 °C with 5% CO_2 at 85% humidity.

2.3. Generation of flag fusion protein expression vectors

PCR derived amplicons of the IEX-1 full length cDNA (5′-primer: *ggatcc*tgtcactctcgcagctgcca; 3′primer: *gtcgac*ttagaaggcggccgggtgtt) and truncated IEX-1 variants (5′ΔN25-primer: *GGATCC*CGGCGGGGCTCCGGT; 3′ΔC25-primer: *GTCGAC*CTAGAGGACGGGGGACACA) were first TA-cloned into pCR2.1 (Invitrogen; Karlsruhe, Germany) and then via BamHI/SalI restriction sites into the Flag-expression vector pcDNA-Flag2B (Stratagene, Mannheim, Germany). Using primers for the generation of an amplification-created MluI restriction site, an IEX-1 construct was produced lacking the putative nuclear localization sequence (HRKR, pos.58–61). After cloning into pcDNA-Flag2B via BamHI/XhoI restriction sites all constructs were subcloned into pCDNA3.1 in order to get identical expression vectors and constructs were checked by automated DNA-sequencing (3730xL DNA Analyzer, Applied Biosystems, Foster City, USA).

2.4. Cell transfection

Cells (25,000/well), plated directly in 12-well plates, were grown overnight and then incubated with 1.0 ml of OptiMEM I (Invitrogen). Transfection (1 µg of plasmid) was done under serum-free conditions for 6 h using ExGen500 (Fermentas) according to the manufacturer's protocol. Further treatment was usually commenced the next day. For immunoprecipitation, cells were seeded at a density of 750,000 cells/plate (9 cm) and submitted to the same transfection procedure as described above, adjusted to 10 µg of plasmid and higher volumes. Plasmids used were: pcDNA3.1-IEX-1, pcDNA3.1 (control), pcDNA3.1-Flag-IEX-1, pcDNA3.1-Flag-ΔNLS-IEX-1, pcDNA3.1-Flag-ΔN25-IEX-1, pcDNA3.1-Flag-ΔC25-IEX-1.

For knock down of IEX-1, cells were seeded into 12 well plates (2×10^5 cells/well) and grown overnight, then transfection with 12 µl/well RNAiFect reagent (Qiagen) and 2 µg/well of either Stealth negative control siRNA (Invitrogen) or Stealth IEX-1 siRNA (Invitrogen) was performed. After overnight transfection, cells were used for indicated assays. For knock down of RelA/p65, cells were seeded into 12 well plates (2×10^5 cells/well) and grown overnight, then transfection with 12 µl/well RNAiFect reagent (Qiagen) and 2 µg/well of either Stealth negative control siRNA (Invitrogen) or Stealth RelA/p65 siRNA (Invitrogen) was performed for 48 h.

2.5. Western blotting

Cellular lysates were prepared as described previously [12,26]. Samples were heated (95 °C) for 5 min and submitted to SDS-PAGE using 4–20% Tris–glycine gradient gels (Cambrex). After electroblotting onto polyvinylidene difluoride membrane, blots were blocked for 1 h with 3% nonfat milk powder, 0.1% bovine serum albumin in TBS (Blotto) at room temperature. Blots were exposed to the

primary antibodies diluted in Blotto overnight at 4 °C. After three washes with Blotto, blots were exposed to the appropriate horseradish peroxidase-conjugated secondary antibody diluted in Blotto for 1 h. Blots were then washed three times in TBS, developed with Super-Signal West Dura solution (Pierce), and analyzed with the Chemidoc system (BioRad, Munich., Germany).

2.6. GST pulldown experiments

pGEX4T3-GST-IEX-1 (a generous gift from Dr. F. Porteu, Paris, France), pGEXKT-GST-RelA/p65(1–306), pGEXKT-GST-RelA/p65(304–551) or pGEX4T2-GST were expressed in *Escherichia coli* BL21(DE3)pLysS (Invitrogen) and handeled as described [26,38]. For pulldown assays, cell lysates were prepared and precleared with bovine serum albumin–Sepharose 4B. Supernatants were then incubated with GST-IEX-1, GST-RelA/p65(1–306), GST-RelA/p65(304–551) or GST coupled to glutathione–Sepharose, or with glutathione–Sepharose 4B only. Further handling and Western blot analysis were carried out as described above.

2.7. Immunoprecipitation

HeLa cells seeded in 10-ml dishes at 4×10^6 cells/dish and grown overnight were transfected with the indicated FLAG-IEX-1 constructs or with the empty vector. The next day, cells were washed with ice-cold PBS, scraped off the plates, and pelleted by centrifugation. Cells were lysed in 300 µl of buffer containing 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 30 mM NaF, and a mixture protease and phosphatase inhibitors (protease inhibitor mixture and PICII, Sigma) by vortexing three times for 45 s. After preclearing of lysate by incubation with protein A/G-agarose (Santa Cruz Biotechnology) and centrifugation, FLAG-IEX-1 complexes and RelA/p65 compexes were precipitated using 2 µg anti-M2 or mouse anti-p65 antibody respectively for 2 h and protein A/G-agarose for 1 h. For precipiation of endogenous IEX-1 a polyclonal rabbit anti-IEX-1 antibody was used [11]. Precipitates were washed three times, denatured by boiling in 2x Laemmli sample buffer, and separated by SDS-PAGE. After transfer onto polyvinylidene difluoride membrane, samples were probed for precipitated IEX-1 constructs using mouse anti-M2 or goat anti-IEX-1 antibody and for RelA/p65 complexes using rabbit anti-p65 antibody, after stripping, for co-precipitated IEX-1 constructs or RelA/p65 mouse anti-M2 or rabbit anti-p65 antibodies were used.

2.8. Caspase assay

For the detection of caspase-3/-7 activity, a homogeneous luminescent assay was performed according to the manufacturer's (Promega) instructions.

2.9. FITC-AnnexinV/PI apoptosis assay

Annexin V and propidium iodide labeling was performed according to the manufacturer's (ApoAlert, BD-Clontech; Heidelberg, FRG) instructions. Flow cytometry was carried out on a Galaxy fluorescence flow cytometer (Dako,

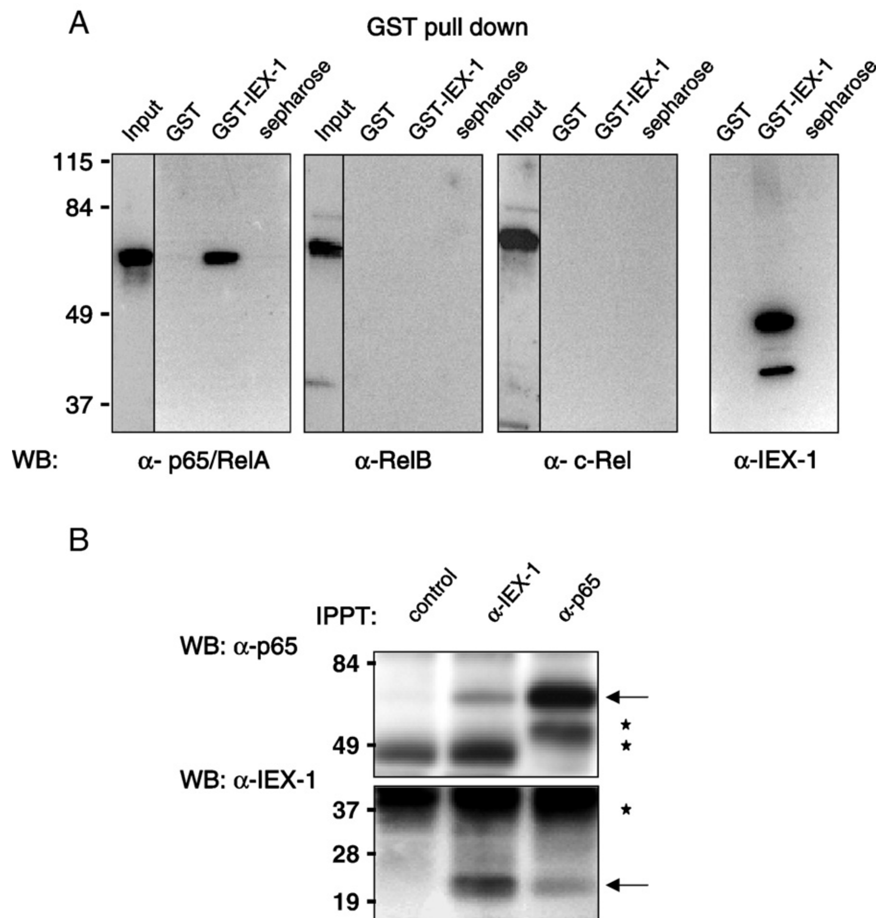

Fig. 1. IEX-1 interacts with the RelA/p65 subunit of the Rel-family. (A) Cellular extracts of HeLa cells were submitted to GST pulldown assays using GST-IEX-1 bound to sepharose (GST-IEX-1). For specificity control, pulldown assays with GST bound to sepharose (GST) or glutathione–Sepharose (sepharose) alone were conducted in parallel. Pulldown samples were analysed by Western blot using the following antibodies: anti-RelA/p65, anti-RelB, anti-c-Rel and anti-IEX-1. (B) HeLa cells were treated with with 10 ng/ml TNF-α (1 h) for induction of endogenous IEX-1 protein. Then, cell lysates were immunoprecipitated (IP) with either an anti-IEX-1 antibody, an anti-p65 antibody or IgG as control. Proteins were visualized by Western blot (WB) using either anti-p65 (upper panel) or anti-IEX-1 (lower panel). Nonspecific bands corresponding to immunglobulin heavy and light chains are indicated with asterisks. Representative results for three independent experiments are shown.

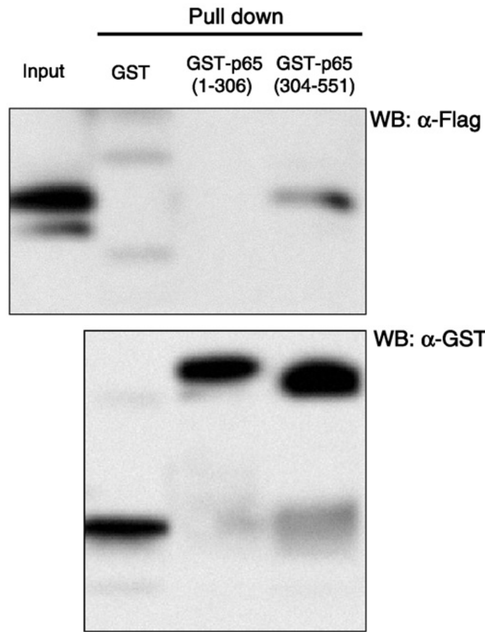

Fig. 2. IEX-1 interacts with the C-terminus of RelA/p65. Cellular extracts of HeLa cells transfected with IEX-1-Flag were submitted to GST pulldown assays using either GST bound to sepharose (GST), GST-p65 (1–306) bound to sepharose (GST-p65(1–306)) or GST-p65 (304–551) bound to sepharose (GST-p65(304–551)). Western blot analysis of pulldown samples were performed using antibodies against IEX-1 (upper panel) and GST (lower panel). A sample of input material is shown in the left lane. A representative result for three independent experiments is shown.

Hamburg, FRG) and data were processed by FloMax software (Dako). Those cells exhibiting high staining with AnnexinV were regarded as being apoptotic.

2.10. Luciferase assay and GAL4 reporter assay

Cells were seeded in a 96-well plate at a density of 1.5×10^4 (HeLa) or 8×10^4 (HEK293) per well. The next day, cells were transfected with 35 ng of each flag tagged IEX-1 construct, or pcDNA3.1 empty vector (control) together with 12 ng of 3× NF-κB-luciferase reporter plasmid (BD-Clontech) and 3 ng of pRL-TK *Renilla* reporter plasmid (Promega, Madison, WI) for normalization of transfection efficiency and cell viability. After 18 h of incubation, medium was exchanged by medium containing TNF-α (10 ng/ml) or pure medium. After 18-h incubation, cells were washed in PBS and lysed in 1× passive lysis buffer (Promega). Dual luciferase assay was performed using a dual luciferase reporter assay system (Promega) and a Genios Pro luminometer (Tecan, Crailsheim, Germany). GAL4 reporter assays were performed by co-transfecting 0.5 μg of a pFA-CMV-p65 plasmid (kind gift of Lienhard Schmitz, Giessen, Germany), containing the Gal4 DNA-binding domain and its nuclear localization sequence fused to the p65 TAD, and 50 ng/ml of a pFR-Luc firefly reporter construct (Stratagene) under the control of a synthetic reporter bearing 5 yeast GAL4-binding sites. Results are expressed as multiples of the level of activation obtained with the vector control.

2.11. RNA isolation and RT-PCR

Total RNA was isolated using the RNeasy Kit from Qiagen (Hilden, Germany) and reverse-transcribed into single-stranded cDNA, as described previously [11]. 2 μl of cDNA were subjected to PCR (95 °C/3 min; 95 °C/1 min, 55 °C/1 min, 72 °C/40 s for 16–24 cycles followed by a final extension at 72 °C for 10 min) with gene-specific primers at a final concentration of 0.5 μM: IEX-1(forward) 5′-CTC CGC TCG GCT CAC CAT-3′/IEX-1 (reverse) 5′-AGT GCG GGG AGT CCA GTT AGA AGG-3′ or β-actin for control using primers from BD Biosciences Clontech (via Takara, Köln, Germany) or for OAS-1 as control using primers from Invitrogen. 10 μl of the PCR reaction were subjected to an 8% polyacrylamide gel electrophoresis and visualized by ethidium bromide staining.

2.12. Chromatin immunoprecipitation (ChIP) assays

ChIP analysis was performed essentially as described [39]. Primers used for PCR were: Bcl-2: forward, 5-TGGAGCGCTGAGTTCTGCAT-3 and reverse, 5-GAC-AGGTGACTCTGCACCGTTTTA-3; Bcl-xL: forward, 5-CCTCTCCCGA-CCTGTGATAC-3 and reverse, 5-CCCCCGTCTTCTCCGAAATG-3; cIAP1: forward, 5-CAGAGAGAAGCAAGCATCGGAT-3 and reverse, 5-ATCC-GATGCTTGCTTCTCTCTG-3 cIAP2: forward, 5-GTGTGTGTGGTT ATTACCGC-3 and reverse, 5-AGCAAGGACAAGCCCAGTCT-3; GAPDH: forward, 5-AG CGCAGGCCTCAAGACCTT-3 and reverse, 5-AAGAA-GATGCGGCTGACTGT-3. Amplified DNA was visualized by agarose gel electrophoresis and visualized by ethidium bromide staining.

2.13. Densitometry

Bands were quantified by QUANTITY ONE software (BioRad).

2.14. Statistics

Data are presented as mean± s.d. and analyzed by Student's *t*-test. A *p*-value <0.05 (indicated as * in the Figures) was considered as statistically significant.

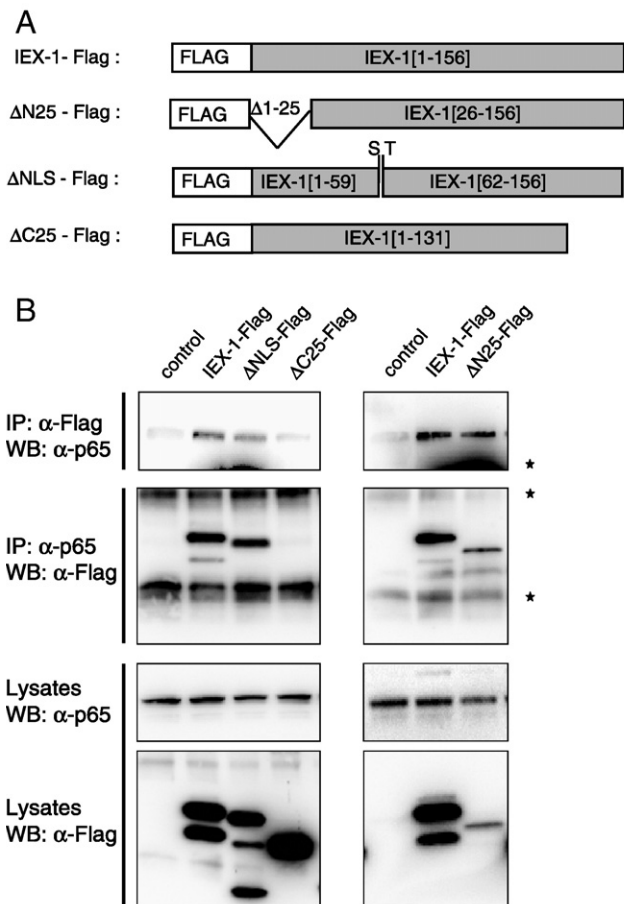

Fig. 3. The C-terminus of IEX-1 is essential for the interaction with RelA/p65. (A) Scheme of the IEX-1-Flag constructs. (B) IEX-1-Flag constructs were transiently transfected into HeLa cells and cell lysates were immunoprecipitated (IP) with either anti-Flag antibody (upper panel) or anti-p65 antibody (2nd panel) and proteins visualized by Western blot (WB) using either anti-p65 (upper panel) or anti-Flag (2nd panel). The input material (lysates) is shown in the lower two panels. Nonspecific bands corresponding to immunoglobulin heavy and light chains are indicated with asterix. A representative result for three independent experiments is shown.

3. Results

3.1. IEX-1 interacts with RelA/p65

To delineate the molecular cross talk between IEX-1 and the NF-κB signalling pathway [12], pull down experiments with an GST-IEX-1 fusion protein were conducted using cellular extracts from HeLa cells and GST-IEX-1 coupled to glutathione–Sepharose. It could be shown that GST-IEX-1 specifically interacts with RelA/p65, as verified by Western blot (Fig. 1A), whereas no other protein of the canonical NF-κB pathway could be detected. Beside IκBα or p50 (data not shown), the other TAD

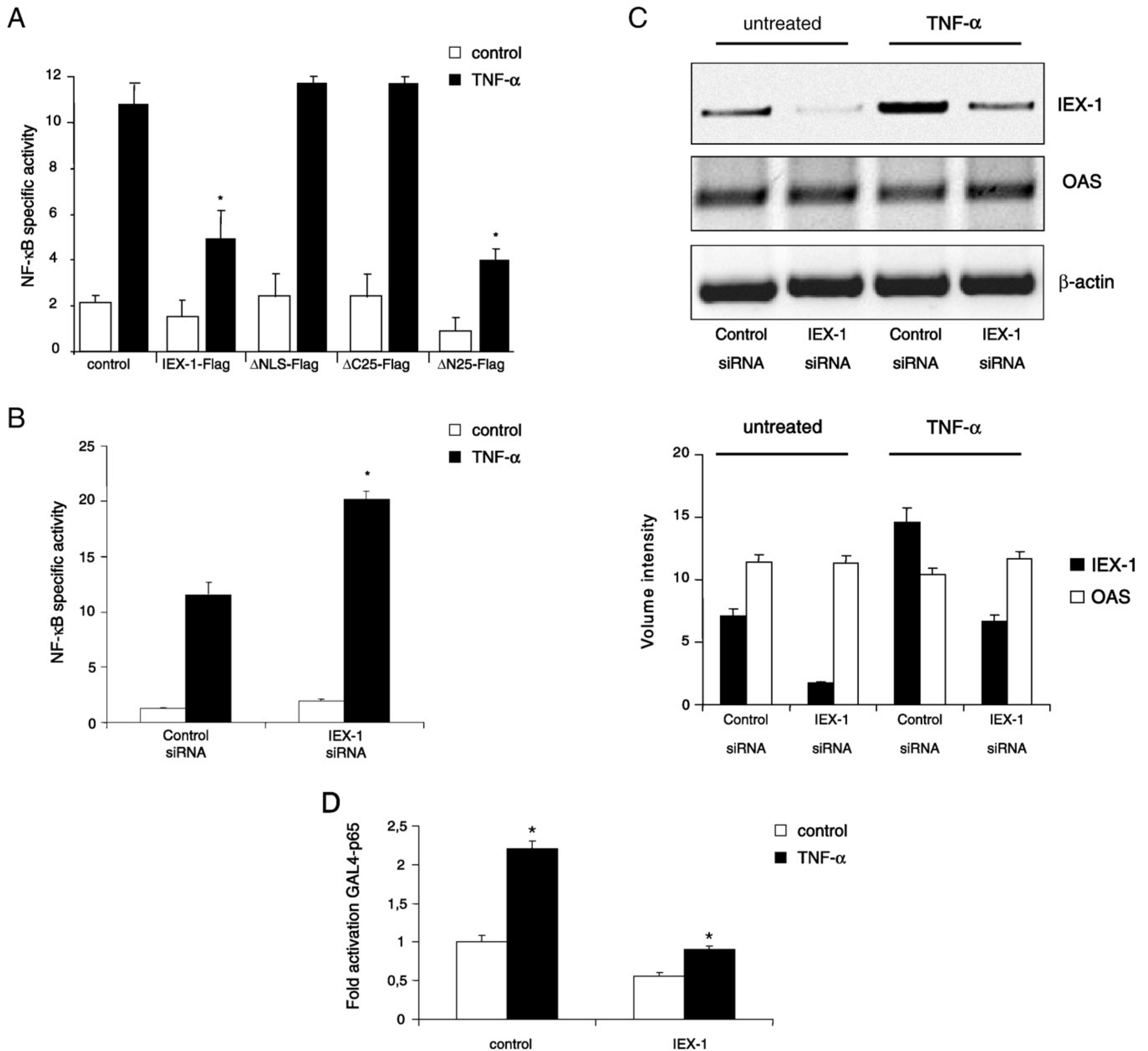

Fig. 4. Interaction of IEX-1 with RelA/p65 mediates NF-κB inhibition. (A) HeLa cells were cotransfected with pcDNA3.1 (control) or the IEX-1-Flag constructs together with pNFκBluc or pGL3 (as control) and with pRLTK. Transfectants were either left untreated or were incubated for 16 h with 10 ng/ml TNF-α. Then, the expression of firefly luciferase and renilla luciferase in total cellular extracts was measured. Firefly luciferase expression was normalized to the amount of renilla luciferase and data represent the NF-κB specific luciferase expression (expressed as pNFκBLuc/pGL3Luc-ratio). Means± s.d. of three independent experiments performed in triplicate are shown. * indicates p-value < 0.05. (B) HeLa cells were cotransfected with control siRNA or IEX-1 siRNA in combination with pNFκBLuc or pGL3 (as control) together with pRLTK followed by incubation for 16 h in the absence or presence of 10 ng/ml TNF-α. Then, expression of firefly luciferase and renilla luciferase was measured. Firefly luciferase expression was normalized to the amount of renilla luciferase and data represent the NF-κB specific luciferase expression (from the pNFκBLuc/pGL3Luc-ratio). Means± s.d. of three independent experiments performed in triplicate are shown, * indicates p-value < 0.05. (C) cDNA from Hela cells transfected with a control siRNA or the siRNA directed against IEX-1 was subjected to RT-PCR analysis for detecting an efficient knock-down of IEX-1 mRNA expression levels. OAS amplification was used to rule out a concomitant induction of an interferon-type off target effect. Amplification of β-actin served as equal loading control. The lower panel shows a graphical representation of the means± s.d. of the mRNA amount as determined by densitometry of four independent experiments. (D) HEK293 cells were transiently transfected with the indicated plasmids and the 5× GAL4-luc reporter plasmid. Extracts were assayed for luciferase activity 48 h after the transfection. Results are expressed as multiples of the level of activation obtained with the vector control. Means± s.d. of three independent experiments performed in triplicate are shown. * indicates p-value < 0.05.

containing NF-κB family members RelB and c-Rel were not detected in GST-IEX-1 pull down experiments (Fig. 1A). In both controls (glutathione–Sepharose alone or GST protein coupled to glutathione–Sepharose), no reactivity for any of the analysed proteins, including RelA/p65, was detected.

In order to verify a direct interaction of the endogenous IEX-1 protein with RelA/p65, HeLa cells were treated with TNF-α (1 h) for the induction of endogenous IEX-1 protein [11] and lysates were immunoprecipitated with IEX-1 or RelA/p65 antibodies, or with IgG as control (Fig. 1B). In IEX-1 immunoprecipitates, RelA/p65 protein, together with the endogenous IEX-1 protein, was detected by Western blotting and, vice versa, the RelA/p65 immunoprecipitated material contained the endogeneous IEX-1 protein. In contrast, neither IEX-1 nor RelA/p65 was detected in IgG control precipitates, thus confirming specificity of the direct interaction of endogenous IEX-1 with RelA/p65.

3.2. The C-terminus of RelA/p65 containing the TAD interacts with IEX-1

The RelA/p65 subunit of NF-κB consists of two main structural regions, the RHD located at the N-terminus and the TAD located at the C-terminus of the molecule. To identify which of these regions of RelA/p65 interacts with IEX-1, GST pulldown experiments with N- or C-terminal RelA/p65 fragments fused to GST were performed with cellular extracts from IEX-1-Flag transfected HeLa cells. As shown in Fig. 2, the C-terminal part of RelA/p65 (amino acids 304–551) harboring the TAD was able to pull down IEX-1-Flag. In contrast, no specific interaction was observed when the N-terminus of RelA/p65 (amino acids 1–306) was used for the pull down experiments. Western blotting with an anti-GST antibody confirmed that comparable amounts of each GST-construct were used for the pull down experiments (lower part of Fig. 2).

3.3. Identification of the RelA/p65-binding region within the IEX-1 protein

To determine which region of IEX-1 binds to RelA/p65, Flag-tagged deletion constructs of IEX-1 were generated lacking 25 amino acids of the N or C-terminus termed ΔN25 and ΔC25 respectively. Furthermore, a mutant lacking the NLS was used, termed ΔNLS (schematic diagram shown in Fig. 3A). Comparable expression of these proteins was confirmed by Western blot analysis (Fig. 3B, lysates). Lysates of HeLa cells transfected with these expression plasmids were immunoprecipitated with a Flag or p65 antibody. Precipitated proteins were analysed by Western blotting, using either Flag antibody to detect the IEX-1 constructs

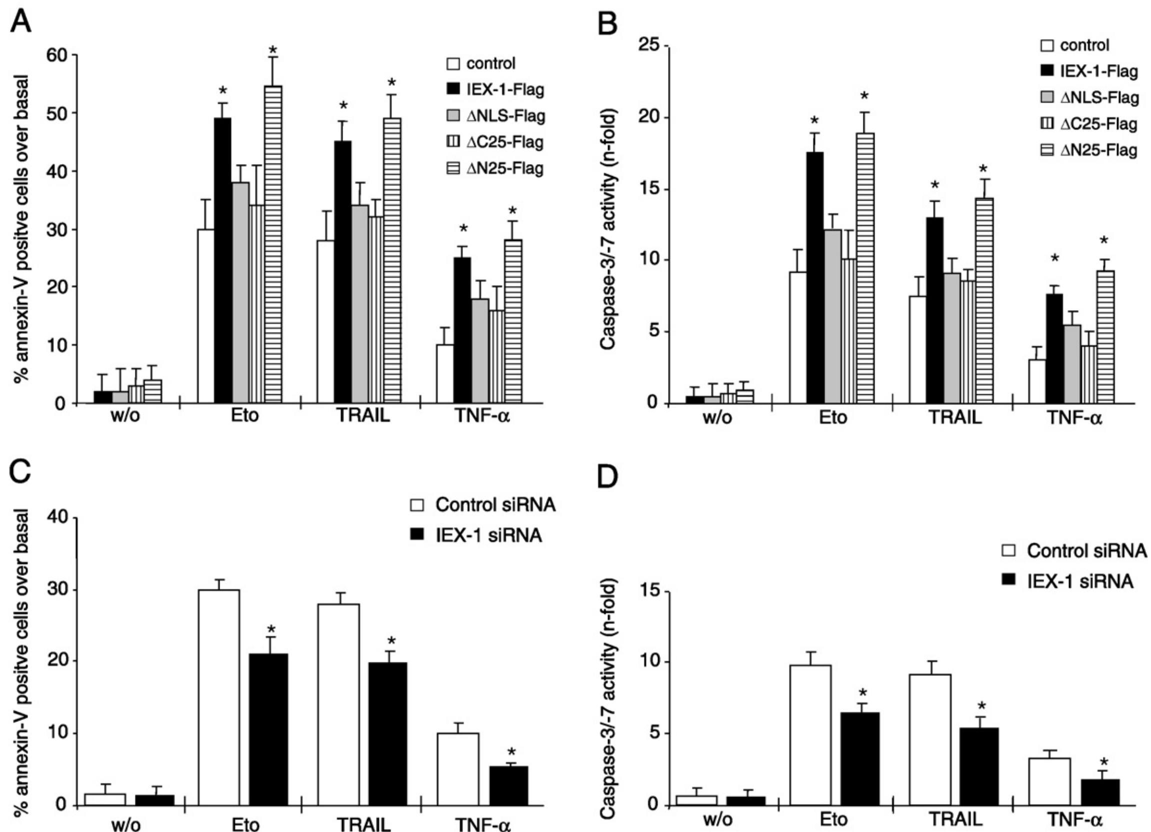

Fig. 5. Interaction of IEX-1 with RelA/p65 mediates sensitization for apoptotic stimuli. HeLa cells transfected with pcDNA3.1 (control) and the IEX-1-Flag constructs as indicated (A,B) or with control siRNA and IEX-1 siRNA (C,D) were exposed to various death stimuli for 24 h: 2 µg/ml etoposide (Eto), 100 ng/ml TRAIL and 10 ng/ml TNF-α. Apoptotic cells were detected by annexin V staining and subsequent fluorescence flow cytometry (A,C) or by a luminescent caspase-3/-7 assay (B, D). Data express the percentage of apoptotic cells of total cell number (A and C) or the n-fold induction of caspase-3/-7 activity compared to untretaed control (B and D). Means± s.d. of five independent experiments are shown, * indicates p-value < 0.05.

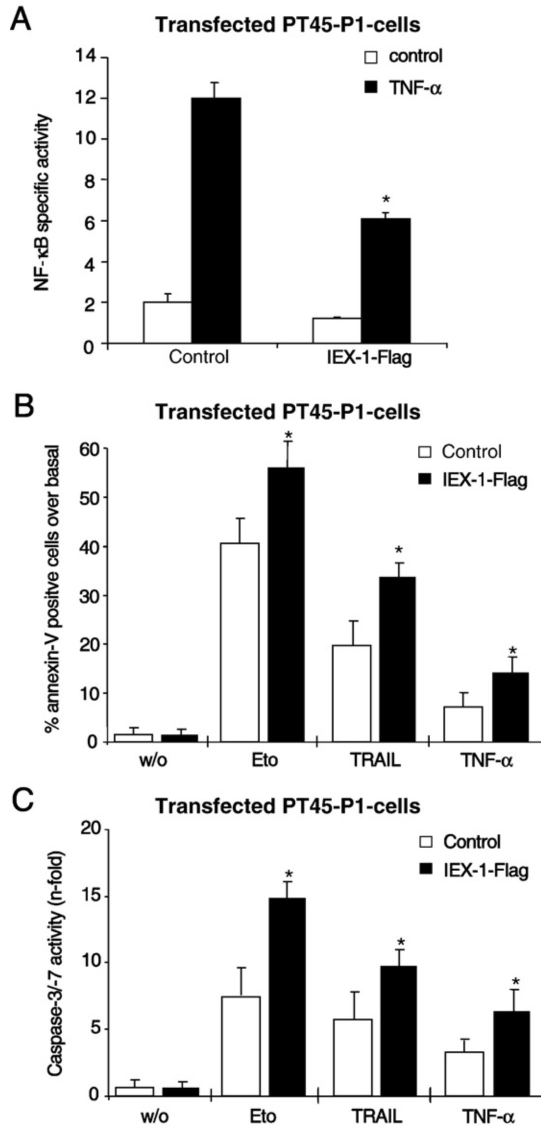

Fig. 6. Repressive Effect of IEX-1 is Not Cell Type-specific. (A) PT45-P1 cells were cotransfected with pcDNA3.1 (control) or IEX-1-Flag together with pNFκBluc or pGL3 (as control) and with pRLTK. Transfectants were either left untreated or were incubated for 16 h with 10 ng/ml TNF-α. Then, the expression of firefly luciferase and renilla luciferase in total cellular extracts was measured. Firefly luciferase expression was normalized to the amount of renilla luciferase and data represent the NF-κB specific luciferase expression (expressed as pNFκBLuc/pGL3Luc-ratio). Means± s.d. of three independent experiments performed in triplicate are shown. * indicates p-value < 0.05. (B, C) PT45-P1 cells transfected with pcDNA3.1 (control) or IEX-1-Flag were exposed to various death stimuli for 24 h: 2 μg/ml etoposide (Eto), 100 ng/ml TRAIL and 10 ng/ml TNF-α. Apoptotic cells were detected by annexin V staining and subsequent fluorescence flow cytometry (B) or by a luminescent caspase-3/-7 assay (C). Data express the percentage of apoptotic cells of total cell number (B) or the n-fold induction of caspase-3/-7 activity compared to untretaed control (C). Means± s.d. of five independent experiments are shown, * indicates p-value < 0.05.

or p65 antibody to detect RelA/p65. As shown in Fig. 3B, full length IEX-1-Flag was capable to coprecipitate endogenous RelA/p65, and ΔNLS-Flag or ΔN25-Flag coprecipitated RelA/p65 to a quite similar extent. Accordingly, Western blotting with a Flag antibody revealed coprecipitation of these IEX-1 constructs together with RelA/p65. In contrast, the ΔC25-Flag was unable to coprecipitate RelA/p65 and, vice versa, RelA/p65 failed to interact with this construct. From this coprecipitation pattern, it can be concluded that the C-terminus of IEX-1 is crucial for the interaction with RelA/p65.

3.4. Nuclear interaction of IEX-1 with RelA/p65 mediates repression of NF-κB signaling

We next analysed whether the interaction of IEX-1 with RelA/p65 accounts for inhibition of NF-κB signaling by IEX-1. Since the TAD of RelA/p65 is located in the C-terminus, NF-κB dependent transactivation was measured in HeLa cells transfected with the IEX-1-Flag constructs. Luciferase assays showed (Fig. 4A) that the full length IEX-1 construct and ΔN25-Flag which already inhibited the basal NF-κB activity significantly reduced the TNF-α induced NF-κB transactivation. Interestingly, not only deletion of the C-terminus of IEX-1 abolished this inhibitory effect, but also the deletion of the NLS (Fig. 4A). This indicates that the nuclear interaction of IEX-1 with RelA/p65 is a prerequisite for its inhibitory effect on NF-κB.

Furthermore, knock down of IEX-1 expression by siRNA significantly amplified TNF-α induced NF-κB activity in HeLa cells as shown by luciferase assay (Fig. 4B). RT-PCR experiments

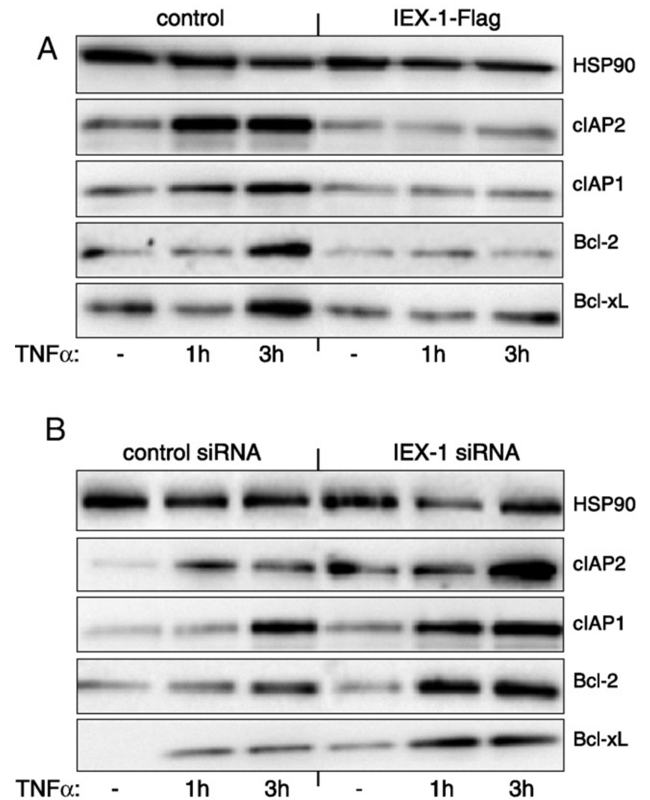

Fig. 7. IEX-1 interferes with the expression of anti-apoptotic NF-κB target genes. HeLa cells transfected with pcDNA3.1 (control) and the IEX-1-Flag construct (A) or with control siRNA and IEX-1 siRNA (B) were exposed to TNF-α for indicated periods. Total protein extracts were submitted to Western blotting for the analysis of Bcl-2, Bcl-xL, cIAP1 and cIAP2 expression, using HSP90 as control. Representative results from three independent experiments are shown.

(Fig. 4C) confirmed that the siRNA directed against IEX-1 is capable to down regulate basal IEX-1 expression and to inhibit TNF-α mediated induction of IEX-1. To exclude an unspecific interferon response mediated by the siRNA [40,41], expression of the 2′,5′-oligoadenylate synthetase (2,5-OAS) was also analysed. As shown in Fig. 4C, the siRNA directed against IEX-1 induced no significant changes in 2,5-OAS mRNA levels as compared with the control siRNA, indicating that the effects mediated by the siRNA were not mediated by off-target effects.

Based on the GST pulldown experiments, our data indicated that IEX-1 represses NF-κB dependent gene expression by interacting with the C-terminal TAD of the RelA/p65 subunit. To confirm this inhibitory interaction, a C-terminal fragment of RelA/p65 (amino acids 286–551) including the TAD and fused to the GAL4 DNA binding region was used for GAL4 assays. As shown in Fig. 4D, cotransfection of GAL4-RelA/p65 together with IEX-1 expression plasmid resulted in a reduction of the basal and TNF-α induced reporter gene expression.

3.5. IEX-1 dependent sensitization for apoptotic stimuli is mediated by NF-κB inhibition

To determine whether the interaction of IEX-1 with RelA/p65 and the resulting inhibition of NF-κB dependent transactivation is required for its effects on cellular survival, HeLa cells were transfected either with flagged IEX-1 and the flagged deletion constructs or with IEX-1 siRNA. Transfected cells were left untreated or were treated for 24 h with either etoposide, TRAIL or TNF-α. Cell death was determined by annexin-V staining followed by fluoresence flow cytometry analysis (Fig. 5A and C) or by measurement of caspase-3/-7 activity using a homogenous luminescent assay (Fig. 5B and D).

As shown in Fig. 5A and B, transfection of Flag-IEX-1 sensitized the cells against all apoptotic stimuli. Compared to empty vector transfected cells, Flag-IEX-1 and ΔN25-Flag expressing cells showed a significant increase in the number of annexin-V positive cells as well as in caspase-3/-7 activity after treatment with etoposide, TRAIL or TNF-α. In contrast, cells expressing ΔC25-Flag did not show an increased apoptosis in comparison with cells transfected with the empty vector and, moreover, transfection with the ΔNLS-Flag also produced no apoptosis sensitizing effect. The sensitization to apoptotic stimuli by IEX-1 was comparable to the effects of siRNA mediated knock down of RelA/p65 expression (supplemental Fig. S1). Conversely, the siRNA mediated knock down of IEX-1 expression significantly decreased the anti-cancer drug or death ligand induced apoptosis, as shown in Fig. 5C and D.

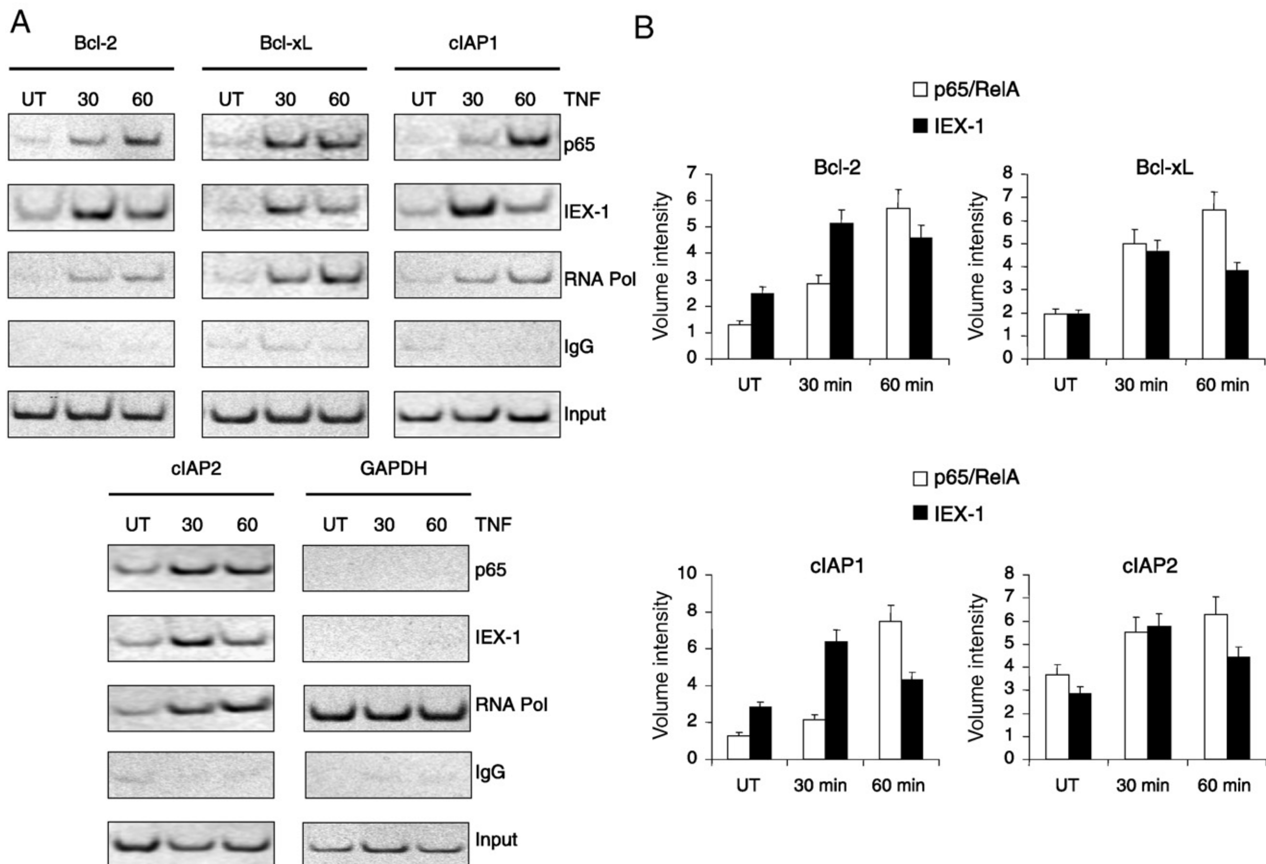

Fig. 8. IEX-1 associates with the NF-κB-regulated promoters of anti-apoptotic genes. ChIP assays were performed on nuclear extracts from HeLa cells either untreated or treated with TNF-α for the indicated times. (A) Immunoprecipitated DNA was subjected to PCR using primers specific to the indicated promoters and amplified DNA was visualized by agarose gel electrophoresis. All experiments were performed a minimum of three times. (B) A graphical representation of the means ± s.d. of the amount of the specific NF-κB/DNA complex as determined by densitometry of three independent experiments.

3.6. Repressive effect of IEX-1 is not cell type-specific

Since the role of NF-κB in the resistance of pancreatic carcinoma cells is well established and since IEX-1 has been shown to be involved in the regulation of proliferation of certain pancreatic cancer cell lines [12,42] similar experiments were also performed in the pancreatic carcinoma cell line PT45-P1. We could show that the ability of IEX-1 to repress RelA/p65-mediated transcription and to sensitize cells for apopototic stimuli is not limited to HeLa cells. PT45-P1 cells were transfected as indicated and assayed for reporter gene activity (Fig. 6). Expression of IEX-1 in PT45-P1 cells caused an inhibition of TNF-α induced RelA/p65-dependent reporter gene expression. Furthermore, analysis of apoptotic cell death by annexin-V staining followed by fluoresence flow cytometry analysis (Fig. 6B) or by measurement of caspase-3/-7 activity (Fig. 6C) revealed that IEX-1 is able to sensitize pancreatic cancer cell lines to death-ligand and chemotherapeutic drug induced cell death. These results confirmed that IEX-1 is able to repress RelA/p65-mediated transcriptional activation and sensitizes cells to apoptotic stimuli and that this effect is not cell type-specific.

3.7. IEX-1 interferes with the expression of anti-apoptotic NF-κB target genes

We next analysed the effects of IEX-1 on the expression of established anti-apoptotic NF-κB target genes in HeLa cells transfected with flagged IEX-1 as well as with IEX-1 siRNA by Western-blot analysis. We could show that overexpression of IEX-1 slightly reduced basal expression of Bcl-2, Bcl-xL, cIAP1 and cIAP2 and significantly inhibited the induced expression of these genes upon TNF-α treatment (Fig. 7A). Conversely, downregulation of IEX-1 through transfection with IEX-1 siRNA led to a higher basal and TNF-α induced expression level of this set of anti-apoptotic NF-κB target genes (Fig. 7B).

3.8. IEX-1 associates with the NF-κB-regulated promoters of anti-apoptotic genes

To determine if IEX-1 is associated with the promoters of these anti-apoptotic NF-κB target genes, ChIP experiments were performed with HeLa cells either left untreated or treated with TNF-α for the indicated periods. As shown in Fig. 8, IEX-1 associated with the promoters of Bcl-2, Bcl-xL, cIAP1 and cIAP2 in untreated cells. After treatment with TNF-α for 30 min, an increased association of IEX-1 with the promoters of these genes could be noted that paralleled the kinetics of RelA/p65 (Fig. 8). In contrast to RelA/p65, IEX-1 began to dissociate from the promoters after 60 min of TNF-α treatment. The association of RNA Pol II with these promoters was used as a positive control and as predicted, neither IEX-1 nor RelA/p65 were associated with the constitutively expressed GAPDH promoter (Fig. 8). In accordance with the observed NF-κB inhibition by IEX-1, overexpression of IEX-1 also inhibited the recruitment of RelA/p65 to the anti-apoptotic NF-κB target genes promoters (data not shown).

4. Discussion

The pleiotropic effects of IEX-1 on cell death and differentiation critically depend on the cellular context [23–30,32–37,11,12,43,44], yet the mechanisms involved are poorly understood. From our present study, we now propose a molecular mechanism of how IEX-1 inhibits activation of the transcription factor NF-κB and thereby compromizes anti-apoptotic protection. We could demonstrate that IEX-1 directly interacts with the NF-κB subunit RelA/p65, along with its functional repression, whereas IEX-1 does not directly bind to any other Rel-family member or known component of the canonical NF-κB signaling pathway in our assays. In support of this repressive effect of IEX-1 on RelA/p65 dependent transactivation, we found that IEX-1 specifically interacts with the C-terminal domain of RelA/p65, containing the transactivation domain (TAD) but not with the N-terminus of the gene including the Rel homology domain (RHD), which is shared by all Rel-family members. Such an exclusive binding to the TAD of RelA/p65 has been also reported for nuclear cofactors like p300 and inhibitors like IκBepsilon [45–50]. Furthermore, we demonstrate that the C-terminus of IEX-1 is crucial for the complexation with RelA/p65. Consistent with an interaction of IEX-1 with the C-terminal TAD domain of RelA/p65, IEX-1 was able to repress GAL4-RelA/p65 (amino acids 286–551) dependent transcription.

Addressing the controversy about the effects of IEX-1 on apoptotic signaling and to further dissect the involved molecular mechanisms, we systematically investigated the impact of truncation and deletion variants of IEX-1 on pro-and anti-apoptotic signaling pathways.

Even though recent reports showed an implication of the N-terminal part of IEX-1 in the regulation of the ERK pathway and IEX-1 as an anti-apoptotic downstream effector of ERK [33–36], there is evidence for pro-apoptotic functions of this gene mediated by negative regulation of the NF-κB and Akt pathways [12,28]. Besides the proposed anti-apoptotic function depending on the N-terminus of IEX-1 [29,33,35], nuclear localization of IEX-1 is supposed to be crucial for its pro-apoptotic effects [26,29]. Strikingly, whilst the C-terminus of IEX-1 has recently been reported to be dispensible from the triggering effect on apoptosis [29], we clearly showed that the sensitizing effect of IEX-1 on death ligand or anti-cancer drug induced apoptosis requires the twenty five C-terminal amino acids. The reason for this discrepancy may be given by the fact that in the above study quite distinct apoptotic conditions were chosen. Neither combined CHX/TNFα nor serum withdrawal induced cell death are affected by RelA/p65 mediated transactivation as it is the case during death ligand (without CHX) and anti-cancer drug induced apoptosis. Obviously, this particular pro-apoptotic action of IEX-1 which relies on the interference with RelA/p65 depends on its intact C-terminus, whereas the effect of IEX-1 on CHX/TNFα and serum deprivation induced apoptosis exhibits distinct structural requirements.

Our results indicated that the capability of IEX-1 to interact with RelA/p65 is essential for the observed NF-κB inhibiting and pro-apoptotic properties of IEX-1. Interestingly, deletion of the NLS in the IEX-1 protein sequence did not affect the

binding of IEX-1 to RelA/p65, but abrogated its inhibitory effect on NF-κB activation and the subsequent sensitization to apoptotic stimuli. By analysing the expression of anti-apoptotic NF-κB target genes, we were able to show that IEX-1 interferes with the basal and the TNF-α induced expression of the anti-apoptotic NF-κB target genes Bcl-2, Bcl-xL, cIAP1 and cIAP2. The Bcl-2 family of proteins and the IAP family of proteins have been shown to prevent apoptosis induction in part via direct inhibition of caspase-3,-7, and -9 [51]. Upregulation of Bcl and cIAP expression in mammalian cells is in part achieved by NF-κB mediated transactivation and has been suggested to participate in the anti-apoptotic pathways elicited by death ligands and chemotherapeutic drugs [1,6,51].

From our data, it can be assumed that IEX-1 may function as a transcriptional corepressor protein affecting both basal and inducible NF-κB-dependent transcription through a direct interaction with the C-terminal TAD of the RelA/p65 subunit. This assumption is supported by ChIP assays demonstrating that, during TNF-α treatment, IEX-1 is initally recruited to the promoters of Bcl-2, Bcl-xL, cIAP1 and cIAP2 exhibiting the same kinetics like RelA/p65, but IEX-1 dissociates with faster kinetics from these promoters than RelA/p65. In addition to the moderate amount of IEX-1 already bound under basal conditions, more IEX-1 is recruited to these promoters after 30 min of TNF-α treatment. Thereby, IEX-1 could not only repress basal expression of Bcl-2, Bcl-xL, cIAP1 and cIAP2, but also contributes to regulation of their inducible expression. Accordingly, IEX-1 knock down leads to an enhanced basal and TNF-α-induced expression of this subset of anti-apoptotic NF-κB target genes. In fact, several corepressors, including HDACs, SIRT1, SMRT and DEK, are associated in unstimulated cells with the promoters of NF-κB target genes and remain at the promoter in response to TNF-α [18,20,21,52]. Therefore, it is likely that many different corepressors are associated with promoters even under activation conditions, implying that these proteins function during activation, possibly helping to maintain a proper level of transcription and to counteract the activity of coactivators.

The precise mechanism by which IEX-1 represses NF-κB activity is not yet known. NF-κB dependent signaling is regulated at several cytoplasmatic steps [16,53] mainly involving phosphorylation dependent proteasomal degradation of the IκBs. In addition to the reported effects of IEX-1 on the activity of the 26S proteasome [12,25], this study indicates a novel function of IEX-1 by physical interaction with RelA/p65. The fact that the direct interaction of IEX-1 with RelA/p65 leads to inhibition of NF-κB dependent signaling in the nucleus indicates that this gene might be instrumental in explaining some of the context dependent effects reported [11,26,29,33]. At this, IEX-1 could sterically compete with co-activators for the same binding site on RelA/p65 thereby inhibiting RelA/p65 dependent transactivation, as reported for SNIP1 [38], or IEX-1 could interfere with post-translational modification of RelA/p65, as indicated by the recent observation that IEX-1 interferes with nuclear phosphorylation of RelA/p65 on Ser 536 [12]. RelA is also modified by acetylation at key lysine residues. Site-specific acetylation controls different biological functions of NF-κB including DNA binding, transcriptional activity and assembly with IκB-α. Some histone deacetylases like SIRT1 [19] have been shown to inhibit basal as well as inducible NF-κB activation by directly interacting and interfering with the acetylation of RelA/p65. Furthermore, corepressor like BMRS1 that mediates recruitment of HDAC1 to RelA/p65 inhibits NF-κB activity and thereby sensitizes cells for apoptotic stimuli in a similar manner as IEX-1 [54]. Such a post-translational modification of transcription factors through IEX-1 might also explain the observed anti-apoptotic functions of the gene, since co-factors like SIRT1 can also act as a pro-or anti-apoptotic factor within the same cell [19]. Together with findings from recent studies, the present data allow to propose a dual mode of action exhibited by IEX-1. Under growth favourable conditions and in the presence of certain growth factors, IEX-1 is involved in ERK1/2 and PP2A regulated processes that promote cellular survival [29,33–36]. In contrast, if growth inappropiate conditions prevail – i.e. death ligand treatment or DNA damaging insults – IEX-1 exerts its pro-apoptotic potential [24–26,11,12,28,29,32] depending on the nuclear localization of IEX-1 [26,29] and including the repressive interaction with RelA/p65. This action of IEX-1 – itself being a NF-κB target gene – might be part of a counterregulatory and selfterminating process of NF-κB, and as one major consequence thereof, NF-κB dependent anti-apoptotic protection is getting lost. Future work will focus on identifying the nuclear mechanisms on the chromatin level by which IEX-1 interferes with NF-κB target gene expression and thereby controls cell survival.

Acknowledgements

The authors would like to thank Tanja Kaacksteen for excellent technical support. This study was supported by grants of the German Research Society (DFG/SFB415) and the NGFN2 (BMBF) Pathway mapping.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamcr.2007.12.010.

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