Tyrosine phosphatase inhibition triggers sustained canonical Serine-dependent NF κ B activation via Src-dependent blockade of PP2A

Sandra Barisic, Claudia Schmidt, Henning Walczak, Dagmar Kulms

► To cite this version:

Sandra Barisic, Claudia Schmidt, Henning Walczak, Dagmar Kulms. Tyrosine phosphatase inhibition triggers sustained canonical Serine-dependent NF κ B activation via Src-dependent blockade of PP2A. *Biochemical Pharmacology*, 2010, 80 (4), pp.439. 10.1016/j.bcp.2010.04.028 . hal-00601169

HAL Id: hal-00601169

<https://hal.science/hal-00601169>

Submitted on 17 Jun 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Title: Tyrosine phosphatase inhibition triggers sustained canonical Serine-dependent NF κ B activation via Src-dependent blockade of PP2A

Authors: Sandra Barisic, Claudia Schmidt, Henning Walczak, Dagmar Kulms

PII: S0006-2952(10)00302-3
DOI: doi:10.1016/j.bcp.2010.04.028
Reference: BCP 10545

To appear in: *BCP*

Received date: 17-2-2010
Revised date: 26-4-2010
Accepted date: 27-4-2010

Please cite this article as: Barisic S, Schmidt C, Walczak H, Kulms D, Tyrosine phosphatase inhibition triggers sustained canonical Serine-dependent NF κ B activation via Src-dependent blockade of PP2A, *Biochemical Pharmacology* (2008), doi:10.1016/j.bcp.2010.04.028

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

**Tyrosine phosphatase inhibition triggers sustained canonical Serine-
dependent NF κ B activation via Src-dependent blockade of PP2A**

Sandra Barisic¹, Claudia Schmidt¹, Henning Walczak², Dagmar Kulms^{1*}

¹Institute of Cell Biology and Immunology, University of Stuttgart, 70569 Stuttgart, Germany

Sandra.Barisic@izi.uni-stuttgart.de

Schmidt.cla@web.de

²Tumour Immunology Unit, Division of Medicine, Imperial College London, London W12

ONN, UK

h.walczak@imperial.ac.uk

*Corresponding author: Dagmar Kulms

University of Stuttgart

Institute of Cell Biology and Immunology

Allmandring 31

70569 Stuttgart, Germany

Phone: +49-711-68569299

Fax: +49-711-68567484,

Email: Dagmar.Kulms@izi.uni-stuttgart.de

Abstract

Activation status of Tyr-kinase Src as well as of the transcription factor NF κ B is a decisive criterion for the onset of cancer and in conveying radio-resistance. While the activation status of Src is Tyr-phosphorylation dependent, NF κ B activation requires Ser phosphorylation of its cytosolic inhibitor, I κ B α . Since constitutive NF κ B activation was linked to tumor maintenance, its tight regulation is mandatory.

We provide evidence that inhibition of pan-Tyr phosphatase activity by orthovanadate is translated via Src to inhibition of Ser phosphatase PP2A, thereby changing the physiologic response of the cell. In particular we unravelled a new sequence of molecular interactions linking initial activating Tyr416 phosphorylation of Src not to Tyr42-dependent phosphorylation and degradation of I κ B α , but to sustained Ser177/181 phosphorylation of I κ B α kinase IKK β following IL-1 stimulation. Consequently, sustained IKK β activation provides for chronic canonical I κ B α degradation, thereby eliciting constitutive NF κ B activation. As the critical translator of Tyr to Ser phosphorylation we identified Ser/Thr phosphatase PP2A. We show that the catalytic subunit PP2Ac serves as a Src substrate with Tyr307 phosphorylation leading to its catalytic inhibition. Additionally to the known survival pathways triggered by Src, Src-mediated canonical and persistent NF κ B activation may fortify its tumorigenic effects.

Keywords: IKK β / I κ B α / NF κ B / PP2A / Src-kinase / Interleukin-1

1. Introduction

Dysregulated activity of the non receptor Tyr-kinase Src family members, as well as of the transcription factor Nuclear factor kappa B (NF κ B) have been implicated in cancer development. Aberrant growth factor driven activity of Src, a designated proto-oncogene, triggers survival signals predominantly by inducing the PI3K/Akt/mTOR and Ras/Raf/MEK/Erk pathways resulting in cell cycle progression, angiogenesis and other aspects of tumorigenesis, including NF κ B activation (reviewed in [1]; [2;3]). Canonical NF κ B (p65/p50) is mostly activated by pro-inflammatory mediators including IL-1 through receptor dependent initiation of Ser-dependent MAPK cascades resulting in activation of a multi subunit IkB-kinase (IKK) complex consisting of IKK α , - β and - γ . Ser177/181 phosphorylation of IKK β consequently catalyzes phosphorylation of the NF κ B inhibitor IkB α at Ser32/36, leading to its polyubiquitination and proteasomal degradation. Liberated NF κ B binds to responsive promoter elements and induces activation of multiple genes involved in inflammation, proliferation, angiogenesis and anti-apoptotic signalling [4;5]. Proper cellular function is assured by early NF κ B-mediated resynthesis of IkB α representing a negative regulatory feedback loop [6]. Recently we demonstrated a crucial role for the Ser/Thr phosphatase PP2A in enabling this feedback loop as it mediates dephosphorylation of IKK β Ser177/181 following IL-1 stimulation, thereby allowing re-accumulation of IkB α , as a prerequisite for NF κ B termination [7;8]. Specific inhibition of the catalytic subunit of PP2A, PP2Ac, resulted in preservation of Ser177/181 phosphorylation of IKK β , continuous downstream phosphorylation and, as a consequence, chronic degradation of newly synthesized IkB α , thereby causing inhibition of the negative feedback loop, hence, sustained NF κ B activation [7].

Reoxygenation is an essential process in sensitizing hypoxic tumor cells to radiotherapy [9]. However, *in vivo* tumor cells and surrounding tissues coexist with

inflammatory cells participating in the tumor-host immune response. These cells release proinflammatory cytokines like IL-1 [10]. Consequently, IL-1-induced NF κ B activation may interfere with radiosensitization of tumor cells by upregulating antiapoptotic genes [11].

Following this line, treatment of cells with the Tyr-phosphatase inhibitor orthovanadate (OVA), mimicking reoxygenation [12-14], was shown to result in NF κ B activation via an alternative pathway involving Tyr42 phosphorylation of I κ B α [14-16]. Similarly, direct Src-dependent phosphorylation of I κ B α at Tyr42 was shown to trigger I κ B α degradation and NF κ B activation in different cell lines [17-19], being even enhanced by OVA treatment [14-16]. Less evidence exists showing that Src activation may cause Ser 32/36 phosphorylation-dependent I κ B α degradation by alternatively phosphorylating upstream IKK β at Tyr188/199 [20;21]. Therefore, OVA may contribute to aberrant NF κ B activation by stabilizing Tyr phosphorylation of Src, I κ B α and even IKK β [16;17;22].

Investigating the essential negative feed back regulation of NF κ B in epithelial cells following IL-1 stimulation, we made the surprising observation that co-stimulation with OVA fully abrogated I κ B α resynthesis. We document the underlying mechanism to be independent of Tyr phosphorylation of either IKK β or I κ B α . Instead, it involved the canonical pathway via sustained Ser phosphorylation of IKK β and I κ B α , respectively. We demonstrate that enhanced cellular Tyr-phosphorylation, e.g. in cancer development, may also have a strong impact on Ser-phosphorylation-dependent pathways. In this particular case PP2Ac being the critical translator of the phosphorylation cascade from Tyr to Ser via Src-dependent inhibitory phosphorylation of PP2Ac at Tyr307, which causes neutralization of its role as a negative regulator of IKK β . Hence, we here identified a new and alternative pathway utilized by Src that causes chronic canonical NF κ B activation via inhibition of PP2Ac, likely contributing to cancer initiation/progression as well as to radio-resistance.

2. Materials and Methods

Unless otherwise stated, results of phosphatase assays are presented as mean $\pm$ SD of 3 independently performed experiments. For statistical analysis student's t-test was performed. Immunoprecipitation, WB analysis, *in vitro* kinase assays show one representative out of 3 independently performed experiments.

2.1. Cells and reagents

The human epithelial carcinoma cell line KB (ATCC) was cultured in RPMI 1640, 10% FCS. Subconfluent cells were stimulated in colourless medium with 2% FCS. Recombinant human IL-1 β (R&D Systems) was applied at 10 ng/ml and Na-Orthovanadate (Sigma) at 1 mM 2 h prior to IL-1 β stimulation. CalyculinA (Merck) was added at 5 nM and MG132 (Merck) at 30 μ M to the cells. Specific kinase inhibitors were purchased from Calbiochem. Recombinant human iz-TRAIL protein, N-terminally fused to a isoleucine-zipper motif in order to constitutively build the trimerised active form [23] was kindly provided by Dr. Henning Walczak, Department of Immunology, Division of Medicine, Imperial College London.

2.2. Determination of Cell Death.

16 h after stimulation cells were detached from dishes, and apoptosis was measured by a Cell Death Detection ELISA (Roche). The enrichment of mono- and oligonucleosomes released into the cytoplasm of cell lysates is detected by biotinylated anti-histone- and peroxidase-coupled anti-DNA-antibodies and is calculated as follows: absorbance of sample cells/absorbance of control cells. The enrichment factor of 2 corresponds to 10 % apoptotic cells as determined by AnnexinV staining followed by FACS analysis.

2.3. Plasmids and Transfection of cells

Based on a IKK β wt-pEYFP-C1 plasmid the following Y to F mutants were generated by site directed mutagenesis using Pfu-ultra polymerase (Stratagen, La Jolla, CA) followed by DpnI digestion (Fermentas Inc., Glen Burnie, MD) according to the manufacturers instruction: Y188F, Y199F, Y205F, Y261F, Y294F, Y397F, Y497F, Y188/199F. The same method was applied to create Y42F and Y305F mutations in pcDNA3 based plasmids encoding wt I κ B α . pcDNA3-based Src variants wt-GFP, CA and KD were kindly provided by Dr. Hausser and Dr. Olayioye, University of Stuttgart, Germany. GST-I κ B α -5-55 was kindly provided by Dr. Storz, Mayo Clinic, Jacksonville, USA. PP2Ac-240-309 was amplified from pcDNA3-PP2Ac via PCR and cloned into pGEX-4T-2 for GST fusion and purification using glutathion-sepharose 4B (GE-Healthcare) followed by elution with 50 mM Tris, pH 8.0, 10 mM glutathion.

For ectopic expression of proteins 6x10⁶ cells were transfected by electroporation at 1200 μ F and 250 V (EasyjecT-plus, Peqlab) in ice cold RPMI medium w/o FCS with 25 μ g of the respective plasmids. Transfection efficacy ranged between 70 and 80 %.

For knock down experiments respective sequences were generated and purchased from MWG. Scrambled: 5'-UAgAAUUAUCCUCAACAgtt-3'; PP2Ac: 5'-gAggUUCgAUgUCCAgUUATT-3'; PKD1: 5'-gUCgAgAgAAgAggUCAAATT-3' and PKD3: 5'-AgAAUAAUgUgCACUgUgATT-3'. 0.8x10⁵ cells were transfected with 50 pmol siRNA using Lipofectamin 2000 (Invitrogen) according to the manufacturer's instructions. After 48 h knock down was documented by Western-blot analysis using an antibody against PP2Ac (# 05-421, clone 1D6, Upstate).

2.4. Immunoprecipitation, WB analysis and in vitro kinase assay

Cells transiently transfected with Src-CA and Src-KD, respectively, were lysed in lysis buffer (50 mM Hepes, pH 7.5; 150 mM NaCl; 10% glycerol; 1% Triton-X-100; 1.5 mM MgCl₂; 1 mM EGTA; 100 mM NaF; 10 mM pyrophosphate, 0.01% NaN₃ and Complete[®]

protease inhibitor cocktail (Roche)) for 15 min on ice. Endogenous PP2Ac and NFκB (p65) were immunoprecipitated using specific antibodies (# 05-421, clone 1D6, Upstate and C-20, sc-372, Santa Cruz) and A/G-plus agarose (Santa Cruz) over night. Precipitates were analyzed by Western-blotting using antibodies against p-Tyr (PY99, Santa Cruz), the N-terminus of PP2Ac (PP2Ac II; 280740R, Invitrogen) and NFκB (F6, sc-8008, Santa Cruz). For WB analysis cells were lysed in lysis buffer as above. 80 µg protein extracts were subjected to SDS-PAGE and Western-blotting and detected with antibodies against IκB-α, P-IκBα-Ser32/36, P-IKKβ-Ser177/181, IKKβ, PP2Ac, (L35A5, 5A5, 16A6, 2C8, # 2038, Cell Signaling (PP2Ac I) and 280740R, Invitrogen (PP2Ac II)), Src (PC 12-301, Upstate), P-Src-Tyr416 (PK1109, Calbiochem), PKD (C-20; sc-639, Santa Cruz), α-tubulin (DM1A, Neomarkers), and GST (GE-Healthcare). For kinase assay immunoprecipitation of PP2Ac was carried out as above. 1 µg of GST-fused purified IκBα(5-55) and PP2Ac(240-309) peptides were incubated with immunoprecipitated Src (antibody for IP: # 2108, Cell Signaling) and 2 µCi [³²P]-γ-ATP in kinase buffer (50 mM Tris, pH 7.4, 10 mM MgCl₂, 2 mM DTT) for 20 min at 37 °C, denaturated for 5 min at 95 °C and analysed autoradiographically on 10 % SDS-PAGE. Subsequently the gel was blotted and analysed for protein expression with respective antibodies.

2.5. Phosphatase assay

PP2Ac was immunoprecipitated (antibody for IP: # 05-421, clone 1D6, Upstate) from cells ectopically expressing the empty vector, Src-CA or Src-KD as above. 10 µg of cell lysates or were diluted in 74 µl phosphatase assay buffer (50 mM Tris/HCl, pH 7.0; 100 µM CaCl₂) and incubated with 6 µl Threonine phosphopeptide (#P-152, Biomol) yielding a final concentration of 75 µM for 5 min at 30°C. 20 µl malachite green solution (Bio Assay Systems) was added and absorption measured at different time points at 650 nm. Phosphatase

activity of un-irradiated cells was determined to be 100%. As an assay standard a serial dilution of 40 μ M phosphate (Bio Assay Systems) was used.

2.6. Semiquantitative RT-PCR analysis

Total RNA was extracted from cells using GIT-buffer followed by phenol/chloroform extraction utilizing Phase Lock Heavy tubes (Eppendorf AG). 1.5 μ g of total RNA was reverse transcribed with an AMV Reverse Transcriptase kit (Promega). The following primers were used in a 20 μ l reaction utilizing the RedTaq polymerase system from Sigma. GAPDH: F: 5'-TgATgACATACCgAAggTggTgAAg-3'; R: 5'-TCCTTggAggCCATgTAggCCAT-3'; xIAP: F: 5'-gAAAACCTATCTgggAAgCAGAg-3'; R: 5'-CgAATATTAAgATTCCggCCCA-3'; cIAP2: F: 5'-CTggATgCTgTTTCCACAgA-3'; R: 5'-gAgTTgCAGTgCCATTCTCA-3'.

2.7. Electro mobility shift assay (EMSA)

Following stimulation cells were harvested and nuclear proteins extracted as described before [24]. The NF κ B consensus oligo nucleotide (sc-2505; Santa Cruz) was end-labeled using [γ ³²P] ATP and T4 polynucleotide kinase (MBI Fermentas, Ontario, Canada), followed by column-purification (QIAquick Nucleotide Removal Kit, Qiagen, Hilden, Germany). Binding reactions were carried out in a 20 μ l volume containing 15 μ g protein extract, 4 μ l 5x binding buffer (20 mM HEPES, pH 7.5; 50 mM KCl; 2.5 mM MgCl₂; 20 % (w/v) ficoll; 1 mM DTT), 2 μ g poly[dIdC]; 2 μ g BSA, and 70.000 cpm of ³²P-labeled NF κ B consensus oligo nucleotide for 20 min at RT. Samples were separated on a 4 % native PAGE at 150 V for 2.5 h and detected by autoradiography.

3. Results

3.1. Co-treatment of cells with IL-1+OVA causes abrogation of the negative feedback loop of NFκB and results in continuous expression of anti-apoptotic genes.

IL-1 stimulation is controlled by a negative regulatory feedback loop which is mediated by NFκB-dependent resynthesis of IκBα starting 90 min after initial degradation and being completed after 2 h. Since reoxygenation is a prerequisite for successful radiotreatment of hypoxic tumors [9], but chronic activation of NFκB is known to confer radioresistance in a variety of tumors [11], we were interested in revealing the behaviour of NFκB in response to reoxygenation, mimicked by adding the tyrosine phosphatase inhibitor orthovanadate (OVA) to transformed KB cells. Upon pre-treatment of these cells with OVA for 2 h, however, IκBα reappearance was completely abrogated (Fig. 1A) and active NFκB persisted within the nucleus over time as documented by EMSA (Fig. 1B). As a consequence, maintained NFκB activation caused prolonged transcription of the NFκB-responsive [25;26] anti-apoptotic genes like XIAP and cIAP2 for at least 4 h instead of only 1 or 2 h, respectively, following treatment with IL-1 only (Fig. 1C). This may enforce an anti-apoptotic phenotype of tumor cells exposed to e.g. radiotherapy. Correspondingly, OVA+IL-1 treatment was shown to significantly reduce apoptosis induced by the death ligand TRAIL (Fig. 1D). This effect seems to be NFκB dependent, because it could completely be antagonized in cells ectopically expressing a super-repressor mutant of IκBα in which the serine residues 32/36, essential for canonical NFκB activation, were substituted by alanine (IκBα-S32/36A). Data hinted at canonical IκBα degradation to be involved in physiological effects triggered by OVA.

3.2. OVA-induced inhibition of IκBα recurrence is independent of tyrosine phosphorylation of either IKKβ or IκBα.

Tyr phosphorylation of IKK β was described to represent an alternative way of downstream I κ B α degradation [20;21]. To test whether Tyr phosphorylation of IKK β was responsible for OVA-inhibited reappearance of I κ B α , we exchanged tyrosine residues at positions 188, 199, 205, 261, 294, 397, 497 and 188/199 of IKK β to phenylalanine by site directed mutagenesis. We then investigated resynthesis of I κ B α upon IL-1 and OVA co-treatment in cells over-expressing each of these IKK β mutants. However, in all cases I κ B α remained absent in cells co-stimulated with IL-1+OVA (Fig. 2A), indicating continuous degradation of resynthesized I κ B α to follow molecular mechanisms independent of Tyr phosphorylation of IKK β .

Other reports claim that the tyrosine kinase Src mediates Tyr42 phosphorylation of I κ B α thereby triggering an alternative pathway of I κ B α degradation and consequently NF κ B activation [14-19]. We therefore analyzed the effect of I κ B α -Y42F mutation on both, its initial IL-1-induced degradation, and its failure to reaccumulate within the cytoplasm upon OVA co-treatment. As Tyr305 phosphorylation of I κ B α was implicated in hepatitis C virus induced NF κ B activation [27], we also included an I κ B α -Y305F mutant in our analysis. Although initial degradation of I κ B α -Y42F was slightly delayed (Fig. 2B), neither this mutant nor the I κ B α -Y305F variant (Fig. 2C) showed any different behaviour when compared to endogenous or ectopically expressed I κ B α -wt. In particular, both mutants failed to reappear at later times, indicating that Src-dependent phosphorylation of I κ B α at Tyr42 or Tyr305 is not primarily responsible for inhibition of I κ B α recurrence. Hence, OVA-induced inhibition of I κ B α reappearance seems to be independent of Tyr phosphorylation of IKK β and I κ B α .

3.3. OVA-induced inhibition of I κ B α reappearance is dependent on canonical serine phosphorylation of I κ B α and coincides with sustained IKK β activation.

Since Tyr phosphorylation did not target IKK β or I κ B α directly, we next investigated the impact of canonical Ser phosphorylation on I κ B α stability upon IL-1 + OVA treatment. We therefore ectopically expressed the I κ B α -S32/36A being incapable of canonical NF κ B activation. We found that whereas reappearance of endogenous I κ B α was inhibited by OVA-treatment the mutant protein remained unaffected, indicating that IL-1+OVA-induced abrogation of the negative feedback loop of NF κ B follows the conservative canonical pattern involving Ser32/36 phosphorylation of I κ B α (Fig. 3A). To make sure that indeed Ser32/36 phosphorylation of newly synthesized I κ B α precedes its immediate post-translational and proteasomal degradation, proteasome inhibition was undertaken to capture resynthesized and Ser-phosphorylated I κ B α . As expected, the proteasome inhibitor MG132 applied 30 min prior to IL-1 stimulation, prevented initial I κ B α degradation and Ser32/36 phosphorylated I κ B α was detected. Adding MG132 15 min after I κ B α + OVA treatment, i.e. at a time when initial I κ B α degradation is completed, also yielded Ser32/36 phosphorylated I κ B α (Fig. 3B). This strongly suggests that OVA-induced inhibition of I κ B α resynthesis is caused by immediate canonical degradation of the resynthesized protein. This assumption was further strengthened by the observation that Ser177/181 phosphorylation of IKK β remained elevated upon cotreatment with OVA at this time (Fig. 3B). Formal evidence for prolonged IKK β activity was obtained from *in vitro* kinase assays with IKK β immunoprecipitated from IL-1/OVA stimulated cells utilizing a GST-purified I κ B α (5-55) fragment as substrate. Initial phosphorylation of IKK β following IL-1 +/- OVA treatment for 15 min caused *in vitro* phosphorylation of the I κ B α fragment as well as phosphorylation and degradation of cellular I κ B α . Sustained IKK β activation after 2 h of IL-1+ OVA stimulation was reflected by prolonged IKK β phosphorylation, its ability to *in vitro* phosphorylate I κ B α (5-55), and lack of I κ B α recurrence at the cellular level (Fig. 3C). Together, these data indicated that IL-1+OVA-

induced inhibition of the negative feedback loop for NF κ B follows the canonical Ser phosphorylation-dependent activation pattern rather than causing Tyr phosphorylation-dependent alternative degradation of resynthesized I κ B α . Nevertheless, as OVA is a specific inhibitor of Tyr phosphatases, Tyr phosphorylation is required upstream in the signalling cascade to maintain the activation/phosphorylation status of IKK β . Yet, the target of this Tyr phosphorylation required to keep IKK β in its active form remained to be determined.

3.4. Inhibition of potential upstream targets of I κ B α do not reverse its lack of recurrence upon IL-1 + OVA treatment

As stated above, OVA has been described to mediate I κ B α degradation via Src-dependent Tyr phosphorylation. In fact, treatment of epithelial cells with the Tyr phosphatase inhibitor OVA caused activation of the Tyr-kinase Src, evident from enhanced Tyr416 autophosphorylation with or without IL-1 co-stimulation (Fig. 4A). In the cell system studied here, however, not Tyr phosphorylation but rather canonical Ser phosphorylation of both IKK β and I κ B α seems to take place upon co-stimulation of cells with IL-1 + OVA. We therefore scrutinized other putative targets of Src, which might potentially interfere with I κ B α resynthesis. Src activates PI3K and Akt which, upon hyperactivation, were shown to promote chemoprevention in an NF κ B-dependent manner [28]. A different study revealed recruitment and activation of PI3K to be dependent on Tyr479 phosphorylation of the cytosolic IL-1 receptor domain, resulting in improved IL-1 signalling [29]. In our system IL-1 + OVA-induced blockade of I κ B α recurrence, however, remained completely unaffected by chemical inhibition of PI3K, MAPK, JNK and Akt, respectively (Fig.4B).

In response to oxidative stress, PKC activation was shown to promote PKD-dependent NF κ B activation in a Ser phosphorylation-dependent fashion [30;31]. Since PKC activation requires Tyr phosphorylation, Src might trigger sustained canonical I κ B α degradation via this

alternative pathway. This possibility could, however, also be excluded by the use of specific PKC inhibitors as well as PKD knock down experiments (Fig. 4C and D). Additionally, free radical formation [32], alternative IKK γ Ser86 phosphorylation [33;34] or Src-mediated tyrosine phosphorylation of NF κ B [35;36] itself could be ruled out as inducers of sustained NF κ B activation in our system (Fig. 4E, F and G).

3.5. OVA-induced activation of the Tyr-kinase Src causes tyrosine phosphorylation and inhibition of PP2Ac.

Another potential candidate responsible for IL-1+OVA-induced chronic NF κ B activation is the Ser/Thr phosphatase PP2A. We recently showed PP2A to be essential for tuning down IKK β activity, thereby contributing to the negative feedback loop of NF κ B following IL-1 treatment [7;8]. Overexpressing a constitutively active variant of Src (Src-CA) resulted in Tyr-phosphorylation of the catalytic subunit PP2Ac, which was further enhanced in cells co-treated with OVA (Fig. 5A). Phosphorylation of PP2Ac at Tyr307 was documented with a pTyr specific antibody and additionally with an antibody which recognizes the C-terminal epitope comprising amino acids 295-309 and fails to detect PP2Ac whenever Tyr307 phosphorylation takes place (PP2Ac I). Tyr307 phosphorylation of PP2Ac is known to cause inhibition of this phosphatase [37] and may therefore facilitate the prolonged IKK β activation, due to sustained Ser-177/181 phosphorylation, as observed upon IL-1 + OVA treatment. Immunoprecipitation experiments further confirmed interaction of PP2Ac and Src. Simultaneously, they specified Tyr-phosphorylation of PP2Ac to only take place in cells overexpressing constitutively active Src (Src-CA) but not in cells overexpressing a kinase-dead (Src-KD) variant of Src (Fig. 5B). An *in vitro* kinase assay confirmed PP2Ac to be a specific target of Src (Fig. 5C). Finally, we performed an *in vitro* phosphatase assay to investigate the impact of Src-dependent Tyr phosphorylation on PP2Ac activity. This

demonstrated that basal PP2Ac activity was significantly enhanced in cells ectopically expressing Src-KD, whereas it was clearly decreased in cells expressing Src-CA (Fig. 5D). Thus, OVA-induced Src activation inhibits PP2Ac activity by phosphorylation at Tyr307, presumably resulting in decreased dephosphorylation of IKK β with the consequence of chronic activation of this kinase.

3.6. OVA treatment results in Src and PP2Ac to cooperate in extending IKK β activation, thereby causing degradation of resynthesized I κ B α and abrogation of the negative feedback loop of NF κ B.

To finally link PP2Ac inhibition to sustained IKK β activation and lack of I κ B α recurrence, we firstly documented dephosphorylation of IKK β to be PP2Ac-dependent. PP2Ac inhibition by the specific inhibitor calyculin A or by siRNA driven knock down resulted in strong Ser177/181 phosphorylation of IKK β coinciding with lack of I κ B α recurrence after 2h, being most pronounced when combined (Fig. 6A). Secondly, we documented the effect of Src-CA overexpression on the phosphorylation status of PP2Ac and IKK β as well as on accumulation of I κ B α . This effect was even boosted by over-expression of Src-CA. In parallel, overexpression of Src-CA resulted in inhibitory Tyr phosphorylation of PP2Ac as documented by two different antibodies – one detecting pTyr, and one recognizing only non-phosphorylated PP2Ac (PP2Ac I). Equal loading of PP2Ac was controlled with a PP2Ac antibody recognizing the N-terminus of PP2Ac (II) which remains unaffected by Tyr phosphorylation. Accordingly, the time point of strongest PP2Ac phosphorylation, 2 h after IL-1+OVA treatment, closely correlated with strongest Ser177/181 phosphorylation of IKK β , comparable to the IKK β phosphorylation status required for initial I κ B α degradation 15 min after stimulation (Fig. 6B). Although the phosphorylation level of IKK β is lower after 2 h of OVA+IL-1 treatment it appears to be sufficient to prevent re-

accumulation of re-synthesized I κ B α over time. Additional evidence is given by the fact that the Src inhibitor herbimycin could fully abolish prolonged – low level - IKK β phosphorylation, thereby allowing for stable I κ B α resynthesis upon OVA+IL-1 treatment for 2 h (Fig. 6C). To finally prove that Src is the crucial inducer of Tyr-dependent, but finally canonical and Ser phosphorylation –mediated degradation of newly synthesized I κ B α , we performed the reverse experiment. We overexpressed a kinase dead variant of Src (Src-KD) which we could demonstrate to facilitate reappearance of I κ B α (Fig. 6D). In summary, OVA+IL-1-induced activation of Tyr kinase Src causes Ser-phosphorylation dependent continuous degradation of I κ B α , thereby abrogating the negative feedback regulation of NF κ B.

4. Discussion

Since uncontrolled NF κ B activity has been linked to the development and maintenance of tumors, by upregulation of anti-apoptotic genes [38;39], tight regulation by the negative regulatory feedback loop is mandatory. Along this line, NF κ B was found to be constitutively active in many cell lines derived from hematopoietic or solid tumors [5]. Moreover, inappropriate regulation of NF κ B is claimed to be directly responsible for multiple diseases including neurodegenerative diseases, arthritis, and psoriasis [40;41]. Due to its anti-apoptotic properties NF κ B activation also appears to confer chemo- and radioresistance [11]. Thus, the signalling pathway involved in canonical NF κ B activation serves as a target for anti-cancer interventions mostly via IKK and/or proteasome inhibition [42].

Constitutive activity of the proto-oncogene Src, activates different survival pathways, some of them involving downstream NF κ B activation (reviewed in [1]; [2;3]). Accordingly, strategies interfering with Src-activation, like use of adenin-mimetics, represent therapeutic strategies to fight malignancies and immunological disorders [42]. Besides the canonical

pathway, alternative ROS formation-induced Tyr42 phosphorylation of I κ B α was shown to cause NF κ B activation being triggered in a Src-dependent [14;15;17] or Src-independent manner [43-45]. Src-dependent Tyr42 phosphorylation of I κ B α was found to be even enhanced when cells were co-stimulated with OVA [15;32]. In this context, I κ B α degradation-dependent [16] and –independent [14] mechanisms of NF κ B liberation have been described, while the contribution of I κ B α Ser32/36 phosphorylation in this scenario is still discussed [16;46].

We have recently reported co-stimulation of cells with IL-1 and UVB to result in complete inhibition of the negative regulatory feedback loop of NF κ B, being due to UVB-induced inhibition of the Ser/Thr phosphatase PP2Ac [7]. Costimulation of cells with IL-1 and the Tyr phosphatase inhibitor OVA, however, presented an identical phenotype of negative feedback abrogation over hours and caused persistant NF κ B activation. In contrast to several reports claiming OVA-induced NF κ B activation through Src family member dependent Tyr phosphorylation of Tyr42 [17-19] or Tyr305 [27] of I κ B α , we found IL-1 + OVA induced NF κ B activation as well as inhibition of I κ B α resynthesis to be independent of Tyr phosphorylation of either I κ B α or IKK β [20;21] but to follow the canonical Ser phosphorylation-dependent pattern. Accordingly, Tyr phosphorylation needs to be translated into Ser phosphorylation involving canonical or alternative kinases upstream of IKK β . In this context, the Tyr-kinase Src was shown to become activated upon IL-1 + OVA stimulation. After ruling out a number of putative Src substrates to interfere with I κ B α degradation [28-34] Ser/Thr phosphatase PP2A was found to be the critical component. PP2A is known to modulate NF κ B activity [47]. While IKK-PP2A complex formation was proposed to facilitate TNF-induced phosphorylation of IKK β [48], more evidence exists favouring inhibition of PP2A to promote activation of NF κ B. In this context direct chemical PP2A inhibition with calyculinA or ocadaic acid provoked I κ B α phosphorylation and degradation [11;49;50].

Moreover, following TNF treatment PP2A was shown to interact with IKK γ to down-regulate IKK β activity [33;51].

Inhibitory Tyr307 phosphorylation of the catalytic subunit PP2Ac can be mediated by Src itself and other Src family members [37]. Src-dependent Tyr307 phosphorylation of PP2Ac was shown to take place *in vitro* and *in vivo*, being strongest upon IL-1 + OVA stimulation, and coincided with loss of phosphatase activity of about 50%. Overexpression of Src-KD in turn failed to phosphorylate PP2Ac and correlated with increased PP2Ac activity (+30%), critically linking Src-dependent Tyr phosphorylation to its catalytic inactivation. While PP2Ac inhibition via siRNA knock down and/or calyculinA treatment impeded I κ B α recurrence upon IL-1 treatment due to chronic IKK β phosphorylation, Src-CA overexpression provided the final link of Tyr307 PP2Ac phosphorylation to continuous Ser177/181 IKK β phosphorylation and abrogation of I κ B α resynthesis, being most pronounced in IL-1 + OVA treated cells. In full accordance, Src inhibition by herbimycin as well as overexpression of Src-KD was shown to antagonize the OVA effect, allowing I κ B α reappearance, thus corroborating the findings from the inverse experiments.

Based on results obtained from the present study, we propose an alternative mechanism by which uncontrolled activity of Src may amplify its oncogenic potential by additional PP2Ac mediated persistent canonical NF κ B activation: IL-1 stimulation predominantly causes canonical NF κ B activation via Ser177/181 phosphorylation of IKK β followed by Ser32/36 phosphorylation-dependent proteasomal degradation of I κ B α . At the same time liberated NF κ B initiates a negative regulatory feedback loop involving I κ B α resynthesis. Under normal conditions PP2A-mediated continuous IKK β dephosphorylation assures stabilization of the resynthesized NF κ B inhibitor, thereby terminating NF κ B activity [7;8]. Tyr-phosphatase inhibition by OVA in parallel stabilizes Tyr416 phosphorylation of Src. Activated Src subsequently inhibits PP2Ac by Tyr307 phosphorylation, thereby aborting PP2A driven

dephosphorylation of IKK β . Prolonged IKK β activation consequently triggers continuous canonical elimination of newly synthesized I κ B α . Thus, abrogation of the negative feedback loop of NF κ B causes prolonged expression of antiapoptotic genes, which might confer resistance against anti-tumor interventions (Fig. 7). Since many tumor cells themselves but especially inflammatory cells surrounding the tumor can release IL-1 [10] cells by the proposed mechanism may escape anti cancer treatments like radiotherapy.

5. Acknowledgements

We especially thank N. Peters for excellent technical assistance. This work was funded by the German Research Foundation (DFG, KU 1981/1-1).

6. References

- [1] Ma WW and Adjei AA, Novel agents on the horizon for cancer therapy. *CA Cancer J Clin* 2009;59:111-37.
- [2] Benati D and Baldari CT, SRC family kinases as potential therapeutic targets for malignancies and immunological disorders. *Curr Med Chem* 2008;15:1154-65.
- [3] Rucci N, Susa M, Teti A, Inhibition of protein kinase c-Src as a therapeutic approach for cancer and bone metastases. *Anticancer Agents Med Chem* 2008;8:342-9.
- [4] Aggarwal BB, Nuclear factor-kappaB: the enemy within. *Cancer Cell* 2004;6:203-8.
- [5] Luque I and Gelinas C, Rel/NF-kappa B and I kappa B factors in oncogenesis. *Semin Cancer Biol* 1997;8:103-11.
- [6] Delhase M, Hayakawa M, Chen Y, Karin M, Positive and negative regulation of IkappaB kinase activity through IKKbeta subunit phosphorylation. *Science* 1999;284:309-13.
- [7] Barisic S, Strozyk E, Peters N, Walczak H, Kulms D, Identification of PP2A as a crucial regulator of the NF-kappaB feedback loop: its inhibition by UVB turns NF-kappaB into a pro-apoptotic factor. *Cell Death Differ* 2008;15:1681-90.
- [8] Witt J, Barisic S, Schumann E, Allgower F, Sawodny O, Sauter T, Kulms D, Mechanism of PP2A-mediated IKKbeta dephosphorylation: a systems biological approach. *BMC Syst Biol* 2009;3:71.
- [9] Dewhirst MW, Cao Y, Moeller B, Cycling hypoxia and free radicals regulate angiogenesis and radiotherapy response. *Nat Rev Cancer* 2008;8:425-37.
- [10] Luger TA and Schwarz T, Therapeutic use of cytokines in dermatology. *J Am Acad Dermatol* 1991;24:915-26.
- [11] Tergaonkar V, Pando M, Vafa O, Wahl G, Verma I, p53 stabilization is decreased upon NFkappaB activation: a role for NFkappaB in acquisition of resistance to chemotherapy. *Cancer Cell* 2002;1:493-503.

- [12] Beraud C, Henzel WJ, Baeuerle PA, Involvement of regulatory and catalytic subunits of phosphoinositide 3-kinase in NF-kappaB activation. *Proc Natl Acad Sci U S A* 1999;96:429-34.
- [13] Hehner SP, Hofmann TG, Ratter F, Droge W, Schmitz ML, Inhibition of tyrosine phosphatases antagonizes CD95-mediated apoptosis. *Eur J Biochem* 1999;264:132-9.
- [14] Imbert V, Rupec RA, Livolsi A, Pahl HL, Traenckner EB, Mueller-Dieckmann C, Farahifar D, Rossi B, Auberger P, Baeuerle PA, Peyron JF, Tyrosine phosphorylation of I kappa B-alpha activates NF-kappa B without proteolytic degradation of I kappa B-alpha. *Cell* 1996;86:787-98.
- [15] Crevecoeur J, Merville MP, Piette J, Gloire G, Geldanamycin inhibits tyrosine phosphorylation-dependent NF-kappaB activation. *Biochem Pharmacol* 2008;75:2183-91.
- [16] Mukhopadhyay A, Manna SK, Aggarwal BB, Pervanadate-induced nuclear factor-kappaB activation requires tyrosine phosphorylation and degradation of IkappaBalpha. Comparison with tumor necrosis factor-alpha. *J Biol Chem* 2000;275:8549-55.
- [17] Fan C, Li Q, Ross D, Engelhardt JF, Tyrosine phosphorylation of I kappa B alpha activates NF kappa B through a redox-regulated and c-Src-dependent mechanism following hypoxia/reoxygenation. *J Biol Chem* 2003;278:2072-80.
- [18] Jalal DI and Kone BC, Src activation of NF-kappaB augments IL-1beta-induced nitric oxide production in mesangial cells. *J Am Soc Nephrol* 2006;17:99-106.
- [19] Ponnappan S, Uken-Trebilcock G, Lindquist M, Ponnappan U, Tyrosine phosphorylation-dependent activation of NFkappaB is compromised in T cells from the elderly. *Exp Gerontol* 2004;39:559-66.
- [29] Huang WC, Chen JJ, Inoue H, Chen CC, Tyrosine phosphorylation of I-kappa B kinase alpha/beta by protein kinase C-dependent c-Src activation is involved in TNF-alpha-induced cyclooxygenase-2 expression. *J Immunol* 2003;170:4767-75.

- [21]Huang WC, Chen JJ, Chen CC, c-Src-dependent tyrosine phosphorylation of IKKbeta is involved in tumor necrosis factor-alpha-induced intercellular adhesion molecule-1 expression. J Biol Chem 2003;278:9944-52.
- [22]Roskoski R, Jr., Src protein-tyrosine kinase structure and regulation. Biochem Biophys Res Commun 2004;324:1155-64.
- [23]Ganten TM, Koschny R, Sykora J, Schulze-Bergkamen H, Buchler P, Haas TL, Schader MB, Untergasser A, Stremmel W, Walczak H, Preclinical differentiation between apparently safe and potentially hepatotoxic applications of TRAIL either alone or in combination with chemotherapeutic drugs. Clin Cancer Res 2006;12:2640-6.
- [24]Strozyk E, Poppelmann B, Schwarz T, Kulms D, Differential effects of NF-kappaB on apoptosis induced by DNA-damaging agents: the type of DNA damage determines the final outcome. Oncogene 2006;25:6239-51.
- [25]Wang CY, Mayo MW, Korneluk RG, Goeddel DV, Baldwin AS, Jr., NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science 1998;281:1680-3.
- [26]Wang Y, Chan S, Tsang BK, Involvement of inhibitory nuclear factor-kappaB (NFkappaB)-independent NFkappaB activation in the gonadotropic regulation of X-linked inhibitor of apoptosis expression during ovarian follicular development in vitro. Endocrinology 2002;143:2732-40.
- [27]Waris G, Livolsi A, Imbert V, Peyron JF, Siddiqui A, Hepatitis C virus NS5A and subgenomic replicon activate NF-kappaB via tyrosine phosphorylation of IkappaBalpha and its degradation by calpain protease. J Biol Chem 2003;278:40778-87.
- [28]Zhang X, Jin B, Huang C, The PI3K/Akt pathway and its downstream transcriptional factors as targets for chemoprevention. Curr Cancer Drug Targets 2007;7:305-16.

- [29] Marmiroli S, Bavelloni A, Faenza I, Sirri A, Ognibene A, Cenni V, Tsukada J, Koyama Y, Ruzzene M, Ferri A, Auron PE, Toker A, Maraldi NM, Phosphatidylinositol 3-kinase is recruited to a specific site in the activated IL-1 receptor I. *FEBS Lett* 1998;438:49-54.
- [30] Storz P and Toker A, Protein kinase D mediates a stress-induced NF-kappaB activation and survival pathway. *EMBO J* 2003;22:109-20.
- [31] Storz P, Doppler H, Toker A, Protein kinase Cdelta selectively regulates protein kinase D-dependent activation of NF-kappaB in oxidative stress signaling. *Mol Cell Biol* 2004;24:2614-26.
- [32] Schieven GL, Kirihaara JM, Myers DE, Ledbetter JA, Uckun FM, Reactive oxygen intermediates activate NF-kappa B in a tyrosine kinase-dependent mechanism and in combination with vanadate activate the p56lck and p59fyn tyrosine kinases in human lymphocytes. *Blood* 1993;82:1212-20.
- [33] Palkowitsch L, Leidner J, Ghosh S, Marienfeld RB, Phosphorylation of serine 68 in the IkappaB kinase (IKK)-binding domain of NEMO interferes with the structure of the IKK complex and tumor necrosis factor-alpha-induced NF-kappaB activity. *J Biol Chem* 2008;283:76-86.
- [34] Wu ZH, Shi Y, Tibbetts RS, Miyamoto S, Molecular linkage between the kinase ATM and NF-kappaB signaling in response to genotoxic stimuli. *Science* 2006;311:1141-6.
- [35] Bijli KM, Minhajuddin M, Fazal F, O'Reilly MA, Plataniias LC, Rahman A, c-Src interacts with and phosphorylates RelA/p65 to promote thrombin-induced ICAM-1 expression in endothelial cells. *Am J Physiol Lung Cell Mol Physiol* 2007;292:L396-L404.
- [36] Kang JL, Jung HJ, Lee K, Kim HR, Src tyrosine kinases mediate crystalline silica-induced NF-kappaB activation through tyrosine phosphorylation of IkappaB-alpha and p65 NF-kappaB in RAW 264.7 macrophages. *Toxicol Sci* 2006;90:470-7.

- [37]Chen J, Parsons S, Brautigan DL, Tyrosine phosphorylation of protein phosphatase 2A in response to growth stimulation and v-src transformation of fibroblasts. J Biol Chem 1994;269:7957-62.
- [38]Cortes SM, Rodriguez F, V, Sanchez P, I, Perona R, The role of the NFkappaB signalling pathway in cancer. Clin Transl Oncol 2008;10:143-7.
- [39]Philip M, Rowley DA, Schreiber H, Inflammation as a tumor promoter in cancer induction. Semin Cancer Biol 2004;14:433-9.
- [40]Foxwell B, Browne K, Bondeson J, Clarke C, de Martin R, Brennan F, Feldmann M, Efficient adenoviral infection with IkappaB alpha reveals that macrophage tumor necrosis factor alpha production in rheumatoid arthritis is NF-kappaB dependent. Proc Natl Acad Sci U S A 1998;95:8211-5.
- [41]Grilli M and Memo M, Nuclear factor-kappaB/Rel proteins: a point of convergence of signalling pathways relevant in neuronal function and dysfunction. Biochem Pharmacol 1999;57:1-7.
- [42]Lin A and Karin M, NF-kappaB in cancer: a marked target. Semin Cancer Biol 2003;13:107-14.
- [43]Gloire G, Charlier E, Rahmouni S, Volanti C, Chariot A, Erneux C, Piette J, Restoration of SHIP-1 activity in human leukemic cells modifies NF-kappaB activation pathway and cellular survival upon oxidative stress. Oncogene 2006;25:5485-94.
- [44]Gloire G, Legrand-Poels S, Piette J, NF-kappaB activation by reactive oxygen species: fifteen years later. Biochem Pharmacol 2006;72:1493-505.
- [45]Schoonbroodt S, Ferreira V, Best-Belpomme M, Boelaert JR, Legrand-Poels S, Korner M, Piette J, Crucial role of the amino-terminal tyrosine residue 42 and the carboxyl-terminal PEST domain of I kappa B alpha in NF-kappa B activation by an oxidative stress. J Immunol 2000;164:4292-300.

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
- [46] Chen F, Demers LM, Vallyathan V, Ding M, Lu Y, Castranova V, Shi X, Vanadate induction of NF-kappaB involves IkappaB kinase beta and SAPK/ERK kinase 1 in macrophages. *J Biol Chem* 1999;274:20307-12.
- [47] Li S, Wang L, Berman MA, Zhang Y, Dorf ME, RNAi screen in mouse astrocytes identifies phosphatases that regulate NF-kappaB signaling. *Mol Cell* 2006;24:497-509.
- [48] Kray AE, Carter RS, Pennington KN, Gomez RJ, Sanders LE, Llanes JM, Khan WN, Ballard DW, Wadzinski BE, Positive regulation of IkappaB kinase signaling by protein serine/threonine phosphatase 2A. *J Biol Chem* 2005;280:35974-82.
- [49] Sun SC, Maggirwar SB, Harhaj E, Activation of NF-kappa B by phosphatase inhibitors involves the phosphorylation of I kappa B alpha at phosphatase 2A-sensitive sites. *J Biol Chem* 1995;270:18347-51.
- [50] Sung SJ and Walters JA, Stimulation of interleukin-1 alpha and interleukin-1 beta production in human monocytes by protein phosphatase 1 and 2A inhibitors. *J Biol Chem* 1993;268:5802-9.
- [51] Hong S, Wang LC, Gao X, Kuo YL, Liu B, Merling R, Kung HJ, Shih HM, Giam CZ, Heptad repeats regulate protein phosphatase 2a recruitment to I-kappaB kinase gamma/NF-kappaB essential modulator and are targeted by human T-lymphotropic virus type 1 tax. *J Biol Chem* 2007;282:12119-26.

Figure Legends

Figure 1. OVA causes abrogation of the negative feedback loop of NF κ B and expression of anti-apoptotic genes. **(A)** KB cells were preincubated or not with OVA (1 mM) for 2 h and then stimulated with IL-1 (10 ng/ml) for the indicated time points. I κ B α protein level was documented by Western-blot analysis. **(B)** Cells were treated as in (A). At the indicated time points nuclear proteins were extracted, and NF κ B activation documented by electrophoretic mobility shift assay using an NF κ B consensus oligo nucleotide. **(C)** Cells were treated as under (A). At the indicated time points RNA was extracted and transcription of cIAP2 and xIAP determined by semiquantitative RT-PCR analysis.

Figure 2. OVA-induced inhibition of I κ B α resynthesis is independent of Tyr phosphorylation of either IKK β or I κ B α . **(A)** Cells were transfected with different single Y to F mutants and a double mutant of IKK β respectively. After stimulation with IL-1 (10 ng/ml) alone or IL-1 + OVA (1 mM, -2 h) for 2 h I κ B α resynthesis and IKK β expression level was evaluated by Western-blot analysis. **(B)** Cells were transfected with the empty vector or the respective plasmid overexpressing I κ B α wt-GFP or I κ B α -Y305F-GFP or **(C)** I κ B α -Y42F. 24 h later cells were preincubated or not with OVA (1 mM) for 2 h and stimulated with IL-1 (10 ng/ml) for 15 min and 2 h, respectively. Western-blot analysis revealed the cellular status of endogenous and ectopically expressed I κ B α variants. Equal loading was monitored by reprobing the respective membrane with an α -tubulin antibody.

Figure 3. OVA-induced inhibition of I κ B α resynthesis is dependent on canonical Ser phosphorylation of I κ B α and coincides with sustained IKK β activation. **(A)** KB cells were transfected with the empty vector or the respective plasmid overexpressing I κ B α S32/36A. 24 h later cells were preincubated or not with OVA (1 mM) for 2 h and stimulated with IL-1 (10

ng/ml) for 15 min and 2 h, respectively. Western-blot analysis revealed the cellular status of endogenous and ectopically expressed mutant I κ B α . Equal loading was monitored by α -tubulin. **(B)** The I κ B α level after IL-1 (10 ng/ml) only and IL-1 + OVA (1 mM) treatment was trapped by preincubating cells with the proteasome inhibitor MG132 (30 μ M). At the indicated time points following IL-1 stimulation Ser phosphorylation as well as overall protein status of IKK β and I κ B α was determined by Western-blot analysis with α -tubulin serving as loading control. **(C)** Cells stably expressing IKK β -GFP were stimulated with IL-1 (10 ng/ml) alone or in combination with OVA (1 mM, -2 h) for 15 min or 2 h. IKK β -GFP was immunoprecipitated and subjected to an *in vitro* kinase assay with a purified GST-I κ B α (5-55) peptide. I κ B α , phospho-IKK β and IKK β statuses were determined by Western-blot analysis. GST and α -tubulin served as loading controls. Phosphorylation status of IKK β was calculated using Image Quant software.

Figure 4. Potential role of different upstream targets in IL-1+OVA-induced continuous I κ B α degradation. **(A)** Cells were stimulated with IL-1 (10 ng/ml) or with IL-1 + OVA (1 mM, -2 h). After 15 min and 2 h the Y416 phosphorylation status of Src was correlated to the cellular I κ B α level by Western-blot analysis and calculated using Image Quant software. **(B)** KB cells were left untreated or incubated with PI3K inhibitor (LY294002; 50 μ M), MAPK inhibitor (PD980569; 100 μ M), JNK inhibitor (SP600125; 10 μ M) or Akt inhibitor II (5 μ M) for 1 h. Subsequently cells were pretreated with OVA (1 mM) for 2 h and stimulated with IL-1 (10 ng/ml) for 2 h. I κ B α status was documented by Western-blot analysis. **(C)** Cells were incubated with PKC inhibitors Gö 6976 (5 μ M) and Gö 6983 (10 μ M), respectively, for 1 h. After treatment with OVA+IL-1 for 2 h, I κ B α status was analyzed by Western-blotting. **(D)** Cells were transfected with scrambled siRNA or siRNA specifically knocking down PKD1 and PKD3, respectively. 48 h later cells were treated with OVA+IL-1 for the indicated times,

IkB α and PKD status were documented by Western-blot analysis. **(E)** Cells were preincubated or not with radical scavengers PDTC (100 μ M) and N-Ac (20 mM) for 1 h. IkB α status was analysed by Western-blotting, 2 h after IL-1 or IL-1+OVA stimulation. **(F)** Cells were transfected with the empty vector (pcDNA-3) or the respective plasmid encoding wt-IKK γ or a S86A-mutant of IKK γ . 24 h post transfection cells were stimulated with IL-1 alone or costimulated with OVA as indicated and IkB α status documented by Western-blot analysis. **(G)** NF κ B was immunoprecipitated from untreated or IL-1+ OVA treated cells. Tyr phosphorylation and NF κ B were analyzed by Western-blotting.

Figure 5. Src activation following OVA treatment causes Tyr phosphorylation and inhibition of PP2Ac. **(A)** Cells were transfected with the empty vector or the respective plasmid overexpressing a constitutive active variant of Src (Src-CA). 24 h later cells were preincubated or not with OVA (1 mM) for 2 h and stimulated with IL-1 (10 ng/ml) for 2 h. Phosphorylation of PP2Ac was documented with an anti-pTyr antibody and verified with an antibody recognizing PP2Ac only in its dephosphorylated but not in its phosphorylated form (PP2Ac I). **(B)** Cells were transfected with Src-CA or a Src kinase dead variant (Src-KD). After 24 h PP2Ac was immunoprecipitated and its phosphorylation status scrutinized with a pTyr specific antibody. **(C)** 24 h after transfection Src was immunoprecipitated from cells expressing either Src-wt-GFP or Src-KD IKK β -GFP and subjected to an *in vitro* kinase assay with a purified GST-PP2Ac(240-309) peptide. Src expression levels and GST-PP2Ac input were evaluated by Western-blot analysis. **(D)** 24 h after transfection of Src-CA or Src-KD, endogenous PP2Ac was immunoprecipitated and subjected to an *in vitro* phosphatase assay using a Threonin-phosphopeptide as a substrate. * $p \leq 0.05$; ** $p \leq 0.005$. Immunoprecipitation of PP2Ac and expression levels of Src variants was determined by western-blot analysis.

Figure 6. Src and PP2Ac cooperate to extend IKK β activation, thereby causing degradation of resynthesized I κ B α (A) KB cells were transfected with scrambled siRNA or siRNA specifically knocking down PP2Ac. 48 h later cells were treated with IL-1 (10 ng/ml) or cotreated with calyculinA (5 nM) and IL-1. After 2 h PP2Ac knock down, phosphorylation status of IKK β and protein level of I κ B α were determined by Western-blot analysis. α -tubulin served as loading control. (B) Cells were transfected with the empty vector or the respective plasmid encoding Src-CA. 24 h later cells were stimulated with IL-1 + OVA as indicated and the phosphorylation status of IKK β and PP2Ac (pTyr; PP2Ac I), expression of Src and protein level of IKK β , I κ B α and PP2Ac (PP2Ac II) were monitored by Western-blot analysis with α -tubulin showing equal loading. (C) Cells were left untreated or pre-incubated with the Src inhibitor herbimycin (3 μ g/ml) for 1 h. Subsequently, cells were incubated with IL-1 only for 15 min and 2 h respectively, or co-stimulated with OVA and IL-1 for 2 h. Phosphorylation status of IKK β and protein level of I κ B α were determined by Western-blot analysis. An antibody against α -tubulin served as loading control. (D) Cells were transfected with the empty vector or the respective plasmid encoding Src-KD. 24 h later cells were stimulated with IL-1 + OVA as indicated and Src expression as well as I κ B α level displayed by Western-blot analysis. Equal loading was monitored by an α -tubulin antibody.

Figure 7. Mechanism of OVA-mediated inhibition of I κ B α resynthesis. Stimulation of KB cells with IL-1 predominantly causes canonical NF κ B activation via transient S177/181 phosphorylation of IKK β followed by S32/36 phosphorylation and consequently proteasomal degradation of I κ B α . NF κ B-dependent I κ B α resynthesis is warranted by PP2A-mediated dephosphorylation and consequently inhibition of IKK β . Upon costimulation of cells with OVA, however, activating Y416 phosphorylation of Src causes inhibitory Y307

phosphorylation of PP2Ac, resulting in extended phosphorylation and activation of IKK β

consequently providing for continuous phosphorylation and degradation of newly synthesized

I κ B α .

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7