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Tyrosine phosphatase inhibition triggers sustained canonical Serine-dependent NFκB activation via Src-dependent blockade of PP2A

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Abstract

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

We provide evidence that inhibition of pan-Try 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 IκBα, but to sustained Ser177/181 phosphorylation of IκBα kinase IKKβ following IL-1 stimulation. Consequently, sustained IKKβ activation provides for chronic canonical IκBα degradation, thereby eliciting constitutive NFκB 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 NFκB activation may fortify its tumorigenic effects.

Keywords: IKKβ / IκBα / NFκB / 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 (NFκB) 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 NFκB activation (reviewed in [1]; [2;3]). Canonical NFκB (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 IκB-kinase (IKK) complex consisting of IKKα, -β and -γ. Ser177/181 phosphorylation of IKKβ consequently catalyzes phosphorylation of the NFκB inhibitor IκBα at Ser32/36, leading to its polyubiquitination and proteasomal degradation. Liberated NFκB 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 NFκB-mediated resynthesis of IκBα 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 IKKβ Ser177/181 following IL-1 stimulation, thereby allowing re-accumulation of IκBα, as a prerequisite for NFκB termination [7;8]. Specific inhibition of the catalytic subunit of PP2A, PP2Ac, resulted in preservation of Ser177/181 phosphorylation of IKKβ, continuous downstream phosphorylation and, as a consequence, chronic degradation of newly synthesized IκBα, thereby causing inhibition of the negative feedback loop, hence, sustained NFκB 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 NFκB 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 NFκB activation via an alternative pathway involving Tyr42 phosphorylation of IκBα [14-16]. Similarly, direct Src-dependent phosphorylation of IκBα at Tyr42 was shown to trigger IκBα degradation and NFκB 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 IκBα degradation by alternatively phosphorylating upstream IKKβ at Tyr188/199 [20;21]. Therefore, OVA may contribute to aberrant NFκB activation by stabilizing Tyr phosphorylation of Src, IκBα and even IKKβ [16;17;22].

Investigating the essential negative feed back regulation of NFκB in epithelial cells following IL-1 stimulation, we made the surprising observation that co-stimulation with OVA fully abrogated IκBα resynthesis. We document the underlying mechanism to be independent of Tyr phosphorylation of either IKKβ or IκBα. Instead, it involved the canonical pathway via sustained Ser phosphorylation of IKKβ and IκBα, 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 IKKβ. Hence, we here identified a new and alternative pathway utilized by Src that causes chronic canonical NFκB 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 ± 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-1β (R&D Systems) was applied at 10 ng/ml and Na-Orthovanadate (Sigma) at 1 mM 2 h prior to IL-1β stimulation. CalyculinA (Merck) was added at 5 nM and MG132 (Merck) at 30 µM 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 IKKβwt-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 IκBα. pcDNA3-based Src variants wt-GFP, CA and KD were kindly provided by Dr. Hausser and Dr. Olayioye, University of Stuttgart, Germany. GST-IκBα-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 gluthation-sepharose 4B (GE-Healthcare) followed by elution with 50 mM Tris, pH 8.0, 10 mM gluthation.

For ectopic expression of proteins $6x10^6$ cells were transfected by electroporation at 1200 µF and 250 V (EasyjecT-plus, Peqlab) in ice cold RPMI medium w/o FCS with 25 µg 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'-UAgAAUUAUUCCUCAACAgTT-3'; PP2Ac: 5'-gAggUUCgAUgUCCAgUUATT-3'; PKD1: 5´-gUCgAgAgAAgAggUCAAATT-3` and PKD3: 5´-AgAAUAUUgUgCACUgUgATT-3`. $0.8x10^5$ 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 $MgCl_2$; 1 mM EGTA; 100 mM NaF; 10 mM pyrophosphate, 0.01% NaN_3 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-blottting 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 immunoprcipitated Src (antibody for IP: # 2108, Cell Signaling) and 2 µCi [^{32}P]-γ-ATP in kinase buffer (50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 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 $CaCl_2$) 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 µM 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 µg of total RNA was reverse transcribed with an AMV Reverse Transcriptase kit (Promega). The following primers were used in a 20 µl reaction utilizing the RedTaq polymerase system from Sigma. GAPDH: F: 5'-TgATgACATACCgAAggTggTgAAg-3'; R: 5'-TCCTTggAggCCATgTAggCCAT-3'; xIAP: F: 5'-gAAAACTATCTgggAAgCAgAg-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 NFκB consensus oligo nucleotide (sc-2505; Santa Cruz) was end-labeled using [γ^{32}P] 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 µl volume containing 15 µg protein extract, 4 µl 5x binding buffer (20 mM HEPES, pH 7.5; 50 mM KCl; 2.5 mM $MgCl_2$; 20 % (w/v) ficoll; 1 mM DTT), 2 µg poly[dIdC]; 2 µg BSA, and 70.000 cpm of ^{32}P-labeled NFκB 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 IKKβ was described to represent an alternative way of downstream IκBα degradation [20;21]. To test whether Tyr phosphorylation of IKKβ was responsible for OVA-inhibited reappearance of IκBα, we exchanged tyrosine residues at positions 188, 199, 205, 261, 294, 397, 497 and 188/199 of IKKβ to phenylalanine by site directed mutagenesis. We then investigated resynthesis of IκBα upon IL-1 and OVA co-treatment in cells over-expressing each of these IKKβ mutants. However, in all cases IκBα remained absent in cells co-stimulated with IL-1+OVA (Fig. 2A), indicating continuous degradation of resynthesized IκBα to follow molecular mechanisms independent of Tyr phosphorylation of IKKβ.

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

3.3. OVA-induced inhibition of IκBα reappearance is dependent on canonical serine phosphorylation of IκBα and coincides with sustained IKKβ activation.

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

induced inhibition of the negative feedback loop for NFκB follows the canonical Ser phosphorylation-dependent activation pattern rather than causing Tyr phosphorylation-dependent alternative degradation of resynthesized IκBα. 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 IKKβ. Yet, the target of this Tyr phosphorylation required to keep IKKβ in its active form remained to be determined.

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

As stated above, OVA has been described to mediate IκBα 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 IKKβ and IκBα 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 IκBα resynthesis. Src activates PI3K and Akt which, upon hyperactivation, were shown to promote chemoprevention in an NFκB-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 IκBα recurrence, however, remained completely unaffected by chemical inhibiton of PI3K, MAPK, JNK and Akt, respectively (Fig.4B).

In response to oxidative stress, PKC activation was shown to promote PKD-dependent NFκB activation in a Ser phosphorylation-dependent fashion [30;31]. Since PKC activation requires Tyr phosphorylation, Src might trigger sustained canonical IκBα 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 IKKγ Ser86 phosphorylation [33;34] or Src-mediated tyrosine phosphorylation of NFκB [35;36] itself could be ruled out as inducers of sustained NFκB 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 NFκB activation is the Ser/Thr phosphatase PP2A. We recently showed PP2A to be essential for tuning down IKKβ activity, thereby contributing to the negative feedback loop of NFκB 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 IKKβ 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 IKKβ with the consequence of chronic activation of this kinase.

3.6. OVA treatment results in Src and PP2Ac to cooperate in extending IKKβ activation, thereby causing degradation of resynthesized IκBα and abrogation of the negative feedback loop of NFκB.

To finally link PP2Ac inhibition to sustained IKKβ activation and lack of IκBα recurrence, we firstly documented dephosphorylation of IKKβ 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 IKKβ coinciding with lack of IκBα 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 IKKβ as well as on accumulation of IκBα. 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 IKKβ, comparable to the IKKβ phosphorylation status required for initial IκBα degradation 15 min after stimulation (Fig. 6B). Although the phosphorylation level of IKKβ is lower after 2 h of OVA+IL-1 treatment it appears to be sufficient to prevent re-

accumulation of re-synthesized IκBα over time. Additional evidence is given by the fact that the Src inhibitor herbimycin could fully abolish prolonged – low level - IKKβ phosphorylation, thereby allowing for stable IκBα 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 IκBα, we performed the reverse experiment. We overexpressed a kinase dead variant of Src (Src-KD) which we could demonstrate to facilitate reappearance of IκBα (Fig. 6D). In summary, OVA+IL-1-induced activation of Tyr kinase Src causes Ser-phosphorylation dependent continuous degradation of IκBα, thereby abrogating the negative feedback regulation of NFκB.

4. Discussion

Since uncontrolled NFκB 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, NFκB was found to be constitutively active in many cell lines derived from hematopoietic or solid tumors [5]. Moreover, inappropriate regulation of NFκB is claimed to be directly responsible for multiple diseases including neurodegenerative diseases, arthritis, and psoriasis [40;41]. Due to its anti-apoptotic properties NFκB activation also appears to confer chemo- and radioresistance [11]. Thus, the signalling pathway involved in canonical NFκB 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 NFκB 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 IκBα was shown to cause NFκB activation being triggered in a Src-dependent [14;15;17] or Src-independent manner [43-45]. Src-dependent Tyr42 phosphorylation of IκBα was found to be even enhanced when cells were co-stimulated with OVA [15;32]. In this context, IκBα degradation-dependent [16] and –independent [14] mechanisms of NFκB liberation have been described, while the contribution of IκBα 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 NFκB, 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 NFκB activation. In contrast to several reports claiming OVA-induced NFκB activation through Src family member dependent Tyr phosphorylation of Tyr42 [17-19] or Tyr305 [27] of IκBα, we found IL-1 + OVA induced NFκB activation as well as inhibition of IκBα resynthesis to be independent of Tyr phosphorylation of either IκBα of IKKβ [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 IKKβ. 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 IκBα degradation [28-34] Ser/Thr phosphatase PP2A was found to be the critical component. PP2A is known to modulate NFκB activity [47]. While IKK-PP2A complex formation was proposed to facilitate TNF-induced phosphorylation of IKKβ [48], more evidence exists favouring inhibition of PP2A to promote activation of NFκB. In this context direct chemical PP2A inhibition with calyculinA or ocadaic acid provoked IκBα phosphorylation and degradation [11;49;50].

Moreover, following TNF treatment PP2A was shown to interact with IKKγ to down-regulate IKKβ 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 phoshporylation 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 IκBα recurrence upon IL-1 treatment due to chronic IKKβ phosphorylation, Src-CA overexpression provided the final link of Tyr307 PP2Ac phosphorylation to continuous Ser177/181 IKKβ phosphorylation and abrogation of IκBα 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 IκBα 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 NFκB activation: IL-1 stimulation predominantly causes canonical NFκB activation via Ser177/181 phosphorylation of IKKβ followed by Ser32/36 phosphorylation-dependent proteasomal degradation of IκBα. At the same time liberated NFκB initiates a negative regulatory feedback loop involving IκBα resynthesis. Under normal conditions PP2A-mediated continuous IKKβ dephosphorylation assures stabilization of the resynthesized NFκB inhibitor, thereby terminating NFκB activity [7;8]. Tyr-phosphatase inhibition by OVA in parallel stabilizes Tyr416 phorylation of Src. Activated Src subsequently inhibits PP2Ac by Tyr307 phosphorylation, thereby aborting PP2A driven

dephosphorylation of IKKβ. Prolonged IKKβ activation consequently triggers continuous canonical elimination of newly synthesized IκBα. Thus, abrogation of the negative feedback loop of NFκB 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

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Figure Legends

Figure 1. OVA causes abrogation of the negative feedback loop of NFκB 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. IκBα 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 NFκB activation documented by electro mobility shift assay using an NFκB 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 IκBα resynthesis is independent of Tyr phosphorylation of either IKKβ or IκBα. **(A)** Cells were transfected with different single Y to F mutants and a double mutant of IKKβ respectively. After stimulation with IL-1 (10 ng/ml) alone or IL-1 + OVA (1 mM, -2 h) for 2 h IκBα resynthesis and IKKβ expression level was evaluated by Western-blot analysis. **(B)** Cells were transfected with the empty vector or the respective plasmid overexpressing IκBαwt-GFP or IκBα-Y305F-GFP or **(C)** IκBα-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 IκBα variants. Equal loading was monitored by reprobing the respective membrane with an α-tubulin antibody.

Figure 3. OVA-induced inhibition of IκBα resynthesis is dependent on canonical Ser phosphorylation of IκBα and coincides with sustained IKKβ activation. **(A)** KB cells were transfected with the empty vector or the respective plasmid overexpressing IκBαS32/36A. 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 IκBα. Equal loading was monitored by α-tubulin. **(B)** The IκBα 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 µM). At the indicated time points following IL-1 stimulation Ser phosphorylation as well as overall protein status of IKKβ and IκBα was determined by Western-blot analysis with α-tubulin serving as loading control. **(C)** Cells stably expressing IKKβ-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. IKKβ-GFP was immunoprecipitated and subjected to an *in vitro* kinase assay with a purified GST-IκBα(5-55) peptide. IκBα, phospho-IKKβ and IKKβ statuses were determined by Western-blot analysis. GST and α-tubulin served as loading controls. Phosphorylation status of IKKβ was calculated using Image Quant software.

Figure 4. Potential role of different upstream targets in IL-1+OVA-induced continuous IκBα 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 IκBα level by Western-blot analysis and calculated using Image Quant software. **(B)** KB cells were left untreated or incubated with PI3K inhibitor (LY294002; 50 µM), MAPK inhibitor (PD980569; 100 µM), JNK inhibitor (SP600125; 10 µM) or Akt inhibitor II (5 µM) 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. IκBα status was documented by Western-blot analysis. **(C)** Cells were incubated with PKC inhibitors Gö 6976 (5 µM) and Gö 6983 (10 µM), respectively, for 1 h. After treatment with OVA+IL-1 for 2 h, IκBα 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,

IκBα and PKD status were documented by Western-blot analysis. **(E)** Cells were preincubated or not with radical scavengers PDTC (100 µM) and N-Ac (20 mM) for 1 h. IκBα 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-IKKγ or a S86A-mutant of IKKγ. 24 h post transfection cells were stimulated with IL-1 alone or costimulated with OVA as indicated and IκBα status documented by Western-blot analysis. **(G)** NFκB was immunoprecipitated from untreated or IL-1+ OVA treated cells. Tyr phosphorylation and NFκB were analyzed be 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 IKKβ-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 anaysis.

Figure 6. Src and PP2Ac cooperate to extend IKKβ activation, thereby causing degradation of resynthesized IκBα **(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 IKKβ and protein level of IκBα 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 IKKβ and PP2Ac (pTyr; PP2Ac I), expression of Src and protein level of IKKβ, IκBα 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 µg/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 IKKβ and protein level of IκBα 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 IκBα level displayed by Western-blot analysis. Equal loadingwas monitored by an α-tubulin antibody.

Figure 7. Mechanism of OVA-mediated inhibition of IκBα resynthesis. Stimulation of KB cells with IL-1 predominantly causes canonical NFκB activation via transient S177/181 phosphorylation of IKKβ followed by S32/36 phosphorylation and consequently proteasomal degradation of IκBα. NFκB-dependent IκBα resynthesis is warranted by PP2A-mediated dephosphorylation and consequently inhibition of IKKβ. 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 IKKβ consequently providing for continuous phosphorylation and degradation of newly synthesized IκBα.

Figure 1

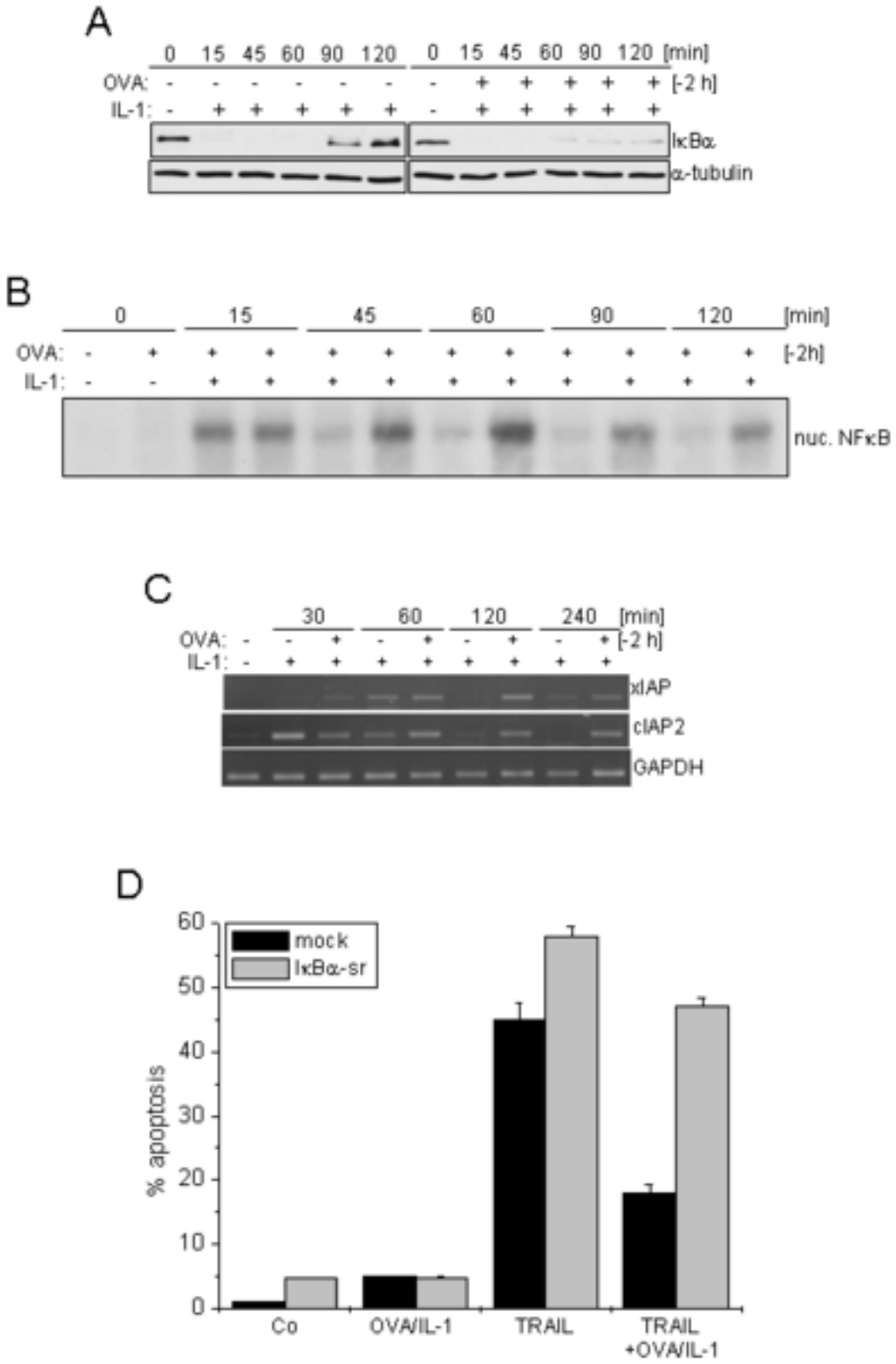

Figure 2

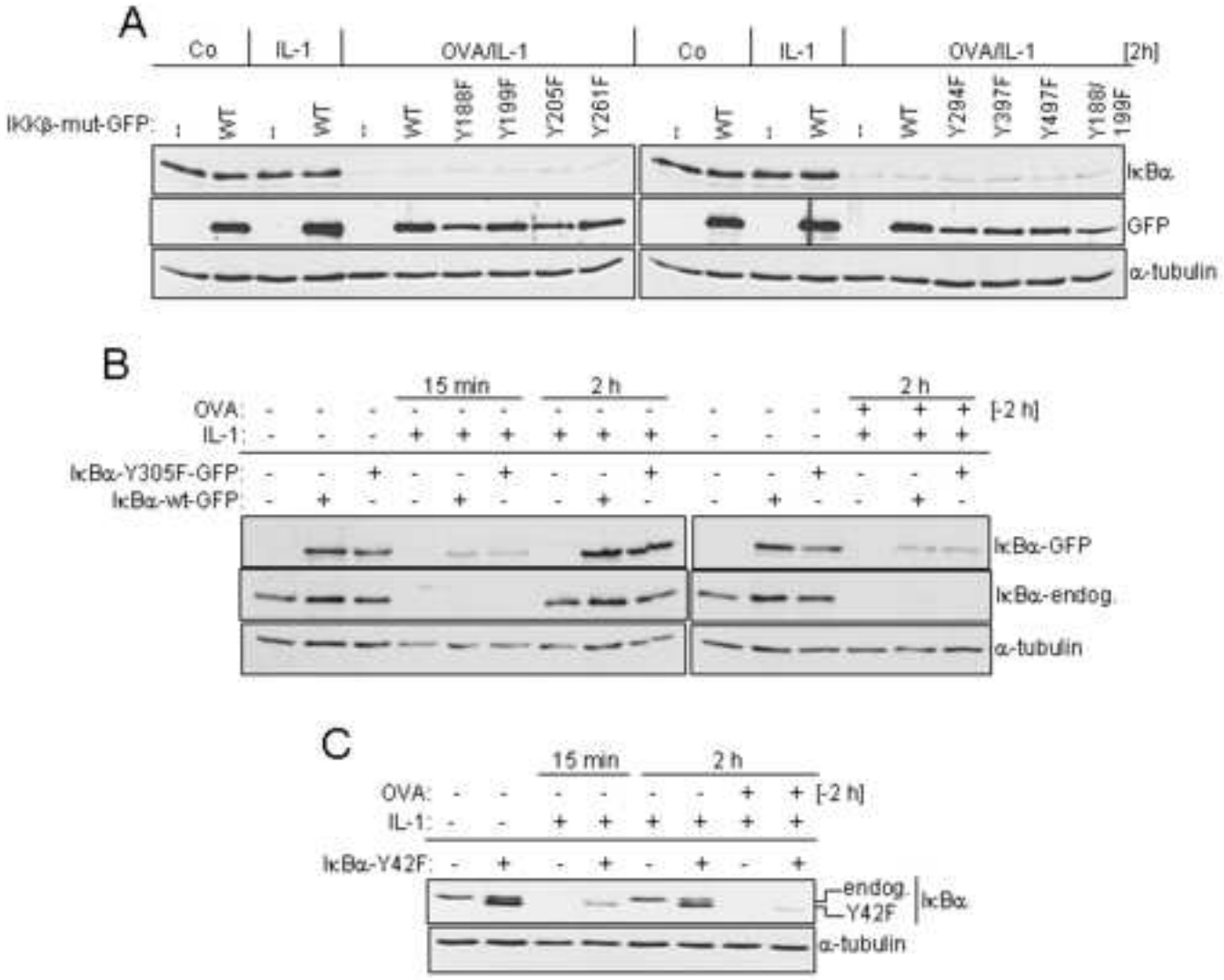

Figure 3

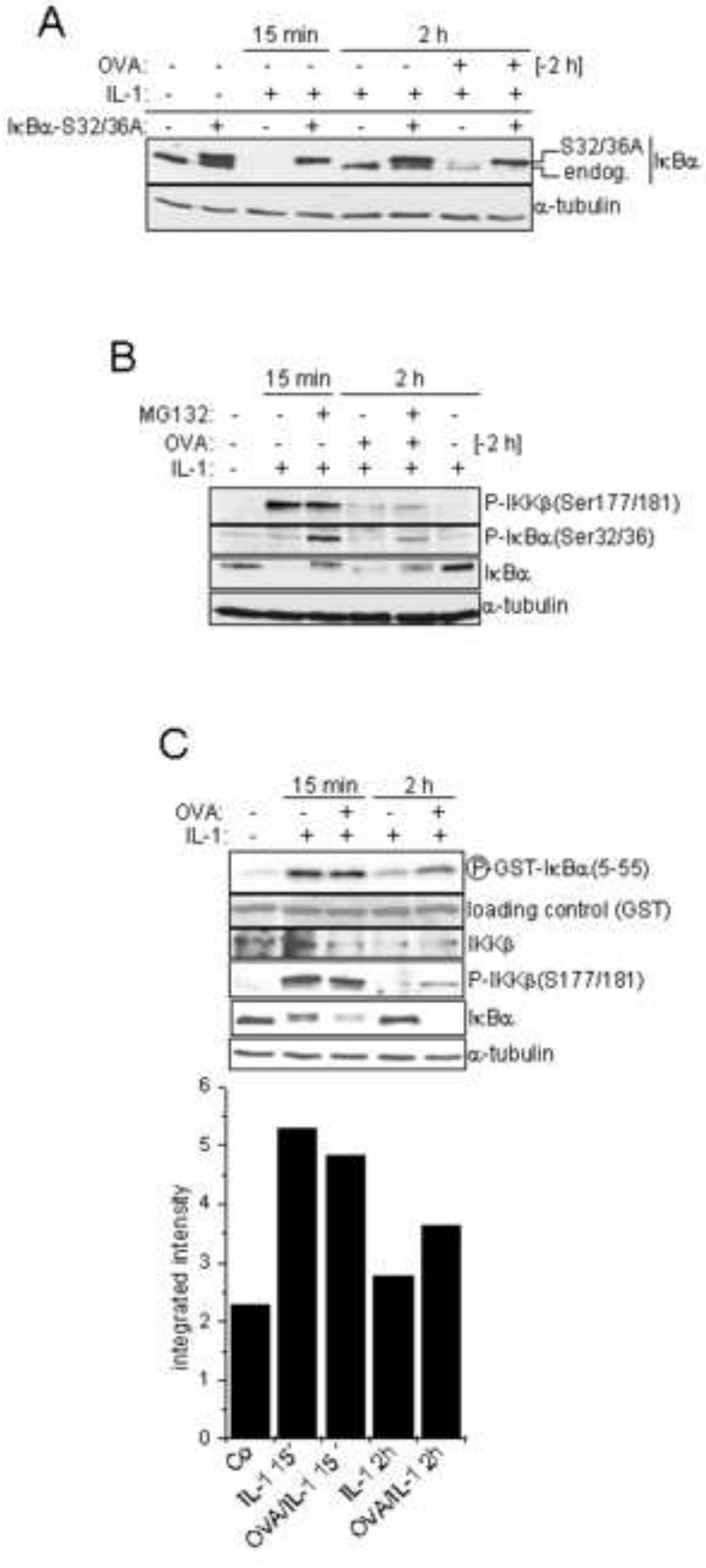

Figure 4

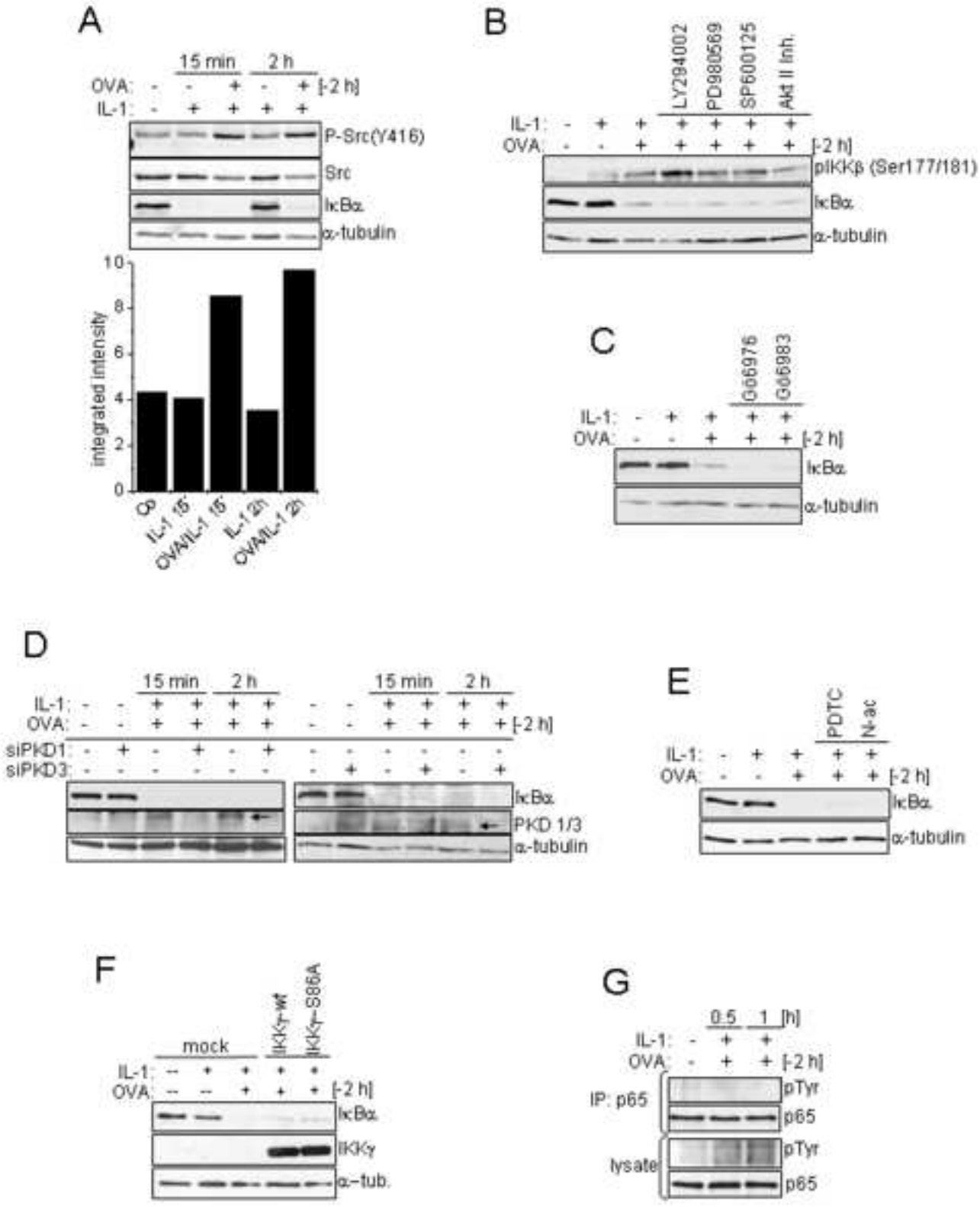

Figure 5

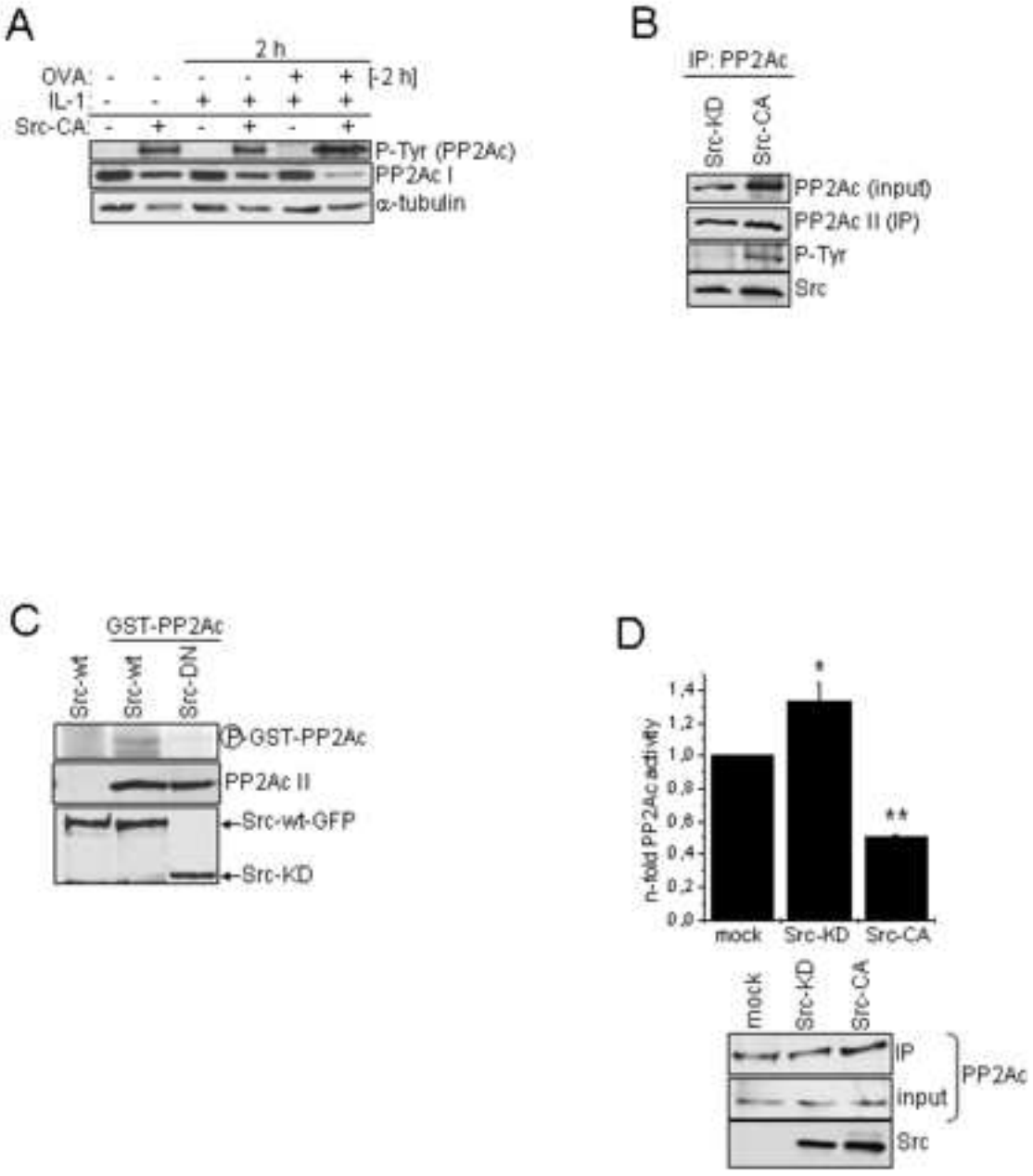

Figure 6

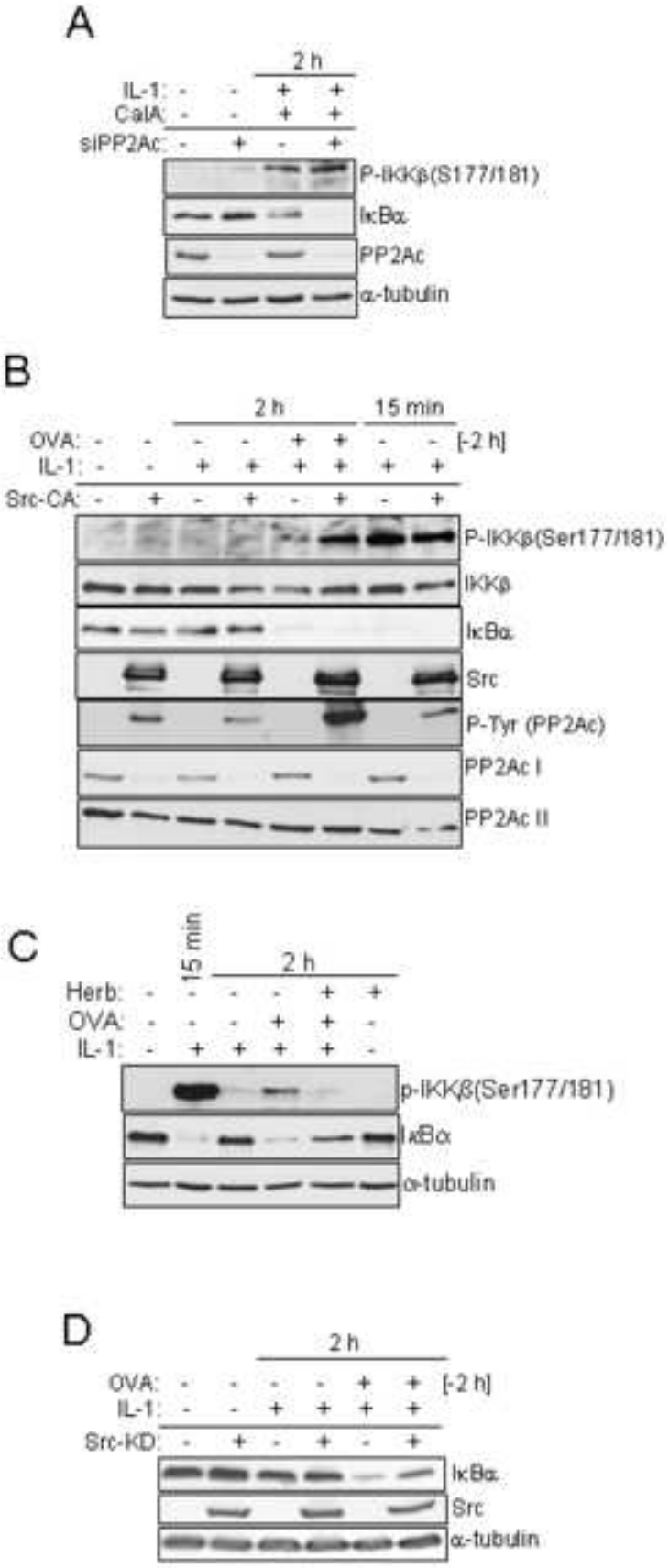

Figure 7