Die Rolle der Ser/Thr Phosphatase PP2A in der Regulation des Transkriptionsfaktors NFκB

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2. Witt J., Barisic S., Schumann E., Allgöwer F., Sawodny O., Sauter T., Kulms D. (2009). Mechanism of PP2A-mediated IKKβ dephosphorylation: a systems biological approach. *BMC Systems Biology,* accepted June 10, 2009.

3. Barisic S., Schmidt C., Kulms D.: Tyrosine kinase Src inhibits Ser/Thr phosphatase PP2A to trigger sustained canonical NFκB activation downstream of IL-1. Received, March, 2009

Inhaltsverzeichnis

1 Abkürzungsverzeichnis

Abb	Abbildung
Akt	Ser/Thr protein kinase known as protein kinase B
APAF-1	Apoptotic protease activating factor-1
ARF	ADP-ribosylation factor
ATR	Ataxia telangiectasia and rad-3-related kinase
Bax	Bcl-2 associated protein X
Bcl-2	B-cell CLL/lymphoma 2
CHK-1	CHEK1, cell cycle checkpoint kinase
cIAP	Cellular inhibitor of apoptosis protein
CYLD	cylindromatosis protein
DD	Death domain
DISC	Death-inducing signalling complex
DMBA	Dimethylbenzanthracen
DNA	Desoxyribonukleinsäure
EGF	Epidermal growth factor
FACS	Fluorescent activated cell sorting
FADD	Fas associated with death domain
FLIP	FLICE inhibitory protein
HAT	Histon Acetyltransferase
HDAC	Histon Deacetylase
HLH	Helix-Loop-Helix
HTLV-1	Human T-lymphotropic virus type 1
HIV	Human immunodeficiency virus
I-CAM	Inter-cellular adhesion molecule
IL-1	Interleukin-1
IL-1R	Interleukin-1 Rezeptor
IκB	Inhibitor of κB
IKK	IκB kinase
IRAK1	IL-1 receptor-associated kinase
LPS	Lipopolysaccharid

MAPK	Mitogen activated protein kinase
MEKK	MAPK kinase kinase
MMP-9	Matrix metallopeptidase 9
MyD88	Myeloid differentiation factor 88
NFκB	Nuclear factor kappa B
NLS	Nuclear localization sequence
OVA	Na-Orthovanadat
PI3K	Phosphatidylinositol 3 kinase
PKC	Protein kinase C
PKD	Protein kinase D
PMA	Para-Methoxyamphetamin
PP2A	Protein phosphatase 2A
RIP	Receptor interacting protein
RHD	Rel-Homologie-Domäne
ROS	Reactive oxygen species
Ser	Serin
TAB2	TAK-1-binding protein
TAK1	Transforming growth factor-β-activated kinase 1
TNF	Tumor necrosis factor
TNF-R1	Tumor necrosis factor-receptor 1
TRADD	Tumor necrosis factor receptor-associated death domain protein
TRAF	Tumor necrosis factor receptor-associated factor
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
Tyr	Tyrosin
v.a.	Vor allem
V-CAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
z. Bsp.	Zum Beispiel

2 Zusammenfassung

Der Transkriptionsfaktor NFκB kann durch eine Vielzahl von Stimuli, wie durch das pro-inflammatorische Zytokin IL-1, aktiviert werden. NFκB ist generell dafür bekannt, dass es die Transkription von Genen verstärkt, deren Produkte das Überleben der Zelle fördern und die Apoptose inhibieren. In diesem Zusammenhang konnte beobachtet werden, dass die TRAIL-induzierte Apoptose durch eine IL-1-vermittelte NFκB-Aktivierung stark vermindert werden kann. Paradoxerweise, wird die durch UVB-Strahlung ausgelöste Apoptose durch Ko-Stimulation mit IL-1 nicht reduziert, sondern sogar noch verstärkt. Dieser Effekt konnte auf eine NFκB-abhängige Repression anti-apoptotischer Proteine und auf eine erhöhte Freisetzung des Zytokins TNF zurückgeführt werden. Sezerniertes TNF induziert durch autokrine Bindung an den TNF-R1 den apoptotischen Signalweg, wodurch die UVB-induzierte Apoptose additiv verstärkt wird. Ein Ziel dieser Arbeit war es, die molekularen Mechanismen, die für die beobachtete NFκB-abhängige Freisetzung von TNF verantwortlich sind, aufzuklären. In unstimulierten Zellen liegt NFκB, durch Bindung an IκBα, inaktiv im Zytoplasma vor. Nach der IL-1-vermittelten Phosphorylierung der Ser/Thr Kinase IKKβ an Ser177/181 wird IκBα phosphoryliert, polyubiquitiniert und umgehend proteasomal degradiert. Daraufhin wird NFκB aktiviert und induziert neben seinen Zielgenen, auch die Transkription von IκBα. Nach kurzer Zeit beenden resynthetisierte IκBα-Moleküle, durch eine erneute Bindung an NFκB, dessen Aktivität. Überraschenderweise findet nach Stimulation von Zellen mit IL-1 und UVB keine Re-Synthese von IκBα mehr statt. Es konnte gezeigt werden, dass die daraus resultierende konstitutive Aktivierung von NFκB für eine massiv erhöhte Transkription von TNF und somit auch indirekt für dessen Sezernierung verantwortlich ist. Unsere Untersuchungen ergaben, dass die Re-Synthese von IκBα dadurch inhibiert wird, dass IκBα zwar NFκB-abhängig transkribiert, das resynthetisierte Protein aber anschließend sofort wieder phosphoryliert und proteasomal degradiert wird. Als Ursache hierfür wurde eine kontinuierliche Phosphorylierung und damit Aktivität von IKKβ festgestellt. Verantwortlich für diese chronische IKKβ Phosphorylierung ist die UVB-induzierte Inhibierung der Ser/Thr Phosphatase PP2A, die so als ein neuer und kritischer Modulator der IKKβ-Aktivität identifiziert werden konnte.

Die Tyrosinkinase c-Src ist an der Regulation vieler zellulärer Prozesse, die das Wachstum und das Überleben der Zelle fördern, beteiligt. Eine unkontrollierte Aktivität dieses Proto-Onkogens spielt, vorwiegend durch die Aktivierung des PI3K/Akt-, Ras/MAPK-, aber auch des NFκB-Signalweges, eine bedeutende Rolle bei der Entstehung vieler Arten von Tumoren. In der vorliegenden Arbeit konnte ein bisher unbekannter Mechanismus der Src-vermittelten konstitutiven NFκB-Aktivierung nachgewiesen werden. Unter Verwendung des Tyr-Phosphatase Inhibitors Orthovanadat kommt es in Kombination mit IL-1 Stimulation zunächst zu einer stabilen aktivierenden Tyr416 Phosphorylierung von Src. Diese wiederum inaktiviert PP2A, durch deren Phosphorylierung an Tyr307, die gleichzeitig IL-1-vermittelte IKKβ-Aktivität verlängert wird. Folglich wird auch in diesem Fall resynthetisiertes IκBα phosphoryliert und proteasomal abgebaut, wodurch die NFκB-abhängige Transkription anti-apoptotischer Gene massiv verstärkt wird.

Summary

The transcription factor can be activated by a variety of stimuli, such as the pro-inflammatory cytokine IL-1. NFκB is generally known to control the expression of many genes that promote cell survival and protect cells from apoptosis. Accordingly, IL-1-mediated NFκB activation significantly reduces TRAIL-induced apoptosis. Paradoxically, UVB-induced apoptosis is not inhibited but even enhanced upon co-stimulation with IL-1. This effect was associated with NFκB-dependent repression of anti-apoptotic genes and sustained secretion of TNF which additively induces apoptosis upon autocrine activation of TNF-R1. The aim of this study was to elucidate the molecular mechanisms underlying NFκB-dependent enhancement of TNF release. In resting cells, inactive NFκB is kept in the cytoplasm by binding to its inhibitor IκBα. Upon stimulation with IL-1, the Ser/Thr Kinase IKKβ becomes phosphorylated at Ser 177/181, which in turn causes phosphorylation of IκBα, leading to its subsequent polyubiquitination and proteasomal degradation. As a result, NFκB becomes activated and induces the transcription of target genes, including the one encoding its inhibitor IκBα. Newly synthesized IκBα consequently binds to NFκB again, thereby terminating its transcriptional activity. Surprisingly, recurrence of IκBα is completely blocked following co-stimulation with IL-1 and UVB, consequently leading to constitutive NFκB activation. We could show permanent NFκB activity to be a prerequisite for enhanced TNF transcription and release. Furthermore our studies revealed lack of IκBα recurrence not to be a result of inhibition of NFκB-dependent transcription, but to be mediated by immediate phosphorylation and proteasomal degradation of newly-synthesized IκBα due to persistent IKKβ phosphorylation and activation. In this context, we revealed the Ser/Thr phosphatase PP2A to play a crucial role in the control of IKKβ activity. Furthermore, we could show that UVB-induced inhibition of PP2A is responsible for constitutive IKKβ phosphorylation and activation.

The Tyrosine-Kinase c-Src is involved in the regulation of many cellular processes, such as growth and survival. Uncontrolled activity of the proto-oncogene Src has been linked to the development of many different types of tumors, mainly by inducing activation of the PI3/Akt, Ras/MAPK, but also the NFκB activating pathways. Utilizing

the Tyr-phosphatase inhibitor orthovanadate combined with IL-1 stimulation we could demonstrate stable and activating Tyr416 phosphorylation of Src to occur. Consequently, stably activated c-Src induces the inhibitory Tyr307 phosphorylation of PP2A, thereby maintaining the IL-1-mediated activity of IKKβ. Accordingly, also under these circumstances, resynthesized IκBα undergoes phosphorylation and proteasomal degradation, finally leading to a strong increase of NFκB-dependent transcription of anti-apoptotic genes.

3 Einleitung

3.1 Apoptose: Der programmierte Zelltod

Apoptose ist ein streng kontrolliertes „Selbstmordprogramm“ der Zelle und spielt eine wichtige Rolle bei der Formgebung von Organen während der Embryonalentwicklung, der Regulation des Immunsystems und bei der Homöostase von Geweben. Desweiteren werden irreversibel geschädigte und mit Pathogenen infizierte Zellen zugunsten des umliegenden Gewebes eliminiert. Schlüsselenzyme der Apoptose sind die Caspasen, Aspartat-spezifische Cysteinyl-Proteasen, die als inaktive Vorstufen konstitutiv exprimiert und durch proteolytische Spaltung aktiviert werden. Zu Beginn der Apoptose aktivierte Initiator-Caspasen (wie Caspase-8 und -9) lösen durch Spaltung von Effektor-Caspasen die Aktivierung einer Caspase-Kaskade aus, die schlussendlich zur Spaltung von spezifischen Todessubstraten führt (Strasser et al., 2000; Thornberry und Lazabnik, 1998; Salvesen und Dixit, 1997). Die Proteolyse dieser Proteine ist wiederum verantwortlich für die charakteristischen morphologischen und biochemischen Veränderungen der apoptotischen Zelle, wie die Kondensation des Chromatins, die Fragmentierung der DNA und das Auflösen der Zellen in membranumschlossene Vesikel, den „apoptotic bodies“, welche anschließend von Makrophagen und Nachbarzellen durch Phagozytose beseitigt werden (Callahan et al., 2000; Rathmell et al., 1999; Enari et al., 1998; Arends und Wyllie, 1991).

Die Apoptose kann generell über verschiedene Mechanismen eingeleitet werden. Der intrinsische Signalweg wird charakterisiert durch eine Permeabilisierung der Mitochondrienmembran und einer anschließenden Freisetzung von Cytochrom C welches mit APAF-1 (apoptotic protease activating factor-1) das sogenannte Apoptosom bildet (Zamzani und Kroemer, 2001). In diesem Protein-Komplex kommt es zur autokatalytischen Pro-Caspase-9 Prozessierung, wodurch die Caspase-Kaskade und somit die Apoptose initiiert wird (Danial und Korsmeyer, 2004; Korsmeyer et al., 2000; Zou et al. 1999).

Der extrinsische apoptotische Signalweg wird dagegen durch die Aktivierung von Todesrezeptoren vermittelt. Zu den bekanntesten Rezeptoren zählen CD95, die TRAIL- (Tumor necrosis factor-related apoptosis-inducing ligand) Rezeptoren und

der TNF-R1 (tumor necrosis factor receptor 1), die allesamt zur TNF-Rezeptor-Superfamilie gehören. Sie besitzen, neben einer cysteinreichen extrazellulären Domäne, eine hoch homologe intrazelluläre Sequenz, die als „Todesdomäne“ (death domain, DD) bezeichnet wird (Schmitz et al., 2000; Ashkenazi et al., 1998). Nach Bindung des jeweiligen entsprechenden Liganden kommt es zur Multimerisierung des Rezeptors und zur Ausbildung des DISC (death-inducing signalling complex), der Proteine enthält, welche durch proteolytische Spaltung die Aktivierung der Caspase-Kaskade ermöglichen (Peter und Krammer, 2003; Muzio et al., 1998).
Natürliche Gegenspieler des programmierten Zelltodes sind die anti-apoptotischen Proteine, die das apoptotische Signal entweder abschwächen oder sogar vollständig blockieren können (Salvesen und Duckett, 2002; Krueger et al., 2001; Deveraux et al., 1999). Während FLIP (FLICE inhibitory protein) die Aktivierung von Initiator-Caspasen an Todesrezeptoren, wie Caspase-8 inhibiert, hemmen die Mitglieder der IAP- (inhibitor of apoptosis protein) Familie vorwiegend Effektor-Caspasen beider apoptotischer Signalwege (Salvesen und Duckett, 2002; Krueger et al., 2001; Deveraux et al., 1999).

3.2 Intrazelluläre Signalwege des TNF-Rezeptor 1

Der TNF-R1 wird konstitutiv auf der Oberfläche vieler Zellen exprimiert. Nach Bindung seines Liganden TNF ist TNF-R1 nicht nur in der Lage, den programmierten Zelltod auszulösen, sondern auch die Aktivierung eines Signalwegs zu initiieren, der das Überleben der Zelle fördert. Die Art der TNF-induzierten Zellantwort wird dabei durch die Zusammensetzung der an den Rezeptor rekrutierten Protein-Komplexe bestimmt (Micheau und Tschopp, 2003).
Durch die Bindung der Adaptorproteine TRADD (tumor necrosis factor receptor-associated death domain protein) und FADD (Fas associated with death domain) an den Rezeptor wird der DISC ausgebildet, woraufhin die Initiator-Caspase-8 autokatalytisch aktiviert und das apoptotische Signal, je nach Zelltyp, entweder direkt durch Spaltung von Effektor-Caspasen weitergeleitet, oder additiv durch Aktivierung des intrinsischen, mitochondrialen Signalwegs verstärkt wird (Korsmeyer et al., 2000; Scaffidi et al., 1999; Li et al., 1998) (Abb1). Im Gegensatz dazu, führt die Bildung eines Protein-Komplexes, der aus den Adaptorproteinen TRADD, den Kinasen RIP

(receptor interacting protein) und TRAF2 (tumor necrosis factor receptor-associated factor 2) besteht, zur Aktivierung von Transkriptionsfaktoren, wie NFκB (nuclear factor kappa B) (Baud und Karin, 2001; Hsu et al., 1996). Da NFκB die Transkription vieler Gene aufreguliert, die als Gegenspieler der Apoptose, wie cIAP, FLIP und Bcl-2, bekannt sind, fördert die Aktivierung dieses Signalweges die Überlebensfähigkeit der Zelle (Catz und Johnson, 2001; Wang et al., 1998; Kreuz et al., 2001).

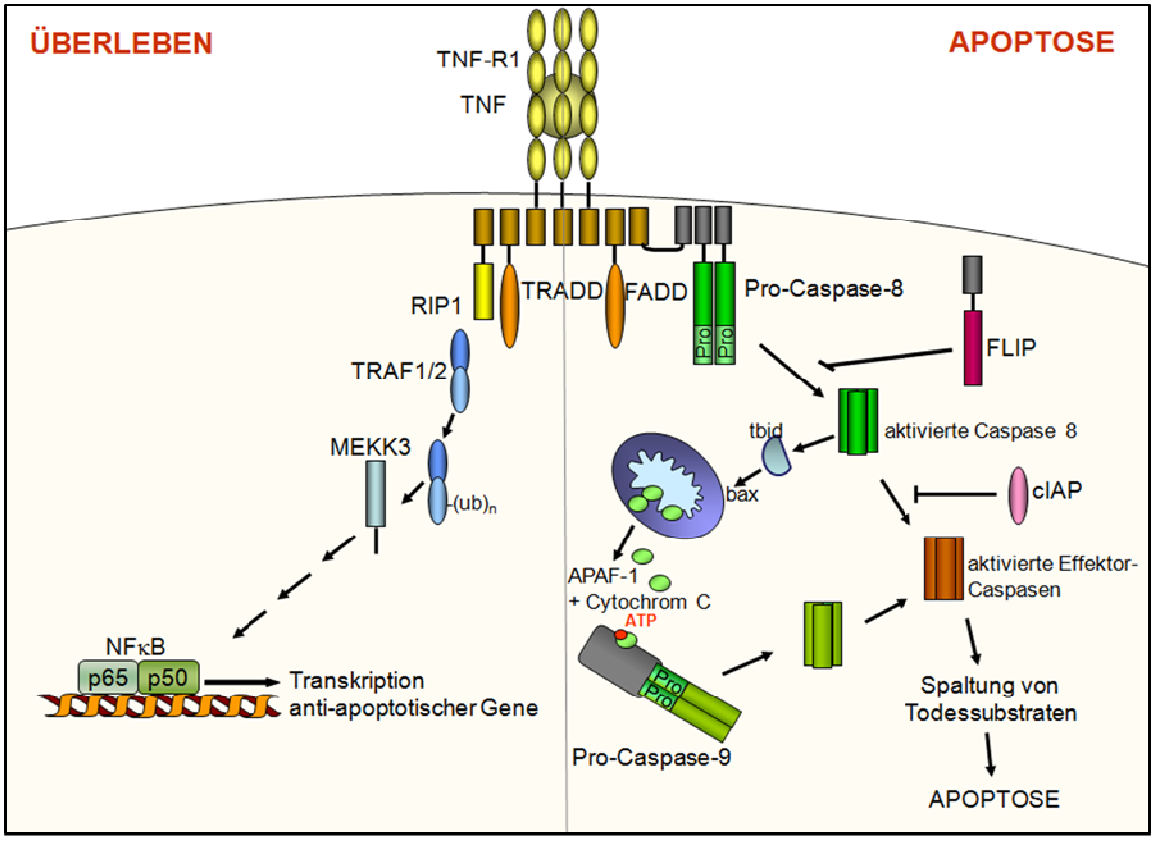

Abb-1: TNF-R1-vermittelte Signalwege
Der apoptotische Signalweg wird initiiert durch die Rekrutierung des Adaptorproteins FADD und der Pro-Caspase-8 an das primäre Adaptorprotein TRADD. Nach autokatalytischer Aktivierung von Caspase-8 werden Effektor-Caspasen aktiviert und das apoptotische Programm eingeleitet. In Folge einer Caspase-8-abhängigen Spaltung von bid, wird die Interaktion von tbid mit bax ermöglicht, wodurch eine Permeabilisierung der Mitochondrienmembran ausgelöst wird. Das freigesetzte Cytochrom C bildet mit APAF-1 das Apoptosom, ein Protein-Komplex, das die autokatalytische Aktivierung von Caspase-9 vermittelt, wodurch das apoptotische Signal verstärkt wird. Der NFκB-Signalweg wird dagegen durch die Bildung eines Protein-Komplexes an TRADD ausgelöst, der aus den Proteinen RIP und TRAF1/2 besteht.

3.3 Der Nukleäre Faktor kappa B

3.3.1 Strukturelle Eigenschaften von NFκB

Der Transkriptionsfaktor NFκB kontrolliert die Transkription einer Vielzahl von Genen, die an der Regulation von Entzündungsprozessen, Angiogenese, Proliferation und Apoptose beteiligt sind und die das Wachstum bzw. das Überleben der Zelle fördern (Karin und Lin, 2002; Li und Verma, 2002; Baldwin, 2001; Chen et al., 1999; Ghosh et al., 1998; Barnes und Karin, 1997) (Abb-2).

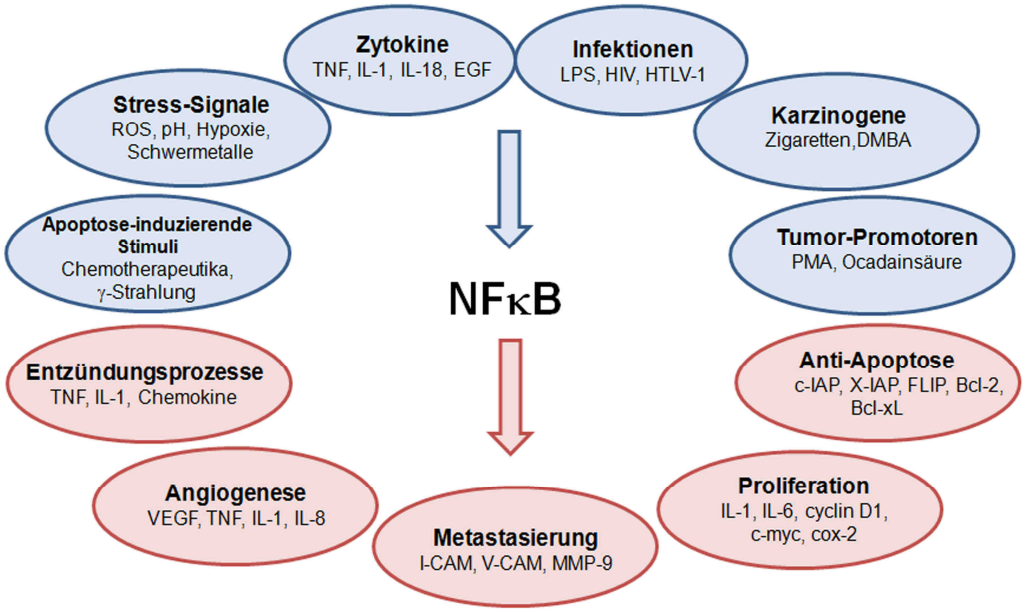

Abb-2: Überblick über die möglichen Induktoren des NFκB-Signalweges und dessen funktionelle Einflüsse auf die Zelle (Darstellung nach Aggarwal, Cancer Cell, 2004)

Die Familie der NFκB/Rel-Proteine umfasst fünf strukturell verwandte Mitglieder, p65 (RelA), p50, p52, RelB und c-Rel, die sich, je nach Funktion, zu einem homo- oder heterodimeren Transkriptionsfaktor NFκB verbinden. Das Heterodimer p65/p50 kommt dabei am häufigsten vor und wird allgemein mit dem Begriff NFκB in direkte Verbindung gebracht. Alle NFκB Proteine besitzen eine C-terminale Region, die Rel-Homologie-Domäne (RHD), welche für die Dimerisierung, die DNA-Bindung und die Interaktion mit inhibitorischen κB-Proteinen (IκB) verantwortlich ist. Zudem enthalten sie die Kernlokalisierungs-Sequenz (nuclear localization sequence, NLS), die

allerdings in unstimulierten Zellen durch die Interaktion mit IκBs verdeckt ist, wodurch NFκB in inaktiver Form im Zytoplasma lokalisiert ist (Ghosh et al., 1998; Jacobs und Harrison, 1998).
NFκB kann sowohl durch klassische, wie auch durch alternative Signalwege aktiviert werden, die durch eine Vielzahl von Stimuli, wie z.Bsp. entzündungsfördernde Zytokine (TNFα, Interleukin-1), virale und bakterielle Produkte, physikalischen (UV-Strahlung) und oxidativen Stress und einige Chemotherapeutika (Karin und Ben-Neriah, 2000) aktiviert werden (Abb-2).

3.3.2 Die Aktivierung von NFκB durch Interleukin-1

Interleukin-1 (IL-1), welches zu den wichtigsten pro-inflammatorischen Zytokinen gehört und eine bedeutende Rolle in akuten und chronischen Entzündungsprozessen spielt, vermittelt die klassische Aktivierung von NFκB (p65/p50) (Dinarello, 1997).
Die Bindung des IL-1 an seinen Rezeptor löst eine Signalkaskade von aktivierten Protein-Kinasen aus, die in nur wenigen Minuten zu einer Aktivierung des IKK-Komplexes (IκB kinase complex) führt (Abb-3). Dieser hochmolekulare Komplex besteht aus den zwei katalytischen Untereinheiten IKKα (IKK1), IKKβ (IKK2) und der regulatorischen Untereinheit IKKγ (NEMO) (Rothwarf et al., 1998; Yamaoka et al.; 1998; Zandi et al., 1997). Obwohl die Ser/Thr-Kinasen IKKα und IKKβ hohe Sequenz- und Struktur-Homologien aufweisen, gibt es Beweise dafür, dass vorherrschend IKKβ für die Aktivierung des NFκB im klassischen Signalweg verantwortlich ist (Li et al., 1999; Tanaka et al., 1999). Die Aktivierung von IKKβ erfolgt durch eine Phosphorylierung an Ser177/181, die neben der Kinase TAK1 im IL-1-Signalweg, durch eine Vielzahl anderer Kinasen, wie MEKK3, PKC, PKD, RIP und Akt, vermittelt werden kann (Sato et al., 2005; Hayden und Ghosh, 2004; Storz und Toker, 2003; Lallena et al., 1999). Die aktivierte Kinase IKKβ phosphoryliert den Inhibitor von NFκB, IκBα an zwei spezifischen Serin-Resten (Ser32/36), wodurch IκBα durch eine SCF^{bTrCP}-Ligase polyubiquitiniert und umgehend proteasomal degradiert wird (Winston et al., 1999; Yaron et al., 1998; Traenckner et al., 1995; Alkalay et al., 1995). Aufgrund der nun freiliegenden NLS transloziert NFκB in den Zellkern und bewirkt dort in Verbindung mit unterschiedlichen Ko-Aktivatoren die

Transkription von spezifischen Zielgenen, wie cIAP, FLIP und dem eigenen Inhibitor IκBα. Nach etwa 1,5 Stunden translozieren die neu-synthetisierten IκBα-Moleküle in den Zellkern und lösen durch die Bindung an NFκB die Dissoziation des Transkriptionsfaktors von der DNA und den Rücktransport ins Zytoplasma aus (Sachdev et al., 1998; Arenzana-Seisdedos et al., 1997). Die Aktivität von NFκB wird somit durch die Induktion einer negativen Rückkopplungsschleife terminiert (Sun et al., 1993).

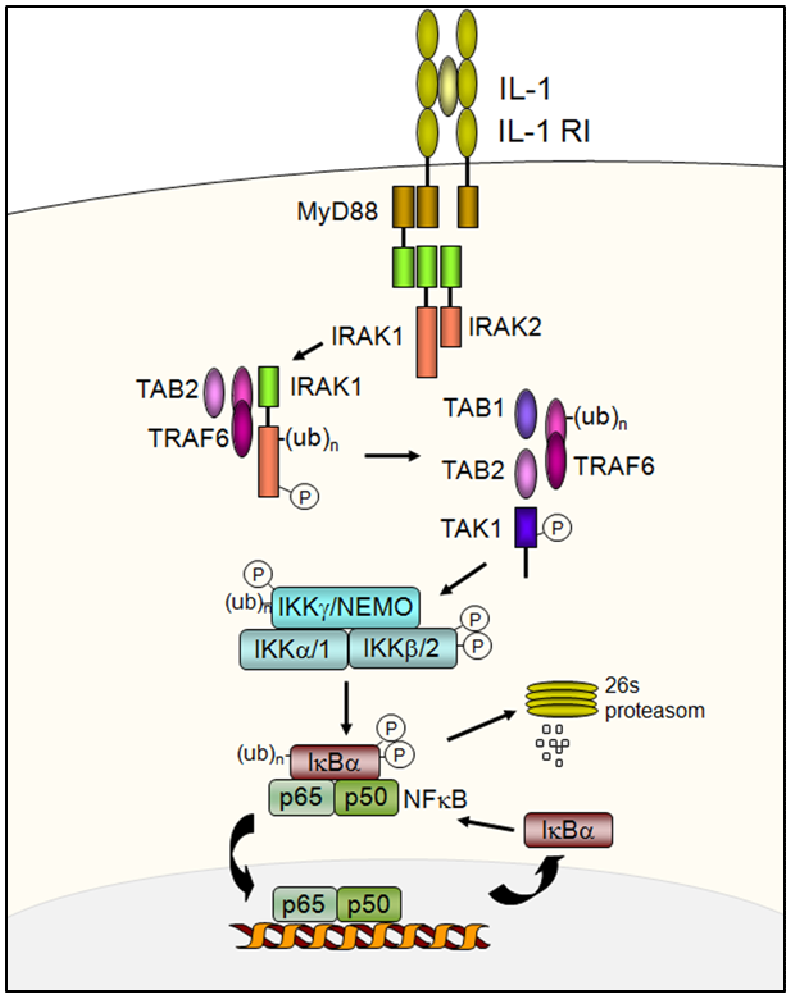

Abb-3: Schematische Darstellung der IL-1-induzierten klassischen NFκB-Aktivierung
Durch Aktivierung des IL-1-Rezeptors werden die Adaptorproteine MyD88 und IRAK an die intrazelluläre Domäne rekrutiert, woraufhin IRAK1 phosphoryliert wird, den Komplex verlässt und mit TRAF6 interagiert. Nach Polyubiquitinierung von TRAF6 und Rekrutierung von TAB2 wird die Kinase TAK1 aktiviert. TAK1 phosphoryliert eine Untereinheit des IKK-Komplexes, IKKβ, wodurch die Kinase aktiviert wird und die Phosphorylierung von IκBα katalysiert. Im Anschluss daran, wird IκBα polyubiquitiniert und proteasomal degradiert, wodurch NFκB in den Zellkern transloziert und die Transkription seines eigenen Inhibitors induziert. Re-synthetisiertes IκBα beendet daraufhin durch Bindung an NFκB dessen Aktivität im Zellkern.

3.3.3 Die Regulation der NFκB-Aktivität

Da NFκB die Transkription von vielen Genen verstärkt, die an der Regulation von Entzündungsprozessen beteiligt sind, welche die Proliferation der Zelle fördern und die Apoptose inhibieren, wird eine Fehlregulation der NFκB Aktivität mit einer Vielzahl von Krankheiten wie Multipler Sklerose, Rheumatoider Arthritis, entzündlichen Darmerkrankungen und der Entstehung von Krebs in Verbindung gebracht (Satoh et al., 2007; Li und Verma, 2002; Karin und Ben-Neriah, 2000; Feldmann et al., 1996). Aus diesen Gründen bildet die Erforschung der molekularen Mechanismen der NFκB-Regulierung seit Jahren einen Schwerpunkt vieler Studien.
Die Ubiquitin-Proteasen CYLD (cylindromatosis protein) und A20 sind als wichtige Regulatoren des NFκB-Signalwegs bekannt. Während CYLD durch De-ubiquitinierung von TRAF-Proteinen die Aktivierung von NFκB verhindert, löst A20, durch seine Funktion als Ubiquitin-Ligase und -Protease, die Degradierung von RIP aus, woraufhin die Aktivität von NFκB im TNF-Signalweg terminiert wird (Wertz et al., 2004; Kovalenko et al., 2003; Trompouki et al., 2003). Um eine konstitutive NFκB-Aktivität zu verhindern, ist, neben der Expression von negativen Regulatoren, vor allem auch die Internalisierung von Oberflächenrezeptoren essentiell, da sie für die Terminierung des aktivierenden Signals verantwortlich sind (Bonizzi et al., 1997).
Der IKK-Komplex nimmt eine bedeutende Rolle in der Regulation der Aktivität des Transkriptionsfaktors ein. So konnte gezeigt werden, dass durch post-translationale Modifikationen von IKKγ, wie Ubiquitinierung und Phosphorylierung, der IKK-Komplex stabilisiert und dessen Kinase-Aktivität reguliert werden kann (Sebban et al., 2006). Weiterhin wird vermutet, dass die Inaktivierung von IKKβ auf eine Autophosphorylierung der Helix-Loop-Helix (HLH) Region im C-terminalen Bereich zurückzuführen ist (Delhase et al., 1999). Im Gegensatz dazu weisen Ergebnisse anderer Studien daraufhin, dass möglicherweise Phosphatasen den Phosphorylierungs-Status und damit die Aktivität von IKKβ kontrollieren. In diesem Zusammenhang wird die Funktion der Ser/Thr-Phosphatase PP2A, welche an der Regulation vieler zellulärer Prozesse, wie der Signaltransduktion, dem Zellwachstum und der Protein-Synthese beteiligt ist, kontrovers diskutiert (Kray et al., 2005; Fu et al., 2003; Yang et al., 2001; DiDonato et al., 1997). PP2A existiert als Heterotrimer, das aus einer strukturellen A-, einer katalytischen C- und einer von verschiedenen regulatorischen B-Untereinheiten zusammengesetzt ist. Die Aktivität der

Phosphatase wir u.a. durch die Phosphorylierung an Tyr307, die durch Mitglieder der Src-Familie der Tyrosinkinasen induziert werden kann, reguliert (Chen et al., 1992).
Die Tyrosinkinase c-Src spielt in der Entwicklung und Progression von Tumoren eine wichtige Rolle. Die Kinase, die schon früh als Proto-Onkogen identifiziert wurde, ist in über 80% humaner Kolonkarzinomen überexprimiert und zeigt eine stark überhöhte Aktivität in vielen Arten von Krebs, wie Brust-, Lungen-, und Prostata-Krebs (Summy und Gallick, 2003; Frame, 2002; Talamonti et al., 1993). Da c-Src an der Aktivierung von Signalwegen, wie dem Ras/MAPK und PI3K/Akt-Signalweg beteiligt ist, die v.a. das Wachstum und das Überleben der Zelle fördern, kann eine konstitutive Aktivität der Tyrosinkinase eine Invasion und Metastasierung von Krebszellen begünstigen (Benati und Baldari, 2008; Yarden und Sliwkowski, 2001).
Interessanterweise scheint c-Src auch an der Aktivierung von NFκB beteiligt zu sein. Während Huang et al. vermuten, dass die Tyrosinkinase durch Aktivierung des IKK-Komplexes den klassischen NFκB-Signalweg induziert, deuten andere Studien darauf hin, dass NFκB alternativ über eine Src-vermittelte Phosphorylierung von IκBα an Tyr42 aktiviert werden kann (Jalal und Kone, 2006; Fan et al., 2003; Beraud et al., 1999). Diese Hypothese wird unterstützt von der Beobachtung, dass der Protein-Tyrosin-Phosphatase-Inhibitor Orthovanadat (OVA) sowohl die aktivierende Tyr416-Phosphorylierung von Src, als auch die Tyrosin42-Phosphorylierung von IκBα stabilisieren und somit eine Aktivierung des Transkriptionsfaktors NFκB vermitteln kann (Roskoski, 2004; Fan et al, 2003; Mukhopadhyay et al., 2000). Der Verdacht liegt demnach nahe, dass eine unkontrollierte Aktivität von Src zusammen mit einer Src-vermittelten konstitutiven Aktivierung von NFκB das Risiko für die Entstehung entarteter Zellen und die Bildung maligner Tumore immens erhöhen könnte.

3.3.4 NFκB: ein Vermittler von anti- und pro-apoptotischen Zellantworten

Eine konstitutive Aktivität von NFκB spielt nicht nur bei der Entstehung von Krebs eine Rolle, sondern kann auch Resistenzen gegenüber der Behandlung von Tumorzellen mit diversen Therapeutika vermitteln (Li und Verma, 2002; Arlt et al., 2001; Kordes et al. 2000; Krappmann et al., 1999; Wang et al., 1999). In den letzten Jahren sind die Todesrezeptoren der TRAIL-Rezeptorfamilie und ihr Ligand TRAIL in

den Mittelpunkt vieler Studien gerückt. Da TRAIL in vielen Tumorzelllinien Apoptose induziert, während gesundes Gewebe weitgehend verschont bleibt, gilt es als ein viel versprechender Kandidat für die tumorselektive Krebstherapie (Wajant et al., 2002; Walczak et al., 1999). Berücksichtigt man allerdings, dass Tumorzellen *in vivo* häufig von Immunzellen umgeben sind, die pro-inflammatorische Zytokine, wie IL-1, sezernieren, könnte die Rezeptor-induzierte Apoptose durch eine gleichzeitige NFκB-Aktivierung beeinträchtigt werden. Tatsächlich konnte gezeigt werden, dass Epithelzellen und transformierte Keratinozyten, aufgrund der IL-1-vermittelten NFκB-Aktivierung, resistent gegenüber TRAIL-induzierter Apoptose werden (Kothny-Wilkes et al., 1998). Wie erwartet, wurde diese Resistenz durch die NFκB-abhängige Aufregulierung von Genen, die für die anti-apoptotischen Proteine FLIP und cIAP kodieren, ausgelöst (Kothny-Wilkes et al., 1998; Kothny-Wilkes et al., 1999). Hierbei scheint es sich aber nicht um einen allgemeingültigen Mechanismus zu handeln, da eine durch UVB-Strahlung ausgelöste Apoptose durch die Zugabe von IL-1 nicht reduziert, sondern im Gegensatz dazu noch deutlich verstärkt wird (Kothny-Wilkes et al., 1999; Pöppelmann et al., 2005).

Die UVB-induzierte Apoptose kann generell auf mehreren Wegen induziert werden. Neben einer Liganden-unabhängigen Aktivierung von Todesrezeptoren und die Bildung von reaktiven Sauerstoff-Spezies, die den intrinsischen apoptotischen Signalweg aktivieren können, wird die Apoptose hauptsächlich durch UVB-vermittelte Schädigungen der DNA ausgelöst (Kulms et al., 1999 und 2002). Mittlerweile konnte gezeigt werden, dass das Transkriptionsverhalten von NFκB durch DNA-Schäden verändert werden kann. So wurde jüngst beobachtet, dass in Folge einer Stimulation von Zellen mit DNA-schädigenden Agenzien, wie Doxorubicin und UVC-Strahlung, die NFκB-abhängige Transkription von anti-apoptotischen Genen deutlich vermindert ist. Diese Repression war auf eine alternative ARF-induzierte und ATR-, CHK-1-abhängige Phosphorylierung von NFκB/p65 zurückzuführen, wodurch die Bindung von p65 an identischen Promotoren mit Ko-Repressoren, wie HDAC1 (Histon Deacetylase1) anstelle von Aktivatoren (Histon-Acetyltransferase, HAT) vermittelt wurde (Campbell et al., 2004, Rocha et al., 2003 und 2005). Nach einer Ko-Stimulation von Zellen mit UVB und IL-1 kommt es dem zufolge zu einer NFκB-abhängigen signifikanten Repression von anti-apoptotischen Genen, wie cIAP und FLIP, und ebenfalls von Genen, die für die TRAF-Proteine TRAF-1, -2 und -6

kodieren, die unter klassischen Bedingungen durch NFκB aktiviert werden würden (Pöppelmann et al., 2005).

Des Weiteren ist die NFκB-abhängige Sezernierung des Zytokins TNF von entscheidender Bedeutung für die Verstärkung der UVB-induzierten Apoptose. Sezerniertes TNF induziert autokrin oder parakrin die Aktivierung des TNF-R1 auf der Zelloberfläche. Aufgrund der NFκB-abhängigen Repression anti-apoptotischer Proteine und TRAF-Proteine, wird ausgehend vom TNF-R1, anstelle der Aktivierung von NFκB, nun der extrinsische apoptotische Signalweg eingeleitet, wodurch die UVB-induzierte Apoptose additiv verstärkt wird (Pöppelmann et al., 2005) (Abb-4).

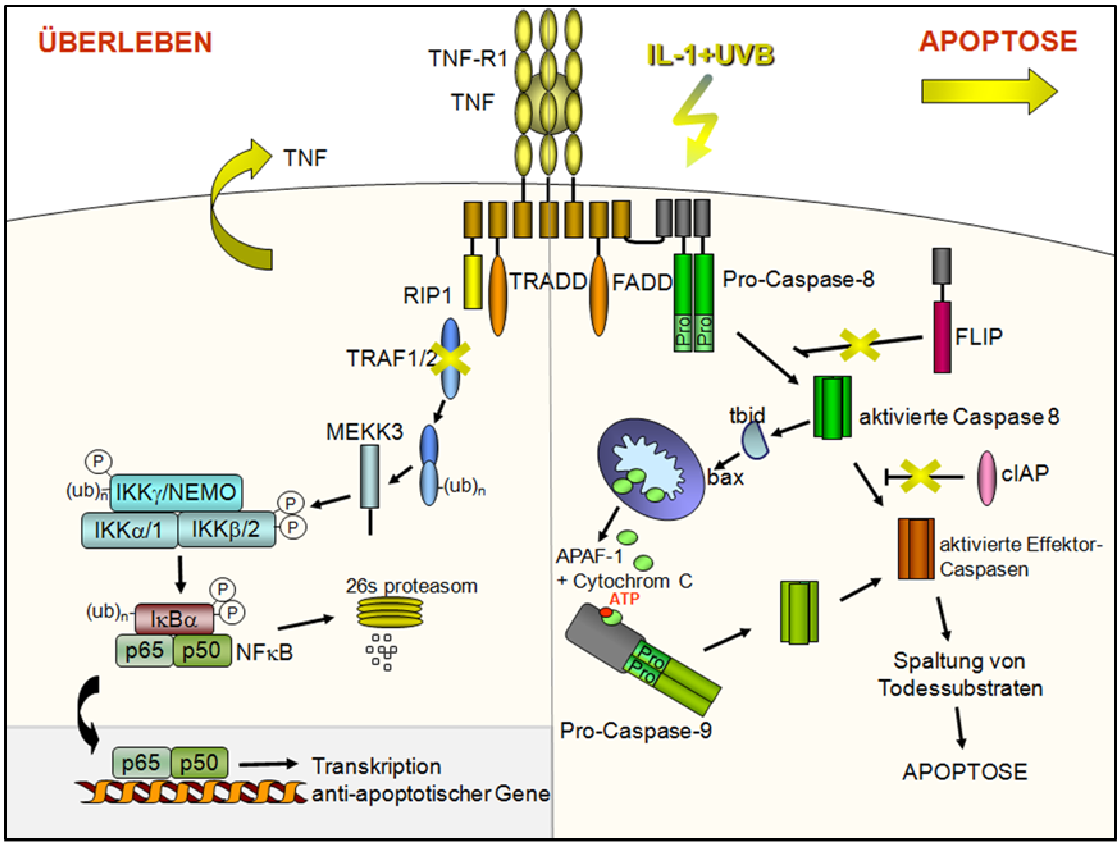

Abb-4: Verstärkung der UVB-induzierten Apoptose durch autokrine Aktivierung des TNF-R1. Durch die NFκB-abhängige Repression von TRAF und anti-apoptotischer Gene nach Ko-Stimulation mit IL-1+UVB, wird das Gleichgewicht zwischen anti- und pro-apoptotischen Signalwegen zugunsten der apoptotischen Signaltransduktion verschoben (nach Pöppelmann et al., 2005).

Obwohl die Repression der anti-apoptotischen Gene durch eine alternative Interaktion von NFκB mit HDACs erklärt werden konnte, sind die Mechanismen für

die NFκB-abhängige massiv erhöhte Expression und Sekretion von TNF noch unklar (Campbell et al., 2004).
Es gibt Hinweise darauf, dass die Freisetzung von TNF nach Stimulation mit IL-1 und UVB durch den UVB-induzierten DNA-Schaden vermittelt sein könnte. So konnte jüngst gezeigt werden, dass eine Sekretion von TNF nach Stimulation von Zellen mit IL-1 nur in Verbindung mit DNA-schädigenden Stimuli auftritt, die in ähnlicher Weise wie UVB, DNA-Basen-Modifikationen hervorrufen (Strozyk et al., 2006). Die Ursache für die gesteigerte Sekretion von TNF konnte allerdings noch nicht geklärt werden. Da *tnf* ebenfalls ein NFκB responsives Gen ist, könnte man vermuten, dass eine verstärkte NFκB-abhängige Transkriptionsleistung dafür verantwortlich sein könnte.

Das Ziel der vorliegenden Arbeit war es, die molekularen Mechanismen der IL-1-vermittelten Verstärkung der UVB-induzierten Apoptose im Detail aufzuklären. Dabei stand die Beantwortung der Frage, welche molekularen und/oder zellulären Veränderungen für die NFκB-abhängige Aufregulierung und Sezernierung von TNF verantwortlich sind, im Vordergrund. Darüber hinaus sollten genauere Erkenntnisse über die differenzielle Regulierung der NFκB-Aktivität im IL-1-Signalweg in der humanen Epithelzelllinie KB gewonnen, sowie weitere Modulatoren des Transkriptionsfaktors identifiziert werden. Im Hinblick auf die Entwicklung neuer differenzierter und spezifischerer therapeutischer Ansätze ist ein besseres Verständnis über das Zusammenspiel verschiedener apoptotischer Signalwege mit NFκB-Signalwegen unabdingbar. Dieser „cross talk“ sollte deshalb im Rahmen der vorliegenden Arbeit genauer aufgeschlüsselt werden.

4 Ergebnisse und Diskussion

Der Transkriptionsfaktor NFκB kontrolliert die Transkription einer Vielzahl von Genen, die v.a. an der Regulation der Proliferation, Entzündungsreaktionen und Apoptose beteiligt sind. NFκB ist vorherrschend dafür bekannt, dass es durch die Aufregulierung anti-apoptotischer Gene den apoptotischen Prozess entweder abschwächen oder sogar vollständig inhibieren kann. In diesem Zusammenhang konnte beobachtet werden, dass TRAIL-induzierte Apoptose durch eine IL-1-vermittelte NFκB-Aktivierung stark vermindert wird (Kothny-Wilkes et al., 1998 und 1999). Im Gegensatz dazu, wird eine durch UVB ausgelöste Apoptose durch Ko-Stimulation mit IL-1 nicht reduziert, sondern sogar noch verstärkt (Kothny-Wilkes et al., 1999). Obwohl in beiden Fällen der Transkriptionsfaktor NFκB eine entscheidende Rolle spielt, vermittelt seine Aktivierung völlig unterschiedliche Zellantworten.

Es konnte gezeigt werden, dass der Erhöhung der UVB-induzierten Apoptose eine massive Sezernierung von TNF und eine NFκB-abhängige Repression von Genen, die für die anti-apoptotischen Proteine cIAP, FLIP und die Proteine der TRAF-Familie kodieren, zugrunde liegt. Freigesetztes TNF kann im Anschluss an seine Sezernierung autokrin oder parakrin an TNF-R1 binden und aufgrund der reduzierten Proteinmenge von cIAP, FLIP und den TRAF-Proteinen, additiv Apoptose induzieren (Kothny-Wilkes et al., 1999; Pöppelmann et al., 2005).

Es gibt einige Studien, die belegen, dass eine TNF-vermittelte Aktivierung des apoptotischen Signalweges mit einer Internalisierung des TNF-R1 einhergeht (Schneider-Brachert et al., 2004; Micheau und Tschopp, 2003). Die beobachtete Abnahme der TNF-R1-Expression auf der Oberfläche von Zellen nach IL-1+UVB-Stimulation, bestätigt die Ergebnisse früherer Arbeiten, dass, ausgehend vom TNF-R1, der apoptotische Signalweg aktiviert wird. Darüberhinaus konnte durch eine Inhibierung der TNF-vermittelten Signaltransduktion, durch Blockierung des TNF-R1 mithilfe eines antagonistischen Antikörpers gezeigt werden, dass die Verstärkung der UVB-induzierten Apoptose tatsächlich TNF vermittelt ist (Pöppelmann et al., 2005; Kothny-Wilkes et al., 1999; Barisic et al., 2008, siehe Manuskript I)

Die massive Freisetzung von TNF spielt somit eine wichtige Rolle in der NFκB-vermittelten pro-apoptotischen Zellantwort. Ein Ziel dieser Arbeit war es daher, die

Mechanismen, die für beobachtete NFκB-abhängige massive Sezernierung von TNF verantwortlich sind, aufzuklären.

Generell wird die Aktivität von NFκB durch die Induktion einer negativen Rückkopplungsschleife reguliert und zeitlich begrenzt. So leitet die Stimulation von Zellen mit IL-1 alleine oder in Kombination mit TRAIL innerhalb weniger Minuten die initiale Degradierung des NFκB-Inhibitors IκBα durch das Proteasom ein, wodurch NFκB aktiviert und die Transkription von IκBα NFκB-abhängig initiiert wird. Nach etwa 1,5 Stunden liegt resynthetisiertes IκBα-Protein wieder in der Zelle vor und beendet durch eine erneute Bindung an NFκB dessen Aktivität (Sachdev et al., 1998; Winston et al., 1999; Yaron et al., 1998; Traenckner et al., 1995; Alkalay et al., 1995). Überraschenderweise konnte nach einer Ko-Stimulation von Zellen mit IL-1 und UVB keine Resynthese von IκBα beobachtet werden. In diesem Zusammenhang konnte festgestellt werden, dass die daraus resultierende konstitutive Aktivierung von NFκB, für die über Stunden währende hohe Transkriptionsrate von TNF verantwortlich ist und somit die Voraussetzung für die spätere massive Sezernierung dieses pro-apoptotischen Zytokins bildet. Weiterhin konnte die beobachtete Inhibierung der negativen regulatorischen NFκB-IκBα-Rückkopplungsschleife nicht etwa auf eine NFκB-abhängige reduzierte Transkriptionsrate von IκBα, sondern auf eine sofortige proteasomale Degradierung von neu-synthetisierten IκBα-Molekülen zurückgeführt werden (Barisic et al., 2008, siehe Manuskript I).

Ein Abbau von IκBα kann generell durch verschiedene Signale eingeleitet werden. Neben der Phosphorylierung von IκBα an den klassischen Ser32/36 durch IKKβ im IL-1-Signalweg, kann die Degradierung des Proteins grundsätzlich auch durch eine alternative Phosphorylierung an Tyr42 oder sogar unabhängig von diesen Phosphorylierungen, wie z.Bsp. infolge von UV-Strahlung, ausgelöst werden (Fan et al., 2003; Kato et al., 2003; Traenckner et al. 1995). Eigene Untersuchungen ergaben, dass im Fall der inhibierten Resynthese, der proteasomale Abbau von IκBα nicht etwa durch alternative Mechanismen, sondern klassisch durch eine kanonische IKKβ-vermittelte Ser32/36 Phosphorylierung eingeleitet wird.

Generell ist die Kinase IKKβ nach Stimulation mit IL-1 nur sehr transient an Ser177/181 phosphoryliert und damit aktiviert, um die Stabilisierung von resynthetisiertem IκBα und somit die Inaktivierung von NFκB zu ermöglichen. Im Gegensatz dazu, führt eine zusätzliche Bestrahlung von Zellen mit UVB zu einer

permanenten Phosphorylierung und Aktivierung von IKKβ und damit zu einer kontinuierlichen Phosphorylierung und proteasomalen Degradierung von neu-synthetisiertem IκBα.

Die Ser/Thr-Kinase IKKβ vermittelt in vielen Signalwegen die Aktivierung des Transkriptionsfaktors NFκB und gilt deshalb als eine zentrale Kontrollstelle. In diesem Zusammenhang wurde eine Vielzahl von Kinasen identifiziert (TAK1, MEKK1, PKC, PKD, RIP, Akt), die durch Phosphorylierung von IKKβ an Ser177/181 deren Aktivierungsstatus beeinflussen (Sato et al., 2005; Hayden und Ghosh, 2004; Storz und Toker, 2003; Lallena et al., 1999; Nemoto et al., 1998). Da UVB-Strahlung generell die Aktivität von Molekülen beeinflussen kann, wäre es aus diesem Grund durchaus möglich, dass die permanente Aktivität von IKKβ durch eine zusätzliche UVB-vermittelte Aktivierung dieser IKKβ-Kinasen bedingt ist. Doch weder eine reduzierte Expression dieser Kinasen durch die Verwendung spezifischer siRNA, noch deren Inhibierung durch Chemikalien, hatte einen Einfluss auf die Aktivität von IKKβ oder die Wiederkehr von IκBα nach Ko-Stimulation der Zellen mit IL-1 und UVB. Auch die Möglichkeit, dass die konstitutive IKKβ-Phosphorylierung durch eine verlängerte Aktivität des IL-1-Rezeptors ausgelöst werden könnte, wurde durch FACS-Analysen und Simulationen anhand eines mathematischen Modells ausgeschlossen (Witt et al., 2009, siehe Manuskript II).

Wenn nicht die zusätzliche Aktivität einer alternativen Kinase für die gesteigerte Aktivität von IKKβ verantwortlich ist, dann könnte die Inaktivierung einer entsprechenden Phosphatase durch UVB von entscheidender Bedeutung sein. In diesem Zusammenhang zeigte es sich, dass PP2A, eine Ser/Thr Phosphatase, die an der Regulation vieler zellulärer Prozesse, wie der Signaltransduktion und der Proteinsynthese beteiligt ist, eine wichtige Rolle spielt. Es gibt viele Studien, die daraufhin hinweisen, dass PP2A als negativer Regulator die Aktivität von NFκB beeinflussen kann. Allerdings basieren diese Vermutungen meist nur auf der Beobachtung, dass Phosphatase-Inhibitoren, die vorwiegend die Aktivität von PP2A blockieren, die Phosphorylierung und Degradierung des NFκB-Inhibitors IκBα einleiten (Chen et al., 1995; Traenckner et al.,1995). Obwohl es einige Studien gibt, die PP2A mit einer Regulierung des IKK-Komplexes in Zusammenhang bringen, wird die Rolle der Phosphatase in diesem Prozess kontrovers diskutiert (Didonato et al., 1997).

So vermuten Kray et al., dass erst durch die Bindung von PP2A an den IKK-Komplex eine Signal-induzierte Aktivierung von IKKβ und somit die initiale Degradierung von IκBα ermöglicht wird. Im Gegensatz dazu, gehen andere Studien von einer PP2A-abhängigen negativen Regulierung des IKK-Komplexes aus. In diesem Zusammenhang konnte beobachtet werden, dass eine Interaktion von PP2A mit IKKγ, einer Untereinheit des IKK-Komplexes, mit einer Reduktion der Aktivität von IKKβ nach Stimulation von Zellen mit TNF einherging (Hong et al., 2007; Fu et al., 2003). Unsere Untersuchungen ergaben allerdings, dass PP2A nicht mit IKKγ, sondern kontinuierlich mit IKKβ assoziiert ist. Im Rahmen dieser Arbeit resultierte eine verminderte Expression von PP2A in Zellen, die nur mit IL-1 stimuliert wurden, in einer permanenten Phosphorylierung und Aktivierung von IKKβ *in vivo* und *in vitro* und in einer Degradierung von resynthetisiertem IκBα ohne zusätzlichen Einfluss durch UVB (Barisic et al., 2008, Manuskript I). Interessanterweise konnte unter diesen Bedingungen, ähnlich wie nach einer Stimulation mit IL-1+UVB, eine erhöhte Transkription von TNF und die Sezernierung des neu-gebildeten Proteins beobachtet werden. Die Tatsache, dass die Phosphatase-Aktivität von PP2A durch eine Bestrahlung mit UVB *in vitro* deutlich reduziert werden konnte, lässt die Vermutung zu, dass die IL-1+UVB-induzierte konstitutive NFκB-Aktivierung durch eine UVB-vermittelte Inhibierung von PP2A ausgelöst wird.

Aufgrund der Ergebnisse unserer Untersuchungen konnte PP2A nicht nur eindeutig als Regulator von NFκB im IL-1-Signalweg identifiziert, sondern auch dessen kritische Rolle in der IL-1-vermittelten Verstärkung der UVB-induzierten Apoptose aufgedeckt werden. Ausgehend von diesen Ergebnissen und mit Hilfe der mathematischen Modellierung wurde folgendes Modell zu den Mechanismen der NFκB-Regulierung im IL-1-Signalweg und die Auswirkung von UVB-Strahlen auf diesen Prozess entwickelt.

Die Phosphatase PP2A liegt schon in unstimulierten Zellen assoziiert an IKKβ vor und verhindert durch stetige Dephosphorylierung der Kinase eine möglicherweise unterschwellig auftretende Phosphorylierung und Aktivierung von IKKβ. Nach Bindung des Zytokins IL-1 an seinen Rezeptor, wird innerhalb nur weniger Minuten die Phosphorylierung von IKKβ massiv induziert, wodurch die kontinuierliche PP2A-vermittelte Dephosphorylierung phenotypisch überdeckt wird. Nach initialer, IKKβ-abhängiger proteasomaler Degradierung von IκBα und Aktivierung von NFκB, kommt

es zur Internalisierung des IL-1R, wodurch eine weitere, durch den Rezeptor-induzierte Phosphorylierung von IKKβ verhindert wird. Durch die andauernde stetige PP2A-vermittelte Dephosphorylierung von IKKβ wird daraufhin die stabile Resynthese von IκBα und damit die Inaktivierung von NFκB ermöglicht. Im Falle einer Ko-Stimulation von Zellen mit IL-1 und UVB bleibt, aufgrund der UVB-vermittelten Inhibierung von PP2A, IKKβ permanent aktiv, wodurch IκBα unmittelbar nach dessen Synthese wieder phosphoryliert und durch das Proteasom abgebaut wird. Aufgrund der ausbleibenden Terminierung der NFκB-Aktivität, verbleibt der Transkriptionsfaktor konstitutiv aktiviert im Zellkern.

Interessanterweise tritt eine vollständige Inhibierung der negativen regulatorischen NFκB-Rückkopplungsschleife, aufgrund eines proteasomalen Abbaus von neu-synthetisiertem IκBα, nicht nur nach Ko-Stimulation von Zellen mit IL-1 und UVB, sondern auch nach Zugabe von Orthovanadat (OVA), einem Tyrosin-Phosphatase-Inhibitor, auf. Obwohl eine daraus resultierende konstitutive Aktivierung von NFκB, auch mit einer Verstärkung der Transkription von TNF und einer Sezernierung des Proteins einhergeht, konnte keine signifikant erhöhte apoptotische Zellantwort, wie sie nach einer Stimulation mit IL-1+UVB auftritt, beobachtet werden (Anhang-1).
Dies dürfte einerseits darauf zurückzuführen sein, dass OVA kein typischer apoptotischer Stimulus für Epithelzellen ist und andererseits, dass vermutlich aufgrund eines fehlenden DNA-Schadens, wie er durch UVB induziert wird, die Transkription von Genen, die für die anti-apoptotischen Proteine cIAP und FLIP kodieren, nicht etwa vermindert sondern verstärkt wird (Anhang-2). Da in diesem Fall eine konstitutive NFκB-Aktivität ein Risiko für die Entstehung von Krebs darstellt, war ein weiteres Ziel dieser Arbeit, die Mechanismen, die für die OVA-IL-1-vermittelte permanente NFκB-Aktivität verantwortlich sind, aufzuklären.
Da die Regulierung von NFκB im klassischen Signalweg rein durch Serin- und Threonin-Phosphorylierung vermittelt wird und über eine Beteiligung von Tyrosin-Phosphorylierungen wenig bekannt ist, wäre es deshalb durchaus möglich, dass die fehlende Resynthese von IκBα nach OVA+IL-1-Stimulation, durch eine Aktivierung alternativer Signalwege vermittelt sein könnte.
Bis heute gibt es eine Vielzahl von Studien, die belegen, dass OVA die Aktivierung von NFκB in Zellen auslösen kann, ein Prozess, der unabhängig von den Ser32/36-

Phosphorylierungen, über eine alternative Phosphorylierung von IκBα an Tyr42 initiiert wird. Obwohl beobachtet werden konnte, dass diese Phosphorylierung die Dissoziation des Inhibitors IκBα von NFκB vermitteln kann, wird die OVA-induzierte Aktivierung des Transkriptionsfaktors meist durch eine proteasomale Degradierung des Tyrosin-phosphorylierten-IκBα ausgelöst (Mukhopadhyay et al., 2000; Imbert et al., 1996). Entgegen all dieser Studien, die zeigen, dass für die OVA-vermittelte NFκB-Aktivierung die Phosphorylierung von IκBα an Tyr42 essentiell ist, belegen unsere Untersuchungen eindeutig, dass diese für die beobachtete Inhibierung der negativen regulatorischen NFκB-IκB-Rückkopplungsschleife nach OVA+IL-1-Stimulation nicht von essentieller Bedeutung ist (Barisic et al., 2009, siehe Manuskript III).

Vielmehr konnte festgestellt werden, dass wiederum eine konstitutive Aktivität von IKKβ für die Phosphorylierung von resynthetisiertem IκBα an Ser32/36 und dessen Degradierung durch das Proteasom verantwortlich ist, ein Vorgang, der auch nach Stimulation von Zellen mit IL-1 und UVB auftritt.

Unter den potentiellen Kandidaten, die Tyrosin-abhängig eine zusätzliche Aktivierung von IKKβ initiieren können, ist die Tyrosinkinase c-Src die vielversprechendste, da sie die Induktion von NFκB auslösen und durch eine OVA-vermittelte Phosphorylierung an Tyr416 aktiviert werden kann (Roskoski, 2004). Auch unsere Untersuchungen haben bestätigt, dass die aktivierende Tyrosin-Phosphorylierung von Src nach Ko-Stimulation von Zellen mit OVA und IL-1 verstärkt wird.

Obwohl c-Src die Aktivierung von NFκB vorwiegend über IKK-unabhängige Mechanismen, wie der Phosphorylierung von IκBα an Tyr42, vermitteln kann (Jalal und Kone, 2006; Fan et al., 2003; Abu-Amer et al., 1998), gibt es einige Studien, die belegen, dass IKKβ in diesen Prozess involviert ist (Romashkova und Makarov, 1999). In diesem Zusammenhang konnten Huang et al. zeigen, dass durch eine Src-vermittelte Phosphorylierung von IKKβ an Tyr188/199 der IKKβ-abhängige proteasomale Abbau von IκBα eingeleitet werden kann. Weiterhin wurde beobachtet, dass c-Src für eine PKD- (Protein kinase D) vermittelte Aktivierung von IKKβ verantwortlich ist, ein Prozess, dessen genaue Mechanismen allerdings noch unklar sind (Storz und Toker, 2003).

Obwohl wir durch *knock-down*-Studien und die Verwendung von chemischen Inhibitoren zeigen konnten, dass keiner dieser bekannten Mechanismen für die

OVA+IL-1-vermittelte konstitutive Aktivierung von IKKβ und Degradierung von resynthetisiertem IκBα verantwortlich ist, deuteten die Ergebnisse dieser Studie eindeutig daraufhin, dass Src an diesem Prozess beteiligt ist. So resultiert eine Überexpression von dominant-negativem Src in einer partiellen Wiederkehr von IκBα nach Stimulation von Zellen mit OVA+IL-1. Darüberhinaus wurde beobachtet, dass die OVA-IL-1-vermittelte permanente Aktivität von IKKβ durch die Überexpression einer konstitutiv aktiven Form von Src sogar noch verstärkt werden kann (Barisic et al., 2009, siehe Manuskript III).

Die Suche nach möglichen Src-Substraten, welche die Aktivität von IKKβ beeinflussen könnten, führte schließlich erneut zur Ser/Thr-Phosphatase PP2A, die in unserer Studie als ein neuer und wichtiger Modulator von IKKβ im IL-1-Signalweg identifiziert wurde. PP2A kann durch eine Phosphorylierung an Tyr307, die interessanterweise durch Mitglieder der Src-Kinase Familie vermittelt werden kann, inaktiviert werden (Chen et al., 1992). Tatsächlich konnten wir durch *in vivo*- und *in vitro*-Studien zeigen, dass in Folge der Überexpression einer konstitutiv aktiven Form von c-Src, nicht nur die inaktivierende Tyr-Phosphorylierung von PP2A verstärkt, sondern auch deren Phosphatase-Aktivität deutlich reduziert wird. Bei einer Überexpression der dominant negativen Form von Src kommt es dabei zu gegensätzlichen Effekten (Barisic et al., 2009, Manuskript III).

Schlussfolgernd ergibt sich somit folgendes Modell der OVA-IL-1-vermittelten konstitutiven Aktivierung von NFκB: Durch die OVA-vermittelte Stabilisierung der aktivierenden Tyr416 Phosphorylierung der Tyrosinkinase c-Src, wird PP2A durch eine Src-abhängige Tyr307-Phosphorylierung inaktiviert, wodurch die IL-1-induzierte IKKβ-Ser177/181-Phosphorylierung und damit deren Aktivität verlängert wird. Folglich wird IκBα unmittelbar nach seiner Synthese an Ser32/36 phosphoryliert und durch das Proteasom abgebaut, worauf NFκB konstitutiv aktiv im Zellkern verbleibt und die Transkription von NFκB-abhängigen Zielgenen, wie cIAP und FLIP, verstärken kann.

Die Tyrosinkinase Src gilt als Proto-Onkogen und ist, wenn es zu dessen Dysregulation kommt, an der Aktivierung von Signalwegen, welche v.a. das Wachstum, die Proliferation und das Überleben der Zelle fördern, beteiligt (Frame et al., 2002; Bjorge et al., 2000; Thomas und Brugge, 1997). Eine unkontrollierte Src-

Aktivität spielt somit nicht nur eine bedeutende Rolle bei der Entstehung vieler Arten von Tumoren, sondern ist auch in Prozesse wie Invasion und Metastasierung von Krebszellen involviert (Benati und Baldari, 2008; Summy und Gallick, 2003). Da Src zudem noch die Aktivierung von NFκB induzieren kann, ist es durchaus möglich, dass das kanzerogene Potential von Src unter diesen Bedingungen erheblich erhöht werden kann. In der vorliegenden Arbeit konnte ein bisher unbekannter Mechanismus der Src-vermittelten NFκB-Aktivierung nachgewiesen werden, ein Prozess, in dem die Ser/Thr-Phosphatase PP2A involviert ist. PP2A wurde anhand unserer Studien als neuer und kritischer Regulator der IKKβ- und somit auch indirekt der NFκB-Aktivität im IL-1 Signalweg identifiziert. Da eine Inhibierung von PP2A eine konstitutive Aktivierung von NFκB zur Folge haben kann, könnte dies die maligne Transformation von Zellen begünstigen. Aufgrund dieser Beobachtungen, wäre PP2A als potentielles Zielmolekül für die Tumortherapie sehr interessant.

Das Gleichgewicht zwischen Überleben und Zelltod wird durch viele miteinander interagierende Signalwege beeinflusst. Die Regulation dieses Gleichgewichts erfordert dabei ein koordiniertes und präzises Zusammenspiel vieler pro-und anti-apoptotischer Faktoren. Störungen dieses Gleichgewichtes können u.a. die Entstehung neurodegenerativer Erkrankungen oder die Bildung und Progression von Tumoren begünstigen. Der Transkriptionsfaktor NFκB ist generell dafür bekannt, dass er die Transkription von Genen verstärkt, welche die Proliferation und die Überlebensfähigkeit der Zelle fördern (Karin und Lin, 2002; Baldwin et al., 2001). Eine unkontrollierte NFκB-Aktivität steht somit häufig in Zusammenhang mit der Entstehung von malignen Tumoren und der Entwicklung von Resistenzen von Tumorzellen gegenüber Chemotherapeutika (Li und Verma, 2002; Arlt et al., 2001; Kordes et al. 2000; Krappmann et al., 1999; Wang et al., 1999). Studien der letzten Jahre jedoch haben gezeigt, dass NFκB auch an der Vermittlung pro-apoptotischer Zellantworten beteiligt sein kann, und damit potentiell auch als Tumorsuppressor fungieren kann (Pöppelmann et al., 2005; Zheng Y. et al., 2001; Kothny-Wilkes et al., 1998 und 1999). Mittlerweile wurde eine Vielzahl von Signalwegen entdeckt, die eine Aktivierung von NFκB initiieren können. Dabei wird deutlich, dass ein Zusammenspiel vieler verschiedener Faktoren, wie die Gesamtheit der induzierenden Stimuli, der Zeitpunkt und der Zelltyp bestimmen, welche Gene

transkribiert werden und welche Zellantwort vermittelt wird. Durch die bedeutende Rolle von NFκB in vielen Krankheiten ist das Interesse an Inhibitoren von NFκB-Signalwegen immer noch sehr groß. Da NFκB unter bestimmten Bedingungen jedoch auch als Tumorsuppressor wirken kann, könnte infolge einer Inhibierung des Transkriptionsfaktors durch Therapeutika, die Entstehung oder das Wachstum von Tumoren in einigen Geweben sogar begünstigt werden. Für eine effiziente Tumortherapie ist es deshalb erforderlich, ein besseres Verständnis über die Zusammenhänge zwischen apoptotischen und NFκB-induzierenden Signalwegen und deren Regulierung zu gewinnen. Die vorliegende Arbeit liefert hierzu nicht nur essentielle neue Erkenntnisse über die molekularen Mechanismen der Regulation von NFκB im IL-1-Signalweg, sondern eröffnet somit auch neue Implikationen und mögliche molekulare Angriffspunkte für die moderne differentielle Krebstherapie.

I Identification of PP2A as a crucial regulator of the NFκB feedback loop: Its inhibition by UVB turns NFκB into a pro-apoptotic factor

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Abstract

Although NFκB usually exerts anti-apoptotic activity, upon activation by interleukin-1 (IL-1) it enhances ultraviolet-B radiation (UVB)-induced apoptosis. This paradoxical effect is associated with NFκB-dependent pronounced secretion of tumor necrosis factor α (TNF) which activates TNF-R1 in an autocrine fashion to enhance UVB-induced apoptosis. We demonstrate that sustained TNF transcription in UVB + IL-1-treated cells involves complete abrogation of the negative feedback loop of NFκB preventing IκBα resynthesis, hence allowing uncontrolled NFκB activity. We show that IκBα is not transcriptionally inhibited but resynthesized protein is immediately marked for degradation due to persistent IKKβ activity. Continuous IKKβ phosphorylation and activation is caused by UVB-mediated inhibition of the phosphatase PP2A. This study demonstrates that the cellular response to different NFκB activators may be converted to the opposite reaction when both stimuli act in concert. Our data sheds new light on the significance of negative feedback regulation of NFκB and identifies PP2A as the key regulator of this process.

Introduction

The transcription factor NFκB is involved in many cellular responses. It comprises 5 proteins: p50/p105, p52/p100, p65, c-Rel and RelB which exist as homo- and heterodimers, p65/p50 being the most abundant form. In unstimulated cells, NFκB is sequestered in the cytoplasm through interaction with the inhibitory protein IκBα which masks its nuclear localisation signal (1). NFκB (p65/p50) is mostly activated by pro-inflammatory mediators including TNF, IL-1 or LPS. Activated receptors mediate activation of a multi subunit IκB-kinase (IKK) complex consisting of IKKα, -β and -γ. Activated IKK acts through phosphorylation of IKKβ at Ser177/181, subsequently catalyzing phosphorylation of IκBα at Ser32/36, leading to its polyubiquitination and proteasomal degradation. Released NFκB translocates into the nucleus to activate responsive genes, among these the one encoding IκBα (2). Nuclear export of resynthesized IκBα is more potent than import, allowing cytosolic localisation of the inactive complex, thus creating a negative feedback loop (3).

Since NFκB serves many different functions, tight regulation by the negative feedback loop is crucial. Only highly controlled and transient expression of NFκB-driven genes ensures proper function. Uncontrolled NFκB activity is linked to transformation, proliferation, suppression of apoptosis, and metastasis (4;5). Thus, strategies interfering with signalling pathways activating NFκB have become major targets for anticancer interventions (6).

We have previously shown that stimulus dependent activation of NFκB can result in completely opposite effects. Stimulation with IL-1 protects keratinocytes and epithelial cells from cytotoxic effects of death ligands in an NFκB-dependent manner via up-regulation of anti-apoptotic cFLIP and cIAPs (7). In contrast, stimulation with IL-1 and UVB resulted in NFκB-dependent down-regulation of anti-apoptotic genes and simultaneous up-regulation of pro-apoptotic TNF. Both events act in concert to enhance the predominantly intrinsic pathway triggered by UVB through additional activation of the extrinsic pathway via TNF-R1 (8). NFκB-dependent repression of anti-apoptotic genes in response to DNA-damaging agents was shown to be facilitated by $p14^{ARF}$-induced association of p65 with the gene silencer histone deacetylase-1 (HDAC1) at different promoters (9), turning NFκB into a repressor of

anti-apoptotic genes via ARF-induced ATR- and CHK1-dependent phosphorylation of p65 (10;11).

One proposed mechanism is that UVB drives the pro-apoptotic effect of NFκB via general repression of anti-apoptotic genes. However, additional mechanisms exist, since NFκB upon IL-1 + UVB co-treatment significantly enhances TNF expression compared to IL-1 alone. Although a mechanism underlying UV-mediated repression of NFκB-regulated genes has been proposed, the mechanism by which UVB accelerates NFκB-dependent TNF transcription remains unclear. Here we show that activation of TNF gene by UVB in IL-1-treated cells relies on complete inhibition of the negative feedback loop involving IκBα resynthesis, hence allowing uncontrolled NFκB activity. Lack of IκBα production is not transcriptionally regulated but caused by persisting IKKβ activity, allowing continuous degradation of *de novo* synthesized IκBα. Enhanced activity of IKKβ is caused by UVB-mediated inhibition of phosphatase PP2A, a potential negative regulator of IKKβ (12). This study identifies UVB to directly affect the negative feedback loop thereby changing the physiological properties of NFκB. It also demonstrates that biologic effects induced by different NFκB activators may tremendously differ when both act in concert.

Results

Co-stimulation with IL-1 inhibits TRAIL- but enhances UVB-induced apoptosis via NFκB activation

Utilizing the epithelial cell line KB, co-stimulation with IL-1 was shown to desensitize cells from death receptor-induced apoptosis, whereas UVB-induced apoptosis was significantly enhanced due to NFκB-dependent TNF production and down-regulation of anti-apoptotic genes (8). EMSA revealed that differential NFκB responses (Fig. 1a and c) were neither due to changes in intensity of activation nor to activation of alternative NFκB activating pathways or to differences in heterodimer composition (Fig. 1b). Accordingly, investigating the molecular mechanism underlying these differential cellular responses to NFκB p50/p65 is of particular interest.

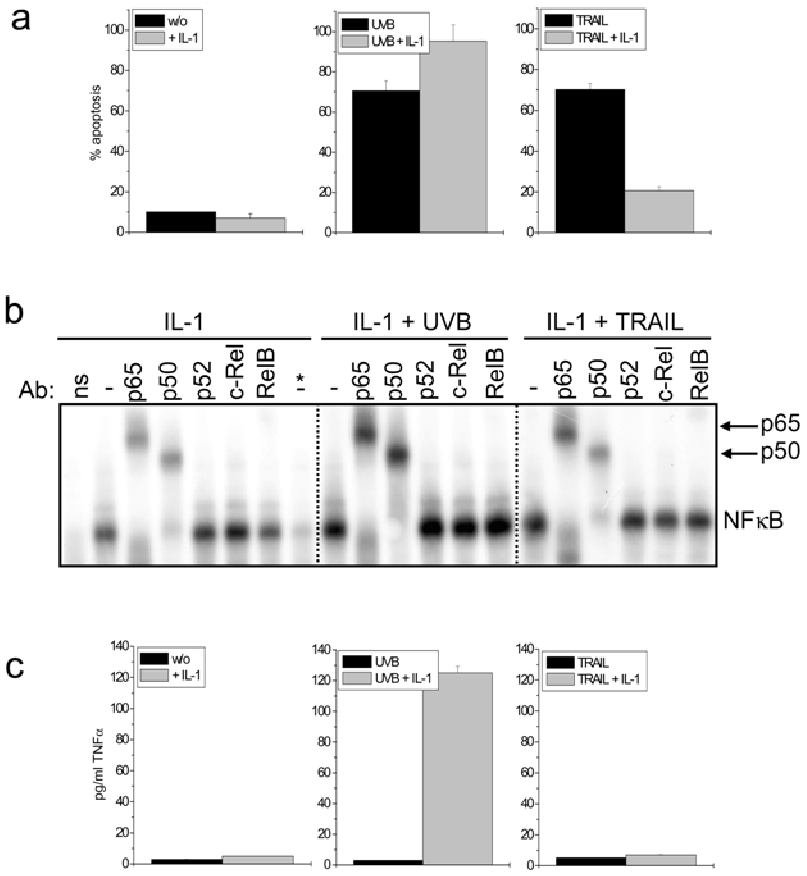

Figure 1 - IL-1 causes activation of the p65/p50 irrespective of the apoptotic co-stimulus. IL-1-induced enhancement of UVB-induced apoptosis coincides with TNF production. Cells were stimulated with IL-1, UVB, TRAIL, or co-stimulated with IL-1 and any of the apoptotic stimuli. **(a)** After 16 h apoptosis was determined in a CDD. **(b)** After identical stimulation for 30 min nuclear translocation of NFκB was analyzed by EMSA and heterodimer composition in a supershift assay using antibodies against p65, p50, p52, cRel and RelB. ns = non-stimulated cells. * = UVB irradiation alone. **(c)** TNF in supernatants of cells analysed in (A) was determined in a TNF ELISA.

IL-1 enhances UVB-induced apoptosis via autocrine TNF-TNF-R1 signalling

Semiquantitative PCR analysis confirmed that enhanced TNF secretion upon IL-1 + UVB stimulation (Fig. 1c) relies on sustained and elevated TNF transcription resulting in accumulation of TNF protein in supernatants over time, finally allowing autocrine TNF-R1 activation resulting in additive apoptotic signalling (Fig. 2a).

TNF-R1-mediated apoptosis but not NFκB-dependent, non-apoptotic responses depends on internalization of the receptor signalling complex (13;14). Accordingly, FACS analysis revealed a non-apoptotic TNF dose (5 ng/ml) to not result in

detectable loss of membrane TNF-R1 staining, whereas an apoptotic dose (100 ng/ml) caused a marked decrease of membrane TNF-R1 expression after 2 h, indicative of receptor internalization and induction of apoptotic pathways (Fig. 2b). Thus, TNF-R1 internalization should precede IL-1 + UVB-induced induction of autocrine TNF-mediated apoptosis. UVB exposure alone produced a slow and only partial reduction in TNF-R1 membrane levels, whereas IL-1 + UVB caused a rapid loss of TNF-R1 at 2 h, which further decreased after 4 h (Fig. 2b). Confirming the critical role of autocrine TNF signalling, IL-1-mediated enhancement of UVB-induced apoptosis was blocked with an antagonistic TNF-R1 antibody (15) (Fig. 2c).
Data show that enhancement of TNF secretion is largely regulated via persistent transcription of TNF gene suggesting sustained NFκB activation.

Inhibition of IκBα reappearance is responsible for sustained NFκB activity and elevated TNFα production

IL-1 stimulation typically results in early, but transient NFκB activation with maximum degradation of IκBα between 15 and 30 min. Rapid transcriptional activation of the IκBα gene and resynthesis of the protein result in replenishment of cytosolic IκBα after 1-2 h, finally terminating NFκB activity (3). This kinetics applies for KB cells upon co-treatment with IL-1 and TRAIL (Fig. 3a). In contrast, co-stimulation with IL-1 + UVB completely abrogated reappearance of IκBα protein in the cytoplasm for at least 4 h (Fig. 3a). At this time point cells are irreversibly committed to undergo apoptosis (data not shown). Correspondingly, EMSA revealed that high NFκB levels persisted in the nucleus upon IL-1 + UVB co-treatment for several hours, whereas upon IL-1 treatment, NFκB vanished from the nucleus with kinetics matching reappearance of IκBα in the cytoplasm (Fig. 3a, b). This suggests that prolonged production of TNF is regulated at the level of IκBα turnover. Therefore, we mimicked inhibition of IκBα resynthesis by pre-incubation of cells with cycloheximide (CHX). At a concentration higher than 1 μg/ml, CHX completely inhibited IκBα *de novo* protein synthesis after 2 h (Fig. 3c), when IκBα resynthesis is usually completed (Fig. 3a). CHX-mediated inhibition of IκBα recurrence caused elevated TNF transcription in IL-1 treated cells even 4 h after stimulation. At this time point TNF transcription had almost ceased in the absence of CHX (Fig. 3d) due to IκBα resynthesis and

termination of NFκB activity. These results support that inhibition of the negative feedback loop drives NFκB-dependent TNF production.

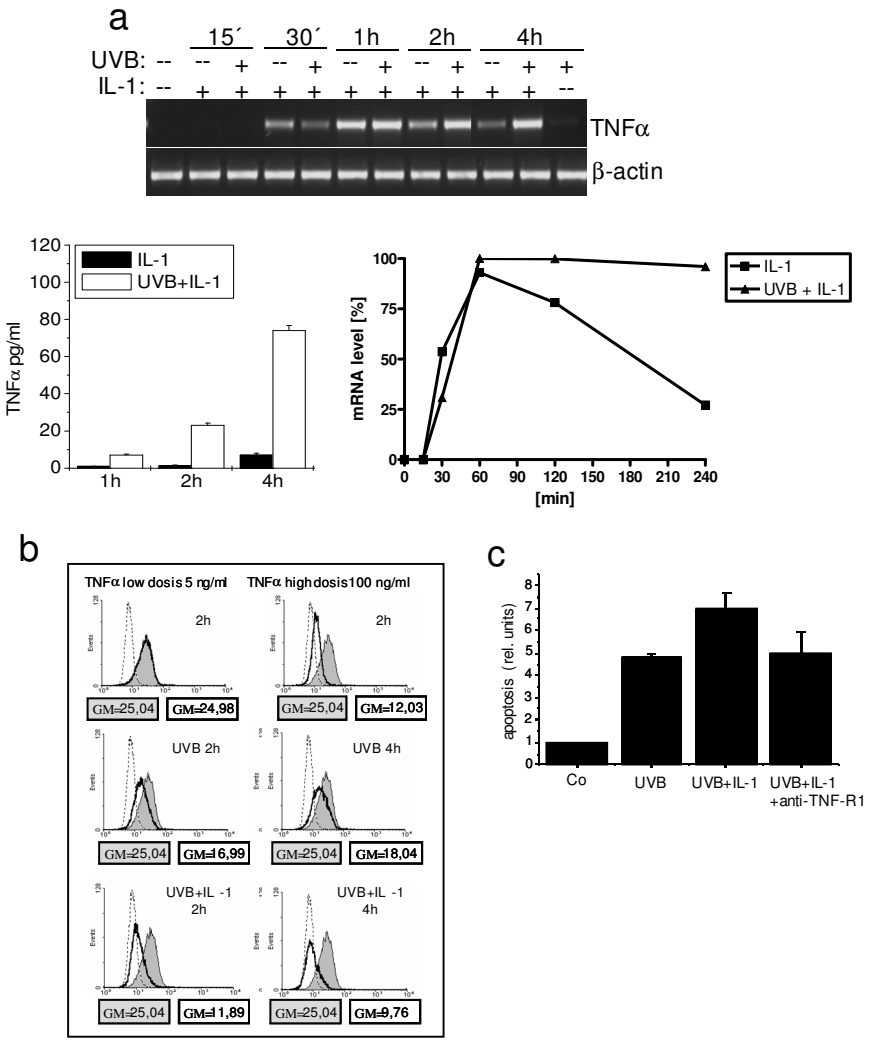

Figure 2 - Sustained transcription upon UVB + IL-1 causes release of TNF and autocrine activation of TNF-R. **(a)** Cells were stimulated with IL-1 or in combination with UVB as denoted. TNF transcription was determined by semiquantitative RT-PCR and analyzed densitometrically. Release of TNF to supernatants of the cells was determined in a TNF ELISA. **(b)** Cells were exposed to UVB, co-treated with IL-1 + UVB or stimulated with 5 ng/ml or 100 ng/ml TNF for 2 h. Cell surface expression of TNF-R1 was determined by FACS analysis. Dashed line presents isotype control. Grey shaded curve shows basic TNF-R1 expression (projected into every histogram) and the bold line TNF-R1 expression of treated cells. **(c)** Cells were stimulated with UVB or UVB + IL-1 with or without pre-incubation for 1 h with an antagonistic TNF-R1 antibody (H-398, 20 µg/ml). 16 h later apoptosis was measured by CDD-ELISA.

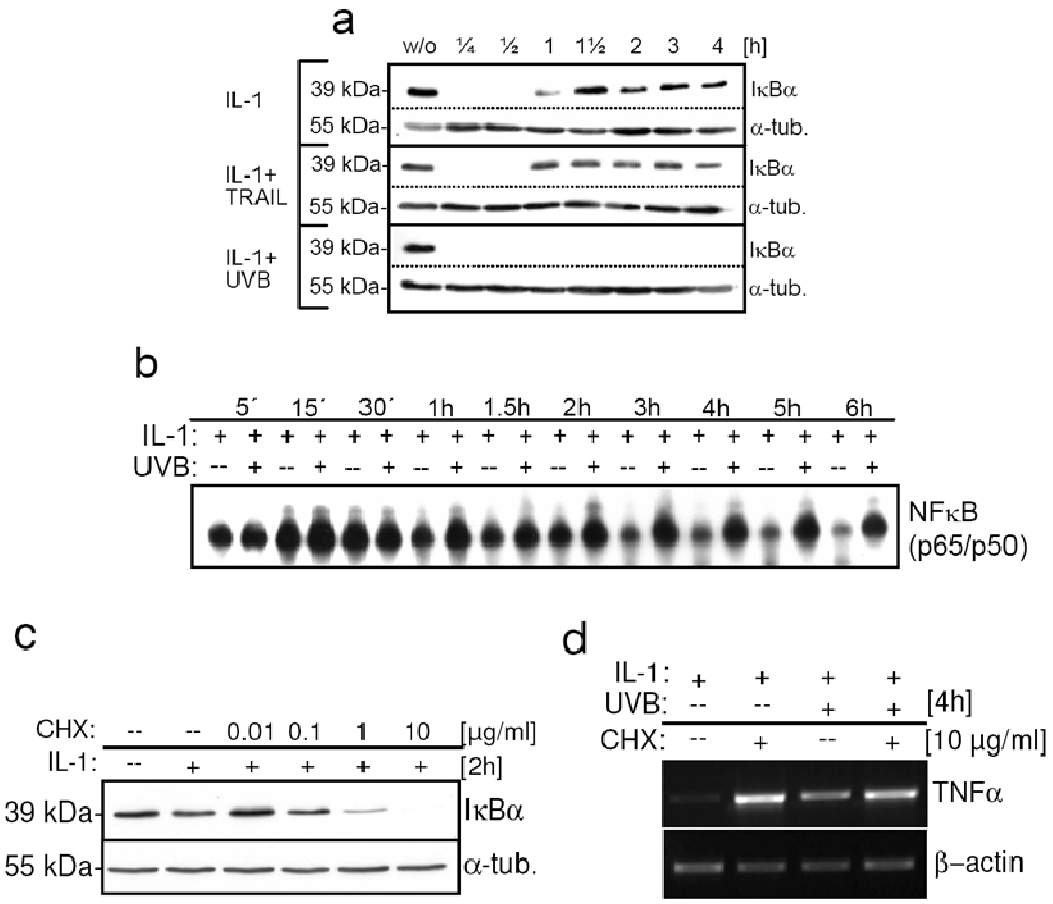

Figure 3 - Sustained TNF transcription is due to lack of IκBα resynthesis. **(a)** Cells were stimulated with IL-1, or in combination with either UVB or TRAIL. Cytosolic protein extracts were analysed for IκBα degradation and resynthesis by WB. **(b)** Cells were treated with IL-1 or co-stimulated with IL-1 + UVB as indicated. Nuclear localization of NFκB was monitored by EMSA. **(c)** Cells were pre-incubated with CHX for 1 h and stimulated with IL-1. 2 h later the IκBα status was documented by WB. **(d)** Cells were stimulated with IL-1 with or without UVB for 4 h. Resynthesis of IκBα protein was blocked by pre-treatment with CHX (10 µg/ml) for 1 h. Transcription efficacy of TNF was determined by semiquantitative RT-PCR.

Inhibition of IκBα reappearance is due to its immediate post-translational proteasomal degradation

Since NFκB can change from an activator to a repressor for selected genes (8), loss of IκBα reappearance might be regulated at the transcriptional level. However, IL-1-mediated transcription pattern of IκBα remained unchanged upon co-stimulation with UVB (Fig. 4a), suggesting that inhibition of IκBα reappearance is regulated post-transcriptionally, affecting either translation or protein stability.

Once designated by IKKβ-mediated phosphorylation IκBα is proteasomally degraded. Addition of proteasome inhibitor MG132 1 h before IL-1 or IL-1 + UVB stimulation, prevented initial IκBα degradation. Adding MG132 15 min after stimulation and initial IκBα degradation, resynthesized IκBα reappeared within the cytoplasm (Fig. 4b), indicating that translation was not impaired. Thus, failure to accumulate a critical level of IκBα sufficient to limit NFκB activity appears to result from immediate and continuous proteasomal degradation of newly synthesized protein. To scrutinize whether immediate IκBα degradation follows conserved patterns or is due to any alternative mechanism the Ser32/36 phosphorylation status of newly synthesized IκBα captured in the cytoplasm by MG132 application was analyzed. Corresponding to initial stimulation, resynthesized IκBα was phosphorylated at Ser32/36 marking it for degradation and causing sustained NFκB activation (Fig. 4c).

Assessing the role of IKKβ in continuous IκBα degradation, blocking its activity by the selective inhibitor BAY-11-7082 resulted in reappearance of IκBα in IL-1 + UVB treated cells (Fig. 4d), strongly suggesting constitutive IKKβ activation to be responsible for controlling IκBα reappearance. Since IKKβ requires phosphorylation at Ser177/181 for activation (2), we monitored IKKβ phosphorylation status (Fig. 4e). Initial IκBα degradation coincided with strong IKKβ Ser177/181 phosphorylation, irrespective of co-stimulation with UVB, indicating IL-1 to be the predominant trigger. IKKβ phosphorylation stayed stable for up to 1 h. After 2 h of IL-1 stimulation, IKKβ phosphorylation had completely vanished allowing stabilization of resynthesized IκBα. In contrast, IKKβ remained phosphorylated in cells co-stimulated with IL-1 + UVB after 2 h, preventing IκBα resynthesis. *In vitro* kinase assay with IKKβ immunoprecipitated from cells 2 h after co-stimulation with IL-1 + UVB, using a purified IκBα (5-55) fragment confirmed high IKKβ activity at this time point (Fig. 4f). Together, these data demonstrate that phosphorylation of resynthesized IκBα upon stimulation with IL-1 + UVB for 2 h is a direct downstream effect of continuous IKKβ phosphorylation and activity.

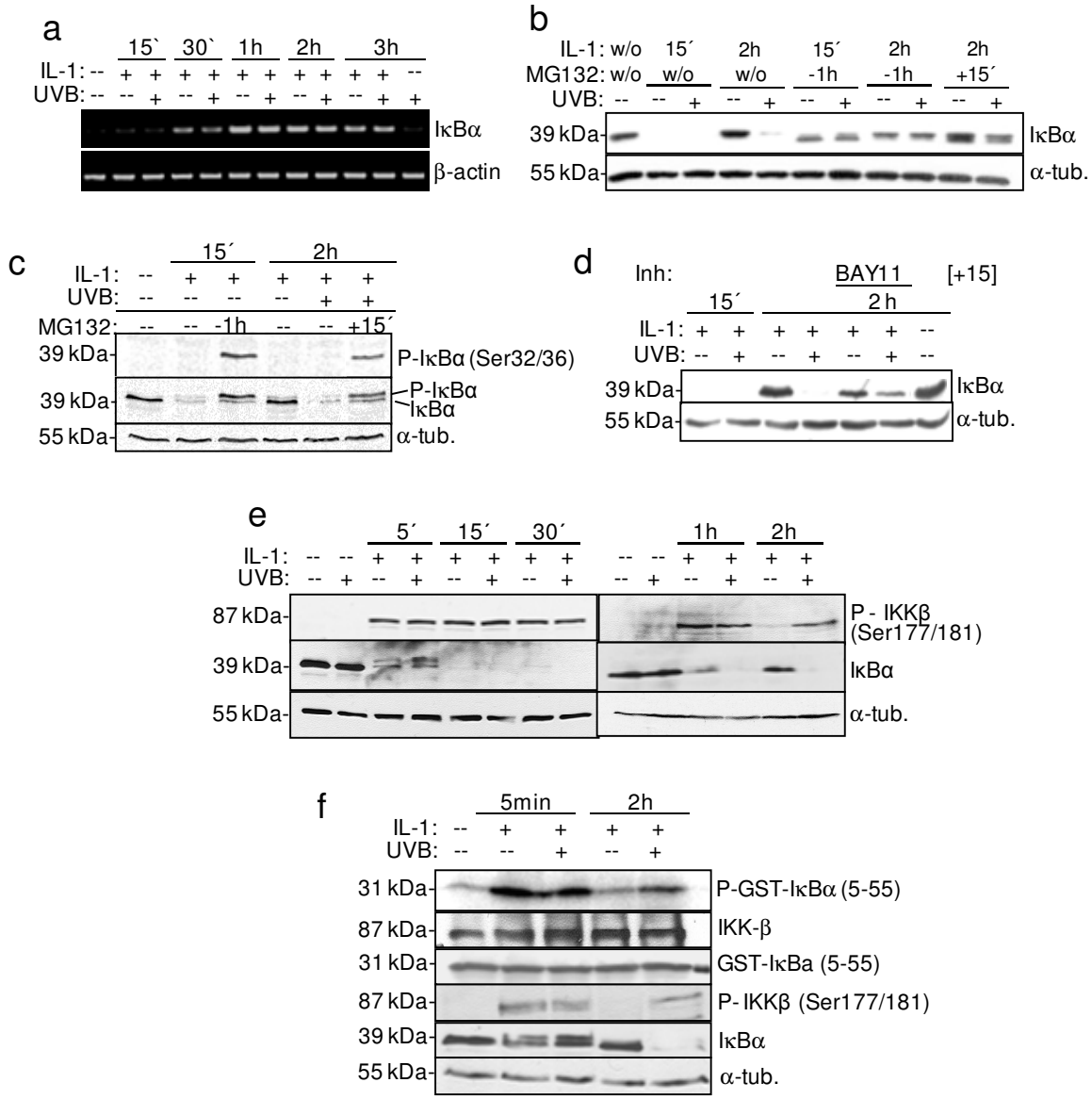

Figure 4 - Failure of IκBα resynthesis is due to continuous IKKβ phosphorylation. **(a)** Cells were stimulated with IL-1 or IL-1 + UVB. At the indicated time points IκBα transcription was analyzed by semiquantitative RT-PCR. **(b)** Cells were stimulated with IL-1 or co-stimulated with IL-1 + UVB. MG132 was added 1 h prior to (-) or 15 min after (+) IL-1/UVB stimulation. Cytosolic IκBα status was determined by WB. **(c)** Cells were stimulated with IL-1 or IL-1 + UVB. MG132 was added 1 h prior to (-) or 15 min after (+) IL-1/UVB stimulation. Specific Ser-Phosphorylation of IκBα was monitored and confirmed by reprobing the stripped membrane with an IκBα antibody. **(d)** Cells were stimulated with IL-1 or IL-1 + UVB as indicated. BAY-11-7082 (30 µM) was added 15 min following initial IL-1/UVB stimulation. Resynthesis of IκBα was detected by WB. **(e)** Cells were stimulated with IL-1 with or without UVB as annotated and the phosphorylation status of IKKβ as well as IκBα degradation determined by WB. **(f)** Cells stably expressing IKKβ-GFP were stimulated with IL-1 or IL-1 + UVB for 5 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, and phospho-IKKβ statuses were determined by WB.

Cooperation of IL-1 and UVB signalling is a prerequisite for enhanced apoptosis

Inhibition of IκBα reappearance seems to be the molecular trigger efficiently enhancing UVB-induced apoptosis. Since UV may induce NFκB activation itself (3;16), prolonged activation might result from additive IκBα degradation following the transient IL-1 signal. Investigating the kinetics of IκBα degradation and NFκB activity at 300 J/m^2 UVB used in this study, UVB alone caused a rather weak and delayed NFκB response after 10 to 16 h, coinciding with only partial IκBα degradation (Fig. 5a). However, at 2 h neither IκBα degradation nor NFκB translocation into the nucleus was detected, suggesting cooperative signalling.

Hence, we investigated the time window of cooperative signalling required to convert IL-1 signalling to pro-apoptosis by UVB. Cells pre-exposed for various length of time (15 min to 8 h) to IL-1 were irradiated with UVB. Alternatively, IL-1 was added after irradiation for identical time intervals (15 min to 8 h) and apoptosis measured 16 h following UVB. IL-1-mediated enhancement of apoptosis was recorded in a narrow window around UVB treatment, ranging from 30 min before to 1 h after irradiation (Fig. 5b, upper panel) and coincided with pronounced TNF release (Fig. 5b, lower panel). This confirms the close interrelationship between TNF production and IL-1-mediated acceleration of UVB-induced apoptosis, revealing critical temporal constraints of cooperative signalling.

To translate tight co-stimulation to interference with IκBα reappearance, cells were stimulated with IL-1 and harvested after 2 h, when cytosolic IκBα levels have regained pre-stimulation levels. Additionally, cells were UVB irradiated in intervals from 5 min up to 120 min prior to harvest (Fig. 5c). WB analysis revealed that IκBα reappearance was only inhibited when the time span between IL-1 and UVB stimulation did not exceed 30 min, closely matching the temporal constraints for TNF production and apoptosis (Fig. 5b).

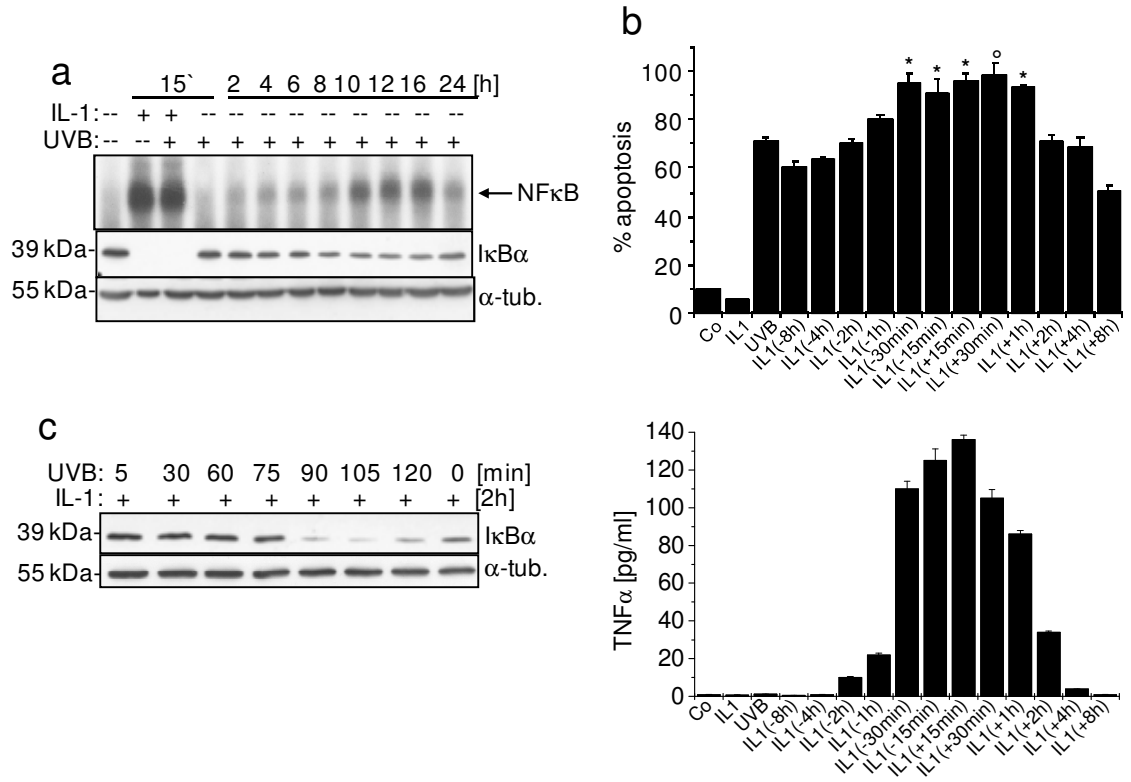

Figure 5 - Cooperation of IL-1 and UVB affects apoptosis, TNF production and inhibition of IκBα resynthesis. **(a)** Cells were stimulated with IL-1, UVB or with IL-1 + UVB for 15 min. Nuclear translocation of NFκB was determined by EMSA. In parallel, corresponding cytosolic IκBα status was determined by WB. **(b)** Cells were stimulated with UVB and co-treated with IL-1 for 8 h, 4 h, 2 h, 1 h, 30 min, 15 min before (-) as well as after (+) UVB exposure. 16 h after UVB exposure apoptosis was measured by CDD-ELISA and TNF secretion analyzed in a TNF ELISA. * significant $p < 0.05$; ° significant $p < 0.01$ **(c)** Cells were treated with IL-1 for 2 h and exposed to UVB at the indicated time points prior to cell harvest. Cytosolic IκBα status was determined by WB.

UVB-induced inhibition of PP2A is responsible for persistent IKKβ activity

We next examined how IKKβ phosphorylation is maintained in IL-1 + UVB co-treated cells. Screening for IKKβ upstream targets that might become either activated or inhibited by UVB, thereby causing chronic IKKβ phosphorylation, revealed that chemical inhibition or siRNA knock-down of potential IKKβ activators (in particular TAB1/TAK, JNK, RIP1, MAPK, AKT, PKD, PKCδ, PI3K, CK II, DNA-PK (including ATM and ATR) IKKγ and p38) as well as ROS scavenging (PDTC, GSH, NAC, Trolox), IKKγ Ser85Ala mutation and over-expression of inhibitors CYLD and A20 all failed to allow IκBα reappearance in IL-1 + UVB treated cells (see Fig. S1).

Therefore it seemed that IL-1 + UVB cooperate to maintain IKKβ activity by inhibiting its dephosphorylation. So far inactivation (12) as well as activation (17) of IKKβ have been described to be mediated by the Ser/Thr-phosphatase PP2A *in vitro*. PP2A is a multi-subunit protein with PP2Ac being the catalytic subunit. Indeed, co-immunoprecipitation revealed that endogenous PP2Ac was constitutively associated with IKKβ, proposing a functional relationship (Fig. 6a). To elucidate the role of PP2A as a potential negative regulator of IKKβ and to mimic the assumed UVB-mediated inactivation, the effect on IκBα degradation in IL-1-treated cells was studied upon specific inhibition of PP2Ac by calyculinA and/or siRNA mediated knock-down. The results fully confirm the critical role of PP2A as a negative regulator of IKKβ. Each, knock-down of PP2Ac and specific inhibition resulted in persistent IKKβ phosphorylation and IκBα degradation upon IL-1 stimulation, with combined treatment producing the strongest effect (Fig. 6b). *In vitro* kinase assay with PP2Ac knock-down cells confirmed that IKKβ in IL-1 only treated cells induced a persistent IκBα phosphorylation (Fig. 6c). Furthermore, knock-down of PP2Ac allowed a sustained TNF production at the mRNA and the protein level (Fig. 6d, e), supporting the functional relationship between cooperative inhibition of PP2Ac and TNF production finally leading to enhancement of apoptosis.

Finally, we investigated the effect of UVB on PP2Ac activity. *Ex vivo* irradiation of whole cell lysates with UVB followed by an *in vitro* phosphatase assay with a Threonin-phosphopeptide revealed overall phosphatase activity to be reduced to approximately 80 % of untreated controls (Fig. 6f). Phosphatase activity of PP2Ac immunoprecipitated from UVB-exposed cells was found to be strongly reduced to about 35 % of the activity in control cells, supporting the concept that continuous IKKβ phosphorylation and subsequent IκBα degradation in IL-1 + UVB treated cells is a result of PP2Ac inhibition. Conclusively, our data reveal 2 major findings: First they identify PP2Ac to be a novel cellular target of UVB and second they unravel chronic NFκB activation to be involved in cell killing instead of cell survival. Consequently, combination of PP2Ac inhibition and DNA-damaging chemotherapeutic drugs may have broader implications in cancer therapy.

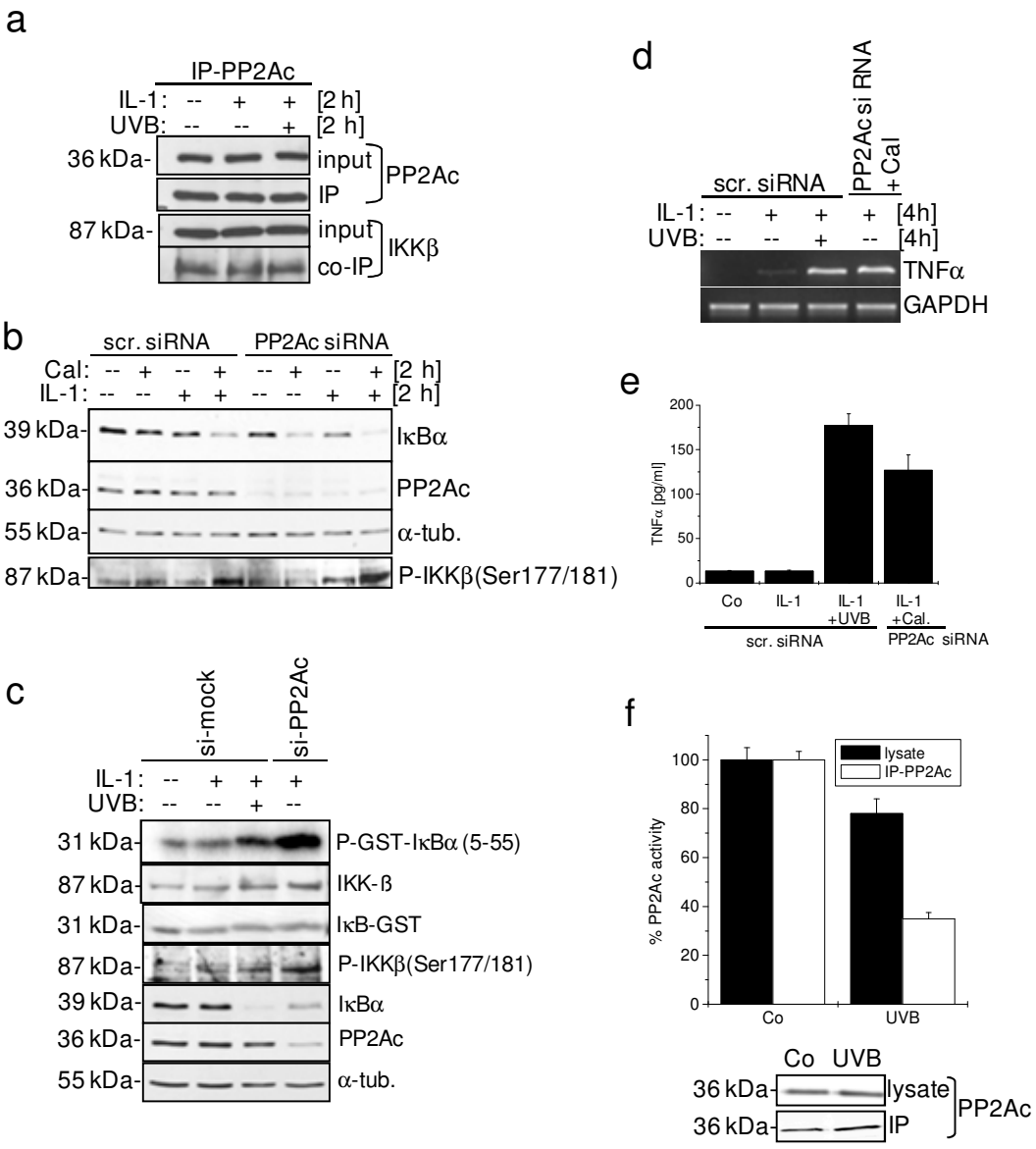

Figure 6 - Loss of PP2Ac results in constitutive activation of IKKβ and production of TNF. **(a)** KB cells were stimulated with IL-1 or IL-1 + UVB for 2 h. PP2Ac was immunoprecipitated and coprecipitation of IKKβ is shown by WB. **(b)** Cells were transfected with scrambled- or PP2Ac-siRNA and stimulated with IL-1 and/or calyculinA (5 nM). After 2 h cytosolic IκBα and phospho-IKKβ statuses as well as the PP2Ac knock down were documented by WB. **(c)** Cells stably expressing IKKβ-GFP were transfected with scrambled or PP2Ac-siRNA and stimulated with IL-1 or UVB + IL-1 for 2 h. IKKβ-GFP was immunoprecipitated and subjected to an *in vitro* kinase assay with a purified GST-IκBα(5-55) peptide. IκBα and phospho-IKKβ status were determined by WB. **(d)** Cells were transfected with scrambled- or PP2Ac-siRNA and stimulated with IL-1 and/or calyculinA (5 nM) and stimulated as indicated. After 4 h semiquantitative RT-PCR was performed to determine the efficacy of TNF transcription. **(e)** Cells were treated as in (D). After 16 h TNF secretion was measured in a TNF ELISA. **(f)** Whole cell lysates were left untreated or irradiated *ex vivo* with UVB. In parallel cells were mock treated or irradiated with UVB and PP2Ac was immunoprecipitated. Lysates and precipitates were subjected to an *in vitro* phosphatase assay using a Threonin-phosphopeptide as a substrate.

Discussion

UVB radiation may serve as a carcinogen by activation of skin oncogenes or inactivation of tumor suppressors and by repression of cell based immune responses that are generally able to eliminate highly antigenic skin tumors (18). In contrast, UVB-induced DNA damage is a prerequisite for execution of apoptosis, leading to elimination of cancer-prone cells, thereby protecting the skin from keratinocyte transformation (19;20). The molecular switch, however, has not yet been identified. It has long been known that human keratinocytes are a potent source of IL-1 (21) which becomes activated and released in response to UVB (22;23). In this respect keratinocytes serve an important role in the induction of skin immune responses under physiological and pathological conditions like sunburn and other inflammatory skin diseases (22;24). According to our findings UVB-induced apoptosis of keratinocytes and epithelial cells is significantly enhanced upon co-treatment with IL-1 *in vitro* (7;8;25). Correspondingly, UVB-induced apoptosis of keratinocytes *in vivo* may be facilitated by concomitant IL-1 signals from neighbouring cells. We therefore propose that the signal cross talk at the level of keratinocytes is of patho/physiological relevance in differential skin responses to UVB.

In this study we shed light on the molecular mechanism underlying the paradoxical finding that IL-1-mediated NFκB activation causes acceleration of UVB-induced apoptosis. Our findings account for sustained transcriptional up-regulation of TNF to be a prerequisite for TNF secretion and autocrine activation of TNF-R1 to additively enhance UVB-induced apoptosis, corroborating previous findings (7;8). Opposing effects of TNF coincide with compartmentalization of activated TNF-R1 complexes (13;14). TNF-R1 mediates a pro-apoptotic response only after internalization of the complex, whereas membrane-anchored TNF-R1 generates NFκB activation (14). The observed TNF-R1 internalization upon IL-1 + UVB treatment is in full accordance with a predominating apoptotic response initiated by autocrine TNF. UVB alone also induced TNF-R1 internalisation, however with a different kinetics and to a lesser extent, which might result from the ability of UV to induce ligand-independent activation of death receptors (26;27).

We show that initial IL-1-governed degradation of IκBα is required for NFκB activation. Upon co-stimulation with UVB slowly increasing amounts of resynthesized IκBα protein continuously become degraded due to constitutive IKKβ phosphorylation

caused by UVB-induced inhibition of its negative regulator PP2Ac. Through this mechanism the negative feedback loop for NFκB is abolished resulting in sustained NFκB activation. Under these specific conditions NFκB persists to up-regulate TNF and to repress anti-apoptotic genes finally producing pro-apoptotic effects (Fig.7a, b).

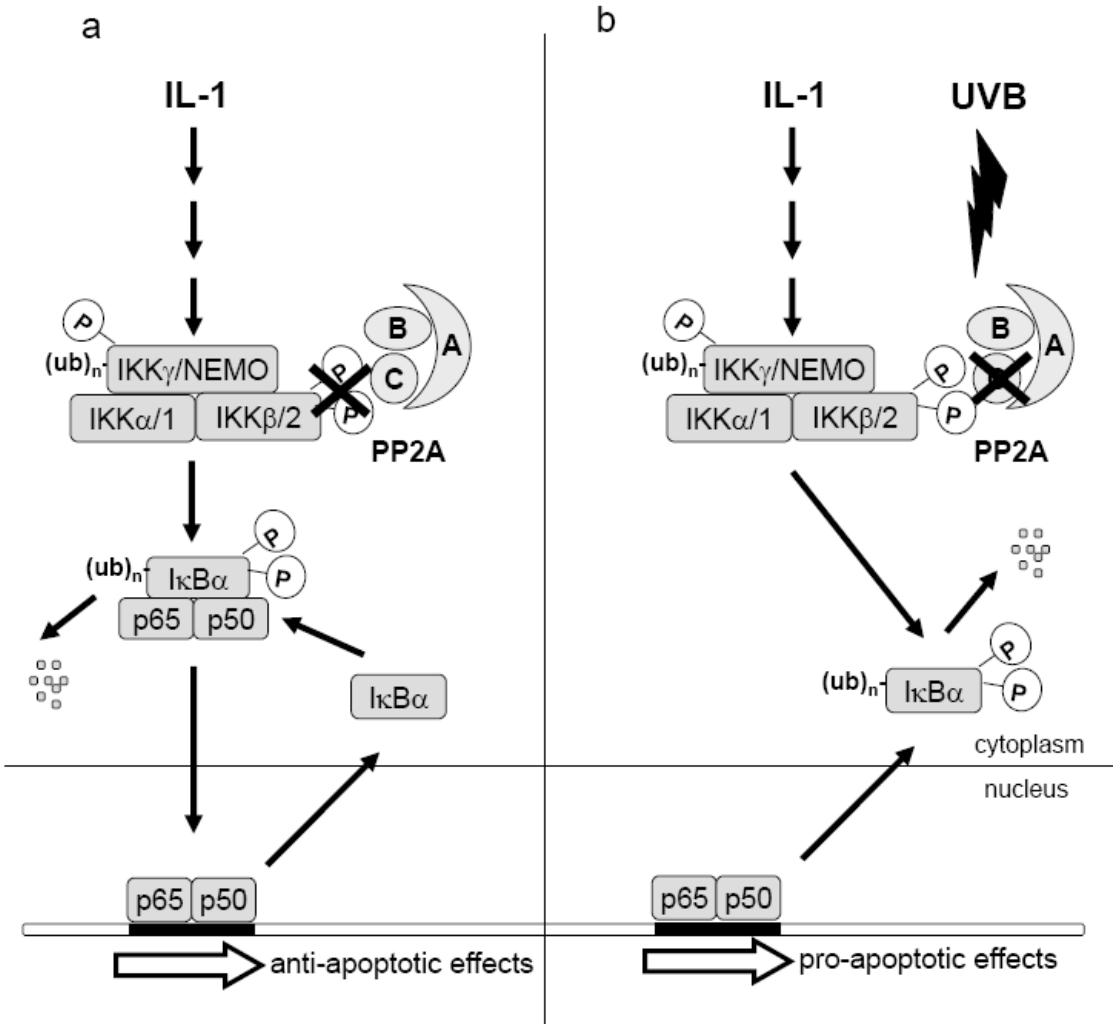

Figure 7 - Scheme of IL-1 + UVB-induced interruption of the negative feedback loop of NFκB. **(a)** Upon IL-1 stimulation IKKβ becomes phosphorylated at Ser 177/181 followed by subsequent phosphorylation of IκBα at Ser 32/36 causing its proteasomal degradation. Released NFκB translocates into the nucleus to mediate resynthesis of IκBα. In parallel, PP2A dephosphorylates IKKβ which allows for stabilization of resynthesized IκBα within the cytoplasm. **(b)** In case of co-stimulation with UVB, initial IκBα degradation remains unaffected but UVB-mediated inactivation of PP2Ac results in continuous phosphorylation and activation of IKKβ. Consequently, persistent phoshorylation and degradation of IκBα disrupts the negative feedback loop of NFκB. Prolonged NFκB activation in this specific physiological context triggers enhancement of apoptosis via repression of anti-apoptotic genes and continuous and elevated expression of TNF.

Since TNF is not a strong inducer of apoptosis but of NFκB-mediated survival signals in epithelial cell lines, down-regulation of anti-apoptotic proteins (FLIP, cIAP) and signal transducers (TRAF 1, 2, 6) complement to trigger apoptotic pathways under the given conditions (8). Our findings account for selective transcriptional up-regulation of TNF as a prerequisite for TNF secretion and autocrine TNF-R1 activation. NFκB activity could escape termination upon IL-1 + UVB stimulation due to lack of IκBα resynthesis. The IκBα gene itself is regulated by NFκB and post-degradational *de novo* synthesis of IκBα serves as a negative feedback regulation terminating NFκB (28). Consequently, failure of IκBα resynthesis coincided with prolonged nuclear localization of NFκB. Sustained NFκB activity seems to account for elevated TNF transcription, since inhibition of IκBα protein synthesis with CHX after initial degradation resulted in increased TNF mRNA expression upon IL-1 stimulation.

Unlike the TNF transcription pattern, the IL-1-dependent IκBα transcription profile remained unchanged in UVB-irradiated cells. Peculiarly, immediate post-translational degradation of resynthesized IκBα was found to cause lack of its cytosolic reappearance. UVC and UVB alone can induce NFκB activation, but no common mechanism became apparent. IKK and IκBα degradation-dependent and -independent mechanisms have been described and the time courses vary remarkably (16;28;29). In our hands, UVB alone triggered NFκB activation only strongly delayed and was accompanied by only partial degradation of IκBα excluding the effect of IL-1 + UVB-mediated instability of resynthesized IκBα to be just additive.

Our kinetic studies identified a critical time window (30 min before until 1 h after UVB) of co-stimulation to be essential for the enhancing effect of IL-1 on UVB-induced TNF secretion and apoptosis. Identical kinetics applied for IκBα degradation, revealing that the temporal constraints of this cooperative signalling affect IκBα protein levels.

HDAC inhibitor trichostatin A was reported to potentiate TNF-mediated activation of several NFκB-driven genes, being associated with delayed reappearance of IκBα and prolonged IKK activity (30). Enhanced proteasomal degradation of IκBα in our setting also appeared to be linked to signals upstream of its phosphorylation, because it could be counteracted by inhibition of IKKβ. Correspondingly, resynthesized IκBα captured by proteasome inhibition after IL-1+ UVB treatment

displayed phosphorylation of Ser32/36 residues . This implied that IκBα degradation was not due to alternative, possibly UVB-induced, cleavage of IκBα, and was consolidated by the observation that stability of IκBα Ser32/36Ala mutants remained unaffected by IL-1 + UVB (Fig. S2). Kinetic analysis finally revealed that IKKβ dephosphorylation at Ser177/181 in IL-1 only treated cells fully matched reappearance of IκBα in the cytoplasm after 2 h. In contrast, IKKβ remained phosphorylated in cells treated with IL-1 + UVB, thereby preventing IκBα resynthesis. Data allowed two interpretations: either UVB activates an upstream kinase which constitutively phosphorylates IKKβ or it inactivates a phosphatase, thereby preventing its dephosphorylation. PP2A is known to modulate NFκB activity (31), its precise role in NFκB-dependent cellular responses, however, remains controversial. While IKK-PP2A complex formation was suggested to be required for TNF-induced IKKβ phosphorylation and IκBα degradation (17), more evidence exists connecting inhibition of PP2A to NFκB activation: PP2A inhibition with calyculinA or ocadaic acid both resulted in IκBα phosphorylation and proteasomal degradation without additional cytokine treatment (32;33). Correspondingly, ocadaic acid treatment of monocytes caused IL-1 production linking PP2A inhibition to NFκB-dependent responses (34). Moreover, PP2A was shown to interact with IKKγ to down-regulate activation of IKKβ following TNF treatment (35;36). We show that the catalytic subunit PP2Ac, associates with IKKβ. Furthermore, siRNA-mediated knock-down of PP2Ac and specific inhibition by calyculinA resulted in degradation of IκBα 2 h after IL-1 stimulation *in vivo* and *in vitro*, in both cases coinciding with chronic IKKβ phosphorylation, mimicking the cellular response to IL-1 + UVB. Stimulus-dependent recruitment of PP2A to IKK can be ruled out, since endogenous PP2Ac co-precipitated with IKKβ even in un-stimulated cells. But, irradiation of cell lysates with UVB reduced total cellular phosphatase activity by ~ 20 % indicating that UVB generally affects phosphatase activity. However, the majority of phosphatases remained unaffected, provided by the fact that the substrate used was not PP2A-specific, but can be processed by other phosphatases present in cell lysates. Yet, immunoprecipitated PP2Ac proved to be particularly sensitive to UVB, resulting in ~ 65 % inhibition of its catalytic activity.

Suppression of PP2A was shown to cooperate with oncogenic changes to promote cell transformation (37). Suppression of PP2A in IL-1 treated cells presumably could also result in tumor progression due to constitutive NFκB activation. In combination with UVB, however, NFκB promotes pro-apoptotic pathways and may exert completely different effects on tumor growth.

In conclusion, the present study attributes a crucial role to PP2A in maintaining the negative feedback loop terminating NFκB. Additionally, we unravel a novel mechanism by which persistent NFκB activation plays an unexpected role as a pro-apoptotic mediator and is a vivid example for the importance of signalling contexts for overall cellular responses.

Because of NFκB´s crucial role in physiology and pathophysiology, NFκB-activating pathways are in the focus of therapeutic interventions (38). Combinations of targeted therapies with radio- and chemotherapeutics are in progress to advance the treatment of cancer and inflammatory diseases (38;39). Therefore, an integrated understanding of cellular responses to NFκB appears mandatory. This requires knowledge about the context-dependent behaviour of NFκB-triggered signalling pathways and molecules to pin-point suitable targets, like PP2Ac, to improve therapeutic strategies.

Methods

Unless otherwise stated, results of CDD- and TNFα-ELISA are presented as mean ± SD of 3 independently performed experiments. For statistical analysis student's t-test was performed. WB analysis, EMSAs, *in vitro* kinase assays and RT-PCR analysis shown represent one out of 3 independently performed experiments. For details see supplemental data.

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. UVB irradiation (300 J/m^2) was performed with TL12 fluorescent bulbs (290-320 nm, Philips). Apoptosis was triggered with 80 ng/ml Iz-TRAIL (40). Recombinant human

IL-1β (R&D Systems) was applied at 10 ng/ml. Transfection of cells with siRNA (MWG) was carried out in Lipofectamin2000 (Invitrogen). For semiquantitative RT-PCR cDNA was generated as described before (25) using the RedTaq system (Sigma). Proteasome inhibition was achieved with MG132 (25 µM, Merck) or lactacystin (15 µM, Merck). IKKβ-inhibitor BAY-11-7082 was applied at 30 µM (Merck). PP2Ac inhibitor calyculinA (Cell Signaling) was added at 5 nM. TNF was measured with a TNF ELISA (BioSource), apoptosis with a Cell Death Detection (CDD)-ELISA (Roche). The enrichment of nucleosomes released into the cytoplasm by factor 2 corresponds to 10 % apoptotic cells as determined by AnnexinV FACS analysis.

EMSA and supershift analysis

Following stimulation cells were harvested and nuclear extracts were generated as described before (25) using a NFκB consensus oligo nucleotide (sc-2505; Santa Cruz). For supershift assays the following antibodies recognizing p65, p50, p52, c-Rel, and RelB were used: sc-109X, sc-7178X, sc-7386, sc-70X, sc-226X (Santa Cruz).

Immunoprecipitation, WB analysis and in vitro kinase assay

Cells 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 was immunoprecipitated using a specific antibody (# 05-421, clone 1D6, Upstate) and A/G-plus agarose (Santa Cruz) overnight. Precipitates were analyzed by WB using antibodies against PP2Ac and IKKβ (10A9B6, Imgenex). For WB analysis cytosolic and nuclear protein extracts were detected with antibodies against IκB-α, P-IκBα-Ser32/36, P-IKKβ-Ser177/181, PP2Ac (L35A5, 5A5, 16A6, # 2038, Cell Signaling), IKKβ (10A9B6, Imgenex), and α-tubulin (DM1A, Neomarkers). For kinase assay immunoprecipitation of IKKβ-YFP was carried out as above. GST-IκBα (5-55) was purified with GSH-sepharose-4B (Amersham) and incubated with IKKβ and [^{32}P]-γ-ATP in kinase buffer and analyzed by SDS-PAGE and autoradiography.

FACS analysis

To monitor membrane bound TNF–R1, FACS analysis was performed as described before (25) using 1 µg of anti-TNFα T1 mouse IgG1 (H398) per $2x10^5$ cells and an goat anti-mouse IgG conjugated to Phycoerythrin (Santa Cruz). Cells were analyzed in FACS-Calibur flow cytometer (Becton Dickinson) and using WinMDI 2.8 software.

Phosphatase assay

10 µg of cell lysates or immunoprecipitated PP2Ac 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 (final conc. 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.

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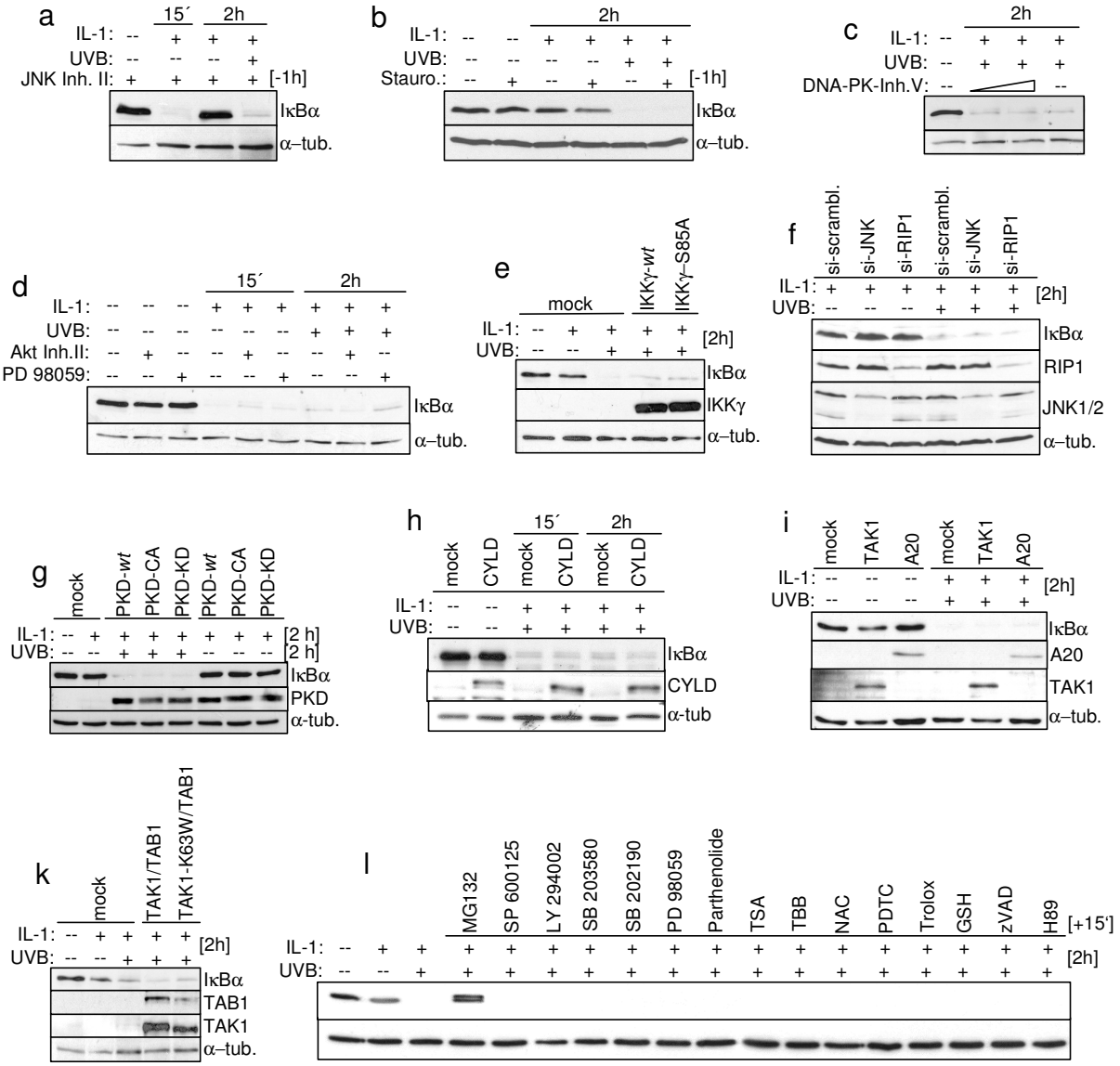
Supplement

Supplemental Figure 1. Inhibition of various potential upstream targets does not reverse lack of IκBα recurrence upon IL-1 + UVB treatment.



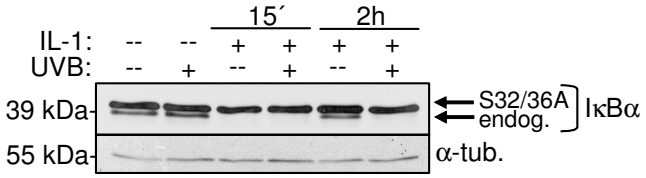
A variety of upstream kinases have been described to cause NFκB activation or to modulate NFκB activity. To rule out that activation of any of these additional or alternative signalling pathways contribute to IL-1 + UVB-induced continuous IκBα degradation, chemical inhibition or siRNA knock down was performed.

(a) C-Jun kinase (JNK) was shown to become activated by UV radiation and to be involved in NFκB activation pathway (1;2): Cells were prestimulated with 100 nM JNK inhibitor II cocktail for 1h followed by IL-1 + UVB treatment as indicated. IκBα status was documented

by WB analysis. **(b)** Protein kinase C (PKC) was described to activate NFκB in response to oxidative stress (3): PKC inhibition was achieved by treating cells with 1 µM staurosporin. After 1h cells were stimulated as denoted and IκBα status analyzed by WB. **(c)** Contribution of DNA damage dependent kinases which might potentially mediate NFκB activation (4): DNA damage dependent kinases, including ATM and ATR were inhibited with 100 and 300 µM DNA-PK inhibitor cocktail V (Calbiochem) respectively, 30 min prior to IL-1 + UVB stimulation. After 2 h IκBα status was determined by WB analysis. **(d)** AKT and MAP-kinase signalling pathways are involved in NFκB activation upon different stimulations (5;6): MAP-kinase inhibition was achieved by prestimulation with 50 µM PD 98059 (Calbiochem) and Akt inhibition by pre-treatment with 10 µM Akt-inhibitor II (Calbiochem) for 30 min each. At the indicated time points after IL-1 + UVB stimulation IκBα status was documented by WB analysis. **(e)** IKKγ Ser85 phosphorylation has been described to mediate NFκB activation in response to DNA damage (7): Cells were transfected with a plasmid encoding wt IKKγ or an IKKγ S85A mutant and stimulated with IL-1 + UVB for 2h. IκBα status and IKKγ expression were analysed by WB. **(f)** Besides JNK, the death domain kinase RIP has been described to mediate IKK activation in response to death receptor stimulation (8): JNK1 and RIP1 were knocked down by transfecting cells with siRNA for JNK1 and RIP1 respectively. 2 h following IL-1 or IL-1 + UVB treatment IκBα, RIP1 and JNK1 statuses were determined by WB analysis. **(g)** Protein kinase D (PKD) was found to mediate PKC-induced NFκB activation in response to oxidative stress (9): Cells were transfected with plasmids encoding PKD-*wt*, a constitutive active variant (CA) and a kinase dead (KD) variant respectively (9). After 2h of IL-1 or IL-1 + UVB treatment, cellular IκBα and expression of PKD variants was analysed by WB. **(h)** The ubiquitin protease CYLD negatively regulates the IKK complex via deubiquitination of IKKγ (10;11): Cells were transfected with plasmids encoding CYLD-*wt* (11). 15 min and 2 h following IL-1 + UVB treatment, cellular IκBα and expression of CYLD were determined by WB analysis. **(i)** TAK1 is critically involved in activation of the IKK complex (12) whereas A20 is a negative regulator of NFκB activity (13): Cells were transfected with plasmids encoding TAK1 and A20 respectively. After 2 h of IL-1 + UVB treatment, cellular IκBα as well as expression of TAK1 and A20 was documented by WB analysis. **(k)** The TAB/TAK complex was identified to be crucially involved in IL-1 as well as TNF- driven NFκB activation (12;14) : Cells were cotransfected with plasmids encoding TAK1 and TAB1 or with plasmids encoding a kinase-inactive mutant of TAK1 (12) and TAB1. After 2 h of IL-1 + UVB treatment, cellular IκBα and expression of TAK1 and TAB1 was monitored by WB analysis. **(l)** For the following experiment all inhibitors were added to the cells 15 min after IL-1 + UVB cotreatment, the time point when initial IκBα degradation is completed. IκBα resynthesis was monitored after 2 h by WB analysis. Inhibitors were used against: 26S proteasome (25 µM MG132); JNK (25 µM SP 600125); PI3-kinase (20 µM LY 294002 (15)); p38 (20 µM SB 203580 or SB 202190 (16)); MAPK (50 µM PD 98059 and 20 µM Parthenolide); Histone deacatylase (500 nM TSA (17)); casein kinase II (30 µM TBB (18)); reactive oxygen species (10 mM NAC, 50 µM PDTC, 10 mM Trolox, 500 µM GSH; reviewed in (19)); caspases (25 µM zVAD (20)); PKA and PKD (20 µM H89 (21)). All inhibitors were obtained from Merck-Calbiochem, but zVAD from Enzymes Systems Products. si RNA was purchased from MWG.

All experiments shown represent one out of three independently performed experiments.

Supplemental Figure 2. Immediate degradation of resynthesized IκBα protein is inhibited via phosphorylation of conserved Ser32/36.



Cells stably expressing an IκBα-Ser32/36Ala mutant were stimulated for 15 min or 2 h with IL-1 and/or UVB. The status of endogenous *wt* IκBα and mutated IκBα was determined by WB. Only resynthesis of wt IκBα (Ser32/36) was committed to immediate post translational degradation, whereas mutated IκBα (Ser32/36Ala) was protected.

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II Mechanism of PP2A-mediated IKKβ dephosphorylation: a systems biological approach

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Abstract

Background

Biological effects of nuclear factor-κB (NFκB) can differ tremendously depending on the cellular context. For example, NFκB induced by interleukin-1 (IL-1) is converted from an inhibitor of death receptor induced apoptosis into a promoter of ultraviolet-B radiation (UVB)-induced apoptosis. This conversion requires prolonged NFκB activation and is facilitated by IL-1 + UVB-induced abrogation of the negative feedback loop for NFκB, involving a lack of inhibitor of κB (IκBα) protein reappearance. Permanent activation of the upstream kinase IKKβ results from UVB-induced inhibition of the catalytic subunit of Ser-Thr phosphatase PP2A (PP2Ac), leading to immediate phosphorylation and degradation of newly synthesized IκBα.

Results

To investigate the mechanism underlying the general PP2A-mediated tuning of IKKβ phosphorylation upon IL-1 stimulation, we have developed a strictly reduced mathematical model based on ordinary differential equations which includes the essential processes concerning the IL-1 receptor, IKKβ and PP2A. Combining experimental and modelling approaches we demonstrate that constitutively active,

but not post-stimulation activated PP2A, tunes out IKKβ phosphorylation thus allowing for IκBα resynthesis in response to IL-1. Identifiability analysis and determination of confidence intervals reveal that the model allows reliable predictions regarding the dynamics of PP2A deactivation and IKKβ phosphorylation. Additionally, scenario analysis is used to scrutinize several hypotheses regarding the mode of UVB-induced PP2Ac inhibition. The model suggests that down regulation of PP2Ac activity, which results in prevention of IκBα reappearance, is not a direct UVB action but requires instrumentality.

Conclusions

The model developed here can be used as a reliable building block of larger NFκB models and offers comprehensive simplification potential for future modeling of NFκB signaling. It gives more insight into the newly discovered mechanisms for IKK deactivation and allows for substantiated predictions and investigation of different hypotheses.

The evidence of constitutive activity of PP2Ac at the IKK complex provides new insights into the feedback regulation of NFκB, which is crucial for the development of new anti-cancer strategies.

Background

Nuclear factor κB (NFκB) (p65/p50) is a transcription factor of central importance in inflammation and anti-apoptotic signaling [1]. Since constitutive activation of NFκB was shown to contribute to the maintenance of a range of cancers by inducing expression of anti-apoptotic genes [2-4], manifold approaches were made to develop new anti-cancer strategies based on NFκB inhibition [3, 5]. Canonical activation of NFκB by the pro-inflammatory cytokine interleukin-1 (IL-1) requires activation of the inhibitor of κB (IκBα) kinase complex (IKK), especially phosphorylation of the catalytic subunit IKKβ at Ser 177/181 [6]. Phosphorylated IKKβ consequently phosphorylates IκBα at Ser 32/36, leading to its poly-ubiquitination and proteasomal degradation. Liberated NFκB translocates into the nucleus to activate transcription of responsive genes [6]. Accordingly, co-stimulation of cells with IL-1 was shown to

inhibit death ligand-induced apoptosis via up-regulation of anti-apoptotic genes and their products [7, 8]. In contrast, ultraviolet-B radiation (UVB)-induced apoptosis was not inhibited but significantly enhanced upon co-stimulation with IL-1. This process on the one hand was associated with NFκB-dependent repression of anti-apoptotic genes. On the other hand, it coincided with long term transcriptional up-regulation followed by pronounced release of tumor necrosis factor α (TNFα), which activates the death receptor TNF-R1 in an autocrine fashion, thereby enhancing UVB-induced apoptosis [9]. Both effects were shown to be NFκB dependent, indicating that UVB is capable to persistently convert NFκB function from an inhibitor into a promoter of apoptosis. This newly-discovered UVB-mediated pro-apoptotic activity of NFκB appears of utmost importance, because it challenges the dogma of NFκB inhibition as a general approach to fight cancer. In contrast, the new evidence provides a basis for alternative approaches in cancer therapy combining induction of DNA damage with NFκB activation rather than inhibition. It is therefore of prime interest to unravel the detailed mechanisms underlying this complex feedback regulation of the NFκB system.

In order to fully convert the cellular NFκB response from anti- to pro-apoptotic functions, transient NFκB activation appears to be insufficient. In fact, recent data revealed a prolonged IL-1-induced nuclear activity of NFκB in epithelial cells co-treated with UVB to be responsible for switching the cellular response towards a pro-apoptotic phenotype [10]. It is generally accepted that activation of NFκB triggers transcription of IκBα, thereby inducing resynthesis of its inhibitor in a negative regulatory feedback loop [11]. This negative feedback loop was shown to be completely abrogated in cells co-treated with IL-1 and UVB, caused by immediate phosphorylation and proteasomal degradation of the newly synthesized protein [10]. Instant phosphorylation of resynthesized IκBα was facilitated by continuously activated IKKβ. Chronic Ser 177/181 phosphorylation of IKKβ was due to UVB-induced inhibition of the catalytic subunit of the Ser-Thr phosphatase PP2A (PP2Ac) [10]. As a consequence, active NFκB persists in the nucleus for several hours providing sufficient time to fundamentally change the transcriptional program and physiologic response of the cell.

The exact molecular mechanisms and kinetics, however, underlying PP2A-mediated IKKβ dephosphorylation in response to IL-1 as well as UVB-induced PP2A inhibition

remain to be determined. Either constitutively active or signal activated PP2A may modulate IKKβ activity. To identify the responsible mode of action we developed a mathematical model of IKKβ phosphorylation based on experimental data.

Several models describing NFκB activation following IKK phosphorylation have been published to date [12], most of them based on the seminal model of Hoffmann et al. [13]. Since these models mainly focus on IκB/ NFκB kinetics, they often do not explicitly describe receptor kinetics. A problem of these generally large models, which is often exacerbated by a comparatively sparse experimental data basis, is that parameters are frequently functionally related and can therefore not be determined unambiguously: the parameters are not identifiable [14]. Particularly, the amount of additional parameters and the system complexity increase tremendously when the IκB / NFκB part of the model influences IKK or when the signaling cascade is extensively modeled. For example, studies of Cho et al. [15] and Park et al. [16] presented very detailed models of TNF-mediated signaling upstream of IκBα, including receptor kinetics and IKK activation. While this approach is very valuable for theoretical studies, the estimation of reliable parameter values would require a huge amount of experimental data. Consequently, Werner et al. [17] did not fit their model to IKK activity data but rather used these data as an input for the IκB/ NFκB module. Cheong et al. [18] assessed the problem in a more mathematical way and modeled IKK kinetics by activation and deactivation functions without direct biological counterpart. Those approaches avoid most identifiability problems, but do not provide an insight into the activation and deactivation processes of IKK. A model for IL-1 receptor signaling exemplifying the identifiability issue has been proposed recently, but lacks experimental validation [19]. In sum, existing models contribute to a deeper understanding of the phosphorylation dynamics of IKKβ, but an experimentally validated model for IL-1 signaling with fully identifiable parameters including the essential biological processes has not been devised to date.

Focusing on IKKβ phosphorylation following IL-1 stimulation, we present a model with pronounced modularity to warrant reusability of either the entire model or some parts of it. The individual modules are not connected by mass flows, but by unidirectional signal flows, and can therefore also be considered decoupled from the remaining model. Particular attention is paid to a careful simplification resulting in a very good identifiability of the parameters. We use a combination of modeling and experimental

methods to analyze the system behavior, and provide evidence that constitutively active PP2A continuously dephosphorylates IKKβ, thereby sustaining the negative feedback loop for NFκB and maintaining proper cellular function.

Results

UVB inhibits IκBα reappearance via continuous phosphorylation of IKKβ

Initiation of transient NFκB activation upon IL-1 stimulation (10 ng/ml) requires Ser177/181 phosphorylation of IKKβ. Subsequent IκBα phosphorylation and proteasomal degradation is completed at the latest after 15 min. Reappearance of IκBα after 90 min perfectly matches dephosphorylation of IKKβ and disappearance of NFκB from the nucleus (Fig. 1).

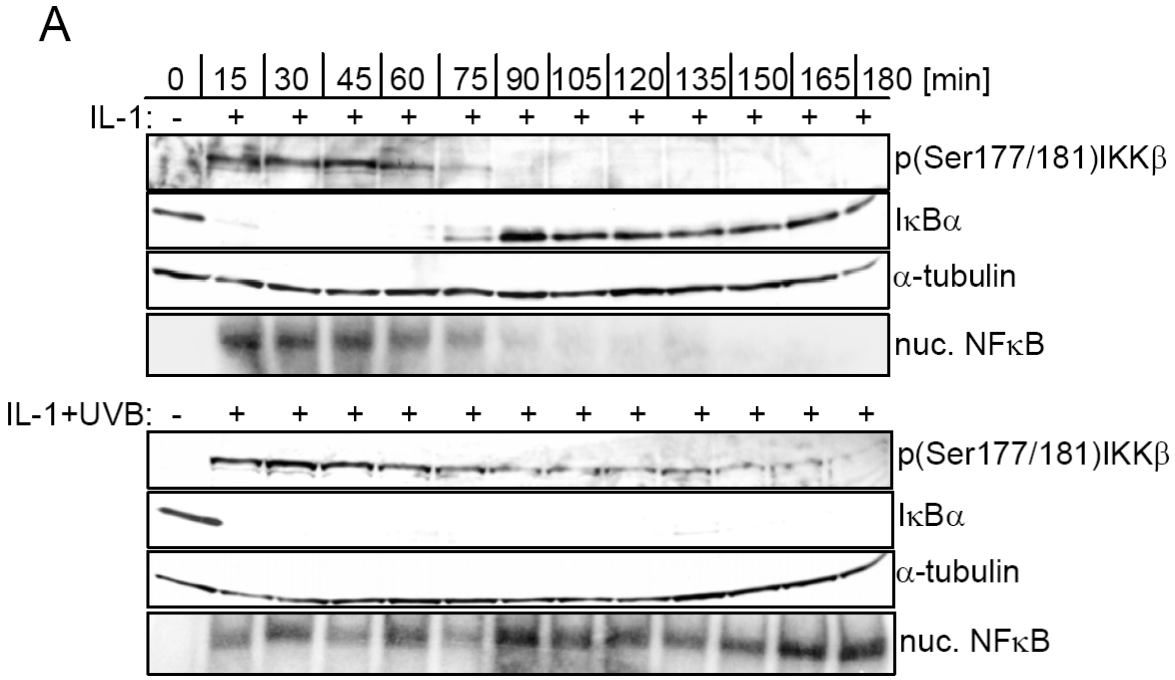

Figure 1 - Failure of IκBα reappearance coincides with continuous IKKβ phosphorylation and NFκB activation.
KB cells were left untreated or stimulated with 10 ng/ml IL-1 alone or in combination with UVB (300 J/m^2). At the indicated time points cytosolic as well as nuclear protein extracts were generated. Cytosolic extracts were analysed for the phosphorylation status of IKKβ and IκBα degradation by Western-blotting. Equal loading was monitored with an antibody against α-tubulin. In parallel, nuclear translocation of NFκB was determined by EMSA with an NFκB specific oligonucleotide. Data shown represent one out of three independently performed experiments.

Costimulation with UVB, however, results in complete inhibition of IκBα reappearance and NFκB termination. Under these conditions the phosphorylation status of IKKβ is somewhat lowered after 90 min but still remains at elevated levels over hours, ensuring continuous phosphorylation and degradation of gradually upcoming levels of resynthesized IκBα (Fig. 1).

The activation status of PP2A at the IKK complex

PP2A is a ubiquitously expressed Ser/Thr phosphatase which is involved in a wide range of cellular processes, only a very small fraction being responsible for IKKβ regulation, meaning that overall measured PP2A activity may differ from the specific local activation status. In general, two mechanisms of PP2A activity may explain the observed phosphorylation status of IKKβ (Fig. 2A). Either inactive PP2A is recruited to the IKK complex in unstimulated cells and becomes activated with a certain delay following IL-1 treatment. Activated PP2A then terminates IKKβ activity, thereby allowing for stabilization of resynthesized IκBα. Alternatively, a constitutively low level of activated PP2A continuously counteracts the IKKβ phosphorylation and thus activity state (Fig. 2A). Previous data from our lab revealed PP2Ac to be constitutively recruited to IKKβ, even in unstimulated cells [10]. Here, the total cellular PP2Ac activity was determined after 15 min of IL-1 treatment, reflecting initial IκBα degradation, and after 2 h, representing the time point of full IκBα resynthesis (see Fig. 1). Phosphatase activity assay did not reveal any significant changes in overall cellular PP2Ac activity at the different time points measured (Fig. 2B), allowing to assume that PP2A located at the IKK complex might also be constitutively active. In order to investigate whether this assumption is consistent with the observed IKKβ phosphorylation pattern and because PP2Ac amounts recruited to IKK are too small to be reliably analyzed separately, we designed an ordinary differential equation model resuming the essential processes based on the assumption of constitutive PP2A activity.

However, to rule out that PP2A may additionally target IκBα directly for dephosphorylation, phosphorylated IκBα was captured by addition of the proteasome inhibitor MG132. 15 min after IL-1 stimulation, IKK is strongly phosphorylated and IκBα disappeared due to proteasomal degradation. Addition of MG132 consequently

resulted in appearance of non-degradable, phosphorylated IκBα, evident from the shifted IκBα band (Fig. 2C). Two hours later, IKKβ was completely dephosphorylated and IκBα reappeared as described before. Upon proteasome inhibition IκBα stayed phosphorylated over the observed time period, indicating that PP2A primarily targeted IKKβ and not IκBα, the latter, if at all, with a significantly slower kinetics (Fig. 2C).

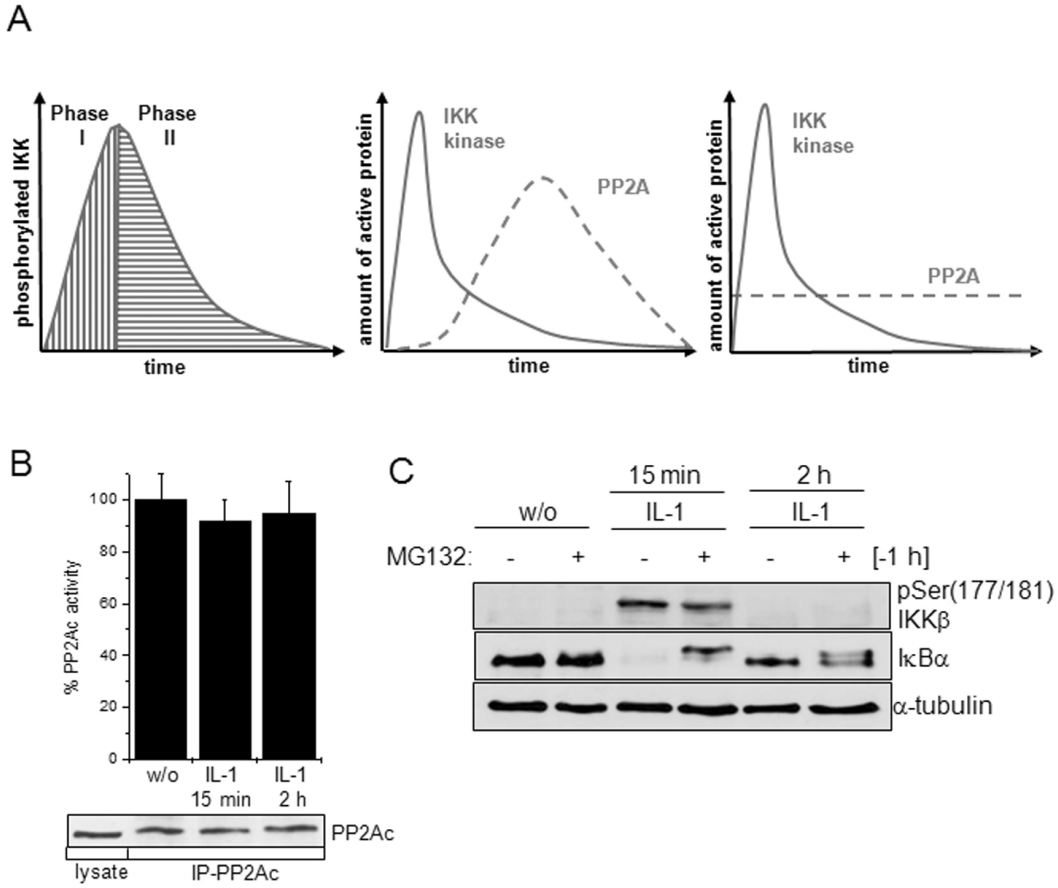

Figure 2 - Hypotheses of PP2A activity.
(A) The IKK phosphorylation curve consists of two phases. Phase I is defined by an increase of phosphorylated IKK, indicating a predominant kinase activity. Phosphorylated IKK decreases in phase II, indicating a predominant phosphatase activity. Both activation of PP2A by IL-1 and constitutive PP2A activity could explain this behaviour. Scaling is different for PP2A and the IKK kinase. **(B)** Cells were left untreated or were stimulated with IL-1 for 15 min and 2 h respectively. Subsequently, PP2Ac was immunoprecipitated and subjected to an *in vitro* phosphatase assay using a threonine-phosphopeptide as a substrate. Pooled data of three independently performed experiments are depicted. **(C)** Cells were pre-stimulated or not with 20 µM MG132 for 1 h. Subsequently cells were left untreated or stimulated with IL-1 for 15 min or 2 h. Phosphorylation status of IKKβ and protein status of IκBα were determined by Western-blot analysis using α-tubulin as loading control.

IKK phosphorylation following IL-1 stimulation can be modeled decoupled from the downstream processes

Our model is based on the following biochemical reactions:

$$\mathrm{ILR} \xrightarrow{\mathrm{ka \cdot il}} \mathrm{ILRc} \xrightarrow{\mathrm{ki}}$$

$$\mathrm{IKK} \xrightarrow{\mathrm{kp \cdot ILRc}} \mathrm{IKKp}$$

$$\mathrm{IKKp} \xrightarrow{\mathrm{kdp \cdot PP2A}} \mathrm{IKK}$$

$$\mathrm{PP2A} \xrightarrow{\mathrm{uv \cdot kuv}}$$

where ILRc and ILR represent the IL-1 receptor with and without bound IL-1, respectively, IKKp and IKK denote phosphorylated and unphosphorylated IKKβ, respectively, and PP2A reflects active PP2Ac at the IKK complex. The model structure is shown in Fig. 3A. The signification of the model parameters is depicted in Table 1.

These reactions imply that IKK phosphorylation can be considered independently of the downstream processes. In contrast, the vast majority of mathematical models for NFκB signaling pathways are based on the one by Hoffmann et al. [13], representing phosphorylation of IκBα as transient complexing of phosphorylated IKK with IκBα, where only uncomplexed IKK can be deactivated. In these models, IKK phosphorylation kinetics can therefore not be considered independently of the IκBα/NFκB kinetics. However, complexing of IKK with IκBα can also be approximated as a catalytic influence of IKK on IκBα, using the quasi-steady-state approximation [20] for all complexes involving IKK. Considering the model by Lipniacki et al. [21], it can be shown that the behavior of the reduced model is almost identical to the kinetics of the original one (Additional file 1, I). This finding offers a significant simplification potential for future modeling of NFκB signaling. Since the further coupling of IKKβ and NFκB via the A20 feedback only exists for TNFα induced NFκB signaling [22], but has been shown to be negligible for IL-1 induced NFκB signaling [23], we can consider IKKβ kinetics independently of the downstream processes involving NFκB.

Assuming mass action kinetics, an initially completely uncomplexed receptor, initially unphosphorylated IKK and constitutively active PP2A, the system can now be written in terms of differential equations as follows:

$$\frac{d\,ILR(t)}{dt} = -ka \cdot il(t) \cdot ILR(t), \quad ILR(0)=1$$
$$\frac{d\,ILRc(t)}{dt} = ka \cdot il(t) \cdot ILR(t) - ki \cdot ILRc(t), \quad ILRc(0)=0$$
$$\frac{d\,IKKp(t)}{dt} = kp \cdot ILRc(t) \cdot IKK(t) - kdp \cdot PP2A(t) \cdot IKKp(t), \quad IKKp(0)=0$$
$$\frac{d\,PP2A(t)}{dt} = -kuv \cdot uv(t) \cdot PP2A(t), \quad PP2A(0)=1$$

with IKK (t) = 1 – IKKp(t).

The inputs *il(t)* and *uv(t)* are step functions: *il(t)* is 0 µM for $t < 0$, and 0.000588 µM for $t \geq 0$, corresponding to the experimentally applied dose of 10 ng/ml. With 300 J/m^2 UVB stimulation, *uv(t)* is the Heaviside function, $uv(t) = 0$ for $t < 0$, and $uv(t) = 1$ for $t \geq 0$. Consequently, without UVB stimulation $uv(t) \equiv 0$. The model was scaled to the experimental data using a unique scaling factor for all IKK observations. An upper bound of 0.095 s^{-1} was imposed on *kp*, based on biophysical considerations (see Additional file 1, II). No bounds were assumed for the remaining parameters.

The hypothesis of constitutive PP2A activity is consistent with the experimental data

The model assuming constitutive PP2A activity (Fig. 3A) was fitted to the experimentally determined IKKβ phosphorylation pattern following stimulation with IL-1 (10 ng/ml), with or without UVB co-stimulation. The simulation results match the experimental data remarkably well (χ^2 = 10.46 + 13.99 = 24.45; Fig. 3B and Table 1), particularly when considering the relatively small system order. In the following, this model structure with these parameters is referred to as the reference scenario. Compared to experimental data, peak concentrations are reached much more rapidly in the simulation than in the experimental data. This effect can be attributed to the choice of the objective function rather than to limitations induced by the model structure: since the standard deviations are higher during the first 30 minutes, higher deviations are tolerated in this time interval. Indeed, a visually more satisfactory fit with peak concentrations shortly after 15 minutes is possible with a slightly larger χ^2 value (28.09, see Additional file 2) and very similar values for *ka*, *ki*, *kuv* and *kdp*. Results presented show that constitutive PP2A activity is indeed consistent with the observed IKKβ phosphorylation pattern.

A similar model developed for investigating the alternative hypothesis of initially inactive PP2Ac, however, yielded comparable results (see Additional file 3), so that none of the two possible mechanisms can be excluded based on modeling results. Consequently, experimental data are consistent with the principle of either hypothesis.

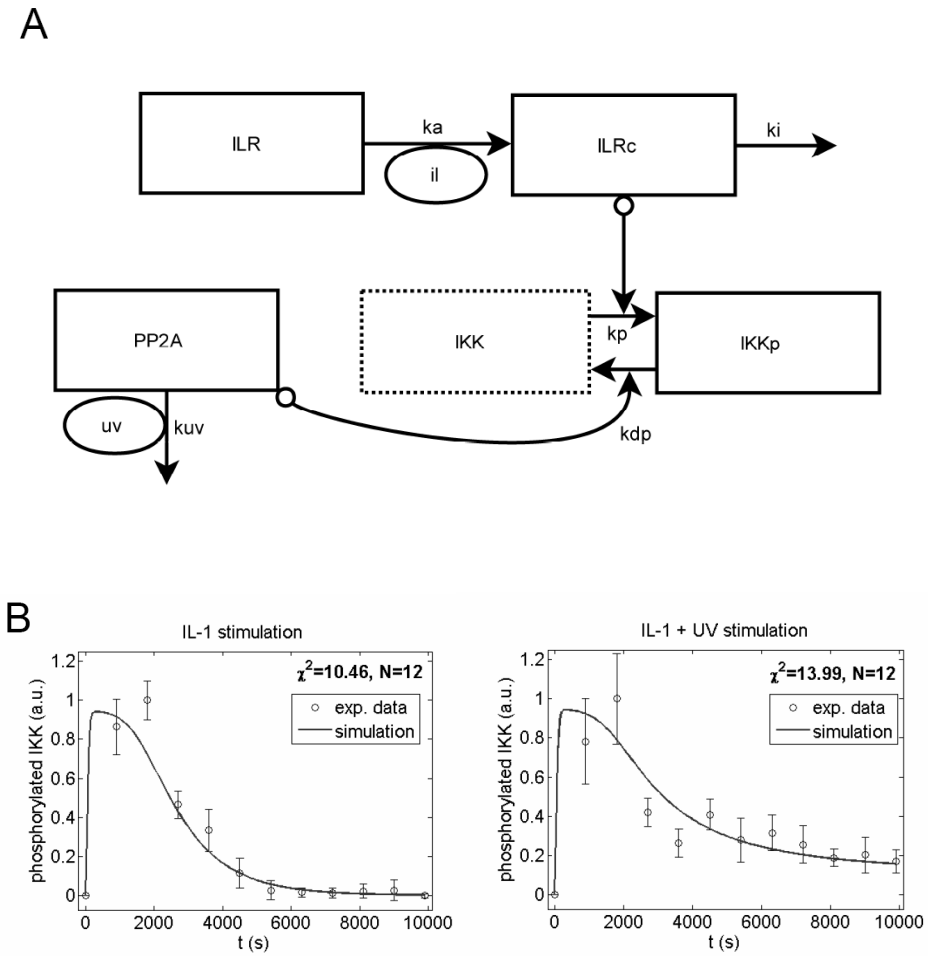

Figure 3 - Schematic representation and simulation results of the mathematical model of the activation status of PP2Ac at the IKK complex.
(A) The IL-1 receptor and IL-1 form the IL-1 receptor complex which triggers IKKβ phosphorylation. PP2Ac dephosphorylates phosphorylated IKKβ and is deactivated by UVB radiation. Ellipses denote model inputs, boxes with solid lines denote state variables, and boxes with dotted lines denote species whose concentration can be calculated from the state variables by mass conservation relations. Normal arrows denote mass flows following mass action kinetics, circles at the arrow tail signify that the involved species participates catalytically in the reaction (signal flow). **(B)** The model assuming constitutive PP2Ac activity matches the experimental data calculated from Fig. 1 well (χ^2 = 10.46 + 13.99 = 24.45). Experimental data are shown as mean and standard deviation of three independently performed experiments.

variable	process	value	unit
ka	association of IL-1 to the receptor	6.7	$(\mu M \cdot s)^{-1}$
ki	internalization of the receptor complex	0.0034	s^{-1}
kp	phosphorylation of IKK	0.095	s^{-1}
kdp	dephosphorylation of IKKp	0.00076	s^{-1}
kuv	deactivation of PP2A under UVB	0.00024	s^{-1}
IKKscale	scaling of the model to the data	0.96	-

Table 1 - Model parameters in the reference scenario
Note that the unit µM occurs only in the reaction involving IL-1, since all state variables are dimensionless and can be interpreted as fraction of the total initial protein.

Experimental data substantiate the constitutive activity of PP2A

To finally address the activation status of PP2Ac and its impact on initial IKKβ phosphorylation we performed PP2Ac knock-down experiments. As a prerequisite, a sensitive IL-1 dose had to be determined allowing observation of minor changes in IκBα levels and IKKβ phosphorylation. Performing dose-response experiments ranging from 10 ng/ml to 0.1 ng/ml IL-1 for 15 min revealed 0.5 ng/ml to be the sensitive dose of choice upon which initial IκBα degradation has only partially taken place (Fig. 4A). Using this sensitive dose of 0.5 ng/ml IL-1, delayed IKKβ phosphorylation was observed compared to treatment with 10 ng/ml (compare Fig. 1 and Fig. 4B). Investigating very short sampling intervals finally disclosed IKKβ phosphorylation to start very early 1 min after IL-1 treatment (Fig. 4B and 4C). However, when PP2Ac was knocked down by siRNA, very early phosphorylation of IKKβ was enhanced, being most pronounced after 30 min of treatment (Fig. 4C; for one representative Western Blot see Additional file 4). These data clearly indicate PP2A to be constitutively active when located at the IKK complex. Furthermore, the model demonstrates that no additional mechanism is required to explain the experimental data.

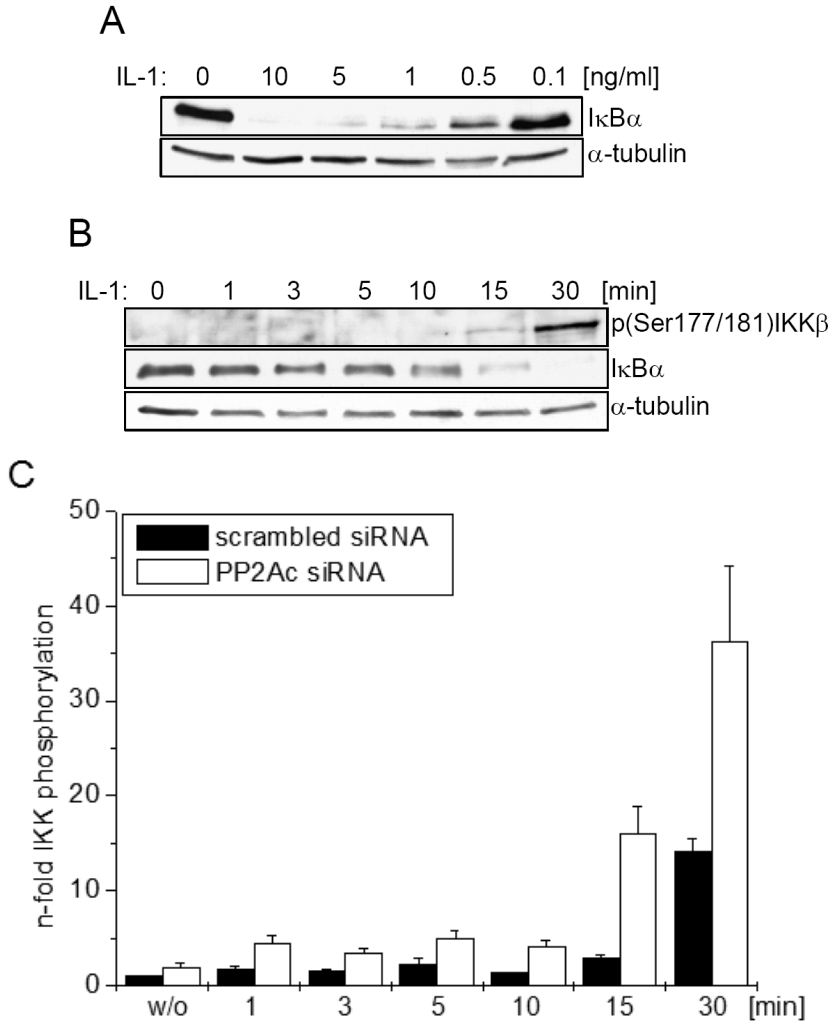

Figure 4 - A sensitive IL-1 dose reveals PP2Ac to be constitutively active at the IKK complex. **(A)** Cells were left untreated or stimulated with decreasing doses of IL-1 for 15 min as indicated. The status of the initial IκBα degradation was determined by Western-blot analysis. **(B)** Cells were left untreated or stimulated with a sensitive dose of 0.5 ng/ml as derived from (A) for the indicated time points. Initial phosphorylation status of IKKβ as well as degradation of IκBα was documented by Western-blot analysis. **(C)** Cells were transfected with scrambled siRNA or siRNA specifically knocking down PP2Ac. 48 h later, cells were stimulated with 0.5 ng/ml IL-1 for the indicated time points, and phosphorylation status of IKKβ, degradation of IκBα and protein level of PP2Ac were analysed by Western-blot. In each analysis α-tubulin served as loading control. IKKβ phosphorylation was quantified using Image Quant software. Pooled data of three independently performed experiments are summarised. One representative Western-blot analysis is shown as Additional file 4.

The model parameters are very well identifiable

In order to determine whether the estimated parameter values are unique, we conducted an identifiability analysis as described in the *Methods* section. The χ^2

value of the best 10 % of the fits was consistently 24.45. The distribution of the parameter values clearly shows that the parameters can be uniquely determined for the given observations within the very large investigated parameter range, as the variance in the parameter sets yielding the best χ^2 values is extremely low (Fig. 5A). The parameter *kp* thereby attains its upper bound, so that the upper bound estimate affects the parameter identification. However, all parameters except *kp* itself are robust against variations of the upper bound in a range of at least two orders of magnitude (see Additional file 1).

When considering the influence of noise in the experimental data, the parameter values still remain meaningful: the lower and upper bounds of the 95% confidence intervals do not vary by more than a factor of 2 for any of the parameters (Table 2). Consequently, the determined parameter values allow for some predictions concerning the occurring processes: Firstly, the model parameters suggest that internalization of the receptor complex occurs within a few minutes. Furthermore, a fast association of IL-1 to the receptor is predicted, which is confirmed by literature data [24] (see also Additional file 1, III).

The model rejects the hypothesis of immediate complete PP2A deactivation.

Finally, the model also makes predictions about the mode of PP2A deactivation. Direct deactivation of PP2Ac by UVB radiation (e.g. by destroying the active centre) would be expected to occur almost immediately. Since a relatively slow decrease of PP2A activity $(t_{1/2} = \frac{\ln 2}{kuv} = 48\,\mathrm{min})$ is predicted, the model suggests an indirect and gradual effect of UVB on PP2Ac. In order to exclude the possibility of an immediate deactivation of PP2Ac in the model, we tested the special case $kuv \to \infty$ (or equivalently *kdp = 0* for UVB radiation). The resulting fits clearly show that the model cannot match the experimental data if we assume immediate PP2A deactivation (Fig. 5B). This indicates that UVB-induced gradual PP2Ac inactivation is an indirect effect that requires instrumentality by other molecules.

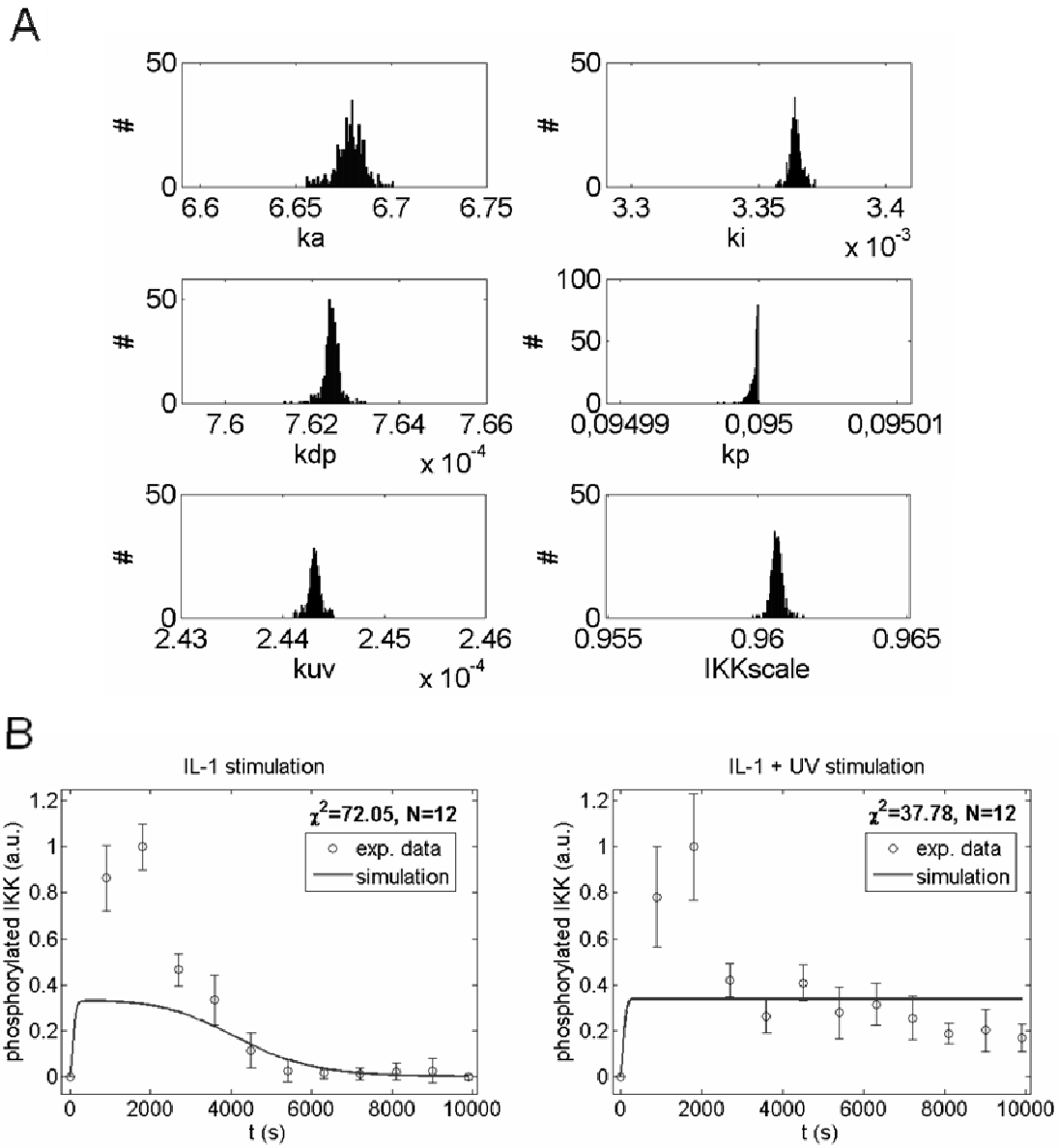

Figure 5 - Model parameters are very well identifiable and suggest indirect deactivation of PP2Ac by UVB

(A) The histograms show the parameter values of the best 10% out of 4000 fits. The initial parameter values for these fits were obtained by randomly varying the parameter values of the reference scenario (Tab. 1) by up to 4 orders of magnitude, as described in the *Methods* section. **(B)** The model rejects the hypothesis of immediate UVB induced PP2Ac deactivation. Assuming *kdp* = 0 in case of UVB stimulation, the experimental data cannot be reproduced (χ^2 = 72.05 + 37.78 = 109.83).

variable	95%-confidence interval	unit
ka	[5.0, 8.5]	$(\mu M \cdot s)^{-1}$
ki	[0.0025, 0.0042]	s^{-1}
kp	[0.095, 0.095]	s^{-1}
kdp	[0.00060, 0.0011]	s^{-1}
kuv	[0.00016, 0.00032]	s^{-1}
IKKscale	[0.78, 1.2]	-

Table 2 - 95%-confidence intervals of the parameters in the reference scenario
Confidence intervals were obtained by fitting to perturbed experimental data and determining the 2.5% and 97.5% quantiles of the resulting parameter sets, as described in the *Methods* section

IL-1 receptor internalization is fast and unaffected by UVB radiation.

IL-1-induced NFκB activation is terminated by IL-1 receptor (IL-1R) internalization, thereby limiting the duration of the exogenous input [25]. Although our data strongly indicate PP2Ac to be constitutively active at the IKK complex regulating the phospho-IKKβ turn over, prolonged IL-1 receptor (IL-1R) activation by UVB may alternatively influence the phospho-IKK status. To investigate whether UVB interferes with the kinetics of IL-1R internalization, and to validate the model prediction of fast internalization, FACS analysis was performed. Comparing cells treated with IL-1 and IL-1 + UVB, respectively, revealed IL-1R internalization to follow almost identical kinetics, starting very early after 5 min and being completed latest after 60 min (Fig. 6A), indicating changes of IL-1R internalization to be irresponsible for prolonged IKKβ phosphorylation. Since the high standard deviation between the independently performed experiments did not allow for a definitive rejection of the hypothesis, we additionally used the model to scrutinize this hypothesis: We altered the model by assuming that UVB radiation does not affect IKKβ dephosphorylation, but alters the internalization of IL-1R (for model equations see Additional file 1, IV). The resulting fits (Fig. 6B) are considerably worse (χ^2 = 24.77 + 25.35 = 50.12) than those of the original model, so that the model suggests rejection of this hypothesis. The experiment also allows validation of the prediction of fast receptor internalization. The

model predicts a receptor complex internalization half-life of $\frac{\ln 2}{ki} = 3.4\,\text{min}$ and a very rapid decrease of the total non-internalized receptor (Additional file 5). This nicely corresponds to the experimental observation that more than half of the receptor is internalized after 5 min (Fig. 6A) and thus presents another indication for the validity of the model.

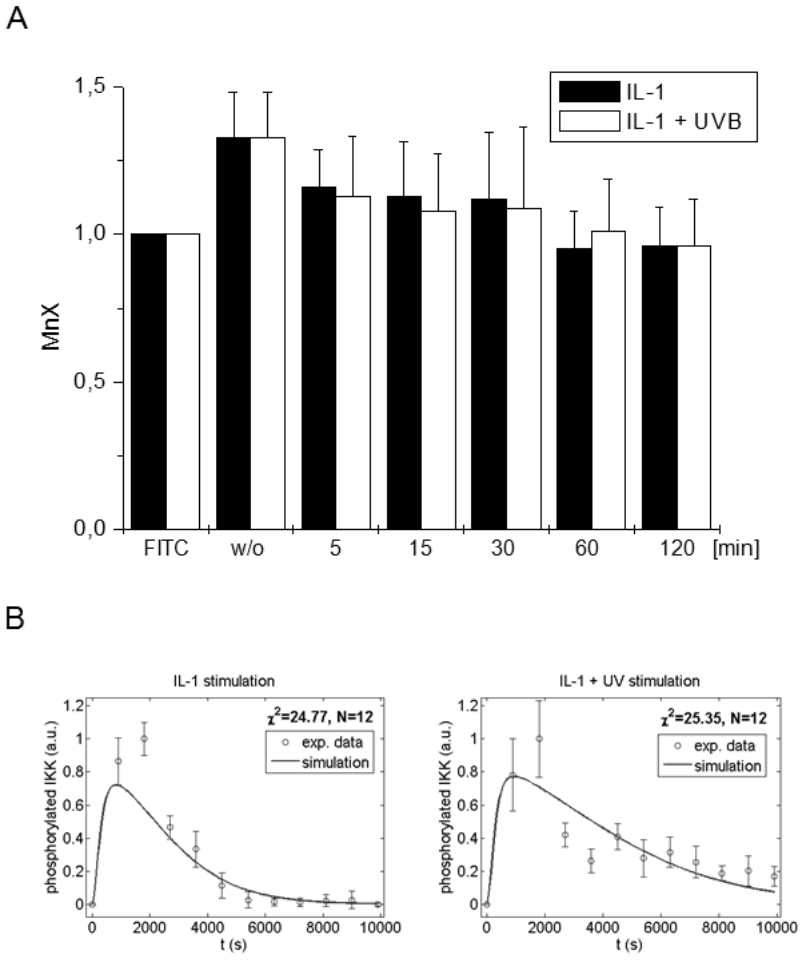

Figure 6 - UVB does not alter IKKβ phosphorylation via delay of IL-1 receptor internalization. **(A)** Cells were left untreated or stimulated with IL-1 (10 ng/ml) alone or in combination with UVB (300 J/m^2) for the indicated time points. Subsequently, IL-1 receptor expression was analyzed by flow cytometry using an IL-1 receptor specific antibody compared to an isotype control of the secondary antibody (FITC). The mean fluorescence intensity (MnX) of three independently performed experiments of this control was set as 1. Receptor expression levels were determined respectively and the MnX of three independently performed experiments compared to FITC control is presented. Consequently, an MnX of 1 in the experiments represents a completely internalized or degraded receptor. **(B)** The hypothesis of receptor internalization leads to a considerably inferior fit quality (χ^2 = 24.77 + 25.35 = 50.12). The model largely diverges from the experimental data for the 30 min value following IL-1 stimulation and systematically underestimates the last 5 data points following IL-1 + UVB stimulation.

Discussion

Tight regulation of NFκB by the negative feedback loop involving post-degradational resynthesis of IκBα is mandatory to ensure proper cellular function. Constitutive NFκB activation is linked to transformation, proliferation, suppression of apoptosis, and metastasis [1, 26]. Thus, strategies interfering with the signaling pathways activating NFκB have become major targets for anti-cancer interventions [27]. The negative feedback loop controlling NFκB activity is critically regulated by the phosphorylation status of the upstream kinase IKKβ. Phosphorylation of Ser 177/181 is a prerequisite for initial phosphorylation and degradation of IκBα. *Vice versa*, dephosphorylation of these IKKβ serine residues is required to prevent phosphorylation of resynthesized IκBα being a prerequisite for NFκB termination [6]. Concerning the molecular mechanism underlying UVB-induced abrogation of the negative feedback loop, we have recently discovered Ser-Thr-phosphatase PP2A to be crucially involved in tuning the phosphorylation status of IKKβ. Impeding IκBα reappearance upon co-stimulation with UVB was linked to UVB-induced inhibition of PP2Ac, causing chronic IKKβ phosphorylation followed by downstream phosphorylation and degradation of resynthesized IκBα [10]. Understanding the general PP2Ac/IKKβ cross talk at the IKK complex, the mode of interference of UVB with PP2Ac activity and the chronology of events leading to full abrogation of the negative feedback loop is of high importance under therapeutic aspects.

Conflicting data about the contribution of PP2A in modulating NFκB activity exist, while most of the reports connect inhibition of PP2A to NFκB activation [10, 28-32]. Less evidence exists suggesting IKK-PP2A complex formation to be a prerequisite for TNF-induced phosphorylation of IKKβ and degradation of IκBα [33]. Developing a new reduced mathematical model strictly relying on experimental data, we could confirm the critical role of PP2A in antagonizing IKKβ phosphorylation and consequently NFκB activity. By this means we were able to unravel the most evident activation status of PP2A at the IKK complex and to predict a mechanism underlying UVB-induced PP2Ac inactivation:

In unstimulated cells PP2A is most likely associated with the IKK complex in a constitutively active fashion, where it presumably controls dephosphorylation of randomly activated IKKβ. Accordingly, we propose the following model of PP2A

function at the IKK complex: In case of IL-1 mediated IL-1 receptor activation, the initial signal input causing IKKβ phosphorylation overrules constitutive PP2A activity, resulting in a net shift towards increase in phosphorylated, i.e. active, IKKβ species, which reach a peak after approximately 15 min. At this time point, IκBα degradation is completed, but the exogenous IL-1 signal is already close to terminated due to rapid IL-1R internalization. Continuing PP2A activity thus results in a reversion of the IKKß status towards an unphosphorylated, inactive species, which subsequently allows accumulation of resynthesized IκBa. In case of PP2Ac inhibition by co-stimulation with UVB, however, the dephosphorylation of IKKβ is strongly impaired, resulting in persistent degradation of newly synthesized IκBα, thereby causing abrogation of the negative feedback loop. Persistent nuclear NFκB activity then promotes pro-apoptotic instead of anti-apoptotic responses under these specific conditions [10].

A constitutive PP2A activity is also consistent with the elusive IKK inactivation mechanism in the model of [18] and may present a promising candidate for the yet unknown fast IKK inactivation mechanism following TNFα stimulation. We could substantially improve our previous modeling approach for IL-1 induced IKK phosphorylation [19] and now present a validated model with well identifiable parameters that incorporates recent findings about the IKK deactivation process. It is independent of the downstream processes and only contains processes strictly necessary to describe the observed dynamics.

The present model structure contains several simplifying assumptions. Particularly, neither hyperphosphorylation nor constitutive protein synthesis and degradation are included, in contrast to other models, e.g. [21]. As shown in detail in Additional file 1, V, these simplifications are consistent with biological considerations and further supported by the fact that a model including these extensions does not perform better than the original model. This finding does not refute the different model structure chosen by Lipniacki et al. [21] but rather indicates that the mechanism of IKKβ deactivation following IL-1 stimulation may differ from that following TNFα stimulation.

Besides the validated predictions concerning the fast receptor kinetics, the model predicted that deactivation of PP2A located at the IKK following UVB radiation does not occur directly through UVB modifications of PP2A. This prediction is consistent

with data from Barisic et al. [10], where total cellular PP2Ac activity is decreased, but still discernable following 2 h of UVB radiation. Although direct inactivation by UVB-mediated destruction of aromatic amino acids in the catalytic centre of Tyr-phosphatases has been reported [34, 35] this does not seem to be the case for Ser/Thr-phosphatase PP2A. Accordingly, indirect deactivation of PP2A by UVB is most likely facilitated by other mediators yet to be identified.

Conclusions

We developed a model of IKKβ phosphorylation with well identifiable parameters that is independent of the downstream processes. This model can be used as a reliable building block for the input of NFκB models investigating the mechanisms associated with the persistent activation of NFκB, which results in pro-apoptotic behavior when combined with UVB and other DNA damaging agents [9, 10, 36, 37]. In contrast to the vast majority of mathematical models for the NFκB signaling pathway, the present model considers IKK phosphorylation independently of IκBα and NFκB. This decouples IKK phosphorylation kinetics from IκBα kinetics and allows for a considerable reduction of the system order. The obtained small model allows for reliable determination of biological parameters such as IKKβ dephosphorylation half-life or half-life of UVB induced PP2Ac deactivation, which are often difficult to obtain experimentally. The simplifying assumptions on which the model is based are supported by biological and model-based reasoning as well as by the good fits to the experimental data and model validation, thus offering a comprehensive simplification potential for future modelling of NFκB signalling. Furthermore, model expansions are very easy to implement, due to the modular model structure which links different modules by signal flows only. These expansions could involve modeling of the omitted proteins in the signaling cascade such as IRAK or TRAF6 [38], or the system behavior for variable UVB doses. For long term studies, constitutive protein synthesis and degradation could be taken into account. Model expansions might also be required for low IL-1 stimulations where a variable IL-1 level or effects in the signaling cascade could play a more prominent role. Combining experimental and modeling approaches sheds new light on the dynamics of IKKβ phosphorylation and the understanding of the negative feedback loop regulation of NFκB. With respect to

tumor maintenance and progression resulting from constitutive NFκB activation [39, 40], the specific cellular activation status of PP2A should be considered by support of mathematical models, and may consequently help to elucidate alternative therapeutic targets to fight individual cancers.

Methods

Cells and reagents

The human epithelial carcinoma cell line KB (ATCC) was cultured in RPMI 1640, 10% FCS. Subconfluent cells were stimulated in colorless medium with 2% FCS. UVB irradiation (300 J/m^2) was performed with TL12 fluorescent bulbs (290-320 nm, Philips). Recombinant human IL-1β (R&D Systems) was applied at 10 ng/ml and 0.5 ng/ml, respectively, as indicated. PP2Ac knock down was facilitated by transfecting 6 x 10^5 cells with 50 pmol siRNA: 5`-GAGGUUCGAUGUCCAGUUA-3` (MWG) using Lipofectamine 2000 (Invitrogen) 48 h prior to stimulation. Scrambled siRNA 5´-UAGAAUUAUUCCUCAACAG-3´ served as negative control.

Western-blot analysis

Cells were harvested via the hot lysis method. Briefly at the indicated time points supernatants were aspirated and adherent cells lysed by addition of 100 µl of hot (95°C) laemmli buffer followed by sonication and incubation at 95°C for further 10 min. After centrifugation, supernatants were collected and 15 µl of each protein samples subjected to 10-12 % SDS-PAGE, blotted onto nitrocellulose membranes and incubated with antibodies directed against pSer177/181-IKKβ, IκBα and PP2Ac (16A6, L35A5, 2038; Cell Signaling) respectively. Equal loading was monitored by reprobing membranes with an antibody against α-tubulin (DM1A, Neomarkers) and HRP conjugated secondary antibodies (Amersham, Buckinghamshire, UK). Bands were visualized applying chemiluminescence SuperSignal®detection system (PIERCE). Relative IKK phosphorylation was quantified densitometrically from Western Blots and normalized against α-tubulin levels using Image Quant™ software. An initial phosphorylation level of 0 for unstimulated IKK was assumed. Time series were scaled in a way that their mean equaled one, subsequently pooled,

and finally scaled in a way that the mean maximal value of both pooled time series equaled one.

FACS analysis

To monitor levels of membrane bound IL-1R, FACS analysis was performed utilizing 0.5 µg of an anti IL-1R mouse IgG (551388, Becton Dickinson) in 100 µl PBS/1% BSA per 5 x 10^5 cells and a goat anti mouse IgG conjugated to FITC (F9137, Sigma). Cells were analyzed in an EPICS® XL-MCL flow cytometer (Coulter, Miami, USA). Excitation wavelength used for FITC was 488 nm. The emitted green fluorescence (λ_{max} 520 nm) was detected using (FL-1) band pass filter. 20.000 cells were analysed for each sample. Data analysis was performed using the WIN/MDI 2.8 software.

Phosphatase assay

Cells were lysed as described above. Endogenous PP2Ac was immunoprecipitated using a specific antibody (PC 12-301, Upstate) and A/G-plus agarose (Santa Cruz) overnight. Immunoprecipitated PP2Ac was 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 (final conc. 75 µM; Biomol) 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. Equal amounts of immunoprecipitated PP2Ac were monitored by Western-blot analysis following the phosphatase assay compared to cell lysate with a specific anti PP2Ac antibody (2038, Cell Signaling).

Model Analysis

The MATLAB (The MathWorks) based software tool box PottersWheel 1.6 [41] was used for the solution, optimization and analysis of the ordinary differential equation system. The χ^2 value was chosen as objective function, with

$$\chi^2(\theta) = \sum_{i=1}^{N} \frac{(y_i - y(t_i; \theta))^2}{\sigma_i^2}$$

where y_i is data point i with standard deviation σ_i and $y(t_i; \theta)$ is the model value at time point i for the parameter vector θ. Initial parameter values prior to the first optimization were arbitrarily chosen as 0.05 for all parameters. Minimization was performed using the FitBoost routine, which combines a trust region and a simulated annealing approach. Additionally, 4000 fits with the trust region approach were performed, each starting from the parameter values of the currently best fit randomly disturbed by up to 4 orders of magnitude (pwF3 routine), thus covering a range of 8 orders of magnitude.

In order to assess the reliability of the obtained best fit parameters, we investigated whether they can be uniquely determined for the given experimental data (identifiability analysis) and how perturbations of the experimental data within the measuring accuracy affect the parameter estimation (confidence intervals).

Identifiability analysis was performed by conducting 4000 independent fits with the trust region method, each starting from the parameter values of the best fit randomly disturbed by up to 4 orders of magnitude (pwF2 routine). The best 10 % of the fits were selected for analysis. For estimation of the confidence intervals, 500 new data sets were generated by adding an $N(0, \sigma_i^2)$ distributed error term to each data point of the original data, where σ_i is the experimentally determined standard deviation at time point i. Separate fitting was performed for each data set, using the FitBoost routine. Additionally 200 fits were performed with the pwF3 routine with random perturbations of up to 2 orders of magnitude, in order to reduce the computational effort. The 2.5% and 97.5% quantiles of the resulting parameter sets were determined.

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Additional files

Additional file 1: Supplementary information about the modelling procedure.

I) IKK kinetics can be modeled without considering IKK-IκBα and IKK-IκBα-NFκB complexes

We consider the model of Lipniacki et al. [1] as an example system. In this system, degradation of IκBα is modeled via the formation of IKK-IκBα and IKK-IκBα-NFκB complexes, from which IκBα is then degraded. We show that this mechanism can be substantially simplified. We consider the following differential equations of the original model [1]:

$$\begin{aligned}
\frac{d\ IKKa(t)}{dt} &= T_R \cdot k_1 \cdot IKKn(t) - k_3 \cdot IKKa(t) - T_R \cdot k_2 \cdot IKKa(t) \cdot A20(t) - k_{deg} \cdot IKKa(t) - \\
&\quad a_2 \cdot IKKa(t) \cdot IkB\alpha(t) + t_1 \cdot IKKaI\kappa B\alpha(t) - a_3 \cdot IKKa(t) \cdot IkB\alpha NF\kappa B(t) + \\
&\quad t_2 \cdot IKKaI\kappa B\alpha NF\kappa B(t) \\
\frac{d\ IKKaI\kappa B\alpha(t)}{dt} &= a_2 \cdot IKKa(t) \cdot IkB\alpha(t) - t_1 \cdot IKKaI\kappa B\alpha(t) \\
\frac{d\ IKKaI\kappa B\alpha NFkB(t)}{dt} &= a_3 \cdot IKKa(t) \cdot I\kappa B\alpha NF\kappa B(t) - t_2 \cdot IKKaI\kappa B\alpha NF\kappa B(t) \\
\frac{d\ NF\kappa B(t)}{dt} &= c_{6a} \cdot IkB\alpha NF\kappa B(t) - a_1 \cdot NF\kappa B(t) \cdot IkB\alpha(t) + \\
&\quad t_2 \cdot IKKaIkB\alpha NF\kappa B(t) - i_1 \cdot NF\kappa B(t)
\end{aligned}$$

Since the value of the parameters t_1 and t_2 is "any large" [1], we can assume that IKKaIκBα(t) and IKKaIκBαNFκB(t) will approximate a value close to zero after a short relaxation period [2], namely

$$IKKaI\kappa B\alpha(t) = \frac{a_2 \cdot IKKa(t) \cdot IkB\alpha(t)}{t_1}$$

$$IKKaI\kappa B\alpha NF\kappa B(t) = \frac{a_3 \cdot IKKa(t) \cdot I\kappa B\alpha NF\kappa B(t)}{t_2}$$

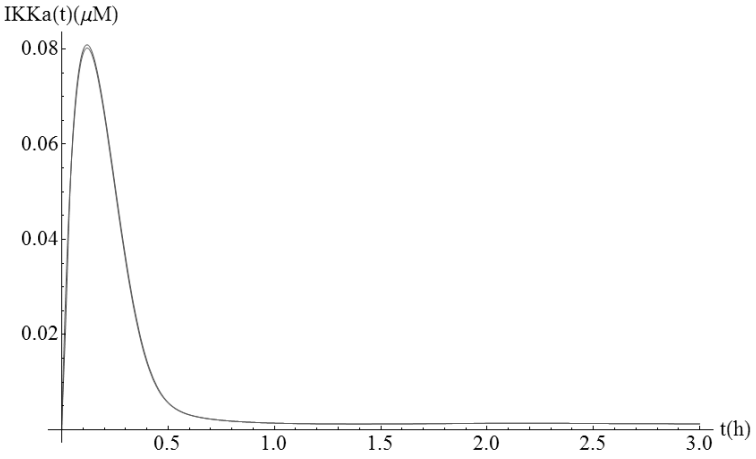
Replacing these arguments in the original equations, we obtain the reduced order representation

$$\frac{d\ IKKa(t)}{dt} = T_R \cdot k_1 \cdot IKKn(t) - k_3 \cdot IKKa(t) - T_R \cdot k_2 \cdot IKKa(t) \cdot A20(t) - k_{\deg} \cdot IKKa(t)$$

$$\frac{d\ NF\kappa B(t)}{dt} = c_{6a} \cdot IkB\alpha NF\kappa B(t) - a_1 \cdot NF\kappa B(t) \cdot IkB\alpha(t) + a_3 \cdot IKKa(t) \cdot IkB\alpha NF\kappa B(t) - i_1 \cdot NF\kappa B(t)$$

The differential equations not mentioned here can be adopted unchanged from Lipniacki et al. [1]. Simulation results using the parameters from the original paper of Lipniacki et al. [1] confirm that the behavior of the original and the reduced model are almost identical (Fig. A1).

After this simplification, IKKa(t) is only coupled to the downstream part of the model by the feedback via A20. In contrast to the TNF stimulation modeled by Lipniacki et al. [1], A20 has been shown to be negligible in IL-1 induced signaling [3] (see also part V of this Additional file), so that the IKK kinetics following IL-1 stimulation can be modeled without considering the IκBα-NFκB part of the model.



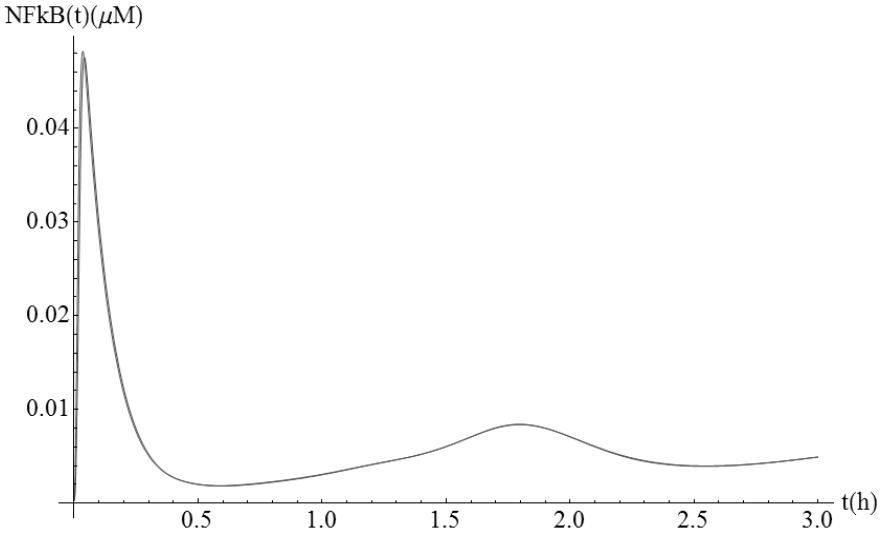


Figure A1 - Comparison of the original model of Lipniacki et al. [1] with its proposed simplification

Simulation results of the original model from Lipniacki et al. [1] (blue) and the reduced model (red) are exemplarily shown for IKKa(t) and NFκB(t). Parameters for both models were taken from Lipniacki et al. [1]. The model and its simplification show an almost identical behavior: the differences vanish due to thickness of the lines. Results for other state variables exhibit similarly small deviations.

II) Determination of an upper bound for the parameter kp

When fitting the model to the experimental data, the parameter *kp* tends to increase to unrealistically high values. We therefore approximate an upper bound for realistic *kp* values based on biophysical considerations as follows:

We assume that the rate determining step in the signaling cascade consists of one diffusion- controlled reaction (e.g. phosphorylation of IKK by TAK1). All other reactions are supposed to proceed with sufficiently high velocity, or to be included in the rate constant *ka* (formation of the receptor complex). The reaction rate constant *k* of a diffusion controlled reaction can be roughly estimated by

$$k = \frac{8RT}{3\eta}$$

where R is the gas constant (8.3145 J K^{-1} mol^{-1}), T is the temperature (K) and η is the viscosity (kg m^{-1} s^{-1}) [4]. For the cytosol, we assume a viscosity of about $6 \cdot 10^{-3}$ kg m^{-1} s^{-1} [5]. Therefore, for a temperature of 37°C = 310.15 K, we obtain $k = 1.15 \cdot 10^{6}$ J m s kg^{-1} mol^{-1} = $1.15 \cdot 10^{3}$ μM^{-1} s^{-1}.

As the state variable ILRc is normalized, this normalization has to be included into the rate constant. Up to 10 000 IL-1 receptors per cell have been reported [6], which is 0.0083 µM, assuming a cell volume of $2 \cdot 10^{-12}$ l [7]. Multiplying by the rate constant, we obtain k = 9.5 s^{-1}. We hereby neglect possible amplification or attenuation factors during the signaling cascade.

In any case, the obtained value is likely to be much overestimated: it only considers one of the reactions in the signaling cascade which are required to phosphorylate IKK. Furthermore, it implies that the collision of the two molecules always results in a reaction, whereas only a small part of the protein surface is in fact reactive.

We assume that the latter induces the largest overestimation and therefore assume additionally that only 10 % of each molecule's surface is reactive. We therewith obtain kp = 9.5 · 0.1 · 0.1 s^{-1} = 0.095 s^{-1}.

It is clearly visible that this procedure can only provide rough estimates. As the optimal *kp* value is exactly on the upper bound, it has to be considered uncertain in spite of its very good identifiability. However, the choice of the upper bound only marginally influences the values of the other parameters, as shown in Table A1.

	kp_{max} = 0.0095 s^{-1}	kp_{max} = 0.095 s^{-1}	kp_{max} = 0.95 s^{-1}	unit
ka	5.0	6.7	8.8	$(\mu M \cdot s)^{-1}$
ki	0.0027	0.0034	0.0045	s^{-1}
kp	0.0095	0.095	0.95	s^{-1}
kdp	0.00072	0.00076	0.00078	s^{-1}
kuv	0.00023	0.00024	0.00025	s^{-1}
IKKscale	1.21	0.96	0.91	-

Table A1 - Parameter values of the best fit of the reference model with different upper bounds for *kp*

III) The association rate predicted by the model is in accordance to literature data

A study investigating the IL-1 binding behavior in human fibroblasts [8] reports an IL-1β association rate constant of $8 \cdot 10^{-7}$ $(M \cdot min)^{-1}$ at 8°C. Correcting the obviously

inversed sign in the exponent[1], the measured value corresponds to 1.33 $(\mu M \cdot s)^{-1}$. Since kinetic constants generally increase significantly with rising temperature, this value is in accordance to the predicted value for *ka,* which is 6.7 $(\mu M \cdot s)^{-1}$ at 37°C. Note that this value is not affected if different initial concentrations of the IL-1 receptor are assumed.

IV) Model equations for the hypothesis of altered internalization

The hypothesis of an effect of UVB on internalization is modeled using the following equations:

$$\frac{d\,ILR(t)}{dt} = -ka \cdot il(t) \cdot ILR(t), \quad ILR(0) = 1$$
$$\frac{d\,ILRc(t)}{dt} = ka \cdot il(t) \cdot ILR(t) - ki \cdot ILRc(t), \quad ILRc(0) = 0$$
$$\frac{d\,IKKp(t)}{dt} = kp \cdot ILRc(t) \cdot (1 - IKKp(t)) - kdp \cdot IKKp(t), \quad IKKp(0) = 0$$

Note that these equations correspond to the original model with PP2A(t) ≡ 1. Thereby, the parameter *ki* may have different values with and without UVB stimulation.

V) Motivation for the simplifying model assumptions

Besides the assumptions made in the *Results* section, the model (Fig. 3A) contains several implicit assumptions, which we will specify and motivate in the following:

The amount of IL-1 was assumed to be much higher than the amount of the IL-1 receptor, reflecting the experimental conditions. IL-1 concentration was therefore approximated as constant after stimulation.

Dissociation of IL-1 from the receptor complex was not considered in the model: For dissociation of IL-1 from the receptor, a rate constant of $2.8 \cdot 10^{-5}$ s^{-1} can be calculated based on biological considerations [9]. This corresponds to a half-life of about 7 hours, so that this process is much unlikely to be relevant, especially when considering the fast kinetics at the receptor.

The internalized IL-1 receptor was assumed to be inactive and was therefore regarded as degraded in the model. Since the aim was to create a simple model, the

[1] The given rate constant would imply that assuming a constant IL-1 concentration of 10 ng/ml, or 0.000588 µM, and mass action kinetics without dissociation, internalization or degradation, IL-1 has bound to half of the receptors after $\frac{\ln 2}{0.000588\mu M \cdot 8 \cdot 10^{-7} (M \cdot \min)^{-1}} = 2.8 \cdot 10^{9}\, a$

very fast kinetics of the signalling cascade leading to IKK phosphorylation was not considered.

Since there are no mass flows, but only signal flows between the modules describing the dynamics of the proteins ILR/ILRc, IKK/IKKp and PP2A, absolute concentrations for these proteins could implicitly be incorporated into the signaling rate constants without loss of generality. Initial concentrations of 1 were therefore assumed for all proteins, so that each state variable reflects the fraction of the total initial concentration of the respective protein.

Compared to other models of IKK phosphorylation (e.g. [1]), several processes are not considered in the present model, namely inactivation of IKK by hyperphosphorylation and constitutive protein synthesis and degradation. This can be motivated by biological as well as model-based arguments:

Constitutive protein synthesis and degradation usually occur on a time scale much larger than the one relevant for signal transduction, which suggests that it is not essential to model them.

As to hyperphosphorylation, Lipniacki et al. assume A20-mediated and spontaneous hyperphosphorylation of IKK following TNF stimulation, leading to IKK deactivation [1]. However, Cheong et al. doubt that early IKK inhibition is mediated by A20 [10]. Following IL-1 stimulation, seemingly contradictory results have been reported for the effect of A20. While no change of the IκBα kinetics can be observed in IL-1 treated A20-/- cells [3], NFκB activity following IL-1 stimulation increases significantly in cells overexpressing A20 [11].

These results can be reconciled by assuming a very weak influence of A20 on IKK following IL-1 stimulation in WT cells. Therefore, only spontaneous but not A20 dependent hyperphosphorylation of IKKp needs to be considered in a model for IL-1 induced IKK phosphorylation. In view of the experimental data for IKK phosphorylation (Fig. 1), however, hyperphosphorylation without subsequent dephosphorylation also seems unlikely since it would suggest a permanently elevated level of (hyper-) phosphorylated IKK, which is not consistent with the experimental data.

Though the given biological arguments all indicate that the mentioned processes are negligible, we also developed an extended model version including constitutive protein synthesis and degradation for all proteins and spontaneous IKK

hyperphosphorylation, in analogy to the model of Lipniacki et al. [1]. The structure of the complete, unreduced model reads

$$\frac{d\,I\tilde{L}R(t)}{dt} = k_{syntilr} - k_{degilr}\cdot I\tilde{L}R(t) - k_a\cdot il(t)\cdot I\tilde{L}R(t)$$

$$\frac{d\,I\tilde{L}Rc(t)}{dt} = k_a\cdot il(t)\cdot I\tilde{L}R(t) - k_i\cdot I\tilde{L}Rc(t)$$

$$\frac{d\,I\tilde{K}K(t)}{dt} = k_{syntikk} - k_{degikk}\cdot I\tilde{K}K(t) - \tilde{k}_p\cdot I\tilde{L}Rc(t)\cdot I\tilde{K}K(t) + \tilde{k}_{dp}\cdot P\tilde{P}2A(t)\cdot I\tilde{K}Kp(t)$$

$$\frac{d\,I\tilde{K}Kp(t)}{dt} = -k_{degikk}\cdot I\tilde{K}Kp(t) - k_{hyp}\cdot I\tilde{K}Kp(t) + \tilde{k}_p\cdot I\tilde{L}Rc(t)\cdot I\tilde{K}K(t) - \tilde{k}_{dp}\cdot P\tilde{P}2A(t)\cdot I\tilde{K}Kp(t)$$

$$\frac{d\,P\tilde{P}2A(t)}{dt} = k_{syntpp2a} - k_{degpp2a}\cdot P\tilde{P}2A(t) - k_{uv}\cdot uv(t)\cdot P\tilde{P}2A(t)$$

$$I\tilde{L}R(0) = \frac{k_{syntilr}}{k_{degilr}}$$

$$I\tilde{L}Rc(0) = 0$$

$$I\tilde{K}K(0) = \frac{k_{syntikk}}{k_{degikk}}$$

$$I\tilde{K}Kp(0) = 0$$

$$P\tilde{P}2A(0) = \frac{k_{syntpp2a}}{k_{degpp2a}}$$

where additionally to the notation used in the reference model, k_{synt} and k_{deg} denote constitutive synthesis and degradation rate constants of the respective proteins and k_{hyp} denotes the rate constant for spontaneous hyperphosphorylation. Rate constants or state variables with a tilde will be used with a different scaling in the following.

We now normalize the state variables $I\tilde{L}R(t), I\tilde{K}K(t)$ and $P\tilde{P}2A(t)$ such that ILR(0) = 1, IKK(0) = 1 and PP2A(0) = 1, and additionally normalize the remaining state variables correspondingly, i.e.

$$I\tilde{L}R(t) = ILR(t)\cdot\frac{k_{syntilr}}{k_{degilr}}$$

$$I\tilde{L}Rc(t) = ILRc(t)\cdot\frac{k_{syntilr}}{k_{degilr}}$$

$$I\tilde{K}K(t) = IKK(t)\cdot\frac{k_{syntikk}}{k_{degikk}}$$

$$I\tilde{K}Kp(t) = IKKp(t)\cdot\frac{k_{syntikk}}{k_{degikk}}$$

$$P\tilde{P}2A(t) = PP2A(t)\cdot\frac{k_{syntpp2a}}{k_{degpp2a}}$$

Substituting this in the original system, the normalized system now reads

$$\frac{d\,ILR(t)}{dt} = k_{degilr} - k_{degilr} \cdot ILR(t) - k_a \cdot il(t) \cdot ILR(t)$$
$$\frac{d\,ILRc(t)}{dt} = k_a \cdot il(t) \cdot ILR(t) - k_i \cdot ILRc(t)$$
$$\frac{d\,IKK(t)}{dt} = k_{degikk} - k_{degikk} \cdot IKK(t) - \tilde{k}_p \cdot \frac{k_{syntilr}}{k_{degilr}} \cdot ILRc(t) \cdot IKK(t) + \tilde{k}_{dp} \cdot \frac{k_{syntpp2a}}{k_{degpp2a}} \cdot PP2A(t) \cdot IKKp(t)$$
$$\frac{d\,IKKp(t)}{dt} = -k_{degikk} \cdot IKKp(t) - k_{hyp} \cdot IKKp(t) + \tilde{k}_p \cdot \frac{k_{syntilr}}{k_{degilr}} \cdot ILRc(t) \cdot IKK(t) - \tilde{k}_{dp} \cdot \frac{k_{syntpp2a}}{k_{degpp2a}} \cdot PP2A(t) \cdot IKKp(t)$$
$$\frac{d\,PP2A(t)}{dt} = k_{degpp2a} - k_{degpp2a} \cdot PP2A(t) - k_{uv} \cdot uv(t) \cdot PP2A(t)$$
$$ILR(0) = 1$$
$$ILRc(0) = 0$$
$$IKK(0) = 1$$
$$IKKp(0) = 0$$
$$PP2A(0) = 1$$

If we now define

$$k_p = \tilde{k}_p \cdot \frac{k_{syntilr}}{k_{degilr}}$$
$$k_{dp} = \tilde{k}_{dp} \cdot \frac{k_{syntpp2a}}{k_{degpp2a}}$$

we get

$$\frac{d\,ILR(t)}{dt} = k_{degilr} - k_{degilr} \cdot ILR(t) - k_a \cdot il(t) \cdot ILR(t)$$
$$\frac{d\,ILRc(t)}{dt} = k_a \cdot il(t) \cdot ILR(t) - k_i \cdot ILRc(t)$$
$$\frac{d\,IKK(t)}{dt} = k_{degikk} - k_{degikk} \cdot IKK(t) - k_p \cdot ILRc(t) \cdot IKK(t) + k_{dp} \cdot PP2A(t) \cdot IKKp(t)$$
$$\frac{d\,IKKp(t)}{dt} = -k_{degikk} \cdot IKKp(t) - k_{hyp} \cdot IKKp(t) + k_p \cdot ILRc(t) \cdot IKK(t) - k_{dp} \cdot PP2A(t) \cdot IKKp(t)$$
$$\frac{d\,PP2A(t)}{dt} = k_{degpp2a} - k_{degpp2a} \cdot PP2A(t) - k_{uv} \cdot uv(t) \cdot PP2A(t)$$
$$ILR(0) = 1$$
$$ILRc(0) = 0$$
$$IKK(0) = 1$$
$$IKKp(0) = 0$$
$$PP2A(0) = 1$$

Note that the parameters $k_{syntilr}$, $k_{syntikk}$ and $k_{syntpp2a}$ do not occur any more in the normalized model. However, the normalization does not cause a loss of generality, since IKKp(t) is a scaled representation of $\tilde{IKKp}(t)$, and only relative values of IKKp(t) are observable.

Fitting of the normalized model to the experimental data leads to $k_{degilr} = k_{degikk} = k_{degpp2a} = k_{hyp} = 0$, after extensive search (4000 fits with the trust region approach) within a range of 8 orders of magnitude around initial parameter values of 0.001 for all rate constants. Naturally, the χ^2 value and the remaining parameters are the same as given in the reference scenario. The biological reasoning is therewith confirmed by the fitting results, which reveal that the additionally assumed processes are best integrated into the model when assuming that they do not occur at all.

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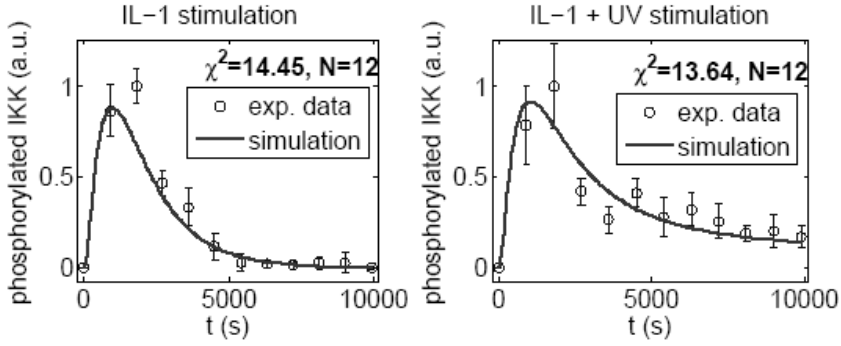
Additional file 2: Simulation results of the reference model with different parameterization.



The fit is visually more acceptable but slightly worse (χ^2 = 14.45 + 13.64 = 28.09) compared to the original parameters. The parameter values are *ka* = 5.1 $(\mu M \cdot s)^{-1}$, *ki* = 0.0027 s^{-1}, *kdp* = 0.00071 s^{-1}, *kp*=0.0037 s^{-1}, *kuv* = 0.00022 s^{-1}, *IKKscale* = 1.8. Note that except for *kp* and the scaling factor, the values are very similar to those of the reference scenario, supporting the reliability of the determined values.

Additional file 3: Simulation results for the alternative model with delayed PP2A activation.

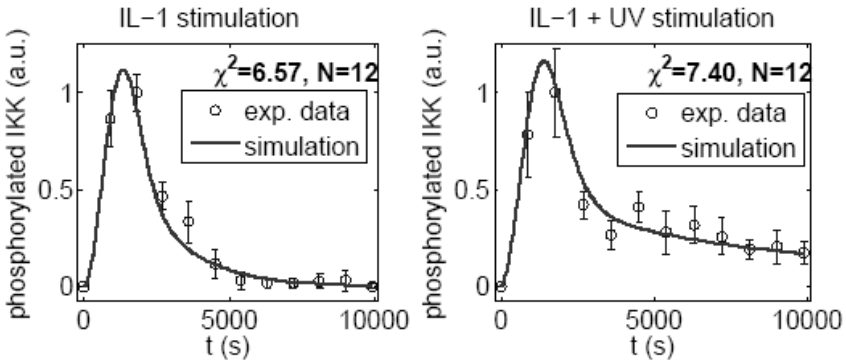
The alternative hypothesis of delayed PP2A activation produces better fits than the reference model (Fig. 3B). However, both χ^2 values do not allow for a rejection of the respective model, so that neither model can be rejected based on modeling results.

Furthermore, the alternative model also comprises two additional degrees of freedom. Considering this, the quality of the fits is about comparable to the fit quality of the reference scenario. The delayed activation was modeled as a Hill-type kinetics,

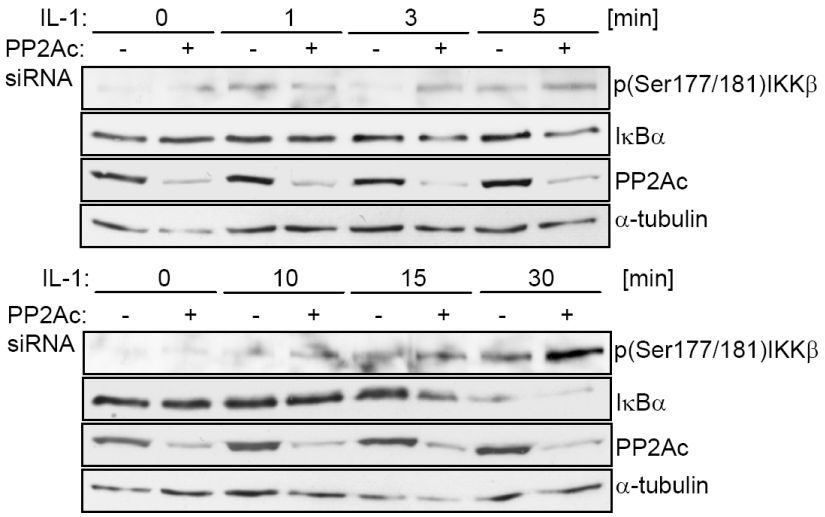
$$\frac{\mathrm{d\,PP2A(t)}}{\mathrm{d\,t}} = \mathrm{PP2An(t)} \cdot \mathrm{il} \cdot \frac{\mathrm{Vmax} \cdot \mathrm{t}^2}{\mathrm{K}^2 + \mathrm{t}^2} - \mathrm{uv} \cdot \mathrm{kuv} \cdot \mathrm{PP2A(t)}, \quad \mathrm{PP2A(0)} = 0$$

$$\frac{\mathrm{d\,PP2An(t)}}{\mathrm{d\,t}} = -\mathrm{PP2An(t)} \cdot \mathrm{il} \cdot \frac{\mathrm{Vmax} \cdot \mathrm{t}^2}{\mathrm{K}^2 + \mathrm{t}^2}, \quad \mathrm{PP2An(t)} = 1$$

where PP2An(t) is the inactive form of PP2A. Note that this formulation of the kinetics is only applicable for $t \geq 0$ and constant inputs *il* and *uv*. The time point of half maximal activation velocity *K* was assumed to occur between 30 and 60 min, 1800 s ≤ *K* ≤ 3600 s. The resulting parameter values are: *ka* = 1.4 $(\mu M \cdot s)^{-1}$, *ki* = 0.00081 s^{-1}, *kdp* = 0.0049 s^{-1}, *kp*=0.0016 s^{-1}, *kuv* = 0.00045 s^{-1}, *Vmax* = 7.7 $(\mu M \cdot s)^{-1}$, *K* = 3600 s, scale*IKK* = 3.4. The unusual unit of *Vmax* is due to the fact that PP2An(t) is dimensionless, whereas *il* has the unit µM.

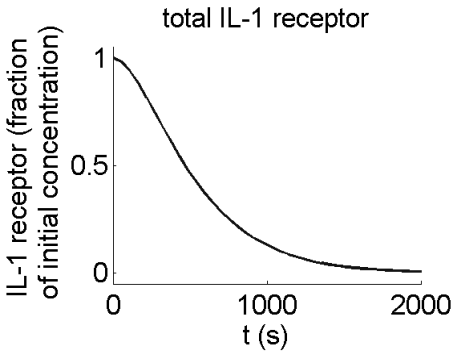


Additional file 4: Representative Western Blot Analysis



Representative Western Blot of PP2Ac-dependent IKKβ phosphorylation.
Cells were transfected with scrambled siRNA or siRNA specifically knocking down PP2Ac. 48 h later, cells were stimulated with 0,5 ng/ml IL-1 for the indicated time points, and the phosphorylations status of IKKβ, degradation of IκBα and protein level of PP2Ac were analysed by Western-blotting. In each analysis α-tubulin served as loading control. Data shown represent one out of three independently performed experiments.

Additional file 5: Simulation results for IL-1 receptor internalisation



Shows the amount of total IL-1 receptor (ILR + ILRc) in the reference scenario.
The time course of total IL-1 receptor (ILR + ILRc) following IL-1 stimulation in the reference scenario corresponds qualitatively to the experimental observations (Fig. 6A).

III Tyrosine kinase Src inhibits Ser/Thr phosphatase PP2A to trigger sustained canonical NFκB activation downstream of IL-1

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Abstract

The activation status of the Tyr-kinase Src as well as of the transcription factor nuclear factor kappaB (NFκB) is a decisive criterion for the onset of cancer. While the activation status of Src is Tyr phosphorylation dependent, NFκB activation requires Ser phosphorylation of its cytosolic inhibitor, IκBα, as a prerequisite of its ubiquitination and proteasomal degradation. Utilizing the Tyr phosphatase inhibitor orthovanadate (OVA), we unravelled a new sequence of molecular interactions linking initial activating Tyr416 phosphorylation of Src to sustained Ser177/181 phosphorylation of the IκBα kinase IKKβ following IL-1 stimulation. As the critical translator of Tyr to Ser phosphorylation we identified the Ser/Thr phosphatase PP2A. Previous studies from our lab revealed PP2A mediated dephosphorylation and inhibition of IKKβ activity upon IL-1 treatment, constituting, together with resynthesized IκBα, a negative feedback loop which terminates NFκB activity. We here show that the catalytic subunit PP2Ac serves as a Src substrate with Tyr307 phosphorylation leading to inhibition of its catalytic activity. This in turn results in sustained IKKβ phosphorylation at Ser177/181 which provides for chronic IκBα degradation and consequently persistent NFκB activation. In addition to the known survival pathways triggered by Src, Src-mediated canonical and sustained NFκB activation may fortify the tumorigenic effects of dysregulated Src by constanty upregulating anti-apoptotic genes. Since PP2A seems to be the nodal point in this

amplifying signal transduction pathway, its specific inhibition should be implemented in the development of new therapeutic strategies in cancer treatment.

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 (reviewed in (1); 2, 3). NFκB (p65/p50) is mostly activated by pro-inflammatory mediators including IL-1 through receptor dependent activation of a multi subunit IκB-kinase (IKK) complex consisting of IKKα, -β and -γ. Following canonical activation, Ser177/181 phosphorylation of IKKβ catalyzes Ser32/36 phosphorylation of the NFκB inhibitor IκBα, leading to its polyubiquitination and proteasomal degradation. Liberated NFκB binds to responsive promoter elements within the nucleus causing activation of multiple genes involved in inflammation, proliferation, angiogenesis and anti-apoptotic signalling (4, 5). Accordingly, proper cellular function is assured by early NFκB-mediated resynthesis of IκBα representing a negative regulatory feedback loop (6). Recent data from our lab revealed the Ser/Thr phosphatase PP2A to be critically involved in the dephosphorylation of IKKβ Ser177/181, thereby allowing accumulation of resynthesized IκBα levels as a prerequisite for NFκB termination following IL-1 stimulation (7). Technical inhibition of the catalytic subunit of PP2A, PP2Ac, instead resulted in chronic IKKβ Ser177/181 phosphorylation, continuous downstream phosphorylation and consequently degradation of newly synthesized IκBα thus causing sustained NFκB activation (7). Accordingly, strategies interfering with both signalling pathways have become major targets for anticancer interventions and serve as potential therapeutic targets in malignancies and immunological disorders (8). Following IL-1 stimulation, however, Src activation could also directly be linked to NFκB activation. Less evidence exists that this process is trigged by alternative Src-dependent Tyr188/199 of IKKβ followed by canonical IκBα degradation (9, 10). In

contrast, direct Src-dependent phosphorylation of IκBα at Tyr42 was shown to represent an alternative way of IκBα degradation and NFκB activation in numerous cell lines (11, 12, 13). The latter pathway was shown to be strongly consolidated upon treatment with the Tyr-phosphatase inhibitor orthovanadate (OVA) (14, 15, 16). In this context, OVA can stabilize activating Tyr416 autophosphorylation of Src as well as IκBα Tyr-42 phosphorylation (11, 16, 17).

Investigating the essential negative feedback regulation of NFκB in epithelial cells following IL-1 stimulation, we observed that costimulation with OVA fully abrogated IκBα resynthesis. The underlying mechanism was found to be independent of either IKKβ or IκBα Tyr phosphorylation but clearly affected the canonical pathway, involving Ser32/36 phosphorylation of IκBα by IKKβ. Rather, Src-dependent phosphorylation at Tyr307 caused inhibition of PP2Ac, a negative regulator of IKKβ. This ensured prolonged IKKβ Ser177/181 phosphorylation, resulting in continuous Ser32/36 dependent IκBα degradation. The here identified new mechanism of Src-dependent chronic NFκB activation may contribute to cancer initiation/progression and should be considered in the context of development of anticancer strategies.

Results

OVA-induced inhibition of IκBα resynthesis is independent of Tyrosine phosphorylation of either IKKβ or IκBα.

According to the negative regulatory feedback loop following IL-1 stimulation, NFκB-initiated resynthesis of IκBα starts 90 min after initial degradation and is completed after 2 h. Upon pretreatment of cells with the tyrosine phosphatase inhibitor orthovanadate (OVA), however, IκBα resynthesis was completely abrogated (Fig. 1A). Since Tyr phosphorylation of IKKβ has been described to represent an alternative way of downstream IκBα degradation (9, 10), tyrosine residues at positions 188, 199, 205, 261, 294, 397, 497 and 188/199 were exchanged to phenylalanine by site directed mutagenesis and the resynthesis of IκBα upon IL-1 and OVA cotreatment was investigated in cells overexpressing each of the IKKβ mutants. After 2 h of cotreatment IκBα remained absent (Fig. 1B) indicating other

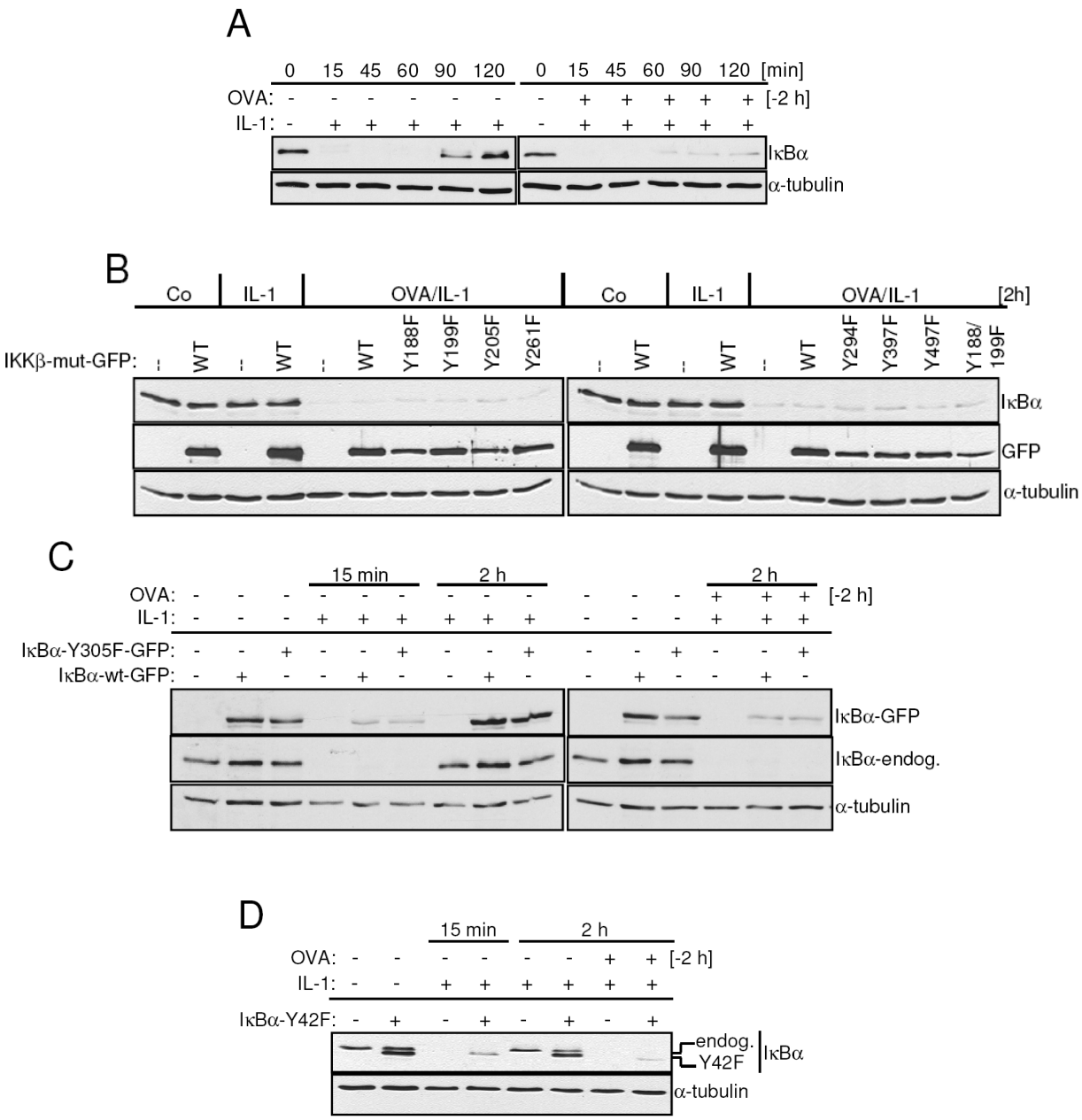

Figure 1 - OVA-induced inhibition of IκBα resynthesis is independent of Tyr phosphorylation of either IKKβ or IκBα. **(A)** KB cells were preincubated or not with OVA (1 mM) for 2 h and then stimulated with IL-1 (10 ng/ml) for the indicated time points. The IκBα protein levels were documented by Western-blot analysis. **(B)** 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. **(C)** Cells were transfected with the empty vector or the respective plasmid overexpressing IκBα–wt-GFP or IκBα-Y305F-GFP or **(D)** 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.

mechanisms than IKKβ-Tyr phosphorylation to cause OVA-induced IκBα degradation. Due to multiple reports proclaiming direct OVA-induced and tyrosine kinase Src-mediated Tyr42 phosphorylation of IκBα as an alternative to trigger its degradation and consequently NFκB activation (11, 12, 13, 14, 15, 16), the effect of Y42F mutation in IκBα on both its initial, IL-1-induced degradation as well as on its failure to reaccumulate within the cytoplasm upon OVA cotreatment was analyzed. Moreover, since Tyr305 phosphorylation was implicated in hepatitis C virus induced NFκB activation (18), a IκBαY305F mutant was also included to investigate potential alternative tyrosines to be targeted by OVA. Although initial degradation of IκBα-Y42F was slightly delayed (Fig. 1D), neither this mutant nor the IκBα-Y305F variant showed any different behaviour when compared to endogenous or ectopically expressed IκBα-wt. In particular, both mutants failed to reappear at later time points, indicating that this alternative pathway of Src dependent NFκB activation is not involved (Fig. 1C and D).

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

Ectopic expression of an IκBα mutant in which the two serine residues 32/36, essential for canonical NFκB activation were substituted by alanine (IκBα-S32/36A) revealed that the mutant remained unaffected, whereas reappearance of endogenous IκBα was inhibited by OVA-treatment. (Fig. 2A). If indeed Ser32/36 phosphorylation of newly synthesized IκBα followed by its proteasomal degradation is responsible for its disappearance upon IL-1 and OVA stimulation, Ser-phosphorylated IκBα should become detectable upon proteasomal inhibition. 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, a time point when initial IκBα degradation is already completed, also yielded Ser32/36 phosphorylated IκBα, suggesting OVA-induced inhibition of IκBα resynthesis following canonical IκBα degradation (Fig. 2B). This assumption was further strengthened by the observation that Ser177/181 phosphorylation levels of IKKβ remained elevated upon cotreatment with OVA at this time point (Fig. 2B) and may consequently allow for continuous

downstream phosphorylation and degradation of newly synthesized IκBα. Formal evidence for prolonged IKKβ activity was obtained in *in vitro* kinase assays of IKKβ immunoprecipitated from IL1/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 cellular IκBα phosphorylation and degradation. 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. 2C).

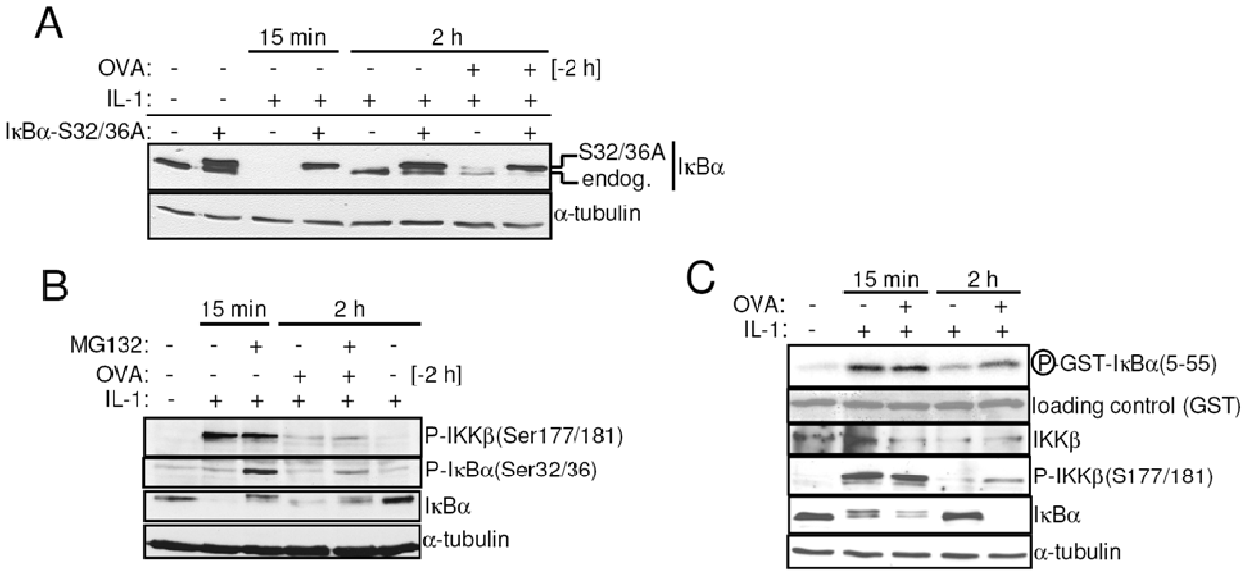

Figure 2 - OVA-induced inhibition of IκBα resynthesis is dependent on canonical Ser phosphorylation of IκBα and coincides with sustained IKKβ activation. **(A)** KB cells were transfected with the empty vector or the respective plasmid overexpressing IκBα–S32/36A. 24 h later cells were preincubated or 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 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.

Tyr-kinase Src activation follows OVA treatment and causes Tyr phosphorylation and inhibition of PP2Ac.

OVA has been described to mediate IκBα degradation via Src-dependent Tyr phosphorylation of this NFκB inhibitor. In fact, treatment of cells with the Tyr phosphatase inhibitor OVA caused activation of the Tyr-kinase Src, evident from enhanced Tyr416 autophosphorylation (Fig. 3A). In the cell system studied here, however, not Tyr phosphorylation but rather canonical Ser phosphorylation of IκBα seems to take place upon costimulation of cells with IL-1 + OVA. We therefore scrutinized other potential targets of Src, which might interfere with IκBα resynthesis. A potential candidate is the Ser/Thr phosphatase PP2Ac, which we could recently show to be essentially involved in tuning down IKKβ activity, thereby contributing to the regulatory feedback loop of NFκB following IL-1 treatment (7). Overexpressing a constitutively active variant of Src (Src-CA) resulted in Tyr-phosphorylation of PP2Ac, which was further enhanced in cells cotreated with OVA (Fig. 3B). Phosphorylation of PP2Ac was documented by Western-blot analysis with a pTyr specific antibody as well as with an antibody only recognizing non-phosphorylated but not Tyr-307 phosphorylated PP2Ac (Fig. 3B).Tyr307 phosphorylation of PP2Ac is known to cause inhibition of this phosphatase (19) 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 specified Tyr-phosphorylation of PP2Ac only to take place in cells overexpressing constitutively active (Src-CA) but not in cells overexpressing a kinase-dead (Src-KD) variant of Src (Fig. 3C), and an *in vitro* kinase assay confirmed PP2Ac to be a specific target of Src (Fig. 3D). Finally, an *in vitro* phosphatase assay was performed to investigate the impact of Src-dependent Tyr phosphorylation on PP2Ac activity. As expected, basal PP2Ac activity was shown to be clearly enhanced in cells ectopically expressing Src-KD, whereas it was significantly decreased in cells expressing Src-CA (Fig. 3E). Data so far indicated that OVA-induced phosphorylation of Src increased its activity thereby triggering Tyr307 phosphorylation and consequently inhibition of PP2Ac, finally resulting in decelerated dephosphorylation of IKKβ.

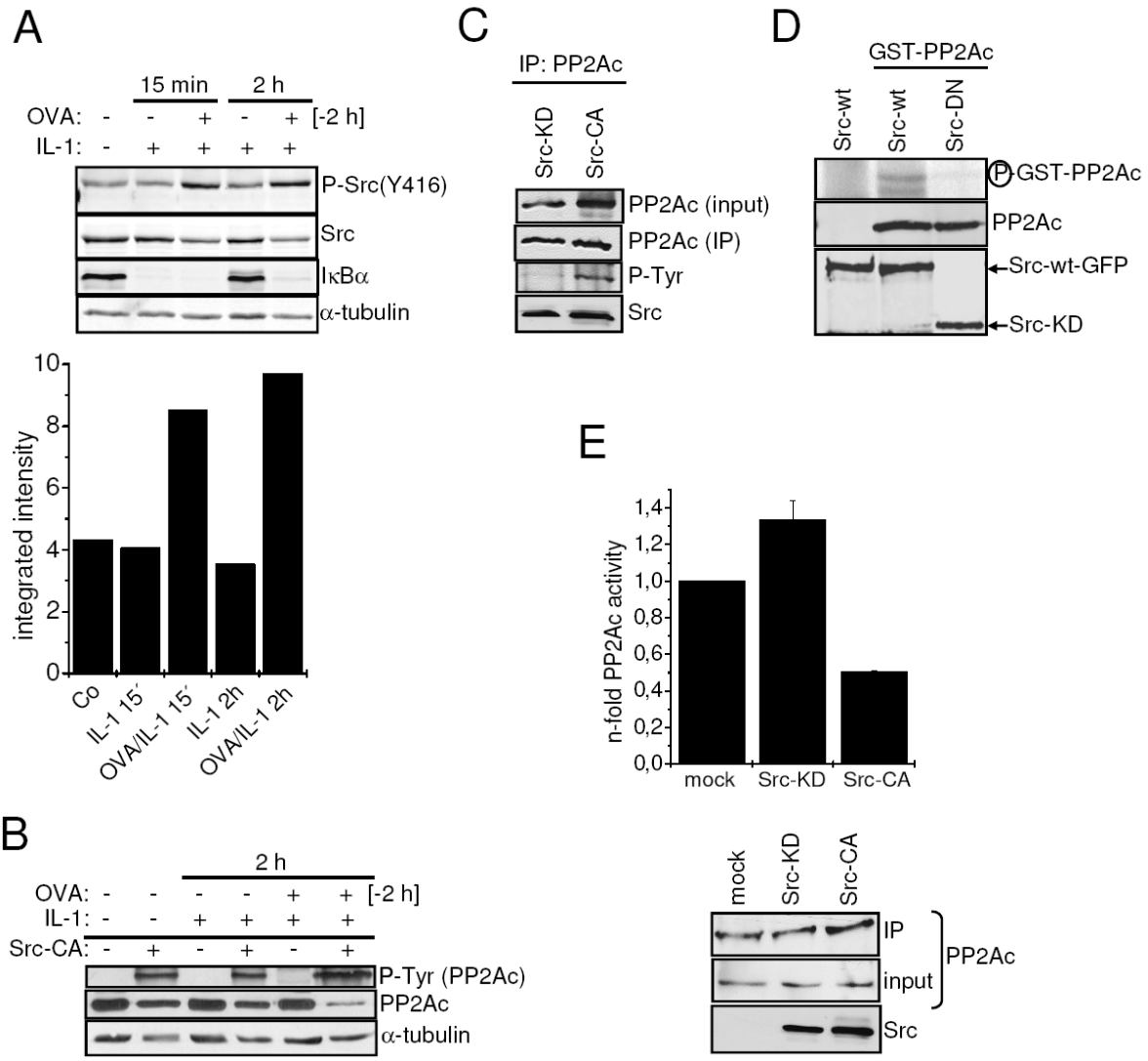

Figure 3 - Src activation following OVA treatment causes Tyr phosphorylation and inhibition of PP2Ac. **(A)** Cells were stimulated with IL-1 (10 ng/ml) or with IL-1 + OVA (1 mM, -2 h). After 15 min and 2 h the Y416 phosphorylation status of Src was correlated to the cellular IκBα level by Western-blot analysis and calculated using Image Quant software. **(B)** 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). **(C)** 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. **(D)** 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. **(E)** 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. Immunoprecipitation of PP2Ac and expression levels of Src variants was determined by western-blot analysis.

Upon OVA treatment Src and PP2Ac cooperate to extend IKKβ activation, thereby causing degradation of resynthesized IκBα, and abrogation of the negative feedback loop of NFκB.

To finally link Src-dependent PP2Ac inhibition to sustained IKKβ activation and lack of IκBα recurrence, we firstly documented PP2Ac-dependent dephosphorylation of IKKβ. PP2Ac inhibition by the specific inhibitor cayculinA as well as siRNA driven knock down PP2Ac resulted in strong Ser177/181 phosphorylation of IKKβ and lack of IκBα recurrence after 2h, being most pronounced upon combination of both inhibitory means (Fig. 4A). The corresponding effect was achieved by overexpression of Src-CA. On the one hand, overexpression resulted in inhibitory Tyr phosphorylation of PP2Ac as documented by two different antibodies – one detecting p-Tyr, and one recognizing only unphosphorylated but not phosphorylated PP2Ac. On the other hand, the time point of strongest PP2Ac phosphorylation closely correlated with the enhancement of IKKβ Ser177/181 phosphorylation initiated by IL-1 + OVA (2 h) stimulation (Fig. 4B). Finally, overexpression of Src-KD facilitated reappearance of IκBα (Fig. 4C).

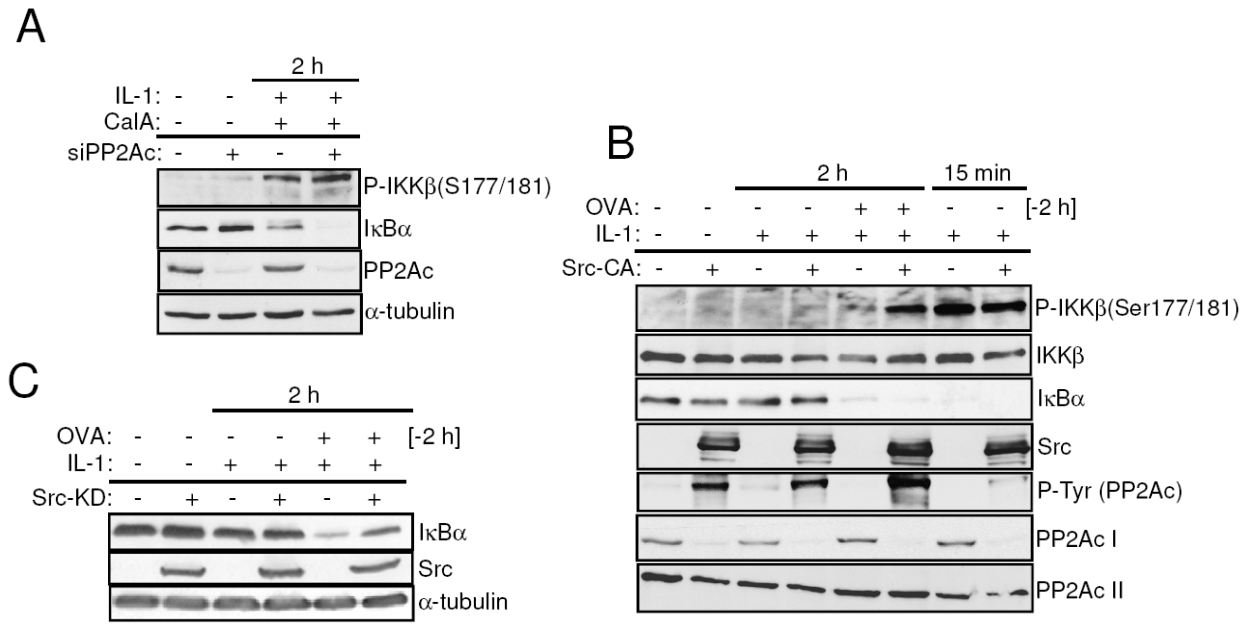

Figure 4 - Src and PP2Ac cooperate to extend IKKβ activation, thereby causing degradation of resynthesized IκBα. **(A)** KB cells were transfected with scrambled siRNA or siRNA specifically knocking down PP2Ac. 48 h later cells were treated with IL-1 (10 ng/ml) or cotreated with calyculinA (5 nM) and 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 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 loading was monitored by an α-tubulin antibody.

Discussion

Based on results obtained here, we propose the following mechanism underlying failure of IκBα reappearance in IL-1 + OVA treated epithelial cells: Tyr-phosphatase inhibition by OVA stabilizes Y416 phosphorylation of Src. Activated Src consequently inhibits PP2Ac by Y307 phosphorylation, thereby terminating PP2A driven dephosphorylation of IKKβ. Prolonged IKKβ activation consequently triggers continuous downstream Ser23/36 phosphorylation of newly synthesized IκBα, being the signal for immediate ubiquitination and proteasomal degradation, thus causing abrogation of the negative feedback loop of NFκB (Fig. 5).

Since uncontrolled NFκB activity has been linked to the development and maintenance of tumors, especially of those derived from chronic inflammation (20, 21) 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 due to mutations of IκBα, enhanced proteasomal activity or constitutive cytokine expression (5). Moreover, inappropriate regulation of NFκB is claimed to be directly responsible for many other diseases including neurodegenerative diseases, arthritis, and psoriasis (22, 23). NFκB activation also appears to confer chemo- and radioresistance in cancer treatment (24). Thus, strategies which interfere with the signalling pathways activating NFκB have become major targets for cancer interventions comprising mostly IKK and/or proteasome inhibition (8).

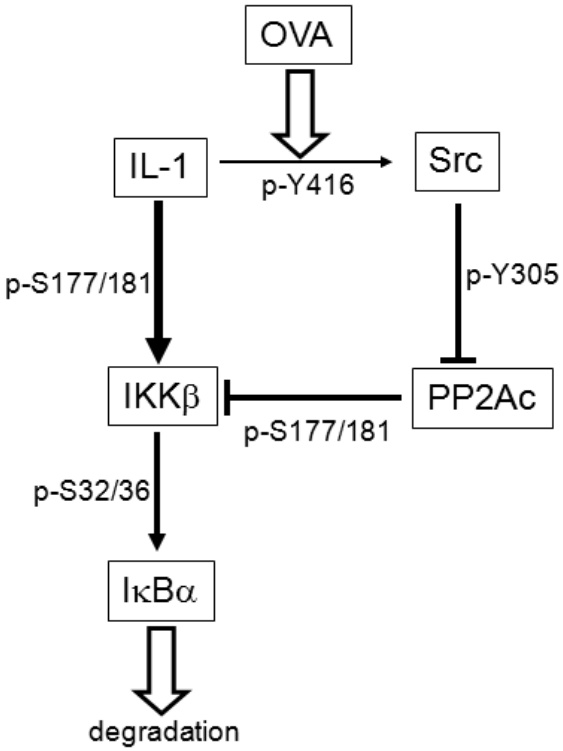

Figure 5 - Mechanism of OVA-mediated inhibition of IκBα resynthesis. Stimulation of KB cells with IL-1 predominantly causes transient S177/181 phosphorylation of IKKβ followed by S32/36 phosphorylation and consequently proteasomal degradation of IκBα. NFκB-dependent IκBα resynthesis is warranted by PP2A-mediated 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.

We have recently reported costimulation 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 kinase PP2Ac, consequently causing continuous Ser177/181 phosphorylation of IKKβ, constantly marking resynthesized IκBα for immediate proteasomal degradation (7). Costimulation of cells with IL-1 and the Tyr phosphatase inhibitor OVA, however, presented an identical phenotype of negative feedback abrogation over hours. In contrast to several reports claiming OVA-induced NFκB activation through Src family member dependent Tyr phosphorylation of IκBα (Tyr42 (11, 12, 13) or Tyr305 (18)), we found IL-1 + OVA induced NFκB activation as well as inhibition of IκBα resynthesis to be completely

independent of Tyr phosphorylation of either IκBα of IKKβ (9, 10) but to follow the canonical pattern. Trapping resynthesized IκBα within the cytoplasm by MG132 mediated proteasomal inhibition revealed sustained classical IκBα Ser32/36 as well as IKKβ Ser177/181 phosphorylation. Tyr phosphorylation dependent canonical NFκB activation may, however, occur by interfering with canonical as well as alternative upstream targets of NFκB signalling. In this context, the Tyr-kinase Src seems to play a major role, because its autophosphorylation at Tyr416, representing active Src, was found to be significantly enhanced upon IL-1 + OVA stimulation (25). Src activates PI3K and Akt, which upon hyperactivation were shown to promote chemoprevention in an NFκB-dependent manner (26). 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 (27). In our system IL-1 + OVA-induced blockade of IκBα resynthesis, however, remained fully unaffected by chemical inhibiton of PI3K, MAPK, JNK and Akt respectively (Fig. S1 A). In response to oxidative stress, PKC activation was shown to promote PKD-dependent NFκB activation in a Ser phosphorylation-dependent fashion (28, 29). Since PKC activation requires Tyr phosphorylation, Src might trigger sustained canonical IκBα degradation via this alternative pathway. This possibility could, however, also be rejected by the use of specific PKC inhibitors as well as PKD knock down experiments (Fig. S1 B and C). Additionally, free radical formation (30), alternative IKKγ Ser86 phosphorylation (31, 32) or Src-mediated tyrosine phosphorylation of NFκB (33, 34) itself could be ruled out as inducers of sustained NFκB activation in our system (Fig. S1 D, E, F).

Another putative target of Src-dependent Tyr phosphorylation that might interfere with NFκB signalling is the Ser/Thr phosphatase PP2A, which was previously shown to modulate IKKβ activity (7). Inhibitory Tyr307 phosphorylation of the catalytic subunit PP2Ac can be mediated by Src itself and other Src family members (19). Tyr phoshporylation of PP2Ac was shown to take place upon Src overexpression *in vitro* and *in vivo,* being strongest following IL-1 + OVA stimulation and coincided with loss of phosphatase activity of about 50%. Phosphorylation at Tyr307 can be deduced from Western-blot analysis shown in Fig. 3B and 4B, because the antibody used (PP2Ac I) binds to the C-terminal epitope comprising amino acids 295-309 and failed to detect PP2Ac whenever Tyr phosphorylation could be detected in parallel. Binding

of a different PP2Ac antibody recognizing the N-terminus of PP2Ac in contrast, remained unaffected by Tyr phosphorylation. Overexpression of Src-DN in turn failed to phosphorylate PP2Ac and correlated with increased PP2Ac activity (+30%), critically correlating Src-dependent Tyr phosphorylation to its catalytic inactivation. While PP2Ac elimination via siRNA knock down and/or calyculinA treatment impeded IκBα recurrence upon IL-1 only 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 upon IL-1 + OVA stimulation. Overexpression of Src-DN was shown to antagonize the OVA effect, allowing IκBα reappearance, thus corroborating the findings from the other experimental approaches. Together, the presented data unravel a new mechanism by which uncontrolled activity of the oncogene Src may amplify its oncogenic potential by additional PP2Ac mediated persistent canonical NFκB activation. This might by of particular importance in tumors being surrounded by immune cells constantly releasing proinflammatory cytokines like IL-1. Consequently, PP2Ac inhibition should be implemented in strategies developing new anti cancer therapies.

Methods

Unless otherwise stated, result of phosphatase assay is 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 representataive out of 3 independently performed experiments.

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.

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.

For knock down experiments respective sequences were generated and purchased from MWG. Scrambled: 5'-UAgAAUUAUUCCUCAACAgTT-3'; PP2Ac: 5'-gAggUUCgAUgUCCAgUUATT-3'; $0.8x10^5$ cells were transfected 50 pmol siRNA with Lipofectamin 2000 (Invitrogen) according to the manufacturers instructions. After 48 h knock down was documented by Western-blot analysis using an antibody against PP2Ac (# 05-421, clone 1D6, Upstate).

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 was immunoprecipitated using a specific antibody (# 05-421, clone 1D6, Upstate) and A/G-plus agarose (Santa Cruz) over night. Precipitates were analyzed by Western-blot using antibodies against p-Tyr (PY99, Santa Cruz) and the N-terminus of PP2Ac (PP2Ac II; 280740R, Invitrogen). 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), 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.

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.

Acknowledgements

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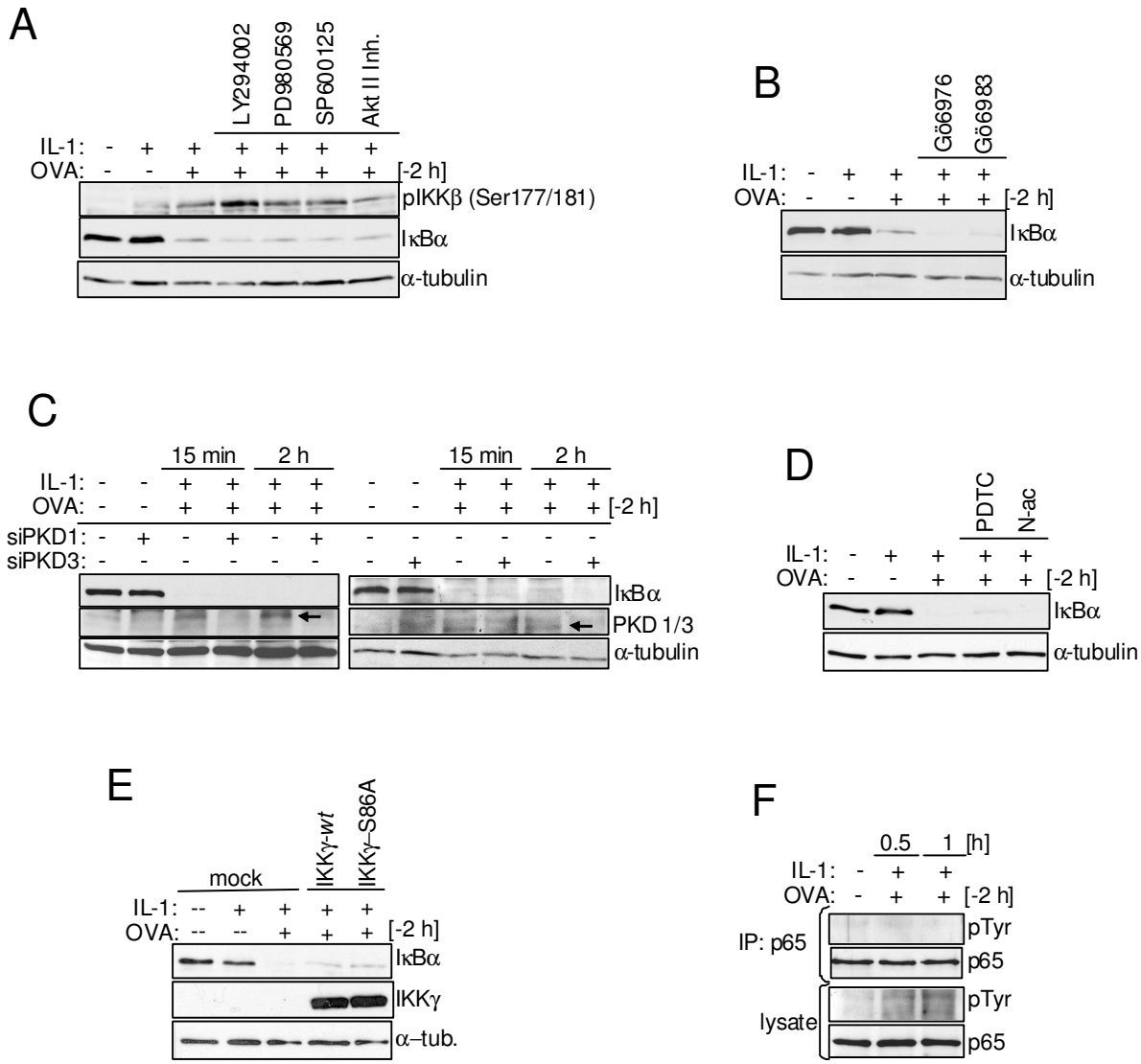
Supplement

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

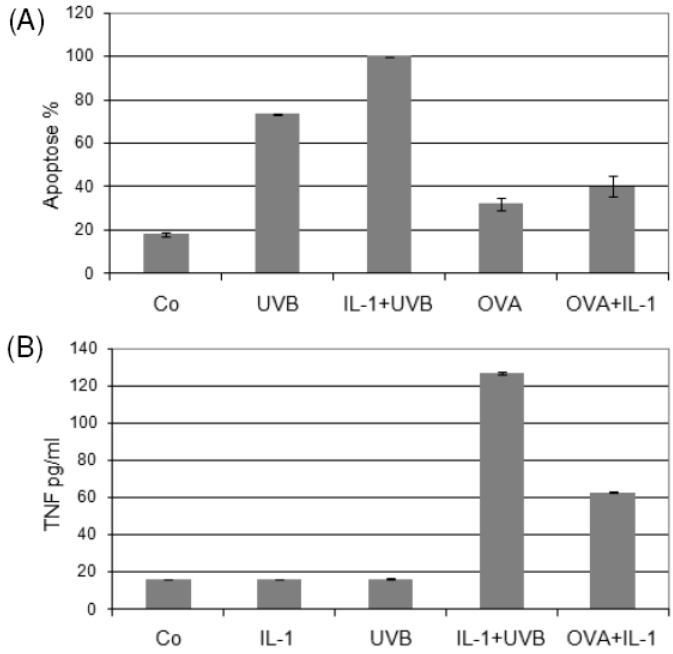


Supplemental Figure 1 - A variety of upstream targets have been described to cause alternative IκBα degradation, thereby modulating NFκB activity. To rule out that activation of any of these additional or alternative signalling pathways contribute to IL-1 + OVA-induced continuous IκBα degradation, chemical inhibition or siRNA knock down was performed.
(A) 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, all inhibitors obtained from Calbiochem) 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 (L35A5, Cell Signaling). **(B)** Cells were incubated with PKC inhibitors Gö 6976 (5 µM) and Gö 6983 (10 µM; both Calbiochem), respectively, for 1 h. After prestimulation with OVA (1 mM) for 2 h and subsequent IL-1 (10 ng/ml) treatment for 2 h IκBα status analyzed by Western-blot. **(C)** PKD1 (5´-gUCgAgAgAAgAggUCAAATT-3`) and PKD3 (5´-AgAAUAUUgUgCACUgUgATT-3`) were knocked down using 50 pmol siRNA (MWG) and Lipofectamine2000 (Invitrogen). After OVA+IL-1 (1 mM _10 ng/ml) stimulation for the indicated times, IκBα and PKD (C-20; sc-639, Santa Cruz) status was documented by Western-blot analysis. **(D)** Cells were preincubated or not with radical scavengers PDTC (100 µM, Calbiochem) and N-Ac (20 mM, Calbiochem) for 1 h. IκBα status was analysed by Western-blotting, 2 h after IL-1 (10 ng/ml) or IL-1 + OVA (1 mM) stimulation. **(E)** 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 (10 ng/ml) alone or costimulated with OVA (1 mM) as indicated and IκBα status documented by Western-blot analysis. **(F)** NFκB was immunoprecipitated (C-20; sc-372, Santa Cruz) form untreated cells or cells stimulated with IL-1 (10 ng/ml) + OVA (1 mM) for the indicated times. Tyr phosphorylation (PY99, Santa Cruz) and NFκB were analyzed be Western-blotting.

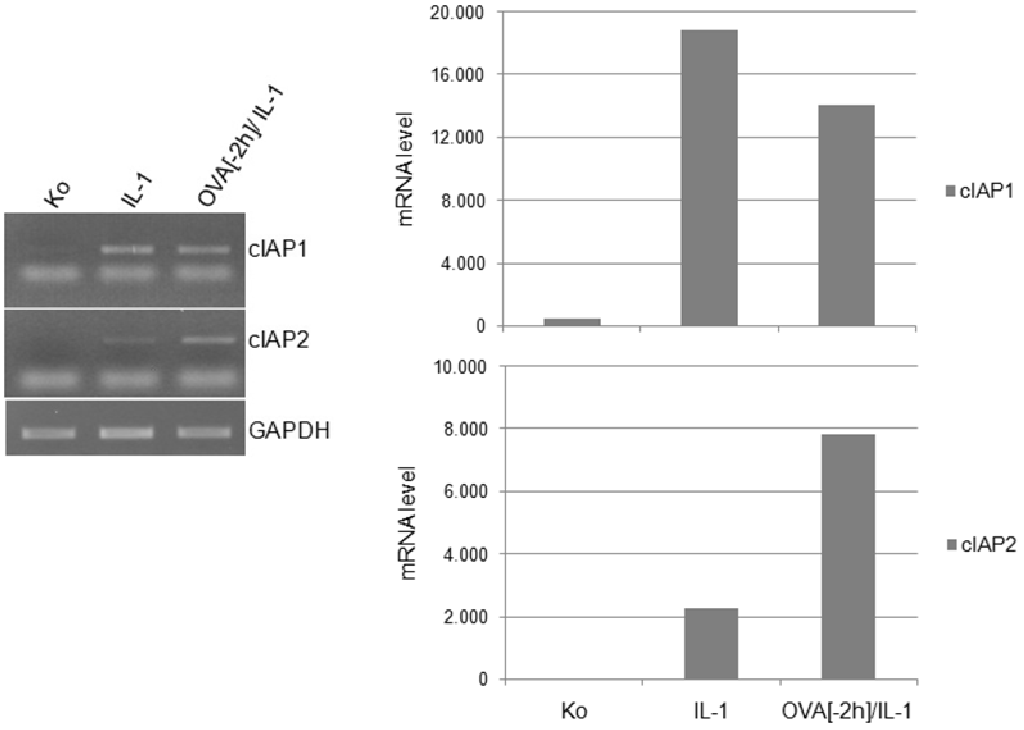
6 Anhang

6.1 Betrachtung der Apoptoserate und TNF-Freisetzung nach OVA+IL-1-Stimulation



Anhang-1: OVA+IL-1-vermittelte Freisetzung von TNF korreliert nicht mit einer signifikanten Erhöhung der Apoptoserate wie nach einer Stimlation mit IL-1+UVB. **(A)** Ergebnis des *Cell Death Detection ELISA* (Roche) nach 16-stündiger Stimulation der humanen Epithelzelllinie KB mit 10ng/ml IL-1 (R&D Systems), 30mJ/cm^2 UVB und 1mM Na-Orthovanadat (Sigma). **(B)** Die Konzentration des TNFα–Proteins im Zellüberstand wurde mittels ELISA (Immunotools) gemessen. Stimulations-bedingungen, siehe (A). Die Ergebnisse zeigen eins von drei unabhängig voneinander durchgeführten Experimenten.

6.2 Nachweis der mRNA-Expression von Mitgliedern der cIAP-Familie nach OVA+IL-1- Stimulation



Anhang-2: Nachweis der mRNA-Expression von Mitgliedern der cIAP-Familie nach Stimulation von Zellen mit OVA+IL-1
RT-PCR-Analyse 4 Stunden nach Stimulation der Zelllinie KB mit 10ng/ml IL-1 (R&D Systems). Die Zugabe von 1mM Na-Orthovanadat erfolgte 2 Stunden vor IL-1-Stimulation.
Die Quantitative Auswertung des mRNA-Levels wurde mithilfe der Software *ImageJ* erstellt.

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8 Wissenschaftliche Veröffentlichungen

1. Witt J., Husser S., Kulms D., Barisic S., Sawodny O., Sauter T. (2007). Modeling of IL-1 induced NF-kappaB signaling and analysis of additional UVB influence. *SICE 2007 Annual Conference* 1: 1353-1358.

2. Barisic S., Strozyk E., Peters N., Walczak H., Kulms D. (2008). 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* 15: 1681-1690.

3. Witt J., Barisic S., Schumann E., Allgöwer F., Sawodny O., Sauter T., Kulms D. (2009). Mechanism of PP2A-mediated IKKβ dephosphorylation: a systems biological approach. *BMC Systems Biology,* accepted June 10, 2009

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10 Lebenslauf

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Eidesstattliche Erklärung:

Hiermit erkläre ich, dass ich die vorgelegte Dissertation

Die Rolle der Ser/Thr Phosphatase PP2A in der Regulation des Transkriptionsfaktors NFκB

selbständig verfasst und keine anderen als die angegebenen Hilfsmittel und Quellen verwendet habe.

Ferner erkläre ich, dass diese Arbeit in gleicher oder ähnlicher Form keiner anderen Prüfungsbehörde als Prüfungsleistung vorgelegt wurde.

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