Mini Review

TNF signaling: early events and phosphorylation

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Abstract. Tumor necrosis factor-α (TNF) is a major mediator of apoptosis as well as immunity and inflammation. Inappropriate production of TNF or sustained activation of TNF signaling has been implicated in the pathogenesis of a wide spectrum of human diseases, including cancer, osteoporosis, sepsis, diabetes, and autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, and inflammatory bowel disease. TNF binds to two specific receptors, TNF-receptor type I (TNF-R1, CD120a, p55/60) and TNF-receptor type II (TNF-R2, CD120b, p75/80). Signaling through TNF-R1 is extremely complex, leading to both cell death and survival signals. Many findings suggest an important role of phosphorylation of the TNF-R1 by number of protein kinases. Role of TNF-R2 phosphorylation on its signaling properties is understood less than TNF-R1. Other cellular substrates as TRADD adaptor protein, TRAF protein family and RIP kinases are reviewed in relation to TNF receptor-mediated apoptosis or survival pathways and regulation of their actions by phosphorylation.

Key words: Tumor necrosis factor α — Phosphorylation — Signaling — Receptors — Kinases

Abbreviations: AP-1, activator-protein 1; CARD, caspase recruitment domain; CD, cluster of differentiation; CD95, designation of cell surface antigen/cell death receptor (APO-1/Fas); CD95L or FasL, ligand of the CD95 receptor; DD, death domain; DISC, death-inducing signaling complex; DNA, deoxyribonucleic acid; DR, death receptor; Erk, extracellular signal-regulated kinase; FADD, fas-associated DD protein; IKK, IκB kinase; IκB, inhibitory protein of NF-κB; IL-1, interleukine-1; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κB; NGF, neuronal growth factor; PEA-15, phosphoprotein enriched in astrocytes; PI3K, phosphatidylinositide 3'-OH kinase; PKC, protein kinase C; PKC-δ, protein kinase C-δ subtype; PM, plasmatic membrane; PTPase, protein tyrosine phosphatase; RIP, receptor-interacting protein; SODD, silencer of DD; TANK, TRAF family member-associated NF-κB activator; TNF, tumor necrosis factor-α; TRADD, TNF receptor-associated DD protein; TRAF, TNF receptor-associated factor; TRAK, TNF receptor-associated kinase; TNF-R1, TNF-receptor type I; TNF-R2, TNF-receptor type II.

TNF and their receptors

Tumor necrosis factor-α (TNF) is a major mediator of apoptosis as well as immunity and inflammation. TNF is primarily produced by activated macrophages as a type II transmembrane 26 kDa protein arranged in stable homotrimers, which are proteolyticaly cleaved to a soluble 17 kDa homotrimeric cytokine by the metalloprotease TACE (TNF converting enzyme) (Black et al. 1997). Inappropriate production of TNF or sustained activation of TNF signaling has been implicated in the pathogenesis of a wide spectrum of human diseases, including cancer, osteoporosis, sepsis, diabetes, and autoimmune diseases such as multiple sclerosis, rheumatoid arthritis, and inflammatory bowel disease. TNF binds to two specific receptors, TNF-receptor type I (TNF-R1, CD120a, p55/60) and TNF-receptor type II (TNF-R2, CD120b, p75/80). They are single transmembrane glycoproteins with 28% homology mostly in their extracellular domain. TNF-R1 as well as TNF-R2 contain four cysteine-rich repeats in their extracellular domain and worthy of note is finding that the extracellular domains of both receptors

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can be proteolytically cleaved to soluble receptor fragments with neutralizing potential (Wallach et al. 1991). Their intracellular sequences are largely unrelated, with almost no homology between each other, and early work suggested delineation of their signaling functions (Grell et al. 1995). It is needed to mention that TNF receptors belong to members of the TNF/NGF (neuronal growth factor) receptor superfamily and comprise of at least 27 members including lymphotoxin receptor, CD40, RANK and decoy or death receptors (DRs). The DRs contain an intracellular domain named death domain (DD). Human DRs include TNF-R1 (CD120a, p55-R), CD95 (Fas, Apo1), TNF receptor-related apoptosis-mediating protein (TRAMP, DR3, Apo3), TNF-related apoptosis-inducing ligand receptor 1 (TRAIL-R1, DR4, Apo2), TRAIL-R2 (DR5, KILLER, TRICK2), death receptor 6 (DR6, TR7), NGF receptor (NGF-R), ectodermal dysplasia receptor (EDA-R) (Ashkenazi and Dixit 1998; French and Tschopp 2003). Stimulation of these receptors leads to DD aggregation in their cytoplasmic tail with adaptor proteins, which also comprise DD in their C-terminal end. Then they oligomerize and form "death inducing signaling complex", named DISC (Kischkel et al. 1995). Because this area is very extensive the interest of this review is focused only to TNF receptors.

TNF-R1

The signaling trough TNF-R1 initiates the majority of TNF's biological activities. Ligand-dependent trimerisation of the receptor seems to be still the key event for triggering the signal, however, this process now appears more complex because the distal cysteine-rich domains of both receptors mediate homophilic interaction of receptor molecules in the absence of ligand. This action mediates a distinct domain from the domain formatting the major contacts with ligand, named pre-ligand-binding assembly domain (PLAD) (Chan et al. 2000). Thus PLAD domains may therefore keep receptors in a silent, homomultimerized state and antagonize spontaneous autoactivation upon overexpression. But, generally, ligand binding to the receptor complex induces activating conformational changes and allows the formation of subsequent complexes. These comprise of activated receptor and other signaling molecules thus transmitting extracellular signal from plasma membrane to targeted cell effectors.

The intrinsic property of the DD of TNF-R1 to self-aggregate is prevented by binding of the silencer of DD (SODD) protein. SODD has been proposed to prevent constitutive TNF-R1 signaling in the absence of TNF, inhibiting intrinsic self-aggregation properties of the DDs and maintaining TNF-R1 in inactive, monomeric state. After TNF treatment, SODD is released from TNF-R1, permitting the recruitment of proteins such as TNF receptor-asociated DD protein (TRADD) and TNF receptor-associated factor 2 (TRAF2) to the active TNF-R1 signaling complex (Jiang et al. 1999). The experiments on mice congenitally deficient in *sodd* gene suggest that SODD is critical for the regulation of TNF signaling (Takada et al. 2003). These authors have shown that mice lacking SODD produced larger amounts of cytokines in response to *in vivo* TNF challenge. TNF-induced activation of nuclear factor κB (NF-κB) was accelerated in SODD-deficient cells, but TNF-induced c-Jun N-terminal kinase (JNK) activity was slightly repressed. However, the results of other authors do not support the concept of a unique, non-redundant role of SODD in the functions of TNF-R1 (Endres et al. 2003), therefore, the role of SODD in TNF-R1 signaling remains still enigmatic.

Signaling through TNF-R1 is extremely complex, leading to both cell death and survival signals. In the classic signaling model, binding of TNF to TNF-R1 leads to the recruitment of adaptor protein TRADD, which acts as a scaffold for recruitment of receptor-interacting protein (RIP), TRAF2 and fas-associated DD protein (FADD) (Chen and Goeddel 2002). RIP and TRAF2 regulate both anti-apoptotic and pro-inflammatory pathways and FADD regulates TNF-induced apoptosis. Recent studies suggest that TNF triggers the rapid assembly of TNF-R1-TRADD-RIP-TRAF2 signaling complex at the level of the plasma membrane (complex I, Figure 1). This complex controls the activation of NF-κB but not apoptosis. Formation of complex I is transient since a large portion of TRADD-RIP-TRAF2 dissociate from TNF-R1 within an hour and form a second complex (complex II, Figure 1) with FADD and caspase 8/10 in the cytosol (Micheau and Tschopp 2003). Apoptosis could be activated by complex II under conditions in which the signal from complex I fails to activate NF-κB.

Studies with TRAF2- and RIP-deficient mouse embryonic fibroblasts have shown that TRAF2 and RIP can be recruited to the TNF-R1 signaling complex independently. TRAF2 is sufficient to recruit the IκB kinase (inhibitory protein of NF-κB kinase; IKK) to the TNF-R1 complex upon TNF stimulation and that activation of requires the presence of RIP in the same complex (Devin et al. 2000).

How phosphorylation regulates these signaling properties of the TNF-R1? To date, many findings suggest an important role of phosphorylation of the TNF-R1 by number of protein kinases. The studies with TNF-activated human neutrophils uncovered a significant role of protein kinase C-δ (PKC-δ) and phosphatidylinositide 3'-OH kinase (PI3K) in regulation of TNF-R1 signaling. In these cells, TNF triggers degranulation and release of reactive oxygen radicals (Kilpatrick et al. 2000, 2004). Both kinases form a signal complex with TNF-R1 in response to TNF. PKC-δ recruitment requires both PKC-δ and PI3K activity, whereas PI3K recruitment is PKC-δ independent. This suggests that PI3K acts upstream of PKC-δ. The inhibition of either PI3K or PKC-δ decreases

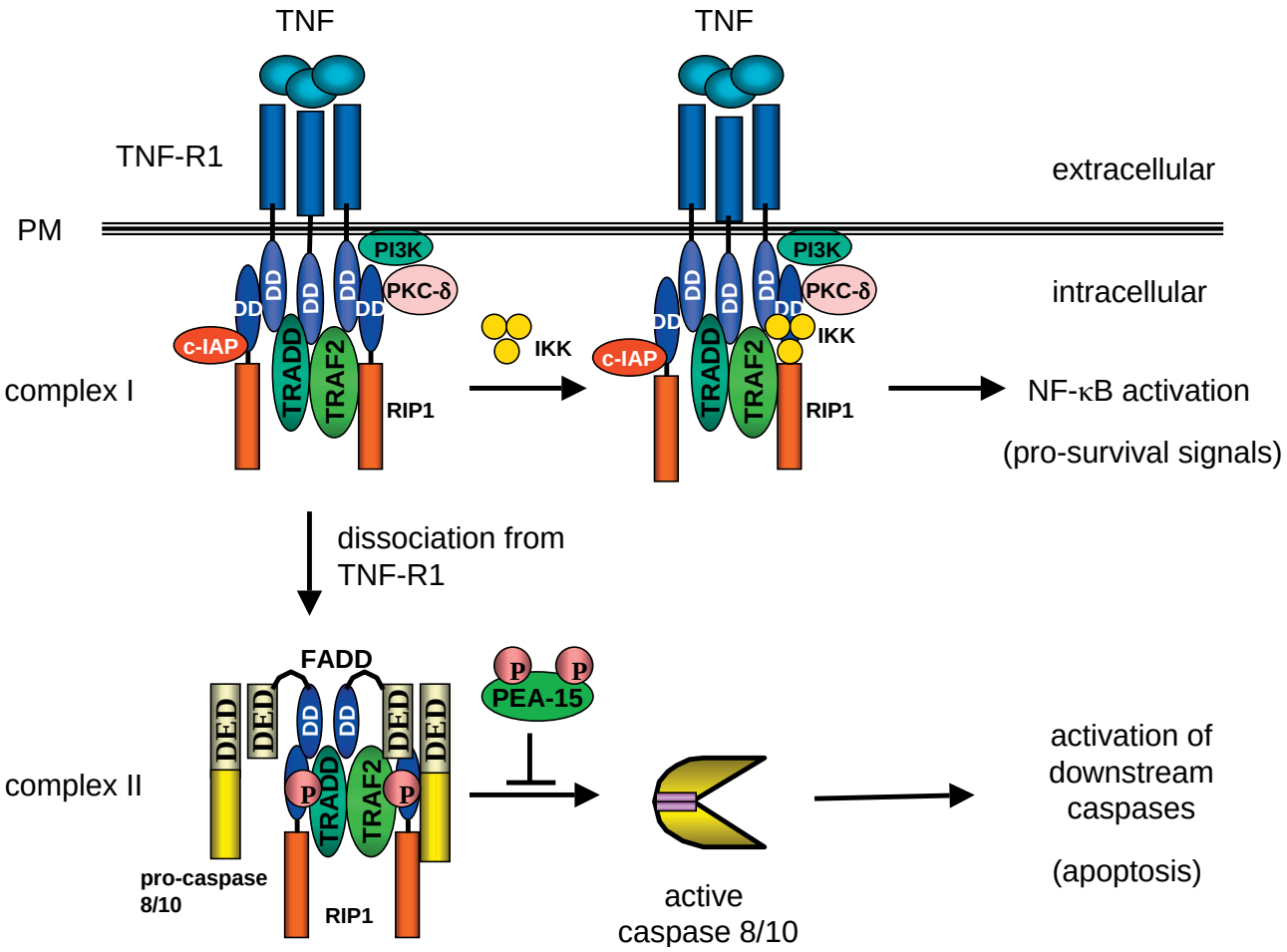

Figure 1. TNF-R1 signaling.

TNF-mediated recruitment of RIP and TRAF2 to TNF-R1 but TRADD recruitment is enhanced. Consequently, PKC-δ and PI3K are positive regulators of TNF-mediated association of TRAF2 and RIP with TNF-R1. Conversely, these kinases are negative regulators of TRADD association. These results suggest that PKC-δ and PI3K regulate TNF antiapoptotic signaling at the level of the TNF-R1 through control of assembly of a TNF-R1-TRADD-RIP-TRAF2 complex I (Figure 1).

Darnay et al. (1994) revealed the interaction of TNF-R1 associated kinase (or p60TRAK) with cytoplasmic domain of TNF-R1 in U-937 cells. Its kinase activity is strongly enhanced by TNF stimulation of the receptor and phosphorylates TNF-R1. Their data demonstrate that residues 344-397 of the cytoplasmic domain of the TNF-R1 are sufficient to bind p60TRAK. In the more recent study (Darnay and Aggarwal 1997), authors have shown that inhibition of PTPase causes a protein tyrosine kinase to bind and phosphorylate Tyr-331 which is located in the DD of the TNF receptor. Additionally, PTPase inhibition results in inactivation of p60TRAK activity, and this may play a role in TNF-mediated growth modulation and NF-κB activation.

The results published in paper of Van Linden et al. (2000) also strongly suggested that TNF-R1-threonine-phosphorylation is provided by another important serine/threonine kinase. Indeed, the MAPK p42/Erk2 has been revealed to be responsible for this action. Preferred residues are Thr-236 and Ser-270 and primary phosphorylation at these sites appears to enable subsequent phosphorylation at Ser-240 and Ser-244, although the level of phosphorylation at these latter two sites is less than the preferred sites. Further investigation using HeLa cells has revealed that the phosphorylation of TNF-R1 alters its subcellular localization and can thereby result in variation of its signaling properties. Moreover, when TNF-R1 is phosphorylated it relocalizes from the Golgi complex to tubular structures of the endoplasmic reticulum and recruits Bcl-2. In this way, oncogenic activation of MAPK p42/Erk2 may serve for the

inhibition of apoptotic activity of TNF-R1 and thus can be prefered NF-κB-dependent pro-survival responses (Cottin et al. 1999, 2001).

Takada and Aggarwal (2004) have recently demonstrated association of spleen tyrosine kinase with both TNF receptors after TNF stimulation. Although this kinase does not phosphorylate TNF receptors, its activation plays an essential role in TNF-induced activation of JNK, p38 MAPK, p44/p42 MAPK, NF-κB, and induction of apoptosis. However, pharmacologicaly inhibited TNF-induced spleen tyrosine kinase activation leads to the suppression of TNF-induced activation of JNK, p38 and p44/p42 MAPK.

In summary, the TNF-R1 appears to have multiple signaling pathways, either pro-apoptotic or anti-apoptotic. TNF triggers both serine and threonine phosphorylation of the TNF-R1 and multiple kinases are in turn involved in this TNF-R1 modification, what may mediate different functional effects. The exact role of TNF-R1 phosphorylation in relation to its signaling properties is cell-type specific, thus the molecular mechanism for cross-talk between TNF-mediated apoptosis, NF-κB and JNK signaling is not completely understood and remains to be the target for further studies.

TNF-R2

While TNF-R1 is constitutively expressed in most tissues, TNF-receptor type II (TNF-R2, CD 120b, p75) is highly expressed and typically found in cells of the immune system. Both TNF receptors show less than 25% identity and their common homology equals with their homology to other members TNF/NGF receptor family. TNF-R2 contains similar extracellular domain as TNF-R1 but the difference of these two receptors rests in their cytoplasmic domains. While TNF-R1 has a so-called DD region, which is essential for induction of apoptosis, TNF-R2 does not have it. Compared with TNF-R1, the function of TNF-R2 is less understood.

Both receptors can be stimulated by membrane-bound TNF, whereas proteolytically processed soluble TNF predominantly signals *via* TNF-R1 (Grell et al. 1995, 1998b). The extracellular domain of both receptors can be proteolytically cleaved and soluble receptor fragments generated in this manner have neutralizing potential in TNF actions (Wallach et al. 1991). Activation of TNF-R2 appears to be proliferative on T cells (Tartaglia et al. 1991; Grell et al. 1998a; Kim and Teh 2001), neuroprotective on ischemia-reperfusion-induced damage of mice retinal cells (Fontaine et al. 2002), or anti-apoptotic (Horie et al. 1999). Other reports suggest its involvement in death signaling, although it lacks the DD. So-called "ligand passing" from TNF-R2 to TNF-R1, can probably be the appropriate mechanism for the induction of cell death by TNF-R2 (Tartaglia et al. 1993). However, other reports suggest that TNF-R2 contributes to cell death independently of TNF-R1 *via* interaction with RIP (Pimentel-Muinos and Seed 1999; Cusson et al. 2002). In the presence of RIP, TNF-R2 triggers cell death, whereas in the absence of RIP, TNF-R2 activates the transcription factor NF-κB. TNF-R2 activates NF-κB probably by recruiting TRAF1 and TRAF2 to the receptor (Rothe et al. 1995b). Rothe and coworkers (1995a) have also shown that TNF-R2-TRAF signaling complex contains two cellular inhibitors of apoptosis proteins cIAP-1 and cIAP-2 by which can TNF-R2 signaling be modulate in response to TNF.

The role of TNF-R2 phosphorylation on its signaling properties is less understood than that of TNF-R1, but some reports have already been published. In 1992, Pennica and coworkers published a report in which they characterized biochemical properties of TNF-R2. The cytoplasmic region of TNF-R2 contains a high number of Ser, Thr, Pro and Glu residues, but not Tyr residues. Ser-415 seems to be a potential phosphorylation site for PKC. By metabolically labeling of 293/TNF-R2 cells with [^{32}P] orthophosphate in the presence or absence of TNF followed by immunoprecipitation with TNF-R2 polyclonal antiserum and SDS-PAGE analysis has been shown that TNF-R2 is the phosphoprotein. TNF-R2 phosphorylation appears to be TNF independent, because no significant difference in the amount of $^{32}P_i$ incorporation was observed in the presence or absence of TNF. Phosphoamino acid analysis of ^{32}P-labeled immunoprecipitated TNF-R2 also revealed that TNF-R2 is phosphorylated largely on Ser residues (Pennica et al. 1992). Later, Darnay et al. (1994) revealed interaction of TNF-R2-associated kinase (or p80TRAK) with cytoplasmic domain of TNF-R2 in U-937 cells. Its kinase activity is strongly enhanced by TNF stimulation of the receptor. This kinase can phosphorylate both TNF-R2 and TNF-R1 . Using deletion mutants in the TNF-R2 cytoplasmic domain, 44 residues (354–397) were identified in this domain, which were sufficient for binding of p80TRAK. It was also the region that contained the phosphorylation site for p80TRAK. Phosphoamino acid analysis revealed phosphorylation primarily on serine residues. In addition to p80TRAK, also purified casein kinase 1 was found to be able to phosphorylate and to bind to TNF-R2 residues 354–397 on its cytoplasmic domain (Darnay et al. 1997).

Other authors also have demonstrated the phosphorylation of TRAF-binding region (amino acids 379 to 419) in the cytoplasmic domain of TNF-R2, by unidentified kinase(s) both *in vitro* and *in vivo* (Ng et al. 1998). Transfection of various TNF-R2 mutants into 293EBNA cells and NF-κB luciferase assay suggested significant role of TRAF-binding domain of TNF-R2 in NF-κB activation. Although TRAF-binding domain of TNF-R2 is a major target of kinases, the construct with mutations of all serine and threonine residues in this domain to alanine does not support any role of TNF-R2 phosphorylation for NF-κB activation. Curiously,

this mutant of TNF-R2 was more active in NF-κB activation than unmodified receptor. The same authors therefore suggest that phosphorylation of this domain of TNF-R2 is not only unnecessary for NF-κB activation but, in addition, phosphorylation of serine and threonine can play a negative regulatory role in activation of NF-κB.

Other cellular substrates related to TNF receptor-mediated apoptosis or survival, and regulation of their activity by phosphorylation

TRADD adaptor protein

TRADD is a 34.2 kDa cytosolic adaptor protein which contains an N-terminal TRAF-binding domain and a C-terminal DD along with nuclear import and export sequences that cause shuttling between the cytoplasm and nucleus. The DD interactions allow direct heterodimerization of cytoplasmic TRADD with TNF-R1. The cytoplasmic TRADD directly interacts with TRAF2, RIP or FADD, signal transducers that activate NF-κB or induce apoptosis (Hsu et al. 1995, 1996b). These signaling proteins begin to dissociate from the receptor within minutes of complex formation, in a process that is accompanied by the phosphorylation of TRADD (Jiang et al. 1999). On the other hand, nuclear TRADD is capable to initiate apoptosis by a pathway, which requires active caspase 9. This pathway represents an alternate means by which TRADD can regulate cell death independently of FADD and caspase 8 that occurs from the nucleus rather than the cytoplasm (Bender et al. 2005).

TRAF protein family

The TNF receptor-associated factors (TRAFs) are implicated in a wide variety of biological functions, such as adaptive and innate immunity, embryonic development, stress response, and bone metabolism (MacEwan 2002). These functions are mediated by TRAFs through the induction of cell survival, proliferation, differentiation and death. TRAF family (TRAF1–6) is a phylogenetically conserved group of scaffold proteins that link receptors of the IL-1R/Toll and TNF receptor family to signaling cascades, leading to the activation of NF-κB and mitogen-activated protein kinases (MAPK) (Wajant et al. 2001). The TRAFs are characterized by the presence of typical carboxyl-terminal homology domain of about 180 amino acids, named as TRAF domain. The TRAF domain plays an important role in TRAF function by mediating self-association and upstream interactions with receptors and other signaling proteins (Takeuchi et al. 1996). The interaction of TRAF proteins with receptors has been described in the case of many receptors belonging to TNF receptor superfamily (Wajant et al. 2001). From the aspect of this review is important that TRAF proteins directly interact with TNF-superfamily of receptors which lack a DD, e.g., the interaction of TRAF2 with TNF-R2. On the other hand, TRAF interaction with DD receptors is mediated *via* additional cytoplasmic adaptor proteins, e.g. already mentioned TRADD, which is recruited by TNF-R1. Thus, TRAF2 indirectly interacts also with TNF-R1.

Many of biological effects of the TRAF signaling appear to be mediated through the activation of transcription factors NF-κB and activator-protein 1 (AP-1). As will be mentioned below, NF-κB is activated by IKK, which consists of two kinase subunits IKKα, IKKβ and regulatory subunit IKKγ/NEMO (NF-κB essential modulator) (DiDonato et al. 1997; Zandi et al. 1997). Phosphorylation of IKK and its subsequent degradation leads to the release and translocation of NF-κB to the nucleus and transcription of target genes.

In connection with the NF-κB activation by TRAFs, Henkler and coworkers (2003) have shown that TRAF1 is constitutively associated with IKK *via* its N-terminal domain. Their experimental results also suggest that TRAF1 and its caspase-cleavage product exert their regulatory effects on receptor-induced NF-κB activation not only by modulation of TRAF2 interaction with the receptor but also by directly targeting the IKK.

Besides NF-κB also AP-1 transcription activity is modulated by TRAFs. AP-1 activity is stimulated by MAPK through either direct phosphorylation or transcription of AP-1 components (Karin 1996). The stimulation of AP-1 activity by MAPK may elicit stress responses and promote cell survival or cell death (Shaulian and Karin 2001). More about TRAF proteins and their interactions is reviewed in recent papers (Bradley and Pober 2001; Wajant et al. 2001; Chung et al. 2002).

Just ten years ago Rothe and coworkers (1996) described the isolation and characterization of a novel TRAF-interacting protein, I-TRAF, that binds to the conserved TRAF-C domain. Yeast two-hybrid screening revealed that I-TRAF/TANK, acting as NF-κB activator, interact with the regulatory IKKγ subunit of IKK. This interaction has been confirmed also by Chariot et al. (2002).

Nomura et al. (2000) have published the association of I-TRAF and cytokine inducible IKK (IKK-i). This association is mediated *via* interaction between the N-terminal domain of I-TRAF and the C-terminal portion of IKK-i. Using *in vitro* kinase assay it has been demonstrated that IKK-i phosphorylates I-TRAF in the middle portion that associates with TRAF2. TRAF2 is then released from the I-TRAF/TRAF2 complex, thus allowing NF-κB activation.

RIP kinases

Another group of cellular proteins, which play important role in apoptosis/pro-survival signaling are RIP kinases. In

1995 Stanger and coworkers have discovered a first member of RIP family, RIP1. Using yeast two hybrid screening they observed 74 kDa protein RIP1, which interacted with Fas receptor DD (hence the designation "receptor interacting protein"). To date RIP family comprises of seven members (RIP1–7), revieved in Meylan and Tschopp (2005). The members of RIP family share a common overall structural architecture and also several functional features. They are all involved in cellular stress signaling caused by many pathogens, in inflammation, cell differentiation programs, or DNA damage. Although these stimuli are different, they converge to initiate similar responses, as NF-κB, AP-1 activation or stimulation of cell death.

The first member RIP1, as already mentioned, interacts with Fas/CD95 receptor. A little later has been shown, that RIP1 is also a key component of the TNF-R1 signaling complex (Hsu et al. 1996a). RIP1 is recruited into the TNF-R1 complex by the TNF adaptor protein TRADD upon ligand stimulation and *via* intermediate domain of RIP1 can also interact with TRAF1, TRAF2 and TRAF3 (Hsu et al. 1996a; Inoue et al. 2000; Bradley and Pober 2001) and also with IKK. Henkler and coworkers (2003) demonstrated that RIP associates with IKK only transiently after receptor stimulation by TNF (Figure 1).

RIP1 is serine/threonine kinase, which contains similarly as other RIP family members, three domains including N-terminal kinase domain, intermediate domain, and C-terminal DD (Stanger et al. 1995). Overexpression of RIP1 in 293 cells results in both NF-κB activation and induction of apoptosis (Hsu et al. 1996a), hence a dual role in cell signaling for RIP1 was proposed. Accordingly, the genetic deletion experiments suggested that RIP1 is essential for TNF-induced NF-κB activation (Ting et al. 1996; Kelliher et al. 1998).

Although RIP1 is serine/threonine kinase, its enzymatic activity is not necessary in TNF-induced NF-κB activation, but the kinase domain is reported to be essential for caspase-independent cell death – necrosis, triggered by Fas ligand (Holler et al. 2000), alternatively also for extracellular signal-regulated kinase (Erk) activation but not for activation of stress-activated protein kinases p38 and JNK (SAPKs) (Devin et al. 2003).

As we mentioned, NF-κB can be activated in response to TNF treatment. NF-κB activation requires phosphorylation of IκB by IKK. IKK is recruited to TNF receptor complex through TRAF2 and its activation requires RIP1. Although TRAF2 or RIP1 can be independently recruited to the TNF-R1 complex, neither one of them alone is capable of transducing the TNF signal that leads to IKK activation. It has also been suggested that RIP interacts with the IKK through the regulatory subunit IKK-γ, but apparently this interaction requires TRAF2 (Zhang et al. 2000; Devin et al. 2001) (Figure 1).

RIP2 and RIP3 are related kinases that share extensive sequence homology with the kinase domain of RIP1. RIP2 has a C-terminal caspase activation and recruitment domain termed CARD (McCarthy et al. 1998). CARD domain mediates the interaction of RIP2 with CARD-containing caspase 1 (Thome et al. 1998). RIP2 also activates the Erk pathway. The kinase activity of RIP2 appears to be important in this process because RIP2 directly phosphorylates and activates Erk2 *in vivo* and *in vitro* (Navas et al. 1999).

RIP3 possesses neither DD nor CARD motif, but on the other hand, it possesses a unique C-terminus named RIP homotypic interaction motif by which can bind RIP1, thereby inhibiting RIP1- and TNF-R1-mediated NF-κB activation (Sun et al. 2002). Using *in vitro* kinase screening and immunoprecipitation, Sun et al. (2002) observed recruitment of RIP3 with RIP1 to the TNF-R1 complex after TNF treatment. RIP1 was phosphorylated by RIP3 in RIP1-RIP3 complex and its phosphorylation inhibits TNF-induced NF-κB activation (Sun et al. 2002). Recent knockout of RIP3 was not succesful to demonstrate any alteration in NF-κB activation induced by TNF (Newton et al. 2004). It suggests dispensable function of RIP3 in TNF-induced NF-κB activation and therefore the role of RIP3 remains still rather enigmatic.

Currently two novel splicing variants of RIP3 (RIP3β and RIP3γ) have been discovered by Yang et al. (2005). Both variants possess a truncated N-terminal kinase domain and a distinct and shorter C-terminus. Considering to full-lenght RIP3, different C- and N-termini of RIP3β and RIP3γ cause abrogation of their shuttling between cytoplasm and nucleus and also their apoptosis-inducing activity. Transient expression of either variant was found to downregulate RIP3-mediated apoptosis. Seeing that the ratio of RIP3γ to full-length RIP3 is significantly increased in colon and lung cancers, revealed by real-time PCR, the authors suggest that a major splice form RIP3γ might be associated with tumorigenesis.

RIP4 contains an N-terminal RIP-like kinase domain and a C-terminal region characterized by the presence of 11 ankyrin repeats (Chen et al. 2001; Cariappa et al. 2003). Overexpression of RIP4 leads to activation of NF-κB and JNK and dominant negative forms of TRAF1, TRAF3 and TRAF6 inhibit RIP4-induced NF-κB activation. Together with the evidence that RIP4 is cleaved after Asp340 and Asp378 during Fas/CD95-induced apoptosis, it suggests that RIP4 is involved in NF-κB and JNK signaling. In addition, caspase-dependent processing of RIP4 may negatively regulate pro-survival or pro-inflammatory signals mediated by NF-κB (Meylan et al. 2002).

PEA-15

Phosphoprotein enriched in astrocytes (PEA-15) is a multifunctional protein that modulates signaling pathways, which control cell proliferation and cell death. PEA-15 exists *in vivo*

as three isoforms, non-phosphorylated and phosphorylated on one or two residues. Using phosphoaminoacid analysis it was revealed that PEA-15 phosphorylation occurs on two serine residues. *In vitro* phosphorylation experiments and phosphopeptide mapping revealed that Ser-104 is substrate for PKC (Araujo et al. 1993; Estelles et al. 1996). On the other hand, second phosphorylation site, Ser-116, is phosphorylated by calcium-calmodulin-dependent kinase 2 and protein kinase B (Kubeš et al. 1998; Trencia et al. 2003).

Because PEA-15 possess death effector domain within its N-terminal domain, it is involved in the control of apoptosis (Figure 1). Indeed, PEA-15 protects astrocytes from TNFα-induced apoptosis (Kitsberg et al. 1999). PEA-15 can be recruited to the DISC, where it may prevent processing of pro-caspase 8, but only the doubly phosphorylated form of PEA-15 can be recruited to the DISC and may perform this action (Xiao et al. 2002). Moreover, PEA-15 interacts *in vitro* with FADD and caspase 8, and its expression decreases astrocyte susceptibility to TNFα-induced cell death (Kitsberg et al. 1999). More about PEA-15 activities is reviewed in Renault et al. (2003).

Data summarized in this rewiev indicate that phosphorylation events in TNF signaling play an important role in modulation of cell responses ranging from regulation of apoptosis to cell survival. Important portion of knowledge remains still incomplete or unclear, and therefore it can serve as a base for further investigation.

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