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The interaction of p62 with RIP links the atypical PKCs to NF-κB activation

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The two members of the atypical protein kinase C (aPKC) subfamily of isozymes (ζPKC and λ/ιPKC) are involved in the control of nuclear factor κB (NF-κB) through IKKβ activation. Here we show that the previously described aPKC-binding protein, p62, selectively interacts with RIP but not with TRAF2 *in vitro* and *in vivo*. p62 bridges the aPKCs to RIP, whereas the aPKCs link IKKβ to p62. In this way, a signaling cascade of interactions is established from the TNF-R1 involving TRADD/RIP/p62/aPKCs/IKKβ. These observations define a novel pathway for the activation of NF-κB involving the aPKCs and p62. Consistent with this model, the expression of a dominant-negative mutant λ/ιPKC impairs RIP-stimulated NF-κB activation. In addition, the expression of either an N-terminal aPKC-binding domain of p62, or its C-terminal RIP-binding region are sufficient to block NF-κB activation. Furthermore, transfection of an antisense construct of p62 severely abrogates NF-κB activation. Together, these results demonstrate that the interaction of p62 with RIP serves to link the atypical PKCs to the activation of NF-κB by the TNFα signaling pathway.

Keywords: NF-κB activation/p62/protein kinase C/RIP

Introduction

The transcription factor nuclear factor κB (NF-κB) plays a critical role in a number of cell functions, including key inflammatory and immune responses (Lenardo and Baltimore, 1989; Baldwin, 1996). NF-κB is composed of dimers of different members of the Rel protein family (Baeuerle and Henkel, 1994; Thanos and Maniatis, 1995; Baldwin, 1996). The most classical form of NF-κB is an heterodimer of p50 and p65 (Rel A) (Baeuerle and Henkel, 1994; Thanos and Maniatis, 1995), which is sequestered in the cytosol by IκB that prevents its nuclear translocation and activity (Thanos and Maniatis, 1995; Verma *et al*., 1995). Upon cell stimulation by inflammatory cytokines such as tumor necrosis factor α (TNFα), IκBα is phosphorylated at residues 32 and 36, which trigger the ubiquitination and subsequent degradation of IκB through the proteosome pathway (Verma *et al*., 1995). These events release NF-κB, which translocates to the nucleus where it activates a number of important genes (Baeuerle and Henkel, 1994; Thanos and Maniatis, 1995; Verma *et al*., 1995; Baldwin, 1996). Recently, several groups have identified two IκB kinases (IKKα and IKKβ) responsible for the signal-induced phosphorylation and posterior degradation of IκB (DiDonato *et al*., 1996, 1997; Mercurio *et al*., 1997; Regnier *et al*., 1997; Woronicz *et al*., 1997; Zandi *et al*., 1997). The IKKs bind NIK (Regnier *et al*., 1997; Woronicz *et al*., 1997), a member of the MAPKKK family that interacts with TRAF2 (Malinin *et al*., 1997), linking IκB degradation and NF-κB activation to the TNF receptor complex. However, it seems that the TRAF2–NIK connection is not the only mechanism for the activation of NF-κB. Thus, cells from TRAF2-deficient mice display only minor alterations in the triggering of NF-κB by TNFα but have a completely impaired activation of JNK/SAPK (Lee *et al*., 1997; Yeh *et al*., 1997). In contrast to TRAF2, cells from RIP-deficient mice are severely impaired in their ability to activate NF-κB in response to TNFα, while displaying an intact activation of JNK/SAPK (Kelliher *et al*., 1998). RIP is a death domain kinase that associates with the TNF receptor 1 (TNF-R1) through its interaction with the adapter molecule TRADD (Hsu *et al*., 1996a). However, the kinase activity of RIP is not required for either NF-κB or JNK/SAPK activation (Hsu *et al*., 1996a). Therefore, it seems that the role of RIP could be that of a scaffold in the TNFα signaling pathway. Recently, two other kinases have been shown to regulate NF-κB at the level of the IKKs. Thus, whereas NIK selectively targets IKKα (Ling *et al*., 1998; Nakano *et al*., 1998), MEKK1 activates IKKα and IKKβ (Lee *et al*., 1998) and the atypical PKCs (aPKCs) (Lallena *et al*., 1999) phosphorylate and activate IKKβ *in vitro* and *in vivo*.

The aPKC subfamily of isozymes is composed of two members, ζPKC and λ/ιPKC. Although their mechanism of activation remains to be fully clarified, it seems clear that these kinases are insensitive to classical lipid mediators but can be modulated by protein–protein interactions (Diaz-Meco *et al*., 1996a,b; Puls *et al*., 1997; Izumi *et al*., 1998; Sanchez *et al*., 1998; Kuroda *et al*., 1999). In this regard, both aPKCs, but not the classical or the novel isoforms, bind selectively to the putative scaffold protein p62 (Sanchez *et al*., 1998). This protein is neither a regulator nor a substrate of the aPKC isoforms, but serves to anchor them to intracellular membranes where they may carry out their function (Sanchez *et al*., 1998). The p62 protein has a number of motifs that suggest a role as an adapter, linking the aPKCs to the membrane receptor signaling complexes (Puls *et al*., 1997; Sanchez *et al*., 1998). In light of the data indicating the important contribution of the aPKCs in NF-κB activation through the IKK pathway (Diaz-Meco *et al*., 1993, 1996b; Dominguez *et al*., 1993; Lozano *et al*., 1994; Bjorkoy *et al*., 1995;

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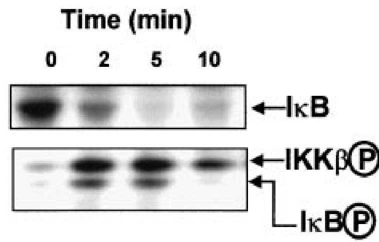

Fig. 1. Activation of IκB degradation and λ/ιPKC in response to TNFα. Cultures of 293 cells were stimulated or not with 30 ng/ml of TNFα. Cell extracts prepared at different times were either fractionated by SDS–PAGE and immunoblotted with an anti-IκB antibody (upper panel) or immunoprecipitated with a selective anti-λ/ιPKC antibody. The immunoprecipitates were incubated with immunopurified IKKβ and recombinant bacterially expressed MBP–IκB$^{1–250}$ to determine the activity of λ/ιPKC as described in the Materials and methods. Essentially identical results were obtained in another three independent experiments.

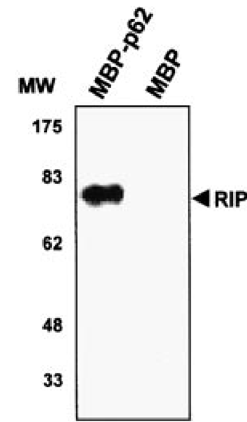

Fig. 2. *In vitro* interaction of p62 with RIP. Purified MBP or MBP–p62 (200 nM) was immobilized on amylose beads and incubated with *in vitro* translated ^{35}S-labeled RIP at 4°C for 1 h. After extensive washing, the recombinant proteins were fractionated by SDS–PAGE followed by autoradiography in an InstantImager. Essentially identical results were obtained in another three independent experiments.

Folgueira *et al*., 1996; Sontag *et al*., 1997; Lallena *et al*., 1999), together with the critical function of RIP in the channeling of signals from the TNF-R complex to NF-κB (Hsu *et al*., 1996a), we have sought to investigate the potential role of p62 as a RIP-binding protein connecting the aPKCs to NF-κB activation by TNFα.

Results

Previous studies have demonstrated that the aPKCs can be activated in response to TNFα (Muller *et al*., 1995). However, ζPKC activity has usually been determined by using either autophosphorylation or the phosphorylation of non-physiologically relevant substrates. The finding that the aPKCs phosphorylate and activate IKKβ *in vitro* and *in vivo* (Lallena *et al*., 1999) permits us to determine now whether or not the aPKCs are activated by TNFα using a more physiological coupled IKKβ/IκBα assay. Thus, 293 cells were stimulated or not with TNFα for different times, after which cell extracts were either fractionated by SDS–PAGE and immunoblotted with an anti-IκB antibody (Figure 1, upper panel) or immunoprecipitated with a selective anti-λ/ιPKC antibody. The immunoprecipitates were incubated with immunopurified IKKβ and recombinant bacterially expressed MBP–IκB$^{1–250}$ to determine the activity of λ/ιPKC toward its physiological substrate. Notably, immunoprecipitated λ/ιPKC from stimulated but not from untreated cells reactivated IKKβ to phosphorylate the N-terminal domain of IκB (Figure 1, lower panel). The activation of λ/ιPKC in these experiments is maximal at 2–5 min, declining at 10 min (Figure 1, lower panel); a time course that is clearly compatible with the TNFα-induced degradation of IκB (compare both panels of Figure 1).

Because RIP is an important intermediary in the TNFα pathway toward the activation of NF-κB, we addressed initially whether RIP could interact with the aPKCs. Despite numerous attempts, we did not detect binding of aPKCs to RIP (not shown). Therefore, we next determined whether the link of aPKCs with RIP could be mediated by the potential adapter p62. Therefore, in the next series of experiments we analyzed the possible *in vitro* interaction of RIP with p62. Thus, p62 bacterially expressed as a maltose binding protein (MBP) fusion protein and immobilized on amylose beads was incubated with radiolabeled, *in vitro*-translated RIP. Following extensive washing with a binding buffer containing 1% Triton X-100 and high salt concentrations (500 mM NaCl), bound MBP–p62 protein and the potentially associated ^{35}S-labeled RIP were boiled in sample buffer and fractionated by SDS–PAGE. The results in Figure 2 demonstrate a reproducible interaction of RIP with p62 but not with MBP alone. Staining of a parallel gel confirms that both reactions contained equal molar amounts of MBP proteins (not shown).

Next, 293 cells were transfected with expression vectors either for Myc-tagged p62, Flag-tagged RIP or both together. Twenty-four hours post-transfection, cell extracts were immunoprecipitated with an anti-Flag antibody and the immunoprecipitates were analyzed by immunoblot with an anti-Myc antibody. The results shown in Figure 3A demonstrate that ectopically expressed p62 co-precipitates with ectopic RIP. RIP has been shown to interact with TRAF2 in co-transfection experiments (Hsu *et al*., 1996a). Therefore, we next determined whether p62 could interact with TRAF2. Cell extracts from 293 cells transfected either with hemagglutinin (HA)-TRAF2, Myc-p62 or both together, were immunoprecipitated with anti-HA antibody, and the immunoprecipitates were analyzed with the anti-Myc antibody. Interestingly, no association of TRAF2 with p62 was detected in either the absence (Figure 3B) or presence of TNFα (not shown). We were interested to determine whether the presence of TRAF2 could affect the ability of p62 to interact with RIP. To address this point, 293 cells were transfected with Flag-RIP along with plasmid control or Myc-p62, with or without HA-TRAF2. Afterwards, expressed RIP was immunoprecipitated with an anti-Flag antibody and the immunoprecipitates were analyzed by immunoblotting with anti-Myc antibody to detect the associated p62. Interestingly, the binding of p62 to RIP was not affected by the presence of TRAF2 (Figure 3C). In order to determine whether TRAF2 binds to RIP in the presence of p62, ectopically expressed TRAF2 was immunoprecipitated with anti-HA antibody and the immunoprecipitates were analyzed by immunoblotting with anti-Flag and anti-Myc antibodies to detect RIP and p62, respectively. The results of Figure 3C show that the

presence of p62 did not affect the interaction of TRAF2 with RIP. Furthermore, p62 was detected in the TRAF2 immunocomplexes only when RIP was co-expressed. Because p62 does not interact directly with TRAF2 (Figure 3C), the results of these experiments indicate that RIP can accommodate both p62 and TRAF2 simultaneously.

In addition, these results together suggest that the RIP–p62 interaction is specific and direct. It is unlikely that this interaction was mediated by an endogenous protein, as expression levels of these compared with the over-expressed proteins were below detection levels (not shown). In order to demonstrate that this interaction also takes place under more physiological conditions, 293 cells were transfected either with an empty plasmid or an expression vector for HA-tagged p62. Twenty-four hours post-transfection, cells were either untreated or stimulated with TNFα for different times. Afterwards, cell extracts were immunoprecipitated with an anti-RIP antibody to immunoprecipitate endogenous RIP, and the co-precipitation of HA-p62 was determined by immunoblot analysis by using an anti-HA antibody. Interestingly, there is a faint but detectable interaction of HA-p62 with the endogenous RIP under basal conditions (Figure 3D). However, this interaction is dramatically increased upon TNFα stimulation (Figure 3D), with a kinetic that is compatible with the induced degradation of IκB and the activation of λ/ιPKC (Figure 1). When the interaction of both endogenous proteins was analyzed, similar results were obtained. Thus, cell extracts from HeLa (Figure 3E) or 293 cells (not shown), either untreated or stimulated with TNFα for different times, were incubated with the anti-RIP antibody to immunoprecipitate endogenous RIP. Afterwards, these immunoprecipitates were analyzed by immunoblotting with an anti-p62 antibody. The results shown in Figure 3E reveal that there is little or no association of endogenous p62 with RIP in unstimulated cells but that this association becomes evident upon TNFα triggering, with a kinetic that is comparable with those of Figures 1 and 3D. Collectively, these data strongly suggest that the RIP–p62 interaction could be the physiological step that allows λ/ιPKC to impinge the TNFα signaling pathway toward IKK/NF-κB activation. Notably, in the experiments in which two or more proteins were ectopically expressed (Figure 3A–C), the presence of TNFα did not increase further their interactions (not shown).

In the next series of experiments, we mapped the domains in both proteins that account for their interaction. Different tagged p62 deletion mutants (Figure 4A) were transfected into 293 cells, after which their association to

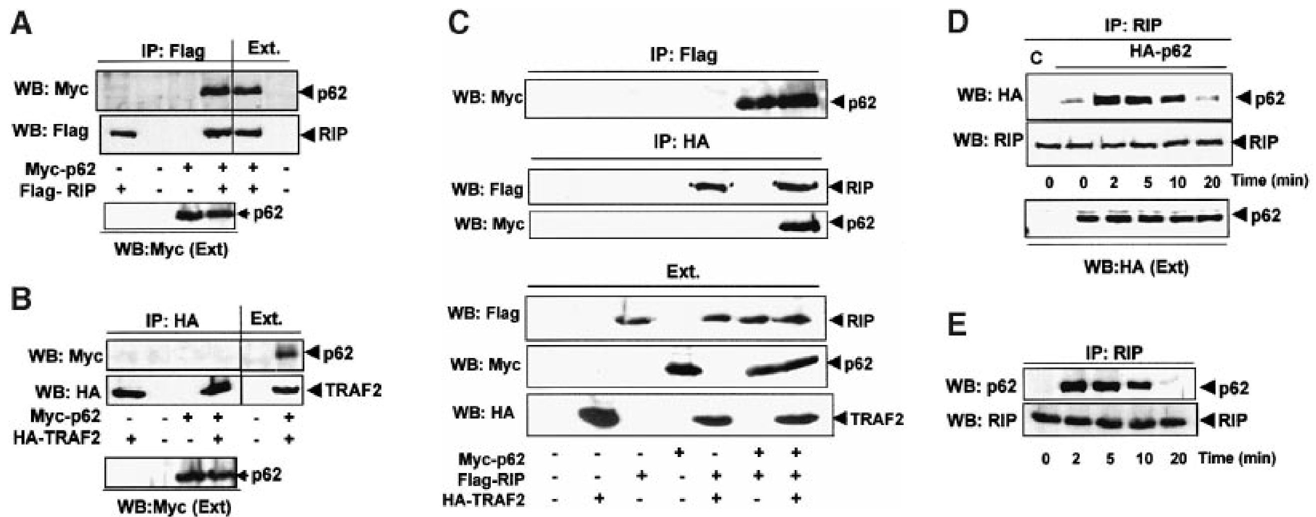

Fig. 3. Interaction *in vivo* of p62 with RIP but not with TRAF2. (**A**) Subconfluent cultures of 293 cells were transfected with 10 μg of either pcDNA3 or expression vectors for either Myc-p62 or Flag-RIP, or both together, and enough empty vector to give 20 μg of total DNA. After transfection (24 h), cell extracts were immunoprecipitated with an anti-Flag antibody, and the immunoprecipitates were analyzed by immunoblotting with anti-Myc or anti-Flag antibodies. An aliquot [one-tenth of the amount of extract (Ext.) used for the immunoprecipitation] was loaded in the gels and analyzed by immunoblotting with the corresponding anti-tag antibodies. (**B**) Subconfluent cultures of 293 cells were transfected with 10 μg of either pcDNA3 or expression vectors for either Myc-p62 or HA-TRAF2, or both together, and enough empty vector to give 20 μg of total DNA. After transfection (24 h), cell extracts were immunoprecipitated with an anti-HA antibody, and the immunoprecipitates were analyzed by immunoblotting with anti-Myc or anti-HA antibodies. An aliquot [one-tenth of the amount of extract (Ext.) used for the immunoprecipitation] was loaded onto the gels and analyzed by immunoblotting with the corresponding anti-tag antibodies. (**C**) Subconfluent cultures of 293 cells were transfected with 10 μg of either pcDNA3 or expression vectors for either Myc-p62, HA-TRAF2, Flag-RIP or different combinations of these plasmids, and enough empty vector to give 30 μg of total DNA. After transfection (24 h), cell extracts were immunoprecipitated with an anti-Flag antibody, the immunoprecipitates were analyzed by immunoblotting with anti-Myc antibody. An equal amount of cell extracts were immunoprecipitated with anti-HA antibody and the washed immunoprecipitates were analyzed by immunoblotting with anti-Myc and anti-Flag antibodies. An aliquot [one-tenth of the amount of extract (Ext.) used for the immunoprecipitation] was loaded in the gels and analyzed by immunoblotting with the corresponding anti-tag antibodies. (**D**) Subconfluent cultures of 293 cells were transfected with 10 μg of either pcDNA3 or an expression vector for HA-p62. After transfection (24 h), cells were either untreated or stimulated with TNFα (30 ng/ml) for different time periods. Afterwards, cell extracts were immunoprecipitated with an anti-RIP antibody, and the immunoprecipitates were analyzed by immunoblotting with anti-HA or anti-RIP antibodies. An aliquot [one-tenth of the amount of extract (Ext.) used for the immunoprecipitation] was loaded onto the gels and analyzed by immunoblotting with the anti-HA antibody. (**E**) Subconfluent cultures of HeLa cells were either untreated or stimulated with TNFα (30 ng/ml) for different time periods. Afterward, cell extracts were immunoprecipitated with an anti-RIP antibody, and the immunoprecipitates were analyzed by immunoblotting with anti-p62 or anti-RIP antibodies. For all experiments, essentially identical results were obtained in another three independent experiments.

endogenous RIP was determined in TNFα-activated cells. Expression of the p62 amino acids 1–266 or 117–439 is sufficient to account for RIP interaction (Figure 5A and B), whereas deletion of amino acids 117–439 (Figure 5B) completely abolishes it. Collectively, these results indicate that the region encompassing amino acids 117–266 seems critical for the binding of p62 to RIP. This sequence includes the ZZ domain, which has recently been described as a novel zinc finger motif of as yet undefined function (Ponting *et al*., 1996). In order to map the domains in the RIP molecule that are critical for the interaction with p62, tagged versions of different RIP deletion mutants (Figure 4B) were transfected along with epitope-tagged p62 in 293 cells, after which the co-precipitation of p62 with the different RIP constructs was determined. The results in

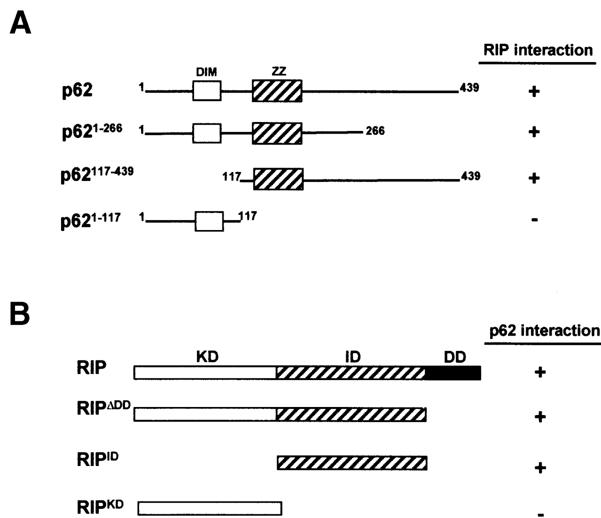

Fig. 4. Schematic representation of the various constructs used for the mapping of the p62–RIP interaction domains. Constructs were prepared as described in the Materials and methods. DIM, dimerization and ζPKC-interacting region; ZZ, zinc finger domain; KD, kinase domain; ID, intermediary domain; DD, death domain.

Figure 6 demonstrate that the death and the kinase domains of RIP are dispensable, whereas the intermediary domain is sufficient, for the interaction with p62. Interestingly, this correlates with the observation that the intermediary domain is sufficient, whereas the death domain of RIP is dispensable, for NF-κB activation (Hsu *et al*., 1996a).

Collectively, these results demonstrate that p62 is the link that connects the aPKCs to RIP. If this model is correct, the expression of p62 should allow the co-precipitation of the aPKCs with RIP in co-transfection experiments. To demonstrate this possibility, 293 cells were transfected with HA-ζPKC along with either control vector or a Myc-p62 expression plasmid with or without Flag-RIP. Cell extracts were immunoprecipitated with an anti-Flag antibody and the immunoprecipitates were analyzed with either an anti-Myc or an anti-HA antibody to detect the associated p62 and ζPKC, respectively. According to the results shown in Figure 7A, ζPKC does not co-precipitate with RIP unless p62 is expressed that is also detected in the anti-Flag (RIP) immunoprecipitates. This indicates that p62 actually forms a bridge between RIP and ζPKC. The same results were obtained with an HA-tagged version of λ/ιPKC (not shown). Recent data from this laboratory have demonstrated the interaction of the aPKCs with IKKβ, which is important for the activation of the NF-κB pathway by TNFα (Lallena *et al*., 1999). Because aPKCs bind to p62, it is conceivable that the interaction of the IKKβ with aPKCs may link IKKβ to p62 and, consequently, to RIP. To address this possibility, 293 cells were transfected with an expression vector for Flag-IKKβ along with either an empty plasmid or an expression vector for HA-p62 with or without Myc-ζPKC. Cell extracts were immunoprecipitated with an anti-Flag antibody and the immunoprecipitates were analyzed with either anti-HA or anti Myc antibodies to detect p62 and ζPKC, respectively. The results of Figure 7B demonstrate that p62 does not co-precipitate with IKK, unless ζPKC is expressed that is also detected in the anti-Flag (IKK)

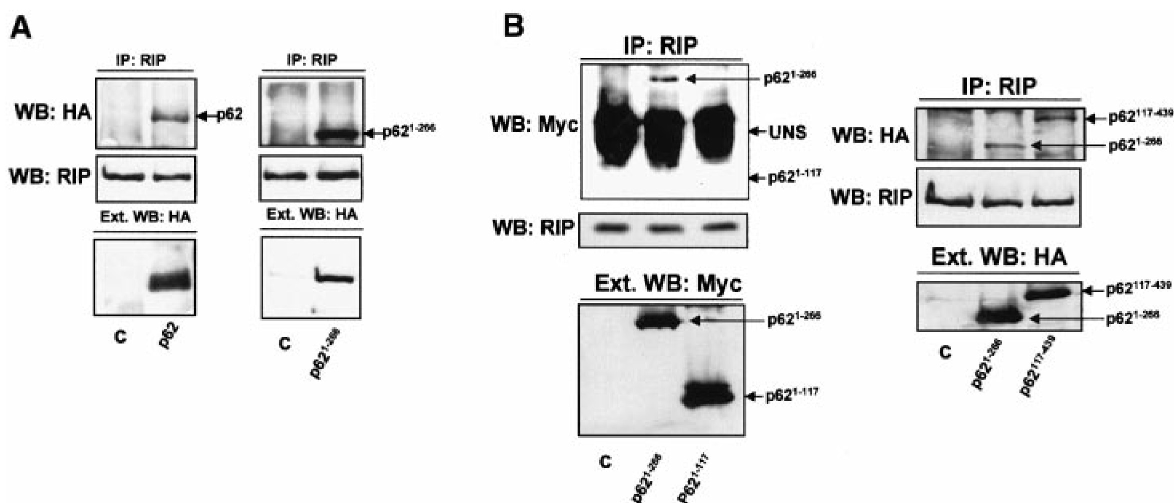

Fig. 5. Interaction of different p62 fragments with RIP *in vivo*. Subconfluent cultures of 293 cells were transfected with 10 μg of either pcDNA3 or expression vectors for either HA-p62 [(**A**), left panel], HA-$p62^{1-266}$ [(A), right panel; (**B**), right panel], Myc-$p62^{1-266}$ [(B), left panel], Myc-$p62^{1-117}$ [(B), left panel], HA-$p62^{117-439}$ [(B), right panel]. After transfection (24 h), cells were stimulated with 30 ng/ml of TNFα for 4 min, and cell extracts were immunoprecipitated with anti-RIP antibody, and the immunoprecipitates were extensively washed, and analyzed by immunoblotting with anti-Myc, anti-HA or anti-RIP antibodies, according to the experiments. An aliquot [one-third of the amount of extract (Ext.) used for the immunoprecipitation] was loaded onto the gels and analyzed by immunoblotting with the corresponding anti-tag antibodies. UNS, unspecific. Essentially identical results were obtained in another three independent experiments.

immunoprecipitates. This indicates that ζPKC bridges IKKβ to p62. The same results were obtained with an HA-tagged version of λ/ιPKC (not shown). Collectively, these results permit one to establish a chain of interactions between RIP and IKKβ that is mediated by p62 and the aPKCs. The association of RIP with the TNF-R1 is mediated through an homotypic interaction of the death domain of the TNF-R1 with that of TRADD (Hsu *et al*., 1996a). To determine whether RIP may act as a link between TRADD and p62, 293 cells were transfected with Myc-TRADD along with either control vector or HA-p62 expression plasmid with or without Flag-RIP. Cell extracts were immunoprecipitated with anti-HA antibody and the immunoprecipitates were analyzed with anti-Myc or anti-Flag antibodies to detect TRADD and RIP, respectively. Results of Figure 7C demonstrate that TRADD co-precipitates with p62 only in cells transfected with RIP which is also detected in the anti-HA (p62) immunoprecipitates. These results are in keeping with the hypothesis that RIP bridges p62 to TRADD. Other studies have demonstrated the recruitment of endogenous RIP to the TNF-R1 complex upon cell stimulation with TNFα (Hsu *et al*., 1996a). To determine whether p62 is also recruited to that complex, cells were untreated or stimulated with TNFα for different times and cell extracts were immunoprecipitated with an anti-TNF-R1 antibody, and the immunoprecipitates were analyzed by immunoblot with either anti-p62 or anti-RIP antibodies. Interestingly, under resting conditions there was no co-immunoprecipitation of p62 or RIP with the TNF-R1. However, a reproducible co-precipitation was detected when the cells were triggered with TNFα (Figure 8), giving a kinetic comparable with that of the association of RIP and p62 (Figure 3D and E).

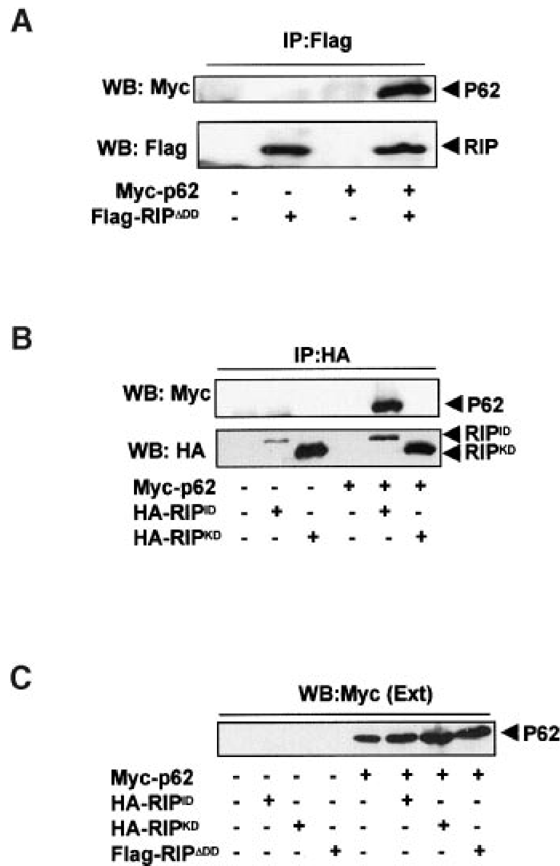

Fig. 6. Interaction of different RIP fragments with p62 *in vivo*. (**A**) Subconfluent cultures of 293 cells in 100-mm-diameter plates were transfected with 10 μg of either pcDNA3 or expression vectors for either Myc-p62 or Flag-RIP$^{\Delta DD}$, or both together, and enough empty vector to give 20 μg of total DNA. After transfection (24 h), cell extracts were immunoprecipitated with an anti-Flag antibody, and the immunoprecipitates were analyzed by immunoblotting with anti-Myc or anti-Flag antibodies. (**B**) Subconfluent cultures of 293 cells in 100-mm-diameter plates were transfected with 10 μg of either pcDNA3 or expression vectors for either Myc-p62, HA-RIP^{ID}, HA-RIP^{KD} or combinations of Myc-p62 with either RIP constructs, and enough empty vector to give 20 μg of total DNA. After transfection (24 h), cell extracts were immunoprecipitated with an anti-HA antibody, and the immunoprecipitates were analyzed by immunoblotting with anti-Myc or anti-HA antibodies. For (A) and (B), essentially identical results were obtained in another three independent experiments. (**C**) An aliquot [one-tenth of the amount of extract (Ext.) used for the immunoprecipitations of (A) and (B)] was loaded in a gel and analyzed by immunoblotting with the anti-Myc antibody.

Collectively, these results suggest that the aPKCs and p62 must play a role in the activation of NF-κB by RIP expression. To address this possibility, 293 cells were transfected with a κB-dependent luciferase reporter gene along with two concentrations of a RIP expression vector either without or with increasing concentrations of a dominant-negative mutant of λ/ιPKC. The results of Figure 9A demonstrate that RIP potently activates NF-κB, consistent with previous data (Hsu *et al*., 1996a), and that the transfection of the dominant-negative mutant of λ/ιPKC completely abolishes that effect. To demonstrate a potential role of p62 in this pathway, we transfected 293 cells with the κB-dependent luciferase reporter along with two mutants of p62. The first mutant, p62$^{117–439}$, binds RIP (Figures 4A and 5B) but does not interact with the aPKCs (Puls *et al*., 1997). The second mutant, p62$^{1–117}$, interacts with aPKCs (Puls *et al*., 1997), but does not bind RIP. We reasoned that the expression of either mutants should inhibit RIP-induced NF-κB, because the N-terminal fragment (p62$^{1–117}$) will form aPKC-containing inactive complexes devoid of RIP, whereas the C-terminal fragment (p62$^{117–439}$) will form likewise inactive RIP complexes lacking aPKCs. The results of Figure 9B demonstrate that this actually appears to be the case as both mutants block RIP-induced, κB-dependent transcriptional activity. These data strongly suggest that the N- and C-terminal domains of p62 mediate separate and essential steps in the NF-κB pathway. In addition, cells transfected with an antisense p62 construct display a significantly impaired RIP-induced activation of NF-κB (Figure 9B). Interestingly, the ability of TRAF2 to activate NF-κB is little or no affected by the λ/ιPKC or p62 mutants, or by the p62 antisense (Figure 9A and B). All these results together reinforce the notion that p62 and the aPKCs are critical players in the activation of NF-κB, probably in the RIP signaling cascade.

Discussion

The two members of the aPKC subfamily of isozymes, namely ζPKC and λ/ιPKC, have recently been shown to be involved in a number of important cellular events, including proliferation and survival (Dominguez *et al*., 1992; Berra *et al*., 1993, 1995; Diaz-Meco *et al*., 1994a,b, 1996a). The mechanisms whereby the aPKCs control these functions most probably involve, at least in part, their

ability to regulate the NF-κB signaling pathway (Diaz-Meco *et al*., 1993, 1994a, 1996b; Dominguez *et al*., 1993; Lozano *et al*., 1994; Bjorkoy *et al*., 1995; Folgueira *et al*., 1996; Sontag *et al*., 1997; Lallena *et al*., 1999). Thus, the blockade of the aPKCs by using either microinjected pseudosubstrate peptide inhibitors (Dominguez *et al*., 1993), antisense oligonucleotides (Dominguez *et al*., 1993; Folgueira *et al*., 1996) or the transfection of kinase-dead dominant-negative mutants of ζPKC or λ/ιPKC (Diaz-Meco *et al*., 1993, 1996b; Lozano *et al*., 1994; Bjorkoy *et al*., 1995; Folgueira *et al*., 1996; Sontag *et al*., 1997), dramatically impairs NF-κB activation. In addition, we have recently demonstrated that the aPKCs bind to the IKKs *in vitro* and *in vivo* (Lallena *et al*., 1999). Importantly, overexpression of ζPKC positively modulates IKKβ but not IKKα activity, whereas the transfection of a ζPKC dominant-negative mutant severely impairs the activation of IKKβ but not of IKKα in TNFα, but not in phorbol 12-myristate 13-acetate (PMA)-stimulated cells (Lallena *et al*., 1999). In addition, recombinant active ζPKC dramatically stimulates *in vitro* the IKKβ but not IKKα activity from unstimulated cells. Taken together, these results indicate that the mechanism whereby the aPKCs contribute to TNFα-induced activation of NF-κB most probably involves the regulation of IKKβ activity. Consistent with these observations, TNFα has been shown to be a potent activator of ζPKC *in vivo* (Muller *et al*., 1995). However, the way the activity of the aPKCs was determined in those experiments relied on the measurement of either the autophosphorylation of the enzyme or its ability to phosphorylate non-physiological substrates. In the study reported here we used a coupled assay to determine the ability of activated aPKCs from TNFα-treated cells to phosphorylate and activate purified IKKβ. Using this methodology, we demonstrate here that TNFα potently activates the aPKCs with a kinetic that is compatible with the induced degradation of IκBα.

Therefore, these results together reinforce the notion that the aPKCs are important components of the TNFα signaling pathway that controls NF-κB activation. A critical unresolved question is the identification of the steps that link the aPKCs to the TNFα receptor signaling complex. The TNF-R1 is a 55 kDa protein with a death domain in its intracellular region that mediates the interaction with a number of adapters that play critical roles in the transmission of the signal from the membrane to the activation of NF-κB and other transcriptional machinaries (Hsu *et al*., 1996a,b; Liu *et al*., 1996; Shu *et al*., 1996; Song *et al*., 1997). Upon cell stimulation with TNFα, the TNF-R1 recruits the protein TRADD that

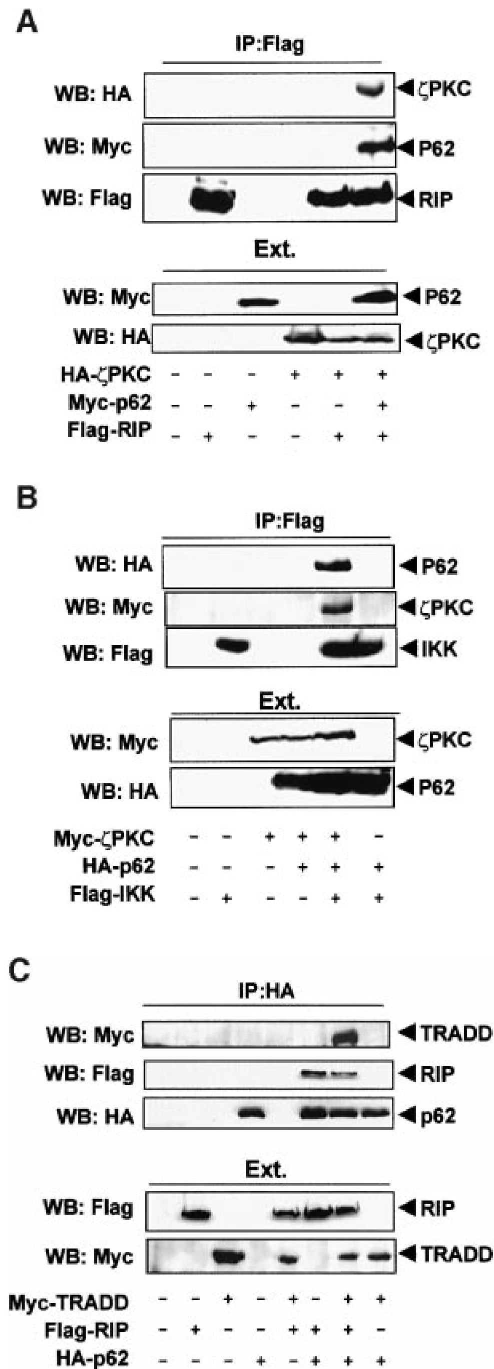

Fig. 7. Role of p62 as a link between TRADD-RIP and ζPKC-IKK complexes. (**A**) Subconfluent cultures of 293 cells were transfected with 10 μg of either pcDNA3 or expression vectors for either HA-ζPKC, Myc-p62, Flag-RIP or different combinations of them, and enough empty vector to give 30 μg of total DNA. After transfection (24 h), cell extracts were immunoprecipitated with an anti-Flag antibody, and the immunoprecipitates were analyzed by immunoblotting with anti-Myc, anti-HA, or anti-Flag antibodies. An aliquot [one-tenth of the amount of extract (Ext.) used for the immunoprecipitation] was loaded onto the gels and analyzed by immunoblotting with the corresponding anti-tag antibodies. (**B**) Subconfluent cultures of 293 cells were transfected with 10 μg of either pcDNA3 or expression vectors for either Myc-ζPKC, HA-p62, Flag-IKK or different combinations of them, and enough empty vector to give 30 μg of total DNA. After transfection (24 h), cell extracts were immunoprecipitated with an anti-Flag antibody, and the immunoprecipitates were analyzed by immunoblotting with anti-Myc, anti-HA, or anti-Flag antibodies. An aliquot [one-tenth of the amount of extract (Ext.) used for the immunoprecipitation] was loaded onto the gels and analyzed by immunoblotting with the corresponding anti-tag antibodies. (**C**) Subconfluent cultures of 293 cells were transfected with 10 μg of either pcDNA3 or expression vectors for either HA-p62, Flag-RIP, Myc-TRADD or different combinations of them, and enough empty vector to give 30 μg of total DNA. After transfection (24 h), cell extracts were immunoprecipitated with an anti-HA antibody, and the immunoprecipitates were analyzed by immunoblotting with anti-Myc, anti-HA or anti-Flag antibodies. An aliquot [one-tenth of the amount of extract (Ext.) used for the immunoprecipitation] was loaded onto the gels and analyzed by immunoblotting with the corresponding anti-tag antibodies. For all experiments, essentially identical results were obtained in another three independent experiments.

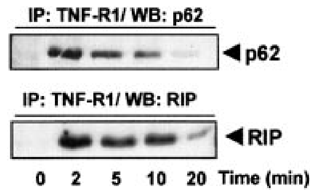

Fig. 8. Recruitment of p62 to the TNF-R1 complex by TNFα stimulation. Cell cultures were unstimulated or treated with TNFα (30 ng/ml) for different times, after which cell lysates were immunoprecipitated with an anti-TNF-R1 antibody. The co-precipitating p62 and RIP were determined by immunoblot analysis with an anti-p62 or anti-RIP antibodies, respectively. This is a representative experiment of another two with similar results.

binds to TRAF2 and RIP. TRAF2 also interacts with the intermediary domain of RIP, giving rise to an inter-connected trimolecular complex involving TRADD, RIP and TRAF2 (Hsu *et al*., 1996a,b). Interestingly, the over-expression of TRAF2 or RIP is sufficient to activate NF-κB and JNK/SAPK (Liu *et al*., 1996). However, only RIP-, but not TRAF2-deficient cells have an impaired NF-κB activation in response to TNFα (Lee *et al*., 1997; Yeh *et al*., 1997; Kelliher *et al*., 1998), indicating that RIP is necessary and sufficient for NF-κB signaling by TNFα. Therefore, in this study we have addressed the potential connection of aPKCs to RIP.

We initially attempted to detect a direct binding of RIP to the aPKCs. However, numerous experiments failed to show any interaction between both molecules. This indicated that if there is a functional link between RIP and the aPKCs, this should be mediated by some additional component. In this regard, the search of scaffold proteins selective for different PKC isotypes is an emerging field of intense research, because they could provide elegant ways for the distinct isoforms to have specific mechanisms of regulation and/or action. The first anchor/scaffold proteins identified for the classical and novel PKCs were the receptor for activated C kinases (RACKs) and A-kinase anchoring proteins (AKAPs), which assemble different signaling components conferring specificity and efficiency to the action of different kinases (Hausken *et al*., 1996; Lester *et al*., 1996; Faux and Scott, 1997; Mochly-Rosen and Gordon, 1998). Recently, two novel proteins have been described to interact with the aPKCs. Thus, Ohno's group has identified atypical PKC isotype-specific interacting protein (ASIP), a mammalian homologue of the *Caenorhabditis elegans* polarity protein Par-3 as an aPKC-interacting protein that serves to localize these kinases to the epithelial tight junctions (Izumi *et al*., 1998). The role played by the aPKCs in the maintenance of the epithelial cell polarity is not yet clear, but this finding reinforces the notion that distinct anchor proteins may serve to locate the aPKCs in different signaling pathways inhibiting the promiscuity and increasing the efficiency of the kinase's actions. In this regard, in neurons, ζPKC seems to interact with the mammalian homologue of UNC-76 (FEZ1), a *C.elegans* protein involved in axonal outgrowth (Kuroda *et al*., 1999). Actually, these authors show a synergistic functional interaction of FEZ1 with ζPKC to promote neuronal differentiation of PC12 cells (Kuroda *et al*., 1999).

We and others had previously identified p62 as a novel aPKC-interacting protein that binds selectively and

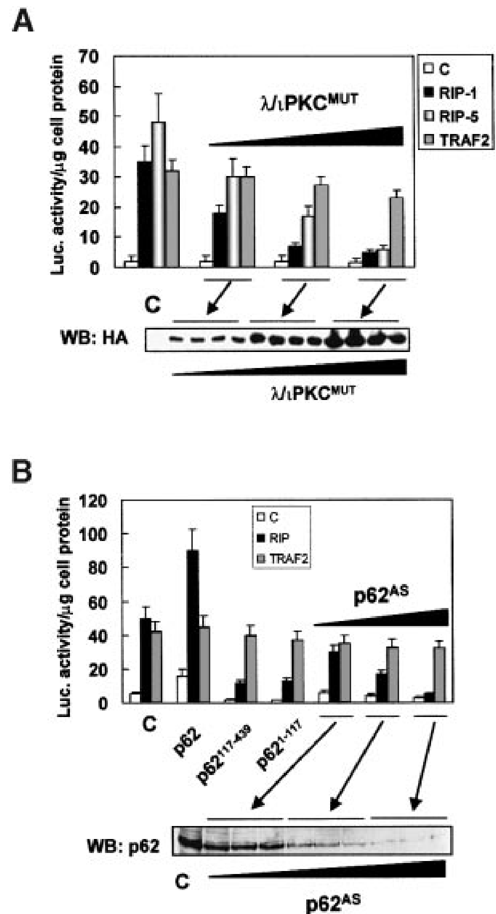

Fig. 9. Role of λ/ιPKC and p62 in NF-κB activation by RIP. (**A**) Subconfluent cultures of 293 cells were transfected with 10 ng of the κB-luciferase reporter gene plasmid along with 1 or 5 µg of RIP, 3 µg of TRAF2, and 0, 2, 5 or 10 µg of a λ/ιPKC dominant-negative mutant (λ/ιPKC^{MUT}), and enough empty vector to give 15 µg of total DNA. After 24 h, extracts were prepared and the levels of luciferase activity were determined as described in the Materials and methods. Results are the mean ± SD of three independent experiments with incubations in duplicate. The panel below is a representative control blot of the expression of the λ/ιPKC mutant from one of the experiments. (**B**) Subconfluent cultures of 293 cells were transfected with 10 ng of the κB-luciferase reporter gene plasmid along with 5 µg of RIP, 3 µg of TRAF2 and 10 µg of the p62 constructs described in Figure 4, and enough empty vector to give 15 µg of total DNA. Parallel cultures were transfected with increasing amounts (2, 5 and 10 µg) of a p62 antisense construct (p62^{AS}). After 24 h, extracts were prepared and the levels of luciferase activity were determined as described in the Materials and methods. Results are the mean ± SD of three independent experiments with incubations in duplicate. The panel below is a representative control blot of the ability of the p62 antisense to reduce p62 levels from one of the experiments.

constitutively to the V1 domains of λ/ιPKC and ζPKC, but not of αPKC or εPKC (Puls *et al*., 1997; Sanchez *et al*., 1998). p62 is neither a regulator nor a substrate of the aPKC isoforms, but has a number of motifs that suggest a function as an adaptor linking the aPKCs to the membrane receptor signaling complexes (Puls *et al*., 1997; Sanchez *et al*., 1998). Therefore, p62 appeared to be a good candidate to connect the aPKCs to more upstream elements in the TNFα signaling pathway. We demonstrate here that the co-transfection of p62 with RIP unveils a potent and selective interaction between both proteins. Interestingly, this interaction becomes TNF-inducible when the binding of the endogenous proteins was

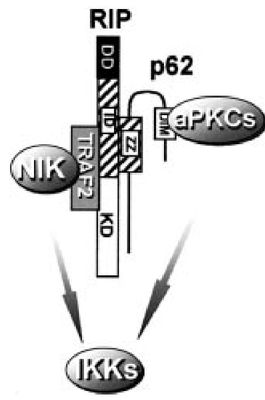

Fig. 10. Model for the involvement of the aPKCs and p62 in RIP-mediated signaling. RIP interacts through its intermediary (ID) and kinase (KD) domains but not through the death domain (DD), with the TRAF domain of TRAF2. The ID of RIP is sufficient to interact with p62. RIP is connected to IKKα through the interaction of NIK with TRAF2, and to IKKβ through the interaction of the aPKCs with the dimerization domain of p62.

measured. This suggests that upon TNFα activation, RIP is able to interact with p62 allowing the aPKCs to participate in the activation of IKKβ and NF-κB. In this regard, we demonstrate here that RIP acts as a bridge linking p62 to TRADD, which is the first step in the TNF-R1 signaling cascade (Hsu *et al*., 1996a,b). In addition, p62 bridges the aPKCs to RIP and the aPKCs link IKKβ to p62. In this way, a signaling cascade of interactions is established from the TNF-R1 involving TRADD/RIP/p62/aPKCs/IKKβ. The intermediary domain of RIP is sufficient to account for the binding of TRAF2 and p62. However, from our data it is clear that these interactions are not antagonistic, existing as a ternary complex that simultaneously involves RIP, p62 and TRAF2. This is of potential functional relevance, because TRAF2 interacts with NIK (Malinin *et al*., 1997), activating IKKα (Regnier *et al*., 1997; Ling *et al*., 1998). Thus, a model can be drawn according to which RIP activates two independent and redundant pathways toward IκB degradation, through TRAF2–NIK and p62–aPKCs (Figure 10). This may explain why the knock-down of RIP abolishes NF-κB activation whereas that of TRAF2 has only a minor impact on this parameter.

Goeddel and co-workers have demonstrated that the ectopic expression of either the kinase or the intermediary domains of RIP is sufficient to induce NF-κB (Hsu *et al*., 1996a). The intermediary domain is also sufficient to account for the interaction of RIP with p62, suggesting the functional relevance of that interaction. Consistent with this notion, the expression of dominant-negative mutants of the aPKCs not only impairs TNFα-stimulated (Diaz-Meco *et al*., 1993, 1996b; Lozano *et al*., 1994; Folgueira *et al*., 1996), but also RIP-stimulated, NF-κB activation (Figure 9). The aPKCs interact with a dimerization domain in p62 that is located upstream of, and close to, the RIP-binding region. Therefore, a model emerges whereby p62 assembles both signaling molecules, aPKC and RIP, through two distinct but proximal modules (Figure 10). The data presented here support this model, as the expression of either the N-terminal aPKC-binding domain or the C-terminal RIP-binding region is sufficient to block NF-κB activation. These results, together with the fact that the aPKCs bind to and activate IKKβ, define a novel mechanism for the TNFα-induced activation of NF-κB.

Materials and methods

Reagents and cell culture

Recombinant human TNFα was purchased from Promega. The monoclonal 12CA5 anti-HA antibody was from Boehringer Mannheim. The rabbit anti-Myc epitope was from Santa Cruz Biotechnologies, Inc. The monoclonal antibodies against λPKC and RIP were both from Transduction Laboratories. The anti-IκB was from Santa Cruz Biotechnologies, Inc. The rabbit affinity-purified anti-p62 has been described previously (Sanchez *et al*., 1998). HeLa and 293 cells were obtained from the American Type Culture Collection (ATCC). Cultures of 293 cells were maintained in high glucose Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS), penicillin G (100 μg/ml) and streptomycin (100 μg/ml) (Flow). HeLa cells were maintained in minimum essential Eagle's medium supplemented with 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate and 10% FCS. Subconfluent cells were transfected by the calcium phosphate method (Clontech, Inc.).

Plasmids

pCDNA3-HA-ζPKC, pCDNA3-HA-λ/ιPKC, pCDNA3-Myc-ζPKC, pCDNA3-Myc-λ/ιPKC, pCDNA3-HA-p62, pCDNA3-Myc-p62 and pMAL-c2-p62 have been described previously (Diaz-Meco *et al*., 1996a,b; Sanchez *et al*., 1998). pRK5-Flag-RIP, pRK5-Flag-IKKβ, pRK5-Myc-TRADD and pCDNA3-HA-TRAF2 constructs were generously provided by D.Goeddel (Tularik, Inc.). The different domains of p62 or RIP were subcloned into pCDNA3-HA, pCDNA3-Flag or pCDNA3-Myc vectors. The antisense p62 was subcloned into pRK5. The pMAL-c2-IκB$^{1–250}$ was obtained from excising a *Bam*HI–*Xba*I fragment from GST–IκBΔC (Lallena *et al*., 1999) and subcloning it into pMAL-c2 (New England Biolabs). MBP, MBP–p62 and MBP–IκB$^{1–250}$ fusions proteins were expressed in *Escherichia coli* and purified by binding to an amylose resin according to the procedures recomended by the manufacturer.

In vitro binding studies

In vitro-translated RIP was prepared by coupled *in vitro* transcription and translation in rabbit reticulocyte lysate (Promega) as described in the manufacturer's protocol and incubated with purified MBP or MBP–p62 in binding buffer [50 mM Tris pH 7.4, 2 mM EDTA, 1 mM EGTA, 2 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 0.5 M NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml of leupeptin and 10 μg/ml of aprotinin]. The MBP fusion proteins with any associated protein were recovered in amylose beads and extensively washed as described previously (Diaz-Meco *et al*., 1996a,b; Sanchez *et al*., 1998). Samples were subjected to SDS–PAGE and autoradiography in an InstantImager (Packard).

Immunoprecipitations

For immunoprecipitations of endogenous proteins, HeLa cells were used. For co-immunoprecipitations, subconfluent 293 cells plated on 10 cm dishes were transfected with the indicated expression plasmids. After transfection (24 h), cells were stimulated or not with 30 ng/ml of TNFα. Cells were then harvested and lysed in PD buffer [40 mM Tris–HCl pH 8.0, 500 mM NaCl, 0.1% Nonidet P-40 (NP-40), 6 mM EDTA, 6 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM PNPP, 300 μM Na_3VO_4, 1 mM benzamidine, 2 M PMSF, 10 μg/ml of aprotinin, 1 μg/ml of leupeptin, 1 μg/ml of pepstatin and 1 mM dithiothreitol (DTT)], and 1 mg of whole-cell lysate was diluted in PD buffer and incubated with 5–10 μg of the indicated antibody. This reaction mixture was incubated on ice for 2 h, and then 25 μl of protein A or G beads were added and the mixture was left to incubate with gentle rotation for an additional 1 h at 4°C. The immunoprecipitates were then washed three times with PD buffer. Samples were fractionated on 8% SDS–PAGE, transferred to Nitrocellulose ECL membrane (Amersham) and subjected to Western blot analysis with the corresponding antibody. Proteins were detected with the ECL reagent (Amersham). To determine the recruitment of p62 to the TNF-R1 *in vivo*, Jurkat cells (10^8) were stimulated with TNFα (30 ng/ml) for different times and cells were extracted and immunoprecipitated with an anti-TNF-R1 antibody (Santa Cruz Biotechnology, Inc.) and the immunoprecipitates were analyzed by

immunoblotting with the anti-p62 antibody or anti-RIP antibody, as a control, as described above.

aPKC activity

Cultures of 293 cells were stimulated or not with 30 ng/ml of TNFα for different times. Cell extracts were prepared in PD buffer as described above, and immunoprecipitated with a monoclonal anti-λPKC antibody. Flag-tagged IKKβ immunocomplexes were isolated as above and washed in kinase buffer. The λPKC immunoprecipitates were incubated with immunopurified IKKβ and recombinant MBP–IκB$^{1-250}$ in kinase buffer (20 mM HEPES pH 7.7, 10 mM β-glycerophosphate, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 10 mM PNPP, 300 μM Na_3VO_4, 1 mM DTT, 10 μM ATP, 1 mM benzamidine, 2 M PMSF, 10 μg/ml of aprotinin, 1 μg/ml of leupeptin, 1 μg/ml of pepstatin and 2 μCi [γ-^{32}P]ATP) at 30°C for 30 min. The kinase reaction was stopped by addition of 5× SDS–PAGE sample buffer, subjected to SDS–PAGE analysis, and visualized in an InstantImager (Packard).

Reporter assays

For reporter gene assays, 293 cells were seeded into 6-well plates. Cells were transfected the following day by the calcium phosphate precipitation method with 10 ng κB–luciferase reporter gene plasmid, and various amounts of each expression construct. The total DNA transfected was kept constant by supplementation with the control vector pCDNA3. After 24 h, extracts were prepared and luciferase activity was determined as described previously (Diaz-Meco *et al*., 1996b).

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