p62 forms a ternary complex with PKCζ and PAR-4 and antagonizes PAR-4-induced PKCζ inhibition

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Abstract It has been reported that prostate apoptosis response-4 (PAR-4) binds to and inhibits protein kinase Cζ (PKCζ) which phosphorylates IκB kinase β (IKKβ) for nuclear factor κB (NFκB) activation, while p62 binds to and recruits PKCζ to the NFκB signaling complex. Thus, a mechanism to coordinate the two binding proteins for the regulation of PKCζ is expected to exist. The present data show that p62 and PAR-4 do not compete for PKCζ binding but directly interact each other and form a ternary complex with PKCζ. Furthermore, p62 not only enhances the catalytic activity of PKCζ but also reactivates catalytically inactive PAR-4-bound PKCζ. As the result, overexpression of p62 protects cells from PAR-4-mediated inactivation of NFκB and apoptotic death. Thus, the regulatory role of p62 for free and PAR-4-bound PKCζ is important in activation of NFκB. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: p62; Prostate apoptosis response-4; Protein kinase Cζ; Nuclear factor κB; Ternary complex; Apoptosis

1. Introduction

Protein kinase Cζ (PKCζ) and ι/λ are the two known members of atypical protein kinase C (aPKC) subfamily [1], and have been implicated in cell proliferation and survival likely through regulation of AP-1 and nuclear factor κB (NFκB) activation pathways respectively [2–4]. In the NFκB activation pathway, PKCζ likely functions as an IκB kinase β (IKKβ) kinase [5]. Due to subtle structural differences in the regulatory domain compared to those of classical and novel PKC isoforms, aPKCs can not be activated by Ca^{2+}, diacylglycerol, or phorbol esters [1], but seem to be regulated by other lipid messengers such as ceramide [6] and phosphatidylinositol 3,4,5-P_3 [7]. In addition, PKCζ is also subjected to modulation by protein regulators including prostate apoptosis response-4 (PAR-4) and p62 [8,9].

The *par*-4 gene has been identified as an inducible gene in neuronal and prostate cancer cells undergoing apoptosis [10]. Its implication in apoptotic cell death has been further supported by induced apoptosis of primary and immortalized cell lines following ectopic expression of PAR-4 [11,12]. Several lines of evidence suggest that the primary role of PAR-4 in apoptotic process is to suppress the NFκB activation pathway by binding to and inhibiting PKCζ [8,13]. Usage of a zinc finger in the regulatory domain of PKCζ for its interaction with PAR-4 [8] indicates that the interaction induces a conformational change and results in inhibition of the catalytic activity of PKCζ.

On the other hand, another PKCζ binding protein p62 has been suggested to play a critical role in PKCζ-mediated NFκB activation in a way that p62 recruits PKCζ to the signaling complex upon ligation of tumor necrosis factor-α (TNF-α), interleukin-1, or nerve growth factor [15–17] without affecting its catalytic activity [14]. For instance, ligation of TNF receptor activates the interaction of p62 with receptor interacting protein (RIP), thus bringing PKCζ to the TNF receptor signaling complex where PKCζ phosphorylates IKKβ [5]. Interestingly, the p62 gene can be induced by various extracellular signals activating NFκB including treatment of cells with PMA, serum, PDGF or oxidative stress [18,19].

All these results suggest the significance of the regulation of PKCζ in balancing the cell death and survival. As both PAR-4 and p62 bind to PKCζ and are products of inducible genes under opposite states of the cell, dying and surviving respectively, qualitative and quantitative control mechanisms for the reciprocal regulation of PKCζ by PAR-4 and p62 are to be expected. In the present study, we have analyzed the regulation of PKCζ in regard to interactions with both PAR-4 and p62.

2. Materials and methods

2.1. cDNA and constructs

cDNAs of PAR-4 and PKCζ were cloned from HeLa cDNA library (Clontech) by PCR. cDNAs were subcloned into pcDNA3.1-Myc/His (Invitrogen) for mammalian cell expression, pEGFPN-1 (Clontech) for green fluorescent protein-tagged expression, pGEX-4T1 (Pharmacia) for glutathione-*S*-transferase (GST) fusion protein expression and pMALp2 (NEB) for maltose binding protein (MBP) fusion protein expression. Truncation mutants of PAR-4 and p62 were generated by PCR using appropriate primers, subcloned into proper expression vectors and used.

2.2. Cell culture and immunoprecipitation

HeLa and U2OS cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). For immunoprecipitation, HeLa cells were lysed in the lysis buffer (50 mM HEPES (pH 7.5), 100 mM NaCl, 20 mM NaF, 1 mM DTT, 1% Triton X-100, 5 mM EDTA, protease inhibitor cocktail (Calbiochem))

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Abbreviations: RIP, receptor interacting protein; TNF, tumor necrosis factor; TRAF, tumor necrosis factor receptor associated factor; PKC, protein kinase C; PAR-4, prostate apoptosis response-4; IKKβ, IκB kinase β; NFκB, nuclear factor κB

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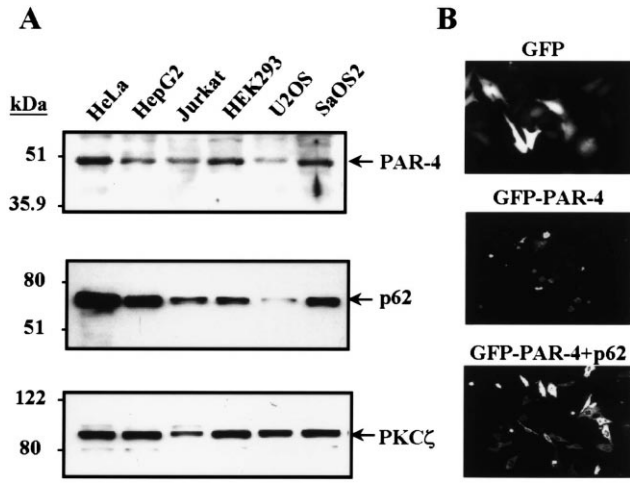

Fig. 1. p62 antagonizes the effect of PAR-4 in apoptotic cell death. A: Expression of PAR-4, p62 and PKCζ in various cell lines. Total lysates of six cell lines were prepared (Section 2), and 50 μg of extracts were separated on a 10% SDS–PAGE and analyzed by Western blot using corresponding antibodies. B: U2OS cells were transfected with pEGFP alone, pEGFP–PAR-4, or pEGFP-PAR-4 and pcDNA1-p62 together. Cells were observed under fluorescence microscope at 48 h post-transfection.

on ice for 10 min. After removing insoluble materials by centrifugation at 4°C, 2 μg each of monoclonal antibodies against PAR-4 (Santa Cruz) or p62 (Transduction Laboratory) was added to the lysate and incubated for 2 h at 4°C. Immune complex was then precipitated by 10 μl of Protein G-agarose (Roche), washed three times with lysis buffer, boiled in SDS sample buffer, and analyzed on the SDS–PAGE followed by Western blot.

2.3. Transfection, NFκB reporter assay, FACS analysis, and microscopic analysis

For NFκB reporter assay, cells in 24-well plates were transfected with 0.4 μg of DNA using lipofectamine-2000 (Gibco-BRL). Fresh DMEM supplemented with 10% FBS was added at 3 h post-transfection. 24 h later, transfectants were treated with TNF-α (20 ng/ml; Calbiochem) for 6 h, and luciferase activity was determined using dual-luciferase assay kit (Promega) according to manufacturer's instructions. For microscopic analysis, cells were transfected with green fluorescent protein-tagged PAR-4 (GFP-PAR-4) and p62 constructs and observed under fluorescence microscope (OLYMPUS IX-70) at 48 h post-transfection. In order to examine the cell death rate, 10^5 transfected cells were harvested, washed twice with PBS, and suspended in 100 μl of Annexin V binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). 5 μl of PE-Annexin V (BD Pharmingen) was then added to the cell suspension and incubated for 15 min at room temperature. After addition of 400 μl of Annexin V binding buffer, cells labeled with Annexin V were analyzed using FACS Calibur (BD Bioscience) with CellQuest software.

2.4. In vitro translation and GST/MBP pull-down assay

In vitro translation was performed using an in vitro quick transcription/translation kit (Promega) in the presence of 15 μCi of [^{35}S]methionine. cDNAs in pcDNA3.1 were used as template. Recombinant glutathione-*S*-transferase-fused PAR-4 (GST–PAR-4) and MBP–p62 were expressed in *Escherichia coli* BL21(DE3) and purified using Glutathione Sepharose-4B (Pharmacia) and amylose resin (NEB), respectively, following manufacturer's instructions. The in vitro translated products in lysis buffer were pulled down by 1 μg of GST–PAR-4 or MBP–p62 bound to glutathione beads or amylose beads, respectively. After a 2-h incubation, proteins bound to the beads were washed three times with lysis buffer, boiled in 20 μl of SDS sample buffer, analyzed on the SDS–PAGE, followed by fluorography.

2.5. In vitro kinase assay

Recombinant PKCζ (Calbiochem) and purified GST–PAR-4 and MBP–p62 were used for in vitro kinase assay. Myelin basic protein (MyBP, Sigma), 10 μCi [γ-^{32}P]ATP (30 Ci/mmol specific activity, Amersham PB10132) and phosphatidyl-serine (50 μg/ml, sigma) were used as substrates. The three recombinant proteins and substrates were incubated in the kinase buffer (20 mM HEPES, pH 7.5, 10 mM β-glycerophosphate, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 10 mM PNPP, 1 mM DTT, 300 μM Na_3VO_4 and protease inhibitor cocktail) for 30 min at room temperature. GST and MBP proteins were used as controls to normalize protein concentration in the reaction mixture. The reaction was stopped by adding 5× protein sample buffer and analyzed on the SDS–PAGE and autoradiography.

3. Results

3.1. p62 antagonizes PAR-4-induced apoptotic cell death

Both PAR-4 and p62 are constitutively expressed in most established cell lines thus far examined (Fig. 1A) despite the fact that both proteins are the products of inducible genes [10,18,19]. Furthermore, over-expression of GFP-PAR-4 but not GFP alone induces apoptotic death of U2OS cells that could be counteracted by co-expression of p62 (Fig. 1B). These results suggest the presence of a balancing system for cell death and survival controlled by PAR-4 and p62. This could be through reciprocal regulation of their shared binding target PKCζ.

3.2. p62 and PAR-4 do not compete for PKCζ but form a ternary complex with PKCζ

The counteractive role of p62 against the pro-apoptotic activity of PAR-4 (Fig. 1B) could be a result of direct competition for PKCζ binding. Interestingly, however, both PAR-4 and PKCζ are found in the immune complex precipitated by anti-p62 antibody (Fig. 2A). Reciprocally, both PKCζ and p62 are present in the complex brought down by using anti-PAR-4 (Fig. 2B), and PAR-4 and p62 in the complex brought down by using anti-PKCζ (data not shown). This suggests a ternary complex formation. Interestingly, recombinant GST–PAR-4 but not GST alone pulls down the in vitro translated

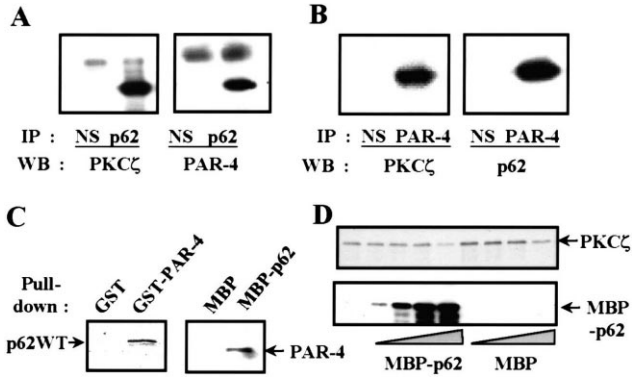

Fig. 2. Ternary complex formation of PKCζ, PAR-4 and p62. A,B: HeLa cell lysate was immunoprecipitated (IP) either with anti-p62 (A) or with anti-PAR-4 (B) antibody, and coprecipitated proteins were analyzed by Western blot (WB) as described in Section 2. Precipitation with non-immune serum (NS) has been used to determine the specificity of antibodies. C: In vitro translated PAR-4 and p62 were incubated in the lysis buffer with MBP–p62 and GST–PAR-4 respectively. After washing, bound proteins were separated on the SDS–PAGE and visualized by fluorography. D: In vitro translated PKCζ was incubated with 1 μg of GST–PAR-4 bound to glutathione beads in the presence of increasing amounts of MBP–p62 (0.5, 1.0, 2.0 and 5.0 μg) or MBP (1.0, 2.0 and 5.0 μg). Bound PKCζ and p62 were separated on the SDS–PAGE and visualized by fluorography for PKCζ and Western blot analysis for MBP–p62.

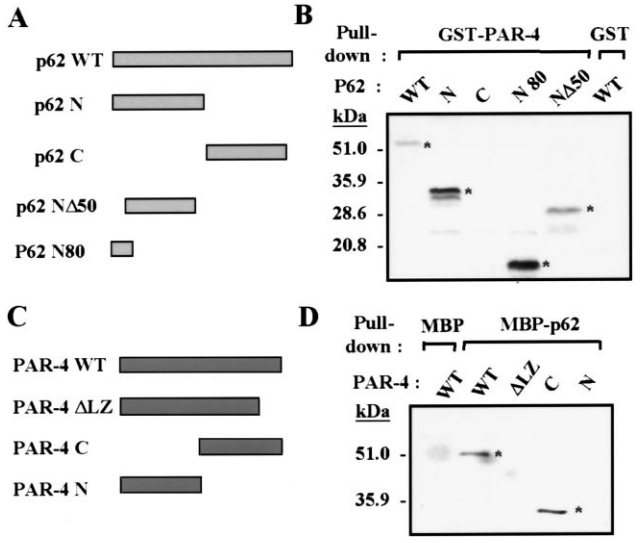

Fig. 3. Leucine zipper domain in PAR-4 and a small region in N-terminus of p62 is critical for interaction. A,C: Schematic diagrams of truncation mutants of p62 (A) and PAR-4 (C) used in domain mapping study. B,D: In vitro translated p62 (B) and PAR-4 (D) proteins were pulled down using GST–PAR-4 and MBP–p62, respectively. Inputs of the in vitro translated p62 and PAR-4 were adjusted to a similar level for each lane. After washing, bound proteins were separated on the SDS–PAGE and visualized by fluorography. Specifically precipitated bands are marked with asterisks. p62 N, amino acids 1–256; p62 C, amino acids 257–439; p62 NΔ50, amino acids 51–256; p62 N80, amino acids 1–80; PAR-4 ΔLZ, amino acids 1–303; PAR-4 C, amino acids 134–342; PAR-4 N, amino acids 1–133.

p62, while MBP–p62, but not MBP alone, precipitates the in vitro translated PAR-4 (Fig. 2C). Furthermore, 1 μg of GST–PAR-4 efficiently pulls down the in vitro translated p62 and PKCζ, but addition of increasing amount of MBP–p62 up to 5 μg does not affect the PAR-4–PKCζ interaction (Fig. 2D). Thus, p62 and PAR-4 do not compete for binding to PKCζ. Rather, p62 and PAR-4 form a ternary complex with PKCζ through direct interactions of all three components. In addition, mapping study shows that a short segment in the N-terminal region (amino acids 50–80) of p62 and the leucine zipper region in the C-terminus of PAR-4 are essential for their interaction (Fig. 3). Thus, the C-terminal 183 amino acids of p62 containing ubiquitin binding site [20] is dispensable for PKCζ binding.

3.3. p62 and PAR-4 have opposite effects on the catalytic activity of PKCζ

Next we analyzed the effect of p62 binding on regulation of the catalytic activity of PKCζ (Fig. 4). Interestingly, recombinant PKCζ shows constitutive kinase activity for MyBP (Fig. 4A, lane 1). This was enhanced by p62 in some extent reproducibly (Fig. 4A, lane 2). On the other hand, PAR-4 inhibited this activity more than three folds, that is consistent with previous reports (Fig. 4A, lane 3 and ref. [8]). Moreover, the inhibited PKCζ catalytic activity by PAR-4 was fully restored, in a dose-dependent manner, by addition of p62 (Fig. 4A, lanes 4 and 5 and Fig. 4B). However, increasing amount of PAR-4 did not inhibit the catalytic activity of PKCζ, which was pre-incubated with p62 (Fig. 4C). These results suggest that p62 indeed modulates the inhibitory activity of PAR-4 for PKCζ and restores the catalytic activity of PAR-4-bound PKCζ by forming a ternary complex.

3.4. p62 restores the PAR-4-mediated suppressed NFκB activity and protects cells from apoptotic death

PKCζ has been recognized as an IKKβ kinase in the TNF-α signaling pathway [5]. Thus, modulation of the catalytic activity of PKCζ would affect TNF-α-induced NFκB activation and thereby the survival of the cell. Indeed, ectopic expression of PAR-4 suppresses TNF-α-induced NFκB activation by approximately four folds (Fig. 5A). Co-expression of wild type or N-terminal 256 amino acids (p62N256) but not C-terminal 183 amino acids (p62C183) of p62 restored the NFκB activity (Fig. 5A). Furthermore, U2OS cells expressing PAR-4 undergoes apoptosis, while cells cotransfected with p62 or p62N256 but not p62C183 were relatively resistant to PAR-4-induced apoptotic cell death (Fig. 5B). These results

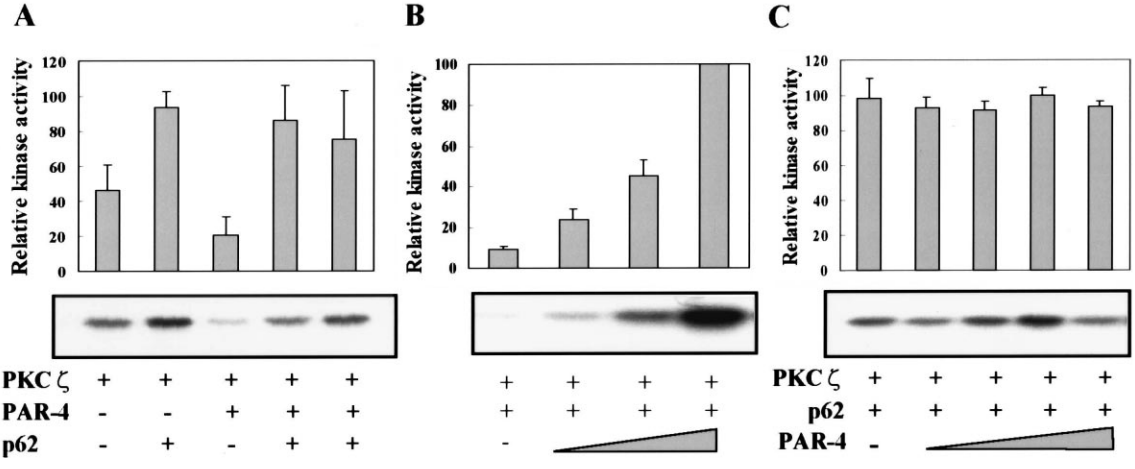

Fig. 4. p62 regulates the catalytic activity of free and PAR-4-bound PKCζ. A: The catalytic activity of recombinant PKCζ (200 ng) in the presence of 200 ng each of PAR-4 and/or p62 was measured as described in Section 2. B,C: PKCζ (200 ng) kinase reaction was performed in the presence of 200 ng of GST–PAR-4 and increasing amounts of MBP–p62 (0, 50, 200 and 400 ng) (B) or in the presence of 200 ng of MBP–p62 and increasing amounts of GST–PAR-4 (0, 50, 100, 200 and 400 ng) (C) for 30 min at room temperature as described in Section 2. Relative radioactivity was measured by densitometry using LAS-1000 (Fuji) and presented as relative kinase activity (the mean ± S.D. of three experiments A: 16.3%, B: 6.0%, C: 4.6%) in bar graphs. Each autoradiogram is the representative of three independent experiments.

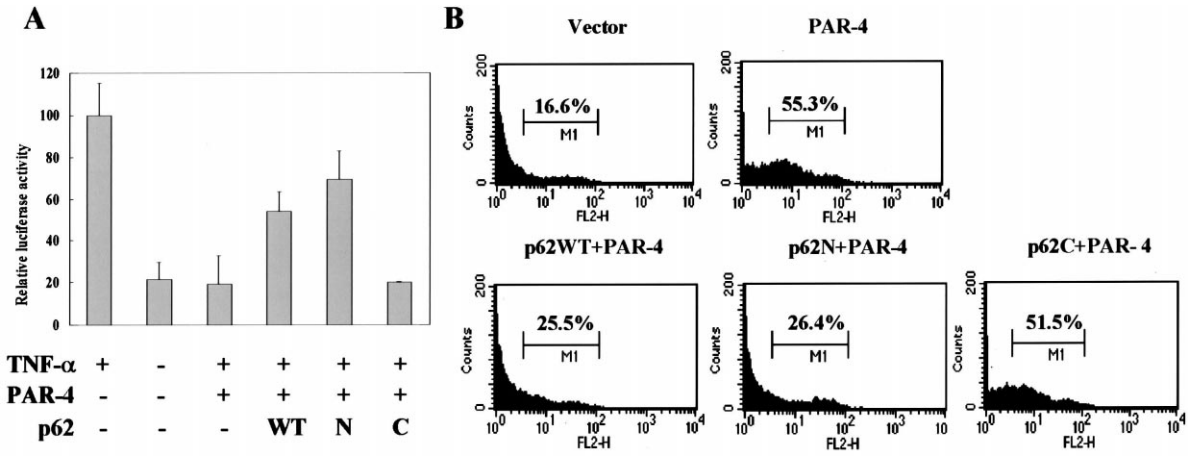

Fig. 5. p62 reverses the PAR-4-mediated NFκB inhibition and cell death. A: Effects of wild type (WT), the N-terminal 256 amino acids (p62N256, N) and the C-terminal 183 amino acids (p62C183, C) of p62 on the TNF-α-induced NFκB activation were compared. NFκB reporter activity of U2OS cells transfected with cDNAs encoding PAR-4 and/or various p62 mutant proteins was measured after treatment of cells with TNF-α (20 ng/ml) for 6 h as described in Section 2. Each data point is a mean of triplicated samples, and the figure is the representative of five independent experiments. B: U2OS cells transfected with cDNAs encoding vector alone or PAR-4 and various p62 proteins (wild type (WT), p62N256 (N) or p62C183 (C)) were analyzed for their apoptotic death by measuring Annexin V-labeled cells following the procedures described in Section 2. Percentage of Annexin V positive cells is marked in each histogram.

are consistent with the facts that binding sites for both PKCζ and PAR-4 are localized in the N-terminal region of p62 (Fig. 3 and ref. [9]) and that p62N256 but not p62C183 reactivates the catalytic activity of PAR-4-bound PKCζ in a manner similar to full length p62 (data not shown). Thus, binding of the N-terminal region of p62 to the PKCζ–PAR-4 complex is sufficient to restore the PKCζ catalytic activity and the pathway for NFκB activation that prevents cells from apoptotic death.

4. Discussion

PAR-4 and p62 are two known PKCζ binding proteins [8,9]. In the previous reports, it was suggested that p62 acts as an adapter that facilitates NFκB activation by linking PKCζ to RIP or tumor necrosis factor receptor associated factor-6 (TRAF-6) [16,17] without affecting the catalytic activity of PKCζ [14]. Furthermore, it was shown that PAR-4 blocks NFκB activation by inhibiting PKCζ activity [8]. Results presented in this study basically support the previous findings; PAR-4 inhibits PKCζ (Fig. 4), suppresses TNF-α-induced NFκB activation, and induces apoptotic cell death (Fig. 5). However, our results suggest that p62 also plays critical roles in the regulation of PKCζ activity in addition to the previously observed adapter function.

Interestingly, in four independent reconstitution experiments using purified recombinant proteins, p62 binding consistently enhanced the catalytic activity of PKCζ up to 50% (Fig. 4A). Furthermore, PAR-4 and p62 did not compete for PKCζ binding. Instead, they interacted directly with each other and also simultaneously bound to PKCζ (Fig. 2). More interestingly, p62 restored the catalytic activity of PAR-4-bound PKCζ by forming a ternary complex (Figs. 2 and 4). Thus, direct regulation of PKCζ and antagonistic function in regard to PAR-4 would represent the roles of p62, at least partly, in activation of NFκB.

However, this model still cannot explain the death of p62-expressing cells caused by an increase of cellular PAR-4 level (Figs. 1 and 5, and ref.[10]). The experiments showed that PAR-4 did not compete with p62 for PKCζ binding (Fig. 2) and that p62-bound PKCζ was active even in the ternary complex with PAR-4 (Fig. 4B,C). Thus, if the adapter function of p62 is an absolute requirement for the PKCζ-mediated NFκB activation, the increased cellular level of PAR-4 would not be expected to affect the NFκB signaling pathway. However, increased PAR-4 level clearly blocked the NFκB activation and induced the apoptotic cell death (Fig. 5). It is thus possible that, although p62 binds to both PKCζ and RIP or TRAF-6 [16,17], the adapter function may not be sufficient in NFκB signaling. Rather, cellular population of catalytically active PKCζ, free and p62 occupied forms, may be important in the signaling where the free PKCζ will be the target of PAR-4-mediated inhibition.

The expression of PAR-4 sensitizes most cell lines to apoptosis by Ca^{2+}, low serum, or TNF-α [8,21,22]. It has also been shown that ectopic expression of PAR-4 alone caused apoptotic death of prostate cancer cells and tumors [23]. Interestingly, U2OS cells underwent spontaneous apoptotic death by ectopic expression of PAR-4 alone without any changes in culture condition (Figs. 1B and 5B). On the other hand, the expression of PAR-4 alone induced apoptosis of HeLa, HEK293, and SaOS2 cells only moderately (data not shown). Particularly low p62 expression level in U2OS cells (Fig. 1A) may have implication for this difference. Sensitization but not direct apoptosis of most cell lines by the PAR-4 expression could be, at least partly, due to the basal activity of Erk which is another regulatory target of PKCζ [24]. As only p62-bound PKCζ is catalytically active when PAR-4 is overexpressed, U2OS cells would have limited PKCζ activity that may be insufficient to maintain the basal Erk activity and to keep the cells surviving. Nevertheless, further analysis of the role of p62 will provide better understanding in PAR-4-mediated apoptosis and sensitization of the cell.

The antagonism between p62 and PAR-4 in respect to PKCζ regulation could be explained by the topology of the binding between the three proteins. The N-terminal amino acids 50–80 and the leucine zipper region of p62 and PAR-4, respectively, are essential for the interaction (Fig. 4). Ap-

parently weaker binding of p62NΔ50 than that of p62N80 to PAR-4 indicates that the further N-terminal region of p62 is important for the efficient binding to PAR-4. The amino acids 41–105 of the rat homolog of p62 and the leucine zipper region of PAR-4 have been shown to be responsible for their PKCζ bindings [8,9]. Furthermore, the zinc finger-like cysteine rich region in the regulatory domain of PKCζ is the binding site for both p62 and PAR-4 [8,9]. The close proximity of binding regions in each component of the ternary complex strongly suggests that there will be distinct changes in conformation of PKCζ by binding of PAR-4, p62, or PAR-4 and p62 together. Nevertheless, although there would be more cellular targets of PAR-4 and p62, further analysis of PKCζ regulation in the context of the ternary complex will provide better understandings in the signaling pathway for NFκB activation and in the cell death and survival.

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