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Cell Signaling and Function Organized by PB1 Domain Interactions

Review

Jorge Moscat,[1,2,4,*] Maria T. Diaz-Meco,[1,2,4] Armando Albert,[3] and Sonsoles Campuzano[1]
[1] Centro de Biología Molecular Severo Ochoa
Consejo Superior de Investigaciones Científicas
Universidad Autónoma, Cantoblanco
28049 Madrid
Spain
[2] Department of Genome Science
Genome Research Institute
University of Cincinnati
2180 East Galbraith Road
Cincinnati, Ohio 45237
[3] Grupo de Cristalografía Macromolecular y Biología Estructural
Instituto de Química-Física Rocasolano
CSIC, Serrano 119
28006 Madrid
Spain

The PB1-domain-containing proteins p62, aPKC, MEKK2/MEKK3, MEK5, and Par-6 play roles in critical cell processes like osteoclastogenesis, angiogenesis, and early cardiovascular development or cell polarity. PB1 domains are scaffold modules that adopt the topology of ubiquitin-like β-grasp folds that interact with each other in a front-to-back mode to arrange heterodimers or homo-oligomers. The different PB1 domain adaptors provide specificity for PB1 kinases to ensure the effective transmission of cellular signals. Also, recent data suggest that PB1 domains may serve to orchestrate signaling cascades not involving other PB1 domains, such as the MEK5-ERK5 and p62-ERK1 interactions.

The PB1s are dimerization/oligomerization domains present in adaptor and scaffold proteins as well as kinases and serve to organize platforms that ensure specificity and fidelity during cellular signaling (Figure 1; http://smart.embl-heidelberg.de/smart/show_many_proteins.pl). A series of very recent studies has provided valuable information on the structural details that govern binding between the different PB1 modules (Hirano et al., 2004; Ito et al., 2001; Leitner et al., 2005; Noda et al., 2003; Terasawa et al., 2001; Wilson et al., 2003; Yoshinaga et al., 2003) and explains how they direct the formation of different macromolecular signaling complexes. Crystallographic and NMR experiments have established the 3D structure of a number of PB1 domains (Hirano et al., 2004, 2005; Muller et al., 2006; Terasawa et al., 2001; Wilson et al., 2003; Yoshinaga et al., 2003). All of them display the topology of a ubiquitin-like β-grasp fold, including six-stranded β-sheets and two α helices (Figure 2A). These structural studies also show that some of them include the OPCA motif, a short sequence containing a cluster of acidic amino acids initially termed AID or octicosapeptide (Ponting et al., 2002), which folds along the hairpin formed by the β strands B4 and B5 and the α helix H2. Other PB1 domains cluster several positively charged residues on the topologically opposite side of the location of the OPCA motif. Finally, another group displays both the OPCA and the positively charged residues.

The interaction between two PB1s will differ depending on whether they have one OPCA, the basic cluster, or both, giving rise to different combinations of homo- and heterodimers. For example, the molecular architecture of two important PB1-PB1 heterodimers has recently been addressed by solving the X-ray structures of the P40phox-p67phox and the PKCλ/ι-Par-6 PB1 domain complexes (Hirano et al., 2005; Wilson et al., 2003). The data show that these interactions involve specific electrostatic contacts between the acidic residues of the OPCA motif located in one side of a given PB1 and the few conserved basic residues of the other PB1 in a front-to-back fashion (Figure 2B). This leads to the classification of the PB1 domains according to their ability to interact with other PB1 domains through the acidic side (A-type), through the basic side (B-type), or through both sides (A-B type). Two clusters of acidic residues of the OPCA motif are buried on the interaction surface of the A-type PB1 domain: the acidic cluster 1 (Ac1), consisting of residues of the loop between β3 and β4; and the acidic cluster 2 (Ac2) that includes residues at the N terminus of the A2 α helix. The B-type PB1 interaction surface involves residues forming the two basic clusters Bc1 and Bc2 that belong to distant secondary structural elements (Figure 2C). The comparative description of the P40phox-p67phox and PKCλ/ι-Par-6 PB1 complexes shows that the formation of salt bridges between Ac1-Bc1 and Ac2-Bc2 residues is the only general structural property governing PB1-PB1 interactions (Hirano et al., 2005). Biochemical and mutagenesis studies have shown that binding between the conserved lysine residue on the B1 β strand included in Bc1, and the acidic residues of the OPCA loop is essential for the oligomerization of PB1 domains (Noda et al., 2003; Wilson et al., 2003; Yoshinaga et al., 2003). The atomic superimposition of the PB1 structures (Sutcliffe et al., 1987) shows that their backbones overlap more than 70 Cα atoms and that the main differences are located in residues that are not involved in the oligomerization, particularly in the area covered by the β strands B2 and B3, and the α helix H2 of the B-type PB1domains. On the other hand, the 3D structure-based alignment of key PB1 domains shows that the residues involved in the interaction between the PB1 domains of aPKC and Par-6 are conserved in the A-type or B-type PB1 domains, respectively. As no other obvious sequence similarities are observed among PB1 domains, it is difficult to establish the structural determinants of the specificity that directs PB1-PB1 interactions. However, since PB1 domains conserve the essential residues that provide the scaffold for their interactions, it is possible to generate templates to build homology models for PB1

*Correspondence: moscatje@ucmail.uc.edu
[4] Present address: Department of Genome Science, Genome Research Institute, University of Cincinnati, 2180 East Galbraith Road, Cincinnati, Ohio 45237.

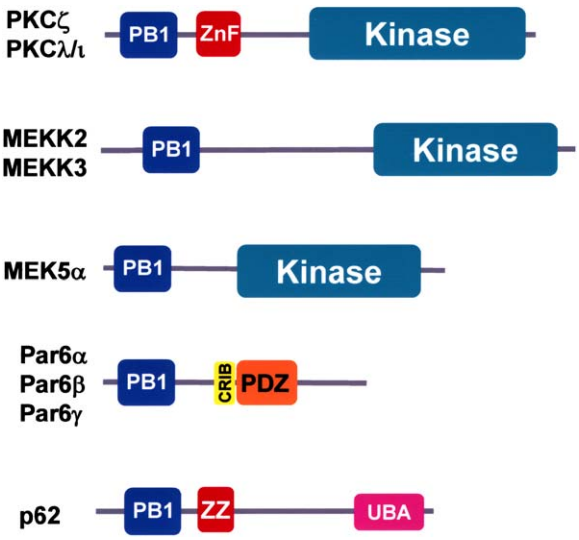

Figure 1. Distinct Modules Regulate the Interactions between Different PB1 Signaling Proteins and Their Targets

ZnF, zinc-finger; CRIB, Cdc42/Rac interactive binding; PDZ, PSD-95/DISCS LARGE/ZO-1; ZZ, atypical ZnF; UBA, ubiquitin associated.

domains of unknown structure, and to predict the structure of unknown heterodimers.

The p62/aPKC Signaling Platform

Two-hybrid screens in yeast, using as bait the first 126 amino acids of PKCλ/ι (which includes its PB1 domain), identified p62 (also known as sequestosome-1) as a partner of this kinase (Moscat and Diaz-Meco, 2000). p62 specifically binds PKCλ/ι and PKCζ and harbors a number of domains that support its role as scaffold in aPKC signaling (Moscat and Diaz-Meco, 2000) (Figure 1). The first clue about the function and mechanism of action of p62 came from the identification of receptor-interacting protein-1 (RIP1) as a p62-interacting protein (Moscat and Diaz-Meco, 2000). RIP1 is a death domain (DD)-containing kinase that interacts, through homotypic interactions, with the DD-containing adaptor TRADD, which also bridges RIP1 to the TNFα receptor-1 (TNFR1) through the interactions of their respective DDs (Chen and Goeddel, 2002). Activated TNFR1 assembles a prosurvival signaling complex involving TRADD, RIP, and the NF-κB and JNK activators TRAF2 and TRAF5 (Chen and Goeddel, 2002) (Figure 3A). Genetic evidence demonstrates that the loss of RIP1, as well as the simultaneous genetic inactivation of TRAF2 and TRAF5, dramatically impairs NF-κB (Meylan and Tschopp, 2005). RIP1 kinase activity is not required for NF-κB activation, but its intermediary region is essential for its TRAF2 binding function that helps to recruit the IKK complex via interaction with its IKKγ regulatory subunit (Chen and Goeddel, 2002; Meylan and Tschopp, 2005) (Figure 3A). Interestingly, p62 also binds the intermediary domain of RIP1 through its ZZ region, further suggesting that p62 is a relevant player in RIP1 function (Moscat and Diaz-Meco, 2000). Consistent with this hypothesis, the knockdown of p62 with an antisense construct severely impairs NF-κB activation in response to TNFα. Biochemical in vitro studies showed that recombinant pure aPKCs were able to phosphorylate the activation-loop residues of IKKβ, but not of IKKα (Moscat and Diaz-Meco, 2000), offering a mechanistic explanation for the potential involvement of the p62/aPKC complex in RIP1-mediated NF-κB activation.

Similar to the TNFR1/TRADD/RIP1/TRAF2 complex, IL-1 activates a module including the IL-1 receptor proximal adaptor MyD88, the kinases IRAK1 and IRAK4, and TRAF6 (Chen et al., 2006) (Figure 3B). The interaction of IRAK1 with TRAF6, once TRAF6 has been phosphorylated by IRAK4 and dislodged from MyD88, serves to assemble a complex including the kinase TAK1 and the scaffold proteins TAB1, TAB2, and TAB3 (Chen et al., 2006). The role of TRAF6 in this pathway has been proven genetically, and p62 has been identified as a relevant TRAF6-interacting partner in IL-1-activated cells (Moscat and Diaz-Meco, 2000). Thus, p62 emerges as a component of both the TNFR1 and IL-1 receptor signaling complex due to its ability to interact with different adaptors of the two complexes (Figure 3B). The p62-TRAF6 interaction involves the TRAF domain of TRAF6 and a short stretch of amino acids in the p62 molecule that conforms to the TRAF binding consensus sequence (Moscat et al., 2006). The role of the p62/aPKC cassette in NF-κB activation is remarkably conserved in *Drosophila*. Thus, the p62 homolog Ref(2)P has a strikingly similar domain organization to that of p62 (Moscat et al., 2006) and has been shown to be required along with DaPKC for the activation of the *Drosophila* homolog of NF-κB in RNAi depletion experiments, both in cell culture and in vivo (Moscat et al., 2006). Two additional isoforms have been described and are named ZIP2 and ZIP3 (Croci et al., 2003; Gong et al., 1999). ZIP2 lacks the TRAF6-interacting sequence, whereas ZIP3 lacks part of the C-terminal region. As both sequences are important for signaling by p62, the expression of these isoforms may have important impacts in p62-mediated signaling. TRAF6 and RIP1 also orchestrate the activation of the MAPK cascades, including those for JNK and p38 (Chen and Goeddel, 2002; Meylan and Tschopp, 2005). However, p62 does not appear to play a role in those signaling cascades (Sanz et al., 1999, 2000).

Genetic and biochemical data demonstrate that TRAF6 is also involved in the RANK pathway (Lomaga et al., 1999), another signaling system that controls, among other functions, the physiology of bone. Cells activated with RANK ligand (RANK-L) trigger RANK-associated TRAF6 molecules to activate NF-κB and JNK, but, in this case, without the intervention of MyD88 or IRAK (Karsenty and Wagner, 2002). Mice deficient in p62 are not osteopetrotic like the TRAF6 or RANK KO mice, but they do show impaired osteoclastogenesis following stimulation by injection of the calciotropic hormone PTHrP. This indicates that p62 is required for efficient osteoclastogenesis under activated or stress conditions (Duran et al., 2004b) (Figure 3B). In vitro studies demonstrate that p62 levels dramatically increase when precursor cells are induced to differentiate into osteoclasts. Also, the loss of p62 severely impairs the sustained phase of NF-κB activation in RANK-stimulated osteoclasts.

The mechanism whereby the p62/aPKC cassette regulates NF-κB by RIP1 and TRAF6 complexes, as well as its relative contribution to this pathway when compared

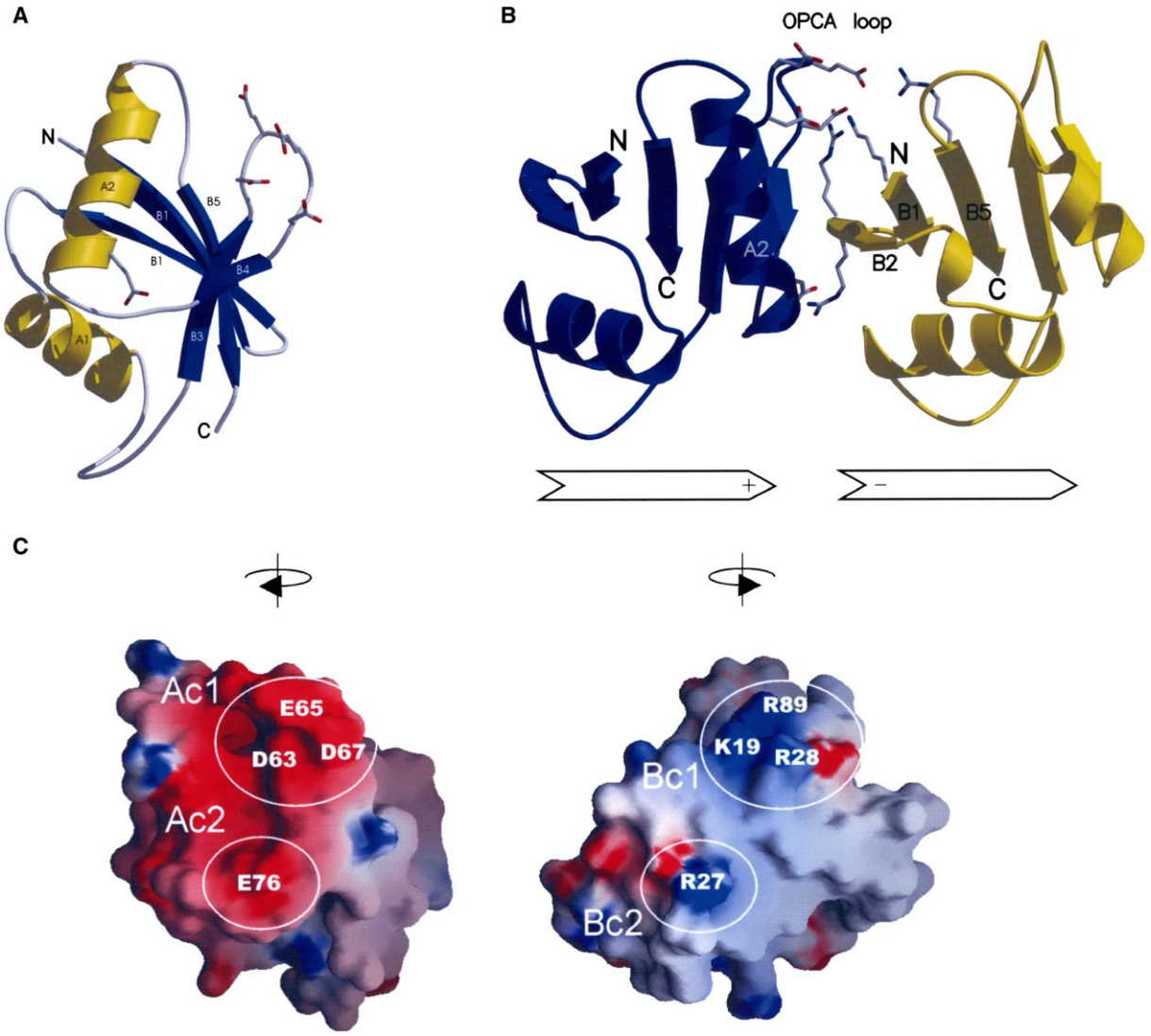

Figure 2. Molecular Structure of the PB1 Domains

(A) Ribbon representation of the structure of the PB1 domain of PKCι (PDB code 1WMH). The conserved acidic residues of the OPCA motif are shown in stick representation.

(B) Schematic representation of the PKCι/Par-6 PB1 domain heterodimer (PDB code 1WMH). The PKCι and Par-6 PB1 domains are depicted in blue and yellow, respectively. The residues responsible for the formation of the acidic and basic clusters are shown in stick representation. A schematic diagram of the front-to-back interaction between PB1 domains is also depicted.

(C) The electrostatic potential on the interaction surface of the PKCι (left) and Par-6 (right) PB1 domains (red, negative; blue, positive) are shown. Each domain is rotated approximately 90 degrees with respect to the view in B in an open book style. The molecular surface is representative of the A-type (PKCι) and B-type (Par-6) domains. The conserved residues involved in the formation of the acidic and basic clusters (Ac and Bc) are labeled and encircled. Figures were produced according to Kraulis (1991) and Merritt and Murphy (1994).

to that of the TAB2/TAK1 module, is a very interesting question. Whereas $p62^{-/-}$ mice display a clear defect in RANK-induced NF-κB activation, $TAB1^{-/-}$ and $TAB2^{-/-}$ cells show normal IL-1 and TNFα-induced NF-κB (Sanjo et al., 2003; Shim et al., 2005). This finding raises the question of what is the adaptor of TAK1 in this pathway. A possible explanation is the presence of TAB3 that may functionally compensate for the loss of TAB2 in the KO mice cells. TAK1 has recently been shown in biochemical and genetic experiments to be required for NF-κB and IKK activation in IL-1- and TNFα-activated cells (Chen et al., 2006; Sato et al., 2005; Shim et al., 2005). The role of the aPKCs as potential IKK kinases in the p62-driven activation of NF-κB in osteoclasts is less clear due to the potential redundancies played by the existence of two highly related aPKCs. In consequence, although cellular and biochemical studies demonstrate that RANK-L triggers the formation of a TRAF6/p62/aPKC complex in osteoclasts (Duran et al., 2004b), $PKC\zeta^{-/-}$ mice do not show alterations in the osteoclastogenic response. This indicates that p62 actions are largely PKCζ-independent but might rely on PKCλ/ι (Duran et al., 2004b). Future studies using conditional $PKC\lambda/\iota^{-/-}$ mice should resolve this important issue.

It is possible that p62 contributes to the activation of NF-κB as part of the RIP1 and TRAF6 complex via a mechanism that is independent of the aPKCs. That is,

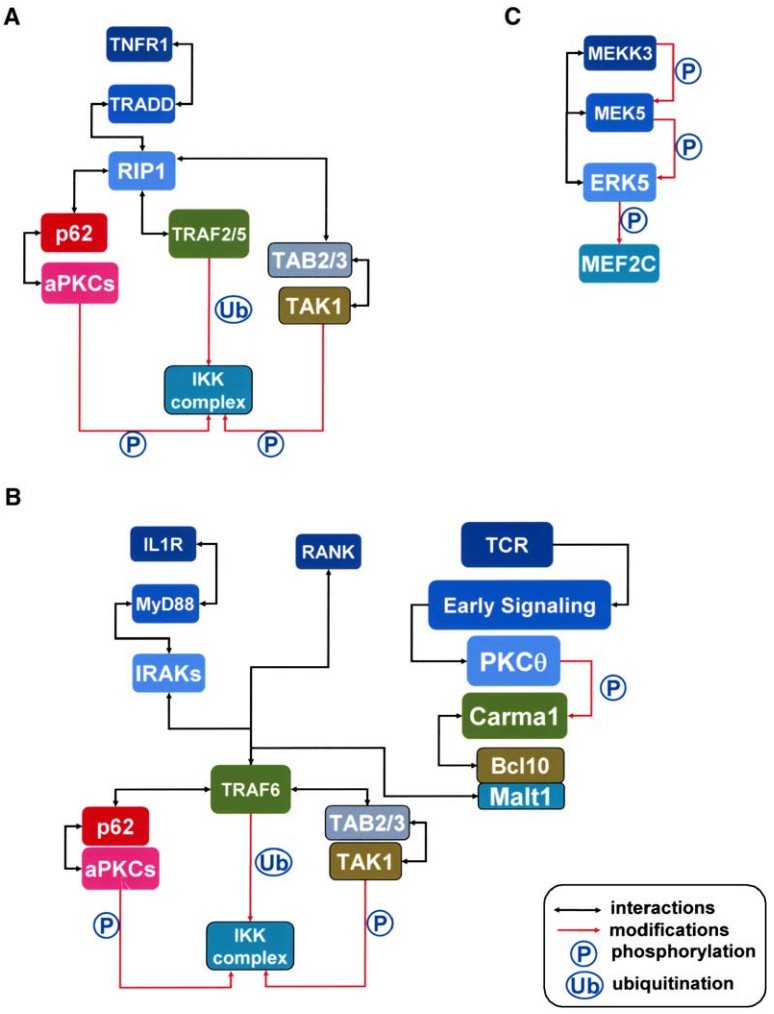

Figure 3. Different Protein Adaptors Regulate Signaling through PB1 Signaling Proteins

(A) Control of IKK activity by the TNFα pathway.

(B) Activation of IKK by the IL-1, RANK, and TCR signaling cascades.

(C) Regulation of MEF2C phosphorylation by the MEKK3 pathway.

the ability of the C-terminal UBA domain of p62 to bind polyubiquitin chains (Wooten et al., 2005), together with evidence that ubiquitination plays a positive role in NF-κB activation through the TRAF6 and RIP1 complexes (Chen et al., 2006), indicates that p62 may be involved in the regulation of the IKK complex via ubiquitin. This notion stems from a series of pioneering in vitro biochemical reconstitution experiments by Chen and coworkers. They identified TRAF6 as an E3 ubiquitin ligase that catalyzes the synthesis of polyubiquitin chains linked through lysine 63 (K63) (Chen et al., 2006; Gao and Karin, 2005) that, in contrast to the more classical K48-linked chains, do not target the polyubiquitinated protein to the proteasome but rather serve as a positive switch in the activation of IKK (Chen et al., 2006). The current model proposes that TAK1 would be activated by the autopolyubiquitination of TRAF6. TAB2 and/or TAB3 has been demonstrated in in vitro biochemical studies to be important in that process, probably through their ability to interact with K63 polyubiquitin chains by means of their well-conserved C-terminal zinc-finger domains (Chen et al., 2006). In the case of TNFα, RIP1 is polyubiquitinated but the E3 ubiquitin ligase is TRAF2 (Meylan and Tschopp, 2005). Another possible target of TRAF6/TRAF2-mediated K63 polyubiquitination is the IKKγ regulatory subunit of the IKK signalsome, which has been shown to be targeted by the T cell receptor adaptor Malt1 (Zhou et al., 2004), likely through TRAF6 (Sun et al., 2004) (Figure 3B). Therefore, a model can be proposed whereby TRAF6 oligomerization leads to its own K63 polyubiquitination as well as that of IKKγ, which is at least a permissive signal for IKK activation by an upstream kinase or by the oligomerization-driven autophosphorylation of the IKK signalsome.

With regard to p62, recent results strongly suggest that its recruitment to the TRAF6 signaling complex facilitates TRAF6 oligomerization and polyubiquitination (Wooten et al., 2005). Interestingly, for p62 to perform that function, in addition to an intact UBA domain and a TRAF6 binding sequence, it also requires the presence of its PB1 domain (Wooten et al., 2005). Whether this is because the aPKC associated to that region also regulates TRAF6 polyubiquitination or because the p62 PB1 plays a structural role in favoring p62-induced TRAF6 oligomerization is not yet clear, but, either way, it highlights the important role of PB1-containing adaptors and kinases in the orchestration of complex interactions during cell signaling in the NF-κB pathway.

Mutations in the p62 gene are the cause of Paget's disease, a genetic bone disorder characterized by aberrant osteoclastogenic activity (Laurin et al., 2002). All the disease-related mutations reported so far are localized

in the UBA domain of p62 (Laurin et al., 2002), which highlights the functional relevance of this region and the likely importance of ubiquitination in the mechanism of action of p62. Although the ability of p62 to participate in the ubiquitination part of the NF-κB activation pathways offers a possible mechanistic explanation for p62's proposed involvement in osteoclastogenesis and bone remodeling, it does not explain the role that the associated aPKC might play in the function of the p62/TRAF6 and p62/RIP complexes. Therefore, the implication of different kinases in the activation of IKK is still open to debate. Prior studies using cells from MEKK2 and MEKK3 KO mice revealed an important participation of these PB1-containing kinases in different phases of IKK activation (Huang et al., 2004; Schmidt et al., 2003).

The genetic evidence available on the role of the aPKCs in IKK and NF-κB activation adds another layer of complexity to the current models. That is, data from PKCζ$^{-/-}$ mice demonstrate that the loss of this kinase impairs the activation of IKK in the lung and liver of mice challenged by injection of *E. coli* lypopolisaccharide and concanavalin A, respectively (Duran et al., 2004a; Leitges et al., 2001). However, at a cellular level, at least in embryo fibroblasts and B cells, PKCζ is not necessary for IKK activation even though it controls the synthesis of κB-dependent genes (Leitges et al., 2001; Martin et al., 2002), most likely by direct phosphorylation of serine 311 (S311) in the RelA transactivating subunit of the NF-κB complex (Duran et al., 2003). This, together with the phosphorylation of S276 (Zhong et al., 1998), serves to recruit the transcriptional coactivator CBP, which allows the efficient transcription of κB-dependent genes (Duran et al., 2003). Since the loss of PKCζ does not impair osteoclastogenesis (Duran et al., 2004b) and since p62$^{-/-}$ osteoclasts display a delayed reduction in IKK activation after stimulation (Duran et al., 2004b), we are tempted to speculate that p62 becomes necessary for NF-κB activation only when it is induced during the intermediate/late steps of cell differentiation, possibly by forming a p62/PKCλ/ι complex. In contrast, PKCζ may use other adaptors to perform its function in cellular systems other than osteoclasts.

Since the requirement of PKCζ for the activation of IKK has only been observed so far in in vivo experiments in whole mice, it is possible that PKCζ is not really an IKK kinase but that it regulates, through its ability to phosphorylate RelA, the synthesis of cytokines that would activate IKK in a feed-forward loop autocrine and/or paracrine manner. In addition, recent data show that PKCζ is also involved in NF-κB-independent pathways. For example, in T cells, PKCζ acts upstream of the nonreceptor tyrosine kinase Jak1 in the IL-4 signaling pathway (Martin et al., 2005). It is unclear whether PKCζ uses its PB1 domain to impinge in this signaling cascade.

The Noninflammatory Functions of p62

When overexpressed, p62 localizes in protein aggregates that stain positively for ubiquitin in immunofluorescence experiments (Bjorkoy et al., 2005). However, under physiological conditions p62 staining results in a punctate pattern that colocalizes with endosomal markers (Geetha et al., 2005; Sanchez et al., 1998), indicating a role for p62 in growth factor receptor internalization. In fact, recent results demonstrate that depletion of p62 impairs the trafficking of TrkA into endocytic vesicles (Geetha et al., 2005) and strongly suggest that K63 polyubiquitination of TrkA, mediated by a p62/TRAF6 complex, controls both its signaling and trafficking. In this regard, previous data indicated that the overexpression of a dominant-negative mutant of PKCλ/ι impaired the translocation of the EGF receptor from late endosomes to lysosomes (Sanchez et al., 1998). Further experiments using p62$^{-/-}$ and PKCλ/ι$^{-/-}$ cells should help to clarify the role of the PB1 module, as well as the potential involvement of the aPKCs in the control of TrkA trafficking.

The PB1 domain may also serve to organize signaling modules that do not necessarily involve PB1-PB1 interactions. For example, ERK5, which lacks a PB1 domain, requires the intact PB1 region of MEK5 for interaction, which also interacts with the PB1 domain of MEKK2/3, orchestrating specific signaling from these kinases to ERK5 in a simple ternary complex (Nakamura et al., 2006; Seyfried et al., 2005). These results provide biochemical evidence for a noncanonical mechanism for protein interactions with certain PB1 domains. In this regard, p62 can bind ERK1 through its PB1 domain, which serves to inhibit, directly or indirectly, ERK activity, modulating adipogenesis and mature-onset obesity (Rodriguez et al., 2006). Consequently, the PB1 domain of p62 plays a dual role, acting positively in the aPKC pathway and inhibiting ERK. It is unclear whether the interaction with the aPKCs may affect p62's role as an inhibitor of ERK, or whether ubiquitination may also be involved in this novel p62 function.

The MEKK2 and MEKK3 PB1 Kinases

Although MEKK2 and MEKK3 interact with MEK5 via their respective PB1 domains (Nakamura et al., 2006), other experiments showed that the aPKCs can also bind MEK5, inducing its activation (Moscat et al., 2006). Interestingly, the aPKCs may act as scaffold proteins helping MEK5 to be activated, most likely by autophosphorylation, whereas in the case of MEKK2/MEKK3 their enzymatic activity is required. It should be noted that the genetic inactivation of MEKK3 in mice gives rise to an embryonic lethal phenotype characterized by defects in angiogenesis and early cardiovascular development (Yang et al., 2000), which is very similar to the phenotypes of mice in which MEK5 (Wang et al., 2005), ERK5 (Hayashi et al., 2004; Yan et al., 2003), or the transcription factor MEF2C (Lin et al., 1997) has been genetically inactivated. Together, these observations establish biochemically and genetically the signaling cascade MEKK3/MEK5/ERK5/MEF2C as physiologically relevant for angiogenesis, most likely at the endothelial cell level (Figure 3C). However, the phenotype of the MEKK2$^{-/-}$ mice does not seem to support its role in this pathway, and further studies are needed for a complete clarification of the potential role of MEKK2 in the immune system (Guo et al., 2002; Leitges et al., 2001). Likewise, one study claims that p62 interacts with MEK5 through their respective PB1 domains (Lamark et al., 2003). However, the phenotype of the p62$^{-/-}$ mice does not support the potential physiological relevance of this interaction (Duran et al., 2004b).

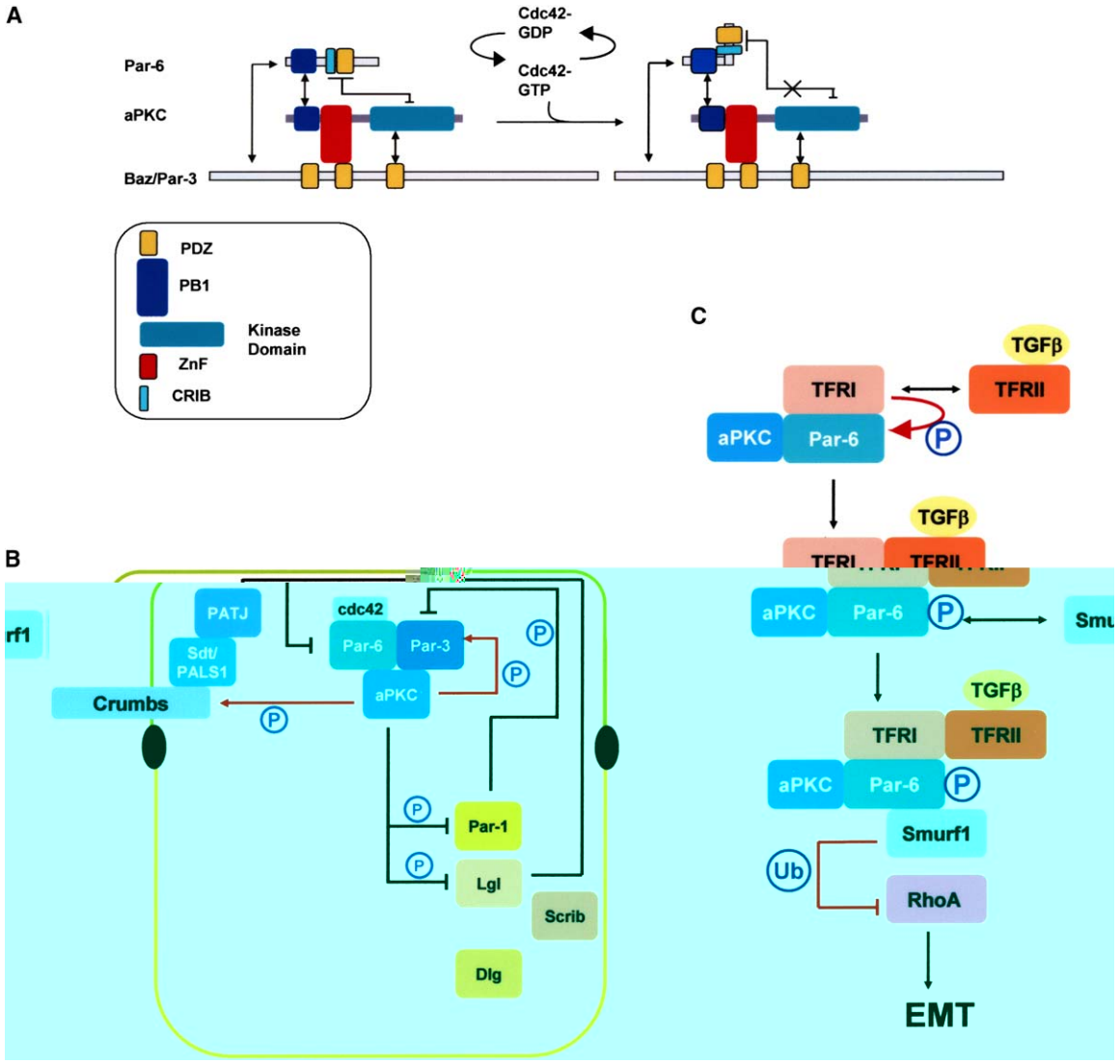

Figure 4. The Par-6/aPKC Signaling Platform

(A) Activation of the signaling complex: binding of activated Cdc42 to Par-6 relieves the inhibitory effect of Par-6 on aPKC kinase activity. The inset shows protein domains, as defined in the legend to Figure 1.

(B) Functional interactions among polarity proteins in an epithelial cell. Apical and basolateral membrane domains are depicted in green and yellow, respectively. The black ovals indicate AJ. Phosphorylation targets of aPKC and Par-1 are indicated.

(C) Involvement of Par-6 downstream of TGFβ in the EMT. TGFβ receptor (TFR)-dependent phosphorylation of Par-6 facilitates the recruitment of the E3 ubiquitin ligase Smurf1 to the receptor complex and facilitates the ubiquitination and degradation of RhoA.

The Par-6/aPKC Signaling Platform

Par-6 is a PB1 multidomain-containing protein (Figures 1 and 4A) initially described in *C. elegans* as essential for the specification of the anterior-posterior axis in the early embryo (Macara, 2004). Mutations in the *Par* genes and in the *PKC-3* gene, encoding the *C. elegans* aPKC, interfere with the first division of the *C. elegans* zygote that ensures unequal partitioning of cytoplasmic determinants between the daughter cells (Macara, 2004). This is a particular case of the more general phenomenon of asymmetric cell division involved in the specification of differential cell fates between sister cells (Doe and Bowerman, 2001). Par-6 has been shown to be central to many biological processes related to polarity, such as apico-basal epithelial cell polarity, cell migration, and axon formation (Bose and Wrana, 2006; Henrique and Schweisguth, 2003; Macara, 2004; Suzuki and Ohno, 2006).

The role of Par-6 in the control of apico-basal epithelial cell polarity has attracted a great deal of attention. The plasma membrane of epithelial cells is subdivided into an apical and a basolateral domain with different protein and lipid compositions (Figure 4B). Membrane domains are separated by the apical junctional complex (tight junction [TJ] in vertebrates), which attaches adjacent epithelial cells to each other and forms a diffusion barrier to prevent free exchange of solutes across the intercellular space (Nelson, 2003). Genetic analysis in *Drosophila* and biochemical studies in polarized mammalian epithelial cell lines are helping to unravel the functional and physical relationships between the more than 30 polarity proteins involved in the establishment