Interaction Codes within the Family of Mammalian Phox and Bem1p Domain-containing Proteins*

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The Phox and Bem1p (PB1) domain constitutes a recently recognized protein-protein interaction domain found in the atypical protein kinase C (aPKC) isoenzymes, α- and βPKC; members of mitogen-activated protein kinase (MAPK) modules like MEK5, MEKK2, and MEKK3; and in several scaffold proteins involved in cellular signaling. Among the last group, p62 and Par6 (partitioning-defective 6) are involved in coupling the aPKCs to signaling pathways involved in cell survival, growth control, and cell polarity. By mutation analyses and molecular modeling, we have identified critical residues at the interaction surfaces of the PB1 domains of aPKCs and p62. A basic charge cluster interacts with an acidic loop and helix both in p62 oligomerization and in the aPKC-p62 interaction. Subsequently, we determined the abilities of mammalian PB1 domain proteins to form heteromeric and homomeric complexes mediated by this domain. We report several novel interactions within this family. An interaction between the cell polarity scaffold protein Par6 and MEK5 was found. Furthermore, p62 interacts both with MEK5 and NBR1 in addition to the aPKCs. Evidence for involvement of p62 in MEK5-ERK5 signaling is presented.

Specific protein-protein interactions mediated by modular protein domains are instrumental for ensuring specificity in cellular signal transduction (1). Proteins involved in signaling generally harbor multiple domains, allowing combinatorial use of these domains as modules in the assembly of specific multi-protein complexes (1, 2). The Phox and Bem1p (PB1) \(^*\) domain is one such evolutionary conserved protein-protein interaction module found in proteins involved in signaling in yeasts, plants, and animals (3–5). Before the extent of the PB1 domain was recognized, a 28-amino acid sequence motif termed the octicosapeptide repeat (OPR) was noted to be present in the mammalian atypical protein kinase C (aPKC) isoforms λ and ε, MEK5, p40phox, p62, and two other mammalian proteins as well as in Drosophila Ref2/2p, yeast Cdc24, and Schizosaccharomyces pombe Scd1 (6). Subsequently, this sequence motif has been denoted Phox and Cdc (PC) or aPKC interaction domain (AID) (7, 8). The OPR/PC/AID motifs are localized within the larger PB1 domains and have now been renamed OPCA motifs (3). However, a subset of PB1 domains does not contain the OPCA motif.

The recently solved structure of the PB1 domain of the yeast protein Bem1p revealed a ubiquitin-like β-grasp fold similar to the Ras-binding domain of c-Raf-1 (4). However, except for a reported interaction between PKC and Ras (9), none of the proteins harboring this domain have been shown to bind to Ras (4). Instead, several proteins containing PB1 domains have been shown to interact with each other. Thus, the scaffold proteins p62 and Par6 (partitioning-defective 6) use their PB1 domains to specifically interact with the PB1 domain of aPKCs (10–14). This way, p62 can recruit the aPKCs into tumor necrosis factor α- and interleukin-1 receptor signaling complexes (15, 16) or target the activity of aPKC to the potassium channel subunit Kvβ2 (17) or to Gβ14 (18). The interaction between p62 and aPKC is evolutionary conserved, since the Drosophila orthologue of p62, Ref2/2p, binds to aPKC (19). The Par6 protein physically links the aPKCs to the Rho family GTPases Cdc42 and Rac1, thus forming signaling complexes involved in cell polarity decisions (12–14, 20).

A PB1 domain-mediated heteromeric interaction between the p40phox and p67phox subunits of the phagocyte NADPH oxidase is important for stimulus-induced production of superoxide (21). Another PB1 domain protein that may potentially be engaged in heteromeric interactions with other PB1-domain proteins is TFG (TRK fused gene). TFG is known as a fusion partner to the nerve growth factor tyrosine kinase receptor TrkA in papillary thyroid carcinoma (22) and to the Alk tyrosine kinase in anaplastic large cell lymphomas (23). The oncogenic fusion gene products encode an N-terminal region of TFG, including the PB1 domain fused to the kinase domain of TrkA or Alk. The PB1 domain is necessary for full transforming activity (24).

PB1 domain interactions may also play an important role in a mitogen-activated protein kinase (MAPK) module consisting of MAP/ERK kinase isoforms (MEK)-2 or -3, MEK5, and ERK5. This MAPK module is implicated in both proliferative and stress-induced signaling (25–30). MEK5 is a specific upstream activator of ERK5. MEK2 and MEK3 bind directly to the MEK5-ERK5 signaling is presented.

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¶ The abbreviations used are: PB1, Phox and Bem1p; aPKC, atypical protein kinase C; OPR, octicosapeptide repeat; PC, Phox and Cdc; AID, α- and βPKC interaction domain; OPCA, OPR, PC, and AID; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, hemagglutinin; HEK, human embryonic kidney; MAPK, mitogen-activated protein kinase; MEF2, myocyte enhancer factor-2; MEK, MAPK/ERK kinase; MEK5, MEK kinase; WT, wild type; IP, immunoprecipitation; WB, Western blot.
to MEK5 via N-terminal regions and activate the kinase by phosphorylation (31, 32). The N-terminal regions of all three proteins contain PB1 domains.

Here, we have examined interactions between mammalian PB1 domain proteins. We have identified critical residues creating the interaction surfaces for the PB1-PB1 interactions between αPKCs and p62 and for the oligomerization of p62. In a systematic screen for PB1-mediated interactions, we found three novel interactions within the family of mammalian PB1 domain proteins.

MATERIALS AND METHODS

Cell Cultures—HeLa cells (ATCC CCL2) were grown in Eagle’s minimum essential medium supplemented with 10% fetal calf serum, non-essential amino acids, 2 mM t-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) (Invitrogen). HEK 293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and the antibiotics described above. Subconfluent HELa and HEK 293 cells were transfected using either the calcium phosphate co-precipitation method or LipofectAmine PLUS (Invitrogen).

Plasmid Constructs—Plasmids used in this work are listed in Table 1. Details on their construction are available upon request. To facilitate the transfer of single CDNA constructs into a variety of expression vectors, CDNA constructs were subcloned into Gateway entry vectors. Expression clones were made as described in the Gateway cloning technology instruction manual (Invitrogen). Destination vectors not obtained from Invitrogen were made by insertion of Gateway cassettes into the polylinker of the desired vectors, using the Gateway Vector Conversion System (Invitrogen). Mutagenesis of plasmid DNA was performed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). All constructs generated by mutagenesis were verified by DNA sequencing using the BigDye sequencing kit (Applied Biosystems). Oligonucleotides for mutagenesis, PCR, and DNA sequencing reactions were obtained from Eurogentec, Belgium.

Yeast Two-hybrid Interactions—The S. cerevisiae strain PJ69-2A (Clontech) was transformed with pGBK7 constructs, and strain Y187 (Clontech) was transformed with pGADT7 constructs. The transformed yeast strains were mated, and diploids were selected by their ability to grow on media lacking leucine and tryptophan. Interactions were scored following plating on media lacking leucine, tryptophan, histidine, and adenine (quadruple dropout medium).

In Vitro Co-immunoprecipitation Experiments—Expression vectors (0.5 μg) for HA-, GFP-, and Myc-tagged proteins were in vitro transcribed/translated in a total volume of 25 μl using the TNT T7 coupled reticulocyte lysate system according to the manufacturer’s protocol (Promega). Twenty μl of in vitro translated 35S-labeled proteins were immediately diluted in 200 μl of ice-cold NET-N buffer (20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, containing 10 μM β-glycerophosphate). The samples were preincubated with a 50% solution of CL-4B Protein A-Sepharose beads in NET-N buffer for 20 min at 4 °C on a rotating wheel and then incubated with 0.1 μg of anti-HA monoclonal antibody (12CA5) for 1 h and for another 30 min in the presence of bovine serum albumin-saturated Protein A-Sepharose beads. The complexes were washed five times with 1 ml of NET-N and resuspended in 15 μl of 2X SDS-polyacrylamide gel load buffer and boiled for 5 min. The samples were resolved on SDS-polyacrylamide gels. 35S-Labeled proteins were detected using a PhosphorImager (Amersham Biosciences). Stripping of membranes was done by incubating in 0.2N NaOH for 10 min before washing and reblocking.

Immunoblotting—Immunoblotting was performed as previously described (33). The following antibodies were used: rabbit anti-GFP (1:1000; Abcam), goat anti-HA (1:1000, Santa Cruz Biotechnology), mouse anti-MEK5 (1:1000; Transduction Laboratories), goat anti-Par6 (1:5000; N-18, Santa Cruz Biotechnology), and peroxidase-conjugated secondary antibodies (1:2000; Santa Cruz Biotechnology). Detection and quantification were performed using ECL chemiluminescence (Amersham Biosciences) and the LumiAnalyst imager and software (Roche Applied Sciences). Stripping of membranes was done by incubating in 0.2 N NaOH for 10 min before washing and reblocking.

Immunofluorescence—Cells grown on 8-well coverglass slides (Nunc) were fixed for 10 min in phosphate-buffered saline containing 4% paraformaldehyde, washed with phosphate-buffered saline, permeabilized with cold methanol for 10 min, and blocked with 3% serum in phosphate-buffered saline for 1 h. Subsequently, cells were incubated at room temperature with primary and secondary antibodies for 60 and 30 min, respectively. The following antibodies were used: mouse anti-Myc (1:200; 9E10; Santa Cruz Biotechnology), mouse anti-HA (1:200; 12CA5; Roche Applied Science), mouse anti-p62 (1:200; Transduction Laboratories), and secondary antibodies conjugated with AlexaFluor 568, 488, or 352 (1:150; Molecular Probes, Inc., Eugene, OR). The mouse anti-MEK5 and anti-p62 (both from Transduction Laboratories) were directly conjugated with AlexaFluor 488 and 555, respectively, using the Zenon labeling kit (Molecular Probes). Images were collected using a Zeiss Axiovert 200 microscope equipped with a LSM510 confocal module and processed using Adobe Photoshop.

Molecular Modeling of PB1 Domains and PB1-PB1 Domain Interactions—The ICM Pro 3.0 program (Molsoft L.L.C., La Jolla, CA; available on the World Wide Web at www.molsoft.com) (36) was utilized for comparative modeling, structure alignment, computer graphics visualizations, protein-protein docking, energy calculations, and calculations of molecular surfaces and electrostatic potentials. An initial model of the PB1 domain (residue range included Ser23–Pro40) of mouse aPKC was built by comparative proteinase digestions and homology modeling in ICM, the sequence alignment shown in Fig. 1, and a structural alignment of the c-Raf-1 Ras-binding domain and Bem1p PB1 structures. The x-ray structure of the Ras-binding domain of c-Raf-1 (Protein Data Bank code 1guu) structure was utilized as a template for the αα or αα, whereas the Bem1p (Protein Data Bank code 1pqg) structure was utilized as a template for the αα or αα. By using the homology module of ICM and a refined aPKC model as a template, models of PB1 domains from 10 other mammalian proteins were constructed. Each model was refined in ICM using energy minimizations, biased probability Monte Carlo simulations of side chain conformations, and local biased probability Monte Carlo simulations of long loops. Rigid body docking simulations were performed, and low energy conformations were selected in a conformational stack for each docking simulation. An ICM global optimization algorithm was applied to refine interface side chains in conformations having the lowest ligand-receptor grid interaction energies. Finally, binding energies including electrostatic and surface terms were calculated for each of the refined complexes.

RESULTS

Mammalian PB1 Domain Proteins

The presently known mammalian PB1 domain proteins, as identified from the SMART domain data base (available on the World Wide Web at smart.embl-heidelberg.de) (37) and confirmed by independent BLAST searches with the isolated do-
Fig. 1. Mammalian PB1 domain proteins. A, domain architecture of mammalian PB1 domain proteins. The different domains and their extents were defined using the SMART domain data base (available on the World Wide Web at smart.embl-heidelberg.de) (37) and by direct sequence alignments to homologous domain sequences. Apart from the common PB1 domain, the following domains/sequence motifs are shown: C1 and ZZ zinc fingers; serine/threonine kinase catalytic domains; ubiquitin-associated (UBA), PDZ (PSD-95, Dlg, and ZO-1/2), Phox (PX), Src homology 3 (SH3), tetrastricopeptide repeats (TPR), Cdc42/Rac-interactive binding (CRIB), and coiled-coil (CC) domains; and polyglutamate (EEE) and proline-rich SH3 binding (PPXP) motifs. The lengths of the proteins in amino acid residues are shown to the right. B, sequence alignment of mammalian PB1 domains. The sequences of the PB1 domain of yeast Bem1p is included, since its structure is known and was used for molecular modeling. The threshold for identity/similarity shading was set to 45% using a PAM250 scoring matrix in the BioEdit program. The extents of the secondary structure elements for the Bem1p structure, the APKC model, and from the NMR data obtained for yeast Cdc24p are shown below the alignment. The aligned sequences are rat PKC, murine APKC, murine MEK5, human TFG, human p62, murine Par6C, human NBR1, human p40phox and p67phox, human MEKK3, and yeast Bem1p. The asterisks indicate insertions in p62 (GKEDA and EPEAAEAAA) and p40phox (EDT). C, structural model of the PB1 domain of murine APKC. The secondary structure elements are numbered from the N-terminal end (N) as $\alpha_1$, $\alpha_2$, for the $\beta$ strands and $\beta_1$, $\beta_2$, for the helices. The locations of the conserved Lys/Arg residue (Lys in APKC) as well as the OPCA loop and helix are indicated. The structure model was made using comparative modeling. The $\beta_1-\beta_2$, sheet from the Ras-binding domain of c-Raf1 and the $\alpha_1-\alpha_2$, helices from the Bem1p structure were used as structural templates (4, 39).

PB1 Domain Interactions

mains, are shown in Fig. 1A. A total of 13 proteins representing nine different domain architectures can be found. All, except p40phox and p67phox, harbor the PB1 domain at their N termini. NBR1 (next to breast cancer 1) and p62 have interesting similarities in their domain architectures, although NBR1 is a much larger protein. The ubiquitin-associated domain of p62 has been shown to bind polyubiquitin noncovalently (38). Five of the PB1 domain proteins are serine/threonine protein kinases, whereas none of the other proteins have been shown to contain any enzymatic activity. Rather, they have features of adapter and scaffold proteins containing additional protein-protein interaction domains such as PDZ, CRIB (Par6), SH3 (p40phox and p67phox), and ZZ (p62 and NBR1). The sequences of the mammalian PB1 domains are aligned in Fig. 1B, along with that of yeast Bem1p. These domains range in size from 78 (NBR1) to 102 amino acids (p62). Both p40phox and p62 contain insertions in two loops before and after $\beta$ strand 2 in the putative secondary structure inferred from the solved Bem1p PB1 domain structure. The overall sequence similarity is low except between the Par6 isoforms, the aPKCs, and MEKK2 and -3. The acidic OPCA motif represents the most conserved sequence feature within the PB1 domains. This motif is found in all but three (p67phox, MEKK2, and MEKK3) of the mammalian PB1 domain proteins.

Molecular models of 11 mammalian PB1 domains were constructed by comparative modeling using the structures of the PB1 domain of yeast Bem1p and the Ras-binding domain of c-Raf1 (4, 39). The three-dimensional model of the PB1 domain of APKC is shown in Fig. 1C. The positively charged Lys/Arg residue in the $\beta_1$ strand is absolutely conserved within the PB1 domain family (Fig. 1B). The 24–26-amino acid-long conserved OPCA motif includes a surface-exposed OPCA loop, flanked by $\beta$ strands $\beta_1$, $\beta_5$, and an OPCA helix as seen in the APKC model. The secondary structure elements of most of the PB1 domain of yeast Cdc24p have been determined by NMR (4). It is noteworthy that the positioning of these elements corresponds very well to our PB1 domain model of APKC (Fig. 1B). Bem1p does not contain any OPCA motif and lacks $\beta$ strand 4. In contrast, the PB1 domain of Cdc24p contains, like the APKC model, an OPCA loop flanked by $\beta$ strands 3 and 4.

The Interaction between aPKCs and p62: Different Requirements for the OPCA Motif and the “Basic Cluster”

We next wanted to determine the molecular surfaces involved in the PB1 domain interactions between the aPKCs and p62. Based on the sequence alignment shown in Fig. 1B and our structure models, we selected 9 residues in the PB1 domain of APKC and 10 residues in the p62 PB1 domain for alanine-scanning mutagenesis. Co-immunoprecipitation assays of in
**PB1 Domain Interactions**

**Fig. 2.** The interaction between the PB1 domains of aPKC and p62 depends on the OPCA motif of aPKC and basic cluster residues of p62. A–C, mutational analyses of the p62-aPKC interaction. HA-tagged λPKC (WT or the indicated mutants) were in vitro co-translated together with Myc-tagged p62 (WT or the indicated mutants) in the presence of [35S]methionine. Immunoprecipitations were performed using an anti-HA antibody. Immunoprecipitated and co-precipitated proteins as well as in vitro translated proteins corresponding to 10% of the input were resolved by SDS-polyacrylamide gel electrophoresis and detected by autoradiography. D, mutation of λPKC in the OPCA loop (D62A) results in loss of co-localization with p62. HeLa cells were co-transfected with GFP- and Myc-tagged fusion constructs. Images were obtained by confocal laser fluorescence microscopy after immunostaining with an anti-Myc antibody. E, yeast two-hybrid analyses of the APKC-p62 interaction. All mutants that were positive with respect to an interaction between λPKC and p62 are indicated by a plus sign, whereas those that did not give any interaction are indicated with a minus sign. Mutants of p62 were tested against WT λPKC, whereas the D69A mutant of p62 was tested against mutants of aPKC. F, model of the murine A PKC-human p62 PB1 domain heterodimer having the lowest interaction energy. Charged residues at the domain interface are displayed (red, negatively charged; blue, positively charged; yellow, aromatic).

In *vitro* translated proteins showed that the basic residue mutations of p62, K7A, R21A/R22A, R21A, and R22A, completely abolished the interaction with λPKC (Fig. 2A). On the other hand, none of the mutations in the OPCA motif of p62 abolished binding of λPKC (Fig. 2A). For λPKC, the picture was exactly the opposite. The R27A and V28A/K29A mutations did not strongly affect the binding of λPKC to p62, whereas the OPCA motif mutants W70A, D72A, E74A, D76A, and E85A showed no or very little binding to p62 (Fig. 2B). Only one of the OPCA motif mutants, Q83A, bound to p62. Clearly, p62 depends on the basic cluster including Lys7, Arg21, and Arg22 for binding to λPKC. Conversely, λPKC is completely dependent on the integrity of critical acidic residues in the OPCA motif for binding to p62.

The scaffold protein p62 has been reported to localize to perinuclear, punctuated structures in the cytoplasm showing some co-localization with late endosomal markers (11). Using an *in vivo* redistribution assay, we confirmed that the highly conserved aspartate residue in the OPCA motif of aPKCs (Asp62 in λPKC, Asp72 in λPKC) is absolutely essential for their interaction with p62 *in vivo*. As shown in Fig. 2D, p62 and GFP-λPKC colocalized in perinuclear, punctuated structures upon co-expression in HeLa cells. However, when the OPCA motif mutant of λPKC, D62A, was analyzed by co-expression with p62, a diffuse cytoplasmic staining pattern characteristic of wild type (WT) λPKC expressed alone resulted. Similar results were obtained when WT and the D72A mutant of λPKC were co-expressed with p62. We also found that the D72A or D62A mutants of the aPKCs did not co-immunoprecipitate with p62 upon overexpression in HEK 293 cells (data not shown).

To confirm the results of the mutation analyses by an independent method, we tested the mutants in the yeast two-hybrid assay. Initially, we were unable to use WT p62 in yeast two-hybrid crosses presumably due to aggregation in the cytoplasm of the yeast cells. However, p62 functions well in this system if it carries a point mutation that weakens self-interaction such as that of a highly conserved aspartate in its OPCA motif (D69A). p62D69A interacted strongly with λPKC and λPKC in two-hybrid crosses and was consequently tested for interaction with λPKC mutants. WT λPKC was also tested for interaction with various p62 mutants. The results of these experiments are summarized below the alignment in Fig. 2E. The two-hybrid data are completely consistent with the results from the *in vitro* co-immunoprecipitation experiments in Fig. 2, A–C.
To interpret our mutation data relative to the putative three-dimensional structures of the PB1 domains, we performed a rigid body docking of the PB1 domain of p62 onto that of APKC. A refined model of the p62-APKC complex with the lowest calculated p62-APKC interaction energy after global optimization of flexible side chains at the ligand/receptor interface is shown in Fig. 2F. The p62 molecule has a basic cluster of positively charged side chains of which five (Lys7, Arg21, Arg69, His66, and Arg82) are localized above the plane of the β-sheet and one (Arg52) at the edge of the β-sheet. The Lys7, Arg21, and Arg52 side chains of p62 are predicted to form salt bridges with acidic residues in APKC, Asp22 and Glu78 in the OPKA loop of APKC interact with Lys7 in p62, Glu68 in the OPKA helix of APKC interacts with Arg21 in p62, whereas Asp96 in the αC-β1 loop of APKC interacts with Arg22 in p62. Since Glu85 in the APKC OPKA helix and Arg18 in the p62 β1-β2 loop are localized in close proximity to each other, an interaction between their side chains is possible. In the p62-APKC model (Fig. 2F), the basic cluster region of p62 is completely occupied by the APKC molecule, whereas the OPKA loop is free and may interact with an additional PB1 domain, such as that of another p62 molecule.

The p62 Self-interaction: Oligomerization Involving Both the OPKA Motif and the “Basic Cluster”

Previous reports have found that p62 forms oligomers (10, 17). We find that this oligomerization is actually mediated by the PB1 domain. To map the critical residues involved in the PB1 domain-mediated self-interaction of p62, we analyzed point mutants in the basic cluster and OPKA motif of p62 using co-immunoprecipitation assays with in vitro translated GFP- and HA-tagged proteins. The K7A, R21A, and D69A single mutants all showed a reduced ability to bind to WT p62 (Fig. 3, A and C). However, the effect of the K7A/D69A double mutant is much more severe (Fig. 3B). This suggests a binding mode where the basic cluster in one molecule interacts with the OPKA loop in the other. This notion is supported by results from experiments testing the ability of mutants of HA-p62 to interact with mutants of GFP-p62 (Fig. 3, A and C). As would be expected, when either the OPKA loop (D69A) or the basic cluster (K7A or R21A) was mutated in both molecules, the interaction was virtually abolished. Notably, the R21A mutation is the only one that completely abrogated the interaction. Surprisingly, the R22A mutant did not negatively influence the interaction between HA- and GFP-tagged p62 molecules. The co-immunoprecipitation data were confirmed by yeast two-hybrid analysis; the single mutants (D69A and K7A) interacted with WT p62 (residues 1–134), whereas the double mutant (K7A/D69A) did not. Furthermore, molecules carrying the same mutation (K7A or D69A) did not interact with each other, whereas molecules carrying mutations in opposite binding surfaces did (Fig. 3D). The single mutants Y67A, D73A, and E82A were unable to interact with the D69A mutant in the yeast two-hybrid assay. This confirms that the acidic binding surface of p62 is created from residues both in the OPKA loop (Tyr67, Asp69, and Asp82) and helix (Glu65) (see also Fig. 2E).

The endogenous p62 protein is located in perinuclear dots in HeLa cells. This pattern is recapitulated and enhanced by overexpression (see Fig. 2D). To test the importance of PB1 domain-mediated self-interactions for the punctuated distribution of p62 in vivo, we transfected HeLa cells with GFP-tagged p62 expression constructs. The striking difference in the distribution of the diffusely, cytosolic located D69A and R21A mutants compared with the dotted appearance of WT p62 and the R22A mutant (controls) is evident from Fig. 3E. A refined model of a p62-p62 dimer, after rigid body docking and global optimization of flexible side chains at the ligand/receptor interface, is shown in Fig. 3F. The basic cluster of one p62 molecule is predicted to form salt bridges with acidic residues in the OPKA loop and helix of a second p62 molecule. Importantly, Lys7 of the basic cluster interacts with Asp96 and Asp32 of the OPKA loop. Arg21 interacts with both Glu65 and Glu82 in the OPKA helix. In the present p62-p62 dimeric model, one of the molecules has an exposed OPKA motif, whereas the other one has an exposed basic cluster that could interact with additional p62 molecules.

Interaction Codes within the Family of Mammalian PB1 Domain Proteins

In order to establish the interaction codes among mammalian PB1 domain proteins, the coding regions of cDNAs for human p62, NBR1, TFG, p40phox, p67phox, MEKK3, murine Par6C, MEK5, APKC, and rat PKC were cloned into entry vectors of the Gateway recombination cloning system (Table I). Initially, we used the GAL4-based yeast two-hybrid system to test the ability of all of the different proteins to interact with each other (Fig. 4). Several previously reported interactions were confirmed. Thus, λ- and γPKC interacted with Par6, p40phox interacted with p67phox, and MEK5 interacted with MEKK3 (Fig. 4). In addition, use of the p62D69A mutant revealed novel interactions between p62 and MEK5 and between p62 and NBR1 (Fig. 4). Another novel interaction indicated by the present data is between MEK5 and Par6. These proteins interacted in two-hybrid crosses, provided that Par6 was fused to the activation domain of GAL4 (Fig. 4).

To further investigate interactions between PB1 domain proteins, interactions were tested by co-immunoprecipitation following in vitro translation of epitope-tagged cDNA expression constructs (Fig. 5). In this assay, WT p62 interacted with APKC (Fig. 5, A and B), NBR1 (Fig. 5, B and C), MEK5 (Fig. 5, B and E), and itself (Fig. 5B). Thus, all interactions observed with p62D69A in the two-hybrid assay system (Fig. 4) were also observed with WT p62 in the co-immunoprecipitation assay. Also, other interactions were confirmed; Par6 interacted with λPKC (Fig. 5, A and D), p40phox interacted with p67phox (Fig. 5G), and MEK5 interacted with MEKK3 (Fig. 5F).

We also tested the abilities of all the PB1 domain proteins to self-interact. A PB1 domain-independent self-interaction has previously been reported for NBR1 and TFG. TFG has been shown to oligomerize via a coiled-coil motif located C-terminal to the PB1 domain (24). NBR1 contains several regions C-terminal to the PB1 domain that mediate self-interaction (40). We observed a weak self-interaction of TFG in the two-hybrid assay (Fig. 4), whereas self-interaction of NBR1 was only observed in the co-immunoprecipitation assay (Fig. 5C). For both the p62 and NBR1 self-interactions, the recovery of precipitated proteins relative to the input amount in the in vitro co-immunoprecipitation assays was 125%, clearly suggesting oligomerization.

Novel Interaction 1: Par6 Interacts with MEK5—Our yeast two-hybrid assays revealed an interaction between the PDZ-domain scaffold protein Par6 and the MAP kinase kinase MEK5 (Fig. 4). The results shown in Fig. 6A demonstrate that the isolated PB1 domain of MEK5 (amino acids 2–112) is sufficient for this and other interactions mediated by full-length MEK5 in the yeast two-hybrid assay. The PB1 domain of MEK5 interacts with full-length Par6, p62, and MEKK3. It also interacts with the isolated PB1 domains of p62 (residues 1–134) and Par6 (residues 1–114). However, it seems as if the PB1 domain of Par6 interacts more weakly than full-length Par6. Notably, the interaction between MEK5 and Par6 was
not detected by co-immunoprecipitation of in vitro translated proteins. To test whether MEK5 and Par6 interact in vivo, we performed co-immunoprecipitation experiments following co-transfection of HEK 293 cells with expression vectors for enhanced green fluorescent protein- and HA-tagged proteins. Clearly, GFP-MEK5 was efficiently co-precipitated with HA-Par6 using an anti-HA antibody. The efficiency of co-precipitation was comparable with that of HA-p62, whereas GFP-MEK5 did not efficiently co-precipitate with HA-APKC (Fig. 6B). Using HeLa cell extracts, we were also able to detect endogenous Par6 in precipitates following immunoprecipitation of endogenous MEK5 (Fig. 6C). To further corroborate these findings, we looked at the ability of Par6 to redistribute GFP-MEK5 into perinuclear, punctuated structures. Par6 localizes to such structures together with the 58-kDa Golgi marker (41). These structures are distinct from the p62-containing structures seen

![Diagram](image-url)

**Fig. 3. Oligomerization of p62 requires both acidic residues of the OPCA motif and residues of the basic cluster.** A and B. mutational analyses of p62-p62 interactions. HA- and GFP-tagged p62 proteins were co-translated in vitro and subjected to immunoprecipitations using an anti-HA antibody, as described in the legend to Fig. 2. 35S-Labeled proteins were quantitated using a PhosphorImager. GFP-p62 mutants are indicated above and HA-p62 mutants below the gels. C, quantitative representation of the interaction data shown in A and B. The amount of 35S-labeled WT GFP-p62 pulled down by WT HA-p62 was set to 100%. The data shown are representative of three independent experiments. The arrowhead and grey line indicate the cut-off level for detecting interaction in the yeast two-hybrid analyses. D, yeast two-hybrid analyses of p62 self-interactions. Full-length D69A, K7A, and D69A/K7A mutants of p62 fused to the GAL4 activation domain (left) were tested against the PB1 domain of WT p62 (residues 1–134) and full-length mutants of p62 fused to the DNA-binding domain of GAL4 (top). Interactions are indicated with a plus sign. E, self-interaction in vivo as visualized by confocal laser fluorescence microscopy of HeLa cells transfected with GFP fusions of p62. Note the loss of perinuclear, punctuated fluorescence for the D69A and R21A mutants. F, model of the human p62 PB1 domain self-interaction. Color coding is as in Fig. 2.
| Vectors | Description | Source |
|---------|-------------|--------|
| pGADT7 | Yeast two-hybrid Ga14 activation domain fusion vector | Clontech |
| pGBK7 | Yeast two-hybrid Ga14 DNA binding domain fusion vector | Clontech |
| pcDNA3-HA | Mammalian HA-tag fusion expression vector, CMV and T7 promoters | Invitrogen (48) |
| pEYFP-C1 | Mammalian EGF fusion expression vector | Clontech |
| pGEX4T-3 | Bacterial GST fusion expression vector, tac promoter | Amersham Pharmacia-Biotech |
| pG5E1bLUC | Mammalian luciferase reporter gene vector for GAL4 fusion proteins | Ref. 47 |
| pGAL-Sap1α | Expression vector for a GAL4 DBD Sap1α fusion protein | Ref. 49 |
| pGAL-MEF2C | Expression vector for a GAL4 DBD MEF2C fusion protein | Ref. 26 |
| pSRαMEK5D | Expression vector for activated murine MEK5 | Ref. 28 |
| pHA-ERK5 | Expression vector for murine ERK5 | Ref. 28 |
| pcRGFL3 | Luciferase reporter for the murine c-jun promoter | Ref. 42 |
| Gateway cloning vectors | | |
| pENTR1A | Entry vector, Kanρ | Invitrogen |
| pENTR2B | Entry vector, Kanρ | Invitrogen |
| pENTR3C | Entry vector, Kanρ | Invitrogen |
| pDONR207 | Donor vector, Gentρ | Invitrogen |
| pDestGADT7 | Donor vector, pGADT7-based, Amp R | This study |
| pDestGBKT7 | Donor vector, pGBKT7-based, Kan R | This study |
| pDestHA | Donor vector, pcDNA3-HA-based, CMV and T7 promoters, Amp R | This study |
| pDestmyc | Donor vector, pcDNA3-myc-based, CMV and T7 promoters, Amp R | This study |
| pcDNA-Dest53 | Donor vector, pEGFP-C1-based, Amp R | Ref. 50 |
| pdEYFP | Donor vector, pEGFP-C1-based, Amp R | This study |
| pDest15 | Donor vector, pEGFP-C1-based, Amp R | This study |
| pDest17 | Donor vector, pEGFP-C1-based, Amp R | This study |
| cDNA constructs made by traditional cloning | | |
| pcDNA3-HA-APKC | Murine APKC in pcDNA3-HA backbone, source of APKC constructs made in this study, used as template for mutagenesis (mutations: R27A, V28A/K29A, W70A, D72A, E74A, D76A, Q83A, and E85A) | Ref. 35 |
| pcDNA3-myc-APKC | Murine APKC in pcDNA3-myc backbone | This study |
| pGEP-AAPKC | Murine APKC in pEGFP-C1 backbone, used as template for mutagenesis (mutation: D72A) | Ref. 35 |
| pGEX-APKC-(1-131) | Murine APKC-(1-131) in pGEX4T-3 backbone | This study |
| pENTR-APKC | Murine APKC in pENTR backbone, used as template for mutagenesis (mutations: R27A, V28A/K29A, W70A, D72A, E74A, D76A, Q83A, and E85A) | This study |
| pcDNA3-HA-APKC | Murine APKC in pcDNA3-HA backbone, source of APKC constructs made in this study | Ref. 48 |
| pGFP-APKC | Murine APKC in pEGFP-C1 backbone, used as template for mutagenesis (mutation: D62A) | Ref. 35 |
| pENTR-APKC | Murine APKC in pENTR backbone, used as template for mutagenesis (mutation: D62A) | This study |
| pGEX-MEK5 | Murine MEK5 in pEGFP-C1 backbone | This study |
| pGEX-MEK5-(1-133) | Murine MEK5 in pGEX4T-3 backbone | This study |
| pGEX-MEK5-(1-131) | Murine MEK5-(1-131) in pGEX4T-3 backbone | This study |
| pENTR-MEK5 | Murine MEK5 in pENTR backbone | This study |
| pENTR-MEK5-(2-112) | Murine MEK5-(2-112) in pENTR backbone | This study |
| pcDNA3-HA-p62 | Human p62 in pcDNA3-HA backbone, source of p62 constructs made in this study | Ref. 11 |
| pcDNA3-myc-p62 | Human p62 in pcDNA3-myc backbone | Ref. 11 |
| pGEX-p62 | Human p62 in pGEX4T-3 backbone | This study |
| pASp62 | Antisense of full-length human p62 in pcDNA3 backbone | This study |
| pENTR-p62 | Human p62 in pENTR backbone, used as template for mutagenesis (mutations: K7A, K13A, R21A, R22A, Y67A, D69A, D71A, D73A, and E82A) | This study |
| pENTR-p62-(1-134) | Human p62-(1-134) in pENTR backbone, used as template for mutagenesis (mutation: D69A) | This study |
| pcDNA3-HA-NBR1 | Human NBR1 in pcDNA3-HA backbone, isolated from KIAA clone KIAA0049 (T, Nagase, Kazusa DNA Research Institute) | This study |
| pENTR-NBR1 | Human NBR1 in pENTR backbone, used as template for mutagenesis (mutations: K12A and D50R) | This study |
| pRK5myc-mPar6 | Murine Par6C in pRKmyc backbone, source for Par6 cDNA constructs made in this study | Ref. 51 |
| pENTR-Par6 | Murine Par6C in pENTR backbone | This study |
| pENTR-Par6-(1-114) | Murine Par6C-(1-114) in pENTR backbone | This study |
| pENTR-TFG | Human TFG5ΔM1 in pENTR backbone, cDNA isolated from human brain cDNA library (Marathon ready, Clontech) | This study |
Novel Interaction 3: The Scaffold Protein p62 Interacts with

Novel Interaction 2: NBR1 and p62 Interact via Their PB1 Domains—NBR1 and p62 share a similar overall domain architecture (Fig. 1A). By yeast two-hybrid analyses and immunoprecipitation of in vitro translated proteins, we show that these two proteins interact (Figs. 4, 5, and 7, A–C). This interaction is dependent on their respective PB1 domains and can be abolished by point mutations. Thus, the D50R mutant (mutated in the OPCA motif of the PB1 domain) of NBR1 does not interact with p62, whereas the basic cluster mutant, K12A, does (Fig. 7A). This was also confirmed by yeast two-hybrid analyses (data not shown). The corresponding point mutations in the PB1 domain of p62 affect the interaction in exactly the opposite manner. Hence, the basic cluster mutant of p62 (K7A) is unable to bind to NBR1 (Fig. 7B), whereas p62D69A binds to NBR1 (Fig. 4). The simplest interpretation of these data is that p62 uses its basic cluster region to interact with the OPCA motif in the PB1 domain of NBR1.

To look for interaction between p62 and NBR1 in vivo, we transfected HEK 293 cells with expression vectors for HA-p62 and GFP-NBR1 followed by immunoprecipitation with anti-HA antibodies. GFP-APKC was used as a positive control for interaction with p62. As seen from Fig. 7C, GFP-NBR1 interacted with HA-p62, whereas the D50R mutant did not. The corresponding mutant in the OPCA loop of the PB1 domain of NBR1 is sufficient for this interaction (Fig. 6D, lower panel).

Novel Interaction 3: The Scaffold Protein p62 Interacts with
MEK5—Recently, a direct interaction involving the PB1 domains of aPKCs and MEK5 was reported (34). However, we were unable to detect such an interaction between aPKCs and MEK5 by in vitro co-immunoprecipitation (Fig. 5A) or by two-hybrid analyses (Fig. 4). Instead, we found a novel interaction between p62 and MEK5. To verify that the p62-MEK5 interaction is direct and to further look for an interaction between aPKCs and MEK5, we performed GST pull-down assays. GST-p62 interacted strongly with both in vitro translated MEK5 (Fig. 8B) and purified recombinant His-MEK5 (Fig. 8C). Actually, the amount of recombinant MEK5 pulled down by GST-p62 was 40-fold above the amount pulled down by GST alone, whereas MEKK3 pulled down twice as much MEK5 as p62 (Fig. 8C). Under the conditions used, we did not observe a significant interaction between MEK5 and in vitro translated full-length aPKC (Fig. 8A) or between MEK5 and the isolated PB1 domains of the aPKCs (Fig. 8C). The input of MEKK3 used in the assay was lower than for the other GST fusion proteins due to a low expression level of GST-MEKK3 in E. coli.

Evidence for an in vivo interaction between p62 and MEK5 in co-transfected HEK 293 cells is shown in Fig. 6B. Immunoprecipitation of HA-p62 gave an efficient co-precipitation of GFP-MEK5. No such co-precipitation was observed with HA-aPKC or HA-γPKC (Fig. 6B and data not shown). Quantitative estimates from three independent experiments showed that 2.2–2.7% of GFP-MEK5 co-precipitated with HA-p62, whereas the amount co-precipitated with HA-aPKC or HA-γPKC (0.1–0.2%) was not higher than the amount of GFP that co-precipitated (0.1%).

Subsequently, we looked at the ability of p62 to redistribute MEK5 upon overexpression in HeLa cells. Indeed, when HA-p62 was co-expressed with GFP-MEK5, GFP-MEK5 was redistributed from the nucleus into p62-containing perinuclear dots (Fig. 8D, top panel). This suggests that the two proteins interact either directly or indirectly in vivo. Co-expression with HA-γPKC did not appear to affect the localization of GFP-MEK5, although the two proteins colocalize to some degree in the cytosol (Fig. 8D). To determine whether endogenous MEK5 and p62 display co-localization, we stained HeLa cells using monoclonal antibodies directly labeled with green and red fluorophores, respectively. Confocal laser microscopy revealed a complete co-localization of endogenous MEK5 and p62 in perinuclear, punctuated structures (Fig. 8D, lower panel). All of these data strongly indicate that MEK5 and p62 interact directly and are part of a protein complex with distinct subcellular localization in vivo.

p62 Forms a Complex with Both aPKCs and MEK5 and Affects ERK5 Signaling

Although aPKCs and MEK5 in our hands do not show a strong direct interaction, they may be part of the same signaling complex, since both interact with the scaffold protein p62. To address this possibility more directly, we first co-transfected HEK 293 cells with expression vectors for HA-tagged p62, GFP-aPKC, and GFP-MEK5 followed by immunoprecipitation of p62 with anti-HA antibodies. As expected, both aPKC and MEK5 co-precipitate with p62 when expressed alone or in combination (Fig. 9A). In similar experiments, HA-MEK5 pulled down GFP-aPKC only when GFP-p62 was co-expressed
Similarly, when HA-aPKC was immunoprecipitated, coexpression of GFP-p62 was necessary to give significant co-precipitation of GFP-MEK5 (Fig. 9C). Clearly, some GFP-MEK5 co-precipitated in the absence of GFP-p62 overexpression (Fig. 9C). This is due to a relatively high level of endogenous p62 in HEK 293 cells (data not shown). Results from titration experiments clearly showed that a threshold level of GFP-p62 was required to observe significant co-precipitation of HA-aPKC and GFP-MEK5 (data not shown). To ensure that our results were not affected in any way by the use of enhanced green fluorescent protein as the tag on two of the three proteins, we performed experiments with three different tags (HA, Myc, and enhanced green fluorescent protein). The results obtained were the same as with two enhanced green fluorescent protein-tagged proteins (data not shown). Altogether, our results suggest that p62, due to its ability to directly bind both proteins, is able to recruit both aPKCs and MEK5 into a common complex.

It has been shown that aPKCs can affect MEK5-ERK5 signaling (34). We therefore asked whether the scaffold protein p62 also is an important actor in the MEK5-ERK5 signaling pathway. To test this possibility, we transfected HeLa cells with a p62 antisense construct to deplete p62 levels. As shown in Fig. 9D, antisense inhibition of p62 expression is very efficient. We found that the total level of p62 was reduced by 46%. With a transfection efficiency of about 50%, there is almost complete inhibition of p62 expression in the transfected cells. The transcription factors Sap1a and MEF2C have both been shown to be activated by ERK5-mediated phosphorylation of their transactivation domains (26, 28). The c-jun promoter is also regulated by ERK5 via a MEF2 binding site (42). Interestingly, antisense inhibition of p62 expression strongly reduced EGF-induced activation of the c-jun promoter, Sap1a, and MEF2C (Fig. 9E). As seen from Fig. 9F, the ability of a constitutive active mutant of MEK5, MEK5D, to activate the c-jun promoter and MEF2C is unaffected by antisense depletion of p62. This suggests that p62 acts upstream of MEK5 in this signaling pathway.

Electrostatic Surface Potentials of PB1 Domains Are Consistent with the Observed Protein-Protein Interactions

Our mutagenesis studies of the aPKC-p62 and p62-p62 interactions implicate electrostatic interactions between acidic residues in the OPCA motif and residues of the basic charge cluster. To further analyze the role of electrostatics, we calculated the electrostatic surface potentials of molecular models of 11 PB1 domains (Fig. 10). The domains fall into three categories. The molecular surfaces of Par6C and p67phox have molecular surfaces with one large electropositive region (β-sheet, α1 helix, and part of the OPCA loop, α2-β2 loop) and large areas with neutral potentials (including the OPCA helix and most of the OPCA loop). Also, MEKK2 and -3 are basically of this category, although their models include areas with negatively charged potentials. In contrast, the molecular surface of p62 can be subdivided into areas with strong electropositive potentials (β1−β3), areas with strong negative potentials (OPCA loop and helix, α1 helix in β2−α3 loop), and areas with neutral potentials. Similar to p62, the p40phox and TFG models include positively charged (ridges on the β-sheet),
neutral, and negatively charged surface potentials, although the exact localization differs in the three models. The electrostatic surface potentials of the different PB1 models (Fig. 10) correlate strongly with the importance of OPCA motif or basic charge cluster residues, respectively, as defined by our mutant analyses of PB1 domain interactions. Ongoing mutagenesis studies further support the validity of the presented surface models. As would be expected, basic charge cluster residues in Par6C are essential for its interaction with aPKCs. Basic charge cluster residues in MEKK3 are essential for its interaction with MEK5, and basic charge cluster residues in p62 are essential for its interaction with MEK5. Finally, residues in the OPCA loop of MEK5 are involved in binding to p62.3

**DISCUSSION**

The recently defined PB1 domains have been implicated as the mediators of important protein-protein interactions in several cellular signaling pathways (see Introduction). These domains are found in a limited set of proteins from yeast to humans. We have identified 13 mammalian proteins containing this domain. To gain more insight into the modes of interaction at the molecular level, we employed mutant analyses and molecular modeling to study the interaction between p62 and aPKCs and the oligomerization of p62. The interaction between the PB1 domains of p62 and aPKCs was shown to depend on electrostatic interactions involving basic cluster residues in p62 and acidic residues in the OPCA loop and helix of aPKCs (Fig. 2F). Similarly, p62 uses its basic cluster to interact with the acidic OPCA loops of MEK5 and NBR1. All of these p62-interacting PB1 domains display strongly electronegative surface potentials (Fig. 10).
PB1 Domain Interactions

The PB1 domain of p62 is the only one of the identified mammalian PB1 domains that mediates oligomerization. The OPCA loop and helix of one p62 molecule is used as the binding surface for the basic cluster of another p62 molecule. In principle, two scenarios are easily envisioned: 1) a dimeric interaction with two p62 molecules reciprocally engaging both binding surfaces in each molecule; 2) a "beads-on-a-string"-like interaction with a chain of p62 molecules interacting via the basic cluster in one molecule and the OPCA loop and helix of the next molecule in the chain. We favor the latter model. It is clear that p62 forms oligomers (10, 17).4 Our mutant analyses show that the ability to form large perinuclear, punctuated structures in cells depends on PB1-mediated self-interactions (see Fig. 3E). Thus, a simple dimeric structure is unlikely. Molecular modeling also suggests a "beads-on-a-string" structure.

All of our present data and the results from studies of the interactions between p40phox and p67phox (8, 21) and between yeast Bem1p and Cdc24p (4, 5, 43) implicate two interaction surfaces in the PB1 domains. The pattern revealed is that one of the interacting partners uses the basic cluster on top of the β-sheet to interact with acidic residues that are solvent-exposed in the OPCA loop and helix of the other partner. PB1 domains without OPCA motifs, like that of p67phox, MEKK2, or MEKK3, use the basic cluster to interact with acidic OPCA motifs in their partners. Par6 has an OPCA motif with few acidic residues compared with aPKCs, MEK5, NBR1, and TFG. This is clearly reflected in the much more electropositive surface potential of Par6 (see Fig. 10). Accordingly, Par6 interacts with the OPCA motifs of aPKCs and MEK5 via basic cluster residues.3

Although several important signaling complexes depend on PB1-PB1 domain interactions, a systematic study to determine the interaction codes within the family of mammalian PB1 domain proteins has not been performed. We therefore did such experiments and uncovered novel interactions between Par6 and MEK5, between p62 and MEK5, and between p62 and NBR1. The interaction between MEK5 and Par6 was readily observed in vivo in yeast and mammalian cells but was not reproducibly detected by in vitro co-immunoprecipitations or GST pull-downs. The reason for this is currently unknown. A post-translational modification may be required for the apparently strong interaction observed in vivo, or it may not be a direct interaction. A conserved third protein may mediate the interaction. Alternatively, a third protein may help stabilize an interaction between Par6 and MEK5.

The biological significance of the interaction between p62 and NBR1 is unknown. Intriguingly, both proteins have a similar domain organization, although NBR1 is twice as big and contains additional domains not present in p62. The NBR1 protein has not been studied to any extent, and no function has been assigned to it. If both proteins act as scaffold proteins and interact with each other, they may build distinct complexes when they are together and apart. Relevant to this notion is our observation that although the D50R mutant of NBR1 cannot bind to and colocalize with p62 in cells, it forms distinct perinuclear speckles. It is potentially interesting that NBR1 can interact with FEZ1 (40). FEZ1 is the mammalian orthologue of the C. elegans protein UNC-76 involved in axonal outgrowth and has previously been found to interact with and act as a substrate for PKC (44).

A direct interaction between aPKCs and MEK5 has been reported to activate MEK5 without a requirement for aPKC kinase activity (34). The interaction was between the N-termi-

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4 T. Lamark, M. Perander, H. Outezen, K. Kristiansen, Aud Øvervatn, E. Michaelsen, G. Bjørkoy, and T. Johansen, unpublished data.

Fig. 8. The scaffold protein p62 interacts with MEK5. A–C, GST pull-downs where 2 μg of the indicated GST fusion proteins were allowed to bind to radiolabeled in vitro translated PKC and MEK5 (A and B) or 200 ng of purified, recombinant His-MEK5 (C). Pulled down proteins were detected by autoradiography (A and B) or immunoblotting (C; upper panel), and the amount of immobilized GST fusion protein was visualized by Coomassie staining (C; lower panel). D, GFP-MEK5 colocalizes with HA-p62 in perinuclear, punctuated structures after co-expression in HeLa cells. Endogenous MEK5 (green) and p62 (red) display co-localization in similar structures in HeLa cells when visualized with directly conjugated monoclonal antibodies. Images were obtained using confocal laser-scanning microscopy.
nal regions of MEK5 and aPKCs. Thus, the PB1 domains of both proteins mediate this interaction. Using co-immunoprecipitation of in vitro translated proteins, GST pull-down of purified, recombinant proteins, co-immunoprecipitation of tagged proteins overexpressed in HEK 293 cells, and yeast two-hybrid analyses, we found no significant direct interaction between MEK5 and aPKCs. Instead, MEK5 interacts directly with p62 in all of these different assays (Figs. 4–6 and 8). Also, the acidic surface potentials of the PB1 domains of both the aPKCs and MEK5 argue against a strong direct interaction.
between aPKCs and MEK5 (Fig. 10). Co-expression studies in HEK 293 cells clearly suggest that efficient co-precipitation of either MEK5 or aPKC occurs when p62 is co-expressed with the two other proteins (Fig. 9, A–C). These observations together with the ability of p62 to oligomerize suggest to us a model where MEK5 and aPKC interact with a chain of p62 molecules. Such a chain may even be branched by interactions with other proteins binding to the ZZ domain and the region between the ZZ and ubiquitin-associated domains of p62. We do not think that a trimer with a 1:1:1 stoichiometry is possible, because aPKC and MEK5 are likely to compete for binding to the basic cluster on a single p62 molecule. Although we did not find a strong direct interaction between aPKCs and MEK5, this does not rule out a weak interaction that could be stabilized by p62 molecules interacting separately with aPKCs and MEK5. In fact, in MAPK signaling modules, the components are often associated via multiple interactions in addition to being tethered by scaffold proteins (45). This assures accuracy and efficiency of signaling.

Using antisense RNA expression to reduce the level of endogenous p62 in HeLa cells, we show that p62 is an important component in MEK5–ERK5-mediated activation of the transcription factors MEF2C and Sap1a following EGF stimulation. EGF-induced activation of the c-jun promoter, which occurs partly via MEF2D activated by ERK5 (42), is also inhibited by depletion of endogenous p62 (Fig. 9). Although unaware of a direct interaction between MEK5 and p62, Geetha and Wooten (46) found that antisense p62 blocked nerve growth factor-mediated activation of ERK5 in PC12 cells. Together with our results, this finding also strongly implicates p62 in ERK5 signaling.

Interestingly, TFG was the only one of all of the proteins analyzed that did not interact with any of the other PB1 domain proteins. Thus, are there more PB1 domain proteins that we have not detected by sequence data base searches due to too low primary sequence homology? Or can PB1 domains also interact with other domains? Both questions are important to answer in further studies of mammalian PB1 domain proteins. 

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