PB1 Domain Interaction of p62/Sequestosome 1 and MEKK3 Regulates NF-κB Activation*

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p62/Sequestosome 1 is a scaffold protein involved in the regulation of autophagy, trafficking of proteins to the proteasome, and activation of NF-κB. p62 encodes an N-terminal PB1 domain in addition to the ZZ domain, TRAF6-binding domain, LC3 interaction region, and ubiquitin-associated domain, each critical for the physiological function of p62. PB1 domains have a β-grasp topology where the front end of one PB1 domain binds the back end of a second PB1 domain. The p62 PB1 domain homodimerizes as well as heterodimerizes with other PB1 domains. The front end of the PB1 domain in p62 binds the PB1 domain of atypical protein kinases C, the MAPK kinase, MEK5, and the NBR1 protein. Other than its role in homodimerization, the rear end acidic cluster region of the p62 PB1 domain had no previous defined binding partners. Herein, we demonstrate that the rear end acidic cluster region of the p62 PB1 domain binds to the rear end basic region of the MAPK kinase kinase, MEKK3. p62 and MEKK3 co-localize in speckles or aggregates that are centers for organizing TRAF6-regulated NF-κB signaling and the assembly of polyubiquitinated proteins sorting to sequestosomes and proteasomes. The p62-MEKK3 complex binds TRAF6, which regulates the ubiquitination of the IKK complex and NF-κB activation. p62 is required for the association of MEKK3 with TRAF6 and short hairpin RNA knockdown of p62 inhibits IL-1 and MEKK3 activation of NF-κB. The rear end acidic cluster of the p62 PB1 domain is used to organize cytosolic aggregates or speckles-associated TRAF6-p62-MEKK3 complex for control of NF-κB activation.

p62/Sequestosome 1 is a scaffold protein of 62 kDa encoding multiple binding domains for proteins involved in signaling, ubiquitination, and autophagy. The N-terminal PB1 (Phox/Bem1p) domain has been shown to bind the PB1 domains of atypical PKCs³ (1), MEK5 (2), and NBR1 (neighbor of BRCA1 gene 1) (2). Additional domains encoded in p62 include the ZZ-type zinc finger domain that binds RIP1 (receptor-interacting protein-1), a TRAF6-binding domain, an LC3 interaction region (3), and a ubiquitin-associated domain (4). p62 is found in cytosolic speckles and aggregates referred to as sequestosomes and is thought to link the autophagy machinery to polyubiquitinated proteins (3). p62 polymerizes via its PB1 domain and binds ubiquitinated proteins via its ubiquitin-associated domain and, via its LC3 interaction region, binds the LC3 protein, which is a member of the autophagosomal machinery (5). p62 also regulates NF-κB activation by its interaction with RIP1 (6) and TRAF6 (7). The p62-TRAF6 complex appears to regulate both phosphorylation (8) and ubiquitination (9, 10) of the IKK complex. Deletion of p62 has been shown to inhibit RANK ligand-stimulated NF-κB activity, resulting in impaired osteoclastogenesis associated with Paget disease of bone (11). p62 also appears important for NF-κB regulation in response to neurotrophic factors (12) and inflammatory cytokines, including IL-1 (7) and tumor necrosis factor α (6). p62 knock-out mice are insulin-resistant and obese, apparently due to a loss of ERK1/2 regulation in adipogenesis (13). Most recently, p62 has been shown to promote tumorigenesis in autophagy-deficient tumor cells by altering NF-κB regulation (14).

The PB1 domain of p62 is critical for many of its defined cellular and physiological functions. PB1 domains regulate protein interactions primarily by forming heterodimers using a β-grasp topology that uses a “front-to-back” arrangement of the two PB1 domains (4). This ubiquitin-like grasp domain uses clusters of basic amino acids in the front of one PB1 domain to bind acidic amino acids in the back of a second PB1 domain. “A-type” PB1 domains have conserved acidic residues, and “B-type” PB1 domains have a conserved lysine or arginine in the basic region. The p62 PB1 domain has both a basic front end and acidic rear end (A-B type PB1 domains) and can therefore homodimerize as well as heterodimerize (2). It is the unique homodimerization of the p62 PB1 domain that confers its ability to form polymers that are a component of aggregates of ubiquitinated proteins in the autophagy pathway. The heterodimerization property of the p62 PB1 domain allows p62 binding of other PB1 domain proteins, such as atypical PKCs, MEK5, and NBR1.

We have previously characterized the role of PB1 domains of the MAPK kinase kinases, MEKK2 and MEKK3 (15). MEKK2 and MEKK3 PB1 domains bind the PB1 domain of the MAPK kinase, MEK5, which phosphorylates and activates the MAPK, ERK5 (16). Although MEKK2 and MEKK3 PB1 domains are highly homologous, they do have differential regulatory properties. We have shown that the MEKK2 PB1 domain has a non-

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p62 and MEKK3 PB1 Domain Binding

canonical function in binding MKK7, a MAPK kinase that activates JNK (17). A substantial literature exists suggesting that MEKK3, but not MEKK2, regulates NF-κB (18–25). The mechanism for how MEKK3 but not MEKK2 could selectively activate NF-κB is not defined. Herein, we demonstrate that the PB1 domains of MEKK3 and p62 heterodimerize using the C-terminal acidic cluster of p62 and the N-terminal basic region of MEKK3. The PB1 domain-dependent MEKK3-p62 complex localizes to cytosolic p62 speckles/aggregates and mediates MEKK3 activation of NF-κB.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—HEK293 and COS7 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C with 7% CO₂. Recombinant human IL-1β was obtained from PeproTech. The mAb (12CA5) against the hemagglutinin (HA) epitope (for immunoprecipitation and staining) was from Roche Applied Science. A mouse mAb for MEKK2 was generated against recombinant MEKK2. Anti-MEKK3 Ab was purchased from Epitomics. Anti-phospho-ERK5 antibody was from Cell Signaling. Poly-(wild type), MEKK3 (wild type or K48A mutant), and MEK5 (wild type, D64A/E65A, or K22A mutant) GST-PB1 domains from HA-p62 PB1 domain (D69A/E70A or K7A) expressing HEK293 cells. The detection was by the same method as stated in A. The bottom panel is an anti-HA Ab blot to show equal input in the pull-down assay. WT, wild type; 69.70DE-AA, D69A/E70A; 7K-A, K7A; 64.5DE-AA, D64A/E65A; 22K-A, K22A; 48K-A, K48A.

DNA Constructs—GST fusions were constructed in pGEX 5X-1, using the cDNA fragments amplified by PCR. His-PB1 domain proteins with a hexahistidine epitope at the N terminus of each protein were engineered by PCR and cloned into pRSET (Invitrogen). The indicated residues were replaced by Ala, using PCR-based mutagenesis, and coding amino acids are described in the figure legends. For lentiviral infections, shRNA for human p62 (TRCN0000007233, -34, -35, -36, and -37) or control shRNA was in pLKO.1 vector (Open Biosystems). For transient expression in HEK293 cells, HA-MEKK2, HA-MEKK3, FLAG-MEKK5, and FLAG-p62 were inserted into the pCMV5 expression vector. HA-p62, provided by Dr. J. Moscat (University of Cincinnati), FLAG-TRAf6, and Xpress-ERK5 were in pcDNA3. HA- and FLAG-p62 PB1 domain (aa 1–107) were inserted in pCMV5. HA-MEKK2-MEKK3 chimeric molecules were generated as a combination of either MEKK2 PB1 domain (aa 1–122) and MEKK3 (aa 124–626) (MEKK2 PB1-MEKK3) or MEKK3 PB1 domain (aa 1–123) and MEKK2 (aa 123–619) (MEKK3 PB1-MEKK2).

Pull-down Assays with GST Fusion Proteins—Different GST fusion proteins were coupled to glutathione beads. Total cell lysates were prepared from HEK293 cells transiently transfected with DNA constructs using a solubilizing buffer. GST fusion proteins on glutathione beads were incubated with 1 mg of HEK293 cell lysate at 4 °C for 2 h. The beads were washed five times with solubilizing buffer, and the protein complex isolated on the beads was subjected to SDS-PAGE and immunoblotting. Surface Plasmon Resonance Assay—GST fusion proteins were purified using glutathione-Sepharose beads. Various His₆ fusion proteins were purified by nickel-His interaction. Analysis was conducted using a Biacore 3000 instrument, and data were evaluated with BIACalculator 3.2 (Biacore). Running buffer contained 10 mM Hepes, pH 7.5, 150 mM NaCl, 3 mM EDTA, 0.0005% Nonidet P-40. Anti-GST Ab was immobilized on a carboxymethylxyltran surface on a CM5 sensorchip (GE Healthcare). Ligands, GST-PB1 domain fusion or GST protein, were engaged with GST fusion proteins on glutathione beads in a titration manner using the BIAcore 3000 instrument. Data were analyzed with BIACalculator 3.2 (Biacore).
captured on an anti-GST Ab-coupled sensorchip were used to see ligand-analyte interaction. After use, antibody-coated sensorchip surfaces were regenerated with 40 μl of 10 mM glycine, pH 1.9, and used for another GST-ligand coupling. Upon coupling a ligand, each GST fusion protein was accumulated until a surface density of 800 response units was achieved. His-PB1

**TABLE 1**

Summary of PB1 domain interactions

| Plus and minus signs indicate positive and negative binding based on the surface plasmon resonance spectroscopy results of Fig. 2, respectively. The K<sub>d</sub> value for specific PB1 domain interactions was calculated according to dose-response measurements with increasing concentrations (5 nM to 5 μM) of His-PB1 domains passed over GST-PB1 domain immobilized on the sensor surface coated with anti-GST Ab, as described under “Experimental Procedures” and the legend to Fig. 2. Data were analyzed using global fitting of association (kₐ) and dissociation (kₐ) rates using BIAevaluation software except p40<sub>phox</sub>-p67<sub>phox</sub> interaction. The affinity in p40<sub>phox</sub>-p67<sub>phox</sub> binding was calculated based on the equilibrium binding measurements at each different concentration of analyte. The K<sub>d</sub> values (nM, except μM in p40<sub>phox</sub>-p67<sub>phox</sub>) are shown in parentheses. NT, not tested. |
|---|---|---|---|---|---|---|
| | GST-K2 WT | GST-K3 WT | GST-K5 WT | GST-p40<sub>phox</sub> | GST-p62 D69A/E70A | p62 K7A |
| His-K2 WT | + | + | + | + | + |
| His-K3 WT | + (25.4 nM) | + (24.8 nM) | + | + | + |
| His-K5 WT | + | + | + | + | + |
| His-p40<sub>phox</sub> | + (3.47 μM) | + (3.47 μM) | + (3.47 μM) | + (5.91 μM) | + (5.91 μM) |
| His-p67<sub>phox</sub> | + (116 nM) | + (116 nM) | + (116 nM) | + (116 nM) | + (116 nM) |
| p62 K7A | + (89.1 nM) | + (19.3 nM) | + (19.3 nM) | + (19.3 nM) | + (19.3 nM) |
| k2 K47A | NT | NT | NT | NT | NT |
| k3 K48A | NT | NT | NT | NT | NT |
| K5 D64A/E65A | NT | NT | NT | NT | NT |
domain proteins were injected at a flow rate of 20 μl/min, and association and dissociation phases were recorded for 10 and 5 min, respectively. Non-specific binding measured by using a flow cell of GST alone was subtracted from the value in a flow cell of GST-PB1 domain fusion protein.

Immunoprecipitation and Immunoblotting—Cells were washed three times with ice-cold phosphate-buffered saline (PBS) and lysed with solubilizing buffer (1% Nonidet P-40, 10 mM Tris, pH 7.5, 150 mM NaCl, 0.4 mM EDTA, 10 mM NaF, 2 mM Na₂VO₄, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml α₁-antitrypsin, and 1 mM phenylmethylsulfonyl fluoride), and cleared supernatants were retained for further processing. Lysates were subjected to immunoblotting or immunoprecipitation with anti-HA, -MEKK2, or -MEKK3 Ab. For immunoprecipitation, immune complexes were collected with protein G- or A-Sepharose beads, separated by SDS-PAGE, and transferred to a nitrocellulose membrane. After blocking, membranes were blotted with the indicated Abs and visualized using the Supersignal West Pico detection system (Pierce). As to protein phosphatase treatment, immunoprecipitate was incubated with λ-protein phosphatase (New England Biolabs) in a 50-μl reaction buffer supplied by the manufacturer for 50 min at 30 °C. For the in vitro kinase assay, immunoprecipitate was incubated with a 50-μl kinase buffer (0.05% Nonidet P-40, 20 mM HEPES, pH 7.5, 50 mM NaCl, 2.5 mM MgCl₂, 20 mM β-glycerophosphate, 0.1 mM Na₂VO₄, 2 mM dithiothreitol, and 0.1 mM ATP) for 50 min at 30 °C. After the reaction, immunoprecipitates were washed with solubilizing buffer three times and provided for further processing.

Immunostaining and Co-localization Analysis—Genes of interest were introduced into COS7 cells on 22-mm square glass coverslips in a 6-well plate using Lipofectamine and Plus reagent (Invitrogen). After 24 h, the cells on coverslips were pulled down with GST-PB1: MEK5 MEKK3

| Pull down from | HA-p62 6970DE-4A | HA-p62 7K-A | HA-MEK5 WT | HA-MEKK3 WT | HA-p62 6970DE-4A | HA-p62 7K-A | HA-MEK5 WT | HA-MEKK3 WT |
|---------------|------------------|-------------|-------------|-------------|------------------|-------------|-------------|-------------|
| Blotting with: | α-HA Ab          | α-HA Ab     | α-MEKK5 Ab  | α-MEKK5 Ab  | α-p62 Ab         | α-p62 Ab    | α-MEKK5 Ab  | α-MEKK5 Ab  |
|               | WCL              | WCL         | WCL         | WCL         | WCL              | WCL         | WCL         | WCL         |
fixed for 10 min with 4% (w/v) paraformaldehyde in PBS at room temperature. Permeabilization was conducted using 0.2% Triton X-100 in PBS for 10 min at room temperature. Non-specific binding was blocked by incubation of coverslips for 1 h in 10% goat serum in PBS. The coverslips were incubated with Abs (either polyclonal anti-HA Ab and anti-FLAG M5 mAb, polyclonal anti-p62 and anti-HA (12CA5) mAb, or polyclonal anti-TRAF6 Ab and anti-p62 or -HA mAb for 1 h at room temperature) and washed with PBS three times for 5 min each time. Bound primary Abs were visualized by incubation with Alexa Fluor 488 goat anti-rabbit Ab and Alexa Fluor 555 goat anti-mouse Ab for 1 h at room temperature, washed, and mounted on glass slides. Imaging was performed using a Zeiss Axiovert 200 M inverted microscope with a 125-watt xenon arc lamp (Sutter Instrument Company, Novato, CA), digital CCD camera (CoolSNAP HQ, Roper Scientific, Tucson, AZ), and Slide- book 5.0 software (Intelligent Imaging Innovations, Denver, CO). An objective (×63 oil 1.25 numerical aperture, Plan Neofluar, Zeiss) was coupled with immersion oil to the bottom face of glass coverslips. Using fluorescein isothiocyanate (for Alexa Fluor 488) and Cy3 (for Alexa Fluor 555) filter sets (Chroma), the images were obtained at 50- and 10-ms exposure with 2 × 2 binning, respectively. For section analyses, the background-subtracted images were transferred to the ImageJ program, and green and red fluorescence intensities in each section were numerically processed. The relative intensity was calculated as a fraction of the maximum number in each section, and the graphs were created using the GraphPad Prism 5 program (La Jolla, CA).

p62 Knockdown in Cells Using Lentiviral shRNAs—HEK293 cells were used as packaging cells for the lentiviral gene transfer system. pLKO.1 vectors and packaging mix (Open Biosystems) were transfected into HEK293 cells. Two days after transfection, the supernatants were collected and diluted with the same volume of fresh complete Dulbecco’s modified Eagle’s medium, and Polybrene was added to the infection medium at 8 μg/ml. The infection mixtures were added to cell cultures seeded on a 6-well plate, incubated overnight, and then replaced with fresh complete Dulbecco’s modified Eagle’s medium containing 8 μg/ml puromycin. The puromycin-resistant cells were isolated and propagated for use in the experiments.

Luciferase Assay—Cells in a 12-well plate were transfected with 140 ng of firefly luciferase reporter construct harboring three tandem NF-κB or MEF2 binding sites and 14 ng of pRL-TK Renilla luciferase construct (Promega) along with the indicated amount of plasmid using Lipofectamine and Plus reagent. The cells were cultured for 24 h, starved of serum for 8 h, harvested in passive lysis buffer, and analyzed using the DualLuciferase reporter assay system (Promega). NF-κB or MEF2C luciferase activity was normalized to Renilla luciferase activity and presented as relative luciferase activity. The data are shown as an average of relative luciferase activity in triplicate with an error bar of S.D.

RESULTS

MEKK3 and p62 PB1 Domains Heterodimerize—In an attempt to define PB1 domain interactions, we have expressed different PB1 domains as either GST fusions or HA-tagged proteins. Because p62 PB1 domains homodimerize as well as heterodimerize, we expressed mutant PB1 domains having the basic region mutation K7A or the acidic cluster mutation D69A/E70A. The K7A and D69A/E70A p62 PB1 domains fail to homodimerize but are able to heterodimerize with specific B-type and A-type PB1 domains, respectively. Our screen with recombinant PB1 domains identified the ability of the p62 PB1 domain to bind the MEKK3 PB1 domain (Fig. 1A). The MEKK3 PB1 domain bound the K7A but not the D69A/E70A PB1 domain of p62. The p40phox PB1 domain, which binds the p67phox PB1 domain in Fig. 2 and Ref. 27, does not bind the p62 PB1 domain, demonstrating the specificity of PB1 domain interactions.

The PB1 domains of MEKK2 and MEKK3 are highly homologous, and both use the front end basic region of their PB1 domains to bind the MEK5 PB1 domain (15). Fig. 1B shows pull-down assays using GST-PB1 domains from MEKK2, MEKK3, and MEK5. The MEKK3 PB1 domain but not the MEKK2 PB1 domain binds the K7A p62 PB1 domain. In contrast to the MEKK3 PB1 domain, the MEK5 PB1 domain binds D69A/E70A p62 PB1 domain. Thus, the MEKK3 PB1 domain binds the C-terminal acidic cluster, and the MEK5 PB1 domain binds the N-terminal basic region of the p62 PB1 domain. This shows that the PB1 domains of MEKK3 and MEK5 use different regions of the p62 PB1 domain for binding, allowing potentially different signaling modules to be organized by p62.

Surface plasmon resonance spectroscopy was used to measure the interactions of PB1 domains for MEKK2, MEKK3, MEK5, p62, p40phox, and p67phox. Fig. 2 characterizes the interactions of the different PB1 domains with the results showing that (i) the basic regions of MEKK2 and MEKK3 PB1 domains bind the acidic cluster of the MEK5 PB1 domain; (ii) the MEK5 acidic cluster binds the basic region of the p62 PB1 domain; (iii) the basic region of MEKK3 and MEKK2 PB1 domains bind the acidic cluster of the p62 PB1 domain; (iv) the p40phox PB1 domain bound only the p67phox PB1 domain, consistent with the specificity of PB1 domain interactions.

The finding that the MEKK2 PB1 domain could bind the p62 PB1 domain using surface plasmon resonance contrasts with the GST-PB1 domain pull-downs in Fig. 1B, where the MEKK2 PB1 domain failed to pull-down with the MEKK2 PB1 domain.
p62 and MEKK3 PB1 Domain Binding

A

MEKK3 WT  p62 WT  p62 7K-A

B

MEKK3 WT vs p62 WT

MEKK3 48K-A vs p62 WT

MEKK3 WT vs p62 7K-A

MEKK3 48K-A vs p62 7K-A

composite  MEKK3  p62

C

MEKK3 WT vs p62 WT

MEKK3 48K-A vs p62 WT

MEKK3 WT vs p62 7K-A

MEKK3 48K-A vs p62 7K-A
PB1 domain did not bind the p62 PB1 domain as detected by immunoblotting in pull-down assays. Dose-response experiments were conducted with increasing concentrations of His-K7A p62 PB1 domain and immobilized GST-MEKK3 or MEKK2 PB1 domain. The PB1 domains of MEKK2 and MEKK3 have similar affinities (~25 nm) for the MEK5 PB1 domain (Table 1). In contrast, the MEKK3 PB1 domain has a 4.6-fold higher affinity for the K7A p62 PB1 domain than the MEKK2 PB1 domain (~19 nm for MEKK3 versus ~89 nm for MEKK2 PB1 domains). Reciprocal experiments where the K7A p62 PB1 domain was immobilized on the sensorchip and the MEKK3 or MEKK2 PB1 domains were used in the flow solution gave similar results (data not shown). This difference in binding affinity most likely accounts for the differential results that are observed in pull-down assays and clearly shows a selectivity of p62 PB1 domain binding to MEKK3 versus MEKK2 PB1 domains.

Specificity of MEKK3-p62 PB1 Domain Interaction—The cumulative findings in Fig. 3 show that the interaction of full-length proteins for MEKK3 and p62 is dependent on their respective PB1 domains. Fig. 3A shows that the MEK5 and MEKK3 PB1 domains bind full-length D69A/E70A and K7A p62, respectively, indicating that the PB1 domains dictate high affinity interaction. Fig. 3B further shows that the basic cluster residue Lys48 in the MEKK3 PB1 domain is required for binding the p62 PB1 domain. In contrast, the MEK5 PB1 domain acidic cluster (DE65) is required for binding the p62 PB1 domain.

Endogenous p62 and MEK5 both bind HA-MEKK3 (Fig. 3C). In contrast, HA-MEKK2 binding to endogenous p62 is not detected using immunoprecipitation assays. However, similar to MEKK3, MEKK2 binds MEK5 by immunoprecipitation, consistent with p62 selectively binding MEKK3, whereas MEK5 binds both MEKK2 and MEKK3. Mutation of K48A in the basic cluster of the MEKK3 PB1 domain inhibits the binding of MEKK3 to endogenous p62 and MEK5 proteins, confirming the role of the basic region in the MEKK3 PB1 domain and front-to-back binding orientation of the MEKK3 PB1 domain with both p62 and MEK5.

Chimeras were synthesized where the MEKK2 and MEKK3 PB1 domains were swapped between the two proteins (Fig. 3, D and E). Endogenous p62 was co-immunoprecipitated with the chimera having a MEKK3 PB1 domain fused with the MEKK2 kinase domain. In contrast, the chimera having the MEKK2 PB1 domain and MEKK3 kinase domain did not bind p62. As predicted, endogenous MEK5 was able to bind both chimeras. The cumulative results are the first demonstration of a PB1 domain interaction with the C-terminal acidic cluster region of p62 other than p62-p62 PB1 domain homodimerization. MEK5, NBR1, and the atypical PKCs bind the N-terminal basic region of p62 (2, 28, 29).

p62 Aggregates Bind MEKK3—p62 readily forms aggregates or speckles in the cytoplasm by oligomerization mediated by its PB1 domain (1, 3). Multiple proteins are recruited to the p62 aggregates, including TRAF6, atypical PKCs, NBR1, caspase-8, NF-κB, and polyubiquitinated proteins (30). Fig. 4A shows that expressed wild type p62 forms characteristic aggregates in the cytoplasm. In contrast, K7A p62 having a disrupted PB1-PB1 domain homodimerization fails to form aggregates because it is unable to form p62 oligomers. Expression of MEKK3 results in a diffuse cytoplasmic staining with an absence of MEKK3 aggregates. Fig. 4B shows that co-expression of MEKK3 and wild type p62 results in the co-localization of MEKK3 in p62 aggregates. For quantification of co-localization (Fig. 4C), intensity profiles for each marker were generated by placing identical linear regions of interest upon both images for each image pair. The intensity values for each pixel were extracted along each line, normalized by setting the peak values to 100%, and then graphed against distance. These traces demonstrate the degree of correlation between the spatial distributions of the two proteins for each condition. The analysis shows that a component of expressed wild type MEKK3 and wild type p62 co-localize in aggregates, as shown in the coordinate peaks of intensity in the upper left panel of Fig. 4C. The lower left panel of Fig. 4C also shows that K7A p62, which is unable to form p62 aggregates but still binds MEKK3, is co-localized with MEKK3 in the cytoplasm. Mutation of MEKK3 or p62 PB1 domains, such that they are unable to heterodimerize, results in a diffuse localization of MEKK3 and loss of co-localization with p62.

Fig. 5 shows the comparative analysis of endogenous p62 co-localization with MEKK3 and MEKK2. Images and quantitation of intensity profiles show that MEKK3 co-localizes in p62 aggregates similar to that shown in Fig. 4. In contrast, MEKK2 is diffusely localized in the cytoplasm and is not found to be concentrated in p62 aggregates. These results confirm the pull-down and immunoprecipitation assays showing that MEK3 but not MEKK2 binds p62 and that MEKK3 is found in p62 cytoplasmic aggregates.

p62 Is Required for MEKK3 Association in TRAF6 Complexes—p62 is well characterized to bind TRAF6 (7, 12). Fig. 6A shows by coimmunoprecipitation of endogenous proteins that MEKK3 but not MEKK2 is in a complex with p62 and TRAF6. Fig. 6B and C shows that the MEKK3 PB1 domain and full-length MEKK3 effectively pull down TRAF6 in a manner requiring its interaction with the p62 PB1 domain. The interaction of TRAF6 and p62 was unaffected by mutation of the p62 PB1 domain (data not shown). The MEKK2 PB1 domain-MEKK3 chimera was used to demonstrate that the MEKK3-endogenous p62-TRAF6 complex was dependent on the MEKK3 PB1 domain (Fig. 6D). Replacement of the MEKK3 PB1 domain with the MEKK2 PB1 domain in the MEKK3 protein results in the loss of p62 binding and association of the chimera in a TRAF6 complex. This chimera, however, retains its ability to bind MEK5 (Figs. 3 and 6D).

FIGURE 4. Localization of p62 and MEKK3 in cells. A, COS7 cells were transfected with HA-MEKK3 or FLAG-p62 (wild type or K7A) individually. Expressed MEKK3 and p62 were stained with polyclonal anti-HA (green) and anti-FLAG (M5) (red) Abs, respectively. Scale bar, 10 μm. B, HA-MEKK3 (wild type or K48A) and FLAG-p62 (wild type or K7A) were simultaneously introduced into COS7 cells and then stained as described in A. Bar, 10 μm. C, lines in B indicate the sections used for the analyses of fluorescence intensity. The relative intensity (y axis) of each fluorescence image along the line (x axis, distance in μm) was calculated as a percentage against the maximum intensity in the section. The images and section analyses shown are representative of duplicate coverslips. WT, wild type; 7K-A, K7A; 48K-A, K48A.
MEKK3-p62 PB1 Domain Interaction Is Required for MEKK3-TRAF6 Complexes in p62 Aggregates—Fig. 7A shows that TRAF6 co-expressed with p62 is in p62 aggregates. Most importantly, Fig. 7B shows that MEKK3 is also co-localized in p62 aggregates with TRAF6. The co-localization of MEKK3 and TRAF6 in p62 aggregates is dependent on a functional MEKK3-p62 PB1-PB1 domain interaction between the two proteins. This is shown with the MEKK3 PB1 domain K48A mutant that disrupts the interaction of MEKK3 with p62.

p62 Is Required for MEKK3 Activation of NF-κB—Using an MEF2C reporter gene readout for ERK5 activation shows that MEKK2 and MEKK3 similarly activate luciferase expression (Fig. 8A), whereas only MEKK3 activates the NF-κB reporter gene (Fig. 8B). Lentivirus-encoded shRNAs were used to knock down p62 protein expression in HEK293 cells (Fig. 8C). The level of knockdown between five different shRNAs targeting p62 varied from 12 to 92% of p62 expression in control cells. Expression of MEKK3 in control and each of the shRNA p62 knockdown clones resulted in similar activation of ERK5 (Fig. 8D). The NF-κB activity in the different p62 shRNA knockdown clones correlated closely with the level of p62 expression, demonstrating that the scaffold protein p62 is essential for NF-κB activation by MEKK3 (Fig. 8E). Thus, p62 is not required for ERK5 activation in response to the expression of MEKK3, but p62 is required for MEKK3 activation of NF-κB.

MEKK3-p62-TRAF6 Endogenous Protein Complex Is Regulated by IL-1—MEKK3, p62, and TRAF6 are in a complex measured by co-immunoprecipitation of the three proteins, using an antibody to MEKK3 (Figs. 6A and 9A). IL-1β stimulation of HEK293 cells resulted in a time-dependent dissociation of MEKK3 from p62 and TRAF6 (Fig. 9A). Fig. 9B demonstrates that the dissociation of MEKK3 from p62 is a function of MEKK3 activation. Phosphatase-treated MEKK3 is in an inactive state, shown by its faster migration on SDS-PAGE (17). In vitro incubation of MEKK3 bound to beads with ATP activates MEKK3 and causes an upward shift on SDS-PAGE (17). Using inactive and activated MEKK3 in pull-down assays shows that activation of MEKK3 resulted in decreased interaction with
both MEK5 and p62, consistent with IL-1β stimulation of cells inducing the dissociation of the MEKK3-p62-TRAF6 complex (Fig. 9A). Fig. 9C further shows that the association of MEKK3 and TRAF6 is dependent on p62 expression. Knockdown of p62 using shRNA inhibits coimmunoprecipitation of TRAF6 with MEKK3. This is consistent with the findings in Fig. 7 that MEKK3 co-localization with TRAF6 requires a functional PB1-domain interaction between MEKK3 and p62. Finally, shRNA knockdown of p62 inhibits IL-1β activation of NF-κB (Fig. 9D), just as loss of p62 inhibits MEKK3 activation of NF-κB.

**DISCUSSION**

The significance of this work is the discovery of a unique function of the C-terminal region of the p62 PB1 domain. Previous characterization of the p62 PB1 domain demonstrated that MEK5, atypical PKCs, and NBR1 bind to the N-terminal basic region of p62 (2, 28, 29). p62 is also able to homodimerize or oligomerize in a PB1 domain-dependent manner, which obviously requires the head-to-tail binding of the N-terminal basic region and C-terminal acidic region of different p62 proteins. Thus, p62 is one of only two known proteins encoding an A-B type PB1 domain, although it has been suggested that atypical PKCs may also have A-B type PB1 domains (31), but we have not found this interaction in our studies. The other protein clearly encoding an A-B type PB1 domain is the TRK-fused gene (2, 32). In contrast, MEK5 encodes an A-type PB1 domain, and MEKK2 and MEKK3 encode B-type PB1 domains.

The shRNA knockdown of p62 expression clearly inhibited MEKK3-mediated NF-κB activation. Previous reports have demonstrated that MEKK3 regulates NF-κB by regulating the activity of the IKK complex (19). Our findings now demonstrate the requirement of p62 for MEKK3 activation of NF-κB, most likely by the p62-MEKK3 complex binding to TRAF6. The fact that atypical PKCs and MEKK3 both bind p62 and both have the ability to activate NF-κB suggests a redundancy in p62 function that is currently not understood. The role of MEK5 binding to p62 is also unclear at the present time because p62 is clearly not required for MEKK3-dependent activation of ERK5. MEKK3 could bind the C-terminal region of the PB1 domain when MEK5 or atypical PKCs occupy the N-terminal region of the p62 PB1 domain. It is likely that there is a mixture

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4 K. Nakamura and G. L. Johnson, unpublished observations.
**FIGURE 7. Colocalization of MEKK3 and TRAF6 in cells.** A, p62-TRAF6 colocalization was shown in COS7 cells transfected with FLAG-TRAF6 together with (lower left panel) or without (upper left panel) FLAG-p62. Expressed TRAF6 and p62 were stained with anti-TRAF6 (green) and -p62 (red) Abs, respectively. A line in a composite image of the lower left panel indicates the section used for an analysis of fluorescence intensity. Section analysis in the right panel is conducted using the same method as for Fig. 5. B, p62-dependent MEKK3-TRAF6 binding. FLAG-TRAF6 and HA-MEKK3 (wild type or K48A, which inhibits PB1 domain interaction with p62) were expressed with or without wild type p62 in the indicated combinations. Localization of TRAF6 (anti-TRAF6 Ab) (green) and MEKK3 (anti-HA (12CA5) Ab) (red) was identified. Each line in the images was subjected to a section analysis of fluorescence intensity. The images and section analyses shown in A and B are representative of duplicate coverslips. Scale bar, 10 μm. WT, wild type; 48K-A, K48A.
of p62 complexes binding atypical PKCs, MEK5, and MEKK3 for the coordinated regulation of the ERK5 and NF-κB pathways.

Previous studies have shown that MEKK3 co-immunoprecipitates with TRAF6 (22). Our findings indicate that this interaction requires p62 linking MEKK3 to TRAF6. It has also been
suggested that MEKK3 is required for tumor necrosis factor α (TNF-α) and IL-1 activation of NF-κB (19, 22), but we do not see the requirement of MEKK3 or p62 for activation of NF-κB in response to tumor necrosis factor α.4 Rather we propose that a major function of MEKK3 is related to its co-localization with p62 in cytoplasmic aggregates for p62-TRAF6-dependent regulation of NF-κB. This complex is clearly regulated by IL-1 for activation of NF-κB.

Interestingly, it is now recognized that autophagy is defective and p62 is overexpressed in many tumor cells (14, 33–35). Normally, autophagy is thought to mediate p62 elimination and to suppress tumorigenesis. However, defective autophagy allows overexpression of p62 that promotes tumorigenesis. The sustained p62 overexpression in tumor cells with defective autophagy appears to dysregulate NF-κB signaling, and altered NF-κB regulation resulting from p62 overexpression is proposed to be a primary mechanism for enhancing tumorigenesis (14). Our studies presented here demonstrate that MEKK3 binds p62 and define the PB1 domain interaction properties for the two proteins. Furthermore, we show that the MEKK3-p62 interaction can occur in cytosolic p62 aggregates or speckles and that the MEKK3-p62 complex with TRAF6 mediates NF-κB activation. It will be interesting to determine in future studies if MEKK3 is involved in dysregulated NF-κB signaling observed with defective autophagy and p62 overexpression in different tumor cells. It is possible that cytokines such as IL-1 play a role in altering NF-κB signaling when autophagy is dysregulated by p62 overexpression.

FIGURE 9. Role of the endogenous MEKK3-p62-TRAF6 complex in IL-1-induced NF-κB activation. A, IL-1 stimulation induces dissociation of the MEKK3-TRAF6 complex. HEK293 cells were treated with IL-1β (10 ng/ml) for the indicated times. After cell lysis, MEKK3 immunocomplexes were analyzed with the immunoblotting of anti-TRAF6, -p62, and -MEKK3 Abs. The expression of each protein is shown in the right panel. B, binding of p62 to MEKK3 in vitro. MEKK3 was immunoprecipitated from cells expressing HA-MEKK3 or empty vector with anti-HA Ab and protein G-Sepharose beads. MEKK3 on the beads was treated with or without protein phosphatase (PP) or used for an in vitro kinase assay (IVK) with ATP for MEKK3 autophosphorylation and activation (shown by upward gel shift of MEKK3). After extensive washing, the MEKK3-bound beads were incubated with lysates from cells expressing FLAG-MEKK5 (wild type) (left) or FLAG-p62 (K7A) (right). Top, FLAG-MEKK5 or -p62 in MEKK3 immunoprecipitates were analyzed with anti-FLAG Ab blotting. Middle, amount of MEKK3 on the beads. The bottom graphs show the relative band intensities of FLAG-MEKK5 and -p62 in MEKK3 immunoprecipitates. C, requirement of p62 for a MEKK3-TRAF6 complex. MEKK3 in p62 knockdown (sh#4) or control (CTR) cells was immunoprecipitated with anti-MEKK3 Ab, and each immunoprecipitate was subjected to the immunoblotting of anti-TRAF6, -p62, and -MEKK3 Abs (left). Expression of each protein is shown in the right panel. D, requirement of p62 for IL-1-induced NF-κB activation. The NF-κB reporter assay was conducted using p62 knockdown (sh#4) and control cells. NF-κB and Renilla luciferase reporter constructs were transfected into shRNA knockdown or a control cell, HEK293 cells. 24 h after transfection, the cells in a serum-free medium were stimulated with the indicated concentration of IL-1β. After 8 h of incubation, cells were harvested, and NF-κB reporter activity was measured against the Renilla reporter (NF-κB/Renilla). The data are shown as the average of the relative luciferase activity ± S.D. (error bars). I.P., immunoprecipitation.
REFERENCES

1. Sanchez, P., de Carcer, G., Sandoval, I. V., Moscat, J., and Díaz-Meco, M. T. (1998) Mol. Cell Biol. 18, 3069–3080
2. Lamark, T., Pedersen, M., Outzen, H., Kristiansen, K., Øvervatn, A., Michaelsen, E., Bjørkøy, G., and Johansen, T. (2003) J. Biol. Chem. 278, 34568–34581
3. Bjørkøy, G., Lamark, T., Brech, A., Outzen, H., Pedersen, M., Øvervatn, A., Stenmark, H., and Johansen, T. (2005) J. Cell Biol. 171, 603–614
4. Moscat, J., Díaz-Meco, M. T., and Wooten, M. W. (2007) Trends Biochem. Sci. 32, 95–100
5. Pankiv, S., Clausen, T. H., Lamark, T., Brech, A., Bruun, J. A., Outzen, H., Øvervatn, A., Bjørkøy, G., and Johansen, T. (2007) J. Biol. Chem. 282, 24131–24145
6. Sanz, L., Sanchez, P., Lallena, M. J., Díaz-Meco, M. T., and Moscat, J. (1999) EMBO J. 18, 3044–3053
7. Sanz, L., Díaz-Meco, M. T., Nakano, H., and Moscat, J. (2000) EMBO J. 19, 1576–1586
8. Lallena, M. J., Díaz-Meco, M. T., Bren, G., Payá, C. V., and Moscat, J. (1999) Mol. Cell Biol. 19, 2180–2188
9. Wooten, M. W., Geetha, T., Seibenhener, M. L., Babu, J. R., Díaz-Meco, M. T., and Moscat, J. (2005) J. Biol. Chem. 280, 35625–35629
10. Martin, P., Díaz-Meco, M. T., and Moscat, J. (2006) EMBO J. 25, 3524–3533
11. Durán, A., Serrano, M., Leitges, M., Flores, J. M., Picard, S., Brown, J. P., Moscat, J., and Diaz-Meco, M. T. (2004) Dev. Cell 6, 303–309
12. Wooten, M. W., Seibenhener, M. L., Mamidipudi, V., Díaz-Meco, M. T., Barker, P. A., and Moscat, J. (2001) J. Biol. Chem. 276, 7709–7712
13. Rodríguez, A., Durán, A., Selloum, M., Champy, M. F., Diez-Guerra, F. J., Flores, J. M., Serrano, M., Auwerx, J., Díaz-Meco, M. T., and Moscat, J. (2006) Cell Metab. 3, 211–222
14. Mathew, R., Karp, C. M., Beaudoin, B., Vuong, N., Chen, G., Chen, H. Y., Bray, K., Reddy, A., Bhanot, G., Gelinas, C., Dipaola, R. S., Karantza-Wadsworth, V., and White, E. (2009) Cell 137, 1062–1075
15. Nakamura, K., and Johnson, G. L. (2003) J. Biol. Chem. 278, 36989–36992
16. Nakamura, K., Uhlik, M. T., Johnson, N. L., Hahn, K. M., and Johnson, G. L. (2006) Mol. Cell Biol. 26, 2065–2079
17. Nakamura, K., and Johnson, G. L. (2007) Mol. Cell Biol. 27, 4566–4577
18. Ellinger-Ziegelbauer, H., Brown, K., Kelly, K., and Siebenlist, U. (1997) J. Biol. Chem. 272, 2668–2674
19. Yang, J., Lin, Y., Guo, Z., Cheng, J., Huang, J., Deng, L., Liao, W., Chen, Z., Liu, Z., and Su, B. (2001) Nat. Immunol. 2, 620–624
20. Matsuda, A., Suzuki, Y., Honda, G., Muramatsu, S., Matsuzaki, O., Nagano, Y., Doi, T., Shimotohno, K., Harada, T., Nishida, E., Hayashi, H., and Sugano, S. (2003) Oncogene 22, 3307–3318
21. Blonska, M., You, Y., Geleziunas, R., and Lin, X. (2004) Mol. Cell Biol. 24, 10757–10765
22. Huang, Q., Yang, J., Lin, Y., Walker, C., Cheng, J., Liu, Z. G., and Su, B. (2004) Nat. Immunol. 5, 98–103
23. Yao, J., Kim, T. W., Qin, J., Jiang, Z., Qian, Y., Xiao, H., Lu, Y., Qian, W., Gulen, M. F., Sizemore, N., DiDonato, J., Sato, S., Akira, S., Su, B., and Li, X. (2007) J. Biol. Chem. 282, 6075–6089
24. Di, Y., Li, S., Wang, L., Zhang, Y., and Dorf, M. E. (2008) Cell Signal. 20, 705–713
25. Sun, W., Li, H., Yu, Y., Fan, Y., Grabiner, B. C., Mao, R., Ge, N., Zhang, H., Fu, S., Lin, X., and Yang, J. (2009) Cell Signal. 21, 1488–1494
26. Wu, H., Naya, F. J., McKinsey, T. A., Mercer, B., Shelton, J. M., Chin, E. R., Simard, A. R., Michel, R. N., Bassel-Duby, R., Olson, E. N., and Williams, R. S. (2000) EMBO J. 19, 1963–1973
27. Kuribayashi, F., Nuno, H., Wakamatsu, K., Tsunawaki, S., Sato, K., Ito, T., and Sumimoto, H. (2002) EMBO J. 21, 6312–6320
28. Wilson, M. L., Gill, D. J., Perisic, O., Quinn, M. T., and Williams, R. L. (2003) Mol. Cell 12, 39–50
29. Hirano, Y., Yoshinaga, S., Ogura, K., Yokoichi, M., Noda, Y., Sumimoto, H., and Inagaki, F. (2004) J. Biol. Chem. 279, 31883–31890
30. Moscat, J., and Díaz-Meco, M. T. (2009) Cell 137, 1001–1004
31. Noda, Y., Kohijima, M., Izaki, T., Ota, K., Yoshinaga, S., Inagaki, F., Ito, T., and Sumimoto, H. (2003) J. Biol. Chem. 278, 43516–43524
32. Roccato, E., Pagliardini, S., Cleris, L., Canevari, S., Formelli, F., Pierotti, M. A., and Greco, A. (2003) Oncogene 22, 807–818
33. Levine, B. (2007) Nature 446, 745–747
34. Mathew, R., Karantza-Wadsworth, V., and White, E. (2007) Nat. Rev. Cancer 7, 961–967
35. Duran, A., Linares, J. F., Galvez, A. S., Wikenheiser, K., Flores, J. M., Diaz-Meco, M. T., and Moscat, J. (2008) Cancer Cell 13, 343–354
PB1 Domain Interaction of p62/Sequestosome 1 and MEKK3 Regulates NF-κB
Activation
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