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The Atypical PKC-Interacting Protein p62 Is an Important Mediator of RANK-Activated Osteoclastogenesis

Short Article

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Summary

The atypical PKCs (aPKCs) have been implicated genetically in at least two independent signaling cascades that control NF-κB and cell polarity, through the interaction with the adapters p62 and Par-6, respectively. P62 binds TRAF6, which plays an essential role in osteoclastogenesis and bone remodeling. Recently, *p62* mutations have been shown to be the cause of the 5q35-linked Paget's disease of bone, a genetic disorder characterized by aberrant osteoclastic activity. Here we show that p62, like TRAF6, is upregulated during RANK-L-induced osteoclastogenesis and that the genetic inactivation of *p62* in mice leads to impaired osteoclastogenesis in vitro and in vivo, as well as inhibition of IKK activation and NF-κB nuclear translocation. In addition, RANK-L stimulation leads to the inducible formation of a ternary complex involving TRAF6, p62, and the aPKCs. These observations demonstrate that p62 is an important mediator during osteoclastogenesis and induced bone remodeling.

Introduction

Bone normal physiology relies on a finely tuned balance between formation and resorption controlled by osteoblasts and osteoclasts, respectively. Pathological bone resorption is produced when this balance is tilted toward an increased osteoclastic activity like in osteoporosis, rheumatoid arthritis, or as a consequence of genetic malfunctions such as in the Paget's disease of the bone (PDB). The latter is a genetic disease characterized by increased osteoclastic activity leading to disorganized bone formation (Roodman, 1996). Recent studies have shown that mutations in the gene coding for the atypical PKC (aPKC) scaffold protein p62 are the cause of the 5q35-linked PDB (Laurin et al., 2002). The aPKCs (λ/ιPKC and ζPKC) are critical mediators in the control of cell survival through the activation of NF-κB and in the regulation of cell polarity (Moscat and Diaz-Meco, 2000; Moscat et al., 2003). The interaction of the V1 domain of the aPKCs with the AID/PB1-containing region of p62 or Par-6 (Moscat and Diaz-Meco, 2000; Ponting et al., 2002) serves to locate these kinases in different signaling cascades that regulate NF-κB and cell polarity, respectively. In response to several stimuli, p62 directly interacts with TRAF6 (Sanz et al., 2000; Wooten et al., 2001), which is essential in the signaling mechanisms leading to the activation of NF-κB in cells triggered by RANK-L, IL-1, or NGF (Cao et al., 1996; Lomaga et al., 1999; Wooten et al., 2001). The canonical NF-κB pathway involves an IKK complex that phosphorylates IκB, which is subsequently targeted for degradation through the proteasome system (Ghosh and Karin, 2002; Li and Verma, 2002). IκB serves to retain NF-κB in the cytoplasm in an inactive complex formed by the transactivating subunit RelA and the transcriptionally inactive p50. Once released from IκB, NF-κB enters the nucleus, where it activates the transcription of a number of genes coding for prosurvival and differentiation proteins and inflammatory cytokines. TRAF6 knockout (KO) mice show impaired NF-κB activation in IL-1-stimulated cells and, interestingly, display an osteopetrotic phenotype (Kobayashi et al., 2001, 2003; Lomaga et al., 1999), which implicates this important adaptor not only in inflammation and innate immunity but also in bone remodeling. As p62 binds TRAF6 (Sanz et al., 2000) and mutations in the *p62* gene are the cause of the PDB (Hocking et al., 2002; Laurin et al., 2002), it is possible that p62, like TRAF6, may be involved in the regulation of osteoclast signaling, which is mainly controlled by the TNFα family cytokine RANK-L. This ligand binds its receptor RANK, activating a TRAF6-dependent mechanism to promote NF-κB and Src activation (Wong et al., 1999), both playing roles in osteoclast function (Franzoso et al., 1997; Iotsova et al., 1997; Soriano et al., 1991). Here we show that p62 is dramatically induced during osteoclastogenesis. The study of mice deficient for this gene reveals that it plays an important role in osteoclast differentiation and NF-κB activation in response to RANK-L in vitro and in osteoclastogenesis in vivo in mice injected with the calciotropic hormone PTHrP. Therefore, our results demonstrate that p62 is an important component in the control of induced bone remodeling.

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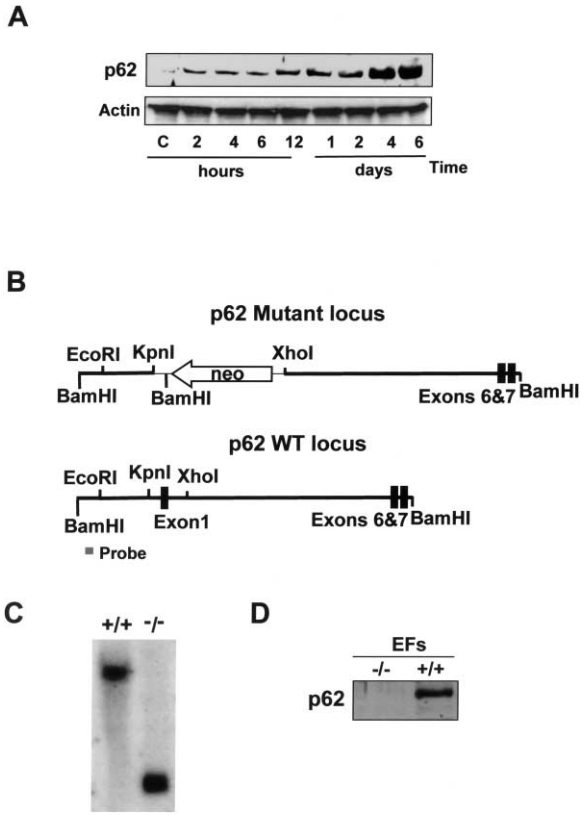

Figure 1. p62 Induction and Gene Targeting at the *p62* Locus

(A) BMDMs were stimulated with M-CSF or M-CSF plus RANK-L for different times, after which cell extracts were prepared and p62 levels determined by immunoblotting. These are representative experiments of another three with similar results.

(B) Restriction map of the *p62* locus (p62 wt); the targeting vector was integrated into the endogenous locus by homologous recombination and gave rise to the mutant allele (p62 Mutant). The boxes indicate exons.

(C) Southern blot analysis of litters derived from heterozygote intercross (+/+, wt; −/− homozygous).

(D) Immunoblot analysis of extracts from EFs wt (+/+) or p62 deficient (−/−).

Results and Discussion

Induction of p62 and Generation of *p62*$^{-/-}$ Mice

In order to determine the potential physiological role of p62, we initially incubated bone marrow-derived macrophages (BMDMs) from wild-type mice with M-CSF and RANK-L for different times, after which cell extracts were prepared and the expression of p62 was analyzed. Interestingly, a detectable increase in p62 levels was observed as early as 2 hr after RANK-L addition, but a much more dramatic rise was seen at day 1, whereas p62 levels remained elevated at days 4 and 6. To further analyze the role of this adaptor protein in vivo, we genetically inactivated the *p62* gene in mice by homologous recombination (Figure 1B). Mice heterozygous for the *p62*-disrupted allele were intercrossed to generate homozygous mutant KO mice. Southern blot analysis of genomic DNA isolated from tails revealed successful disruption of the *p62* gene (Figure 1C). The lack of expression of functional p62 was determined by immunoblotting of extracts from *p62*$^{-/-}$ embryo fibroblasts (Figure 1D).

Role of p62 in Bone Physiology

The p62 KO mice were born in Mendelian proportions and were grossly normal. Six- to eight-week-old p62 KO mice did not show any sign of osteopetrosis. Radiograms of the whole bodies did not display obvious differences between wild-type (wt) and p62 KO mice (data not shown). Histological analysis of sections from long bones (femurs and tibias) from the mutant mice revealed similar cortical thickness as compared to wt, and no alterations were found in the trabecular size and distribution or in the number of osteoclasts (Figure 2A). This suggests that basal osteoclastogenesis is not affected by the loss of p62. However, it is possible that osteoclast normal physiology in vivo under conditions in which bones are challenged by osteoclastogenic stimuli such as the calciotropic hormone PTHrP, which induces osteoclastogenesis by means of the RANK-L pathway (Li et al., 2000), may be impaired in the p62 KO mice. To address this possibility, wt and p62-deficient mice were intraperitoneally injected with 20 μg of recombinant PTHrP per day during 4 days. Afterward, mice were sacrificed, and bone osteoclastogenic activity was determined by histological analysis. Serum was also obtained from these mice, and IL-6 levels were measured by ELISA. Interestingly, the number of TRAP+ osteoclasts below the growth plate in the tibias of PTHrP-injected KO mice was severely reduced as compared to that of wt controls (Figure 2B). Thus, whereas the PTHrP-injected wt mice show 40 ± 5 osteoclasts/mm^2, identically treated KO mice only show 19 ± 3 osteoclasts/mm^2. The sham-treated wt mice show 6 ± 2 osteoclasts/mm^2, similar to what is detected in identically treated KO mice (5 ± 2 osteoclasts/mm^2). Of note, the bone volume (BV/TV) of KO mice is slightly increased as compared with wt mice (tibia wt 12.1 ± 1.5 versus tibia KO 13.9 ± 2.7; femur wt 10.2 ± 0.8 versus femur KO 12.8 ± 1.1). Consistently, there was a small increase in the trabecular number (TbN) in the p62 mutant mice as compared to wt mice (tibia wt 3.5 ± 0.3 versus tibia KO 4.0 ± 0.4; femur wt 3.6 ± 0.3 versus femur KO 4.9 ± 0.5). Also, the trabecular separation (TbSp) was slightly reduced in the mutant mice (tibia wt 259 ± 26 versus tibia KO 223 ± 28; femur wt 258 ± 23 versus femur KO 183 ± 21).

Important Role of p62 in Osteoclastogenesis In Vitro

In good agreement with the TRAP data, IL-6 production in response to PTHrP injection is reduced in the p62 KO mice (Figure 2C). IL-6 synthesis in this experimental setting is produced as a consequence of activated osteoclasts that respond to the RANK-L generated by osteoblasts and other stromal cells stimulated by PTHrP (Lee and Lorenzo, 1999). As PTHrP-induced serum RANK-L levels in p62 KO mice were indistinguishable from those in wt mice (data not shown), it can be inferred

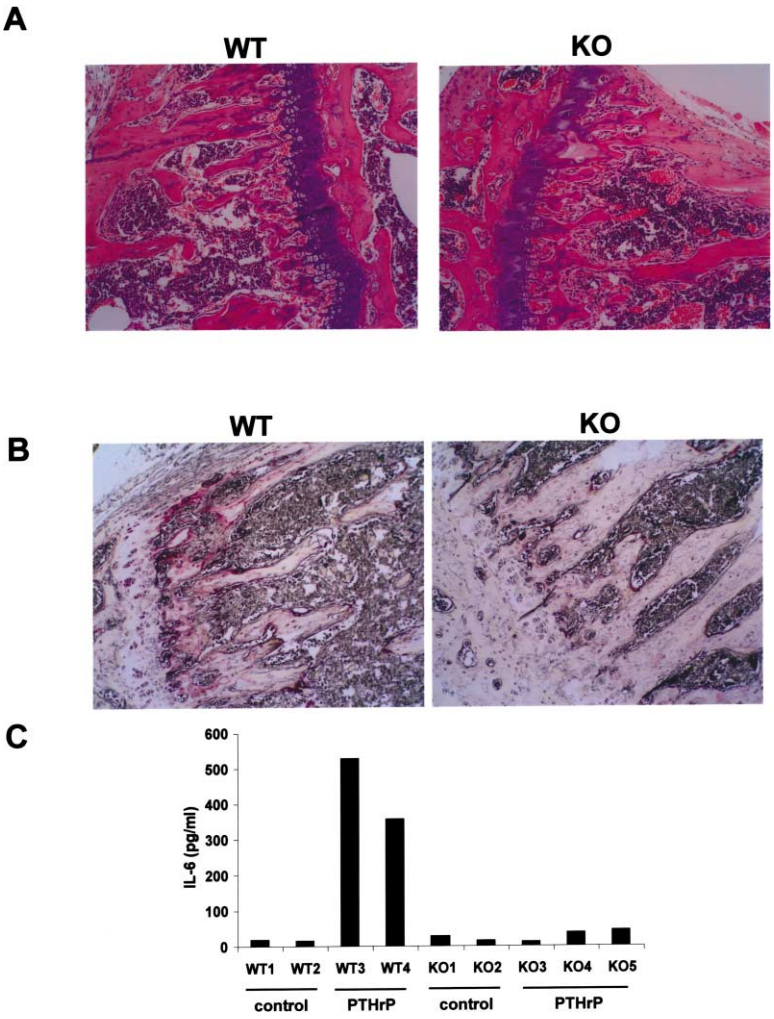

Figure 2. PTHrP-Induced Osteoclastogenesis Is Impaired in p62-Deficient Mice

(A) Hematoxylin-eosin-stained sections of tibias from untreated wt and p62 KO mice show normal bone morphology and basal osteoclastogenesis. (B) TRAP staining on sections of tibias from wt and p62 KO mice challenged with PTHrP. A significant reduction in the osteoclastogenic response to the PTHrP injection was observed in p62-deficient mice. (C) Serum IL-6 levels induced by PTHrP are impaired in the p62 KO mice. These are representative experiments of another three with similar results.

that p62 is a critical intermediary in RANK signal transduction in vivo. To determine whether the loss of p62 would affect osteoclast differentiation in vitro, BMDMs, either wt or *p62*$^{-/-}$, were incubated with M-CSF and RANK-L for 6 days, after which TRAP-positive multinucleated osteoclasts were identified. Wild-type BMDMS gave a robust osteoclastogenic response, which was dramatically inhibited in p62-deficient cultures (Figure 3A, upper panel, and 3B, left panel). These results indicate that p62 is required for an optimal induced osteoclastogenesis in vitro and in vivo. The synthesis of the transcription factor NFATc1 is potently induced during, and is required for, osteoclastogenesis (Takayanagi et al., 2002). To investigate whether the loss of p62 impacts NFATc1 synthesis in this system, BMDMs were incubated as above for different times, after which cell extracts were analyzed by immunoblotting with anti-NFATc1 antibody. The data of Figure 3C (left panel) show that RANK-L induced NFATc1 synthesis in BMDMs wt cultures but that this is dramatically reduced in p62-deficient cells, consistent with the data from the osteoclastogenesis experiments. As a control, induced TRAF6 protein levels produced by RANK-L in the same cultures is not affected by the loss of p62 (Figure 3C, left panel), indicating the specificity of the NFATc1 effects and that p62 does not control TRAF6 synthesis. As p62 binds ζPKC, which has been shown genetically to control NF-κB (Duran et al., 2003; Leitges et al., 2001), we next determined whether this aPKC is also involved in osteoclastogenesis. Results of Figure 3 (3A, lower panel; 3B, right panel; and 3C, right panel) demonstrate that the loss of ζPKC does not inhibit (but even seems to favor) osteoclast formation or NFTAc1 production. Therefore, p62 actions in this cellular system appear to be ζPKC independent.

NF-κB Signaling in p62-Deficient Osteoclasts

Previous results from our and other laboratories have suggested that p62 is necessary for NF-κB activation by linking TRAF6 to IKK stimulation (Sanz et al., 2000). Interestingly, although the early RANK-L-stimulated activation of NF-κB in BMDMs is not inhibited in the p62 KO cells (Figure 4A, left panel), which is consistent with the fact that p62 is nearly undetectable in cultures that have not been exposed to RANK-L for a relatively long period of time, at 24 hr and 48 hr of RANK-L stimulation, NF-κB activation requires p62 (Figure 4A, right panel). This requirement is maintained even at 6 days of culture

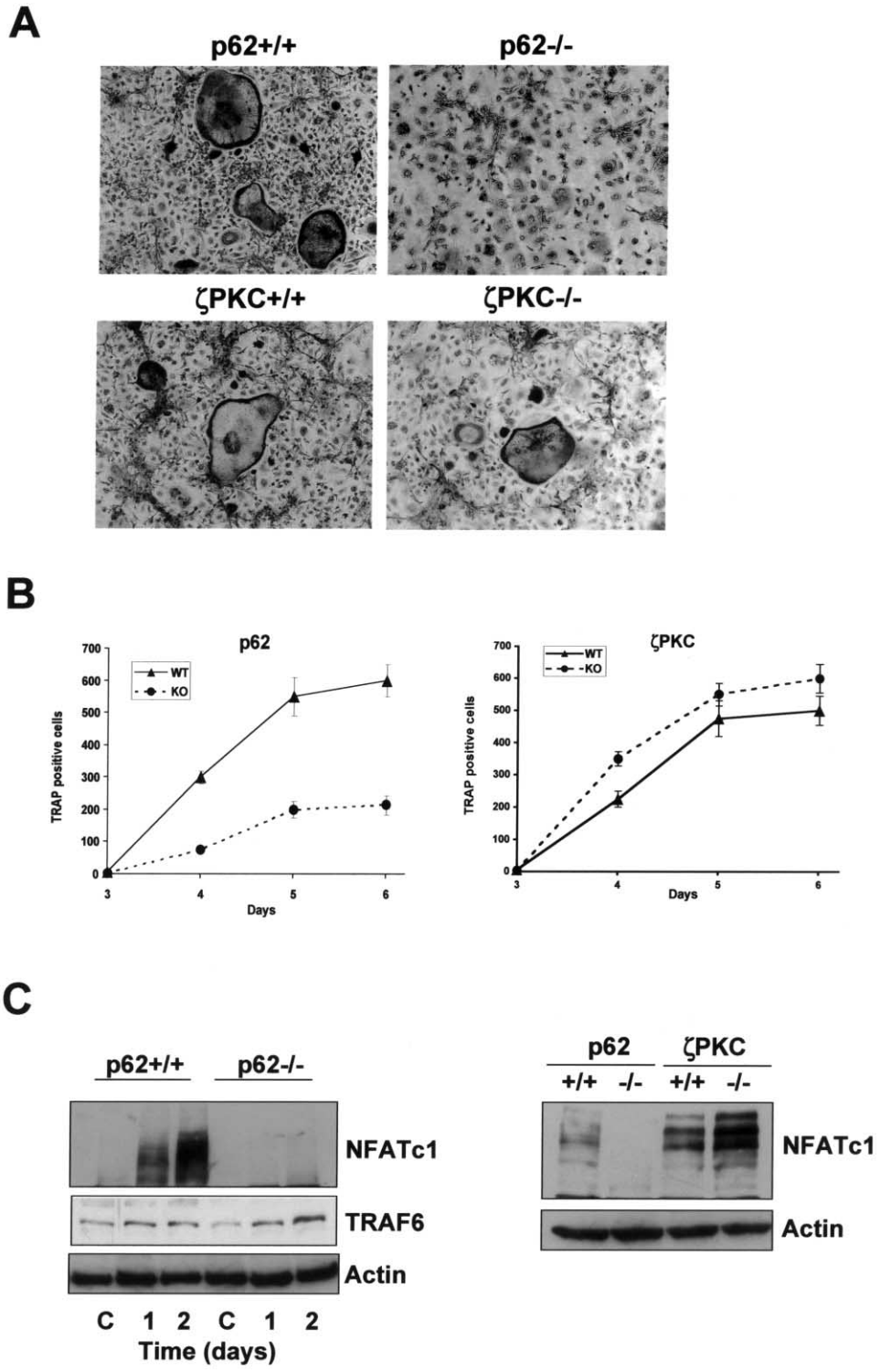

Figure 3. *p62*$^{-/-}$ but Not *ζPKC*$^{-/-}$ Mice Have Defective RANK-L-Mediated Osteoclastogenesis In Vitro

BMDMs from either wt, p62, or ζPKC KO mice (A) were incubated with M-CSF plus RANK-L for 6 days, after which the number of TRAP+ multinucleated cells was determined. (B) Quantitation of TRAP+ multinucleated cells in cultures of the above experiments. Results are the mean ± SD of three independent experiments with incubations in duplicate. (C) (Left panel) BMDMs from wt or p62 KO mice were stimulated with RANK-L for 24 or 48 hr, and the induction of NFTAc1 and TRAF6 was determined by immunoblotting. (Right panel) BMDMs as above were incubated with M-CSF plus RANK-L for 6 days, after which NFATc1 levels were determined as above. These are representative experiments of another three with similar results.

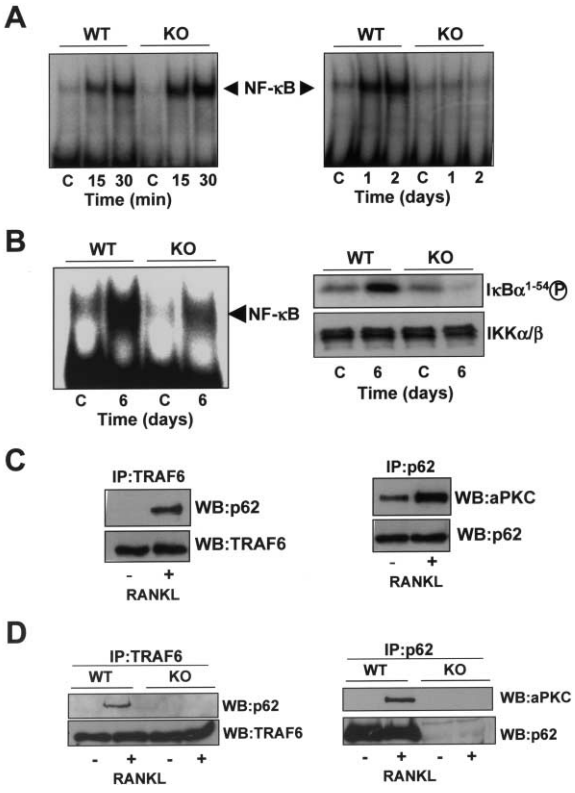

Figure 4. Role of p62 in RANK-L Signaling in Osteoclasts

(A) (Left panel) BMDMs from wt and p62 KO mice were stimulated with RANK-L for 15 and 30 min, after which nuclear extracts were prepared and analyzed by EMSA for nuclear NF-κB binding activity. (Right panel) BMDMs as above were stimulated with RANK-L for 1 and 2 days, after which nuclear NF-κB binding activity was determined. (B) In another set of experiments, BMDMs were incubated with RANK-L for 6 days, after which extracts were prepared for nuclear NF-κB activity (left panel) or immunoprecipitated with an anti-IKKγ antibody and the IKK enzymatic activity was determined. (C) RAW cells were stimulated with RANK-L for 10 min and extracts were immunoprecipitated either with anti-TRAF6 or anti-p62, after which the associated p62 or aPKC, respectively, was detected by immunoblottting with specific antibodies. (D) Extracts from BMDMs incubated as in (B) were immunoprecipitated as above. These are representative experiments of another three with similar results.

(Figure 4B, left panel), consistent with the p62 induction kinetic (Figure 1A). The action of p62 in this pathway is most likely at the level of IKK, since this activity is severely inhibited in p62 KO osteoclasts (Figure 4B, right panel).

TRAF6-p62-aPKC Complex Formation in Response to RANK-L

We next sought to determine whether TRAF6 and p62 interact in response to the stimulation with RANK-L. Thus, the preosteoclast cell line RAW 264.7 (RAW) was treated with RANK-L, cell extracts were prepared and TRAF6 was immunoprecipitated, and the associated p62 was detected by immunoblotting. Results of Figure 4C (left panel) show that RANK-L triggers the formation of a p62-TRAF6 complex. As p62 is a scaffold for the aPKCs, we next determined whether RANK activation promotes the p62-aPKC interaction. Therefore, cell extracts from the above experiment were immunoprecipitated with anti-p62 antibody and immunoblotted with an anti-aPKC antibody. There is a basal association of aPKC with p62 in unstimulated RAW cells that is dramatically induced upon RANK-L addition (Figure 4C, right panel). In addition, in primary BMDMs incubated in the presence of RANK-L as in Figure 4B, a specific association of p62 with TRAF6 and aPKC is readily detectable (Figure 4D). These results indicate that the activation of the RANK pathway promotes the formation of a TRAF6-p62-aPKC complex required for NF-κB activation, NFATc1 synthesis, and osteoclast differentiation. As the *ζPKC*$^{-/-}$ BMDMs do not have osteoclastogenic defects, collectively these results suggest that λ/ιPKC may be the aPKC involved in this pathway. The λ/ιPKC KO is embryonic lethal at very early stages (M.T.D.-M. and J.M., unpublished data) precluding any analysis of osteoclasts. Further studies using conditional KOs when available will make this analysis possible.

The signaling pathways that regulate osteoclast physiology are becoming increasingly investigated because of the great impact that alterations in bone remodeling have in human diseases. Our data identify p62 as a player in this pathway. The fact that induced but not basal osteoclastogenesis is affected by the loss of p62 is reminiscent of the *NIK*$^{-/-}$ mice phenotype (Novack et al., 2003). NIK activates a novel noncanonical NF-κB pathway that is initiated by the phosphorylation and processing of p100 (NF-κB2) to give p52/RelB complexes (Moscat et al., 2003). However, this non-canonical pathway does not appear to be affected in the p62 KO (data not shown), which suggests that both are independent but important cascades for bone remodeling during induced conditions. Recent data revealed that in *fos*$^{-/-}$ mice, bone remodeling and erosion during rheumatoid arthritis are mediated by osteoclasts (Redlich et al., 2002). It is tempting to speculate that, as p62 is important during induced but not basal osteoclastogenesis, its inhibition may be beneficial for the treatment of rheumatoid arthritis with, in principle, relatively minor toxic effects in bone basal physiology. On the other hand, it is interesting to note that the osteoclasts in the pagetic lesions are hyperactivated (Roodman, 1996), which, combined with the data shown here clearly demonstrating that p62 is necessary for osteoclastogenesis, suggests that the pagetic mutations in *p62* lead to a permanently active protein. In fact, in bone resorption assays, the activity of p62 KO osteoclastic cultures is much more reduced than that of parallel wt cultures (data not shown). In connection with this, the ectopic expression of a p62 construct harboring a pagetic mutation is more efficient in activating NF-κB than the expression of a wt construct (see Supplemental Figure S1 at http://www.developmentalcell.com/cgi/content/full/6/2/303/DC1). The precise mechanism whereby the PDB mutations affect p62 function is unclear yet, but the observation that IL-6 synthesis is severely reduced in the p62 KO mice is consistent with the fact that IL-6 levels are elevated in pagetic patients, which is responsible, at least in part, for the osteoclastic pagetic phenotype (Roodman, 1996).

Experimental Procedures

Reagents and Antibodies

Reagents were purchased as follows: soluble RANK-L and M-CSF (Peprotech) and PTHrP (American Peptide). Polyclonal antibodies anti-TRAF6, anti-ζPKC, anti-actin, anti-IKKγ, anti-IKKβ, and the monoclonal antibodies anti-NFATc1 and anti-p52 were purchased from Santa Cruz. The polyclonal affinity-purified anti-p62 was generated in our laboratory. All antibodies were used according to manufacturers' instructions.

Mice

The ζPKC KO mice (ζ*PKC*$^{-/-}$) were described previously (Leitges et al., 2001). Mice genetically deficient for *p62* (*p62*$^{-/-}$) were obtained using standard procedures. A genomic DNA segment containing murine *p62* was identified by screening a commercial library ("Down-to-the-Well," Genome Systems; 129Sv genetic background) by PCR using primers complementary to *p62* exon 1. The targeting construct generated, using the pPNT vector, eliminates exon1 of *p62*. This construct was linearized and electroporated into R1 embryonic stem 129 SvJ (ES) cells. One ES clone, with a targeted *p62* allele detected by Southern blot, was used to produce chimeric male mice which were bred to C57BL/6 female mice, and the offspring was genotyped by Southern blot. EFs were isolated from 13.5 d.p.c. embryos from crosses between *p62*$^{+/-}$ mice. All mice were born and kept under pathogen-free conditions. Animal handling protocols conform to the NIH guidelines.

Calciotropic Hormone Challenge

Age and sex matched wt and p62 KO (6- to 8-week-old) mice were injected intraperitoneal twice daily with either PTHrP (20 μg/day) or vehicle alone (PBS) during 4 days. Long bones (femurs and tibias) and sera were collected 3 hr after the final injection. Bones were fixed overnight in 10% buffered formalin, decalcified in 14% EDTA, embedded in paraffin, sectioned, and stained for hematoxylin-eosin or tartrate-resistant acid phosphatase (TRAP) using a commercial TRAP staining kit (Sigma-Aldrich) according to manufacturer's instructions. The number of osteoclast per ten fields (100× magnification) for five mice in each group was counted. Serum levels of IL-6 were determined by ELISA using the OptiEA-IL-6 kit (BD Biosciences).

Bone Histomorphometry

Bone histomorphometry was performed on standardized sections of proximal tibia and distal femur stained with hematoxylin-eosin, using a semiautomated method on Bioquant Nova Prime Image Analysis System (Bioquant, USA). All measurements were done between 0.5 and 4.5 mm from the growth plate-metaphysal junction. Primary bone parameters were measured at 10×.

Osteoclast Cultures

For in vitro osteoclastogenesis assays, nonadherent bone marrow cells (1×10^6 per well in a 24-well plate) were cultured in α-MEM with M-CSF (10 ng/ml) and soluble RANK-L (100 ng/ml) to generate osteoclasts. Six days later, TRAP+ multinucleated (>3 nuclei) osteoclasts were counted, and cell lysates were prepared to evaluate signaling during osteoclastogenesis. For some experiments, BMDMs generated from bone marrow cells cultured in M-CSF for 5 days were treated with soluble RANK-L for 24 or 48 hr, and whole-cell lysates were analyzed by immunoblotting with the different antibodies. For the coimmunoprecipitation experiments, RAW 264.7 cells were serum starved for 3 hr prior to stimulation with soluble RANK-L (100 ng/ml) for 10 min, and cells extracts were prepared in buffer PD. Immunoprecipitations were carried out as described (Sanz et al., 2000).

Electrophoretic Mobility Shift Assay, In Vitro Kinase Assay, and Luciferase Reporter Assay

EMSA experiments and luciferase reporter assays were performed as previously described (Diaz-Meco et al., 1993). Plasmid pCDNA3HA-p62 has been previously described (Sanchez et al., 1998). The p62 pagetic mutant (pCDNA3HA-p62P392L) was obtained by site-directed mutagenesis (QuickChange Kit, Stratagene). IKK activity was determined in anti-IKKγ immunoprecipitates as described (Lallena et al., 1999).

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