SHP-1 Regulation of p62DOK Tyrosine Phosphorylation in Macrophages

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SHP-1 plays key roles in the modulation of hematopoietic cell signaling. To ascertain the impact of SHP-1 on colony-stimulating factor-1 (CSF-1)-mediated survival and proliferative signaling, we compared the CSF-1 responses of primary bone marrow macrophages (BMM) from wild-type and SHP-1-deficient mice. CSF-1-induced protein tyrosine phosphorylation levels were similar in wild-type and melme BMM, except for the constitutive hyperphosphorylation of a 62-kDa phosphoprotein (pp62) in melme macrophages. pp62 was identified as the RASGAP-associated p62DOK and was shown to be the major CSF-1R-associated tyrosine-phosphorylated protein in CSF-1-treated BMM. p62DOK was found to be constitutively associated with SHP-1 in BMM and in 293T cells, co-expressing p62DOK and either wild-type or catalytically inert SHP-1 (SHP-1 C453S). In both cell types, the interaction of SHP-1 with p62DOK occurred independently of p62DOK tyrosine phosphorylation, but only the tyrosine-phosphorylated p62DOK was bound by SHP-1 C453S in a far Western analysis. These findings suggest a constitutive association of SHP-1 and p62DOK that is either conformation-dependent or indirect as well as a direct, inducible association of the SHP-1 catalytic domain with tyrosine-phosphorylated p62DOK. p62DOK hyperphosphorylation is not associated with altered CSF-1-induced RAS signaling or proliferation. However, whereas wild-type macrophages undergo cell death following CSF-1 removal, melme macrophages exhibit prolonged survival in the absence of growth factor. Thus, p62DOK is a major SHP-1 substrate whose tyrosine phosphorylation correlates with growth factor-independent survival in macrophages.

Colony-stimulating factor-1 (CSF-1) is the primary growth factor regulating the differentiation, survival, and proliferation of cells in the monocyte/macrophage lineage. CSF-1 mediates these effects by binding to the CSF-1 receptor (CSF-1R), a receptor protein tyrosine kinase (PTK) encoded by the c-fms proto-oncogene (reviewed in Ref. 1). CSF-1 treatment of macrophages results in CSF-1R dimerization, activation, and receptor transphosphorylation at specific tyrosines within the cytoplasmic domain, the latter of which mediate recruitment of cytoplasmic signaling molecules containing Src homology 2 (SH2) or phosphotyrosine binding domains (reviewed in Refs. 2 and 3). The activated CSF-1R has been shown to form multimeric complexes with a number of signal-transducing proteins including Shc, Grb2, Sos1, phosphatidylinositol 3-kinase, and Cbl (4–7). However, only the adaptor protein Grb-2 (8) and the p85 subunit of phosphatidylinositol 3-kinase (9) have been demonstrated to associate directly with the receptor via their SH2 domains. In addition, both the SH2 domain-containing protein tyrosine phosphatase, SHP-1 (10, 11), and the recently identified macrophage F-actin-associated protein, MAYP (12), are tyrosine-phosphorylated in response to CSF-1 but do not form stable complexes with the receptor. In myeloid progenitor cells, the CSF-1R has also been reported to interact with the SH2 domain-containing inositol phosphatase, SHIP (13), phospholipase C-γ2 (14), and a recently characterized adaptor protein, Mona (15). In addition to these upstream interactions, CSF-1R signaling also results in the activation of the RAS-mitogen-activated protein kinase pathway (reviewed in Ref. 3). However, the precise mechanisms whereby the activated receptor induces RAS-mitogen-activated protein kinase activation and downstream biological outcomes such as proliferation, survival, and differentiation have not yet been defined.

At present it is also unclear how signals initiated by the activated CSF-1R are modulated. However, the SHP-1 protein tyrosine phosphatase (PTP) represents a particularly attractive candidate for regulating CSF-1 signaling. SHP-1 is a nontransmembrane PTP possessing tandem amino-terminal SH2 domains followed by a single catalytic domain and a short carboxyl-terminal tail. In contrast to the closely related, ubiquitously expressed SHP-2 PTP (reviewed in Ref. 16), SHP-1 is expressed only in epithelial and hematopoietic cells and appears to act predominantly as a negative regulator of growth-promoting cell receptors. SHP-1 binds and inhibits a number of receptor PTKs, such as c-Kit, and regulates cytokine receptors lacking intrinsic tyrosine kinase activity by direct association and dephosphorylation of the associated Jak kinases (reviewed in Ref. 17). SHP-1 also inhibits signaling from both the T- and B-cell antigen receptors, by interactions with both the antigen receptor components, and PTKs such as Lck, ZAP-70, and Syk, required for antigen receptor signaling (17, 18). The inhibitory
effects of SHP-1 on these latter receptors is also realized by interactions with the immunoreceptor tyrosine-based inhibitory motifs (ITIMs) of antigen receptor co-modulators such as FcγRIIB, CD22, CD72 (reviewed in Refs. 19 and 20), and p91/PIR-B (21, 22) in B-cells. Similarly, SHP-1 inhibits T-cell activation and natural killer cell function by associating with the ITIMs of human killer inhibitory receptors (KIRs) or the murine LY49 receptors (reviewed in Refs. 23 and 24) and also associates with a number of leukocyte immunoglobulin-like receptors (reviewed in Ref. 25). Recruitment of SHP-1 to KIRs has been shown to result in the dephosphorylation of critical signaling molecules, such as the adaptor protein SLP-76 (26), which has been shown to result in the dephosphorylation of critical associates with a number of leukocyte immunoglobulin-like receptors and also constitutes tyrosine phosphorylation and cell proliferation (31), and subsequently, pp130 was shown to be a membrane-spanning glycoprotein and a subunit of SHP-1. SHP-1, with substantially reduced catalytic activity (me/me) (reviewed in Ref. 29), participates constitutively with an unidentified 130-kDa phosphotyrosyl protein (pp130) (31). Subsequently, pp130 was shown to be a membrane-spanning glycoprotein and a substate of SHP-1 in macrophages and was identified as comprising p91/PIR-B (32, 33), a recently cloned ITIM-containing inhibitory receptor (34, 35) and SHPS-1 (36), or the SHPS-1-related protein BIT (32). Both SHPS-1 and BIT belong to the Sirpα1 family of signal inhibitory related proteins, which has recently been implicated in the negative regulation of receptor PTK signaling (37). Although two major SHP-1-associated proteins have been identified in macrophages, it is unclear how impaired interactions between these proteins and SHP-1 contribute to the profound dysregulation of macrophages seen in me/me mice.

In the present study, we show that a major substrate for SHP-1 in primary bone-marrow derived macrophages (BMM) is the recently characterized signaling molecule, p62DOK. A 62-kDa phosphoprotein (p62) was originally shown to associate with the p120 RASGTPase-activating protein (GAP) and to be regulated by a number of mitogenic and transforming tyrosine kinases, including the epidermal growth factor and platelet-derived growth factor receptors, src, src, abl, and v-fms (38, 39). More recently p62 was characterized as a constitutively tyrosine-phosphorylated protein in chronic myelogenous leukemia progenitor cells (40) and was subsequently purified, cloned, and demonstrated to be the RASGAP-associated p62, termed p62DOK (for downstream of kinase) (41, 42). Our findings show that p62DOK becomes transiently tyrosine-phosphorylated and associated with the CSF-1 receptor following macrophage treatment with CSF-1. In contrast to control macrophages, SHP-1-deficient macrophages from me/me mice manifest constitutive tyrosine phosphorylation of p62DOK, which appears to represent a direct substrate of SHP-1. This constitutive tyrosine phosphorylation of p62DOK is correlated with the increased growth factor-independent survival of SHP-1-deficient macrophages.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Culture**—Fetal and thalamic bone marrow-derived macrophages (BMM) were obtained from 2- to 3-week-old me/me or me/me' and wild-type littermate control mice as described (43). Briefly, bone marrow cells were incubated for 24 h in a + medium containing essential medium containing 0.292 mg/ml glutamine, 0.02 mg/ml ascorbic acid, 5 x 10^-4 M β-mercaptoethanol, 0.2 g/liter penicillin, and 0.2 g/liter streptomycin supplemented with 15% fetal calf serum (BioWhittaker), 10% WEHI-3 conditioned media, as a source of interleukin-3, and 12 ng/ml CSF-1 (a gift from Chiron, Inc.). Non-adherent primitive mononuclear phagocytes were reseeded in a + media containing 15% fetal calf serum and CSF-1 (120 ng/ml) for 48 h, in order to allow their differentiation to adherent mononuclear phagocytes. The resulting adherent macrophages were passaged once into experimental plates. BAC1.2FS cell line macrophages were maintained in a + media supplemented with 10% newborn calf serum and 36 ng/ml CSF-1. Cells (either BMM or BAC1.2FS) used in immunoprecipitation and immunoblot experiments were cultured until subconfluence (cell/plate) and then incubated for ~20 h in the absence of CSF-1 to allow for CSF-1R up-regulation. Up-regulated cells were stimulated with CSF-1 (360 ng/ml) for various times and then solubilized as described below. Cells used for apoptosis assays were cultured until confluent and then maintained in media without CSF-1 for various times. Fragmented genomic DNA was isolated (44) and separated on a 1.5% agarose gel and stained with ethidium bromide. Chinese hamster ovary cells stably expressing the insulin receptor (45) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.292 mg/ml glutamine, 0.2 g/liter penicillin, 0.2 g/liter streptomycin, and 10% fetal calf serum. Cells used for experiments were cultured until subconfluently maintained in media without serum for approximately 48 h, and then stimulated with 10 ng insulin.

For analysis of BMM growth rates, me/me and wild-type macrophages were plated at 2 x 10^4 cells in 2 ml of a + medium (containing 15% fetal calf serum and 120 ng/ml CSF-1) per 35-mm dish. After 48 h (day 1) the medium was removed, and the cells were rinsed with medium without CSF-1 and refed with media with or without CSF-1. Subsequently the medium was changed every 3 days. On day 6 some of the dishes grown in medium without CSF-1 were refed with medium containing CSF-1. Cell counts were performed daily on triplicate cultures. Adherent macrophages were removed using 0.005% CHAPS (Sigma), diluted in Isoton II (Curtin Matheson Scientific, Inc.), and counted electronically using a Coulter Counter ZM.

**Antibodies**—A polyclonal rabbit antibody was prepared against a GST fusion protein encoding the amino-terminal half of p62DOK, but because of the relatively lower affinity of this antigen it was only used in one experiment (Fig. 4A). For all subsequent experiments, the 2C4 monoclonal antibody that recognizes p62DOK (Babco) was used for immunoprecipitation, and a monoclonal antibody directed against the amino-terminal region of p62DOK (Santa Cruz Biotechnology) was used for immunoblotting. Peptide purified antibodies to CSF-1R have been described previously (46) and were used for both immunoprecipitation and immunoblotting. Antibodies to phosphotyrosine (PY-20) was from Transduction Laboratories, and RAS antibody Y13-259 linked to agarose was from Oncogene Science.

**Immunoprecipitations and Western Blotting**—Cells were solubilized in ice-cold Nonident P-40 solubilization buffer (10 mM Tris-HCl, pH 7.0, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 0.5% Nonident P-40, and 5 μM ZnCl2). Whole cell lysates were normalized for equal total protein using the BCA protein assay reagent kit (Pierce) or by immunoblotting for CSF-1R protein. Lysates containing equivalent amounts of total protein or CSF-1R were used in subsequent experiments. Normalized Nonident P-40 cell lysates were incubated with 10 μg of purified antibodies or with a 1:50 dilution of antiseraum, and the immunoprecipitates were analyzed by SDS-PAGE and Western blotting as described previously (10). For Western blotting all primary antibodies were used at 2 μg/ml and secondary antibodies linked to horseradish peroxidase (Pierce) were diluted 1:5000, except for immunoblotting.
with anti-phosphotyrosine (anti-PY) antibody, where a monoclonal antibody linked to horseradish peroxidase (Transduction Laboratories) was used at a dilution of 1:7500. Resulting signals were detected by ECL (Amersham Pharmacia Biotech).

Expression Constructs and Transfections—Expression plasmids for c-fos, p62DOK, and SHP-1 were obtained by subcloning each of these cDNAs into the pCMV5 vector. The catalytically inactive (C453S) SHP-1 was generated by introducing a point mutation using standard polymerase chain reaction techniques. All expression constructs were sequenced and subsequently tested by transient transfection into human embryonic kidney 293T cells using the standard calcium-phosphate method. Transfected cells were lysed in Nonidet P-40 lysis buffer and analyzed by Western blotting with anti-CSF-1R, anti-p62DOK, and anti-SHP-1 antibodies.

In Vitro Binding Reactions and Far Western Analysis—Glutathione S-transferase (GST) expression plasmids encoding the tandem SH2 domains of SHP-1, full-length wild-type and C453S SHP-1 (47), were transformed into Escherichia coli DH5α, and the fusion proteins were purified from isopropyl-1-thio-β-D-galactopyranoside-induced bacteria with glutathione-conjugated Sepharose beads. The GST fusion protein encoding the RASGAP amino-terminal SH2 domain was from Santa Cruz Biotechnology. For binding studies, 5 µg of each GST fusion protein immobilized on glutathione beads were incubated with 1 µg of protein lysate overnight at 4 °C. The beads and bound proteins were washed four times with Nonidet P-40 lysis buffer, eluted with SDS-PAGE sample buffer, and analyzed by SDS-PAGE followed by anti-PY and anti-p62DOK immunoblotting. For far western analyses, the immobilized GST fusion proteins were eluted by incubation with 10 mM glutathione (Sigma) and the eluted proteins quantitated by SDS-PAGE and Coomassie Blue staining. Western blots were blocked in 5% non-fat dry milk for 2 h at room temperature and then incubated with 1 µg/ml of the appropriate GST fusion protein overnight at 4 °C. Washed blots were incubated with 1 µg/ml of a polyclonal GST antibody (Santa Cruz Biotechnology) for 2 h at room temperature. Washed blots were then incubated with a secondary antibody linked to horseradish peroxidase and developed with ECL.

In Vitro Dephosphorylation Reactions—me/me macrophage protein lysates were treated with 10 mM iodoacetic acid to inactivate irreversibly endogenous PTPs. The lysates were then immunoprecipitated with either p62DOK or α-CSF-1R antibodies, and the resulting immune complexes were collected with protein G-Sepharose (Zymed Laboratories Inc.) and washed three times with Nonidet P-40 lysis buffer, twice in phosphatase wash buffer (10 mM Tris, pH 7.5, 50 mM NaCl, and 0.5% Nonidet P-40), and once in phosphatase buffer (10 mM Tris, pH 7.4, 10 mM EDTA, 0.1% β-mercaptoethanol, 1 mg/ml bovine serum albumin). The immune complexes were then resuspended in phosphatase buffer supplemented with either GST-SHP-1 wild-type or GST-SHP-1 C453S and incubated at 37 °C for various times. The reactions were stopped by washing once in ice-cold wash buffer (without Nonidet P-40) with 10 mM iodoacetic acid and once in ice-cold wash buffer with 100 µM sodium orthovanadate, followed by elution in SDS sample buffer. The reactions were analyzed by SDS-PAGE and anti-PY Western blotting.

Analysis of Nucleotides Bound to RAS—This analysis was performed as described previously (48). Briefly, subconfluent plates of me/me and wild-type BMM were incubated for 16 h in phosphate-free DMEM supplemented with 10% dialyzed fetal calf serum. The plates were rinsed 3 times with phosphate-free DMEM and labeled for 4 h in phosphate-free DMEM supplemented with 10% dialyzed fetal calf serum and 250 µCi/ml [32P]orthophosphate (Amersham Pharmacia Biotech). At the end of the labeling period, the cells were treated with 360 ng/ml CSF-1 for the indicated times, washed 3 times with ice-cold phosphate-buffered saline, and scrapped into 1 ml of lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20 mM MgCl2, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, vortexed, and centrifuged for 20 min. The supernatant was incubated with 10 µg of agarose-immobilized RAS antibody with gentle agitation for 2 h at 4 °C. Immuno precipitates were collected by centrifugation and washed twice with lysis buffer and three times with phosphate-buffered saline. The resulting pellets were resuspended in 25 µl of 0.75 M KH2PO4, pH 3.4, 5 mM EDTA, 0.1 mM GTP, and 0.1 mM GDP and heated at 75 °C for 5 min. Equal counts of the eluates were spotted onto polyethyleneimine-cellulose thin layer plates and developed with 1 M KH2PO4, pH 4.0. The plates were exposed to film for approximately 4 h, and the resulting autoradiographic signals were quantitated by densitometry using the ImageQuant densitometry program (Molecular Dynamics).

**RESULTS**

**pp62 Is the Major Protein Hyperphosphorylated on Tyrosine in me/me Macrophages**—To identify potential substrates of SHP-1 in myeloid cells, bone marrow-derived macrophages (BMM) from wild-type and me/me mice were compared with respect to the pattern of CSF-1-induced protein tyrosine phosphorylation. To this end, wild-type and me/me macrophages were stimulated with CSF-1 for various times, and the cells were then subjected to lysis in Nonidet P-40, followed by SDS-PAGE and immunoblotting with anti-phosphotyrosine (PY) antibody. In both wild-type and me/me cells, CSF-1 stimulation resulted in transient tyrosine phosphorylation of the CSF-1R and several other proteins; the phosphorylation levels peaked at 1 min, and returned to a basal level by 10 min (Fig. 1). The kinetics and level of total protein tyrosine phosphorylation appeared similar in the wild-type and mutant cells, although tyrosine phosphorylation of both the CSF-1R and a phosphoprotein of ~95 kDa was slightly elevated in me/me BMM. Most significantly, however, the control and me/me cells differed substantially with respect to the tyrosine phosphorylation of a 62-kDa phosphoprotein, pp62, one of the major tyrosine-phosphorylated proteins observed in me/me cells. In contrast to its transient and rather modest tyrosine phosphorylation in CSF-1-treated wild-type cells, pp62 appeared significantly more phosphorylated in the CSF-1-treated me/me cells and also remained phosphorylated in the latter cells for a more prolonged period. Even more striking, however, was the constitutive tyrosine phosphorylation of pp62 observed in the absence of CSF-1 only in me/me cells. These results thus identify pp62 as a potential participant in CSF-1R signaling and also indi-

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**Fig. 1.** Increased tyrosine phosphorylation of a 62-kDa phosphoprotein (pp62) in me/me macrophages. Wild-type (Wt) and me/me BMM were incubated with CSF-1 for the indicated times. Nonidet P-40 cell lysates were prepared and analyzed by SDS-PAGE and immunoblotting with anti-phosphotyrosine (α-PY), α-CSF-1R, and α-SHP-1 antibodies. Western blot.
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**Fig. 2. Identification of \( pp62 \) as RASGAP-associated \( p62^{DOK} \).** Nonidet P-40 cell lysates were prepared from wild-type (W) or me/me (M) BMM cultured in the absence (B and D) or presence (A) of CSF-1 or from CHO-IR cells, unstimulated, or stimulated with insulin (C). Nonidet P-40 whole cell lysates (WCL) were analyzed directly by SDS-PAGE and immunoblotting with α-PY (lanes 1, 2, 9, 10, 21, and 22) or subjected to either immunoprecipitation with α-PY antibody (lanes 3, 4, 11, and 12) or α-\( p62^{DOK} \) antibody (lanes 5, 6, 13, 14, 17, and 18), or to an in vitro binding reaction with immobilized GST fusion protein containing the GAP amino-terminal SH2 domain (lanes 7, 8, 15, 16, 19, and 20). The immunoprecipitates and GST-GAP-SH2 complexes were then subjected to SDS-PAGE and α-PY immunoblotting. D, me/me cell lysates prepared either before (lane 21) or after (lane 22) immunodepletion with α-\( p62^{DOK} \) antibody were fractionated by SDS-PAGE and then subjected to α-PY immunoblotting analysis. **WB,** Western blot.

**pp62 Is the RASGAP-associated \( p62^{DOK} \).** The RASGAP-associated \( p62 \) is one of the many proteins that becomes tyrosine-phosphorylated in hematopoietic cells in response to activation of receptor PTKs. A \( p62 \) was originally described as a protein that is tyrosine-phosphorylated by the activated epidermal growth factor receptor and by oncogenic protein tyrosine kinases (38, 39), and subsequently was shown to be regulated by a number of growth factor receptors, including the CSF-1 (49) and insulin (50) receptors. More recently \( p62 \) was shown to be hyperphosphorylated on tyrosine in chronic myeloid leukemic cells expressing the BCR-ABL gene product and a \( v-abl \)-transformed murine precursor B-cell line, and its purification from these cells resulted in the cloning and characterization of \( p62^{DOK} \) (41, 42). Although \( p62^{DOK} \) was previously confused with the Sam68 protein (51), a monoclonal antibody (identified as 2C4) that had been demonstrated to react specifically with the RASGAP-associated \( p62 \) in insulin-stimulated cells (50) has now been shown to recognize \( p62^{DOK} \) (42). To determine whether the 62-kDa phosphoprotein showing increased tyrosine phosphorylation in me/me BMM was \( p62^{DOK} \), Nonidet P-40 lysates of BMM cultured in the presence of CSF-1 were immunoprecipitated with the 2C4 monoclonal antibody, and the precipitated proteins were then subjected to SDS-PAGE and anti-PY immunoblotting analysis. As shown in Fig. 2A, the results of this analysis revealed tyrosine phosphorylation of \( p62^{DOK} \) to be increased in me/me compared with wild-type cells (lanes 5 and 6) and also that the tyrosine-phosphorylated \( p62^{DOK} \) comigrated with the 62-kDa phosphoprotein that was selectively hyperphosphorylated in me/me macrophage lysates (lanes 1 and 2) and anti-PY immunoprecipitates (lanes 3 and 4). To confirm the identity of \( p62 \), proteins in wild-type and me/me cell lysates that specifically bound to a GST fusion protein containing the RASGAP amino-terminal SH2 domain were also subjected to SDS-PAGE and anti-PY immunoblotting (lanes 7 and 8). The results of this analysis indicated that the RASGAP SH2 domain fusion protein, like the anti-\( p62^{DOK} \) antibody, selectively recovered a 62-kDa phosphoprotein showing increased tyrosine phosphorylation in the me/me cells. Similar results were observed when this analysis was repeated using BMM cultured in the absence of CSF-1, a finding that is consistent with the contention that \( pp62 \) represents \( p62^{DOK} \) (Fig. 2B). The monoclonal antibody to \( p62 \) and the GAP SH2 domain fusion protein also selectively recovered an insulin-responsive 62-kDa phosphotyrosyl-containing protein from Chinese hamster ovary cells overexpressing the insulin receptor (CHO-IR cells) (Fig. 2C), the system in which the insulin-responsive \( p62 \) was first described (50). To determine whether \( p62^{DOK} \) accounts for all of the hyperphosphorylated 62-kDa protein detected in me/me macrophages, \( p62^{DOK} \) was first depleted from me/me macrophage lysates by two successive immunoprecipitations with the 2C4 anti-\( p62^{DOK} \) antibody, and the lysates were then immunoblotted with anti-PY. As evident in Fig. 2D, \( p62^{DOK} \) depletion removed the 62-kDa hyperphosphorylated protein from the me/me macrophage lysate. Together, these observations indicate that the \( pp62 \) species showing increased tyrosine phosphorylation in me/me BMM is \( p62^{DOK} \).

**\( p62^{DOK} \) Is a Major CSF-1R-Associated Protein—**Several proteins that are rapidly tyrosine-phosphorylated in the macrophage response to CSF-1 exhibit CSF-1-induced association with the CSF-1R. Among these are the c-cbl proto-oncogene product and Shc (6). To determine whether \( p62^{DOK} \) behaves similarly, anti-CSF-1R immunoprecipitates were subjected to SDS-PAGE and immunoblotted with anti-PY, anti-\( p62^{DOK} \), and anti-CSF-1R antibodies. As shown in Fig. 3, the results of anti-PY immunoblotting analysis revealed a 62-kDa phosphoprotein to be the major tyrosine-phosphorylated protein associated with the CSF-1R in BMM and indicated the kinetics of its tyrosine phosphorylation to be similar to those observed for \( pp62 \) in anti-PY immunoblots of whole cell lysates (Fig. 1). In both cases, \( pp62 \) was found to be hyperphosphorylated in me/me, compared with control CSF-1-treated BMM, and again to be constitutively tyrosine-phosphorylated in me/me but not wild-type BMM. Anti-\( p62^{DOK} \) immunoblotting confirmed the CSF-1R-associated 62-kDa protein to be \( p62^{DOK} \) but also indicated the \( p62^{DOK}/CSF-1R \) association to be comparable in control and mutant cells and to be transiently increased at 1 min after CSF-1 stimulation. Thus, whereas \( p62^{DOK} \) tyrosine phosphorylation is constitutively and inducibly increased in me/me cells, the amount of \( p62^{DOK} \) that binds to the CSF-1R is not altered in the context of the me/me mutation.

**\( p62^{DOK} \) Associates with SHP-1 in Macrophages—**To ascertain whether hyperphosphorylation of \( p62^{DOK} \) in SHP-1-deficient BMM relates to the capacity for SHP-1 to bind and de-
phosphorylate p62DOK, anti-p62DOK immunoprecipitates from BMM cultured with and without CSF-1 were subjected to SDS-PAGE and immunoblotting with α-PY, α-p62DOK, and α-CSF-1R. WB, Western blot.

Fig. 3. Association of p62DOK with the CSF-1R in macrophages. Wild-type (Wt) and me/me BMM were stimulated with CSF-1 for the indicated times. α-CSF-1R immunoprecipitates (IP) of Nonidet P-40 cell lysates were analyzed by SDS-PAGE followed by immunoblotting with α-PY, α-p62DOK, and α-CSF-1R. WB, Western blot.

Fig. 4. SHP-1/p62DOK association in macrophages. A, wild-type (Wt) and me/me BMM were processed as in Fig. 3, and α-p62DOK immunoprecipitates (IP) of the resulting cell lysates were analyzed by SDS-PAGE followed by immunoblotting with α-PY, α-SHP-1, and a polyclonal antibody to p62DOK. B, wild-type and me/me BMM were similarly processed, and α-SHP-1 immunoprecipitates of the resulting lysates were analyzed by SDS-PAGE followed by immunoblotting with α-PY and a monoclonal α-p62DOK antibody. The same lysates were subjected to SDS-PAGE followed by immunoblotting with α-SHP-1 antibody to demonstrate equal loading. Wild-type whole cell lysate (WCL) was used to document the presence and mobility of both p62DOK and SHP-1. WB, Western blot.
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**SHP-1 Dephosphorylates \( \text{p62}^{\text{DOK}} \) in Vitro and in Vivo**—The association of SHP-1 with \( \text{p62}^{\text{DOK}} \) was also detected in the SHP-1 immunoprecipitates from both wild-type and \( \text{me}^{-}/\text{me}^{-} \) lysates (Fig. 4B). Reprobing this blot with a monoclonal antibody to \( \text{p62}^{\text{DOK}} \) revealed this species to be \( \text{p62}^{\text{DOK}} \) and thus indicated that a small fraction of total cellular \( \text{p62}^{\text{DOK}} \) co-immunoprecipitates with SHP-1 from wild-type cells, and also to a slightly greater extent with the mutated SHP-1 found in \( \text{me}^{-}/\text{me}^{-} \) BMM. These results indicate that SHP-1 and \( \text{p62}^{\text{DOK}} \) associate in vivo, and the constitutive nature of the association implies that it is phosphotyrosine-independent.

**C453S SHP-1 Association in 293T Cells**—To confirm the capacity of SHP-1 to dephosphorylate \( \text{p62}^{\text{DOK}} \) in vivo, a transient transfection system was developed involving introduction of \( \text{p62}^{\text{DOK}} \), c-fins, and wild-type or C453S SHP-1 into 293T cells, which normally express negligible levels of SHP-1 and no CSF-1R. SHP-1 C453S was used in these studies as a potential “substrate trap,” a property that has been well established for many PTPs mutated at this catalytic cysteine site. Following transfection with \( \text{p62}^{\text{DOK}} \) and c-fins, alone, or in combination with either wild-type or C453S SHP-1, the transfected cells were incubated with CSF-1R and \( \text{p62}^{\text{DOK}} \). To demonstrate in vitro substrate specificity, the kinetics of SHP-1-mediated dephosphorylation were compared between \( \text{p62}^{\text{DOK}} \) and the CSF-1R, a protein that does not appear to be significantly regulated by SHP-1 in vivo. Both \( \text{p62}^{\text{DOK}} \) and the CSF-1R were immunoprecipitated from CSF-1-stimulated \( \text{me}^{-}/\text{me}^{-} \) macrophages, and the immune complexes were then incubated for various times with recombinant wild-type SHP-1. As shown in Fig. 5B, although both the CSF-1R and \( \text{p62}^{\text{DOK}} \) can serve as substrates for SHP-1 in vitro, \( \text{p62}^{\text{DOK}} \) is much more efficiently dephosphorylated by SHP-1 than is the CSF-1R (Fig. 5B, left panel). Interestingly, these data suggest that \( \text{p62}^{\text{DOK}} \) species associated with the CSF-1R to be an even more efficient substrate of SHP-1 than total cellular \( \text{p62}^{\text{DOK}} \) (Fig. 5B).

**Dephosphorylation in Vivo**—To examine \( \text{p62}^{\text{DOK}} \) association in vivo, BMM were cultured in the presence of CSF-1 until subconfluent and then lysed in Nonidet P-40, a washed \( \text{p62}^{\text{DOK}} \) immunoprecipitates of the lysates were incubated with the indicated amounts of GST fusion proteins containing either the full-length wild-type SHP-1 (WT) or the C453S form of SHP-1 (CS) for 30 min at 37 °C in phosphatase reaction buffer. Reactions were stopped by the addition of SDS sample buffer, and the incubation mixture was analyzed by SDS-PAGE and immunoblotting with anti-PY. B, Nonidet P-40 lysates were immunoprecipitated (IP) with either \( \alpha\text{-CSF-1R} \) antibody (left panel) or \( \alpha\text{-p62}^{\text{DOK}} \) antibody (right panel), and the immune complexes were incubated with recombinant wild-type (WT) SHP-1 (2 μg) for the indicated times at 37 °C prior to analysis as in A. C, the kinetics of SHP-1-mediated \( \text{p62}^{\text{DOK}} \) and CSF-1R (square) dephosphorylation (in B) were compared by plotting the % zero time tyrosine phosphorylation at each time point (determined densitometrically) versus reaction time. WB, Western blot.
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Fig. 6. SHP-1 regulation of p62DOK in 293T cells. 293T cells were co-transfected with c-fms and p62DOK expression constructs, alone (control, lanes 1–4) or in combination with expression constructs encoding SHP-1 WT (lanes 5–8) or SHP-1 C453S (lanes 9–12). Following transfection, cells were cultured for 24 h in complete media and then cultured in low serum (0.5%) containing media for an additional 24 h before stimulation with CSF-1 for the indicated times. Nonidet P-40 cell lysates were prepared and immunoprecipitated (IP) with either α-p62DOK (A) or α-SHP-1 (B), followed by SDS-PAGE and Western blotting (WB) with either α-PY, α-p62DOK, or α-SHP-1 antibody.

Lane: 1 2 3 4 5 6 7 8 9 10 11 12

A

\[
\begin{array}{ccc}
\text{control} & \text{SHP-1 WT} & \text{SHP-1 CS} \\
0 & 1 & 3 & 10 & 0 & 1 & 3 & 10 & 0 & 1 & 3 & 10 \\
95 & 64 & 36 & & & & & & & & & \\
p62DOK \\
\end{array}
\]

I P: α-p62DOK

WB: α-PY

B

\[
\begin{array}{ccc}
\text{control} & \text{SHP-1 WT} & \text{SHP-1 CS} \\
0 & 1 & 3 & 10 & 0 & 1 & 3 & 10 & 0 & 1 & 3 & 10 \\
95 & 64 & 36 & & & & & & & & & \\
\text{SHP-1/ p62DOK} \\
\end{array}
\]

I P: α-SHP-1

WB: α-PY

C453S SHP-1 in Vitro—The above data indicate that SHP-1 and p62DOK associate in vivo but provide no information as to whether this association is direct or indirect. To address this question, a far Western experiment was performed, the rationale being that this approach facilitates analysis of direct protein-protein interactions. This approach was used to derive conclusive evidence that p130Cas is a direct substrate of the Yersinia PTP YopH (53) and that SLP-76 is a direct substrate of SHP-1 (26). Accordingly, p62DOK immunoprecipitates from control and CSF-1-treated 293T cells, co-transfected with c-fms and p62DOK, were separated on SDS-PAGE, transferred to polyvinylidene fluoride membrane, and subjected to either immunoblotting with anti-PY and anti-p62DOK antibodies or, alternatively, to probing with GST fusion proteins containing either the SHP-1 SH2 domains (SH2), the full-length wild-type SHP-1 (SHP-1 WT), or the full-length SHP-1 C453S mutant (SHP-1 C453S). CSF-1R and SHP-1 immunoprecipitates (from CSF-1-stimulated BAC1.2F5 cells) were processed similarly and employed as negative and positive controls, respectively. As illustrated in Fig. 7A, SHP-1 C453S bound p62DOK, but only upon induction of p62DOK tyrosine phosphorylation following CSF-1 treatment (compare lanes 3, 4, 19, and 20). In fact, the data indicate that SHP-1 C453S bound only a portion of the total tyrosine-phosphorylated p62DOK (lane 20). Consistent with published data, SHP-1 C453S also bound the SHP-1-associated 130-kDa phosphoprotein previously identified as PIR-B, and an additional 120-kDa SHP-1-associated phosphotyrosyl protein (pp120) (compare lanes 2 and 18). No association was observed, however, between SHP-1 C453S and the stimulated CSF-1R, despite significant CSF-1R tyrosine phosphorylation (compare lanes 1 and 17). In contrast to the SH2-mediated binding of SHP-1 to PIR-B (compare lanes 10 and 18), SHP-1 binding to p62DOK (lane 20) does not appear to be mediated by its SH2 domains (lane 12). Furthermore, whereas the wild-type SHP-1 bound both PIR-B and pp120, albeit less efficiently than SHP-1 C453S (compare lanes 14 and 18), no association was detected between wild-type SHP-1 and tyrosine-phosphorylated p62DOK (lane 16).

These findings, which indicate direct p62DOK/SHP-1 association to be dependent on p62DOK tyrosine phosphorylation, contrast with the earlier data indicating that in vivo these proteins associate constitutively and thus largely independently of p62DOK phosphorylation. This discrepancy may reflect the existence of additional protein interactions in vivo which facilitate an indirect association of p62DOK and SHP-1, or alternatively an additional direct interaction between SHP-1 and p62DOK, which may not be detectable by far Western analysis. Interestingly, the results of in vitro binding assays, utilizing the same GST fusion proteins immobilized on glutathione beads and incubated with Nonidet P-40 lysates from 293T cells (co-transfected with c-fms and p62DOK), revealed that SHP-1 C453S bound only a small fraction of tyrosine-phosphorylated p62DOK (Fig. 7B, top panel), whereas both SHP-1 C453S and SHP-1 WT bound a more significant but similar amount of total p62DOK in a phosphotyrosine-independent manner (Fig. 7B, bottom panel). Thus, the constitutive association of p62DOK and SHP-1 (Fig. 6) in vivo appears to be mirrored by the in vitro binding data. The failure of SHP-1 C453S to bind a significant amount of tyrosine-phosphorylated p62DOK under non-denaturing conditions in vitro and in vivo suggests that some of the p62DOK phosphotyrosines involved in this interaction may be masked by other binding proteins.

Das Signaling Is Unaltered by the Presence of Hyperphosphorylated p62DOK in mel/mel BMM—Earlier studies identifying tyrosine-phosphorylated p62DOK as a major RASGAP-associated protein following growth factor stimulation, and in several
assays. Cell lysates were incubated with immobilized GST fusion proteins corresponding to those used as probes in lanes 6, 10, 14, in each of the lanes containing SHP-1 immunoprecipitates (lanes 1, 5, 9, 13, and 17) and SHP-1 immunoprecipitates (IP) (lanes 2, 6, 10, 14, and 18) from CSF-1-treated BAC1.2F5 cells were processed similarly. The band migrating at ~55 kDa in each of the lanes containing SHP-1 immunoprecipitates (lanes 6, 10, 14, and 18) represents the rabbit IgG heavy chain. B, in vitro binding assays. Cell lysates were incubated with immobilized GST fusion proteins corresponding to those used as probes in A, and the bound proteins were analyzed by SDS-PAGE followed by Western blotting (WB) with α-PY (top panel) or α-p62DOK (bottom panel) antibody.

Increased Tyrosine Phosphorylation of p62DOK Is Associated with Growth Factor-independent Survival in me/me Macrophages—The correlation of p62DOK tyrosine phosphorylation with cellular transformation by activated tyrosine kinases coupled with our finding that p62DOK is constitutively hyperphosphorylated in me/me BMM suggests that me/me macrophages may exhibit increased proliferative or survival potential in the absence and/or presence of growth factor. Macrophages normally proliferate in response to CSF-1 or granulocyte-macrophage CSF (GM-CSF) and undergo cell death following growth factor removal. Previous studies have indicated that me/me BMM are hyper-responsive to CSF-1 (31) and/or GM-CSF (54), but survival and/or proliferation in the absence of growth factor has not been formally investigated in these cells. To address this issue, me/me and wild-type BMM were cultured at low initial cell density in the presence or absence of CSF-1, and cell numbers were monitored over a 10-day period. The data in Fig. 9A (left panel) show that CSF-1-stimulated me/me and wild-type BMM proliferate at almost identical rates, although the me/me cultures showed a higher final cell density than those established from wild-type mice. However, the most striking findings were observed following CSF-1 removal, at which time the macrophage number in cultures from normal mice markedly decreased, whereas the macrophage number in cultures from me/me mice remained relatively constant. When CSF-1 was added back to me/me and wild-type cells after 6 days of CSF-1 deprivation, me/me, but not wild-type cells, proliferated with approximately the same doubling time as before CSF-1 removal (Fig. 9A, right panel), a result consistent with the capacity of me/me macrophages to survive in the absence of growth factor. An analysis of cell death was also performed by evaluating genomic DNA fragmentation following growth factor removal, and the results revealed me/me macrophages to be resistant to DNA fragmentation following CSF-1 removal (Fig. 9, lanes 6–8); these cells exhibit approximately the same level of DNA fragmentation as seen in wild-type cells cultured in the presence of CSF-1 (lane 1). By contrast, in wild-type macrophages DNA fragmentation characteristic of apoptosis was detected rapidly after CSF-1 removal, peaking at the 24- and 48-h time points (Fig. 9B, lanes 2 and 3). These findings

Fig. 7. SHP-1/p62DOK association in vitro. 293T cells, co-transfected with c-fms and p62DOK expression constructs, were treated in the absence or presence of CSF-1 for 1 min and then lysed in Nonidet P-40. A, far Western analysis. p62DOK immunoprecipitates were separated by SDS-PAGE, transferred to polyvinylidene fluoride membrane, and either Western blotted with α-PY (lanes 3 and 4) or α-p62DOK (lanes 21 and 22), or probed by far Western blot with GST fusion proteins containing GST (lanes 7 and 8), SHP-1 SH2 domains (SH2) (lanes 11 and 12), or the full-length GST (lanes 15 and 16), or the full-length C453S (CS) SHP-1 (lanes 19 and 20). CSF-1R (lanes 1, 5, 9, 13, and 17) and SHP-1 immunoprecipitates (IP) (lanes 2, 6, 10, 14, and 18) from CSF-1-treated BAC1.2F5 cells were processed similarly. The band migrating at ~55 kDa in each of the lanes containing SHP-1 immunoprecipitates (lanes 6, 10, 14, and 18) represents the rabbit IgG heavy chain. B, in vitro binding assays. Cell lysates were incubated with immobilized GST fusion proteins corresponding to those used as probes in A, and the bound proteins were analyzed by SDS-PAGE followed by Western blotting (WB) with α-PY (top panel) or α-p62DOK (bottom panel) antibody.
SHP-1 Regulation of p62DOK Tyrosine Phosphorylation

DISCUSSION

The SHP-1 tyrosine phosphatase has been implicated in CSF-1R signaling and has also been shown to interact with several growth inhibitory receptors in macrophages, namely PIR-B (32, 33) and SHPS-1 (32, 36). At present, however, little is known about the cytosolic signaling effectors with which SHP-1 interacts so as to modulate macrophage behavior. In the current study, we identify the RASGAP-associated protein, p62DOK, as a protein that is tyrosine-hyperphosphorylated in SHP-1-deficient me/me bone marrow macrophages, both constitutively and in response to CSF-1 stimulation. p62DOK was found to exhibit transient tyrosine phosphorylation and increased receptor association following CSF-1 stimulation of normal BMM. The data also revealed the capacity of SHP-1 to dephosphorylate p62DOK in vitro and indicated CSF-1-induced tyrosine phosphorylation of p62DOK to be dramatically reduced in CSF-1R-expressing 293T cells in the presence of wild-type SHP-1 but notably increased in the presence of SHP-1 C453S. Taken together, these findings identify p62DOK as a potential modulator of CSF-1R signaling and indicate a role for SHP-1 in regulating both CSF-1-dependent and -independent tyrosine phosphorylation of p62DOK.

In addition to dephosphorylating p62DOK, small fractions of SHP-1 and p62DOK were also found to be associated in BMM. This association was observed in the absence as well as in the presence of CSF-1 and occurs independently of p62DOK tyrosine phosphorylation. Constitutive association of SHP-1 with p62DOK was also observed in p62DOK/SHP-1-expressing 293T cells, and the association was not enhanced by substituting SHP-1 C453S for wild-type SHP-1. Similarly, the in vitro binding data revealed wild-type and C453S SHP-1 to associate to a comparable extent with p62DOK and again indicated this interaction to be phosphotyrosine- and SH2 domain-independent. By contrast, the results of a far Western analysis indicated that a significant amount of p62DOK bound to C453S, but not wild-type SHP-1, and that this association was phosphotyrosine-dependent, thus demonstrating direct binding of tyrosine-phosphorylated p62DOK to the catalytic domain of SHP-1, consistent with a catalytic role of SHP-1 in p62DOK dephosphorylation. The incapacity of C453S SHP-1 to bind a significant portion of tyrosine-phosphorylated p62DOK in both the in vitro binding experiment and in vivo in 293T cells, could be related to the conformation of the native tyrosine-phosphorylated p62DOK and/or its interactions with other proteins. Conversely, the inability to detect tyrosine phosphorylation-independent binding of SHP-1 to p62DOK in the far Western analysis could indicate that the constitutive association detected in vivo, if direct, is conformation-dependent or, alternatively, that this association is mediated indirectly through interactions of SHP-1 and/or p62DOK with other signaling effectors. p62DOK contains a number of PXXP motifs and a potential pleckstrin homology domain at the amino terminus. Thus, there are many signaling effectors that may potentially subserve the role of a SHP-1/p62DOK “linker.”

Taken together, our findings suggest that the p62DOK-SHP-1 interaction is comprised of two components as follows: a phosphotyrosine-independent, possibly indirect interaction, and the direct catalytic interaction of SHP-1 with p62DOK phosphotyrosines. Interestingly, a similar paradigm has recently been suggested in relation to another SHP-1 substrate, SLP-76, the available in vitro binding data revealing an activation-inducible association of SHP-1 with SLP-76 (26), although these proteins have been shown to constitutively associate in vivo (55). Thus, the phosphotyrosine-independent, constitutive association of SHP-1 with certain substrates in vivo may facilitate its catalytic function. The direct binding of SHP-1 to p62DOK does not appear to involve the SH2 domains of SHP-1, since they did not bind tyrosine-phosphorylated p62DOK in the far Western analysis. Thus, this interaction differs from the phosphotyrosine-dependent association of SHP-1 and p91/PIR-B, an interaction that has been shown to be mediated by both the SH2 and catalytic domains of SHP-1 (32, 33). The absence of stable, phosphotyrosine-dependent SH2 domain binding to p62DOK may explain why, in contrast to p91/PIR-B, p62DOK is not a major SHP-1-binding protein.

p62DOK appears to be highly expressed in the spleen (42) and leukocytes (41) and has been implicated in signaling via the c-Kit (41) and FcγRIIB1 (56) receptors and, based on the current findings, the CSF-1R. Although the mechanisms whereby p62DOK might transduce or modulate signals evoked by these or other growth-regulating receptors remain unclear, the capacity of p62DOK to associate with RASGAP suggests that p62DOK signaling functions may include the regulation of RAS...
activation. As GAP is a negative regulator of RAS, its association with p62DOK might provide a means to suppress or enhance signaling, for example by virtue of promoting GAP recruitment to the RAS complex or, alternatively, sequestering GAP away from RAS (42). Although these possibilities require further investigation, the current data suggest that tyrosine phosphorylation status per se does not influence p62DOK effects on RAS signaling, as RAS activation was not altered in conjunction with the augmented tyrosine phosphorylation of p62DOK observed in me/me macrophages. These findings are consistent with recent data revealing p62DOK overexpression to be associated with increased cell migration but no alteration in insulin-induced activation of mitogen-activated protein kinase (57). Importantly, however, in the current study, GAP was not co-immunoprecipitated with p62DOK from either wild-type or me/me macrophages, so the possibility remains that p62DOK functions in these cells are mediated largely independently of RAS. In this respect, it is worth noting that p62DOK has many tyrosine phosphorylation sites and that these include potential binding sites for the SH2 domains of Crk, Src family kinases, Zap-70, and SHP-2 as well as RASGAP (41, 42). Whether p62DOK roles in CSF-1R or other growth-regulated signaling cascades are mediated through interactions with these or other signaling effectors remains to be determined.

In previous studies, tyrosine phosphorylation of p62DOK has been found to be associated with the transforming capabilities of a number of mitogenic and oncogenic PTKs. In addition, p62DOK has been shown to be constitutively hyperphosphorylated in primary chronic phase chronic myelogenous leukemia blasts (40) and on this basis hypothesized to play a role in development of this disease. Interestingly, earlier studies demonstrated that chronic myelogenous leukemia progenitors and cell lines exhibit growth factor-independent survival, rather than increased rates of cell proliferation (58, 59). These findings are consistent with the current data revealing p62DOK to be constitutively hyperphosphorylated in BMM from me/me mice in which macrophage accumulation represents the most predominant hematopoietic cell defect. This massive overexpansion of the me/me macrophage population has been previously attributed to increased responsiveness of these cells to both CSF-1 (31) and GM-CSF (54). However, in the current study, me/me BMM were found to survive independently of growth factor, and no differences were detected between the proliferative rates of control and me/me cells, although the latter did reach higher final densities than did wild-type cells. Whereas these discrepant findings may reflect differences in the cell populations studied, the current data also raise the possibility that reduced cell death and enhanced survival rather than enhanced proliferative rates underlie the expansion of macrophage populations in SHP-1-deficient mice. This hypothesis is, in fact, consistent with previous data identifying a role for SHP-1 in promoting the spontaneous apoptosis of myelomonocytic U937 cells (60). Since p62DOK tyrosine phosphorylation is increased in SHP-1-deficient BMM, it is possible that p62DOK is involved in regulating survival signals. If so, it is unclear as to which macrophage receptors p62DOK might modulate so as to influence cell survival, although receptors that are modulated by SHP-1 represent potential candidates. These would include, for example, SHPS-1 and PIR-B and, in view of recent data indicating macrophage adhesion to be enhanced in the context of SHP-1 deficiency (57), integrin receptors as well.

Although the capacity of p62DOK to undergo tyrosine phosphorylation is well recognized, the PTKs that target p62DOK tyrosine phosphorylation have not been well defined. The current data revealing p62DOK to be tyrosine-phosphorylated in both a CSF-1-dependent and CSF-1-independent fashion suggest that p62DOK represents a substrate for both the CSF-1R and one or more other PTKs, normally active in the absence of growth factor and that could be regulated by cell surface interactions. Although our data are consistent with a role for SHP-1/p62DOK in regulating signaling cascades governing macrophage survival, it is also possible that this interaction impacts on activation, adhesion, and/or other biological properties of macrophages.

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Note Added in Proof—Experiments performed following acceptance of this manuscript demonstrated that p62DOK can be co-immunoprecipitated with RASGAP from wild-type primary macrophages when GAP is immunoprecipitated with a polyclonal antibody directed against the GAP SH3 domain. An increased amount of p62DOK is co-immunoprecipitated with GAP with me/me macrophages.

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