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Phosphoinositide 3-Kinase–dependent Membrane Recruitment of p62^{dok} Is Essential for Its Negative Effect on Mitogen-activated Protein (MAP) Kinase Activation

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Abstract

A major pathway by which growth factors, such as platelet-derived growth factor (PDGF), regulate cell proliferation is via the receptor tyrosine kinase/Ras/mitogen-activated protein kinase (MAPK) signaling cascade. The output of this pathway is subjected to tight regulation of both positive and negative regulators. One such regulator is p62^{dok}, the prototype of a newly identified family of adaptor proteins. We recently provided evidence, through the use of p62^{dok}-deficient cells, that p62^{dok} acts as a negative regulator of growth factor–induced cell proliferation and the Ras/MAPK pathway. We show here that reintroduction of p62^{dok} into p62$^{dok-/-}$ cells can suppress the increased cell proliferation and prolonged MAPK activity seen in these cells, and that plasma membrane recruitment of p62^{dok} is essential for its function. We also show that the PDGF-triggered plasma membrane translocation of p62^{dok} requires activation of phosphoinositide 3-kinase (PI3-kinase) and binding of its pleckstrin homology (PH) domain to 3′-phosphorylated phosphoinositides. Furthermore, we demonstrate that p62^{dok} can exert its negative effect on the PDGFR/MAPK pathway independently of its ability to associate with RasGAP and Nck. We conclude that p62^{dok} functions as a negative regulator of the PDGFR/Ras/MAPK signaling pathway through a mechanism involving PI3-kinase–dependent recruitment of p62^{dok} to the plasma membrane.

Key words: growth factors • cell proliferation • membrane lipids • signal transduction • protein-serine-threonine kinase

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Introduction

The Dok proteins (downstream of tyrosine kinases) are a newly identified family of adaptor proteins phosphorylated by a wide range of protein tyrosine kinases (PTKs). The family includes the prototype family member, p62^{dok}, which was originally identified as a tyrosine-phosphorylated 62-kD protein associated with p120RasGAP (1, 2), a negative regulator of Ras (3). p62^{dok} is phosphorylated upon activation of receptor tyrosine kinases (RTKs)* (including the epidermal growth factor receptor, platelet-derived growth factor receptor, and insulin receptor), antigen receptors (such as FcγRIIB), and nonreceptor tyrosine kinases (including v-Src, v-Abl, and the p210$^{bcr-abl}$ fusion oncoprotein) (1, 4–9). While the sequence of p62^{dok} suggested that the protein may serve as a docking protein (1, 2), recent studies have shed light on its function as a negative regulator of cell proliferation and the Ras/mitogen-activated protein kinase (MAPK) signaling pathway.

A major contribution towards deciphering the function of p62^{dok} came from studies that described the properties of p62$^{dok-/-}$–derived cells in response to various stimuli. We recently demonstrated that cells of multiple origins, such as bone marrow cells, thymocytes, and mouse embryo fibro-

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**Abbreviations used in this paper:* aa, amino acid(s); MAPK, mitogen-activated protein kinase; MEF, mouse embryo fibroblast; MLV, multilamellar vesicle; PC, phosphatidylcholine; PDGFR, platelet-derived growth factor receptor; PH, pleckstrin homology; PS, phosphatidylserine; PTB, phosphotyrosine binding; RT, room temperature; RTK, receptor tyrosine kinase; SHIP1, Src homology 2 domain–containing inositol 5-phosphatase; WT, wild-type.

Published July 30, 2001

blasts (MEFs), isolated from p62^{dok} knockout mice, showed a higher degree of proliferation upon growth factor stimulation than those from wild-type (WT) mice. Moreover, we observed sustained MAPK activation and prolonged levels of activated, GTP-bound Ras after growth factor removal in p62$^{dok-/-}$ MEF cells (10). In addition, using splenic B cells derived from p62$^{dok-/-}$ mice, Yamanashi et al. provided consistent evidence for a negative role of p62^{dok} in antigen receptor–mediated signaling through suppression of MAPK activity and cell proliferation (7). Taken together, these studies suggest that the negative effect of p62^{dok} on cell proliferation is at least in part due to negatively influencing the Ras/MAPK pathway.

As a docking molecule, p62^{dok} harbors several characteristic motifs and domains. At its N terminus, p62^{dok} contains a pleckstrin homology (PH) domain followed by a phosphotyrosine-binding (PTB) domain, while the COOH-terminal tail of p62^{dok} harbors 10 potential tyrosine phosphorylation sites and 7 PxxP motifs, which may serve as docking sites for SH3 domains (1, 2, 11, 12). The PTB domain of p62^{dok} was recently found to be a functional PTB module and is involved in its association with Src homology 2 (SH2) domain–containing inositol 5-phosphatase (SHIP1) (8, 13), as well as in the homodimerization of p62^{dok} through its interaction with a phospho-tyrosine residue (Tyr 146) (14). Interestingly, mutations in the PTB domain that block homodimerization significantly reduce the ability of p62^{dok} to inhibit v-Src–induced transformation (14). Phosphorylation of the multiple tyrosine residues at the COOH terminus of p62^{dok} have been shown to create docking sites for proteins containing SH2 domains, including Nck, RasGAP, and Csk (1, 4, 8, 13, 15, 16, 17).

Studies on the more recently identified Dok family members, p56^{dok-2} (or DokR and IL-4R interacting protein [FRIP]) and Dok3 (or DokL) (18–22), using overexpression experiments, are consistent with a role for the Dok proteins as negative regulators of cell proliferation and MAPK activation induced by different stimuli (20–24). In addition to containing the motifs and domains featured above for p62^{dok}, p56^{dok-2} has been demonstrated to associate with RasGAP and Nck upon tyrosine phosphorylation (18, 19, 25), and Dok3 with SHIP1 and Csk, but not RasGAP (21, 22).

Despite the identification of the various protein–protein interactions mentioned above, the mechanism by which p62^{dok} negatively interferes with cell proliferation and the Ras/MAPK pathway remains unclear. In particular, the role of the PH domain of p62^{dok} remains to be clarified as to whether it affects the subcellular localization of p62^{dok} in response to growth factors, and in turn influences its biological functions. Furthermore, the functional relevance of the association of p62^{dok} with RasGAP is unclear. It is tempting to speculate that upon growth factor stimulation, p62^{dok} brings RasGAP into the vicinity of Ras in order to attenuate Ras activation, resulting in a decrease in MAPK activity and cell proliferation. Tamir et al. have provided evidence in favor of this model. They overexpressed chimeric receptors containing the extracellular and transmembrane domains of the FcγRIIB fused to the COOH-terminal portion of p62^{dok}, which includes the RasGAP-binding region. Upon BCR/FcγRIIB coaggregation, the chimera was able to recruit RasGAP and suppress MAPK activation (8). However, in contrast to the above model, overexpression of either a p56^{dok-2} mutant deficient in RasGAP binding, or Dok3, which is unable to bind to RasGAP, still resulted in the downregulation of MAPK activity induced by EGFR or v-abl, respectively (22, 23). Although these discrepancies might be caused by differences between DOK family members, stimuli, or cell types, the function of the RasGAP/p62^{dok} complex requires further elucidation using a p62^{dok} mutant deficient in RasGAP binding.

In this study, we first establish the role of p62^{dok} as a negative regulator of the PDGFR/Ras/MAPK pathway by performing rescue experiments and overexpression studies. Subsequently, we further investigate the mechanism by which p62^{dok} exerts its effects. We demonstrate that in response to PDGF, the PH domain of p62^{dok} mediates the recruitment of the protein to the plasma membrane in a phosphoinositide 3-kinase (PI3-kinase) activity-dependent manner, and that this translocation is important for the tyrosine phosphorylation of p62^{dok}, as well as for its function as a negative regulator of the PDGFR/MAPK pathway. We also provide evidence suggesting that the mechanism by which p62^{dok} negatively influences PDGF-triggered MAPK activity is not dependent on the recruitment of RasGAP to the membrane, or displacement of Nck, a known positive regulator of the Ras/MAPK pathway.

Materials and Methods

Plasmid Constructs and Abs. Retroviral pMaRxIV-p62^{dok} wild-type (WT) and p62^{dok}ΔPH constructs, used to generate Rat1 stable cell lines, were obtained by cloning full-length and amino acids (aa) 121–481 of human p62^{dok} into the EcoRI site and EcoRI/XhoI sites of pMaRxIV vector (gift from G. Hannon, Cold Spring Harbor Laboratory). GFP-expressing full-length p62^{dok}, p62^{dok}PH, and p62^{dok}ΔPH constructs were generated by cloning PCR generated fragments of p62^{dok} full-length, aa 1–120 and aa 121–481 into the EcoRI/SalI sites of pEGFP-N_3 (CLONTECH Laboratories, Inc.). The His-tagged p62^{dok} bacterial expression constructs were obtained by cloning PCR generated p62^{dok} fragments into the KpnI/SalI sites of pQE30 (QIAGEN). The retroviral constructs pBabePuro-p62^{dok}WT, p62^{dok}RasGAP binding deficient mutant (p62^{dok}GBD), used in the rescue experiments, were constructed by cloning full-length cDNA and a cDNA containing Tyr→Phe mutations at aa 296, 315, 362, 398, and 409 into the EcoRI site of pBabePuro, respectively. The p62^{dok}ΔPH construct was generated by cloning aa 121–481 of p62^{dok} into EcoRI/SalI sites of pBabePuro (26). myc-tagged p62^{dok}WT and p62^{dok}GBD were obtained by cloning the corresponding cDNAs into the EcoRI/XhoI sites of pcDNA3.1/myc-His vector (Invitrogen). Polyclonal Abs against p62^{dok} were generated against peptides derived from a 15-aa sequence in the COOH terminus (1) and from a 14-aa sequence (LQSQFFGTKRWRKT) in the N-terminal region of human p62^{dok}. Monoclonal Abs were generated against GST-human p62^{dok}ΔPH purified protein. These Abs do recognize rat and mouse p62^{dok}, but with much lower affinity.

Cell Culture, Transfection, and Infection. Rat1 cells, WT MEF cells, and p62$^{dok-/-}$ MEF cells were maintained in DMEM con-

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taining 10% fetal bovine serum. Retroviral gene transfer was performed as described (27). Infected cell populations were selected by culturing in the media described above containing hygromycin (100 μg/ml) for 5 d or puromycin (2.5 μg/ml) for 3 d. For transient transfections, Fugene 6 (Roche Molecular Biochemicals) was used according to the manufacturer's recommendations.

Cell Fractionation. Cells were serum starved for 16 h and stimulated with PDGF (50 ng/ml) for 15 min. When PI3-kinase inhibitors were used, cells were treated with wortmannin (25 nM) or LY292004 (25 μM) for 5 min, and subsequently stimulated with PDGF. Cells were then homogenized in detergent-free buffer (10 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 0.2 M sucrose, 0.5 mM EDTA), and centrifuged at 1,000 *g* for 10 min at 4°C. The pellets were homogenized again and centrifuged as above. The supernatants from both steps were pooled and centrifuged at 100,000 *g* for 40 min to give the S100 (cytosolic) fraction. The corresponding pellet was homogenized in 6 mM Tris-HCl, pH 8.0, 1 mM EDTA, and centrifuged another 40 min at 100,000 *g*. The pellet was homogenized again, and resuspended in 20 mM Tris-HCl, pH 7.5, 1 mM EDTA. This fraction represents the P100 (membrane) fraction. All buffers contained protease and phosphatase inhibitors. An equal amount of protein from each fraction was subjected to immunoprecipitation and Western blot analyses.

Purification of Full-Length $p62^{dok}$, $p62^{dok}$ΔPH, and $p62^{dok}$PH His-tagged Proteins. The bacterially expressed His-tagged $p62^{dok}$, $p62^{dok}$PH, and $p62^{dok}$ΔPH proteins were purified on Ni^{2+} affinity columns according to the manufacturer's instructions (QIAGEN). The protein samples were dialyzed against 50 mM Hepes, pH 8.0, 150 mM NaCl, and stored at −20°C until use.

Lipid Binding Assays. Multilamellar vesicles (MLVs) were used to analyze lipid-binding according to published procedures (28, 29). Synthetic phospholipids were obtained from Avanti Polar Lipids and from Matreya. To prepare MLVs, lipids in chloroform/methanol/water were mixed at the desired ratios and evaporated to dryness at room temperature (RT) in a Rotavapor (Buechi). The lipid film was further desiccated under high vacuum for 2 h in the dark and rehydrated at a concentration of 2 mg/ml in buffer A (10 mM morpholino propane sulfonic acid [MOPS], pH 7.4, 0.1 mM EGTA, 100 mM NaCl, 0.01% Triton X-100) by intermittent gentle shaking over 20 min at RT. MLVs were collected by centrifugation at 15,000 *g* for 10 min at 4°C, and resuspended in the original volume of fresh buffer A. $p62^{dok}$ full-length, $p62^{dok}$PH, and $p62^{dok}$ΔPH His-tagged proteins were diluted to 100 ng/μl in buffer A (supplemented with protease inhibitors) and centrifuged at 15,000 *g* for 10 min at 4°C. The supernatant was used as a protein stock solution. For the binding assay, serial dilutions of MLVs were added to 0.5 μg of protein in a total volume of 100 μl of buffer A, and the reactions were incubated at RT for 30 min. Subsequently, free and lipid-bound proteins were separated by centrifugation at 15,000 *g* for 10 min at 4°C. The supernatant was immediately removed and supplemented with 25 μl 5× SDS loading buffer, whereas the pellet was resuspended in 125 μl 1× SDS loading buffer. The samples were boiled for 3 min at 95°C and 20 μl of each sample was loaded on polyacrylamide gels, which were then processed for Western blotting.

Immunofluorescence Studies. 5×10^4 Rat1 cells were plated on 30-mm round glass coverslips, transfected, and serum starved for 20 h. The coverslips were placed into a chamber that was mounted on a heated stage and kept at 36.5°C. The cells were incubated in serum-free DMEM, and treated with indicated stimuli and PI3-kinase inhibitors. Images were taken and processed using an inverted ZEISS confocal microscope under 100× oil immersion objective and LSM50 software.

MAPK Assay. Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% Triton, 5 mM EDTA, 40 mM β-glycerophosphate, 50 mM NaF, 100 μM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM sodium orthovanadate). Equal amounts of each sample were resolved on 12% SDS-PAGE gels, transferred to nitrocellulose membranes, and immunoblotted with phospho-specific p44/p42 MAPK Ab (New England BioLabs, Inc.) to measure the activation status of MAPK. To verify equal loading, membranes were blotted with p42 MAPK Ab (Santa Cruz Biotechnology, Inc.). Immunoblots were visualized using enhanced chemiluminescence detection reagents (ECL; Amersham Pharmacia Biotech).

Cell Proliferation Assay. Cell proliferation was assessed by [^{3}H]thymidine incorporation assays. WT and $p62^{dok-/-}$ MEF cells, infected with $p62^{dok}$ WT or empty vector (5×10^4 cells/well in 12-well plates), were serum starved for 24 h, and then stimulated by adding 50 ng/ml of PDGF. 12 h later, the cells were pulsed with 5 μCi/ml [methyl-^{3}H]thymidine; and after another 3 h, the cells were washed, trypsinized, and transferred to glass fiber filters followed by scintillation counting.

Results

Suppression of Increased Cell Proliferation and MAPK Activity by Reintroducing $p62^{dok}$ into $p62^{dok}$-deficient Cells. Recently, we inactivated $p62^{dok}$ by homologous recombination and observed that $p62^{dok-/-}$ cells derived from multiple origins exhibited a higher proliferation rate in response to different growth factors when compared with their corresponding WT cells. Moreover, we found that inactivation of $p62^{dok}$ results in sustained activation of Ras and MAPK activity after removal of PDGF in $p62^{dok-/-}$ MEF cells (10). To ultimately prove that $p62^{dok}$ is responsible for the above effects, we reintroduced $p62^{dok}$ into $p62^{dok-/-}$ MEF cells and examined whether the increased cell proliferation and prolonged MAPK activity observed in these cells is reversed. Selected cell populations stably expressing $p62^{dok}$ or empty vector were assessed for [^{3}H]thymidine incorporation in response to PDGF. As shown in Fig. 1 A, reintroduction of $p62^{dok}$ diminished the enhanced proliferation rate of $p62^{dok-/-}$ MEF cells, whereas the empty vector had no effect. To measure MAPK activity, total protein extracts were prepared from cells at the indicated time points, and subjected to Western blot analysis with anti-phospho-p44/p42 MAPK (ERK1/2) Abs. While the duration of MAPK activation was prolonged in $p62^{dok-/-}$ MEF cells expressing the empty vector, similar MAPK profiles were observed for WT cells and $p62^{dok-/-}$ MEF cells expressing $p62^{dok}$ (Fig. 1 B). We also examined the effects of $p62^{dok}$ overexpression on PDGF-triggered MAPK activation using Rat1 stable cell lines expressing empty vector or WT $p62^{dok}$. Expression of $p62^{dok}$, but not empty vector, in Rat1 cells resulted in a reduction in phosphorylation of MAPK after 10 and 20 min of PDGF stimulation (Fig. 1 C). Together, these results confirm that $p62^{dok}$ is a negative regulator of PDGF-triggered cell proliferation and MAPK activation.

In contrast to WT $p62^{dok}$, the introduction of a mutant $p62^{dok}$ lacking the PH domain, $p62^{dok}$ΔPH, into $p62^{dok-/-}$ MEF cells did not suppress the sustained MAPK activity af-

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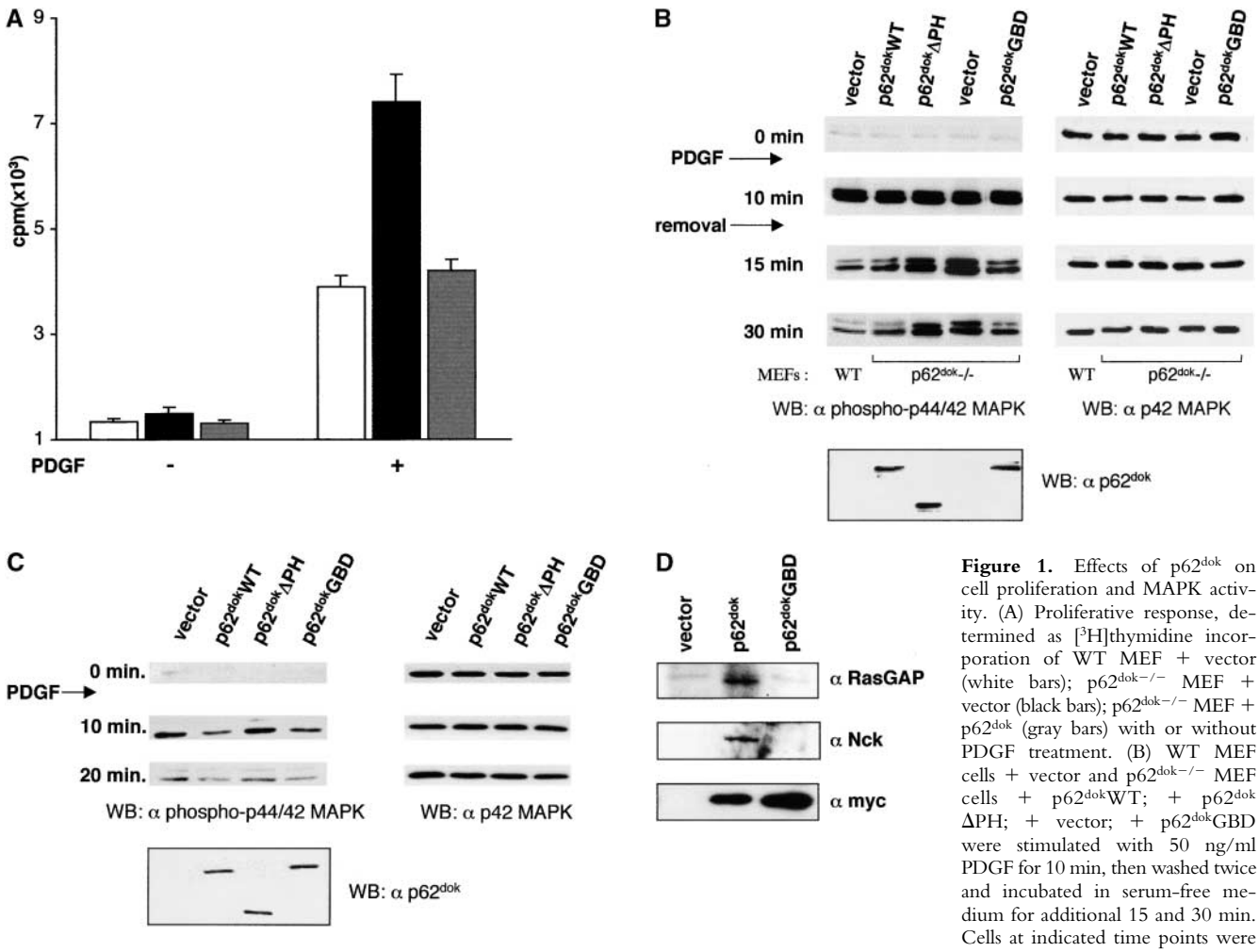

Figure 1. Effects of p62^{dok} on cell proliferation and MAPK activity. (A) Proliferative response, determined as [^{3}H]thymidine incorporation of WT MEF + vector (white bars); p62$^{dok-/-}$ MEF + vector (black bars); p62$^{dok-/-}$ MEF + p62^{dok} (gray bars) with or without PDGF treatment. (B) WT MEF cells + vector and p62$^{dok-/-}$ MEF cells + p62^{dok}WT; + p62^{dok}ΔPH; + vector; + p62^{dok}GBD were stimulated with 50 ng/ml PDGF for 10 min, then washed twice and incubated in serum-free medium for additional 15 and 30 min. Cells at indicated time points were lysed and subjected to Western blot (WB) analysis using polyclonal Abs against phospho-p44/42 MAPK. Abs against p42 MAPK were used to show approximately the same loading of proteins in all lanes, and monoclonal Abs against p62^{dok} were used to show expression of the p62^{dok} construct. (C) Rat1 cells expressing either empty vector; p62^{dok} WT; p62^{dok}ΔPH or p62^{dok}GBD were stimulated with PDGF (50 ng/ml) for 10 and 20 min. Lysates were subjected to Western blot analysis and analyzed for MAPK activity as described in B. All figures are representative of experiments repeated three times. (D) Rat1 cells were transiently transfected with empty vector, myc/His-tagged p62^{dok}WT or myc/His-tagged p62^{dok}GBD constructs, and stimulated with PDGF (50 ng/ml) for 10 min. Total protein extracts were immunoprecipitated with Abs against Myc and subjected to Western blot analysis using Abs against RasGAP and Nck (Transduction Laboratories).

ter PDGF removal (Fig. 1 B). This result was confirmed in Rat1 stable cell lines: overexpression of p62^{dok}ΔPH did not reduce phosphorylation of MAPK after PDGF stimulation (Fig. 1 C). These observations, together with the fact that PH domains have previously been demonstrated to mediate protein–membrane interactions, suggest that plasma membrane recruitment of p62^{dok} is essential for its negative effect on PDGF-triggered MAPK activation. However, the membrane association of p62^{dok} in response to growth factors and the function of its PH domain has until now not been addressed.

The PH Domain Is Essential for PDGF-triggered Translocation of p62^{dok} to the Plasma Membrane. To demonstrate that the PH domain of p62^{dok} is important for its subcellular localization, we initiated experiments examining its localization in response to PDGF. We performed fractionation experiments and examined the distribution of p62^{dok} within single living cells. For the fractionation experiments, we used stable Rat1 fibroblast cell lines expressing WT p62^{dok} or p62^{dok} lacking the PH domain (p62^{dok}ΔPH). These cells were serum starved for 16 h, subsequently treated with PDGF (50 ng/ml) for 15 min, and subjected to classical subcellular fractionation techniques (see Materials and Methods). For the detection of p62^{dok} and p62^{dok}ΔPH, equal amounts of protein of the supernatant (S100) and pellet (P100) fractions were subjected to immunoprecipitation using polyclonal Abs raised against the COOH terminus of p62^{dok} followed by Western blotting using anti-p62^{dok} monoclonal Abs. In the absence of PDGF, more p62^{dok} protein can be detected in the S100 fraction than in the P100 fraction. Upon PDGF stimulation, a major shift of the protein to the P100 fraction can be observed, suggesting that PDGF can trigger the relocalization of p62^{dok} from the cytoplasm to the membrane (Fig. 2). Furthermore, when the same Western blot was stripped and probed with the anti-phosphotyrosine Ab, PY20, a larger pool of phos-

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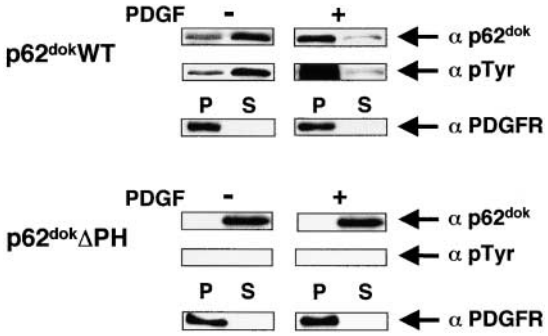

Figure 2. Subcellular localization of p62^{dok}. Rat1 cells expressing p62^{dok}WT or p62^{dok}ΔPH were treated as indicated; S100 (S) and P100 (P) fractions were separated by high-speed centrifugation. Equal amount of proteins of both fractions were immunoprecipitated, using Abs raised against the COOH terminus of p62^{dok}, subjected to SDS/PAGE and immunoblotted with mAb against p62^{dok} (α p62^{dok}) and with mAb PY20 (α pTyr). Abs against PDGFR were used to demonstrate the enrichment of this receptor in the membrane fraction and its exclusion from the cytosolic fraction. All figures are representative of experiments repeated four times.

phorylated p62^{dok} was found in the P100 fraction of PDGF-treated cells when compared with unstimulated cells (Fig. 2). This suggest that p62^{dok} undergoes further phosphorylation once recruited to the plasma membrane. The same results were obtained by directly performing Western blot analysis on the S100 and P100 fractions, rather than first immunoprecipitating p62^{dok}. Moreover, similar results were obtained when PDGF-treated and -untreated p62$^{dok-/-}$ MEF cells expressing p62^{dok}WT were used in our fractionation experiments (data not shown). In contrast, p62^{dok}ΔPH was detected exclusively in the S100 fraction, and remained unphosphorylated regardless of PDGF stimulation (Fig. 2). Surprisingly, although a basal level of tyrosine phosphorylation can be seen for WT p62^{dok} in the cytosolic fraction of untreated cells, this is not the case for p62^{dok}ΔPH. One possible explanation is that the PH domain itself contains a putative tyrosine phosphorylation site and that this site is responsible for the basal level of phosphorylation noted for full-length p62^{dok}.

To further confirm that PDGF stimulates relocalization of p62^{dok} to the plasma membrane, we expressed enhanced green fluorescent protein (EGFP) fusion proteins of either WT p62^{dok}, the PH domain, or p62^{dok} lacking the PH domain in Rat1 cells. These cells were transiently transfected with one of the following GFP-fusion constructs: p62^{dok}WT-GFP, p62^{dok}PH-GFP, p62^{dok}ΔPH-GFP, or GFP alone. Cells were plated onto coverslips, serum starved for 20 h, and stimulated with 50 ng/ml PDGF. Images were collected every 5 min over a period of 20 min using confocal microscopy. The data in Fig. 3 show representative examples of the distribution of p62^{dok}WT-GFP, p62^{dok}PH-GFP, p62^{dok}ΔPH-GFP, and GFP alone before and 10 min after PDGF stimulation. The localization of p62^{dok}WT-GFP and p62^{dok}PH-GFP in unstimulated Rat1 fibroblasts was mainly cytosolic. A rapid translocation of p62^{dok}WT-GFP and p62^{dok}PH-GFP to the plasma membrane was observed upon treatment with PDGF (Fig. 3). Both fusion proteins could be detected at the plasma membrane 5 min after PDGF stimulation. In contrast, no plasma membrane association of p62^{dok}ΔPH-GFP and GFP was observed despite PDGF treatment (Fig. 3).

Together, these results indicate that the PH domain of p62^{dok} is essential to translocate the protein from the cyto-

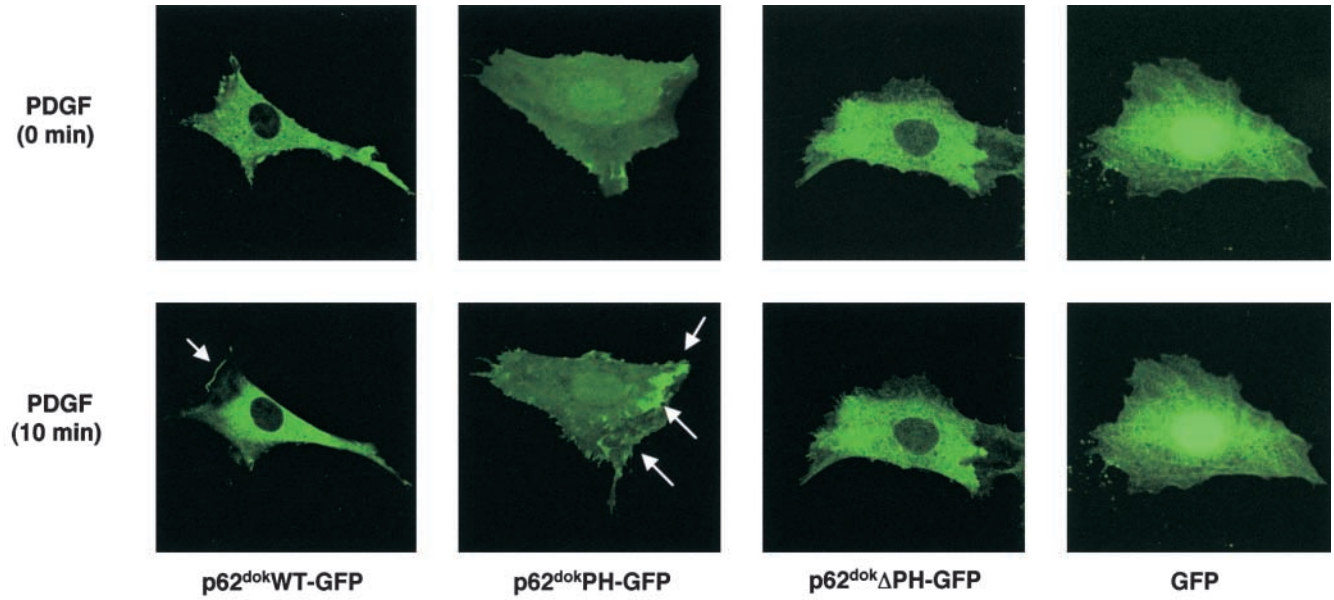

Figure 3. PDGF-induced plasma membrane translocation of p62^{dok} within single living cells. Rat1 cells expressing p62^{dok}WT, p62^{dok}PH-GFP, p62^{dok}ΔPH-GFP, or empty GFP vector were stimulated with 50 ng/ml PDGF after serum starvation. Confocal images taken before and 10 min after PDGF treatment are shown. A translocation to ruffle-like membrane structures can be noted for p62^{dok}WT-GFP and p62^{dok}PH-GFP, but not for p62^{dok}ΔPH-GFP after PDGF treatment. GFP alone as well as the p62^{dok}PH-GFP give nuclear staining, due to their smaller size. All figures are representative of experiments repeated three times.

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plasm to the plasma membrane in response to PDGF, where it undergoes further phosphorylation by certain kinase(s) in the vicinity.

Selective Binding of the PH Domain of p62^{dok} to Phosphoinositides In Vitro. To demonstrate that the PH domain of p62^{dok} binds to phospholipids in the plasma membrane, we performed an in vitro assay using MLVs (28, 29). MLVs are composed of a stack of lipid bilayers encapsulating an aqueous core, and represent a good model for the supramolecular organization of the lipids within the single bilayer of the plasma membrane. MLVs can be collected out of suspension by centrifugation, and hence this allows us to study the partitioning of a protein between the aqueous and the lipid phase by analyzing the contents of the supernatant and the lipid pellet. Recombinant His-tagged full-length p62^{dok}, p62$^{dok}\Delta$PH, and p62^{dok}PH proteins were purified as described in Materials and Methods. We prepared MLVs from either phosphatidylcholine (PC), PC and phosphatidylserine (PC/PS 70/30 mole percent), or a mixture of PC and PS with one of the following phosphoinositides: PtdIns, PtdIns-3,4-P_2, PtdIns-4,5-P_2, or PtdIns-3,4,5-P_3 (PC/PS/phosphoinositide 70/27/3 mole percent). It is noteworthy that the latter indicated percentage of lipid composition is close to that found in the plasma membrane. MLVs were incubated with recombinant proteins, and binding of the proteins to the lipids was detected by Western blot analysis using Abs generated against N- and COOH-terminal regions of p62^{dok}.

Fig. 4 shows that in contrast to p62$^{dok}\Delta$PH, which did not bind to any of the MLVs prepared, full-length p62^{dok} and p62^{dok}PH bound to MLVs containing PtdIns-3,4-P_2, PtdIns-4,5-P_2, or PtdIns-3,4,5-P_3. However, we did not detect any binding of p62^{dok} or p62^{dok}PH to MLVs only consisting of PC or PC/PS, indicating that the presence of phosphoinositides is required for binding. Furthermore, among the phosphoinositides tested, p62^{dok} and p62^{dok}PH did not bind to PtdIns, indicating a preference for highly phosphorylated phosphoinositides, such as PtdIns-3,4-P_2, PtdIns-4,5-P_2, and PtdIns-3,4,5-P_3. These results indicate that the PH domain of p62^{dok} is sufficient and necessary for binding of p62^{dok} to polyphosphoinositides.

Membrane Targeting of p62^{dok} Is Dependent on PI3-Kinase Activity In Vivo. Several lines of evidence support the notion that agonist-triggered membrane recruitment of PH domain–containing proteins is directed by binding of their PH domains to lipid products of activated PI3-kinases (30–32). Numerous growth factors, including PDGF, have been shown to activate members of the PI3-kinase family (33). Once activated, these lipid kinases are capable of modifying position 3 of the inositol ring of phospholipids with a phosphate group, so that four different lipid products can be generated, including PtdIns-3-P, PtdIns-3,4-P_2, PtdIns-3,5-P_2, and PtdIns-3,4,5-P_3. Among these products, PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 have been demonstrated to serve as targets for PH domains in cells (31, 33, 34). Based on our findings, we hypothesized that PDGF-triggered translocation of p62^{dok} to the plasma membrane is regulated by binding of its PH domain to products of PI3-kinase. Hence, we performed in vivo experiments to establish whether the membrane translocation of p62^{dok} is dependent on PI3-kinase activation.

We initially assessed whether inhibitors of PI3-kinase can interfere with the translocation of p62^{dok} to the membrane. Firstly, we performed fractionation experiments in which we treated Rat1 cells stably expressing p62^{dok} with the specific PI3-kinase inhibitors, wortmannin or LY292004 (35), before stimulating them with PDGF. As shown in Fig. 5, addition of 25 nM wortmannin inhibited the PDGF-triggered shift of the p62^{dok} protein from the S100 fraction to the P100 fraction. As a result, less tyrosine phosphorylated p62^{dok} protein is detected in the P100 fraction, when compared with cells not treated with wortmannin. Similar results were obtained when 25 μM LY292004

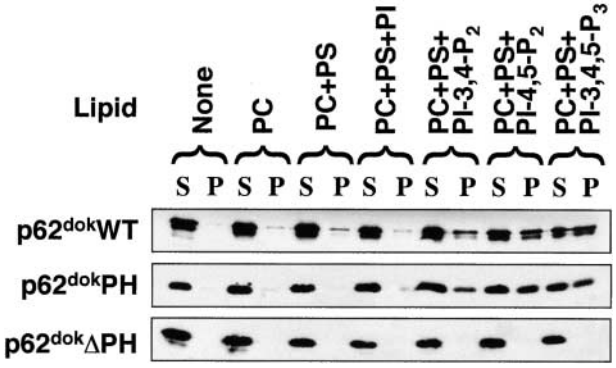

Figure 4. The PH domain of p62^{dok} is necessary and sufficient for binding to polyphosphoinositides. MLVs were prepared from PC, PC/PS (70/30 mole percent), or PC/PS/phosphoinositide (70/27/3 mole percent), and lipids at a nominal total concentration of 200 ng/μl were incubated with 5 ng/μl each of p62^{dok}WT, the PH domain of p62^{dok}, or p62$^{dok}\Delta$PH purified proteins. The aqueous and the lipid phases were separated by centrifugation and the proteins in the supernatant and associated with the pellet were detected by Western blotting. p62^{dok} can bind through its PH domain to lipid products generated by PI3-kinase, as well as to PtdIns-4,5-P_2. The results shown are representative of experiments performed with five independent vesicle preparations and two protein batches.

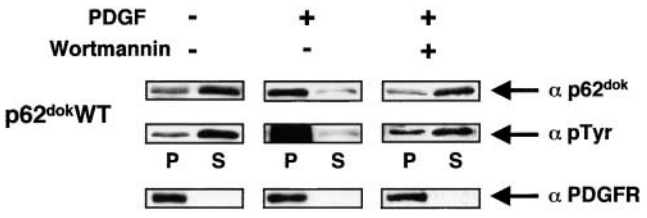

Figure 5. Inhibition of PI3-kinase activity prevents PDGF-triggered plasma membrane translocation of p62^{dok}. Rat1 cells stably expressing p62^{dok} were pretreated with 25 nM wortmannin for 5 min before stimulation with PDGF (50 ng/ml) for 15 min. S100 (S) and P100 (P) fractions were separated by high-speed centrifugation. Equal amount of proteins of both fractions were immunoprecipitated, using Abs raised against the COOH terminus of p62^{dok}, subjected to SDS/PAGE, and immunoblotted with mAb p62^{dok} (α p62^{dok}) and with mAb PY20 (α pTyr). Abs against PDGFR were used to demonstrate the enrichment of this receptor in the membrane fraction and its exclusion from the cytosolic fraction. All figures are representative of experiments repeated four times.

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was added (data not shown). Second, we examined the distribution of p62^{dok}PH-GFP within single living cells as described above. We observed that addition of 25 μM LY292004 to PDGF-pretreated p62^{dok}PH-GFP expressing cells caused an almost complete release of fluorescence from the plasma membrane to the cytosol. As shown in Fig. 6 A, after 10 min incubation with the drug, p62^{dok}PH-GFP was barely detectable at the plasma membrane.

We also investigated whether expression of an activated mutant form of PI3-kinase is sufficient to trigger the translocation of the PH domain of p62^{dok} to the membrane. We cotransfected a membrane-targeted form of p110α PI3-kinase (PI3K-CAAX) and p62^{dok}PH-GFP into Rat1 cells. Expression of this PI3-kinase mutant construct was previously shown to cause the production of PtdIns-3,4,5-P_3 and PtdIns-3,4-P_2, and to induce the formation of lamellipodia and membrane ruffles (36, 37). We noted that expression of PI3K-CAAX drives the recruitment of p62^{dok}PH-GFP (but not of p62^{dok}ΔPH-GFP) to the plasma membrane in serum-starved Rat1 cells (Fig. 6 B), indicating that alterations in lipid composition caused by activated PI3-kinase result in binding of the PH domain of p62^{dok} to the plasma membrane in vivo. In contrast, p62^{dok}PH-GFP was not observed at the plasma membrane in serum-starved cells when cotransfected with the empty vector. Thus, our in vivo experiments indicate that the PH domain–mediated plasma membrane translocation of p62^{dok} is dependent on PI3-kinase activation.

The Negative Effect of p62^{dok} on PDGF-triggered MAPK Activation Is Independent of RasGAP and Nck Association. We have provided considerable evidence that the plasma membrane recruitment of p62^{dok} is essential for its function. However, once at the membrane, the signaling components through which p62^{dok} negatively influences PDGF-elicited MAPK activity have not been well defined. One potential candidate is RasGAP, previously shown to associate with phosphorylated p62^{dok} (1, 4, 8, 13, 16). A plausible mechanism is that, upon PDGF stimulation, p62^{dok} brings RasGAP into the vicinity of Ras at the plasma membrane, thus accelerating the hydrolysis of RasGTP to RasGDP, and in turn decreasing MAPK activity. To address this possibility, we took advantage of the previously mapped RasGAP binding sites on p62^{dok} to generate a mutant deficient in RasGAP binding (p62^{dok}GBD), in which Tyr-296, 315, 362, 398, and 409 have been mutated into phenylalanines (38). We confirmed that this mutant fails to interact with RasGAP in PDGF-treated Rat1 cells (Fig. 1 D). Subsequently, we introduced p62^{dok}GBD into p62$^{dok-/-}$ MEF cells or Rat1 cells to assess whether it can suppress PDGF-elicited MAPK activity. As shown in Fig. 1 B, this mutant could still diminish the prolonged MAPK activity seen in p62$^{dok-/-}$ cells after the

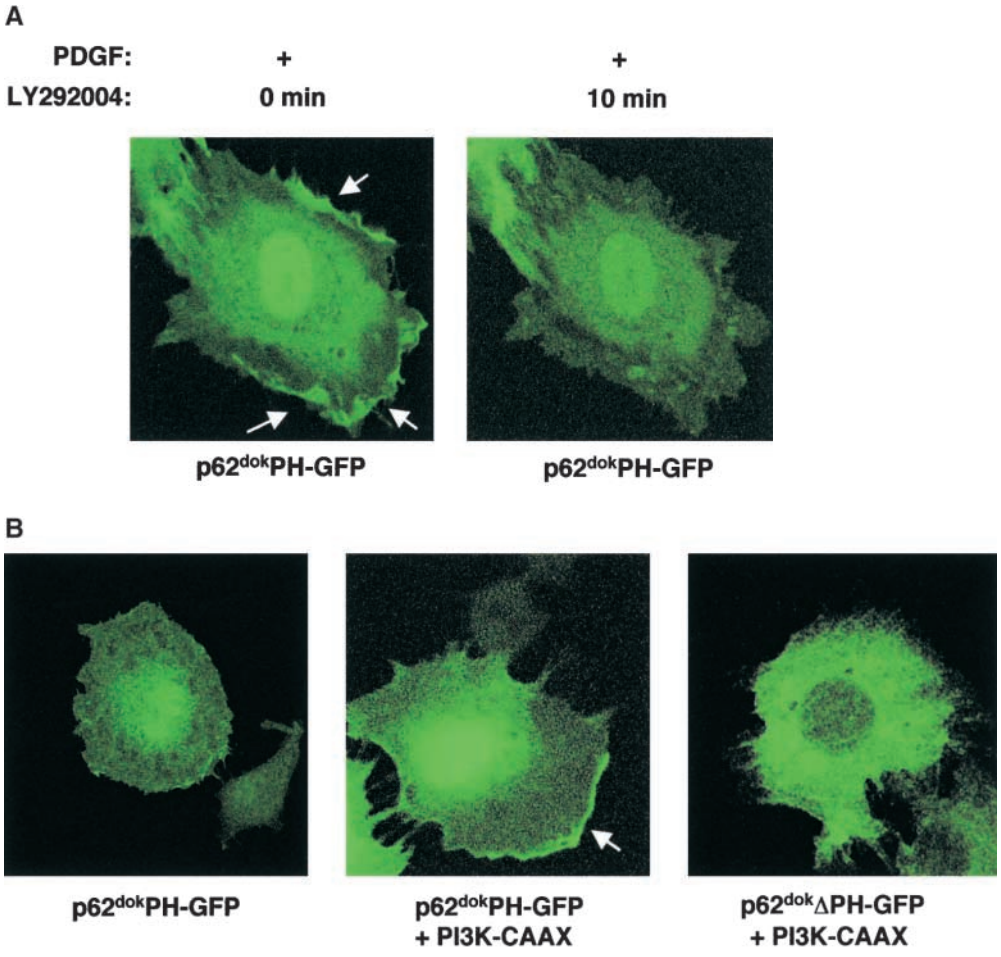

Figure 6. PI3-kinase dependent recruitment of p62^{dok}PH-GFP to the plasma membrane. (A) Effects of the PI3-kinase inhibitors on the localization of p62^{dok}PH-GFP in PDGF-pretreated Rat1 cells. Rat1 cells were transfected with p62^{dok}PH-GFP and treated with PDGF (50 ng/ml) for 10 min. Addition of 25 μM LY292004 reversed plasma membrane translocation. (B) Effects of membrane-targeted PI3-kinase on the localization of p62^{dok}PH-GFP and p62^{dok}ΔPH-GFP. Rat1 cells were cotransfected with a membrane-targeted version of PI3-kinase (PI3K-CAAX) and p62^{dok}PH-GFP or p62^{dok}ΔPH-GFP and serum starved. Expression of PI3K-CAAX triggers the recruitment of p62^{dok}PH-GFP, but not of p62^{dok}ΔPH-GFP, to the membrane. All figures are representative of experiments repeated three times.

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removal of PDGF. Moreover, overexpression of p62^{dok}GBD in Rat1 cells was also able to decrease PDGF-triggered MAPK activity (Fig. 1 C). These results suggest that association of p62^{dok} with RasGAP is not essential for its negative effect on the PDGF-triggered MAPK activation. Furthermore, Noguchi et al. have previously shown that Tyr 361 in mouse p62^{dok} is indispensable for its interaction with Nck (16). The phenylalanine substitution of its human counterpart, Tyr362, which is contained in the p62^{dok}GBD mutant, also abolished the binding to Nck (Fig. 1 D). The latter implies that p62^{dok} also does not exert its effect through its interaction with Nck. Further identification and characterization of p62^{dok}-interacting proteins will be required to define the precise mechanism by which p62^{dok} negatively influences the PDGFR/Ras/MAPK signaling pathway.

Discussion

A central route by which growth factors, such as PDGF, regulate cell proliferation is via the receptor tyrosine kinase (PDGFR), Ras, and MAPK signaling cascade, the disregulation of which has been implicated in numerous cancers (39, 40). The output of this pathway is tightly controlled by both positive and negative regulators. Although the adaptor proteins identified to date mainly function as positive regulators of RTK signaling (12), we have demonstrated that the RasGAP-associated docking protein, p62^{dok}, acts as a negative regulator of the PDGFR/Ras/MAPK signaling pathway. Through the reintroduction of p62^{dok} into p62$^{dok-/-}$ MEF cells, we were able to suppress the increase in MAPK activity and cell proliferation observed in these cells, hence unequivocally showing that p62^{dok} does indeed negatively affect MAPK signaling initiated by RTKs. We further demonstrated that translocation of p62^{dok} to the plasma membrane in response to PDGF is mediated through its PH domain and regulated by PI3-kinase activity, and that this event is essential for its role as a negative regulator. Furthermore, we showed that the inhibition of MAPK activity is independent of RasGAP and Nck binding to p62^{dok}.

Plasma membrane targeting of proteins is often accomplished via protein-lipid and/or protein–protein interactions, which can be mediated by PH and PTB domains (31). PH domains are generally involved in phosphoinositide binding, as in the case of Btk (32), while PTB domains interact with phosphotyrosines on plasma membrane–associated receptors, as in the case of Shc (30). As mentioned above, Dok family members contain an N-terminal PH domain followed by a PTB domain. The presence of these domains strongly implies that these adaptors function at the inner surface of the plasma membrane. We have demonstrated that in response to PDGF, p62^{dok} translocates to the plasma membrane where it can become further phosphorylated. In contrast, p62^{dok} lacking the PH domain is insensitive to PDGF treatment, as it remains cytosolic and is unphosphorylated. Furthermore, upon PDGF stimulation, the PH domain alone of p62^{dok} was able to associate with the plasma membrane, as well as interact with phosphoinositides directly in vitro. Taken together, these data support the idea that the PH domain of p62^{dok} is crucial to anchor the protein to the plasma membrane in order to propagate signaling events initiated by RTKs. In support of this idea, Noguchi et al. showed that the PH domain is essential for insulin-triggered phosphorylation of p62^{dok} and for its effect on cell migration (16). In contrast to our findings above, Tamir et al. suggest that membrane recruitment of p62^{dok} requires the aid of additional molecules. They found that p62^{dok} is phosphorylated during FcγRIIB activation in WT B cells, but not in cells lacking SHIP1. Based on this observation, and that p62^{dok} can interact with SHIP-1 via its PTB domain, the authors proposed that p62^{dok} is recruited to the vicinity of the plasma membrane by SHIP1, which is able to associate with FcγRIIB upon receptor activation (8). As SHIP1 is exclusively expressed in hematopoietic cells, and FcγRIIB belongs to the family of antigen receptors, we conclude that this contradiction is likely to be due to differences in cell and receptor types. Subcellular localization studies of p62^{dok} in B cells will be required to further address this issue. With regard to other Dok family members, abrogation of the phosphorylation of p56^{dok-2} in response to EGF required the mutation of two highly conserved arginines in the PTB domain in addition to the deletion of the PH domain. This implies that interaction of the PTB domain of p56^{dok-2} with a phosphotyrosine within the receptor is required for its localization at the plasma membrane (23). Although Dok proteins share aa sequence similarity and generally behave as negative regulators in cellular signaling, divergence between the Dok family members exist. For example, the different Dok family members have different COOH-terminal sequences, different expression patterns, and are phosphorylated by different receptors. Hence, the mechanism by which different Dok family members negatively regulate signaling cascades important for the control of cell proliferation may involve common, as well as distinct events.

In general, agonist-triggered recruitment of PH domain-containing proteins to the plasma membrane involves activation of PI3-kinase, which results in the production of PtdIns-3,4-P_2 and/or PtdIns-3,4,5-P_3, to which PH domains of the host proteins target and bind (30–32). We have found that the PH domain of p62^{dok} is a target of PI3-kinase lipid products. Using an in vitro MLV lipid binding assay, we have shown that the PH domain of p62^{dok} preferentially bound to polyphosphoinositides, including PI3-kinase generated phosphoinositides, PtdIns-3,4-P_2, PtdIns-3,4,5-P_3, as well as a non–PI3-kinase generated phosphoinositide, PtdIns-4,5-P_2. Given that PI3-kinase products are less abundant in the cell than PtdIns-4,5-P_2, even after agonist treatment (31), one would expect that the PH domain of p62^{dok} would bind more strongly to PI3-kinase products than to PtdIns-4,5-P_2 in vivo, if the former lipid products can target the PH domain to the plasma membrane. However, we must keep in mind that the above assay is an in vitro assay, and that either posttranslational modifications, such as tyrosine phosphorylation, or other factors normally present in vivo may restrict the specificities even further. To address the significance of the binding of p62^{dok} to PI3K products

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in vivo, we used a p62^{dok}PH-GFP construct which we introduced into Rat1 cells. Our data showed that unlike the PH domain of PLCδ (32), the PH domain of p62^{dok} does not localize at the plasma membrane in serum-starved cells. This suggests that it does not have a high enough affinity in vivo to interact with PtdIns-4,5-P_2. We also found that the addition of pharmacological inhibitors of PI3-kinase interfered with the PDGF-triggered localization of p62^{dok} at the membrane. Furthermore, expression of an activated mutant form of PI3-kinase is sufficient to localize p62^{dok}PH-GFP to the plasma membrane. Thus, these in vivo results indicate that targeting of p62^{dok} to the plasma membrane via its PH domain is regulated by PI3-kinase activity in cells.

As an adaptor protein which translocates to the plasma membrane upon PDGF stimulation, it is reasonable to suppose that p62^{dok} aids in the formation of a multiple protein signaling complex at the membrane. Consequently, proteins in this complex would play key roles in regulating cellular signaling initiated by the PDGFR. We found that p62^{dok} lacking the PH domain was not able to suppress MAPK activity when reintroduced into p62$^{dok-/-}$ MEF cells, or when overexpressed in Rat1 cells. This observation strongly supports a model in which p62^{dok} is regulated by translocation to the plasma membrane via its PH domain in order to negatively affect the MAPK pathway. In principle, p62^{dok} may exert its negative effect on the Ras/MAPK pathway by either recruiting negative regulators of the pathway, or by sequestering and inactivating positive regulators. Given these possibilities and known p62^{dok}–interacting proteins, three models are plausible. (a) p62^{dok} links RasGAP to attenuation of Ras signaling. As previously mentioned, the functional significance of the association of p62^{dok} with RasGAP is inconclusive (8, 22, 23). We therefore examined the effects of a p62^{dok} mutant deficient in RasGAP binding, and found that this mutant was still capable of suppressing PDGF-induced MAPK activity in p62$^{dok-/-}$ MEF cells, as well as in Rat1 cells. Thus, our data suggest that p62^{dok} is not dependent on its association with RasGAP to exert its negative effect on MAPK activity. (b) p62^{dok} may act in conjunction with a negative regulator other than RasGAP. Lemay et al. showed that interactions between Dok3 and inhibitory molecules, such as Csk and SHIP1, are important for the negative effect of Dok3 on BCR-triggered cytokine production, which actually does not involve MAPK activation (21). SHIP1 and Csk have been shown to interact with tyrosine-phosphorylated p62^{dok} (8, 13, 17). We can exclude SHIP1 as a possible candidate, given that its expression is restricted to hematopoietic cell lines (41). The involvement of Csk is unclear and awaits the identification of its binding sites on p62^{dok}. However, data by Songyang et al. indicate that Csk is not involved in mediating the negative effect of p62^{dok} on Src-induced transformation (14). (c) p62^{dok} may interfere with the recruitment of positive mediators of Ras signaling, such as Nck and Shc, to activated receptors. Both Nck and Shc have been identified in a protein complex which coimmunprecipitates with p62^{dok} (13, 15, 16). We excluded the possibility of Nck sequestration by using the above p62^{dok}GBD mutant which is also incapable of binding to Nck. However, sequestration of Shc by p62^{dok} remains a possibility, but has not yet been defined. Identification of additional p62^{dok}-interacting proteins will provide us with a more comprehensive view as to how p62^{dok} achieves its negative effect on cellular signaling.

In conclusion, our studies provide insights into the molecular mechanism by which p62^{dok} negatively regulates the RTK/MAPK signaling pathway. We have found that the PH domain–mediated translocation of p62^{dok} to the plasma membrane occurs in a PI3-kinase activity–dependent manner, and is necessary for the effects of p62^{dok} in RTK signaling. Given that p62^{dok} acts downstream of multiple tyrosine kinases, including oncogenic kinases, unraveling the mechanism by which p62^{dok} regulates RTK signaling pathways will help us to understand its role in cell growth control, as well as oncogenic transformation.

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