

Dok-3 sequesters Grb2 and inhibits the Ras-Erk pathway downstream of protein-tyrosine kinases

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Adaptor proteins are essential in coordinating recruitment and, in a few cases, restraint of various effectors during cellular signaling. Dok-1, Dok-2 and Dok-3 comprise a closely related family of adaptor, which negatively regulates mitogen-activated protein kinase Erk downstream of protein-tyrosine kinases (PTKs). Recruitment of p120 rasGAP, a potent inhibitor of Ras, by Dok-1 and Dok-2 appears critical in the negative regulation of the Ras-Erk pathway. However, as Dok-3 does not bind rasGAP, it has been unclear how Dok-3 inhibits Erk downstream of PTKs. Here, we identified Grb2 as a Dok-3-binding protein upon its tyrosine phosphorylation. This interaction required the intact binding motifs of the Grb2 SH2 domain, and a mutant (Dok-3-FF) having a Tyr/Phe substitution at these motifs failed to inhibit Ras and Erk activation downstream of a cytoplasmic PTK Src. Because Grb2 forms a stable complex with Sos, a crucial activator of Ras, these data suggest that Dok-3 restrains Grb2 and inhibits the ability of the Grb2-Sos complex to activate Ras. Indeed, forced expression of Dok-3, but not Dok-3-FF, inhibited the recruitment of the Grb2-Sos complex to Shc downstream of Src, which is an essential event for activation of the Ras-Erk pathway. These findings indicate that Dok-3 sequesters Grb2 from Shc and inhibits the Ras-Erk pathway downstream of PTKs.

Introduction

Activation of protein-tyrosine kinases (PTKs) induces tyrosine phosphorylation of target proteins, which triggers inter- or intra-molecular interactions of proteins (Hubbard & Till 2000). Many such interactions are essential in regulation of various cell activities such as proliferation, differentiation, motility, metabolism, and survival. Although PTKs initiate a wide range of positive signaling cascades, they also evoke negative signaling to avoid inappropriate activation of cells, which may cause abnormalities such as malignancies, developmental disorders, metabolic syndromes, chronic inflammation, or autoimmune reactions. The Ras-Erk pathway is one of the positive signaling cascades that are critical in cell

activation downstream of PTKs. An enormous number of studies have revealed that Ras activity is regulated by a balance between positive and negative regulators, which are guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs), respectively (Lowy *et al.* 1991). For example, the GEFs include Sos and the GAPs include p120 rasGAP. An adaptor protein Grb2 is comprised of a central Src homology 2 (SH2) domain and two flanking Src homology 3 (SH3) domains, and it forms a stable complex with Sos via the SH3 domains, thereby linking recruitment of Grb2 to activation of Ras. As the SH2 domain of Grb2 recognizes a phosphotyrosine (pY)-containing motif of the form pYXN (Kessels *et al.* 2002), many PTKs activate the Ras-Erk pathway by recruiting Grb2 to the motifs of their own or downstream adaptors such as Shc. Furthermore, studies with dominant negative mutants, gene targeting, or siRNA-based knock-down of Shc have revealed a critical role for it in activation of the Ras-Erk pathway downstream of PTKs including epidermal growth factor receptor, platelet-derived growth factor (PDGF) receptor, and a cytoplasmic PTK

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Src (Lai & Pawson 2000; Ravichandran 2001; Faisal *et al.* 2004).

Dok-1 was originally identified as p62^{dok} that is a common substrate of many PTKs (Carpino *et al.* 1997; Yamanashi & Baltimore 1997). Later, Dok-3 was identified as the third member of the Dok-family of proteins (Cong *et al.* 1999; Lemay *et al.* 2000), which have been expanded to six members, Dok-1 to Dok-6, to date (Di Cristofano *et al.* 1998; Nelms *et al.* 1998; Grimm *et al.* 2001; Crowder *et al.* 2004). These proteins work as adaptors in a variety of signaling situations and share structural similarities characterized by N-terminal pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains (Veillette *et al.* 2002), followed by C-terminal SH2-target sites. The PH domain is involved in lipid-protein interactions and indeed Dok-1 translocated to the plasma membrane upon PDGF-treatment of cells in a manner dependent on phosphoinositide (PI)-3-kinase, which facilitated production of 3'-phosphorylated PIs that are binding targets of the Dok-1 PH domain (Zhao *et al.* 2001). The PTB domain is involved in binding to a phosphotyrosine-containing motif of the form NPXpY or the like and is important for interaction of the Dok-family adaptors with upstream PTKs (Songyang *et al.* 2001; Jones & Dumont 1999). Upon tyrosine phosphorylation of the C-terminal SH2-target motifs, the Dok-family proteins bind or recruit SH2-containing molecules (Yamanashi & Baltimore 1997; Van Slyke *et al.* 2005; Robson *et al.* 2004). Among the Dok-family adaptors, Dok-1, Dok-2 and Dok-3 comprise a closely related sub-family, and forced expression of each of these adaptors in cultured cells negatively regulates Erk downstream of PTKs (Cong *et al.* 1999; Jones & Dumont 1999; Wick *et al.* 2001; Van Slyke *et al.* 2005). Experiments with mice lacking Dok-1 and/or Dok-2 demonstrated an indispensable role for the Dok proteins in negative regulation of the Ras-Erk pathway *in vivo* (Yamanashi *et al.* 2000; Niki *et al.* 2004; Yasuda *et al.* 2004; Shinohara *et al.* 2005). In contrast, biological and biochemical functions of Dok-4, Dok-5 and Dok-6 are still controversial (Grimm *et al.* 2001; Cai *et al.* 2003; Crowder *et al.* 2004). Recently, two research groups including ours independently found that Dok-1 and Dok-2 are key negative regulators of cytokine responses and are essential for myeloid homeostasis and suppression of leukemia in living animals (Niki *et al.* 2004; Yasuda *et al.* 2004). We also found that the Dok-family adaptors negatively regulate Toll-like receptor 4 signaling and are essential for homeostasis of the innate immunity to endotoxin, a bacterial lipopolysaccharide (Shinohara *et al.* 2005).

Since Dok-1 and Dok-2 bind p120 rasGAP upon their tyrosine phosphorylation, these adaptors appear to

recruit it as an effector to suppress the Ras-Erk pathway (Nelms *et al.* 1998; Wick *et al.* 2001). Indeed, Dok-1 mutants lacking tyrosine residues responsible for the rasGAP binding have lost these inhibitory effects. However, we previously found that rasGAP binding is not sufficient for the inhibitory function of Dok-1 and others have even reported that it is dispensable for suppression of Erk downstream of the PDGF receptor (Zhao *et al.* 2001; Shinohara *et al.* 2004), suggesting an as yet unidentified mechanism. In fact, Dok-3 does not have a rasGAP-binding motif and does not bind p120 rasGAP. It has recently been reported that forced expression of Dok-3 in a B cell line inhibited production of interleukin-2 upon B cell receptor stimulation, probably by recruiting the lipid phosphatase SHIP (Robson *et al.* 2004). In the particular signaling context discussed here, Dok-3 specifically suppressed activation of JNK but not other effector molecules, including Erk. However, given that forced expression of Dok-3 in 293 cells inhibits Erk activation downstream of PTKs (Cong *et al.* 1999), the molecular mechanism underlying the inhibition of Erk has to be studied. It should also be noted that, unlike Dok-1 and Dok-2, it has been unclear whether Dok-3 inhibits Ras.

Here, we first revealed that forced expression Dok-3 in 293T cells inhibits Ras downstream of Src. To address the mechanism of Dok-3 function, we hypothesized that it interacts with critical regulators of the Ras-Erk pathway besides p120 rasGAP. Thus, we examined proteins that bind Dok-3 upon its tyrosine phosphorylation and found that it is Grb2 that binds Dok-3 in the presence, but not in the absence, of Src. Furthermore, a Dok-3 mutant having a Tyr/Phe substitution in the predicted binding motifs of the Grb2 SH2 domain has lost both its binding activity to Grb2 and its inhibitory effects against Ras and Erk. That forced expression of Dok-3, but not the mutant, inhibited recruitment of the Grb2-Sos complex to Shc downstream of Src indicates that Dok-3 sequesters Grb2 from Shc. Because the recruitment of Grb2-Sos complex to Shc is essential for activation of the Ras-Erk pathway downstream of PTKs, including Src, our findings taken together reveal a novel mechanism in negative regulation of this pathway by the Dok family of adaptors.

Results

Dok-3 suppresses Src-induced activation of Ras

It was previously shown that forced expression of Dok-3 inhibits Erk activation downstream of v-Abl, a constitutively active PTK, but does not inhibit it downstream of a constitutively active form of Ras (Cong *et al.* 1999).

Figure 1 Dok-3 negatively regulates Ras downstream of Src. (A) Forced expression of v-Src phosphorylates Dok-3. 293T cells were transfected with expression plasmids for the indicated proteins, and anti-FLAG immunoprecipitates (IP) were subjected to immunoblotting (IB) with antibodies to phosphotyrosine (pTyr) or FLAG epitope. (B) Forced expression of Dok-3 inhibits activation of Ras. 293T cells were transfected with expression plasmids for the indicated proteins, and whole cell lysates were subjected to RBD pull-down assay for evaluation of activated Ras. Expression levels of H-Ras and Dok-3-FLAG were examined by IB.

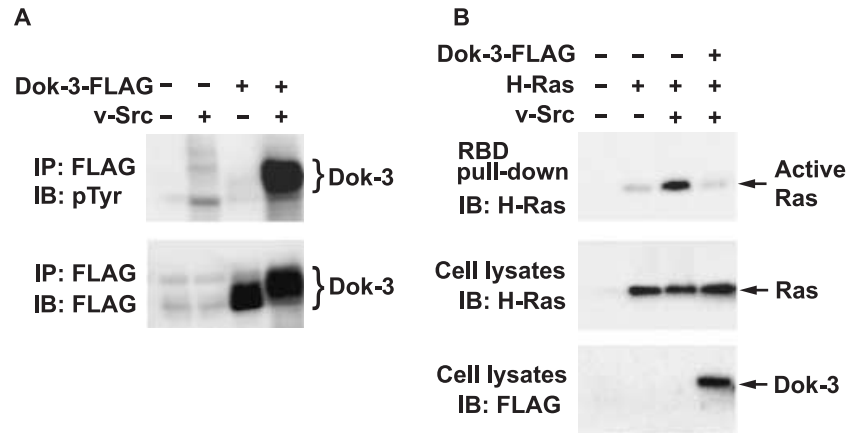


Table 1 Proteins associated with Dok-3 in the presence of Src. Molecular mass (m/z) and charge of each peptide ion observed in LC-MS/MS analysis, together with the assigned amino acid sequence and its position in the corresponding protein are shown

Protein	Peptide (m/z)	Charge	Sequence	Residues (start-end)
Grb2	825.74	3	GACHGQTGMFPRNYVTPVNRNV	196-217
	799.35	2	VLNEECDQNWYK	27-38
Csk	558.34	2	GDVLTIVAVTK	33-43
	515.29	3	VGREGIIPANYVQK	54-67
	835.06	3	VMEGTVAQAQDEFYRSGWALNMK	172-193
	433.74	2	VSDFGLTK	330-337

This appears to suggest a negative role of Dok-3 between v-Abl and Ras. However, Dok-3 is believed to work as a negative regulator upon its tyrosine phosphorylation, and Ras does not induce such modification, making it difficult to define whether Dok-3 functions upstream of Ras or not. To clarify this, we examined Ras activation downstream of a constitutively active PTK, v-Src, which strongly phosphorylated Dok-3 in 293T cells and slightly decreased its mobility in polyacrylamide gel electrophoresis (Fig. 1A). The Ras-binding domain (RBD) pull-down assay clearly showed that forced expression of Dok-3 in 293T cells inhibits v-Src-mediated activation of Ras (Fig. 1B), indicating that Dok-3 is an upstream regulator of Ras downstream of PTKs. Because Dok-3 does not bind p120 rasGAP, this strongly suggests that Dok-3 interacts with as yet unknown molecules involved in the negative regulation of Ras.

Identification of Grb2 as a Dok-3-binding protein downstream of Src

To identify Dok-3-associated proteins upon its tyrosine phosphorylation, FLAG-tagged Dok-3 was exogenously

expressed in 293 cells with or without Src-YF. This mutant is a constitutively active form of Src as it has Tyr/Phe substitution at Tyr-530, which is essential for intramolecular inactivation of catalytic activity. We confirmed that Src-YF strongly phosphorylates Dok-3, as does v-Src, which also lacks the inhibitory tyrosine residue (data not shown). One day after transfection, Dok-3 was immunoprecipitated by agarose-conjugated anti-FLAG antibodies, washed, and eluted with an excess amount of FLAG peptide. The eluted proteins, which included Dok-3 binding partners, were digested with Lys-C endopeptidase (*Achromobacter* protease I) and the cleaved fragments were directly analyzed using the highly sensitive "direct nano-flow LC-MS/MS" system as described in Experimental procedures (Natsume *et al.* 2002; Komatsu *et al.* 2004). Following a database search, a total of six peptides were assigned to MS/MS spectra obtained from four nano-LC-MS/MS analyses for Dok-3-FLAG-associated proteins in the presence, but not in the absence, of Src-YF. These peptide data identified two proteins as Dok-3-associated components downstream of Src: Grb2 and Csk (Table 1). Csk was already known to be associated with tyrosine phosphorylated Dok-1, Dok-2 and Dok-3

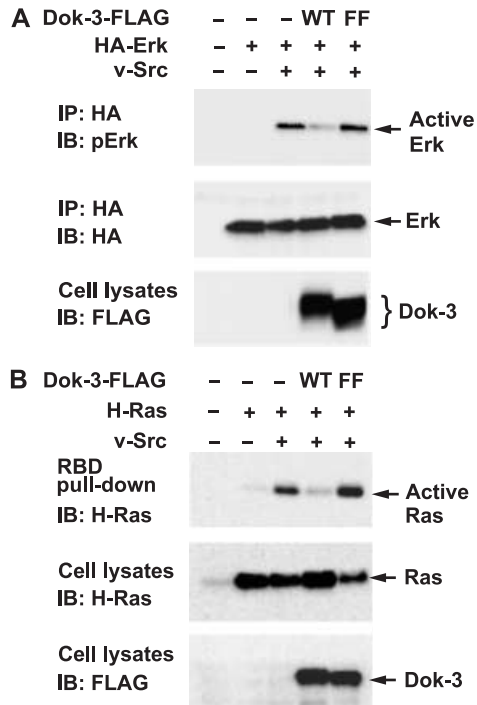


Figure 4 Grb2 binding sites are required for Dok-3 to inhibit the Ras-Erk pathway. (A) Dok-3-FF fails to suppress Erk activation. 293T cells were transfected with expression plasmids for the indicated proteins. Anti-HA IP or whole cell lysates were subjected to IB with the indicated antibodies. (B) Dok-3-FF fails to suppress Ras activation. 293T cells were transfected with expression plasmids for the indicated proteins and subsequent activation of Ras was evaluated as described in Figure 1B.

Moreover, Dok-3-FF having a substitution at these tyrosines completely lacked Grb2 binding activity. Thus, Dok-3 appears to be phosphorylated at Tyr-398 and Tyr-432 by Src so that it can bind Grb2 via the SH2 domain.

It is generally accepted that Grb2 forms a stable complex with Sos, a GEF of Ras, through the SH3 domains, and activates Ras upon recruitment via the SH2 domain by appropriate tyrosine-phosphorylated adaptors or autophosphorylated PTKs. However, it was also reported that a few adaptors bind and sequester Grb2 under certain signaling situations (Hanafusa *et al.* 2002). Therefore, we addressed the question of whether or not Grb2 binding plays a role in the inhibitory function of Dok-3 to the Ras-Erk pathway. We found that Dok-3-FF indeed has lost its ability to inhibit Erk downstream of v-Src (Fig. 4A). Consistently, this mutant did not suppress Ras downstream of v-Src (Fig. 4B), suggesting that Dok-3 sequesters Grb2 and prevents positive regulation of the Ras-Erk pathway downstream of PTKs.

Dok-3 inhibits the recruitment of Grb2-Sos complex to Shc

There is evidence demonstrating an indispensable role of Shc, an adaptor protein having the PTB and SH2 domains, in Erk activation downstream of PTKs (Lai & Pawson 2000; Ravichandran 2001; Faisal *et al.* 2004). Activation of the Ras-Erk pathway via the recruitment of Grb2-Sos complex to Shc is crucial in Src-mediated signaling (Faisal *et al.* 2004). Indeed, ectopic expression of v-Src in 293T cells induced the recruitment of Grb2 to Shc (Fig. 5A). Thus, we examined if forced expression of Dok-3 or Dok-3-FF affects the Shc-Grb2 complex formation in the presence of v-Src and found that Dok-3, but not Dok-3-FF, inhibited it (Fig. 5A). Consistently, Dok-3, but not Dok-3-FF, inhibited the recruitment of Sos to Shc (Fig. 5B). Together, our findings demonstrate that Dok-3 sequesters Grb2 and inhibits the recruitment of Grb2-Sos complex to Shc downstream of PTKs, thereby inhibiting activation of the Ras-Erk pathway.

Discussion

We recently demonstrated that mice lacking either Dok-1 or Dok-2 show a severe and lethal response to bacterial endotoxin, a lipopolysaccharide (Shinohara *et al.* 2005). In addition, mice lacking both adaptors exhibited myeloproliferative disorders, which eventually progressed into myeloproliferative disease resembling human chronic myelogenous leukemia (CML) or chronic myelomonocytic leukemia (Niki *et al.* 2004; Yasuda *et al.* 2004). These *in vivo* studies demonstrated that the Dok-family adaptors are key negative regulators of PTK-mediated signaling and help maintain homeostasis of living animals, suggesting an important role for a closely related protein, Dok-3. As mentioned earlier, Dok-1 and Dok-2 adaptors appear to recruit p120 rasGAP as an essential effector to suppress the Ras-Erk pathway (Nelms *et al.* 1998; Wick *et al.* 2001). However, as Dok-3 does not interact with rasGAP, it has been unclear how it inhibits Erk downstream of PTKs. Here, we demonstrated that forced expression of Dok-3 in 293T cells inhibits activation of Ras downstream of Src (Fig. 1), indicating a rasGAP-independent mechanism for the inactivation of Ras. Indeed, we also demonstrated that Dok-3, upon its tyrosine phosphorylation, binds Grb2 and inhibits the recruitment of Grb2-Sos complex to Shc, thereby inhibiting Ras, downstream of Src (Figs 2-5). Because two tyrosine residues, Tyr-398 and Tyr-432, of Dok-3 were essential for Grb2 binding and because each residue is in the target motif of the Grb2 SH2 domain, association of Dok-3 with Grb2 likely depends upon physical interaction between the SH2

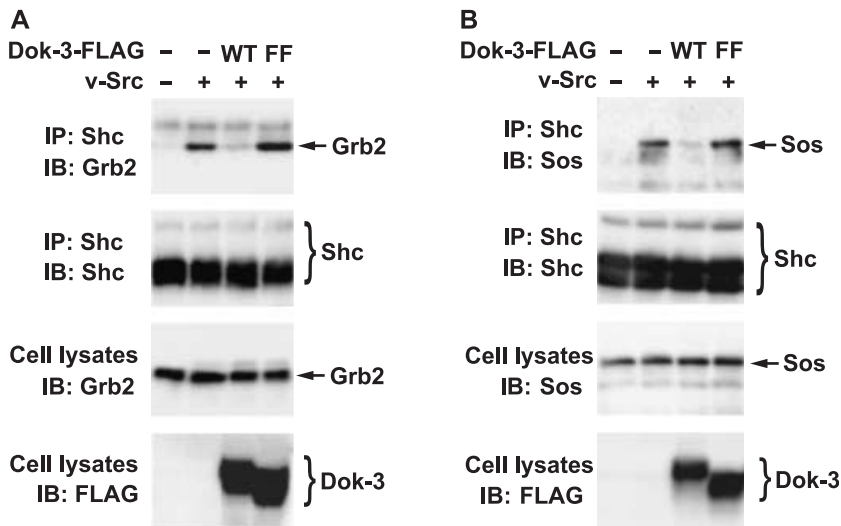


Figure 5 Dok-3 negatively regulates the recruitment of Grb2 and Sos to Shc downstream of Src. (A) Dok-3, but not Dok-3-FF, inhibits the recruitment of Grb2 to Shc. 293T cells were transfected with expression plasmids for the indicated proteins. Anti-Shc IP or whole cell lysates were subjected to IB with the indicated antibodies. (B) Dok-3, but not Dok-3-FF, inhibits the recruitment of Sos to Shc. 293T cells were transfected with expression plasmids for the indicated proteins. Anti-Shc IP or whole cell lysates were subjected to IB with the indicated antibodies.

domain and the target motifs. It is of note that Dok-2 also has an SH2 target motif; however, the binding capability of Dok-2 to Grb2 was very weak in comparison with that of Dok-3 (Fig. 2B). Thus, our findings demonstrate that Dok-3 negatively regulates Ras in a manner distinct from that of Dok-1 and Dok-2. Since these Dok-family proteins are preferentially expressed in hematopoietic cells, the Dok-3 specific pathway probably reinforces the negative regulation of Ras by Dok-1 and Dok-2 in these cells except T cell lineage where Dok-3 expression was virtually undetectable (data not shown).

Dok-1 and Dok-2 are among major phosphotyrosine-containing proteins in leukemic cells of patients with CML, which is caused by a constitutively active PTK termed Bcr-Abl (Wisniewski *et al.* 1999). We and others (Niki *et al.* 2004; Yasuda *et al.* 2004) independently reported that these adaptors work against the aberrant PTK in blastic transformation of CML-like disease in mice. Bcr-Abl has a target motif of the Grb2 SH2 domain including Tyr-177, which is an autophosphorylation site of the PTK. Thus, a Bcr-Abl mutant having a Tyr/Phe substitution at the residue loses its binding capability to Grb2. Interestingly, this amino acid substitution also impairs the transforming potential of Bcr-Abl *in vitro* and *in vivo*, demonstrating an essential role for Grb2 downstream of the PTK (Million & Van Etten 2000). Therefore, in addition to Dok-1 and Dok-2 signaling, Dok-3-mediated negative signaling may also intersect the oncogenic pathway by sequestering Grb2 from the Bcr-Abl oncoprotein, which was shown to phosphorylate Dok-3 (Cong *et al.* 1999; Lemay *et al.* 2000).

SHIP is expressed mostly in hematopoietic cells, where it acts by hydrolyzing inositol metabolites phosphorylated at the 5' position of the inositol ring: namely, PI-(3,4,5)P₃ and PI-(1,3,4,5)P₄. Studies on B cell receptor-mediated signaling with *ex vivo* B cells or B cell lines lacking SHIP have provided evidence that recruitment of SHIP to FcγRIIB inhibits B cell activation by preventing PI-(3,4,5)P₃ accumulation, activation of Btk and Akt, calcium fluxes, and Erk activation (Rohrschneider *et al.* 2000). Recently, Robson *et al.* (2004) reported that stimulation of B cell receptor induced tyrosine phosphorylation of Dok-3 and its binding to SHIP. They also demonstrated that forced expression of Dok-3, but not a mutant lacking the SHIP-binding site, inhibits the JNK-pathway, indicating a role for SHIP in Dok-3-mediated negative signaling. However, the recruitment of SHIP to Dok-3 did not inhibit PI-3-kinase-Akt or Ras-Erk pathway downstream of the B cell receptor. In addition, SHIP was not detected in the Dok-3-associated proteins even in the presence of Src in 293 cells (Table 1). Therefore, it is unlikely that SHIP was responsible for down-regulation of Ras in our experiments. The authors also defined a binding site of another Dok-3 partner, Csk, which inhibits Src and Src-like PTKs by phosphorylating a conserved inhibitory tyrosine residue near the C-terminus of each PTK (Robson *et al.* 2004; Roskoski 2004). Although Csk was detected in the Dok-3 associated proteins in our assay (Table 1), both Dok-3 and Dok-3-FF have an intact Csk-binding site. Moreover, Csk does not inhibit v-Src or Src-YF, because these mutants lack the inhibitory tyrosine residue by the C-terminal mutations as mentioned above. Together, at least in our assay systems, the sequestration

of Grb2 appears to be an essential event in Dok-3-mediated negative regulation of the Ras-Erk pathway. Because recruitment of Grb2 to PTKs or adaptors including Shc and FRS is critical to drive the Ras-Erk pathway in many signaling situations, further studies of Dok-3 function probably will contribute to a better understanding of the molecular bases of negative regulation in many biological events.

Experimental procedures

cDNAs and plasmids

The cDNA for human Dok-3 fused with the FLAG-tag at the C-terminus was amplified by PCR and inserted into the mammalian expression vector pcDNA3.1 (invitrogen) to generate pcDNA-Dok-3. Template Dok-3 cDNA was a kind gift from Sumio Sugano. pcDNA-Dok-3-based expression plasmids for mutants having Tyr/Phe substitutions (Dok-3-YF, FY or FF) were generated by PCR with the following primers: 5'-CAGTCCCATCTTCCACAACGGCCAGGAC-3' and 5'-GTCAGGGTAGAAGGTGTTGCCGTCCTG-3' for YF; 5'-GGCAATGAGCACCTCTTTGAGAACCTGTG-3' and 5'-CCGTTACTCGTGGAGAACTCTTGGACAC-3' for FY; and an appropriate combination of these primers for FF. Mouse Grb2 cDNA was generated by RT-PCR, fused with the hemagglutinin (HA)-tag at the N-terminus, and inserted into the expression vector pCAGGS (Niwa *et al.* 1991). All cDNAs were confirmed by sequencing. The expression plasmids for the following proteins were kind gifts; Erk2 fused with the HA-tag at the N-terminus from Yukiko Gotoh (Wakioka *et al.* 2001); H-Ras from Tomohiro Kurosaki (Hashimoto *et al.* 1999); and v-Src and human c-SrcY530F (Src-YF) from Tadashi Yamamoto (Kim *et al.* 2004).

Cell culture and antibodies

293 and 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The following antibodies were used for immunoblotting and/or immunoprecipitation: mouse monoclonal antibodies (mAbs) to FLAG epitope (M2) from Sigma; mouse mAbs to phosphotyrosine (4G10) and rabbit polyclonal antibodies to Shc from Upstate; rat mAbs to HA epitope (3F10) from Roche; rabbit polyclonal antibodies to phospho-Erk1/2 (pErk) from Cell Signaling; mouse mAbs to Grb2, Sos, or H-Ras from Transduction Laboratories.

Transfection

For liquid chromatography combined with collision-induced dissociation tandem mass spectrometry (LC-MS/MS), 293 cells were transfected with pcDNA-Dok-3 alone or along with pcDNA-Src-YF using Fugene 6 reagent (Roche). Cells were subjected to immunoprecipitation using M2 antibodies to FLAG epitope and an LC-MS/MS analysis at 20–24 h after transfection. Otherwise, 293T cells were transfected with the indicated plasmids by a

standard calcium phosphate method, and subjected to immunoprecipitation or immunoblotting at 48 h after transfection.

Immunoprecipitation and immunoblotting

293T cells were transfected with the indicated plasmids and solubilized with TNN buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1.0% Nonidet P-40, 0.2 mM Na₃VO₄, and 20 µg/mL aprotinin). Cleared lysates were then sequentially incubated with antibodies to FLAG epitope, HA epitope, or Shc, and protein G-Sepharose (Amersham Biosciences). The immune complexes were precipitated and the immunoprecipitates were washed five times with TNN buffer. Proteins in whole cell lysates or immunoprecipitates were separated by SDS/10% or 12% polyacrylamide gel electrophoresis, transferred to PVDF membrane (Bio-Rad Laboratories), and incubated with appropriate antibodies for immunoblotting. The membranes were then incubated with horseradish peroxidase-labeled secondary antibodies (Amersham Biosciences) and the blots were visualized with an ECL system (Amersham Biosciences).

Purification and digestion of Dok-3-associated proteins

The Dok-3-associated proteins were purified and digested as previously described (Natsume *et al.* 2002; Komatsu *et al.* 2004). In brief, 293 cells transfected with pcDNA-Dok-3 alone or along with pcDNA-Src-YF were solubilized with 1 mL of TNE buffer (10 mM Tris-HCl [pH 7.5], 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 1 mM Na₃VO₄, 5 µg/mL aprotinin, 1 mM phenylmethylsulfonyl fluoride, 5 µg/mL leupeptin, 3 µg/mL pepstatinA, and 50 mM NaF). Cleared lysates were then incubated overnight at 4 °C with 20 µL of mAbs (M2)-conjugated agarose. The protein-bound M2-agarose beads were washed extensively with TNE buffer, and the proteins were eluted with FLAG peptide. The isolated proteins (0.2 µg of each) were precipitated using 20 µL of a methanol/chloroform mixture (1 : 1 v/v). After vacuum-drying, the precipitate was digested with 5 µL of *Achromobacter* protease I (40 µM; substrate-to-enzyme ratio 50 : 1) in Tris buffer (50 mM Tris-HCl [pH 9.0], 6 M urea, and 0.005% *n*-octyl glucopyranoside) overnight at 37 °C.

LC-MS/MS analysis

The Dok-3-associated proteins digested with *Achromobacter* protease I were analyzed using a nanoscale LC-MS/MS system as described (Natsume *et al.* 2002; Komatsu *et al.* 2004). In brief, the peptide mixture was applied to a Mightysil-PR-18 (1 µm particle, Kanto Chemical) frit-less column (45 mm × 0.150 mm internal diameter) and separated using a 0%–40% gradient of acetonitrile containing 0.1% formic acid over 30 min at a flow rate of 50 nL/min. Eluted peptides were sprayed directly into a quadrupole time-of-flight hybrid mass spectrometer (Q-Tof *Ultima*, Micro-mass). MS and MS/MS spectra were obtained in a data-dependent mode. Up to four precursor ions above an intensity threshold of 10 counts/s were selected for MS/MS analyses from each survey scan. All MS/MS spectra were searched against protein sequences

of Swiss Prot and RefSeq (NCBI) using batch processes of the Mascot software package (Matrix Science). Criteria for match acceptance were: (1) when the match score was 10 over each threshold, identification was accepted without further consideration; (2) when the difference of score and threshold was lower than 10, or when proteins were identified based on a single matched MS/MS spectrum, the raw data were manually confirmed prior to acceptance; (3) peptides assigned by less than three y series ions and peptides with +4 charge state were all eliminated regardless of their scores.

RBD pull-down assay

A bacterially expressed glutathione S-transferase (GST) fused with the Ras-binding domain (RBD) of human c-Raf-1 (amino acids 1–149), bound with glutathione-sepharose beads was prepared as described (Taylor & Shalloway 1996). 293T cells transfected with the indicated plasmids were solubilized in a lysis buffer (50 mM Tris [pH 7.4], 100 mM NaCl, 10% glycerol, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.2 mM Na₃VO₄, and 20 µg/mL aprotinin). Cleared lysates were then incubated with GST-Raf RBD beads containing 100–200 µg of the fusion protein for 60 min at 4 °C. After extensive washing, proteins bound to the beads were boiled and eluted with SDS-loading buffer (50 mM Tris [pH 6.8], 5 mM EDTA, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol) and were separated by SDS/12% polyacrylamide gel electrophoresis for immunoblotting with mAbs to H-Ras.

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References

- Cai, D., Dhe-Paganon, S., Melendez, P.A., Lee, J. & Shoelson, S.E. (2003) Two new substrates in insulin signaling, IRS5/DOK4 and IRS6/DOK5. *J. Biol. Chem.* **278**, 25323–25330.
- Carpino, N., Wisniewski, D., Strife, A., *et al.* (1997) p62^{dok}: a constitutively tyrosine-phosphorylated, GAP-associated protein in chronic myelogenous leukemia progenitor cells. *Cell* **88**, 197–204.
- Cong, F., Yuan, B. & Goff, S.P. (1999) Characterization of a novel member of the DOK family that binds and modulates Abl signaling. *Mol. Cell. Biol.* **19**, 8314–8325.
- Crowder, R.J., Enomoto, H., Yang, M., Johnson, E.M. Jr & Milbrandt, J. (2004) Dok-6, a novel p62 Dok family member, promotes Ret-mediated neurite outgrowth. *J. Biol. Chem.* **279**, 42072–42081.
- Di Cristofano, A., Carpino, N., Dunant, N., *et al.* (1998) Molecular cloning and characterization of p56^{dok-2} defines a new family of RasGAP-binding proteins. *J. Biol. Chem.* **273**, 4827–4830.
- Faisal, A., Kleiner, S. & Nagamine, Y. (2004) Non-redundant role of Shc in Erk activation by cytoskeletal reorganization. *J. Biol. Chem.* **279**, 3202–3211.
- Grimm, J., Sachs, M., Britsch, S., *et al.* (2001) Novel p62dok family members, dok-4 and dok-5, are substrates of the c-Ret receptor tyrosine kinase and mediate neuronal differentiation. *J. Cell Biol.* **154**, 345–354.
- Hanafusa, H., Torii, S., Yasunaga, T. & Nishida, E. (2002) Sprouty1 and Sprouty2 provide a control mechanism for the Ras/MAPK signalling pathway. *Nature Cell Biol.* **4**, 850–858.
- Hashimoto, A., Kurosaki, M., Gotoh, N., Shibuya, M. & Kurosaki, T. (1999) Shc regulates epidermal growth factor-induced activation of the JNK signaling pathway. *J. Biol. Chem.* **274**, 20139–20143.
- Hubbard, S.R. & Till, J.H. (2000) Protein tyrosine kinase structure and function. *Annu. Rev. Biochem.* **69**, 373–398.
- Jones, N. & Dumont, D.J. (1999) Recruitment of Dok-R to the EGF receptor through its PTB domain is required for attenuation of Erk MAP kinase activation. *Curr. Biol.* **9**, 1057–1060.
- Kessels, H.W.H.G., Ward, A.C. & Schumacher, T.N.M. (2002) Specificity and affinity motifs for Grb2 SH2-ligand interactions. *Proc. Natl. Acad. Sci. USA* **99**, 8524–8529.
- Kim, M., Tezuka, T., Tanaka, K. & Yamamoto, T. (2004) Cbl-c suppresses v-Src-induced transformation through ubiquitin-dependent protein degradation. *Oncogene* **23**, 1645–1655.
- Komatsu, M., Chiba, T., Tatsumi, K., *et al.* (2004) A novel protein-conjugating system for Ufm1, a ubiquitin-fold modifier. *EMBO J.* **23**, 1977–1986.
- Lai, K.M.V. & Pawson, T. (2000) The ShcA phosphotyrosine docking protein sensitizes cardiovascular signaling in the mouse embryo. *Genes Dev.* **14**, 1132–1145.
- Lemay, S., Davidson, D., Latour, S. & Veillette, A. (2000) Dok-3, a novel adapter molecule involved in the negative regulation of immunoreceptor signaling. *Mol. Cell. Biol.* **20**, 2743–2754.
- Lowy, D.R., Zhang, K., DeClue, J.E. & Willumsen, B.M. (1991) Regulation of p21ras activity. *Trends Genet.* **7**, 346–351.
- Million, R.P. & Van Etten, R.A. (2000) The Grb2 binding site is required for the induction of chronic myeloid leukemia-like disease in mice by the Bcr/Abl tyrosine kinase. *Blood* **96**, 664–670.
- Natsume, T., Yamauchi, Y., Nakayama, H., *et al.* (2002) A direct nanoflow liquid chromatography-tandem mass spectrometry system for interaction proteomics. *Anal. Chem.* **74**, 4725–4733.
- Neet, K. & Hunter, T. (1995) The nonreceptor protein-tyrosine kinase CSK complexes directly with the GTPase-activating protein-associated p62 protein in cells expressing v-Src or activated c-Src. *Mol. Cell. Biol.* **15**, 4908–4920.
- Nelms, K., Snow, A.L., Hu-Li, J. & Paul, W.E. (1998) FRIP, a hematopoietic cell-specific rasGAP-interacting protein phosphorylated in response to cytokine stimulation. *Immunity* **9**, 13–24.
- Niki, M., Di Cristofano, A., Zhao, M., *et al.* (2004) Role of Dok-1 and Dok-2 in leukemia suppression. *J. Exp. Med.* **200**, 1689–1695.
- Niwa, H., Yamamura, K. & Miyazaki, J. (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**, 193–199.

- Ravichandran, K.S. (2001) Signaling via Shc family adapter proteins. *Oncogene* **20**, 6322–6330.
- Robson, J.D., Davidson, D. & Veillette, A. (2004) Inhibition of the Jun N-terminal protein kinase pathway by SHIP-1, a lipid phosphatase that interacts with the adaptor molecule Dok-3. *Mol. Cell. Biol.* **24**, 2332–2343.
- Rohrschneider, L.R., Fuller, J.F., Wolf, I., Liu, Y. & Lucas, D.M. (2000) Structure, function, and biology of SHIP proteins. *Genes Dev.* **14**, 505–520.
- Roskoski, R. Jr (2004) Src protein-tyrosine kinase structure and regulation. *Biochem. Biophys. Res. Commun.* **324**, 1155–1164.
- Shinohara, H., Inoue, A., Toyama-Sorimachi, N., *et al.* (2005) Dok-1 and Dok-2 are negative regulators of lipopolysaccharide-induced signaling. *J. Exp. Med.* **201**, 333–339.
- Shinohara, H., Yasuda, T. & Yamanashi, Y. (2004) Dok-1 tyrosine residues at 336 and 340 are essential for the negative regulation of Ras-Erk signaling, but dispensable for rasGAP-binding. *Genes Cells* **9**, 601–607.
- Songyang, Z., Yamanashi, Y., Liu, D. & Baltimore, D. (2001) Domain-dependent function of the rasGAP-binding protein p62Dok in cell signaling. *J. Biol. Chem.* **276**, 2459–2465.
- Taylor, S.J. & Shalloway, D. (1996) Cell cycle-dependent activation of Ras. *Curr. Biol.* **16**, 1621–1627.
- Van Slyke, P., Coll, M.L., Master, Z., Kim, H., Filmus, J. & Dumont, D.J. (2005) Dok-R mediates attenuation of epidermal growth factor-dependent mitogen-activated protein kinase and Akt activation through processive recruitment of c-Src and Csk. *Mol. Cell. Biol.* **25**, 3831–3841.
- Veillette, A., Latour, S. & Davidson, D. (2002) Negative regulation of immunoreceptor signaling. *Annu. Rev. Immunol.* **20**, 669–707.
- Wakioka, T., Sasaki, A., Kato, R., *et al.* (2001) Spred is a Sprouty-related suppressor of Ras signaling. *Nature* **412**, 647–651.
- Wick, M.J., Dong, L.Q., Hu, D., Langlais, P. & Liu, F. (2001) Insulin receptor-mediated p62^{dok} tyrosine phosphorylation at residues 362 and 398 plays distinct roles for binding GTPase-activating protein and Nck and is essential for inhibiting insulin-stimulated activation of Ras and Akt. *J. Biol. Chem.* **276**, 42843–42850.
- Wisniewski, D., Strife, A., Swendeman, S., *et al.* (1999) A novel SH2-containing phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase (SHIP2) is constitutively tyrosine phosphorylated and associated with src homologous and collagen Gene (SHC) in chronic myelogenous leukemia progenitor cells. *Blood* **93**, 2707–2720.
- Yamanashi, Y. & Baltimore, D. (1997) Identification of the Abl- and rasGAP-associated 62 kDa protein as a docking protein, Dok. *Cell* **88**, 205–211.
- Yamanashi, Y., Tamura, T., Kanamori, T., *et al.* (2000) Role of the rasGAP-associated docking protein p62^{dok} in negative regulation of B cell receptor-mediated signaling. *Genes Dev.* **14**, 11–16.
- Yasuda, T., Shirakata, M., Iwama, A., *et al.* (2004) Role of Dok-1 and Dok-2 in myeloid homeostasis and suppression of leukemia. *J. Exp. Med.* **200**, 1681–1687.
- Zhao, M., Schmitz, A.A.P., Qin, Y., Di Cristofano, A., Pandolfi, P.P. & Van Aelst, L. (2001) Phosphoinositide 3-kinase-dependent membrane recruitment of p62^{dok} is essential for its negative effect of mitogen-activated protein (MAP) kinase activation. *J. Exp. Med.* **194**, 265–274.

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