Novel p62dok family members, dok-4 and dok-5, are substrates of the c-Ret receptor tyrosine kinase and mediate neuronal differentiation

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Docking proteins are substrates of tyrosine kinases and function in the recruitment and assembly of specific signal transduction molecules. Here we found that p62dok family members act as substrates for the c-Ret receptor tyrosine kinase. In addition to dok-1, dok-2, and dok-3, we identified two new family members, dok-4 and dok-5, that can directly associate with Y1062 of c-Ret. Dok-4 and dok-5 constitute a subgroup of dok family members that is coexpressed with c-Ret in various neuronal tissues. Activated c-Ret promotes neurite outgrowth of PC12 cells; for this activity, Y1062 in c-Ret is essential. c-Ret/dok fusion proteins, in which Y1062 of c-Ret is deleted and replaced by the sequences of dok-4 or dok-5, induce ligand-dependent axonal outgrowth of PC12 cells, whereas a c-Ret fusion containing dok-2 sequences does not elicit this response. Dok-4 and dok-5 do not associate with rasGAP or Nck, in contrast to p62dok and dok-2. Moreover, dok-4 and dok-5 enhance c-Ret–dependent activation of mitogen-activated protein kinase. Thus, we have identified a subclass of p62dok proteins that are putative links with downstream effectors of c-Ret in neuronal differentiation.

Introduction

The receptor tyrosine kinase c-Ret was discovered as an oncogene and is mutated in patients with multiple endocrine neoplasia (MEN)2A,* MEN2B, and familial medullary thyroid carcinoma cancer syndromes (Takahashi et al., 1985; Donis-Keller et al., 1993; Mulligan et al., 1993; Hofstra et al., 1994). In addition, c-Ret and its ligand glial-derived neurotrophic factor (GDNF) play essential roles in embryogenesis. Ablation of the GDNF or c-Ret genes in mice results in absence or severe hypoplasia of the kidneys. Moreover, development of neural crest cells and their derivatives is impaired and, as a consequence, portions of the enteric nervous system are not formed and the number of neurons of several peripheral ganglia is reduced (Schuchardt et al., 1994; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996). Similarly, inactivating mutations of c-Ret in humans can cause Hirschsprung's disease, which is characterized by hypoplasia of the enteric nervous system (Edery et al., 1994; Romeo et al., 1994). Two different isoforms of c-Ret exist as a result of alternative splicing; both isoforms promote differentiation of PC12 cells when activated (Rossel et al., 1997). Activated c-Ret recruits signaling proteins like Grb7/Grb10, PLCγ, Shc/Enigma and Grb2, which bind to phosphorylated tyrosine residues in its COOH-terminal sequence, Y905, Y1015, Y1062, and Y1096, respectively (Asai et al., 1996; Borrello et al., 1996; Durick et al., 1996; Arighi et al., 1997; Lorenzo et al., 1997; Alberti et al., 1998). Recent work has shown that Shc binding to c-Ret recruits complexes consisting of Grb2, Gab1/2, SHP-2, and phosphotidyl inositol 3 kinase in addition to Grb2-Sos (Besset et al., 2000; Hayashi et al., 2000). The docking site Y1062 of c-Ret constitutes a consensus phosphotyrosine binding (PTB) interaction site and has been shown to be essential for mitogenic signaling by MEN2A and MEN2B c-Ret mutants (Asai et al., 1996). However, the function of Y1062 in morphogenic signaling and recruitment of specific substrates for c-Ret signaling has not been explored.

The specificity of receptor tyrosine kinase signaling has been investigated in great detail. A variety of substrates are

shared by several receptors (Ullrich and Schlessinger, 1990; van der Geer et al., 1994). In addition, specific docking proteins of receptor tyrosine kinases have been discovered which mediate particular biological responses. Insulin receptor substrate (IRS)-1 and IRS-2 are two essential substrates of the insulin receptor and, accordingly, ablation of the *IRS-2* gene in mice results in diabetes (Sun et al., 1991, 1995; Withers et al., 1998). Gab1 is important for signaling of c-Met; *Gab-1*–deficient mice exhibit a phenotype similar to c-Met$^{-/-}$ mice, i.e., embryonal lethality and impaired migration of myogenic precursor cells (Bladt et al., 1995; Weidner et al., 1996; Sachs et al., 2000). Moreover, FRS2 mediates FGF and Trk receptor signaling in cells (Kouhara et al., 1997; Meakin et al., 1999). Similarly, dos in *Drosophila* is essential in sevenless signaling (Herbst et al., 1996; Raabe et al., 1996). These docking proteins contain NH_2-terminal membrane-targeting elements, pleckstrin homology (PH) domains or myristylation sites, and receptor-targeting sequences, PTB or PTB-like domains. In addition, docking proteins harbor multiple consensus binding sites for SH2 and SH3 containing molecules.

Several recent reports implicate the previously identified dok members, p62dok (dok-1), dok-2, and dok-3, in negative regulation of signaling pathways activated by tyrosine kinases. These doks inhibit mitogen-activated protein (MAP) kinase signaling, cell proliferation, and cellular transformation (Cong et al., 1999; Suzu et al., 2000; Tamir et al., 2000). The closely related p62dok and dok-2 may exert their inhibitory effects by recruitment of rasGAP, a negative regulator of ras signaling. Dok-2 can also attenuate EGF receptor (EGFR)-induced MAP kinase activation, independent of its association with rasGAP (Jones and Dumont, 1999). Also, dok-3 is a negative regulator of immune receptor and v-Abl signaling without binding rasGAP, but recruiting SHIP and Csk (Cong et al., 1999; Lemay et al., 2000). The p62dok family members resemble docking proteins in their structure, since they contain PH and PTB domains as well as multiple SH2 and SH3 binding sites (Carpino et al., 1997; Yamanashi and Baltimore, 1997; Di Cristofano et al., 1998; Nelms et al., 1998; Cong et al., 1999). In the present study, we identified a new subgroup of p62dok family members, dok-4 and dok-5, which associate directly with the receptor tyrosine kinase c-Ret. We show that dok-4 and dok-5 can function in c-Ret–mediated neurite outgrowth. In contrast to p62dok and dok-2, dok-4 and dok-5 do not bind rasGAP and play a positive role in activation of the MAP kinase pathway.

Results

### Identification of p62dok family members as interaction partners of c-Ret

To identify new substrates that interact with the activated c-Ret receptor tyrosine kinase, we carried out a modified yeast two-hybrid screen (O'Neill et al., 1994; Weidner et al., 1996). The bait vector encodes the cytoplasmic part of the short isoform of c-Ret (Tahira et al., 1990), which includes Y1062, plus the DNA binding and dimerization domain of the LexA transcription factor. Since this bait dimerizes, its tyrosine kinase is constitutively active and is phosphorylated

on tyrosine residues in yeast (data not shown). By screening a mouse E10.5 cDNA library, we identified p62dok family members, dok-2 and dok-3, and a novel cDNA clone, dok-4, as direct c-Ret binding proteins (Fig. 1 a and data not shown). An additional p62dok family member, dok-5, was identified by low stringency hyridization. By searching Expressed Sequence Tag databases, we have also found human dok-6, but failed to isolate the mouse homologue (data not shown). In the yeast two-hybrid assay, dok-5 and -6 also interact with c-Ret. A mutation of tyrosine 1062 in the c-Ret sequence abolishes binding to all dok family members (Fig. 1 a and data not shown). Moreover, dok proteins bind to c-Ret in a phosphorylation-dependent manner, since a kinase-defective receptor, K758M, does not interact. We also examined interaction of p62dok family members with other tyrosine kinase receptors. Dok-2 binds to c-Ret, Tie-2, and weakly to the EGFR, but not to other receptors like Met, Kit, Fms, Ros, TrkA, ErbB-2, and ErbB-3 (Fig. 1, b and c). Dok-4 displays a similar specificity, except that it does not bind to the EGFR (Fig. 1, a and c; see also below).

We isolated the complete cDNAs of five dok family members, mouse dok-1–5. All dok members contain NH_2-terminal PH and central PTB domains; the PTB domains mediate receptor interaction (see below). The COOH-terminal sequences vary in length and show only limited sequence identity (Fig. 2 a). Dok proteins contain tyrosine residues within consensus binding sites for SH2 domains and several PXXP SH3 binding motifs. The deduced amino acid sequences of the new dok members, dok-4 and dok-5, are shown in Fig. 2, b and c; the sequences of dok-1–3 have been reported previously (Carpino et al., 1997; Yamanashi and Baltimore, 1997; Di Cristofano et al., 1998; Jones and Dumont, 1998; Nelms et al., 1998; Cong et al., 1999). A dendrogram of deduced amino acid sequences of the PTB domains of the doks shows that dok-4/5 and dok-1/2, respectively, are closely related (Fig. 2 d).

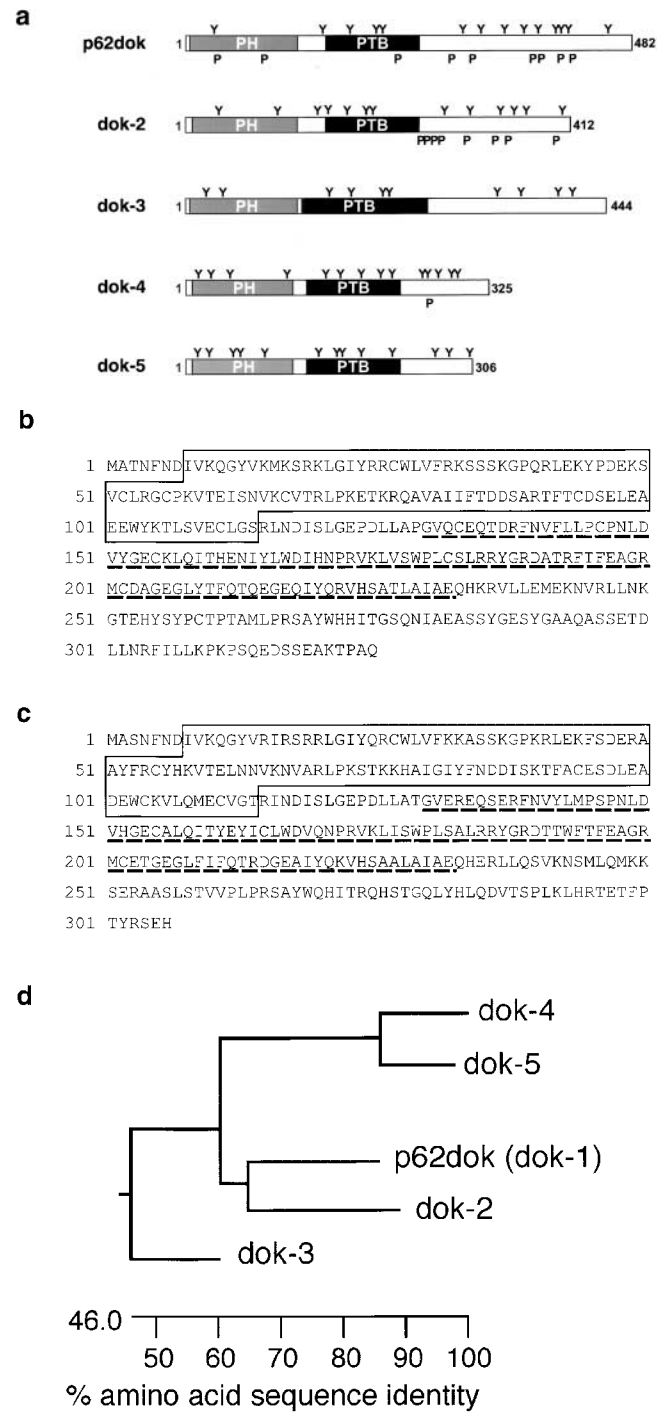### Dok family members are differentially expressed

In situ hybridization of E12.5 and E13.5 mouse embryos showed that dok-4 is expressed in the central and peripheral nervous system and in endothelia (Fig. 3, a, d, g, and h). Sites of expression in the nervous system are the ventral portion of the neural tube and the dorsal root and cranial sensory ganglia. Dok-5 is also expressed in the nervous system, e.g., in the neural tube and the dorsal root and cranial ganglia, but not in endothelia (Fig. 3, b and e). The expression of these doks appears to be specific for neurons, since intense punctate signals are observed in large neuronal cells of the dorsal root ganglia and are absent from the glial cells lining the emerging spinal nerves (data not shown). Potential overlaps between dok-4/5 and c-Ret are thus seen in the ventral part of the neural tube and in the dorsal root, the trigeminal and geniculate ganglia (Fig. 3, c and f). c-Ret expression at this stage of development is restricted to a subset of large neurons; however, the number of small c-Ret neurons increases with age (data not shown; Molliver et al., 1997). In the kidney, dok-4 is expressed in endothelia, whereas c-Ret is expressed in the growing tips of the ureter epithelium (Fig. 3, i and j). The other dok family members, dok-1–3, are mainly expressed in hematopoietic tissues (Carpino et al.,
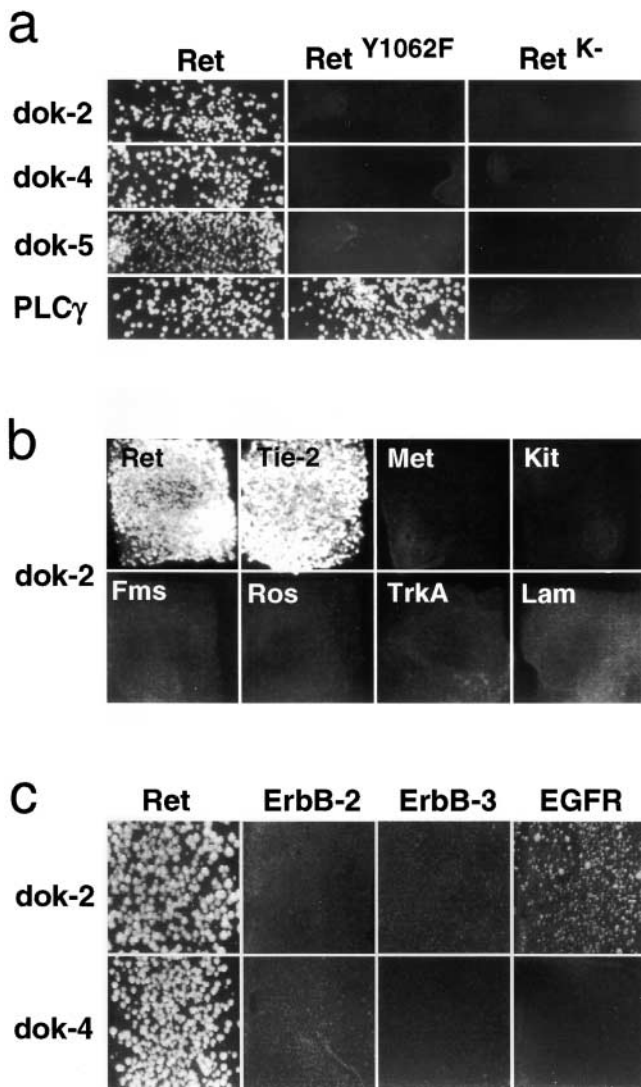
Figure 1. **Interaction of dok family members with c-Ret and other receptor tyrosine kinases in the yeast two-hybrid system.** Growth of yeast on selective medium. (a) Dok-2, -4, and -5 interact with wild-type c-Ret, but not with c-Ret receptors harboring a Y1062F mutation or an inactive kinase (Ret K⁻, K758M; Liu et al., 1996). (b) Dok-2 interacts with c-Ret and Tie-2, but not with other receptor tyrosine kinases tested. Lam, lamin. (c) Dok-2, but not dok-4, interacts weakly with the EGFR but not other members of the EGFR family.

Figure 2. **The dok protein family and newly identified dok members.** (a) Domain structure of dok family members. PH and PTB domains are marked grey and black, and the positions of tyrosine residues (Y) and PXXP motifs (P) are indicated. (b and c) Deduced amino acid sequences of mouse dok-4 and dok-5, respectively. PH domains are boxed and PTB domains are underlined. (d) Phylogenetic tree of dok family members. Sequences of the PTB domains were aligned.

1997; Nelms et al., 1998; Lemay et al., 2000). For instance, dok-2 is found in a punctuate pattern in the embryonal liver, consistent with an expression in hematopoietic precursors (Fig. 3 k and data not shown). Northern blotting indicated that the expression of the various dok mRNAs is maintained in the respective adult tissues (Fig. 4 and data not shown). Dok-4 is expressed broadly in many tissues, such as brain, heart, lung, and kidney, consistent with its expression in endothelia at earlier stages. Dok-5 is specifically expressed in the brain. In contrast, Dok-2 is found in spleen and lung, which are rich in hematopoietic cell types.

The cellular association of c-Ret with dok family members was investigated by coimmunoprecipitation using 293 cells. Dok proteins bind to wild-type c-Ret, which is phosphorylated upon overexpression (Fig. 5 a). The new dok proteins,

dok-4 and dok-5, exhibit apparent molecular weights of 41 and 36 k, respectively (Fig. 5 a). All dok proteins tested are phosphorylated by endogenous c-Ret upon GDNF stimulation, and they also associate with the endogenous c-Ret receptor (shown for dok-2 and dok-5 in Fig. 5 b). Binding of the doks to c-Ret is mediated by their PTB domain and re-
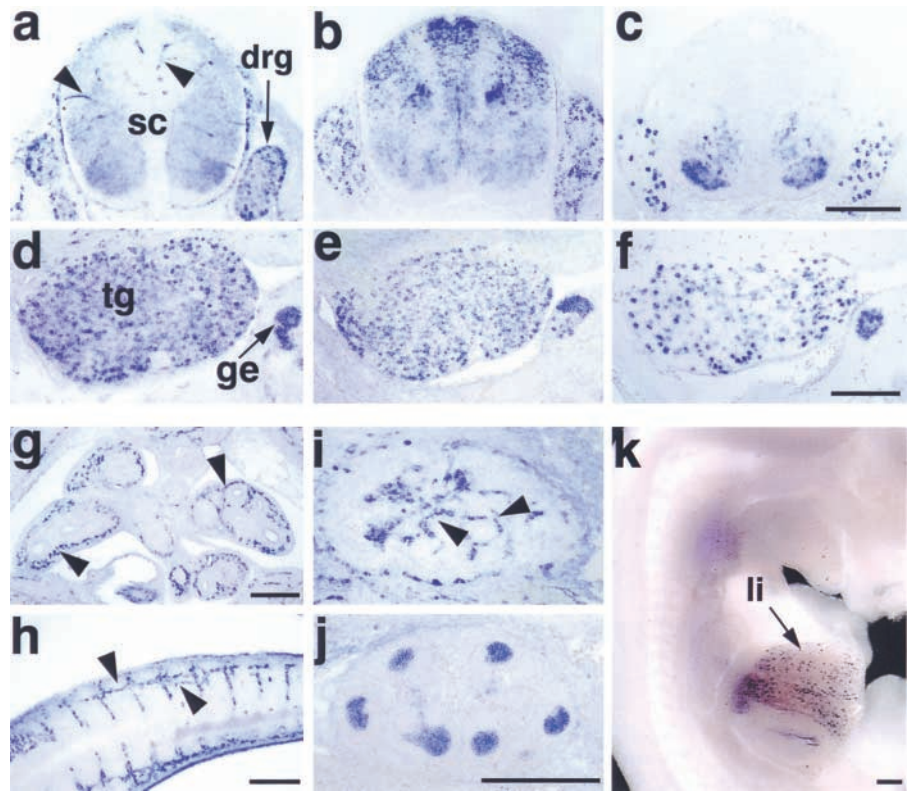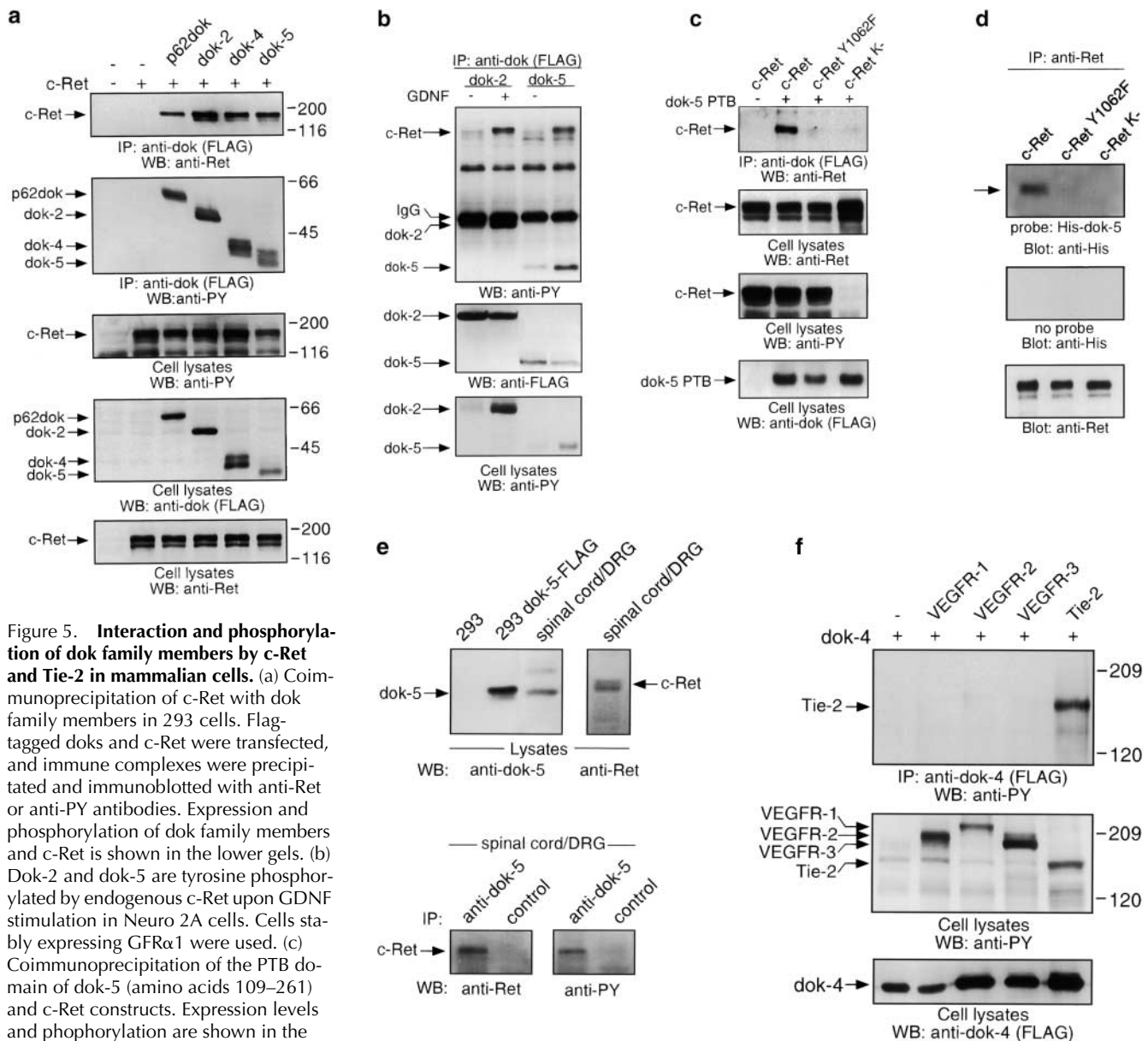
Figure 3. **Expression of dok family members and c-Ret in mouse embryos.** Whole-mount in situ hybridization of E12.5 and E13.5 mouse embryos followed by semithin sectioning. (a, d, and g–i) Labeling with a dok-4–specific probe. Dok-4–positive cells are observed in the ventral part of the spinal cord (sc), the dorsal root ganglia (drg), the trigeminal (tg) and geniculate ganglia (ge), and in endothelia (arrowheads). g–i are sections through lung, tail, and kidney, respectively. (b and e) Labeling with a dok-5–specific probe. (c, f, and j) Labeling with a c-Ret–specific probe. Panel j shows expression in the buds of the ureter epithelium in the kidney. (k) Whole-mount in situ hybridization with a dok-2–specific probe. Dok-2 is specifically expressed in islands of cells within the embryonal liver (li). Bars, 0.2 mm.

quires phosphorylation of Y1062 (shown for dok-5 in Fig. 5 c). Interaction between the doks and c-Ret is direct, as demonstrated by far Western blotting (Fig. 5 d). We could also show interaction of dok-5 and c-Ret in vivo: in extracts of spinal cords and dorsal root ganglia from newborn mice,

c-Ret was coimmunoprecipitated with dok-5 by using a dok-5–specific antiserum (Fig. 5 e). We also tested whether dok-4 associates with endothelial receptor tyrosine kinases. Indeed, dok-4 coprecipitates with Tie-2, but not the VEGF receptors 1, 2, and 3 (Fig. 5 f).
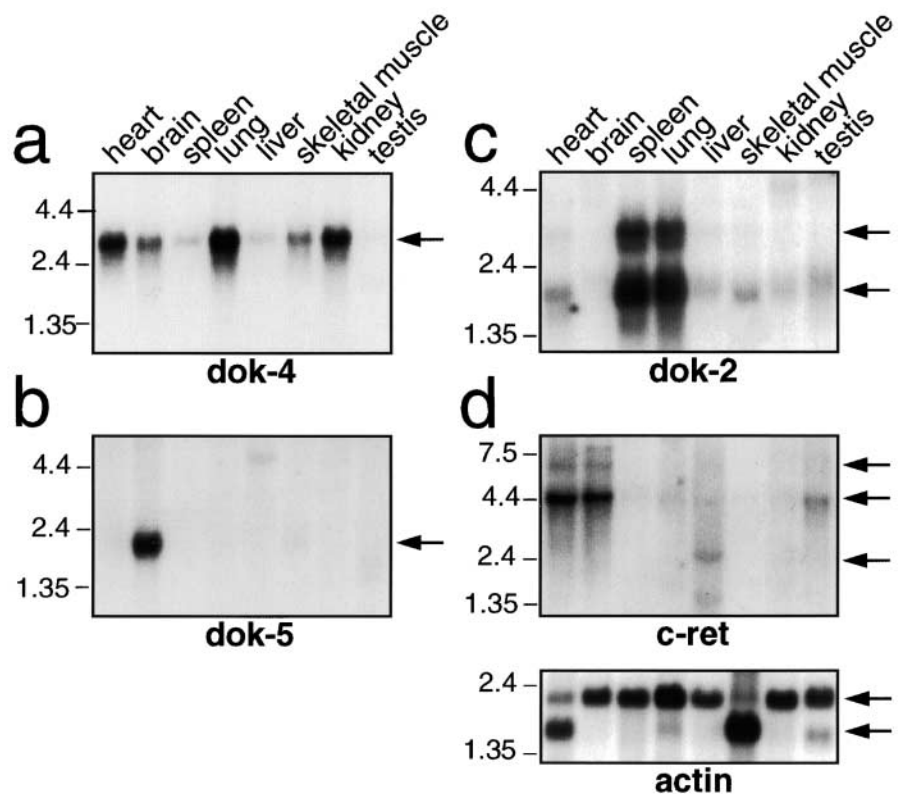
Figure 4. **Northern blotting of dok and c-Ret transcripts in adult mouse tissues.** cDNA probes specific for dok-4 (a), dok-5 (b), dok-2 (c), c-Ret (d), or actin were used. The positions of the transcripts are indicated by arrows.

Figure 5. **Interaction and phosphorylation of dok family members by c-Ret and Tie-2 in mammalian cells.** (a) Coimmunoprecipitation of c-Ret with dok family members in 293 cells. Flag-tagged doks and c-Ret were transfected, and immune complexes were precipitated and immunoblotted with anti-Ret or anti-PY antibodies. Expression and phosphorylation of dok family members and c-Ret is shown in the lower gels. (b) Dok-2 and dok-5 are tyrosine phosphorylated by endogenous c-Ret upon GDNF stimulation in Neuro 2A cells. Cells stably expressing GFRα1 were used. (c) Coimmunoprecipitation of the PTB domain of dok-5 (amino acids 109–261) and c-Ret constructs. Expression levels and phophorylation are shown in the lower gels. (d) Far Western blot demonstrating direct interaction of dok-5 with c-Ret but not mutant c-Ret. (e) A dok-5–specific antibody recognizes dok-5 in transfected 293 cells and in preparations of spinal cord and dorsal root ganglia, which also express c-Ret (top). The anti–dok-5 antibody coimmunoprecipitates phosphorylated c-Ret in extracts of spinal cords and dorsal root ganglia (bottom). (f) Coimmunoprecipitation of Tie-2 and dok-4 in 293 cells. Flag-tagged dok-4 and endothelial receptor tyrosine kinases were transfected, and immune complexes were precipitated and immunoblotted with anti-PY antibodies. Expression of phosphorylated receptors and dok-4 is shown in the lower gels.

Dok-4 and dok-5 mediate c-Ret–dependent neuronal differentiation of PC12 cells

Stimulation of c-Ret, but not the EGFR, induces axonal outgrowth in PC12 cells (Treanor et al., 1996). However, a chimeric receptor that contains the extracellular domain of the EGFR fused to the intracellular domain of c-Ret can induce axonal outgrowth in an EGF-dependent manner (Santoro et al., 1994; Rizzo et al., 1996; see also Fig. 6 a). In contrast, the Y1062F mutant EGFR/c-Ret hybrid, which does not bind dok proteins, is not efficient in inducing axonal outgrowth (Fig. 6 a, see statistics and expression levels of hybrid receptors in Fig. 6 d). To determine if activation of dok family members by c-Ret induces neuronal differentiation, we fused

sequences of dok-4 to an EGFR/c-Ret hybrid that lacks the 22 COOH-terminal amino acids including tyrosine 1062. The dok-4 sequence used also lacks the region encoding the PH domain. Indeed, this fusion receptor, EGFR/c-Ret-dok-4, strongly induces axonal outgrowth of PC12 cells upon stimulation with EGF (Fig. 6, b and d). In addition, receptor fusions that contain only COOH-terminal sequences of dok-4 downstream of the PTB domain, or dok-5, promote neurite outgrowth (Fig. 6, b–d). In contrast, an EGFR/c-Ret hybrid fused to dok-2 is ineffective in this assay (Fig. 6, c and d). These data indicate that the newly identified dok members, dok-4/5, are putative links with downstream effectors of c-Ret involved in neuronal differentiation.
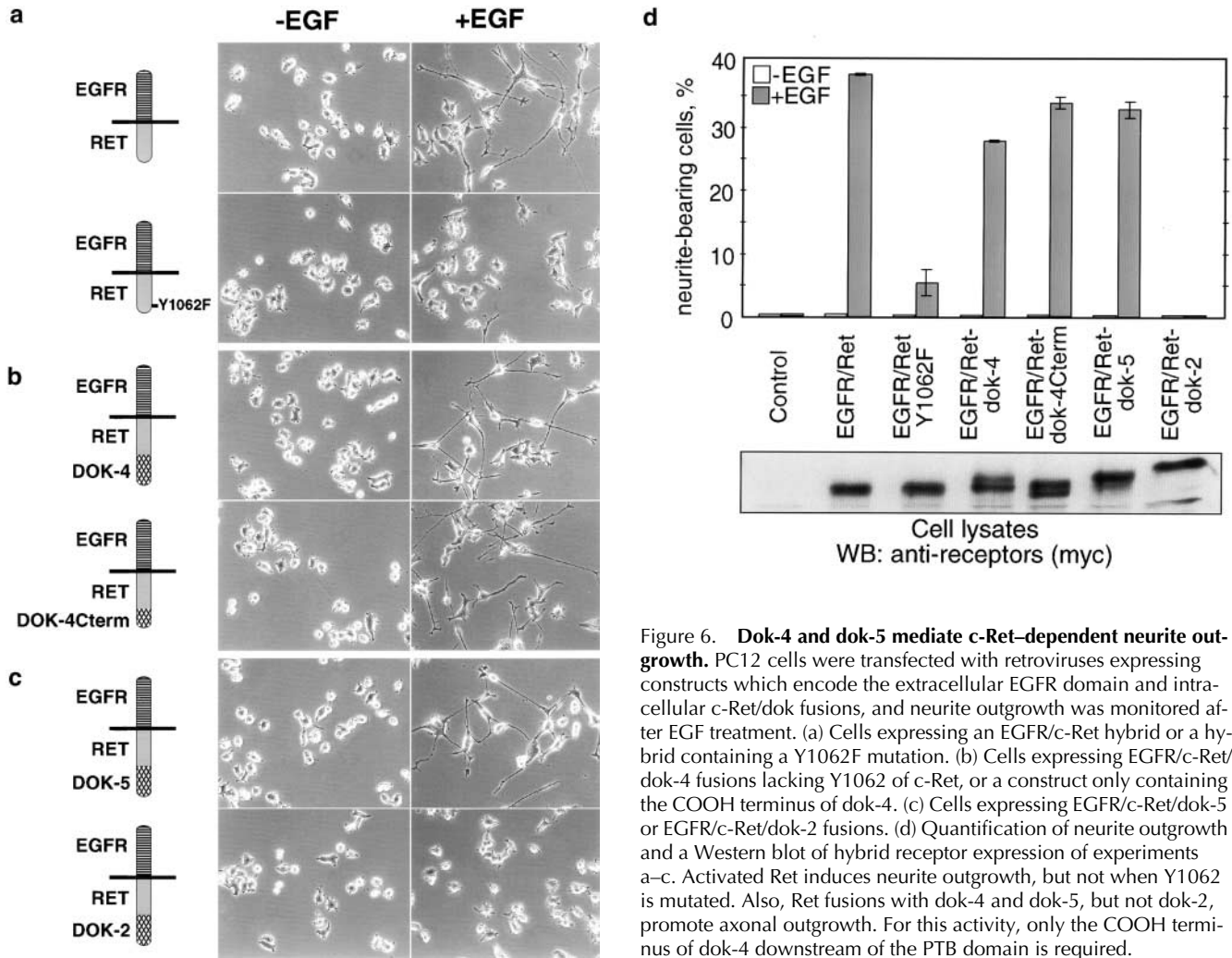
Figure 6. **Dok-4 and dok-5 mediate c-Ret–dependent neurite outgrowth.** PC12 cells were transfected with retroviruses expressing constructs which encode the extracellular EGFR domain and intracellular c-Ret/dok fusions, and neurite outgrowth was monitored after EGF treatment. (a) Cells expressing an EGFR/c-Ret hybrid or a hybrid containing a Y1062F mutation. (b) Cells expressing EGFR/c-Ret/dok-4 fusions lacking Y1062 of c-Ret, or a construct only containing the COOH terminus of dok-4. (c) Cells expressing EGFR/c-Ret/dok-5 or EGFR/c-Ret/dok-2 fusions. (d) Quantification of neurite outgrowth and a Western blot of hybrid receptor expression of experiments a–c. Activated Ret induces neurite outgrowth, but not when Y1062 is mutated. Also, Ret fusions with dok-4 and dok-5, but not dok-2, promote axonal outgrowth. For this activity, only the COOH terminus of dok-4 downstream of the PTB domain is required.

Previous investigations have shown that p62dok and dok-2 associate with rasGAP, and inhibit the MAP kinase pathway (Jones and Dumont, 1999; Tamir et al., 2000; Yamanashi et al., 2000). In contrast, we found that the newly identified dok members, dok-4 and dok-5, do not associate with rasGAP, and also not with Nck (Fig. 7 a). Also, when fused to c-Ret, dok-4 and dok-5 activate the MAP kinase pathway, as demonstrated by Erk1/2 phosphorylation (Fig. 7 b). An Elk-1–dependent promoter is also activated by dok-4/5 (Fig. 7 c), whereas hybrids containing dok-2 are inactive in these assays. Remarkably, expression of dok-2 inhibited GDNF-induced activation of the Elk-1 reporter (Fig. 7 d). Dok-2 also blocked Elk-1 activity induced by MEN2A Ret, an oncogenically activated c-Ret receptor, whereas dok-5 did not (Fig. 7 e). These results suggest that dok-4 and dok-5 are not only differently expressed, but can also activate different signaling pathways than the dok family members identified previously.

Discussion

Here we have identified a novel subfamily of dok multiadapter proteins, dok-4 and dok-5, which are putative links with downstream effectors of c-Ret signaling. Yeast twohybrid analysis and coimmunoprecipitation demonstrated that dok family members, through their PTB domains, bind directly to phosphorylated tyrosyl 1062 of Ret and become phosphorylated themselves. Interaction of doks and c-Ret could also be demonstrated in tissues of the spinal cord and dorsal root ganglia. Many other receptors, such as Met, Kit, Fms, Ros, TrkA, and ErbB-2, do not interact directly with the doks. Dok-4 and dok-5 are coexpressed with c-Ret in neuronal tissues. Moreover, PC12 cells that express c-Ret/dok-4 and c-Ret/dok-5 hybrids produce neurite outgrowth upon stimulation by ligands. These data suggest that the novel doks can mediate signals required for neuronal differentiation.

The expression of the new dok family members overlap with c-Ret in tissues of the central and peripheral nervous system. A function of c-Ret signaling in the nervous system has been demonstrated by genetic experiments. For instance, the number of sensory neurons of dorsal root ganglia and motor neurons of the spinal cord is reduced in GDNF-deficient mice (Moore et al., 1996; Sanchez et al., 1996). Ablation of neurturin, another ligand of c-Ret, leads to loss of cells in dorsal root and trigeminal sensory ganglia (Heuckeroth et al., 1999). In addition, it has been shown that GDNF and c-Ret play an important role in development of the enteric and sympathetic nervous system and the kidney (Schuchardt et al., 1994; Moore et al., 1996; Pichel et al., 1996;
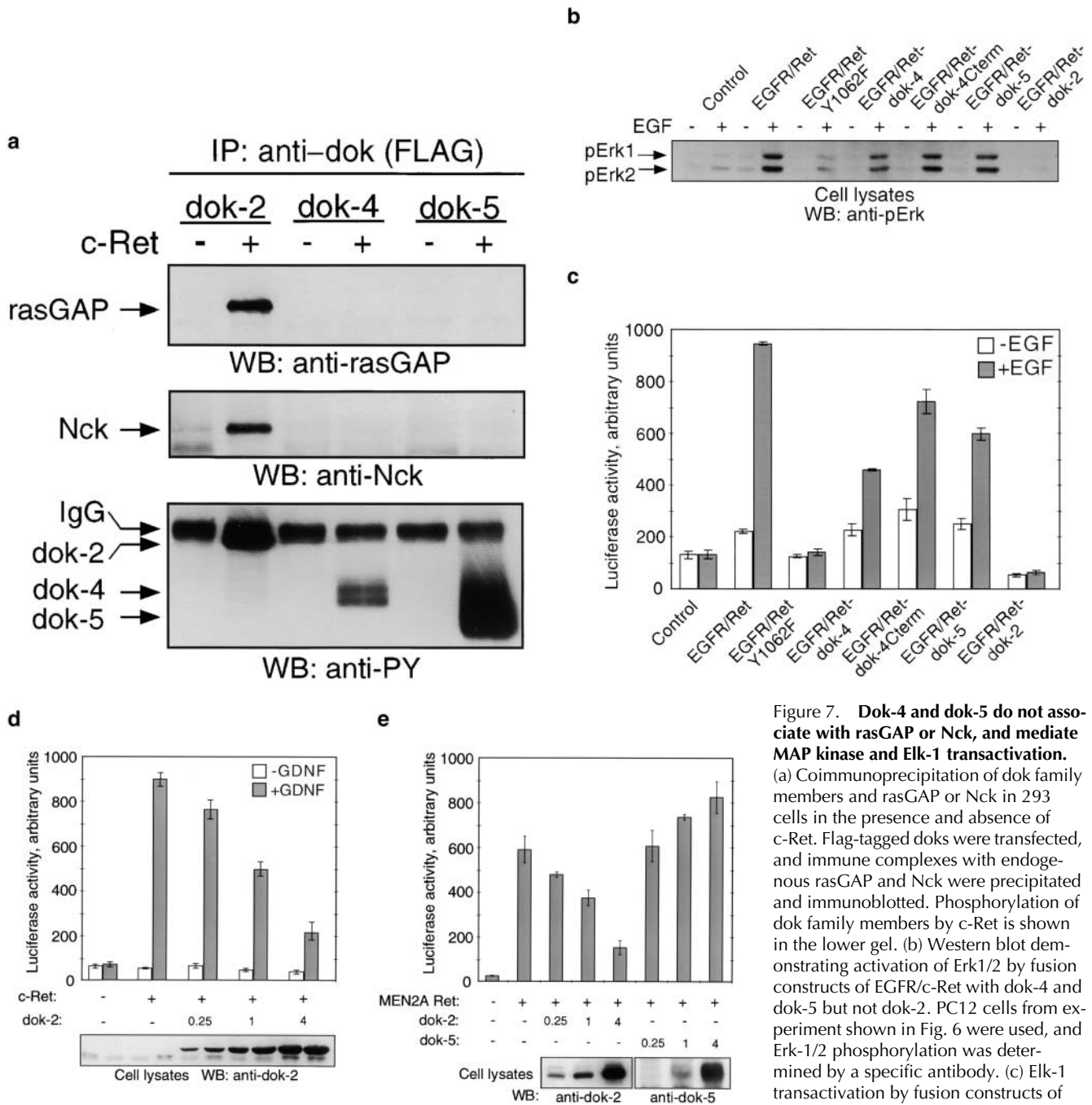
Figure 7. **Dok-4 and dok-5 do not associate with rasGAP or Nck, and mediate MAP kinase and Elk-1 transactivation.** (a) Coimmunoprecipitation of dok family members and rasGAP or Nck in 293 cells in the presence and absence of c-Ret. Flag-tagged doks were transfected, and immune complexes with endogenous rasGAP and Nck were precipitated and immunoblotted. Phosphorylation of dok family members by c-Ret is shown in the lower gel. (b) Western blot demonstrating activation of Erk1/2 by fusion constructs of EGFR/c-Ret with dok-4 and dok-5 but not dok-2. PC12 cells from experiment shown in Fig. 6 were used, and Erk-1/2 phosphorylation was determined by a specific antibody. (c) Elk-1 transactivation by fusion constructs of EGFR/c-Ret with dok-4 and dok-5 but not dok-2. Neuro 2A cells were transiently transfected with the indicated constructs together with Elk-1/Gal4 and Gal4-luciferase reporter plasmids. Cells were treated with EGF for 5 h and Luciferase activity was then determined. (d) Inhibition of c-Ret–induced Elk-1 activation by dok-2. 293 cells were transfected with c-Ret, increasing amounts of dok-2 and reporter plasmids. Cells were stimulated for 5 h with 50 ng/ml GDNF and 0.5 μg/ml soluble GFRα1. (e) Inhibition of MEN2A Ret induced activation of Elk-1 by dok-2 but not dok-5. 293 cells were transfected with Ret C634R, increasing amounts of dok-2 or dok-5, and reporter plasmids. Luciferase activity was determined 48 h posttransfection.

Sanchez et al., 1996). However, in the latter tissues, expression of dok family members does not overlap with that of c-Ret. However, it is possible that additional dok family members that are expressed at these sites exist, or that other adapters take over dok functions. Thus, we suggest that the newly identified dok proteins, dok-4 and dok-5, can mediate c-Ret signals in a subset of neuronal tissues. Dok family members are also expressed in other tissues where c-Ret expression is weak or has not been de-

scribed (Pachnis et al., 1993; Avantaggiato et al., 1994). For instance, dok-4 is strongly expressed in the vascular endothelium. We found that dok-4 can also associate with the endothelial Tie-2 receptor, suggesting that dok-4 may function as a substrate for Tie-2 in endothelia. Tie-2 has already been reported to associate with Dok-2 (Jones and Dumont, 1998); however, endothelial expression of dok-2 is not pronounced and we have not been able to detect expression in this cell type.

Other members of the dok family, dok-1–3, are mainly expressed in hematopoietic tissues. Several recent reports suggest an involvement of these dok proteins in lymphoid signaling: p62dok and dok-2 are strongly tyrosine phosphorylated in Bcr-Abl–transformed myelogenous leukemia cells (Carpino et al., 1997; Yamanashi and Baltimore, 1997; Di Cristofano et al., 1998). Dok-2 (dok-R/FRIP) also binds directly to the IL-4 receptor (Nelms et al., 1998). It is also possible that hematopoietic dok proteins act as c-Ret substrates in lymphoid cells, since recent reports suggest an involvement of c-Ret in hematopoietic differentiation (Wasserman et al., 1995; Gattei et al., 1997, 1998, 1999; Nakayama et al., 1999). Phoshorylation of p62dok after c-Ret activation can also occur in a phosphotidyl inositol 3 kinase–dependent manner (Murakami et al., 1999).

Dok family members have the typical features of multiadapter proteins such as membrane localization sequence (PH domain), receptor interaction domain (PTB domain), and several putative binding sites for downstream substrates (P-tyr and PXXP elements). The importance of direct association of particular substrates with specific receptor tyrosine kinases for activation of various signaling pathways has been demonstrated recently, e.g., IRSs are essential for insulin receptor function, FRS2 is important for fibroblast growth factor receptor and Trk signaling, and Gab-1 is an essential substrate for c-Met (Sun et al., 1991, 1995; Weidner et al., 1996; Kouhara et al., 1997; Sachs et al., 2000; Schaeper et al., 2000). c-Ret can directly associate with all dok family proteins. However, dok family members have distinct expression patterns. Differential expression of these adapter proteins thus adds another layer to the complexity and specificity of signal transduction by receptor tyrosine kinases.

Members of the hematopoietically expressed doks, dok-1 and dok-2, contain long COOH-terminal sequences with many tyrosyls and PXXP motifs adjacent to the PTB domain (e.g., 10 for dok-1). These doks have been shown to associate with rasGAP, c-Abl, and Nck (Holland et al., 1997; Yamanashi and Baltimore, 1997). Several recent reports suggest a negative role of these dok proteins in the regulation of MAP kinase (Nelms et al., 1998; Jones and Dumont, 1999; Noguchi et al., 1999; Yamanashi et al., 2000). The dok-4/5 proteins newly identified here contain short COOH-terminal tails with fewer tyrosyls and few or no PXXP motifs, and do not bind rasGAP or Nck. Moreover, when dok-4 and dok-5 are fused to c-Ret, they strongly induce MAP kinase and Elk-1 transactivation and trigger axonal outgrowth. Thus, the two subfamilies of the dok proteins, dok-1–3 and dok-4/5, appear to take over opposite signaling functions in cells.

Materials and methods

Yeast two-hybrid screens and protein interaction studies
The cDNA encoding the cytoplasmic domain of the short isoform of mouse c-Ret (amino acids 660–1,072) was fused to LexA sequences in the yeast expression vector BTM116 (Weidner et al., 1996). Other LexA-tyrosine kinase hybrids were constructed similarly. In the case of the ErbB family of receptors, COOH-terminal tails were inserted downstream of a LexA–tpr–met construct, which lacks substrate binding sites of Met (EGFR, amino acids 983–1,210; ErbB-2, amino acids 1,005–1,260; ErbB-3, amino acids 980–1,342; tpr-met, amino acids 1–480). Yeast two-hybrid screens were performed in yeast strain L40 using a E10.5 mouse embryo cDNA library (Behrens et al., 1996). For coimmunoprecipitation and tyrosine phosphorylation studies, 293 and Neuro 2A cells were transfected by standard calcium phosphate precipitation, solubilized in lysis buffer (150 mM NaCl, 1mM EDTA, 50 mM Hepes, pH 7.5, 1% Triton X-100, 10% glycerol), and proteins were precipitated with anti-Flag affinity agarose (Sigma-Aldrich) followed by SDS-PAGE and immunoblotting. For far Western blotting, 293 cells were transiently transfected with c-Ret constructs, followed by immunoprecipitation with c-Ret antibodies, SDS-PAGE, and blotting. Membranes were probed with 250 nM purified His-tagged dok-5 protein (amino acids 16–306) and developed with anti-His antibody. Antibodies used were anti-Ret C-19 and anti-rasGAP B4F8 (Santa Cruz Biotechnology, Inc.), anti-Nck and anti-PY PY20 (Transduction Laboratories), anti-Flag Octapeptide (Zymed Laboratories), antiactive MAP kinase (Promega), peroxidase-conjugated anti-His (Sigma-Aldrich), and horseradish peroxidase–conjugated anti-IgG (Jackson ImmunoResearch Laboratories). An antiserum against dok-5 was prepared in rabbits by immunization with a mixture of two peptides (amino acids 247–257 and 292–306) and was affinity purified. Spinal cords and dorsal root ganglia were prepared from P1 mouse embryos, solubilized in lysis buffer, and immunoprecipitations with anti–dok-5 antibodies were performed as described above.

In situ hybridization and Northern blotting
Digoxygenin-labeled RNA transcripts were synthesized with T3 or T7 RNA polymerase using a digoxygenin RNA–labeling kit (Boehringer). Wholemount in situ hybridization was performed as described (Wilkinson, 1992). Probes used were: c-Ret, nt 1–1,445; dok-4, entire coding sequence plus 900 3' nucleotide sequences; dok-5, entire coding sequence plus 180 5' nucleotides; dok-2, nucleotides 469–890. All probes revealed reproducible hybridization patterns when used in antisense orientation, whereas transcripts in sense orientation revealed no specific hybridization. Semithin serial sections (7–15 μm) were prepared from the whole-mounts embedded in Technovit 7100 (Kulzer GmbH). Mouse multiple tissue Northern blots (CLONTECH Laboratories, Inc.) were probed with ^{32}P-labeled cDNA probes specific for dok family members prepared by the Megaprime DNA labeling kit (Amersham Pharmacia Biotech).

Construction of EGFR/c-Ret-dok chimeras and generation of PC12 transfectants
EGFR/c-Ret constructs were inserted into the SalI site of pBabe Puro retroviral expression vector (Morgenstern and Land, 1990); in EGFR/c-Ret Y1062F, tyrosine residue 1,062 was mutated to phenylalanin by PCR. Fusion constructs of EGFR/c-Ret and various dok family members were generated by insertion of dok fragments (encoding amino acids 119–412, dok-2; 113–325, dok-4; 116–306, dok-5; and 233–325, dok-4Cterm) into the XhoI site of c-ret, deleting sequences encoding the last 22 amino acids of c-Ret including Y1062. PC12 cells were infected with high titer stock of a retrovirus that contained the EGFR/c-Ret-dok constructs, and were selected for 5 d with 0.5 μg/ml puromycin. Pools of selected cultures were grown in the presence or absence of 50 ng/ml EGF for 48 h, and neurite outgrowth was quantitated by scoring cells with neurites longer than the size of two cell bodies.

MAP kinase and Elk-1 reporter assays
PC12 cells infected with retroviruses were serum-starved overnight and then treated with and without 50 ng/ml EGF for 9 h. Cells were lysed and assayed for Erk1/2 phosphorylation using the antiactive MAP kinase antibody. Neuro 2A cells were transfected with pBabe EGFR/c-Ret-dok expression plasmids, pFA2-Elk1, pFRLuc (Stratagene), and pSV40lacZ expression vectors. 2 d posttransfection, cells were stimulated with 50 ng/ml EGF for 5 h. 293 cells were transfected with c-Ret or MEN2A Ret (C634R), different amounts of dok-2 or dok-5, and reporter plasmids. Cells were lysed 2 d posttransfection by three freeze and thaw cycles, and extracts were analyzed for β-galactosidase and luciferase activity.

References

Alberti, L., M.G. Borrello, S. Ghizzoni, F. Torriti, M.G. Rizzetti, and M.A. Pierotti. 1998. Grb2 binding to the different isoforms of Ret tyrosine kinase. *Oncogene.* 17:1079–1087.

Arighi, E., L. Alberti, F. Torriti, S. Ghizzoni, M.G. Rizzetti, G. Pelicci, B. Pasini, I. Bongarzone, C. Piutti, M.A. Pierotti, and M.G. Borrello. 1997. Identification of Shc docking site on Ret tyrosine kinase. *Oncogene.* 14:773–782.

Asai, N., H. Murakami, T. Iwashita, and M. Takahashi. 1996. A mutation at tyrosine 1062 in MEN2A-Ret and MEN2B-Ret impairs their transforming activity and association with shc adaptor proteins. *J. Biol. Chem.* 271:17644–17649.

Avantaggiato, V., N.A. Dathan, M. Grieco, N. Fabien, D. Lazzaro, A. Fusco, A. Simeone, and M. Santoro. 1994. Developmental expression of the RET protooncogene. *Cell Growth Differ.* 5:305–311.

Behrens, J., J.P. von Kries, M. Kuhl, L. Bruhn, D. Wedlich, R. Grosschedl, and W. Birchmeier. 1996. Functional interaction of β-catenin with the transcription factor LEF-1. *Nature.* 382:638–642.

Besset, V., R.P. Scott, and C.F. Ibanez. 2000. Signaling complexes and protein-protein interactions involved in the activation of the Ras and PI3K pathways by the c-Ret receptor tyrosine kinase. *J. Biol. Chem.* 275:39159–39166.

Bladt, F., D. Riethmacher, S. Isenmann, A. Aguzzi, and C. Birchmeier. 1995. Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. *Nature.* 376:768–771.

Borrello, M.G., L. Alberti, E. Arighi, I. Bongarzone, C. Battistini, A. Bardelli, B. Pasini, C. Piutti, M.G. Rizzetti, P. Mondellini, M.T. Radice, and M.A. Pierotti. 1996. The full oncogenic activity of Ret/ptc2 depends on tyrosine 539, a docking site for phospholipase Cγ. *Mol. Cell. Biol.* 16:2151–2163.

Carpino, N., D. Wisniewski, A. Strife, D. Marshak, R. Kobayashi, B. Stillman, and B. Clarkson. 1997. p62(dok): a constitutively tyrosine-phosphorylated, GAP-associated protein in chronic myelogenous leukemia progenitor cells. *Cell.* 88:197–204.

Cong, F., B. Yuan, and S.P. Goff. 1999. Characterization of a novel member of the dok family that binds and modulates Abl signaling. *Mol. Cell. Biol.* 19:8314–8325.

Di Cristofano, A., N. Carpino, N. Dunant, G. Friedland, R. Kobayashi, A. Strife, D. Wisniewski, B. Clarkson, P.P. Pandolfi, and M.D. Resh. 1998. Molecular cloning and characterization of p56dok-2 defines a new family of RasGAP-binding proteins. *J. Biol. Chem.* 273:4827–4830.

Donis-Keller, H., S. Dou, D. Chi, K.M. Carlson, K. Toshima, T.C. Lairmore, J.R. Howe, J.F. Moley, P. Goodfellow, and S.A.J. Wells. 1993. Mutations in the RET proto-oncogene are associated with MEN 2A and FMTC. *Hum. Mol. Genet.* 2:851–856.

Durick, K., R.Y. Wu, G.N. Gill, and S.S. Taylor. 1996. Mitogenic signaling by Ret/ptc2 requires association with enigma via a LIM domain. *J. Biol. Chem.* 271:12691–12694.

Edery, P., S. Lyonnet, L.M. Mulligan, A. Pelet, E. Dow, L. Abel, S. Holder, F.C. Nihoul, B.A. Ponder, and A. Munnich. 1994. Mutations of the RET proto-oncogene in Hirschsprung's disease. *Nature.* 367:378–380.

Gattei, V., A. Celetti, A. Cerrato, M. Degan, A. De Iuliis, F.M. Rossi, G. Chiappetta, C. Consales, S. Improta, V. Zagonel, et al. 1997. Expression of the RET receptor tyrosine kinase and GDNFR-α in normal and leukemic human hematopoietic cells and stromal cells of the bone marrow microenvironment. *Blood.* 89:2925–2937.

Gattei, V., M. Degan, D. Aldinucci, A. De Iuliis, F.M. Rossi, F.T. Mazzocco, M. Rupolo, V. Zagonel, and A. Pinto. 1998. Differential expression of the RET gene in human acute myeloid leukemia. *Ann. Hematol.* 77:207–210.

Gattei, V., M. Degan, F.M. Rossi, A. De Iuliis, F.T. Mazzocco, E. Cesa, D. Aldinucci, V. Zagonel, and A. Pinto. 1999. The RET receptor tyrosine kinase, but not its specific ligand, GDNF, is preferentially expressed by acute leukaemias of monocytic phenotype and is up-regulated upon differentiation. *Br. J. Haematol.* 105:225–240.

Hayashi, H., M. Ichihara, T. Iwashita, H. Murakami, Y. Shimono, K. Kawai, K. Kurokawa, Y. Murakumo, T. Imai, H. Funahashi, A. Nakao, and M. Takahashi. 2000. Characterization of intracellular signals via tyrosine 1062 in RET activated by glial cell line-derived neurotrophic factor. *Oncogene.* 19:4469–4475.

Herbst, R., P.M. Carroll, J.D. Allard, J. Schilling, T. Raabe, and M.A. Simon. 1996. Daughter of sevenless is a substrate of the phosphotyrosine phosphatase Corkscrew and functions during sevenless signaling. *Cell.* 85:899–909.

Heuckeroth, R.O., H. Enomoto, J.R. Grider, J.P. Golden, J.A. Hanke, A. Jackman, D.C. Molliver, M.E. Bardgett, W.D. Snider, E.M.J. Johnson, and J. Milbrandt. 1999. Gene targeting reveals a critical role for neurturin in the development and maintenance of enteric, sensory, and parasympathetic neurons. *Neuron.* 22:253–263.

Hofstra, R.M., R.M. Landsvater, I. Ceccherini, R.P. Stulp, T. Stelwagen, Y. Luo, B. Pasini, J.W. Hoppener, H.K. van Amstel, G. Romeo, et al. 1994. A mutation in the RET proto-oncogene associated with multiple endocrine neoplasia type 2B and sporadic medullary thyroid carcinoma. *Nature.* 367:375–376.

Holland, S.J., N.W. Gale, G.D. Gish, R.A. Roth, Z. Songyang, L.C. Cantley, M. Henkemeyer, G.D. Yancopoulos, and T. Pawson. 1997. Juxtamembrane tyrosine residues couple the Eph family receptor EphB2/Nuk to specific SH2 domain proteins in neuronal cells. *EMBO J.* 16:3877–3888.

Jones, N., and D.J. Dumont. 1998. The Tek/Tie2 receptor signals through a novel Dok-related docking protein, Dok-R. *Oncogene.* 17:1097–1108.

Jones, N., and D.J. Dumont. 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.

Kouhara, H., Y.R. Hadari, K.T. Spivak, J. Schilling, S.D. Bar, I. Lax, and J. Schlessinger. 1997. A lipid-anchored Grb2-binding protein that links FGF-receptor activation to the Ras/MAPK signaling pathway. *Cell.* 89:693–702.

Lemay, S., D. Davidson, S. Latour, and A. Veillette. 2000. Dok-3, a novel adapter molecule involved in the negative regulation of immunoreceptor signaling. *Mol. Cell. Biol.* 20:2743–2754.

Liu, X., Q.C. Vega, R.A. Decker, A. Pandey, C.A. Worby, and J.E. Dixon. 1996. Oncogenic RET receptors display different autophosphorylation sites and substrate binding specificities. *J. Biol. Chem.* 271:5309–5312.

Lorenzo, M.J., G.D. Gish, C. Houghton, T.J. Stonehouse, T. Pawson, B.A. Ponder, and D.P. Smith. 1997. RET alternate splicing influences the interaction of activated RET with the SH2 and PTB domains of Shc, and the SH2 domain of Grb2. *Oncogene.* 14:763–771.

Meakin, S.O., J.I. MacDonald, E.A. Gryz, C.J. Kubu, and J.M. Verdi. 1999. The signaling adapter FRS-2 competes with Shc for binding to the nerve growth factor receptor TrkA. A model for discriminating proliferation and differentiation. *J. Biol. Chem.* 274:9861–9870.

Molliver, D.C., D.E. Wright, M.L. Leitner, A.S. Parsadanian, K. Doster, D. Wen, Q. Yan, and W.D. Snider. 1997. IB4-binding DRG neurons switch from NGF to GDNF dependence in early postnatal life. *Neuron.* 19:849–861.

Moore, M.W., R.D. Klein, I. Farinas, H. Sauer, M. Armanini, H. Phillips, L.F. Reichardt, A.M. Ryan, M.K. Carver, and A. Rosenthal. 1996. Renal and neuronal abnormalities in mice lacking GDNF. *Nature.* 382:76–79.

Morgenstern, J.P., and H. Land. 1990. Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res.* 18:3587–3596.

Mulligan, L.M., J.B. Kwok, C.S. Healey, M.J. Elsdon, C. Eng, E. Gardner, D.R. Love, S.E. Mole, J.K. Moore, L. Papi, et al. 1993. Germ-line mutations of the RET proto-oncogene in multiple endocrine neoplasia type 2A. *Nature.* 363:458–460.

Murakami, H., T. Iwashita, N. Asai, Y. Shimono, Y. Iwata, K. Kawai, and M. Takahashi. 1999. Enhanced phosphatidylinositol 3-kinase activity and high phosphorylation state of its downstream signalling molecules mediated by ret with the MEN 2B mutation. *Biochem. Biophys. Res. Commun.* 262:68–75.

Nakayama, S., K. Iida, T. Tsuzuki, T. Iwashita, H. Murakami, N. Asai, Y. Iwata, M. Ichihara, S. Ito, K. Kawai, M. Asai, K. Kurokawa, and M. Takahashi. 1999. Implication of expression of GDNF/Ret signalling components in differentiation of bone marrow haemopoietic cells. *Br. J. Haematol.* 105:50–57.

Nelms, K., A.L. Snow, L.J. Hu, and W.E. Paul. 1998. FRIP, a hematopoietic cell-specific rasGAP-interacting protein phosphorylated in response to cytokine stimulation. *Immunity.* 9:13–24.

Noguchi, T., T. Matozaki, K. Inagaki, M. Tsuda, K. Fukunaga, Y. Kitamura, T. Kitamura, K. Shii, Y. Yamanashi, and M. Kasuga. 1999. Tyrosine phosphorylation of p62(Dok) induced by cell adhesion and insulin: possible role in cell migration. *EMBO J.* 18:1748–1760.

O'Neill, T.J., A. Craparo, and T.A. Gustafson. 1994. Characterization of an interaction between insulin receptor substrate 1 and the insulin receptor by using the two-hybrid system. *Mol. Cell. Biol.* 14:6433–6442.

Pachnis, V., B. Mankoo, and F. Costantini. 1993. Expression of the c-ret proto-oncogene during mouse embryogenesis. *Development.* 119:1005–1017.

Pichel, J.G., L. Shen, H.Z. Sheng, A.C. Granholm, J. Drago, A. Grinberg, E.J. Lee, S.P. Huang, M. Saarma, B.J. Hoffer, H. Sariola, and H. Westphal. 1996. Defects in enteric innervation and kidney development in mice lacking GDNF. *Nature.* 382:73–76.

Raabe, T., E.J. Riesgo, X. Liu, B.S. Bausenwein, P. Deak, P. Maroy, and E. Hafen. 1996. DOS, a novel pleckstrin homology domain-containing protein required for signal transduction between sevenless and Ras1 in *Drosophila*. *Cell*. 85:911–920.

Rizzo, C., D. Califano, G.L. Colucci-D'Amato, G. De Vita, A. D'Alessio, N.A. Dathan, A. Fusco, C. Monaco, G. Santelli, G. Vecchio, M. Santoro, and V. de Franciscis. 1996. Ligand stimulation of a Ret chimeric receptor carrying the activating mutation responsible for the multiple endocrine neoplasia type 2B. *J. Biol. Chem.* 271:29497–29501.

Romeo, G., P. Ronchetto, Y. Luo, V. Barone, M. Seri, I. Ceccherini, B. Pasini, R. Bocciardi, M. Lerone, H. Kaariainen, et al. 1994. Point mutations affecting the tyrosine kinase domain of the RET proto-oncogene in Hirschsprung's disease. *Nature*. 367:377–378.

Rossel, M., A. Pasini, S. Chappuis, O. Geneste, L. Fournier, I. Schuffenecker, M. Takahashi, L.A. van Grunsven, J.L. Urdiales, B.B. Rudkin, G.M. Lenoir, and M. Billaud. 1997. Distinct biological properties of two RET isoforms activated by MEN 2A and MEN 2B mutations. *Oncogene*. 14:265–275.

Sachs, M., H. Brohmann, D. Zechner, T. Müller, J. Hülsken, I. Walther, U. Schaeper, C. Birchmeier, and W. Birchmeier. 2000. Essential role of Gab1 for signaling by the c-Met receptor in vivo. *J. Cell Biol.* 150:1375–1384.

Sanchez, M.P., S. Silos, I.J. Frisen, B. He, S.A. Lira, and M. Barbacid. 1996. Renal agenesis and the absence of enteric neurons in mice lacking GDNF. *Nature*. 382:70–73.

Santoro, M., W.T. Wong, P. Aroca, E. Santos, B. Matoskova, M. Grieco, A. Fusco, and P.P. di Fiore. 1994. An epidermal growth factor receptor/ret chimera generates mitogenic and transforming signals: evidence for a ret-specific signaling pathway. *Mol. Cell. Biol.* 14:663–675.

Schaeper, U., N.H. Gehring, K.P. Fuchs, M. Sachs, B. Kempkes, and W. Birchmeier. 2000. Coupling of Gab1 to c-Met, Grb2, and Shp2 mediates biological responses. *J. Cell Biol.* 149:1419–1432.

Schuchardt, A., V. D'Agati, B.L. Larsson, F. Costantini, and V. Pachnis. 1994. Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. *Nature*. 367:380–383.

Sun, X.J., P. Rothenberg, C.R. Kahn, J.M. Backer, E. Araki, P.A. Wilden, D.A. Cahill, B.J. Goldstein, and M.F. White. 1991. Structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein. *Nature*. 352:73–77.

Sun, X.J., L.M. Wang, Y. Zhang, L. Yenush, J. Myers-MG, E. Glasheen, W.S. Lane, J.H. Pierce, and M.F. White. 1995. Role of IRS-2 in insulin and cytokine signalling. *Nature*. 377:173–177.

Suzu, S., M. Tanaka-Douzono, K. Nomaguchi, M. Yamuda, H. Hayasawa, F. Kimura, and K. Motoyoshi. 2000. p56(dok-2) as a cytokine-inducible inhibitor of cell proliferation and signal transduction. *EMBO J.* 19:5114–5122.

Tahira, T., Y. Ishizaka, F. Itoh, T. Sugimura, and M. Nagao. 1990. Characterization of ret proto-oncogene mRNAs encoding two isoforms of the protein product in a human neuroblastoma cell line. *Oncogene*. 5:97–102.

Takahashi, M., J. Ritz, and G.M. Cooper. 1985. Activation of a novel human transforming gene, ret, by DNA rearrangement. *Cell*. 42:581–588.

Tamir, I., J.C. Stolpa, C.D. Helgason, K. Nakamura, P. Bruhns, M. Daeron, and J.C. Cambier. 2000. The RasGAP-binding protein p62dok is a mediator of inhibitory FcgammaRIIB signals in B cells. *Immunity*. 12:347–358.

Treanor, J.J., L. Goodman, F. de Sauvage, D.M. Stone, K.T. Poulsen, C.D. Beck, C. Gray, M.P. Armanini, R.A. Pollock, F. Hefti, et al. 1996. Characterization of a multicomponent receptor for GDNF. *Nature*. 382:80–83.

Ullrich, A., and J. Schlessinger. 1990. Signal transduction by receptors with tyrosine kinase activity. *Cell*. 61:203–212.

van der Geer, P., T. Hunter, and R.A. Lindberg. 1994. Receptor protein-tyrosine kinases and their signal transduction pathways. *Annu. Rev. Cell Biol.* 10:251–337.

Wasserman, R., Y.S. Li, and R.R. Hardy. 1995. Differential expression of the blk and ret tyrosine kinases during B lineage development is dependent on Ig rearrangement. *J. Immunol.* 155:644–651.

Weidner, K.M., S. Di Cesare, M. Sachs, V. Brinkmann, J. Behrens, and W. Birchmeier. 1996. Interaction between Gab1 and the c-Met receptor tyrosine kinase is responsible for epithelial morphogenesis. *Nature*. 384:173–176.

Wilkinson, D.G. 1992. In Situ Hybridization: A Practical Approach. Oxford University Press/IRL Press, NY. 224 pp.

Withers, D.J., J.S. Gutierrez, H. Towery, D.J. Burks, J.M. Ren, S. Previs, Y. Zhang, D. Bernal, S. Pons, G.I. Shulman, W.S. Bonner, and M.F. White. 1998. Disruption of IRS-2 causes type 2 diabetes in mice. *Nature*. 391:900–904.

Yamanashi, Y., and D. Baltimore. 1997. Identification of the Abl- and rasGAP-associated 62 kDa protein as a docking protein, Dok. *Cell*. 88:205–211.

Yamanashi, Y., T. Tamura, T. Kanamori, H. Yamane, H. Nariuchi, T. Yamamoto, and D. Baltimore. 2000. Role of the rasGAP-associated docking protein p62(dok) in negative regulation of B cell receptor-mediated signaling. *Genes Dev.* 14:11–16.