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 phosphotyrosyl 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 NH2-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 hybridization. By searching Expression 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 NH2-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.,
dok-2 is found in a punctuated 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, but not with c-Ret receptors harboring a Y1062F mutation or an inactive kinase (Ret K1, K758M; Liu et al., 1996). 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.

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 dok to c-Ret is mediated by their PTB domain and

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**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 K1, 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.
quires phosphorylation of Y1062 (shown for dok-5 in Fig. 5c). Interaction between the doks and c-Ret is direct, as demonstrated by far Western blotting (Fig. 5d). 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. 5e). 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. 5f).

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.

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.
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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.
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 two-hybrid 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 (Schiachardt et al., 1994; Moore et al., 1996; Pichel et al., 1996;
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 described (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., 1997; Gatge et al., 1997, 1998, 1999; Nakayama et al., 1999). Phosphorylation of p62dok after c-Ret activation can also occur in a phosphotidylinositol 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 hybrid constructs were constructed similarly. In the case of the Embf 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) coupled by SDS-PAGE and immobilized on nitrocellulose. All procedures reported were performed with 293 cells that 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 16–310) and developed with anti-His antibody. Antibodies were used against anti-Ret C-19 and anti-rasGAP B4F8 (Santa Cruz Biotechnology, Inc.), anti-Nck and anti-Flag (Transduction Laboratories), anti-Flag Octapeptide (Zymed Laboratories), antiaactive MAP kinase (Promega), peroxidase-conjugated anti-His (Sigma–Aldrich), and horseradish peroxidase-conjugated anti-FLAG (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 immunoprecipitated with anti-dok-5 antibodies were performed as described above.

In situ hybridization and Northern blotting

Digoxigenin-labeled DNA transcripts were synthesized with T3 or T7 RNA polymerase using a digoxigenin RNA-labeling kit (Boehringer). Whole-mount 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′ nucleotide sequences; 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. Semi-thin serial sections (7–15 µm) were prepared from the whole-mount embedded in Technovit 7100 (Kulzer GmbH). Mouse multiple tissue Northern blots (CLONTECH Laboratories, Inc.) were probed with 32P-labeled cDNA probes specific for dok family members prepared by the Megaprim 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 Sall 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 µl 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 pM540lacZ 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.

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