Dok-4 regulates GDNF-dependent neurite outgrowth through downstream activation of Rap1 and mitogen-activated protein kinase

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Summary

During development of the central and peripheral nervous systems, neurite extension mediated via glial-cell-line-derived neurotrophic factor (GDNF) and its receptor RET is critical for neuronal differentiation. In the present study, we investigated the role of the RET substrate Dok-4 in neurite outgrowth induced by the GDNF/RET signaling pathway. In TGW neuroblastoma cells, which endogenously express both RET and Dok-4, depletion of Dok-4 through treatment with small interfering RNA resulted in a marked decrease in GDNF-stimulated neurite outgrowth. By contrast, exogenous expression of wild-type Dok-4 induced sustained p44/42 mitogen-activated protein kinase (ERK1/2) activation and enhanced neurite outgrowth. Expression of Dok-4 mutants in which the tyrosine residues at codons 187, 220 and 270, conserved between Dok-4, -5, and -6, were each replaced with a phenylalanine inhibited sustained ERK1/2 activation and neurite outgrowth. We also found that Dok-4 induced a significant activation of the small G protein Rap1 and that expression of a dominant active Rap1 mutant restored neurite outgrowth in Dok-4-depleted cells. By contrast, expression of a dominant negative Rap1 mutant impaired GDNF-stimulated neurite outgrowth from TGW cells. Finally, we found that neurite formation in cultured rat hippocampal neurons was enhanced by the expression of Dok-4. Together, our results suggest that Dok-4, through activation of the Rap1-ERK1/2 pathway, regulates GDNF-mediated neurite outgrowth during neuronal development.

Supplementary material available online at
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Key words: Dok-4, GDNF, RET tyrosine kinase, Rap1, MAPK, Neuronal differentiation

Introduction

In the developing vertebrate nervous system, neurite outgrowth is required for the formation of the highly specific pattern of connections between nerve cells. Neurite outgrowth begins with the activation of membrane receptors by extracellular cues. These receptors then activate intracellular signaling cascades that trigger changes in plasma membrane dynamics, cytoskeleton reorganization, and the transcription of genes essential for neuronal differentiation (Chao, 2003). The molecular mechanisms of neuronal differentiation have been the subject of intensive investigation (Whitford et al., 2002). It has already been established that the activation of p44/42 mitogen-activated protein kinase (ERK1/2) by growth factors can trigger cell growth and/or differentiation. Although transient activation of ERK1/2 is thought to stimulate proliferation, its sustained activation appears to induce differentiation (Marshall, 1995; York et al., 1998). In the rat pheochromocytoma tumor cell line PC12, nerve growth factor (NGF)-dependent neurite outgrowth requires sustained ERK1/2 activation, which is mediated by the activation of Rap1, a small Ras family G protein (Vossler et al., 1997; York et al., 1998; Grewal et al., 2000a; Stork, 2003). However, it remains poorly understood whether the molecular mechanisms responsible for neurite outgrowth are conserved in signaling pathways triggered by other neurotrophic factors.

The tyrosine kinase receptor RET plays an important role in the development of the enteric nervous system and the kidney (Manié et al., 2001; Takahashi, 2001; Airaksinen and Saarma, 2002). It has been demonstrated that members of the glial cell line-derived neurotrophic factor (GDNF) family, which includes GDNF, neuturin, artemin, and persephin, act as RET ligands and function as survival and/or differentiation factors in various central and peripheral neurons. Thus, RET activation is mediated by the binding of these neurotrophic

factors to glycosylphosphatidylinositol-anchored co-receptors termed GDNF family receptors α1-4 (GFRα1-4) (Jing et al., 1996; Treanor et al., 1996; Klein et al., 1997; Airaksinen and Saarma, 2002), which result in the activation of several signaling pathways, including the Ras/ERK, phosphatidylinositol-3 kinase [PI(3)K]/AKT, p38MAPK, phospholipase Cγ, and Rac/c-Jun N-terminal kinase (JNK) pathways (Besset et al., 2000; Hayashi et al., 2000; Hayashi et al., 2001; Segouffin-Carius and Billaud, 2000; Fukuda et al., 2002; Fukuda et al., 2005). Ablation of the *Gdnf* or *Ret* genes in mice results in the absence or severe hypoplasia of the kidneys, enteric nervous system defects, and reduced numbers of some peripheral and central neurons (Schuchardt et al., 1994; Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996). Loss-of-function mutations of the *RET* gene in humans lead to the development of Hirshsprung's disease (Edery et al., 1994; Romeo et al., 1994), a congenital malformation characterized by the absence or decreased number of intrinsic ganglion cells in the gastrointestinal tract. Given the diverse roles of RET in the regulation of neuronal differentiation/maturation in the central and enteric nervous systems, it is important to understand the complex intracellular signaling pathways stimulated by RET activation. However, despite considerable progress in understanding the signaling pathways that regulate RET-dependent cell survival and proliferation, the molecular basis underlying RET-dependent neuronal differentiation is still poorly understood.

Recently, putative signaling proteins Dok-4, -5, and -6 were described as new members of the p62dok (downstream of kinase) family that appeared to mediate RET-mediated neurite outgrowth (Grimm et al., 2001; Crowder et al., 2004). p62dok family proteins were originally identified as substrates of several tyrosine kinases and mediators of several cytokine signaling pathways, and have emerged as a subgroup of an expanding range of signaling molecules composed of N-terminal tandem pleckstrin homology (PH) and phosphotyrosine-binding (PTB) domains (Carpino et al., 1997; Yamanashi and Baltimore, 1997; Cristofano et al., 1998; Cong et al., 1999; Grimm et al., 2001). Dok-4, -5 and -6 constitute a subclass of the p62dok family and are highly expressed in the developing nervous system, as opposed to Dok-1, -2 and -3, which are mainly expressed in hematopoietic tissues (Nelms et al., 1998; Cong et al., 1999; Lemay et al., 2000; Grimm et al., 2001; Cai et al., 2003; Crowder et al., 2004). It has been shown by in situ hybridization of mouse embryos that Dok-4, -5 and -6 colocalize with RET to the ventral part of the neural tube, dorsal root ganglia, cranial sensory ganglia, and/or the ureteric buds of the developing kidney, which suggests that they function downstream of RET in vivo. Previous studies have shown that Dok-4, -5 and -6 directly associate with the tyrosine at position 1062 of RET following phosphorylation in response to GDNF stimulation (Grimm et al., 2001; Crowder et al., 2004), and that overexpression of Dok-4 and -5 promotes neurite outgrowth in rat PC12 cells (Grimm et al., 2001). However, this latter study involved the exogenous expression of an EGFR (epidermal growth factor receptor)/RET chimeric receptor fused at the C-terminus with Dok proteins in PC12 cells.

In the present study, we attempted to characterize the role of endogenous Dok-4 in RET-dependent neurite outgrowth. We used a TGW human neuroblastoma cell line that had the advantage of expressing RET, GFRα-1, and Dok-4 endogenously. Through the use of small interfering RNA (siRNA) specific to Dok-4, we found that Dok-4 was crucial for RET-dependent neurite outgrowth through the induction of sustained ERK1/2 activation. Moreover, we also found a possible function for Dok-4 in activating Rap1, which occurs downstream of RET and regulates neurite outgrowth in TGW cells.

Results

Depletion of Dok-4 attenuates GDNF-stimulated neurite outgrowth in TGW neuroblastoma cells

To facilitate our studies on Dok-4, we generated a polyclonal antibody (Ab) raised against a 19 C-terminal amino acid fragment of Dok-4, and used it to examine Dok-4 protein expression in several cell lines. Western blot analysis revealed that the anti-Dok-4 Ab recognized a specific band with a relative molecular mass of 41 kDa in human TGW neuroblastoma cell lysates that was not detectable in lysates from other neuronal or non-neuronal cell lines including SK-N-SH, SK-N-MC, HEK293, HeLa and TT (Fig. 1Aa). The size of the band detected by our antibody was identical to that obtained for Dok-4 expressed exogenously in HEK293 cells, which indicated the specificity of the antibody. Because TGW cells also endogenously express both RET and GFRα-1 (Nozaki et al., 1998; Hayashi et al., 2000), we used TGW cells to study the physiological roles of Dok-4 in the GDNF/RET signaling pathway.

To further verify the specificity of the anti-Dok-4 Ab and to assess the function of Dok-4 in TGW cells, we employed RNA-mediated interference (RNAi) to suppress Dok-4 expression. Small (21-nucleotide) interfering Dok-4 RNA (Dok-4 siRNA) or a 21-nucleotide irrelevant RNA (control siRNA) were introduced into TGW cells. Western blot analysis showed that transfection with Dok-4 siRNA effectively reduced Dok-4 expression levels by over 90% without affecting expression of the Shc adaptor protein (Fig. 1Ab). Using fluorescently labeled siRNA, we confirmed that the transfection efficiency was about 90-95% (data not shown).

To examine the subcellular localization of Dok-4, we immunofluorescently stained TGW cells with anti-Dok-4 Ab. Results indicated that Dok-4 was mainly localized to the plasma membrane in TGW cells (Fig. 1Ba, left panel). When TGW cells were transfected with Dok-4 siRNA, expression of Dok-4 was almost undetectable, which again indicated the specificity of the immunostaining (Fig. 1Ba, right panel). TGW cells stimulated with GDNF undergo morphological changes and start to extend neurites (Nozaki et al., 1998). Dok-4 was localized to the extended neurites and their tips as well as to the plasma membrane in GDNF-treated cells (Fig. 1Bb), which suggested that Dok-4 constitutively associated with the plasma membrane independent of GDNF stimulation.

We next examined the effect of siRNA-mediated knockdown of Dok-4 expression on neurite outgrowth from TGW cells following GDNF stimulation for 48 hours. Compared with control siRNA-transfected cells, Dok-4 siRNA-transfected cells showed short neurites in response to GDNF (Fig. 1Cc,d). To test whether the addition of exogenous Dok-4 could restore the defective neurite outgrowth observed in knockdown cells, we constructed a V5-fused siRNA-resistant (siRNAr) version

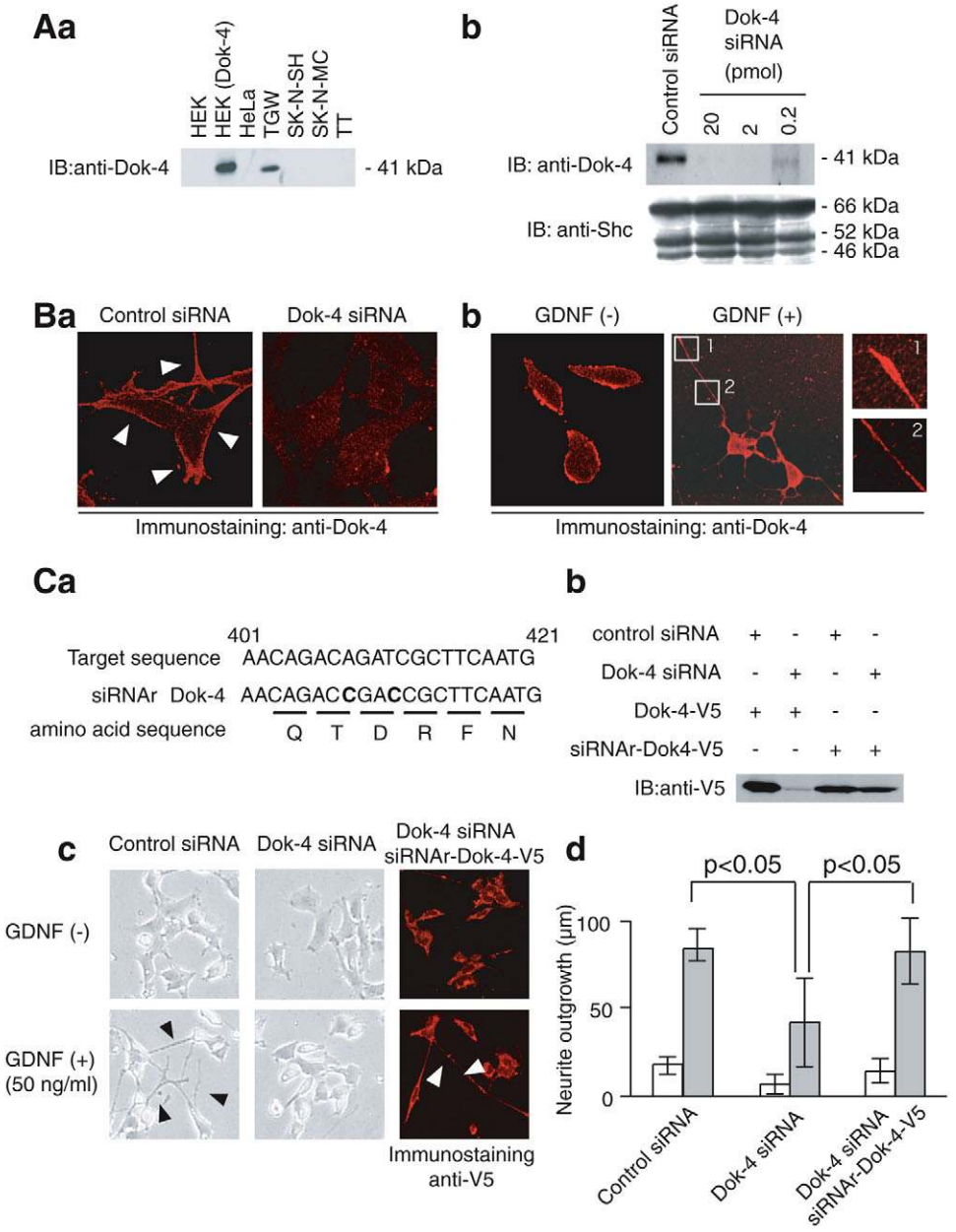

Fig. 1. Knockdown of Dok-4 suppresses GDNF-dependent neurite outgrowth in TGW neuroblastoma cells. (A) (a) Expression of Dok-4 in TGW cells. Lysates from human cell lines were analyzed by western blotting with anti-Dok-4 polyclonal antibody under reducing conditions. HEK (human embryonic kidney), HeLa (human cervical adenocarcinoma), TGW (human neuroblastoma), SK-N-SH (human neuroblastoma), SK-N-MC (human primitive neuroectodermal tumor) and TT (human medullary thyroid carcinoma) cell lines were used. Lysate from HEK293 cells transfected with Dok-4 cDNA was used as a positive control. (b) Depletion of Dok-4 in TGW cells by siRNA. Total cell extracts from control siRNA- and Dok-4 siRNA-transfected TGW cells were subjected to western blot analysis with anti-Dok-4 and anti-Shc antibodies. (B) (a) TGW cells were transfected with control (left panel) or Dok-4 (right panel) siRNA, fixed 72 hours after transfection, and then stained with anti-Dok-4 antibody. Arrowheads denote the localization of Dok-4 to the plasma membrane. (b) TGW cells were serum-starved, incubated with or without GDNF (50 ng/ml) for 48 hours, and then stained with anti-Dok-4 antibody. Boxes 1 and 2 denote a shaft and a tip of an extended neurite which was positively stained with anti-Dok-4 antibody, respectively. (C) Depletion of Dok-4 attenuates neurite outgrowth by TGW cells in response to GDNF. (a) The target sequence of Dok-4 siRNA and the nucleotide substitutions used to generate siRNA-resistant (siRNAr) Dok-4 are indicated. (b) HEK293 cells were transfected with control or Dok-4 siRNA together with Dok4-V5 or siRNAr-Dok4-V5. Dok-4 expression was monitored by western blot analysis. (c) TGW cells transfected with siRNA were serum-starved, incubated with or without GDNF for 48 hours and then visualized by microscopy. TGW cells transfected with both siRNA and siRNAr-Dok4-V5 were also analyzed. (d) Mean of the longest neurite was determined for each culture from measurements of 100 neurons for three different experiments. Each data point represents the mean ± s.e.m. (*P<0.05).

of Dok-4 that harbored silent mutations (Fig. 1Ca,b). TGW cells were transiently transfected with siRNAr-Dok-4-V5, incubated with GDNF for 48 hours, and then immunostained with anti-V5 antibody. As shown in Fig. 1Cc,d, siRNAr-Dok-4-V5 expression fully restored neurite outgrowth in response to GDNF. These results suggested that Dok-4 was a crucial mediator of GDNF/RET signaling-induced neurite outgrowth in TGW cells.

Neurite outgrowth induced by Dok-4 is mediated by the ERK pathway

To examine the effect of Dok-4 overexpression on neurite outgrowth, we established TGW cell lines that stably expressed human wild-type Dok-4 [TGW(Dok-4)] (Fig. 2Aa). Overexpression of Dok-4 markedly enhanced neurite outgrowth by TGW cells after GDNF stimulation (Fig. 2Ab,B). The degree of neurite outgrowth was quantified, and results confirmed using three clones. The Ras/ERK, PI(3)K/AKT, and Rac/JNK-dependent pathways have been implicated in GDNF-mediated cell proliferation, neurite outgrowth, cell migration and/or cytoskeleton remodeling (Ichihara et al., 2004; Kodama et al., 2005). Using selective kinase inhibitors, we examined whether the blockade of each of these pathways could inhibit GDNF-induced neurite outgrowth (Fig. 2Ab,B). Blockade of

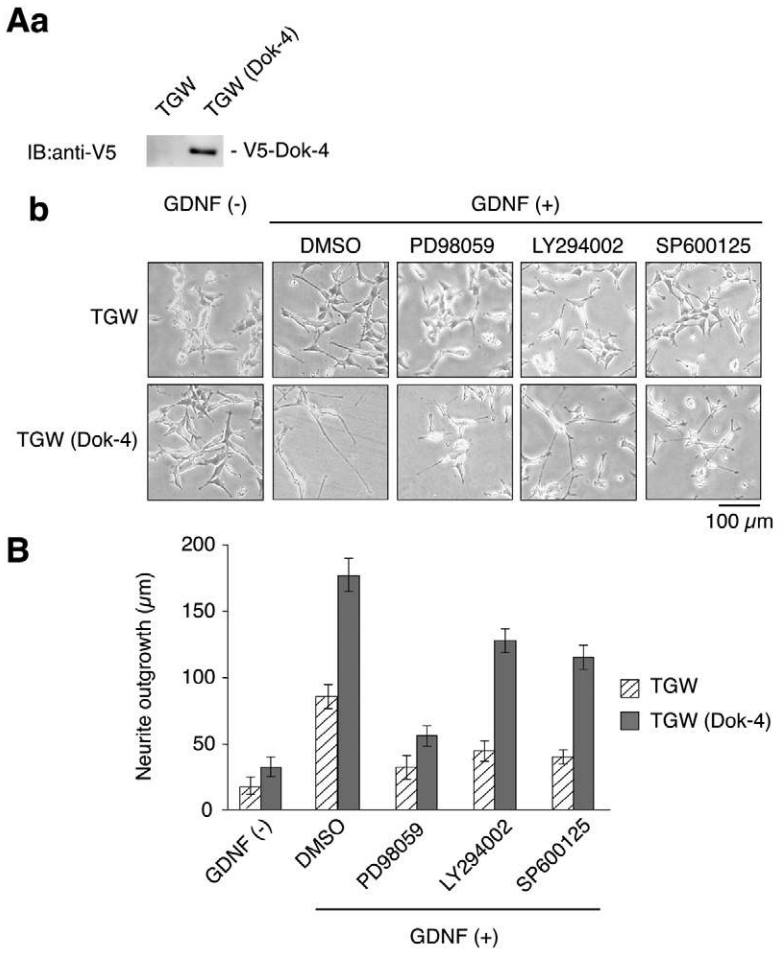

Fig. 2. Expression of Dok-4 induces neurite outgrowth in TGW cells. (A) Neurite outgrowth of TGW cells transfected with Dok-4. (a) Expression of Dok-4V5 was monitored by western blot analysis. (b) TGW cells stably expressing Dok-4 [TGW(Dok-4)] or parental TGW cells (TGW) were serum-starved and incubated for 48 hours with or without GDNF (50 ng/ml) in the presence or absence of DMSO (0.1%), PD98059 (18 μM), LY294002 (15 μM) or SP600125 (20 μM). (B) Mean of the longest neurite was determined for each culture from measurements of 100 neurons for three different experiments. Each data point represents the mean ± s.e.m. (*P<0.05).

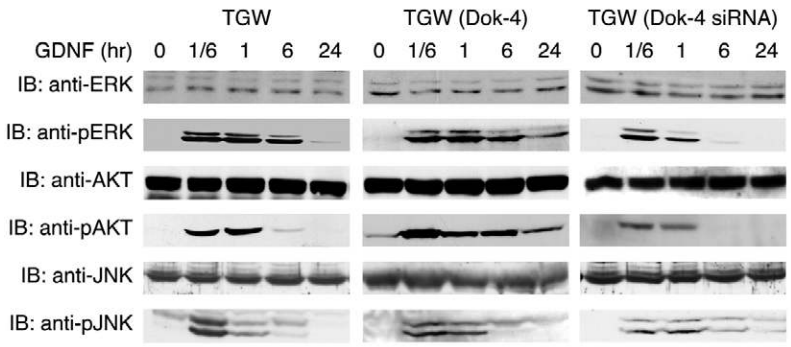

Fig. 3. Dok-4 mediates sustained ERK and AKT activation in the GDNF/RET signaling pathway. TGW cells, TGW cells stably expressing Dok-4 [TGW(Dok-4)], or Dok-4 knockdown cells [TGW(Dok-4 siRNA)] were serum-starved and stimulated with GDNF (50 ng/ml) for the indicated times, and cell extracts subjected to western blot analysis with the indicated antibodies.

the ERK pathway by the MEK1 inhibitor PD98059 was the most effective in preventing neurite outgrowth by TGW(Dok-4) cells following GDNF treatment, whereas the PI(3)K inhibitor LY294002 and the JNK inhibitor SP600125 exhibited only mild to moderate effects. Thus, it appeared that Dok-4-induced neurite outgrowth was highly dependent on the ERK pathway rather than on the PI(3)K or JNK pathways.

In contrast to the effect of Dok-4 on neurite outgrowth from TGW cells, the cell growth was not affected by Dok-4 expression (supplementary material Fig. S1).

Dok-4 induces a sustained activation of ERK in TGW cells

It has already been shown that sustained ERK activation mediated by neurotrophic factors is a hallmark of the differentiating response in neurons, as opposed to the transient activation kinetics observed during cell proliferation (Marshall, 1995; York et al., 1998). Because our data indicated a link between Dok-4 and ERK activation, we attempted to determine whether Dok-4 potentiated sustained ERK activation in response to GDNF in TGW cells. TGW cells that overexpressed Dok-4 [TGW(Dok-4)] were stimulated with GDNF for varying lengths of time under serum starvation conditions, and were then analyzed for ERK activation by western blotting. A high level of ERK phosphorylation was maintained until 24 hours after GDNF stimulation in TGW(Dok-4) cells (Fig. 3 and supplementary material Fig. S2). By contrast, ERK phosphorylation significantly decreased 24 hours after stimulation both in parental TGW cells and in Dok-4 knockdown cells [TGW(Dok-4 siRNA)]. In addition, three independent experiments showed that the level of phosphorylated ERK at 6 hours after GDNF stimulation was considerably lower in TGW(Dok-4 siRNA) cells than in parental TGW cells (Fig. 3). Enhanced activation of AKT but not JNK was also observed in TGW(Dok-4) cells, which suggested that Dok-4 might also have some role in the PI(3)K/AKT signaling pathway.

Conserved tyrosine residues in Dok-4 are crucial for sustained ERK activation and neurite outgrowth

Dok-4 has been reported to undergo tyrosine phosphorylation in cells that overexpress RET (Grimm et al., 2001). However, the role of tyrosine phosphorylation in GDNF/RET signaling has not yet been established. Therefore, we investigated whether Dok-4 contained tyrosine residues important for biochemical and biological responses. While human Dok-4 contains a total of 14 tyrosine residues, only three tyrosines (at amino acid positions 187, 220 and 270) are conserved across species, including human, rat, mouse and *Xenopus laevis*, as well as with Dok-5 and -6 (Fig. 4A). We replaced each of these three tyrosines with

phenylalanine (Y187F, Y220F and Y270F, respectively). As a control, the tyrosine at position 255 in Dok-4, which is not conserved with Dok-5 and -6, was also replaced with phenylalanine (Y255F). Each mutant was stably expressed in TGW cells as a V5 epitope-tagged protein. Dok-4 was immunoprecipitated with anti-V5 antibody after GDNF stimulation, and analyzed by western blot using anti-V5 or anti-phosphotyrosine (4G10) antibody. As shown in Fig. 4Ba, tyrosine phosphorylation of each mutant was detected, suggesting that Dok-4 appears to contain multiple phosphorylation sites. On the other hand, its tyrosine phosphorylation was undetectable in the GDNF-untreated cells (data not shown).

To investigate the role of the conserved tyrosine residues, we examined the effects of the mutant proteins on ERK activation. Activation of ERK and AKT in TGW cells that stably expressed Dok-4 Y187F, Y220F or Y270F was significantly decreased compared to TGW cells that expressed wild-type Dok-4, which suggested that the conserved tyrosine residues were crucial for Dok-4 function (Fig. 4Bb). By contrast, ERK activation in TGW cells expressing Dok-4 Y255F was rather increased compared with parental TGW cells (Fig. 4Bb).

We also examined the phenotypic changes of TGW cells expressing mutant Dok-4 proteins after GDNF treatment. TGW cells that stably expressed mutant Dok-4 were serum-starved for 6 hours, incubated with GDNF for 48 hours, and then examined for the degree of neurite extension (Fig. 5A,B). Consistent with the findings for ERK activation, TGW cells that expressed Y187F, Y220F or Y270F showed no significant neurite outgrowth in response to GDNF compared with parental TGW cells. By contrast, the Y255F mutant led to enhanced neurite outgrowth, although the degree of outgrowth was still lower than that induced by wild-type Dok-4.

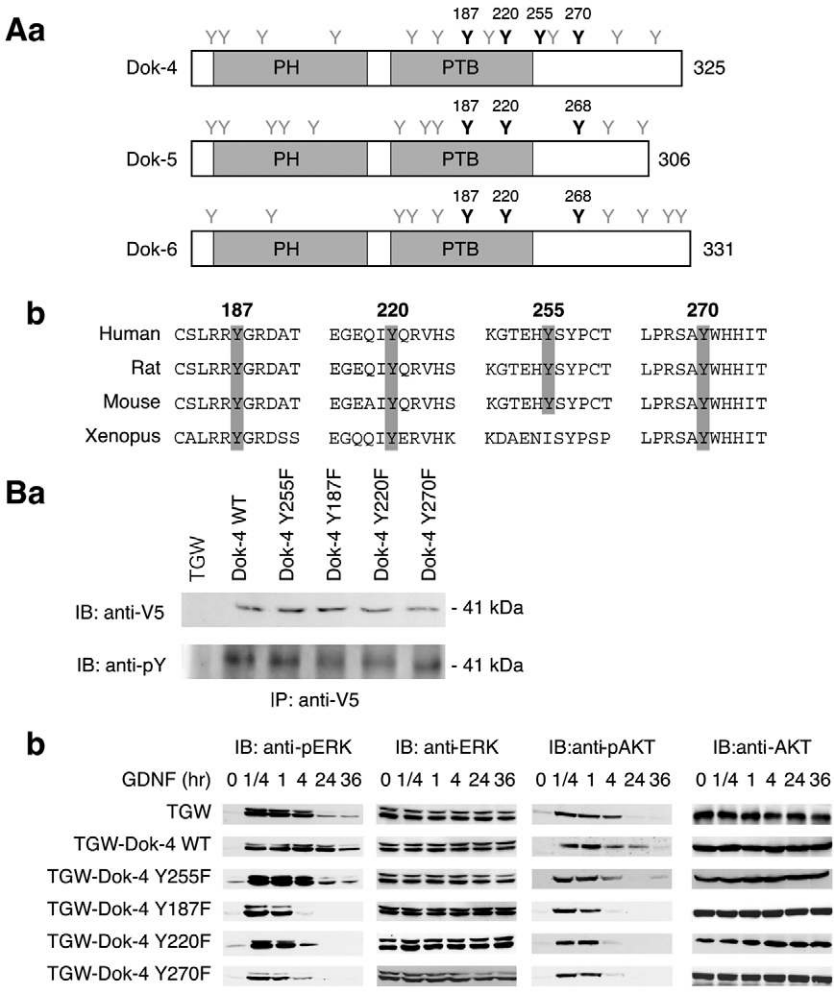

Fig. 4. Conserved tyrosine residues in Dok-4 are important for sustained ERK and AKT activation. (A) (a) Schematic representation of Dok-4, -5 and -6 structures. Y indicates tyrosine residues, and the tyrosine residues conserved among Dok-4-6 are indicated in bold. (b) Y187, Y220 and Y279 are conserved across a wide range of species. (B) (a) Each mutant was stably expressed in TGW cells as a V5 epitope-tagged protein. Dok-4 was immunoprecipitated with an anti-V5 antibody after GDNF stimulation, followed by immunoblotting with anti-V5 or anti-phosphotyrosine antibody. (b) TGW cells and TGW cells stably expressing each mutant were stimulated with GDNF (50 ng/ml) for the indicated times, and cell extracts subjected to western blot analysis with the indicated antibodies.

Dok-4 activates Rap1 downstream of RET

Our findings suggested a link between sustained ERK activation mediated by Dok-4 and neurite outgrowth in TGW cells. As it has been reported that sustained ERK activation is dependent on Rap1 activation during NGF signaling in PC12 cells (York et al., 1998), we investigated whether Rap1 activation was mediated by Dok-4 and induced neurite outgrowth in TGW cells (Fig. 6). As GTP-bound Rap1 is known to associate with high selectivity and specificity to Ral GDP dissociation stimulator (RalGDS) in vitro (Herrmann et al., 1996), we performed a Rap1 activity assay using a GST-RalGDS-RBD (Ras binding domain) fusion protein. Rap1 activation, as determined by the amount of Rap1 bound to RalGDS relative to the total amount of Rap1 in the lysate (Franke et al., 1997), was assessed in TGW(Dok-4), TGW(Dok-4 Y220F) and TGW(Dok-4 siRNA) cells. We also measured the amount of activated Ras bound to Raf-1 RBD by western blot analysis. A significant increase in Rap1 activation was observed in TGW(Dok-4) cells compared with parental TGW cells, TGW(Dok-4 siRNA) cells and TGW(Dok-4 Y220F) cells. Rap1 activation was observed at least until 6 hours after GDNF stimulation in TGW(Dok-4) cells whereas it was undetectable at 1 hour after stimulation in TGW cells (Fig. 6Aa). In contrast, Ras was activated to similar levels in all four cell lines in response to GDNF treatment. Time course of the Ras activation was similar between TGW and TGW(Dok-4) cells (Fig. 6Ab). Thus, Dok-4 expression may induce a constitutive increase in the level of GTP-bound endogenous Rap1.

To test whether Rap1 activation induced neurite outgrowth, TGW cells were transfected with a dominant active Rap1 mutant (RapV12) (Kitayama et al., 1990), in which the glycine

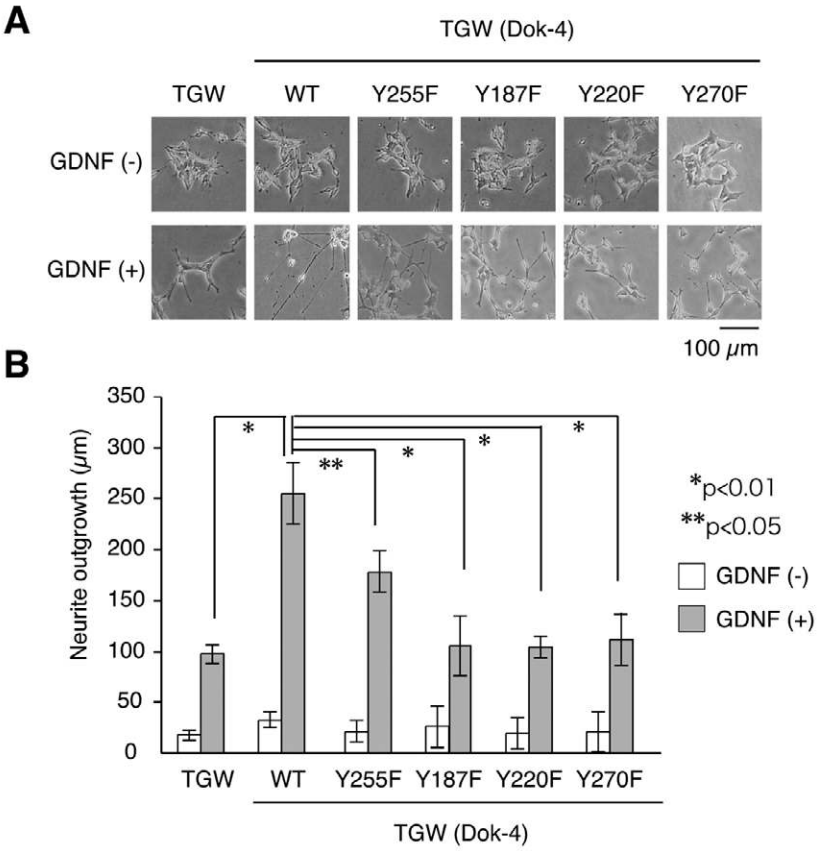

Fig. 5. The conserved tyrosine residues in Dok-4 are important for GDNF-dependent neurite outgrowth in TGW cells. (A) TGW cells and TGW cells stably expressing each mutant were serum-starved and stimulated with GDNF (50 ng/ml) for 48 hours. (B) Mean of the longest neurite was determined for each culture from measurements of 100 neurons for three different experiments. Each data point represents the mean ± s.e.m.

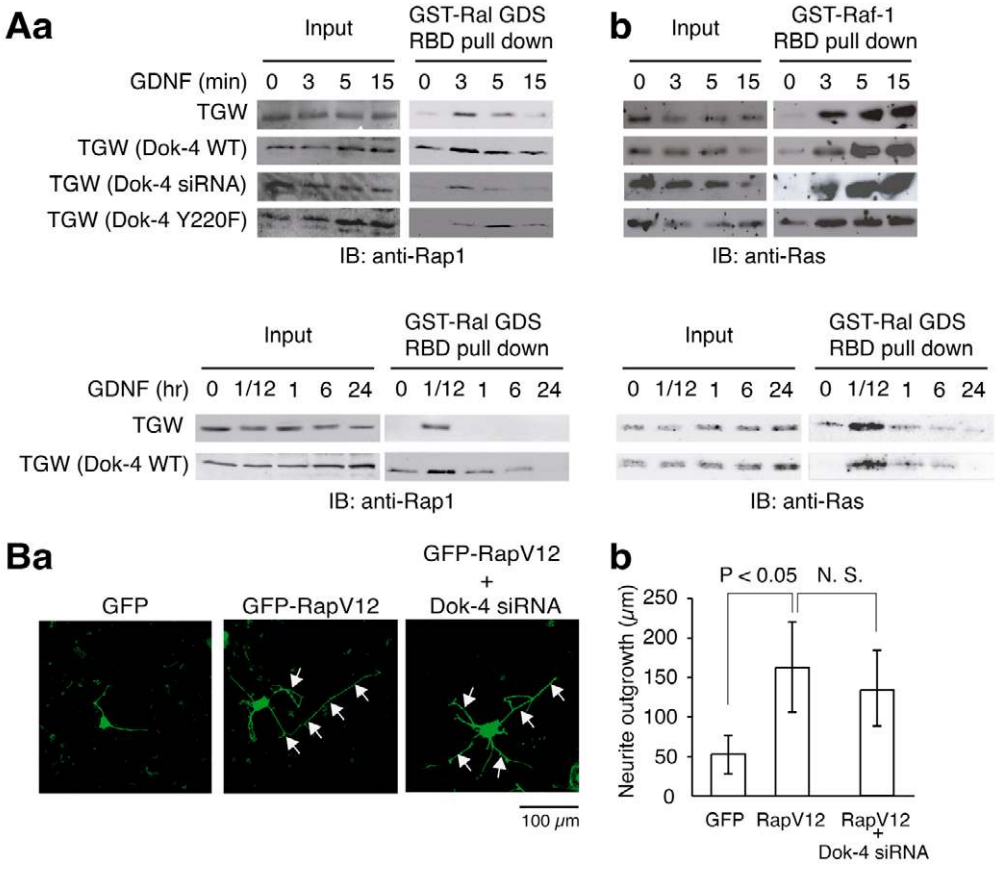

Fig. 6. Dok-4 activates Rap1 that promotes neurite outgrowth in TGW cells. (A) Rap1 and Ras activation in TGW cells expressing Dok-4. Subconfluent TGW cells, TGW cells expressing Dok-4 wild-type or Y220F mutant, or Dok-4 knockdown cells were stimulated with GDNF (50 ng/ml) for the indicated times, thoroughly washed, and lysed. Lysates were clarified and incubated at 4°C with GST-RalGDS RBD (a) or GST-Raf-1 RBD (b) fusion proteins for 1 hour. Beads were washed, SDS sample buffer added, and the proteins subjected to western blot analysis for Rap1 (a) or Ras (b). (B) Rap1 induces neurite outgrowth downstream of Dok-4 in TGW cells. (a) TGW cells or Dok-4 knockdown cells were transfected with either GFP or GFP-RapV12 and incubated for 48 hours. (b) Mean of the longest neurite was determined for each culture from measurements of 50 neurons for three different experiments. Each data point represents the mean ± s.e.m.

at position 12 (within the GTPase domain) was replaced with valine, and neurite length measured after 48 hours (Fig. 6B). Our results indicated that expression of green fluorescent protein (GFP)-tagged RapV12 induced significant neurite outgrowth by TGW cells in the absence of GDNF stimulation compared with control cells that expressed only GFP. Moreover, exogenous expression of RapV12 also enhanced neurite outgrowth in Dok-4 knockdown cells (Fig. 6B). These results indicated that active Rap1 alone is sufficient to induce neurite outgrowth, and that Rap1 appeared to be activated downstream of Dok-4 in TGW cells.

To further confirm the role of Rap1 in neurite outgrowth, TGW cells or TGW(Dok-4) cells were transfected with a GFP-tagged Rap1GAP construct or a GFP-tagged dominant negative Rap1 mutant (RapN17) in which the serine at position 17 was replaced with asparagine. As shown in Fig. 7A,B, GDNF-dependent neurite outgrowth was markedly impaired by expression of these proteins. In addition, sustained ERK activation was significantly decreased in GFP-Rap1GAP or GFP-RapN17-expressing TGW(Dok-4) cells (Fig. 7C).

Dok-4 mediates neurite outgrowth in cultured rat hippocampal neurons

We next investigated the expression of *Dok-4* and *RET* in primary hippocampal neurons from the rat brain by RT-PCR. As shown in Fig. 8A, Dok-4 and RET transcripts were detected in them. Thus, it is possible that Dok-4 plays a role in differentiation of hippocampal neurons. The prepared neurons were transfected with either GFP-fused wild-type or Y220F mutant Dok-4, and incubated for 3 days with or without GDNF. After fixation, neurons were stained with anti-MAP2 antibody, a marker of differentiated neuron dendrites, and then cell morphology analyzed by microscopy. Expression of wild-type but not mutant Dok-4 induced significant neurite outgrowth compared with the control cells in the presence or absence of GDNF (Fig. 8B,C). These results implied that Dok-4 played a crucial role in the differentiation of primary hippocampal neurons. Although enhancement of neurite outgrowth after GDNF stimulation was weak, this may be due to a low level of the RET protein expression in the cultured primary neurons (data not shown). On the other hand, neurite outgrowth in hippocampal neurons was rather decreased by expression of RapGAP or RapN17 (Fig. 8B,C).

Discussion

Roles of Dok-4 in neurite outgrowth induced by GDNF/RET signaling

Neurite outgrowth is an established marker of neuronal differentiation that requires regulated cytoskeletal changes and intracellular signaling cascades (Chao, 2003). To reconstruct the pathways involved in RET-mediated neuronal differentiation and neurite outgrowth, it is necessary to identify the proteins that act downstream of the activated receptor complex. Here, we report that Dok-4, a member of the p62dok adaptor protein family, plays an important role in RET-mediated neurite outgrowth in the TGW cell line. Dok-4 was originally identified from a yeast two-hybrid screen as a protein that interacted with RET (Grimm et al., 2001). It was also clearly shown that Dok-4 was phosphorylated following RET activation and that overexpression of Dok-4 induced RET-dependent neurite outgrowth in PC12 cells (Grimm et al., 2001). However, as this study used a chimeric protein of Dok-4 fused with the extracellular domain of EGFR and

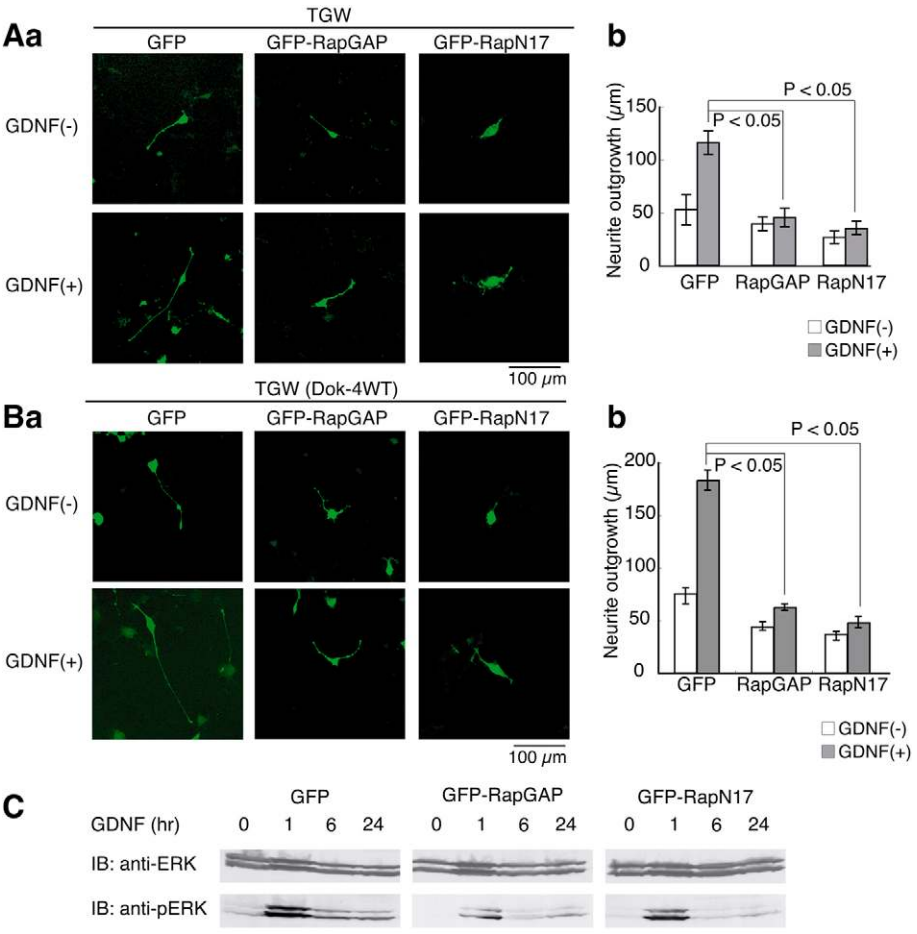

Fig. 7. Dominant negative Rap1 (RapN17) or Rap1GAP impairs neurite outgrowth and sustained ERK activation (A,B) (a) TGW cells (A) or TGW(Dok-4) cells (B) were transfected with GFP, GFP-RapGAP (SPA-1) or GFP-RapN17 and incubated for 48 hours with or without GDNF. (b) Mean of the longest neurite was determined for each culture from measurements of 50 neurons for three different experiments. Each data point represents the mean ± s.e.m. (C) TGW(Dok-4) cells were transfected with GFP, GFP-RapGAP (SPA-1) or GFP-RapN17, serum-starved, and stimulated with GDNF (50 ng/ml) for the indicated times, and cell extracts subjected to western blot analysis with the anti-ERK or anti-phosphoERK antibody.

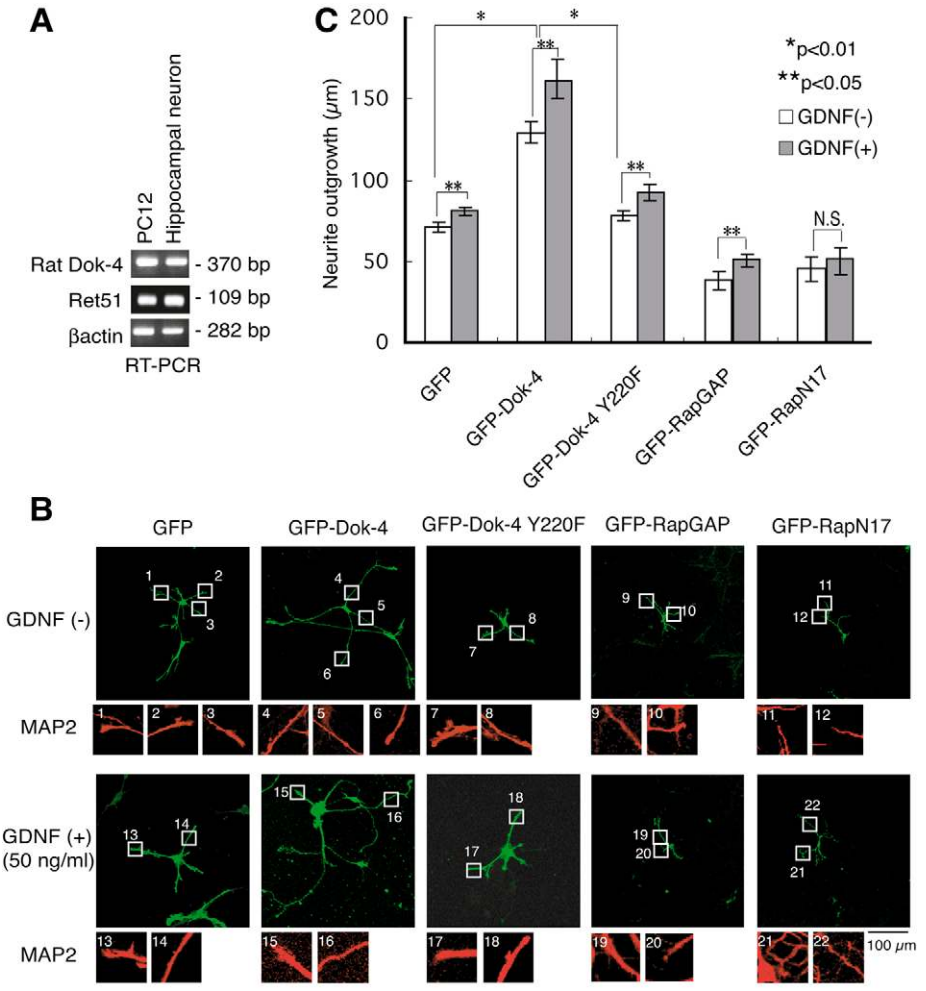

Fig. 8. Dok-4 induces neurite outgrowth in rat hippocampal neurons.
(A) Expression of rat Dok-4 and RET in rat hippocampal neurons. Total RNA was isolated from PC12 cells and rat hippocampal neurons, and RT-PCR performed using rat Dok-4-and RET-specific primers as described in Materials and Methods. β-actin was amplified as a control. (B) Hippocampal neurons were transfected with GFP, GFP-Dok-4 wild-type, GFP-Dok-4 Y220F mutant, GFP-RapGAP (SPA-1) or GFP-RapN17, incubated for 72 hours, and visualized by confocal microscopy. Boxes denote dendrites of neurons identified by MAP2 expression (lower panels).
(C) Hippocampal neurons transfected with the indicated constructs were incubated for 72 hours with or without GDNF (50 ng/ml), and dendrite length quantified. Mean of the longest dendrite was determined for each culture from measurements of 50 hippocampal neurons for three different experiments. Each data point represents the mean ± s.e.m.

the cytoplasmic domain of RET, the role of endogenous Dok-4 in the GDNF/RET signaling pathway remained unclear. In the present study, we found that human neuroblastoma TGW cells endogenously expressed both RET and Dok-4. Knockdown and overexpression experiments showed that Dok-4 was important for RET-induced neurite outgrowth in response to GDNF, and that sustained MAPK activation was enhanced in TGW cells that overexpressed Dok-4 but was decreased in Dok-4 knockdown cells.

Although the mechanism underlying Dok-4-induced neurite outgrowth in TGW cells is not fully understood, it appears to be dependent on phosphorylation mediated by RET activation. Consistent with this view, we found that Dok-4 residues Y187, Y220 and Y270, tyrosines conserved between Dok-4 proteins from a wide range of species as well as with Dok-5 and -6, may act as phosphorylation sites responsible for the sustained ERK activation in response to GDNF (Fig. 4). In addition, these conserved tyrosines were found to be necessary for full Dok-4 activity and induction of marked neurite outgrowth by TGW cells (Fig. 5). As tyrosine phosphorylation was still detected in each Dok-4 mutant tested, it is possible that phosphorylation at multiple tyrosine residues is required for the binding of adaptor or effector proteins to Dok-4. Alternatively, the tyrosines may be involved in maintaining the proper three dimensional structure of Dok-4. Exact determination of the phosphorylation sites in Dok-4 by phosphopeptide mapping or mass spectrometry is the subject of further investigation.

Exogenous expression of Dok-4 also induced neurite outgrowth of rat hippocampal neurons. It is interesting to note that Dok-4 expression showed a marked effect on neurite outgrowth without GDNF stimulation. This suggested that Dok-4 was activated in primary hippocampal neurons under our culture conditions in the absence of GDNF. Despite the presence of Dok-4 transcript in the primary neurons (Fig. 8A), we could not examine the endogenous Dok-4 protein expression in them because our anti-human Dok4 antibody did not recognize rat Dok-4. Thus, further investigation is necessary to elucidate the role of endogenous Dok-4 in neurite outgrowth of rat hippocampal neurons. In addition, a recent study revealed that the GDNF/NCAM pathway promoted axonal growth in rat hippocampal neurons independently of RET (Paratcha et al., 2003), uncovering a possible synergistic interaction between the GDNF/RET and GDNF/NCAM pathways, although the NCAM pathway does seem to be dispensable for organogenesis and nerve regeneration in vivo (Enomoto et al., 2004). It is also interesting to investigate whether Dok-4 is involved in the GDNF/NCAM pathway.

Role of Rap1 in GDNF/Ret signaling pathway

The enhancement of neurite outgrowth in TGW cells that

overexpressed Dok-4 was significantly inhibited by PD98059, which indicated the importance of the ERK pathway in neuronal differentiation. Although it is currently not known which kinase is responsible for the sustained ERK phosphorylation mediated by Dok-4, our results showed that Dok-4 activated Rap1, a small GTPase of the Ras family, and is therefore a candidate mediator of sustained ERK activation (Vossler et al., 1997; York et al., 1998; Grewal et al., 2000a; Stork, 2003). In PC12 cells, an established model of NGF-induced neuronal differentiation, Rap1 is activated by NGF, which then increases the duration of ERK signaling by acting through the related Raf isoform B-Raf, thereby potentiating neuronal differentiation (Vossler et al., 1997; York et al., 1998; Annerén et al., 2000; Grewal et al., 2000b). In megakaryocytes, activation of Rap1, B-Raf, and ERK by thrombopoietin induces megakaryocytic differentiation (Delehanty et al., 2003).

In addition to sustained ERK activation and cell differentiation, Rap1, like Ras, has been implicated in a wide range of biological processes, including cell proliferation and adhesion (Bos et al., 2001; Bos, 2005). It has also been reported that Rap1 functions as a Ras antagonist, opposing various actions of Ras including ERK pathway regulation (Cook et al., 1993; Stork, 2003; Bos, 2005). Although the functional diversity of Rap1 has led to contradictory reports of its effects (Zwartkruis et al., 1998), one of the unique features of Rap1 is its cell-type-specific regulation of ERK (Stork et al., 2003). To our knowledge, the role of Rap1 in the GDNF/RET signaling pathway has not been investigated previously. Our present study showed that GDNF stimulation induced Rap1 activation, which was clearly enhanced by Dok-4 expression. Expression of the constitutively active RapV12 induced neurite outgrowth in the absence of GDNF stimulation and in Dok-4-depleted cells (Fig. 6), which indicated that Rap1, acting downstream of Dok-4, may play a crucial role in the neuronal differentiation of TGW cells. By contrast, expression of the dominant negative Rap1 mutant or Rap1GAP markedly impaired neurite outgrowth from TGW cells as well as from hippocampal neurons. However, as Rap1 activation was not detected in TGW(Dok-4) cells 24 hours after GDNF stimulation (Fig. 6A), it may be unnecessary for maintaining neurite outgrowth. In addition, our study did not elucidate the molecular mechanism by which Dok-4 activated Rap1. Interaction between endogenous Dok-4 and Rap1 or Rap1 guanine nucleotide exchange factors (GEFs) such as C3G and PDZ-GEFs was not detected in immunoprecipitation assays (data not shown). Further experiments are required to determine the mechanism of how Dok-4 activates Rap1.

In addition to Dok-4, Dok-5 and -6 were recently identified as new members of the p62dok family. In contrast to Dok-1-3, which are expressed in hematopoietic tissues (Cristofano et al., 1998; Cong et al., 1999), Dok-4-6 are mainly expressed in the central and peripheral nervous systems, partially colocalized with RET. Together with the finding that Dok-4-6 show only remote sequence similarity to Dok-1-3, it appears that Dok-4-6 constitute a p62dok subfamily. Recent overexpression studies have shown that Dok-5 and -6 also associate with RET and promote neurite outgrowth in rat PC12 and mouse Neuro 2A cells in response to GDNF stimulation, although the role of endogenous Dok-5 and -6 remain to be clearly demonstrated (Grimm et al., 2001; Crowder et al., 2004). Nonetheless, owing to the sequence and structural similarities between Dok-4-6, it is important to clarify the level of functional divergence, synergism, and complementation between these proteins in GDNF/RET-dependent neuronal differentiation.

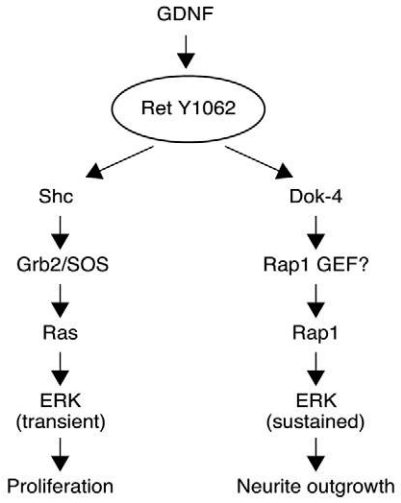

Fig. 9. A proposed model for the involvement of Dok-4 in GDNF/RET signaling. After activation of RET by GDNF, the tyrosine at position 1062 (Y1062) in RET is autophosphorylated, which then recruits Shc or Dok4 adaptor proteins. In one pathway, phosphorylated Shc stimulates the Grb2/SOS/Ras/ERK cascade, thereby inducing transient activation of ERK. Alternatively, activation of Dok-4 leads to increased GTP-bound Rap1 levels, which induces sustained activation of ERK and neuronal differentiation including neurite outgrowth.

In summary, our experiments suggested a model of Dok-4-induced neurite outgrowth (Fig. 9) in which downstream of RET, phosphorylated Dok-4 augments Rap1 activation, which then mediates sustained ERK activation and neurite outgrowth. Many reports have suggested that Shc binding to RET induces the Ras activation responsible for transient ERK activation and cell proliferation. Our additional findings that overexpression of wild-type but not mutant Dok-4 enhanced the phosphorylation of AKT, an important mediator of cell survival, proliferation and migration (Datta et al., 1999; Brazil et al., 2002; Enomoto et al., 2005), supports the idea that Dok-4 is required for the organization of other signaling cascades in neuronal differentiation. Mechanisms of other cellular events regulated by Dok-4 await further investigation.

Materials and Methods

Plasmids

The constitutively active Rap1 (RapV12) construct was generously provided by T. Urano (Nagoya University). The dominant negative Rap1 (RapN17) and Rap1 GTPase activating protein (RapGAP called SPA-1) constructs were provided by K. Kaibuchi (Nagoya University). Human Dok-4 cDNA was isolated from a TGW cell cDNA library. Dok-4 mutants were generated using the QuikChange™ site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. The pcDNA3.1-Dok-4 and pEGFP-Dok-4 constructs were generated as previously described (Murakami et al., 2002). EGFP was fused to the N-termini, and V5 and myc tags were fused to the C-termini of the Dok-4 proteins. The sequences of all constructs were confirmed by DNA sequencing.

Antibodies

Rabbit anti-Dok-4 polyclonal antibody was developed against a 19 C-terminal amino acid fragment of Dok-4 and affinity-purified using the immunizing peptide. Anti-Rap1 polyclonal antibody and anti-Ras monoclonal antibody were purchased

from Upstate. Other antibodies used included anti-p42/44 ERK polyclonal antibody, anti-phospho p42/44 ERK polyclonal antibody, anti-AKT polyclonal antibody, anti-phospho-AKT monoclonal antibody (Cell Signaling Technology) and anti-MAP2 monoclonal antibody (Upstate).

Cell culture and quantification of neurite outgrowth

TGW cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. TGW cells were grown in Dulbecco's modified Eagle's medium supplemented with 8% calf serum. For quantification of neurite outgrowth, TGW cells were cultured for 48 hours in medium with or without 50 ng/ml GDNF or inhibitors (18 μM PD98059, 15 μM LY294002 and 20 μM SP600125, added 60 minutes prior to GDNF stimulation). Neurite length was determined by tracing the entire length of the process, and total length calculated using the software program WinROOF (Mitani Corp., Fukui, Japan). At least 100 TGW cells were evaluated in each culture.

RNA interference

The siRNA-mediated knockdown of Dok-4 protein expression was performed using a previously described method (Enomoto et al., 2005). The targeted sequence that effectively silenced Dok-4 expression was (sense sequence) 5′-AACAGACAGATCGCTTCAATG–3′ (nucleotides 401–421). The sequence was searched against the human genome using BLAST to ensure that only the Dok-4 gene was targeted, and then the 21-nucleotide synthetic duplexes prepared by Qiagen. For siRNA annealing, 20 μM single stranded siRNA was incubated in siRNA Suspension Buffer (QIAGEN) for 1 minute at 90°C, followed by 1 hour at 37°C. TGW cells were transfected with either Dok-4-specific siRNA or 21-nucleotide irrelevant RNA (Qiagen) as a control using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Suppression of Dok-4 in the corresponding transfectants was confirmed by western blot analysis. siRNA-resistant Dok-4 was created by introducing two silent mutations at nucleotide positions 408 and 411 (5′-AACAGACAGATCGCTTCAATG-3′, mutations underlined).

Immunofluorescent staining

TGW cells were plated on collagen I (10 μg/ml, Upstate)-coated coverslips or glass base dishes, and fixed with methanol for 10 minutes and then 10% bovine serum albumin for 10 minutes. The fixed cells were then stained with the indicated antibodies, and fluorescence examined using a confocal laser-scanning microscope (Fluoview FV500, Olympus).

Rat hippocampal neurons were plated on poly-D-lysine (100 μg/ml, Sigma) and laminin (50 μg/ml, IWAKI, Tokyo, Japan)-coated coverslips or glass base dishes, and then fixed with 3.7% formaldehyde in PBS for 10 minutes at room temperature, followed by treatment for 10 minutes with 0.05% Triton X-100 on ice and then 10% bovine serum albumin for 10 minutes. Neurons were immunostained with the indicated antibodies and fluorescence examined using a confocal laser-scanning microscope (Fluoview FV500, Olympus).

Rap1 activity assay

GDNF-treated or untreated TGW cells were lysed on ice for 10 minutes in lysis buffer (50 mM Tris-HCl (pH 7.4), 0.5 M NaCl, 1% NP40, 2.5 mM $MgCl_2$, 10% glycerol, 1 mM sodium orthovanadate, 250 μM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) and the resulting lysates centrifuged at 15,000 ***g*** at 4°C for 5 minutes. Supernatants were added to 50 μg Ral GDS-RBD agarose beads (glutathione-Sepharose beads pre-coupled to GST protein fused with the Ras-binding domain of RalGDS) and incubated at 4°C for 45 minutes with gentle rotation. The beads were then washed four times in lysis buffer and boiled in SDS sample buffer. The amount of GTP-bound Rap1 was analyzed by immunoblotting with anti-Rap1 antibody.

Ras activity assay

GDNF-treated or untreated TGW cells were lysed on ice for 10 minutes in lysis buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, 1% NP-40, 10 mM $MgCl_2$, 1 mM EDTA and 10% glycerol, 1 mM sodium orthovanadate, 250 μM PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin) and the lysates centrifuged at 15,000 ***g*** at 4°C for 5 minutes. Supernatants were added to 5 μg Raf-1 RBD agarose beads (glutathione-Sepharose beads pre-coupled to GST protein fused with the Ras-binding domain of Raf-1) and incubated at 4°C for 45 minutes with gentle rotation. The beads were washed four times in lysis buffer and boiled in SDS sample buffer. The amount of GTP-bound Ras was analyzed by immunoblotting with anti-Ras antibody.

Primary culture of rat hippocampal neurons

Fetuses were obtained at E19 from timed pregnant Sprague Dawley rats (Japan SLC company). Primary hippocampal neurons were prepared according to published protocols (Inagaki et al., 2001; Yoshimura et al., 2005). Briefly, hippocampi were dissociated by treatment with papain (500 μg/ml 60 minutes at 37°C), followed by trituration. Dissociated neurons were seeded (1.5×10^6 cells per 35 mm dish) on glass-based dishes coated with poly-D-lysine (PDL; Sigma) and laminin (Iwaki, Tokyo, Japan) in Neurobasal medium (Gibco) in the presence of supplement B27 (Gibco) and 100 mM L-glutamine, and kept at 37°C under 5% CO_2. At 12 hours after plating, cultured neurons were transfected with either wild-type or mutant Dok-4 using a Neuroporter (Gene Therapy System) according to the manufacturer's instruction. After 3 days culture, neurons were fixed in 3.7% formaldehyde and stained with anti-MAP2 antibody for the morphometric analysis of neurite length.

Dok-4 and Ret RT-PCR

Total RNA was isolated using RNeasy Mini (Qiagen) and cDNA transcribed using Superscript (Invitrogen). RT-PCR was performed with primers specific to rat Dok-4 (5′-GAGCAGACAGATCGCTTCAA-3′ and 5′-CCTGCCTAGGCTTTGG-CTTA-3′), RET (5′-CCGATGGCACTAGCACTGGGTTCC-3′ and 5′-ATTTTG-CCGCTGAGGGTGAAACCA-3′) and actin (5′-CACCACAGCTGAGAGGGA-AAT-3′ and 5′-CCACCAGACAGCACTGTGTTG-3′).

Data analysis

Data are presented as mean ± s.e (standard error). Statistical significance was evaluated by Student's *t*-test.

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