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Analysis of DOK-6 function in downstream signaling of RET in human neuroblastoma cells

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Point mutations and structural alterations of the *RET* tyrosine kinase gene cause multiple endocrine neoplasia type 2 (MEN 2) and papillary thyroid carcinoma, respectively. RET activation by glial cell line-derived neurotrophic factor (GDNF) is essential for the development of the enteric nervous system and the kidney. The signal through RET tyrosine kinase requires several adaptor proteins including the DOK (downstream of kinase) family of proteins. Of the seven members of the DOK protein family, DOK-1, -4, -5, and -6 have been reported to play roles in the GDNF–RET signaling pathway. Although DOK-6 has been shown to bind to RET and promote GDNF-induced neurite outgrowth in mouse Neuro2A cells, DOK-6 function in human cells remains unclear. In the present study, we investigated the role of DOK-6 in GDNF–RET signaling in human cells including neuroblastoma cells. DOK-6 was constitutively localized to the plasma membrane via its pleckstrin homology (PH) domain, and was phosphorylated following RET activation via a *MEN2A* mutation or GDNF stimulation. However, DOK-6 could not significantly affect downstream signaling and neurite outgrowth in human neuroblastoma cells. The binding affinity of the DOK-6 phosphotyrosine-binding (PTB) domain to RET was much lower than that of the DOK-1, DOK-4, and SHC PTB domains to RET. These findings indicate that DOK-6 is involved in RET signaling with less influence when compared with DOK-1, DOK-4, and SHC. (*Cancer Sci* 2010; 101: 1147–1155)

The *RET* proto-oncogene encodes receptor tyrosine kinase (RTK) expressed in a wide variety of neurons and the developing kidney.[1–4] RET tyrosine kinase is activated by stimulation of the glial cell line-derived neurotrophic factor (GDNF) family of ligands including GDNF, neurturin, artemin, and persephin in the presence of glycosylphosphatidylinositol-anchored co-receptors called GDNF family receptor alpha 1–4.[5] Consistent with the expression pattern of RET during embryogenesis, signaling through RET plays crucial roles in the development of the enteric nervous system and the kidney.[6,7] *RET* mutations are responsible for the development of several human diseases including multiple endocrine neoplasia (MEN) type 2A and 2B, familial medullary thyroid carcinoma (FMTC), papillary thyroid carcinoma (PTC), and Hirschsprung's disease.[8–10] *RET* mutations in MEN2A, MEN2B, FMTC, and PTC are gain-of-function mutations, whereas Hirschsprung mutations are loss-of-function mutations.[11–17] Specific tyrosine residues in the RET intracellular domain are phosphorylated by GDNF stimulation or *RET* mutations, which results in the activation of several signaling pathways, including the RAS/ERK (extracellular signal-regulated kinase), PI3K (phosphatidylinositol-3 kinase)/AKT, p38MAPK (mitogen activated protein kinase), phospholipase Cγ, and RAC/JNK (c-Jun N-terminal kinase) pathways.[8–10] These signaling pathways are important for cell proliferation, survival, migration, and invasion.

Signal transduction through RET requires various adaptor proteins such as SHC, FRS2, GRB2, and the DOK (downstream of kinase) family of proteins. These proteins are characterized by the presence of binding domains for protein–protein interaction and lack of specific enzymatic activity.[8–10] DOK proteins (DOK-1–7) are characterized by an N-terminal pleckstrin homology (PH) domain, a phosphotyrosine-binding (PTB) domain in the middle region, and a carboxy-terminal (CT) domain containing several tyrosine residues.[18–25] DOK-1 was originally identified as a common substrate of many RTKs and is a negative regulator of the RAS/ERK pathway, whereas DOK-4 and -5 were shown to positively regulate the RAS/ERK pathway in GDNF–RET signaling.[18,19,22,26–30] Our previous study revealed that the DOK-1 PTB domain binds to phosphorylated tyrosine 1062 in RET activated by GDNF or *MEN2* mutations. DOK-1 overexpression suppressed RAS/ERK activation and enhanced JNK and c-JUN activation.[27] We also showed that DOK-4 overexpression positively regulates GDNF-induced neurite outgrowth in TGW neuroblastoma cells, and that this effect is mediated by activation of the small G protein RAP1 and ERK1/2.[30]

DOK-6 has been identified as a gene showing significant homology to *DOK-4* and *DOK-5* by searching through the Expressed Sequence Tag database. It was also shown that DOK-6 binds to phosphorylated tyrosine 1062 in RET and promotes RET-mediated neurite outgrowth in mouse Neuro2A neuroblastoma cells.[23] However, DOK-6 function in human cells has not yet been elucidated. Therefore, we further characterized the roles of DOK-6 in the GDNF–RET signaling pathway using human neuroblastoma cells.

Materials and Methods

Cell culture and reagents. The SK-N-MC human neuroblastoma cell line was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8% fetal bovine serum (FBS). The TGW human neuroblastoma cell line was grown in RPMI supplemented with 8% FBS. They were maintained at 37°C in a humidified atmosphere of 5% CO_2. Stable transfectants of SK-N-MC cells overexpressing wild-type RET or RET with a *MEN2A* mutation (cysteine 634 was substituted by arginine, C634R) were established as described previously (MC-RET and MC-RET-2A).[31,32] For transient transfection experiments, cells were transfected with expression vectors using Lipofectamine 2000 (Invitrogen, San Diego, CA, USA) according to the

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manufacturer's protocol. The cells were used for analysis 24 h after transfection.

Reverse transcription–PCR (RT–PCR) analysis. Total RNA was isolated from cultured cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany) and cDNA transcripts were then generated using the Superscript III First Strand Synthesis System for RT–PCR (Invitrogen). RT–PCR was performed with primers common to both human and mouse *DOK-6* (sense, 5′-AAGGGAAGGAGAAATGATCTATCAGAAGG-3′; antisense, 5′-TAGCTGAATCCATAGCTGCTGGACCG-3′), *DOK-1* (sense, 5′-CGCCTTTCCGAAAGGCAGCTGG-3′; antisense, 5′-CCATAGCGACGCAACAGAGTGTAGGG-3′), *DOK-4* (sense, 5′-GTGAAGCTCGTCTCGTGGCCCCTCTGC-3′; antisense, 5′-GGAGGTCTGTTTCCGAGCTGGCCTGG-3′) and *DOK-5* (sense, 5′-CAGAGTGTGAAAAACTCGATGCTCC-3′; antisense, 5′-TCAGTGCTCAGATCTGTAGGCTGGAAAAG-3′).

Plasmid construction. The human *DOK-6* (*hDOK-6*) cDNA was amplified by PCR from human brain cDNA and cloned into the pcDNA3.1/myc-His expression vector (Invitrogen). Full-length or truncated *hDOK-6* cDNA encoding amino acids 1–331, 119–331, 1–232, 119–232, 230–331, 1–113, or (1–113) + (230–331) were inserted into the vector pEGFP-C1 (Clontech, Palo Alto, CA, USA) to produce green fluorescent protein (GFP)-fused proteins (GFP-DOK-6, -ΔPH, -ΔCT, -PTB, -CT, -PH, and -ΔPTB, respectively). Full-length or truncated *hDOK-6* cDNA or full-length *hDOK-4* cDNA[30] was inserted into the vector pcDNA3.1(+) (Invitrogen) with a glutathione-*S*-transferase (GST) sequence at the 5′-terminal to produce GST-fused DOK-4 or DOK-6 proteins in cells (GST-DOK-4, GST-DOK-6, -ΔPH, -ΔCT, -PTB, -CT, -PH and -ΔPTB, respectively). The cDNA fragments encoding the PTB domains of hDOK-4, hDOK-6, hSHC, and mouse DOK-1 were cloned into pGEX4T-2 (GE Healthcare, Little Chalfont, UK) for production of the recombinant GST-fused PTB domains in *Escherichia coli* (*E. coli*) DH5α strain. The cDNA fragment cloned into each vector was produced by PCR with PfuUltra HF DNA polymerase (Stratagene, La Jolla, CA, USA). The plasmid DNAs were amplified in *E. coli* DH5α and purified with the PureLink HiPure Plasmid Maxiprep Kit (Invitrogen).

Confocal fluorescent microscopy. For visualization of GFP-fused proteins, cells transiently expressing GFP-fused proteins were grown on collagen I-coated glass base dishes, rinsed twice with PBS and fixed with 3.7% paraformaldehyde in PBS for 10 min at room temperature. The fluorescence images were captured with a Fluoview FV500 confocal laser microscope (Olympus, Tokyo, Japan).

Antibodies. Rabbit polyclonal anti-RET antibody was raised against a synthetic peptide of carboxy-terminal 19 amino acids of the long isoform.[32] Mouse monoclonal anti-phosphotyrosine (pTyr) antibody (4G10) and rabbit polyclonal anti-GFP antibody were purchased from Upstate Biotechnology (Lake Placid, NY, USA) and MBL (Nagoya, Japan), respectively. Mouse monoclonal anti-GST antibody, anti-phospho JNK polyclonal antibody, anti-JNK polyclonal antibody, anti-phospho p42/44 MAPK polyclonal antibody, anti-p42/44 MAPK polyclonal antibody, anti-phospho p38 MAPK polyclonal antibody and anti-p38 MAPK polyclonal antibody were purchased from Cell Signaling Technologies (Beverly, MA, USA). The monoclonal anti-β-actin and anti-c-myc (9E10) antibodies were purchased from Sigma (St. Louis, MO, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively.

Western blot analysis. Cells were harvested using a cell scraper, transferred to microcentrifuge tubes, and disrupted in sodium dodecyl sulfate (SDS) sample buffer (20 mM Tris-HCl [pH 6.8], 5 mM EDTA, 2% SDS, 5% sucrose, 50 μg/mL bromophenol blue, and 2% 2-mercaptoethanol) by sonication. After boiling, the lysates were subjected to SDS–polyacrylamide gel electrophoresis. Proteins were transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After blocking with 5% bovine serum albumin, membranes were probed with the primary antibody, followed by incubation with the secondary antibody conjugated to horseradish peroxidase (Dako, Glostrup, Denmark). After washing, antigen–antibody complexes were detected by the enhanced chemiluminescence system (ECL; GE Healthcare).

Detection of DOK-6 phosphorylation. Cells were transfected with GST-fused DOK-6 expression vectors. Twenty-four hours post-transfection, cells were serum starved for 3 h and then stimulated with GDNF (100 ng/mL). Cells were lysed in Nonidet P-40 (NP-40) lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.5% NP-40, 2 mM sodium orthovanadate, and 1 mM PMSF) supplemented with one tablet of complete protease inhibitor cocktail (Complete Mini; Roche Diagnostics, Mannheim, Germany) per 10 mL, and the resulting cell lysates were clarified by centrifugation. The supernatants were incubated with glutathione-sepharose beads (Glutathione Sepharose 4B; GE Healthcare) overnight at 4°C. After washing the beads, the bound proteins were eluted by boiling in SDS-sample buffer and subjected to western blotting using anti-pTyr or anti-GST antibody.

***In vitro* GST pull-down assay.** To generate recombinant GST-fusion proteins, *E. coli* DH5α transformed with pGEX4T-2 carrying a cDNA fragment for the PTB domain of DOK-6, DOK-1, DOK-4, or SHC was grown in LB medium into the log phase and induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 25°C for 12 h. The bacteria were collected by centrifugation and lysed in NETN buffer (137 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl [pH 8.0], 0.5% NP-40) with sonication. Lysates were clarified by centrifugation and the supernatants were incubated with 25 μL of glutathione-sepharose beads (GE Healthcare) to immobilize the GST-fusion proteins. The MC-RET-2A cells were lysed in NP-40 lysis buffer supplemented with Complete Mini (Roche) and the cell lysates were clarified by centrifugation. The supernatants were incubated with the immobilized GST or GST-fusion proteins on the beads for 6 h at 4°C and the beads were washed with the lysis buffer three times. The proteins bound to the GST-fusion proteins were eluted by boiling in SDS-sample buffer and analyzed by western blotting.

Quantitation of neurite outgrowth. TGW cells were transfected with GFP-fused DOK-6 or the GFP control vector. Twenty-four hours after transfection, cells were serum starved, then stimulated with GDNF (100 ng/mL) for 24 h. Cells were fixed and the neurite outgrowth of the transfected cells was analyzed. For measurement of the neurite length, the entire length of the process was traced and the total length was calculated and analyzed by the software program WinROOF (Mitani, Fukui, Japan). At least 100 transfected TGW cells were evaluated in each culture.

RNA interference. The siRNA-mediated knockdown of *DOK-6* was performed using siRNAs against *hDOK-6* purchased from Integrated DNA Technologies (Coralville, IA, USA). MC-RET cells were transfected with *DOK-6* siRNA or an irrelevant siRNA as a control using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. Suppression of *DOK-6* in the transfectants was confirmed by RT–PCR.

Simulations of docking models and interaction energies. Molecular Operating Environment (MOE) software (Chemical Computing Group, Montreal, Canada) was employed to simulate the homology models of the DOK-1 and DOK-6 PTB domains based on the structural data of the mouse DOK-1 (mDOK-1) PTB domain (1UEF.pdb) and the human DOK-5 PTB domain (1J0W.pdb) respectively, which are registered in the Protein Data Bank. Docking simulations using the ZDOCK SERVER (http://zdock.bu.edu)[33] were performed between the amino acid sequence surrounding pTyr 1062 of the RET long

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isoform and the homology model of the DOK-1 or DOK-6 PTB domain. Docking simulations were also done between the RET phosphopeptide and mDOK-1 PTB domain based on the structural data of 1UEF.pdb for comparison. Among docking simulations regarding each PTB domain, a model of the minimum docking energy was confirmed to be structurally optimized, and its interaction energy was calculated using MOE. The CHARMM force field was used in a series of simulations. Distances between interactive amino acid residues were measured using MOE.

Results

***DOK-6* expression in human and mouse cell lines.** The DOK family of proteins possesses common domains of the N-terminal PH domain and the PTB domain in its middle region (Fig. 1A). To speculate on the biological role of DOK-6 in cells, we first investigated the transcript expression of several *DOK* genes in cell culture lines. Semi-quantitative RT–PCR analysis revealed that *DOK-6* is expressed in all of the human neuroblastoma cell lines (SK-N-MC, TGW, NB1, NB39, SK-N-SH, and SH-SY-5Y) examined and HEK293 cells (Fig. 1B). Expression of *DOK-4,* but not *DOK-1* and *DOK-5,* was also detected in all of these human neuroblastoma cells.

The PH domain of DOK-6 is required for localization to the plasma membrane. To elucidate the localization of DOK-6 in human neuronal cells, we visualized DOK-6 molecules by tagging the DOK-6 protein with GFP. The expression vector pEGFP/DOK-6 was constructed by inserting the full-length and deletion fragments of human *DOK-6* cDNA into the pEGFP-C1 vector, and SK-N-MC cells overexpressing RET (designated MC-RET) were transfected with these constructs. The GFP-fused DOK-6 proteins (GFP-DOK-6, -ΔPH, -ΔCT, -PTB, -CT, -PH, and -ΔPTB) were expressed in MC-RET cells (Fig. 2A,B), and were analyzed for intracellular localization by fluorescence microscopy. As shown in Figure 2(C), GFP-DOK-6 appeared to be mainly localized to the plasma membrane and the cytoplasmic vesicles, whereas GFP alone was distributed throughout the cytoplasm and the nucleus. GFP-fused DOK-6 fragments designated ΔCT, PH, or ΔPTB was also localized to the plasma membrane as observed for full length DOK-6, while those designated ΔPH, PTB, or CT were dispersed throughout the cell (Fig. 2C). These findings indicate that the PH domain is required for the localization of DOK-6 to the plasma membrane. MC-RET cells expressing GFP-fused DOK-6 proteins were stimulated with GDNF and the localization of GFP-fused DOK-6 proteins was analyzed. No significant difference in the localization of GFP-fused proteins was observed before and after GDNF stimulation (data not shown). In addition, when GFP-fused DOK-6 proteins were expressed in parental SK-N-MC cells, their distribution was similar to that in MC-RET cells (Fig. S1).

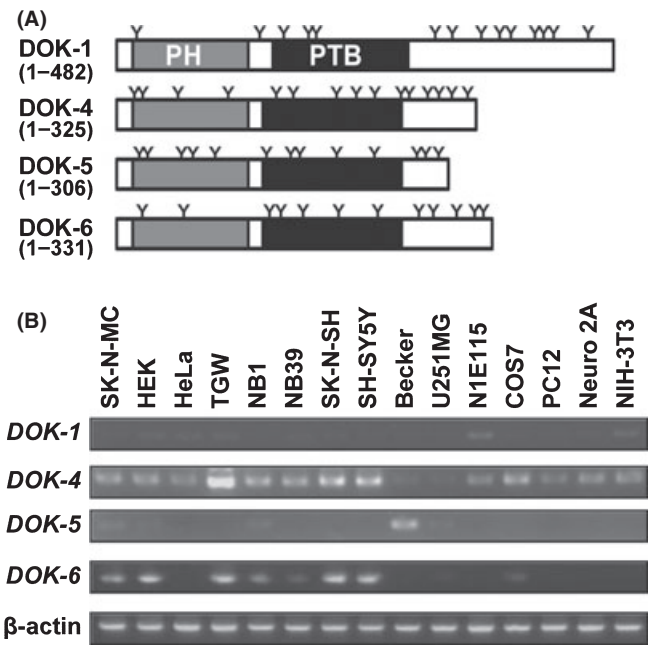

Fig. 1. The expression of *DOK-6* in human cell lines. (A) Schematic illustration of the domain structure of DOK family proteins, DOK-1, -4, -5, and -6. The pleckstrin homology (PH) domain, phosphotyrosine-binding (PTB) domain, carboxy-terminal (CT) domain, and tyrosine residues (Y) are indicated. (B) Expression of *DOK-1, -4, -5,* and *-6* in mammalian cell lines detected by semi-quantitative RT–PCR. The β-actin product was used as an internal control for the amount of RNA.

Both PH and PTB domains of DOK-6 are necessary for its tyrosine phosphorylation downstream of GDNF–RET signaling. Since DOK family proteins have been reported to be phosphorylated downstream of GDNF–RET signaling,[22,23,27,30] we investigated whether DOK-6 was also phosphorylated in MC-RET cells in a RET-dependent manner. MC-RET cells were transfected with the expression vector carrying GST-fused DOK-6 (GST-DOK-6) and stimulated with GDNF (100 ng/mL). Lysates obtained from these cells were subjected to GST pull-down and the bound proteins were analyzed for tyrosine phosphorylation by western blotting with anti-pTyr antibody. Although overexpressed GST-DOK-6 was partly phosphorylated without GDNF stimulation, the phosphorylation level of GST-DOK-6 increased after GDNF stimulation (Fig. 3A). GST-DOK-6 was also phosphorylated at a high level in SK-N-MC cells overexpressing RET with a *MEN2A* mutation, a constitutive active form of RET (MC-RET-2A). To exclude the possibility that tyrosine in GST but not in DOK-6 was phosphorylated, we generated myc (EQKLISEEDL)-tagged DOK-6. Myc-tagged DOK-6 was phosphorylated on tyrosine in MC-RET-2A cells (Fig. S2), confirming that DOK-6 is a downstream target of GDNF–RET signaling. In addition, we found that GST-fused DOK-4 was phosphorylated in a similar manner downstream of GDNF–RET signaling (Fig. S3).

It has been reported that the PH domain of DOK-1 is required for its tyrosine phosphorylation in response to insulin.[26] To investigate which domain of DOK-6 is necessary for GDNF–RET-dependent phosphorylation of DOK-6, we generated expression vectors carrying GST-fused DOK-6 fragments (GST-DOK-6, -ΔPH, -ΔCT, -PTB, -CT, -PH, and -ΔPTB) which were introduced in MC-RET-2A cells (Fig. 3B). The GST-fusion proteins in cell lysates were subjected to GST pull-down, followed by western blotting with anti-pTyr antibody. As shown in Figure 3(C), high-level phosphorylation of full length GST-DOK-6 and -ΔCT was induced by RET-MEN2A, whereas phosphorylation was almost undetectable in GST-ΔPH, -PTB, -CT, -PH, and -ΔPTB proteins. These results suggest that both PH and PTB domains are necessary for DOK-6 phosphorylation.

DOK-6 expression does not significantly affect GDNF-mediated intracellular signaling and neurite outgrowth in human neuroblastoma cells. We analyzed the activation of intracellular signaling after GDNF stimulation in MC-RET cells with or without transient DOK-6 overexpression. MC-RET cells were transfected with the expression vector carrying GFP-DOK-6 or GFP alone and stimulated with GDNF (10 ng/mL; Fig. 4A). Phosphorylation levels of intracellular signaling proteins, including AKT, ERK, JNK, and p38MAPK were compared between MC-RET cells expressing GFP-DOK-6 and those expressing GFP. As shown in Figure 4(C), no significant difference in time-dependent phosphorylation of these signaling

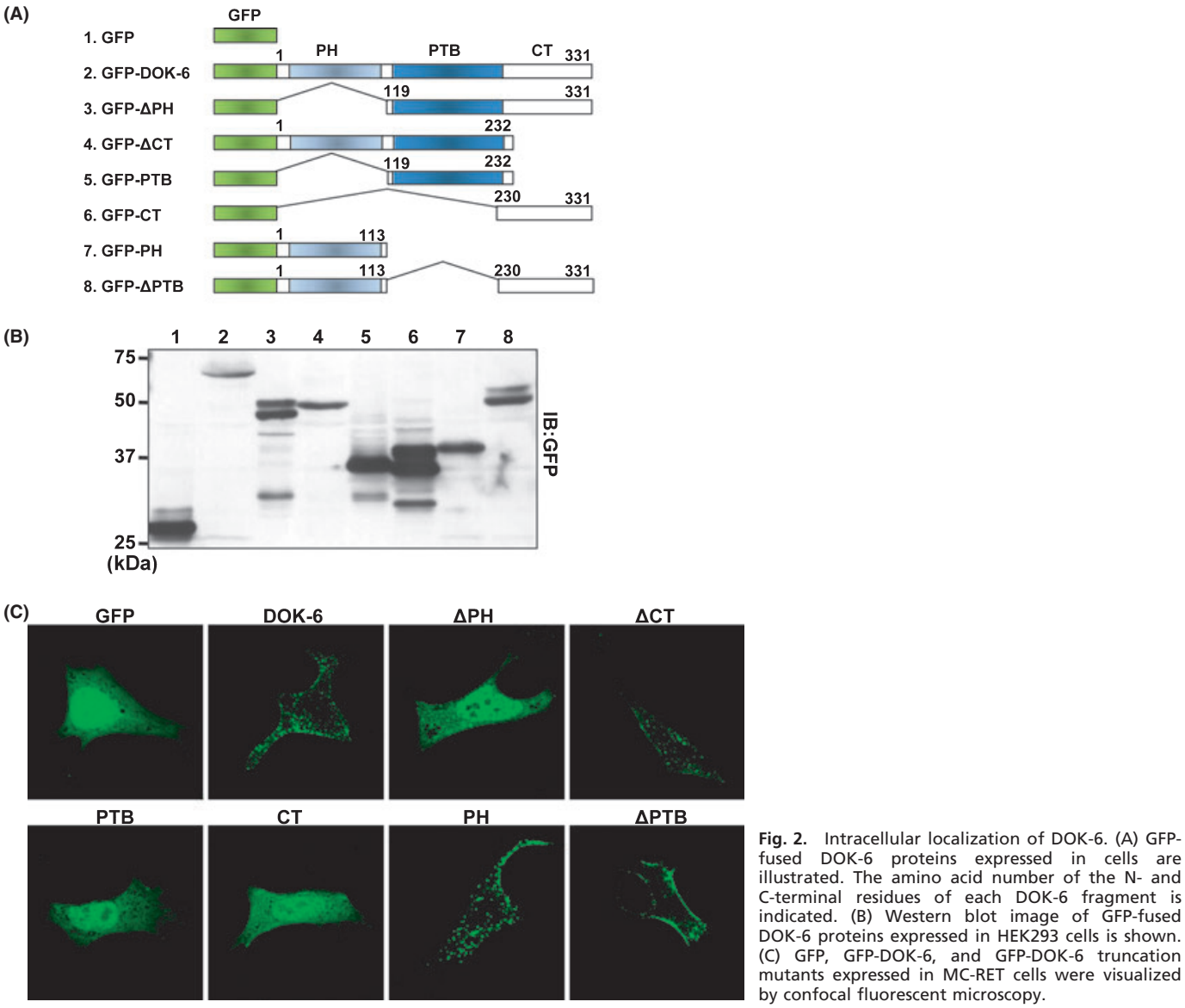

Fig. 2. Intracellular localization of DOK-6. (A) GFP-fused DOK-6 proteins expressed in cells are illustrated. The amino acid number of the N- and C-terminal residues of each DOK-6 fragment is indicated. (B) Western blot image of GFP-fused DOK-6 proteins expressed in HEK293 cells is shown. (C) GFP, GFP-DOK-6, and GFP-DOK-6 truncation mutants expressed in MC-RET cells were visualized by confocal fluorescent microscopy.

molecules was observed between these two cells. Additionally, no significant difference in phosphorylation levels of these molecules was detected between control and DOK-6-specific siRNA-treated MC-RET cells (Fig. 4B,C). As previously reported, when GST-DOK-4 was expressed in MC-RET cells, GDNF stimulation enhanced ERK phosphorylation (Fig. S4). Expression of GST-DOK-4 but not GST-DOK-6 also enhanced ERK phosphorylation in GDNF-stimulated SK-N-SH human neuroblastoma cells (Fig. S4).

We further analyzed GDNF-dependent neurite outgrowth in TGW cells with or without DOK-6 overexpression. TGW cells were transfected with the expression vector carrying GFP-DOK-6 or GFP alone and stimulated with GDNF (100 ng/mL). Neurite outgrowth of TGW cells was assessed under the fluorescence microscope (Fig. 4D). No significant difference of GDNF-dependent neurite outgrowth between the cells with and without DOK-6 overexpression was observed. In addition, knockdown of DOK-6 expression by its siRNA did not show a significant change in neurite outgrowth of TGW cells (Fig. S5). These findings suggest that DOK-6 does not affect GDNF-dependent downstream signaling and neurite outgrowth in human neuroblastoma cells.

The PTB domain of DOK-6 has less potential to bind to RET, compared with those of DOK-1, DOK-4, and SHC. We previously reported that DOK-1, DOK-4, and SHC play crucial roles in GDNF–RET signaling.[27,30,31] We compared the binding affinity of DOK-6 to RET with that of DOK-1, DOK-4, and SHC to RET using an *in vitro* pull-down assay. GST-fusion protein with the PTB domain of DOK-1, DOK-4, DOK-6, or SHC, which is the binding domain with RET, was produced in *E. coli* and immobilized to glutathione-sepharose beads. The beads were incubated with the cell lysate of MC-RET-2A, and the bound proteins on the beads were subjected to western blotting using anti-RET antibody. As shown in Figure 5(A), the binding affinity of the DOK-6 PTB domain to RET-MEN2A protein was much lower than that of the DOK-1, DOK-4, or SHC PTB domain. The fact that SHC is highly expressed in human neuroblastoma cells[31] and both SHC and DOK-6 bind to phosphorylated tyrosine 1062 (pTyr 1062) in RET suggests that the SHC PTB domain strongly competes with the binding of the DOK-6 PTB domain to RET.

To explain the low binding affinity of the DOK-6 PTB domain to RET, we introduced computer simulations and

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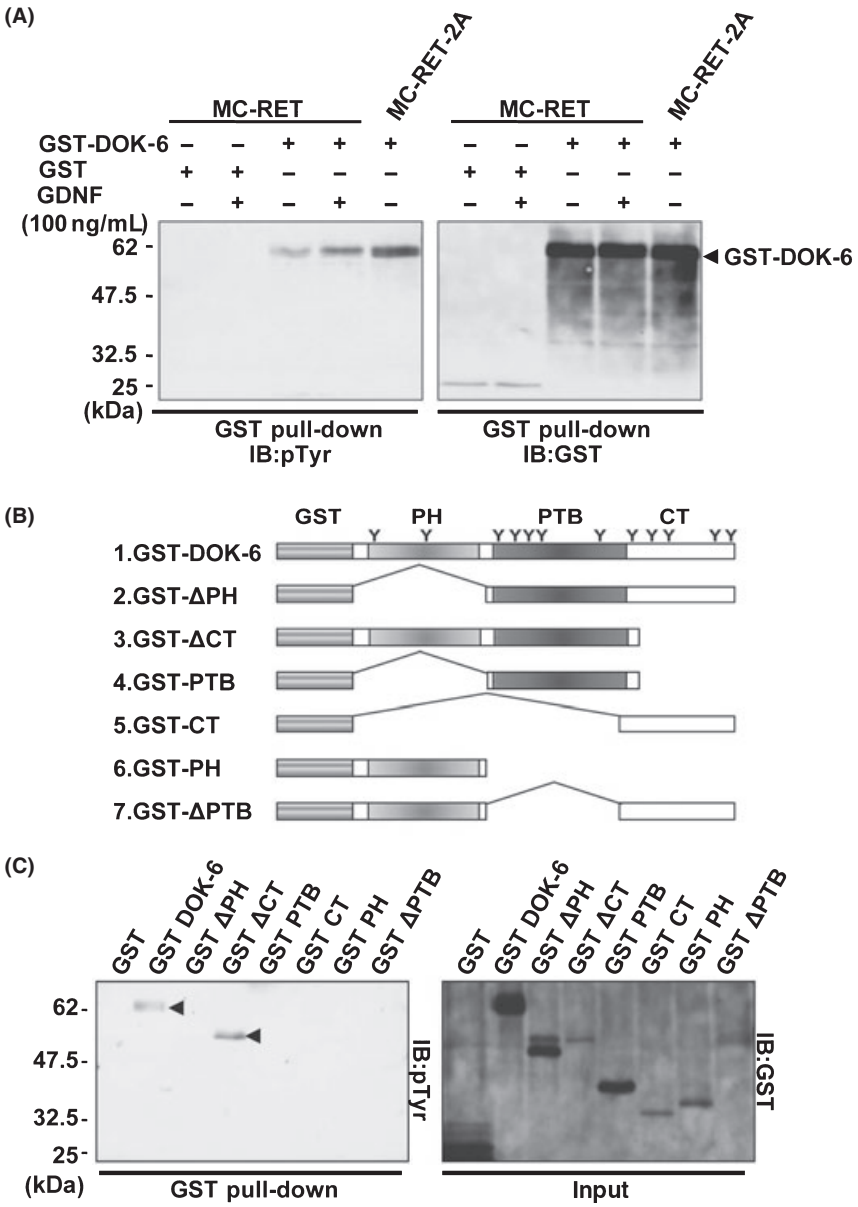

Fig. 3. Both the pleckstrin homology (PH) and phosphotyrosine-binding (PTB) domains are required for DOK-6 phosphorylation in glial cell line-derived neurotrophic factor (GDNF)–RET signaling. (A) MC-RET or MC-RET-2A cells were transfected with the expression vector for GST-DOK-6 or GST alone and then stimulated with GDNF (100 ng/mL) for 10 min as indicated. Their cell lysates were subjected to DOK-6 phosphorylation analysis as described in the Materials and Methods. The left panel shows the blot probed with anti-pTyr antibody, and the right panel shows the blot probed with anti-GST antibody. (B) GST-fused DOK-6 and truncated DOK-6 proteins used in this experiment are illustrated. (C) MC-RET-2A cells were transfected with the expression vector for GST alone, GST-DOK-6 or each GST-DOK-6 truncation mutant, and their cell lysates were subjected to DOK-6 phosphorylation analysis. The left panel is the blot probed with anti-pTyr antibody, and the right panel is the blot probed with anti-GST antibody.

compared the interactions between the RET long isoform and the PTB domain of mouse DOK-1, human DOK-1, human DOK-4, or human DOK-6. The simulations indicated that the interaction energy between RET and the DOK-6 PTB domain is significantly higher than that between RET and the human or mouse DOK-1 PTB domain or the human DOK-4 PTB domain (Table 1), consistent with the *in vitro* pull-down assay demonstrating the low affinity of the DOK-6 PTB domain to RET. Structural or mutational analyses previously reported revealed that pTyr 1062 of RET interacts with DOK-1 PTB domain via arginine (R) 207 and R222 of DOK-1.[27,34] Those arginine residues are also conserved in the DOK-6 PTB domain, but the simulated structural models demonstrated that the interactions of those arginine residues to the pTyr 1062 of RET were weaker in the DOK-6 PTB domain than in the DOK-1 counterpart (Fig. 5B). Simulations also displayed weaker interaction of the DOK-6 PTB domain to RET asparagine (N) 1059 (data not shown). Those structural findings are compatible with the results of calculated interaction energies.

Discussion

To date, seven members of the DOK family of proteins, DOK-1–7, have been identified and shown to serve as substrates of various protein tyrosine kinases, including ABL, SRC, BCR, TIE2, KIT, platelet-derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR), muscle-specific tyrosine kinase (MuSK), and RET.[18,19,22–24,27–30,35–40] DOK-1–3 are preferentially expressed in hematopoietic tissues and act as negative regulators of RAS/ERK signaling. On the other hand, DOK-4–6 are mainly expressed in non-hematopoietic tissues such as the nervous system and embryonic kidneys, and act as positive or negative regulators of RAS/ERK signaling.[20–23,27–30,37,41] DOK-7 is expressed in skeletal muscle and functions as an activator of MuSK.[24,42] Among these seven DOK family proteins, DOK-1, -4, -5, and -6 have been reported to be involved in the GDNF–RET signaling pathway.[22,23,27,29,30] In the present study, we investigated DOK-6 function in GDNF–RET signaling using human neuroblastoma cells. Semi-quantitative RT–PCR revealed that the expression of

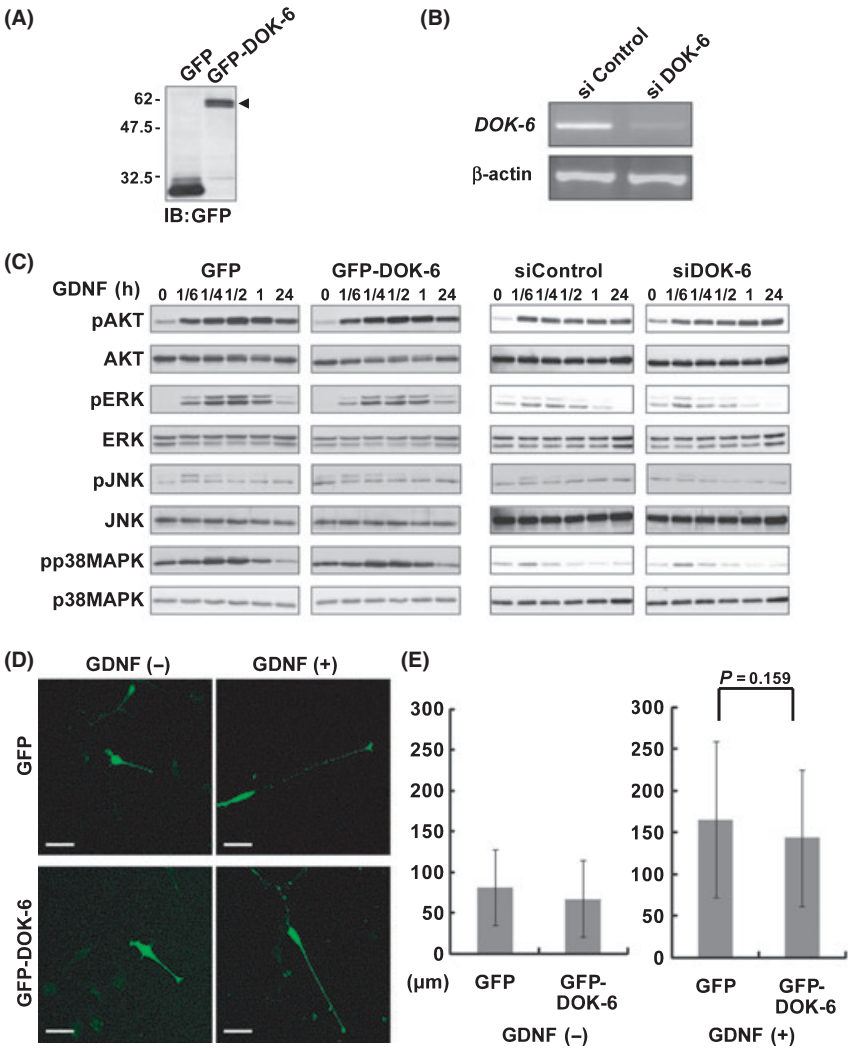

Fig. 4. DOK-6 does not significantly affect signaling downstream of RET in human neuroblastoma cells. (A) Western blot image for exogenous GFP and GFP-DOK-6 expression in MC-RET cells probed with anti-GFP antibody. (B) RT–PCR image for siRNA-mediated *DOK-6* depletion in MC-RET cells. RT–PCR amplification of β-actin was used as an internal control for the amount of RNA. (C) Time course analysis of phosphorylation of signaling molecules downstream of RET in MC-RET cells with GFP or GFP-DOK-6 expression (left panel), or MC-RET cells with *DOK-6* depletion (right panel). Twenty-four hours post-transfection, cells were stimulated by GDNF (10 ng/mL) for the indicated period and then lysed for Western blot analysis. (D) Fluorescent cell images of TGW cells with GFP or GFP-DOK-6 expression with or without GDNF (100 ng/mL) stimulation. (E) Neurite outgrowth of TGW cells with GFP or GFP-DOK-6 expression was assessed with or without glial cell-line derived neurotrophic factor (GDNF) (100 ng/mL) stimulation as described in the Materials and Methods.

DOK-6 was relatively high in several human neuroblastoma cell lines and the HEK293 cell line derived from fetal kidney, suggesting that it plays roles in neuronal cells and kidneys. Consistent with this view, *in situ* hybridization on mouse organs showed that *DOK-6* expression was detected in the developing neocortex, diencephalon, spinal cord, cranial parasympathetic, sensory and sympathetic ganglia, facial motor nucleus, and ureteric bud in the kidney.[23]

We also found that GFP-fused full-length DOK-6 localized to the plasma membrane irrespective of GDNF stimulation, while PH domain-deleted mutants of DOK-6 lost this characteristic and demonstrated diffuse cytosolic distribution in SK-N-MC neuroblastoma cells. This finding indicated that DOK-6 constitutively localized to the plasma membrane via its PH domain, although it has been reported that DOK-4 requires both the PH and PTB domains for its localization to the plasma membrane.[29,43] We confirmed that DOK-6 could undergo phosphorylation by GDNF stimulation and by a constitutively active RET mutant as observed for DOK-4, suggesting that DOK-6 functions downstream of RET. In addition, our studies with deletion mutants of DOK-6 showed that the ΔCT mutant of DOK-6 containing amino acids 1–233, but no other deletion mutants, was tyrosine phosphorylated in MC-RET-2A cells, indicating that both the PH and PTB domains were required for DOK-6 phosphorylation. This suggests that membrane localization of DOK-6 via the PH domain as well as DOK-6 binding to signaling molecules via the PTB domain are necessary for its phosphorylation. Crowder *et al.* reported that DOK-6 is phosphorylated by SRC kinase downstream of RET.[23] However, they also reported that the ΔCT mutant of DOK-6 containing amino acids 1–260 was not phosphorylated by GDNF stimulation in mouse Neuro2A neuroblastoma cells. The discrepancy may be due to the use of MC-RET-2A cells expressing active RET mutant in our experiments.

It has been reported that DOK-4, -5, and -6 promote growth factor-dependent neurite outgrowth in PC12, Neuro2A, or TGW cells, and that DOK-4 induces a sustained activation of ERK in TGW cells.[22,23,30] In the present study overexpression of DOK-6 or siRNA-mediated knockdown of *DOK-6* did not significantly alter GDNF-mediated neurite outgrowth in TGW cells. Consistent with this finding, phosphorylation levels of ERK, AKT, JNK, and p38MAPK induced by GDNF in MC-RET cells were not significantly affected by overexpression or siRNA-mediated knockdown of DOK-6. Since Crowder *et al.*[23] used Neuro2A cells overexpressing GDNF family receptor α1 (GFR α1) to show promotion of neurite outgrowth by DOK-6, the expression level of GFR α1 may affect DOK-6-mediated biological response in neuroblastoma cells.

Interaction between specific phosphorylated tyrosines of RTK and the PTB domain of DOK proteins are required for downstream signaling. We compared the binding affinity of the

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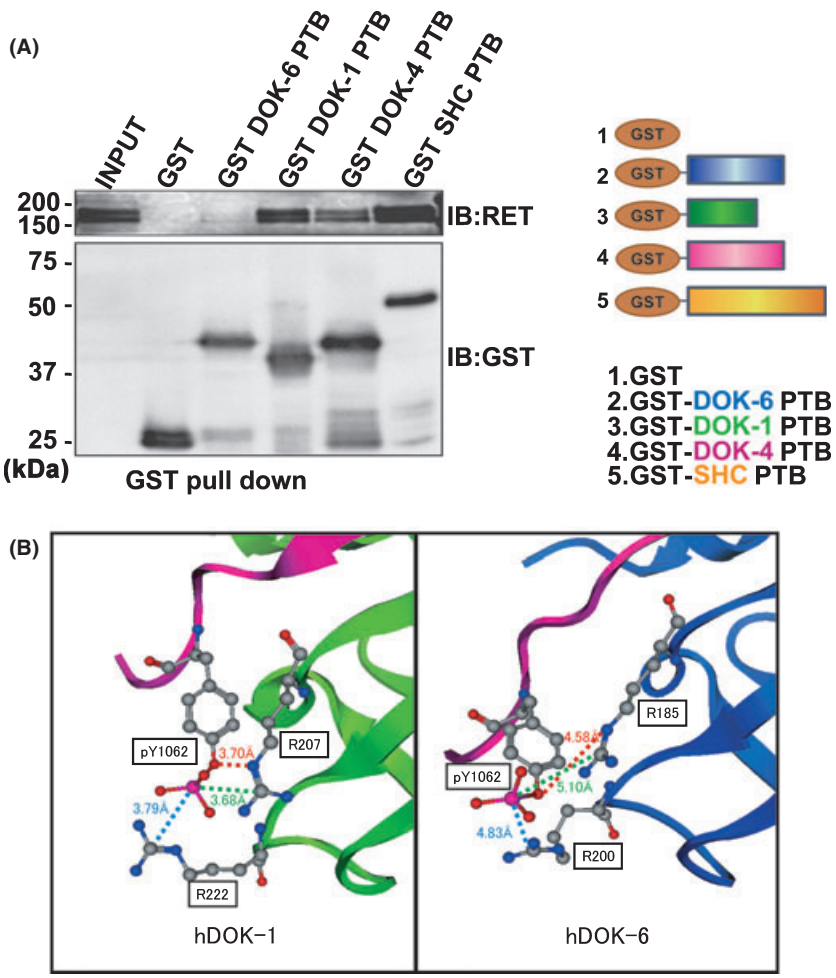

Fig. 5. The binding ability of phosphotyrosine-binding (PTB) domain of DOK-6 with RET. (A) *In vitro* pull-down assay with the GST-fused PTB domains of DOK-6, DOK-1, DOK-4, and SHC was performed using the MC-RET-2A cell lysate. The bound proteins on the glutathione beads were subjected to western blotting with anti-RET antibody (upper panel) and anti-GST (lower panel) antibody. (B) Interaction models between the amino acid sequence surrounding pTyr 1062 of the RET long isoform (pink ribbon) and the PTB domain of DOK-1 (left, green ribbon) or DOK-6 (right, blue ribbon). The protocols of the simulations are described in the Materials and Methods. The atomic distance between pTyr 1062 of RET and interacting arginines of the DOK-1 PTB domain is closer than that of the DOK-6 counterpart, compatible with the difference of interaction energies shown in Table 1.

DOK-6 PTB domain to RET-MEN2A proteins with the binding affinity of the PTB domains of DOK-1, DOK-4, and SHC by GST pull-down assay. Interestingly, the DOK-6 PTB domain has less potential to bind to RET-MEN2A when compared with DOK-1, DOK-4, and SHC. Because SHC is expressed in human neuroblastoma cells at high levels, our finding suggests that the SHC PTB domain strongly competes with the DOK-6 PTB domain for binding to RET. Computer simulations of docking models and interaction energies also displayed the results consistent with the *in vitro* pull-down assay demonstrating the low binding affinity of DOK-6 to RET.

PTB domains tend to fall into two groups: (1) PTB domains that have primary sequence similarity to the SHC PTB domain; and (2) insulin receptor substrate 1 (IRS1)-like PTB domains (PTBi) that have sequence homology to the IRS1 PTB domain.[44] The latter group includes docking proteins such as IRS, DOK, and SNT/FRS2 families. Despite a lack of significant sequence homology between SHC PTB and PTBi domains, the overall structures are similar between these two groups.[44] Our simulation of the SHC PTB domain reported previously indicated that pTyr 1062 of RET interacts with arginine 67 (R67) of the SHC PTB domain.[45] The DOK-1 PTB domain also utilizes arginine residues for pTyr recognition, such that pTyr 1062 of RET interacts with the DOK-1 PTBi domain via R207 and R222.[34] Those arginine residues are conserved in all PTBi domains including IRS, DOK, and the SNT/FRS2 family of proteins.[34] According to a previous publication, structure-based mutational analyses revealed that the DOK-1 PTBi domain specifically interacts with EGFR, but the PTBi domain of DOK-4 or DOK-5 does not.[40] In our present study, the lower binding affinity of the DOK-6 PTBi domain to RET when compared with its DOK-1 counterpart was suggested using simulations of docking models and semi-quantitative interaction energies.

Table 1. Interaction energies between the amino acid sequence surrounding pTyr 1062 of the RET long isoform and the phosphotyrosine-binding (PTB) domain of mouse DOK-1, human DOK-1, human DOK-4, or human DOK-6

	Interaction energies (kcal)
Mouse DOK-1	719.541
Human DOK-1	695.694
Human DOK-4	1464.761
Human DOK-6	2466.893

The methods of calculations are described in the Materials and Methods.

In conclusion, we have investigated molecular roles of the DOK-6 adaptor protein in the GDNF–RET signaling pathway using neuroblastoma cells. We found that although DOK-6 is a plasma membrane protein phosphorylated by GDNF–RET signaling, it has less potential to bind to RET compared with DOK-1, DOK-4, and SHC, and does not significantly affect downstream signaling. These findings suggest that DOK-6 may function in a different manner from other DOK proteins in the GDNF–RET signaling pathway. Further analysis including

identification of binding proteins of DOK-6 is necessary to clarify the roles of DOK-6 in RTK signaling pathways.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Intracellular localization of DOK-6 in SK-N-MC cells. GFP, GFP-DOK-6, and GFP-DOK-6 truncation mutants expressed in SK-N-MC cells were visualized by confocal fluorescent microscopy.

Fig. S2. Myc-tagged DOK-6 phosphorylation. MC-RET-2A cells were transfected with the expression vector for myc alone or DOK-6-myc. Twenty-four hours after transfection, their total cell lysates were subjected to western blot analysis. The left panel shows the blot probed with anti-pTyr antibody, and the right panel shows the blot probed with anti-myc antibody.

Fig. S3. DOK-4 phosphorylation in glial cell line-derived neurotrophic factor (GDNF)–RET signaling. MC-RET or MC-RET-2A cells were transfected with the expression vector for GST-DOK-4 or GST alone and then stimulated with GDNF (100 ng/mL) for 10 min as indicated. Their cell lysates were subjected to DOK-4 phosphorylation analysis as described in the Materials and Methods. The left panel shows the blot probed with anti-pTyr antibody, and the right panel shows the blot probed with anti-GST antibody.

Fig. S4. DOK-4 but not DOK-6 enhances ERK phosphorylation downstream of RET in human neuroblastoma cells. (A,C) Western blot analyses for GST, GST-DOK-4, and GST-DOK-6 expression in MC-RET cells (A) or SK-N-SH human neuroblastoma cells (C). Protein expression was detected with anti-GST antibody. (B,D) Time course analysis of ERK phosphorylation induced by glial cell-line derived neurotrophic factor (GDNF) in MC-RET cells (B) or SK-N-SH cells (D) expressing GST, GST-DOK-6, or GST-DOK-4. Twenty-four hours after transfection, cells were stimulated by GDNF (10 ng/mL) for the indicated times and their lysates were subjected to western blot analysis with anti-ERK or anti-phosphoERK antibody.

Fig. S5. DOK-6 does not significantly affect neurite outgrowth by glial cell line-derived neurotrophic factor (GDNF) in human neuroblastoma cells. (A) SiRNA-mediated *DOK-6* depletion in TGW cells. RT–PCR amplification of β-actin was used as an internal control for the amount of RNA. (B) TGW cells were transfected with siControl or siDOK-6. Twenty-four hours after transfection, cells were serum starved, then stimulated with GDNF (100 ng/mL) for 24 h. Cells were fixed and the images were captured with Olympus IX70 microscope (Olympus, Tokyo, Japan). For measurement of the neurite length, the entire length of the process was traced and the total length was calculated and analyzed by the software program WinROOF (Mitani, Fukui, Japan). At least 100 TGW cells were evaluated in each culture.