Dok-6, a Novel p62 Dok Family Member, Promotes Ret-mediated Neurite Outgrowth*

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Activation of Ret, the receptor-tyrosine kinase for the glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs), results in the recruitment and assembly of adaptor protein complexes that function to transduce signals downstream of the receptor. Here we identify Dok-6, a novel member of the Dok-4/5 subclass of the p62 Dok family of intracellular adaptor molecules, and characterize its interaction with Ret. Expression analysis reveals that Dok-6 is highly expressed in the developing central nervous system and is co-expressed with Ret in several locations, including sympathetic, sensory, and parasympathetic ganglia, as well as in the ureteric buds of the developing kidneys. Pull-down assays using the Dok-6 phosphotyrosine binding (PTB) domain and GDNF-activated Ret indicate that Dok-6 binds to the phosphorylated Ret Tyr1062 residue. Moreover, ligand activation of Ret resulted in phosphorylation of tyrosine residue(s) located within the unique C terminus of Dok-6 predominantly through a Src-dependent mechanism, indicating that Dok-6 is a substrate of the Ret-Src signaling pathway. Interestingly, expression of Dok-6 potentiated GDNF-induced neurite outgrowth in GDNF family receptor α1 (GFRα1)-expressing Neuro2A cells that was dependent upon the C-terminal residues of Dok-6. Taken together, these data identify Dok-6 as a novel Dok-4/5-related adaptor molecule that may function in vivo to transduce signals that regulate Ret-mediated processes such as axonal projection.

Neurotrophic factors influence the development of the nervous system by regulating neuronal size, number, phenotype, and spatial organization. Many neurotrophic factors function by activating receptor-tyrosine kinases (RTKs) via ligand-dependent receptor dimerization and in trans autophosphorylation.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY599248.

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‡ The abbreviations used are: RTK, receptor-tyrosine kinase; GDNF, glial cell line-derived neurotrophic factor; GFL, GDNF family ligand; GFRA, GDNF family receptor α; SH2, Src homology 2; PTB, phosphotyrosine binding; PH, pleckstrin homology; HA, hemagglutinin; FACS, fluorescence-activated cell sorting; GST, glutathione S-transferase; MAP, mitogen-activated protein; EST, expressed sequence tag; RT, reverse transcriptase; FBS, fetal bovine serum.

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ucleus were originally identified as substrates of RTK and non-receptor-tyrosine kinase signaling cascades (27, 28). All Dok family members (Dok-1–5) characterized to date are modular docking proteins consisting of an N-terminal pleckstrin homology (PH) domain, a central PTB domain, and a variable C-terminal region (20, 27–30). The PH and PTB domains enable Dok proteins to couple to tyrosine kinases and target them to the plasma membrane, whereas the unique C terminus contain docking sites for effectors that act to transduce signals. Several reports indicate that Dok-1–3 act as negative regulators of tyrosine kinase-activated signaling pathways (30–32). Dok-1 and Dok-2 may exert these effects through the recruitment of RasGAP, a negative regulator of Ras, whereas Dok-3 acts as a negative regulator of signaling independent of RasGAP (30, 33). Dok-4 and Dok-5 were recently identified as targets of Ret and the insulin receptor (20, 34). In contrast to Dok-1–3, Dok-4 and Dok-5 positively regulate RTK signaling pathways (20, 34). The physiologic processes regulated by Dok proteins remain poorly understood. Dok-1 and Dok-2 are predominantly expressed in tissues of hematopoietic origin and are believed to regulate processes such as immunoreceptor signaling and lymphoid cell phenotype in vivo (31, 35–37). Dok-4 and Dok-5 are highly expressed in non-hematopoietic tissues, in particular, the nervous system. Although no genetic models have yet been developed to understand the function of the Dok-4/5 subfamily of Dok proteins in vivo, ectopic expression of Dok-4 and Dok-5 have been shown to promote Ret-mediated neurite outgrowth in vitro (20).

In this study, we identify and characterize Dok-6, a novel member of the Dok-4/5 subclade of the p62Dok family that can associate with Ret and serves as a substrate of the Ret signaling cascade. We also show that Dok-6 expression in vivo overlaps significantly with that of Ret and that Dok-6 can function to potentiate Ret-mediated neurite outgrowth in vitro.

**EXPERIMENTAL PROCEDURES**

**Identification, Cloning, and Sequence Analysis of Dok-6**—Sequence analysis and cloning was performed essentially as described (38). The nucleotide sequence of mouse Dok-4 (GenBank™ accession number, AF148207) and mouse Dok-5 (GenBank™ accession number AF148208) were used in a BLAST (39) query to identify an EST clone (accession number B1546276) in the dbEST data base corresponding to the 5′-end of a novel human gene of the p62Dok family. This clone was obtained through the I.M.A.G.E. Consortium from Invitrogen (Carlsbad, CA) and sequenced in its entirety in both directions. This EST clone contained the complete cDNA sequence of a novel gene, Dok-6, which has been submitted for review (GenBank™ accession number AT3599248). The open reading frame of Dok-6 was PCR-amplified, cloned into an EcoRV site of pBluescript KS (Stratagene, La Jolla, CA), and total RNA sequences of pDNA3.1 (Invitrogen). A C-terminal hemagglutinin (HA) tag was added to the wild-type Dok-6 cDNA in pDNA3.1 via PCR. An expression vector for a C-terminally deleted Dok-6 mutant (Dok-6ΔC) encoding amino acid residues 1–280 of Dok-6 was similarly constructed using standard PCR techniques. All Dok-6 cDNA constructs were confirmed by sequencing. The chromosomal localization and the deduced genomic organization of Dok-6 gene were determined with the aid of the Ensembl Genome Browser (www.ensembl.org/) by using the complete Dok-6 cDNA sequence as a query against the human genome.

**Quantitative RT-PCR Analysis and In Situ Hybridization**—For quantitative RT-PCR analysis, commercial human total RNAs (Clontech Laboratories, Inc., Palo Alto, CA) and accession numbers human TR3α1 (Invitrogen) extraction from the indicated cell lines were converted to cDNA essentially as described (40). Expression levels of genes were measured by real-time PCR and normalized to 18 S RNA as previously described (41). The partial, complete, and predicted coding sequences used in designing gene and species-specific PCR primers were derived from the following GenBank™ accession numbers: human Dok-5, AF466368; human Dok-4, AF466369; mouse Dok-6, XM_140539; mouse Dok-5, AF418208; mouse Dok-4, AF418207; rat Dok-6, XM_225633. Primer sequences used for the quantitative analysis of each gene are available upon request. For in situ hybridization, digoxigenin-labeled antisense riboprobes corresponding to nucleotides 738–836 of the open reading frame of Dok-6 cDNA were synthesized using T3 RNA polymerase. Fresh frozen sections (30 μm) obtained from E13.5 and E14.5 mouse embryos were incubated with the antisense riboprobe and processed for the detection of the specific Dok-6 signal as previously described (6, 42). No specific hybridization patterns were observed using the corresponding sense riboprobes.

**Plasmid Expression Vectors**—The expression plasmid for the Ret9 isoform was described previously (43). The mouse Dok-5 cDNA was amplified by PCR from mouse brain cDNA using primers to incorporate sequences corresponding to the HA tag at the 3′-end and cloned into the pcDNA3.1 expression vector. A cytomegalovirus promoter-driven expression plasmid mouse Dok-2 cDNA (accession number BC040590) was obtained from ATCC (Manassas, VA) and modified by PCR to contain the HA tag at the 3′-end of the cDNA insert. The sequences of all cDNA constructs were confirmed by DNA sequencing.

**Lentivirus Production**—Lentiviruses were produced essentially as described (44) using a modified transfer vector containing a bicistronic element that permitted expression of both the gene of interest and the yellow fluorescent protein variant, Venus, as a reporter gene (45). The transfer vector, pFUHIV, was constructed by replacing the EGGP gene from FUGW with the IRES cassette from pIRES-EYFP (BD Biosciences Clontech) followed by the Venus gene. The gene of interest (wild-type or mutant Ret isoforms or Dok genes) was inserted into the transfer vector by excising the respective cDNA from plasmid vectors described here or previously (43) and cloned between the ubiquitin-C promoter and IRES sequences of pFUHIV. Lentiviral vectors were produced by cotransfecting the transfer vector pFUHIV containing the gene of interest, the VSVG envelope glycoprotein, and the HIV-1 packaging vector Δ8.9 into 293T cells, and concentrated by centrifugation as described (44, 46). Viral titers were determined by infecting 293T with serial dilutions of virus and evaluating Venus expression in cells by fluorescence microscopy 48–72 h after infection.

**Cell Lines and Transfection**—Neuro2A neuroblastoma cells stably expressing GFP-R1 were produced by transfecting Neuro2A cells with a FLAG and GFP-tagged GFP-R1 expression plasmid (47) in G418 (0.8 mg/ml) resistance. Antibiotic-resistant colonies were screened for GFP-R1 expression using the FLAG antibody (Sigma), and one GFP-R1–positive clone, N2A-s1, was propagated for subsequent use. Expression cassettes for C-terminal HA-tagged versions of Dok-5, Dok-6, or Dok-6ΔC, or an empty vector control were stably introduced into N2A-s1 cells using lentivirus infection. Following lentivirus infection, cells were grown for 1–2 weeks in medium as described (47, 48) and selected using fluorescence-activated cell sorting (FACS) against the Venus marker gene to obtain cells expressing the inserted Dok gene. N2A-s1 cells were seeded into Primaria 10-cm dishes (Falcon, BD Biosciences) at 3 × 10^6 cells/dish 1 day prior to transfection or use in GST pull-down assays. For transfection, expression vectors for the indicated genes were introduced using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer’s instructions, and cells were maintained for 48 h prior to lysis. For both assays, N2A-s1 cells were switched to media containing 0.5% FBS for 1–2 h, then treated with 30 ng/ml GDNF or left untreated for 30 min, lysed, and processed as described. For some experiments, cells were preincubated with the indicated pharmacological agents for 30 min prior to GDNF stimulation.

CHP126 human neuroblastoma cells, which express GFRα1 but are devoid of endogenous Ret expression (43), were used to create cell lines stably expressing wild-type or tyrosine to phenylalanine point mutants of Ret. CHP126 cells were infected with lentiviruses containing wild-type or mutant Ret isoforms grown for 1–2 weeks in growth medium (43), and selected by FACS against the Venus marker gene to obtain similar levels of Ret expression in all stable lines. For experiments involving CHP126-Ret stable cell lines, 4 × 10^6 cells were seeded on 10-cm dishes the day prior to the experiment. Cells were then switched to media containing 0.5% FBS for 2–3 h, treated with 30 ng/ml GDNF or left untreated for 30 min and then lysed and processed as described.

**Antibody Production**—Rabbit polyclonal antibodies to the HA tag sequence (YPYDVPDYA) were produced using the peptide CYPYDVPDYASL. Peptide synthesis, coupling to the keyhole limpet hemocyanin carrier protein, and injection of the immunogen into rabbits was performed by the Animal Biologics Service Center (ABSC, Frederick, MD). The resulting antiserum was tested for production of specific antibodies by enzyme-linked immunosorbent assay (ELISA) as previously described (49) using the immunizing peptide as the antigen. High titer antiserum was affinity-purified by binding antibodies to affinity columns produced by co-

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isopropyl /H9252 ° extracts were cleared of insoluble debris by centrifugation (5 min at rabbit polyclonal anti-HA tag antibodies produced as described (see pull-down assays, supernatants (buffer, eluted by boiling in sample buffer, resolved by SDS-PAGE, ° MM, and bacteria were grown an additional3ha t3 0

was diluted 1:1000; anti-HA, clone 12CA5 (Roche Applied Science) was

protocol; mouse anti-phosphotyrosine (P-Tyr-100; Cell Signaling Technol-

erol; 5 m M MgCl2, 1m M dithiothreitol, 1m M phenylmethylsulfonyl

mM Tris, pH 7.5, 150 m M NaCl, 10% glyc-

with purification buffer (50 mM Tris, pH 7.5, 150 m M NaCl, 1%

be expressed in the bacteriophage T7 expression system (New England

was used for immunoblot analyses were as follows: rabbit anti-

oratories, Inc., West Grove, PA), and processed to detect specific pro-

in TBST (0.1% Tween 20 in Tris-buffered saline), incubated with the

complete 5

and rat (GenBank accession numbers: AK057795, BC0199045, BG471399, BX272778, BX371689), but most contained incom-

sequent proteins (50

Gene Expression, Beverly, MA). The blots were quantified using an Epi Chem II

RESULTS

Identification of Dok-6, a Novel p62 Dok Family Member
Homologous to Dok-4/5—The nucleotide sequences of mouse

Dok-4 (GenBank™ accession number AF418207) and Dok-5 (GenBank™ accession number AF418208) were used as que-

ry to the dbEST data base by using the BLAST search algo-

905 (P-Ret) (43) was diluted 1:1000; phosphotyrosine specific Ret Tyr905 (P-Ret) (43) was diluted 1:1000; one of the human EST sequences identified (I.M-

A.G.E. clone 5260167, accession number B156267) corresponded to a gene that contained significant homology to the 5'-ends of both the Dok-4 and Dok-5 cDNAs, but did not match exactly to any known Dok family member. This I.M-

A.G.E. clone was obtained, sequenced in its entirety, and found to contain the full-length cDNA of a novel p62 Dok family gene, which we have named Dok-6 (GenBank™ accession number AY599248). Using the Dok-6 cDNA as a query, we identified several additional human clones corresponding to the Dok-6 gene (GenBank™ accession numbers: AK057795, 38.3 kDa protein consisting of features common to other Dok family members: an N-terminal PH domain, a central PTB domain, and a unique C-terminal region (Fig. 1A) (20, 27, 29, 30).

Alignment of Dok-6 with Dok-4 and Dok-5 illustrates the high degree of similarity present between these three proteins (Fig. 1B). Dok-6 is ~75 and 78% identical to Dok-4 and Dok-5, respectively, over the regions comprising the PH and PTB domains. In addition to these highly similar regions, Dok-6 shares a short region of homology to Dok-4 and Dok-5 in their otherwise divergent C termini (Fig. 1B). This motif, LPRESAY-WHIT in Dok-6 (where Y is Tyr200), was previously identified in Dok-4 and Dok-5 (34) and may indicate a conserved function or mode of regulation for this subclass of Dok proteins. Most Dok family proteins contain tyrosine residues in their C-terminal regions that lie within recognizable consensus binding sites for SH2 domains and that act as sites for coupling to effector proteins. We attempted to identify specific proteins or signal transduction cascades that the Dok-4/5/6 subfamily may couple to by using this conserved motif as a data base search query (data not shown). However, we were unable to identify any known, functionally characterized genes that may provide in-

one of the most highly conserved regions of the Dok family of proteins, the PH domain. The PH domain of Dok-5 contains five of the six tyrosine residues throughout the phosphotyrosine specific Ret Tyr905 (P-Ret) (43) was diluted 1:1000; rabbit polyclonal antibody against the extracellular domain of Ret (43) was diluted 1:1000; anti-HA, clone 12CA5 (Roche Applied Science) was diluted 1:1000 following reconstitution according to manufacturer’s protocol; mouse anti-phosphotyrosine (P-Tyr-100; Cell Signaling Technol-
We used the Dok-6 cDNA as a query to identify the chromosomal location of the human DOK-6 gene and deduced the exon-intron boundaries within the Dok-6 coding region. DOK-6 was localized to 18q22.2 and found to encompass 0.44 Mb of chromosomal DNA. The DOK-6 gene structure is shown in Fig. 2. DOK-6 consists of eight exons, analogous to the genomic structure recently reported for DOK-4 and DOK-5, and has an identical intron "phase" to human DOK-5 as defined by Favre et al. (53).

Dok-6 Expression Significantly Overlaps with That of Ret—To gain insight into potential functions of Dok-6, we characterized the tissue expression pattern of Dok-6 and those of Dok-4 and Dok-5 using quantitative (q) RT-PCR (Fig. 3 and data not shown). Dok-6 is highly expressed in the central nervous system, in particular, the fetal brain (Fig. 3). The highest level of expression of Dok-6 observed outside the central nervous system occurs in the kidney and testis. Interestingly, Ret function is required for embryonic kidney development and plays a critical role postnatally during spermatogenesis (5). The tissue expression pattern of Dok-6 most closely resembled that of Dok-5. However, unlike Dok-5, Dok-6 is not highly expressed in skeletal muscle and the heart (Refs. 34 and 53 and data not shown). In contrast to Dok-5 and Dok-6, Dok-4 is expressed broadly in variety of tissues (20,34,53 and data not shown). In addition to characterizing Dok-6 expression in tissues, we evaluated its expression in several human neuroblastoma cell lines. We detected Dok-6 expression in several Ret-positive cell lines (NBL-S, SK-N-SH, SH-SYS5Y) as well as Ret-negative cell lines (data not shown). Dok-6 expression in the developing kidney is localized to the ureteric buds, the same site where Ret is expressed during kidney development (54). In contrast, Dok-4 expression occurs in the kidney endothelium during development (20). The site of Dok-5 expression in the kidney is unknown. In addition to the central nervous system and kidney, Dok-6 is also expressed in cranial parasympathetic (sphenopalatine), sensory (trigeminal, nodose, dorsal root), and sympathetic ganglia, and in the facial motor nucleus (Fig. 4, A and B). Intense Dok-6 expression was detected in the brain in the developing neocortex and diencephalon, and in the spinal cord (Fig. 4, A and B). Interestingly, Dok-6 expression in the developing kidney is localized to the ureteric buds, the same site where Ret is expressed during kidney development (54). In contrast, Dok-4 expression occurs in the kidney endothelium during development (20). The site of Dok-5 expression in the kidney is unknown. In addition to the central nervous system and kidney, Dok-6 is also expressed in cranial parasympathetic (sphenopalatine), sensory (trigeminal, nodose, dorsal root), and sympathetic ganglia, and in the facial motor nucleus (Fig. 4, A, C, and D.). Importantly, the expression of Dok-6 during development overlaps with Ret in more locations than any previously characterized p62 Dok family member (3, 20, 27, 29, 30).

Dok-6 Binds to the Ret Receptor Complex via Ret Tyr 1062—The PTB domains of Dok-1, Dok-4, and Dok-5 associate with Ret through interactions involving the phosphorylated Ret (sphenopalatine), sensory (trigeminal, nodose, dorsal root), and sympathetic ganglia, and in the facial motor nucleus (Fig. 4, A, C, and D.). Importantly, the expression of Dok-6 during development overlaps with Ret in more locations than any previously characterized p62 Dok family member (3, 20, 27, 29, 30).

Dok-6 Is a Substrate of Ret

**Fig. 1. Sequence analysis and alignment of Dok-6.** A, deduced amino acid sequence of human Dok-6. Dashed underlined region corresponds to the PH domain of Dok-6; solid underlined region denotes the PTB domain. B, alignment of the amino acid sequences of the human Dok-4, Dok-5, and Dok-6 proteins. Identical residues are boxed. The shared motif present in the divergent C termini of Dok-4, Dok-5, and Dok-6 is shaded.

| Donor | INTRON | Acceptor |
|-------|--------|----------|
| Exon 1 | CTG | GGTAGCTGCTCCAG | ATT TAC CCA GGA TGA |
| Exon 2 | CAT | GGAAGCAGAGTCCACAG | GTA ACT Val Thr |
| Exon 3 | GAG | TCAAGTCTTGAGAGT | AGG CTG GAG |
| Exon 4 | CAT | GAG | AGA ACT TCC |
| Exon 5 | TCA | GAA | AGA GTG |
| Exon 6 | GCC | GGG | CTG |
| Exon 7 | TIG | CAA | CAT CAG |

**Fig. 2. Genomic analysis of human Dok-6.** A, intron-exon junctions in the human Dok-6 gene. Exons are represented by boxes and are scaled to size. Intron (connecting lines) are not to scale. Numbers inside the boxes indicate the number of nucleotides within the coding sequence for each respective exon. B, intron structure of human Dok-6 gene.
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Fig. 3. Dok-6 expression in human tissues. Dok-6 expression was examined in human tissues by quantitative RT-PCR using human Dok-6-specific primers and normalized against 18S rRNA. Fold expression levels are relative to that of Dok-6 in the prostate, which is arbitrarily set to an expression level of 1. The graphed values represent the mean ± S.E. relative expression levels calculated from three independent experiments. Sm. Int., small intestine; Sk. Muscle, skeletal muscle; Mam. Gland, mammary gland.

Fig. 4. Expression of Dok-6 in mouse embryos. In situ hybridization of E13.5 and E14.5 mouse embryonic tissues using a Dok-6 riboprobe. Panel A, transverse section through abdominal region. Dok-6 expression was detected in the spinal cord (sc), dorsal root ganglia (drg), sympathetic chain paraganglia (sp), and in the ureteric buds of the developing kidneys (ub). Panel B, transverse section through the developing brain showing Dok-6 expression in the neocortex (nc), diencephalon (de), and vestibulocochlear ganglion (vc). Panel C, parasagittal section in the head and neck region. Note that Dok-6 is expressed in locations also known to express Ret, including the trigeminal ganglion (tg), nodose ganglion (ng), superior cervical ganglion (scg), sphenopalatine ganglion (spg), and the facial motor nucleus (fmn). Panel D, parasagittal section through the thoracic region showing Dok-6 expression in the dorsal root ganglia (drg) and sympathetic chain ganglia (sg).

Tyr\textsuperscript{1062} residue, the cognate PTB binding site in Ret (20, 21, 55). We investigated whether Dok-6 interacts with the Ret receptor complex by utilizing a pull-down assay with GST fusion proteins containing the Dok-6 PTB domain or the Dok-5 PTB domain as a control. The GST fusion proteins were incubated with lysates containing endogenous Ret from unstimulated or GDNF-stimulated Neuro2A cells stably expressing GFR\textalpha\textsubscript{1} (N2A-\textalpha\textsubscript{1}). Protein complexes associated with the GST fusion proteins were then probed via Western blotting using anti-Ret and anti-phosphotyrosine antibodies (Fig. 5A). The results from these experiments demonstrate that the Dok-6 PTB domain interacts with the Ret receptor complex in a phosphorylation-dependent manner. As expected, the Dok-5 PTB domain also bound to phosphorylated Ret. Probing the protein complexes with anti-phosphotyrosine antibodies revealed that Ret was the predominant phosphoprotein in these cellular lysates that interacted with the Dok-6 PTB domain (Fig. 5A, second panel from top).

Ret is alternatively spliced after sequences that encode the Tyr\textsuperscript{1062} residue to give rise to at least two Ret isoforms, Ret\textalpha\textsubscript{9} and Ret\textalpha\textsubscript{51}, that are believed to perform different functions \textit{in vivo} (43, 47, 56, 57). In a previous study, we demonstrated that Ret\textalpha\textsubscript{9} and Ret\textalpha\textsubscript{51} are differentially phosphorylated at key tyrosine residues, including Tyr\textsuperscript{1062}, and that the protein complexes associated with each Ret isoform are markedly different (43). To test whether the Ret-Dok-6 interaction exhibits Ret isoform selectivity and involves the Ret Tyr\textsuperscript{1062} residue, we performed pull-down assays using the Dok-6 PTB GST fusion protein with lysates from CHP128 neuroblastoma cells stably expressing either wild-type Ret isoforms or isoforms containing tyrosine 1062 (Y) to phenylalanine (F) point mutations (Y1062F) (Fig. 5B). We observed no consistent difference in the association of the Dok-6 PTB domain between isoforms. In addition, Tyr\textsuperscript{1062} was essential for phosphorylation-dependent association of the Dok-6 PTB domain for each Ret isoform.

Ret Phosphorylates Tyrosine Residues within the C Terminal of Dok-6 via Src—All previously characterized p62Dok family members are known to serve as substrates for non-receptor tyrosine kinases and RTKs, including Ret (20, 21). To determine if Dok-6 can function as a substrate for the Ret signaling cascade, we transfected Dok-6 into N2A-\textalpha\textsubscript{1} cells. Activation of Ret by GDNF stimulation resulted in the tyrosine phosphorylation of Dok-6, indicating that Dok-6 is a substrate of Ret or a tyrosine kinase associated with the Ret receptor complex (Fig. 6A). Consistent with previous studies, Dok-5 was also tyrosine-phosphorylated upon Ret activation (20). Mutational analyses of Dok-1–3 revealed that the unique C termini of these proteins contain specific tyrosine residues that become phosphorylated by tyrosine kinases and serve as sites for coupling to effector molecules (21, 30, 35, 55, 58, 59). To determine if Dok-6 tyrosine phosphorylation sites reside within the Dok-6 C terminus, we introduced either Dok-6 or a C-terminally truncated Dok-6 mutant, Dok-6\textalpha\textsubscript{C}, into N2A-\textalpha\textsubscript{1} cells (Fig. 6B). In contrast to full-length Dok-6, Dok-6\textalpha\textsubscript{C} was not tyrosine-phosphorylated upon Ret activation, indicating that Ret-dependent Dok-6 tyrosine phosphorylation occurred within the unique C terminus of Dok-6.

Previously, we demonstrated that the non-receptor tyrosine kinase Src is activated by Ret and that Src is a critical component of the Ret signaling cascade (47, 48). To determine if Dok-6 is a substrate of Ret-activated Src, we transfected Dok-6 into N2A-\textalpha\textsubscript{1} cells and measured the effect of two selective pharmacological inhibitors of Src family kinases, PP2 and SU6656 (60, 61), on Ret-dependent Dok-6 phosphorylation (Fig. 6C). Both Src inhibitors markedly reduced Ret-dependent Dok-6 phosphorylation whereas PP3, an inactive analog of PP2, did not. Together, these data provide compelling evidence that Ret phosphorylates tyrosine residue(s) lying within the C terminus of Dok-6 predominantly through a Src- or Src family kinase-dependent mechanism.

Expression of Dok-6 Potentiates Ret-dependent Neurite Outgrowth—Introduction of GFR\textalpha co-receptors into the Neuro2A neuroblastoma cell line permits GFL-induced Ret activation and subsequent neuronal differentiation (47, 48, 62). To determine whether Dok-6 may affect GFL-dependent neuronal dif-
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The p62Dok family of intracellular adaptor proteins can be divided into two subfamilies based upon sequence similarity, tissue expression patterns, and proposed physiologic functions. Members of the Dok-1–3 subfamily are predominantly expressed in hematopoietic tissues and in vitro studies have implicated these molecules in growth factor, cytokine, and immunoreceptor signaling pathways (27–31, 35). For Dok-1 and Dok-2, in vitro and in vivo experiments suggest that these molecules function to regulate processes such as proliferation and cell migration and may influence the acquisition of hematopoietic cell lineage phenotypes (32, 37, 64, 65). The two previously characterized members of the recently identified Dok-4/5 subfamily are more highly expressed in non-hematopoietic tissues such as muscle and in the nervous system (20, 34, 53). Although the physiologic functions of these family members are unclear, in vitro studies suggest that Dok-4 and Dok-5 may be involved in insulin receptor signaling and regulate cellular processes such as neurite outgrowth (20, 34).

In this study, we provide initial characterization of Dok-6, a new member of the Dok-4/5 subfamily of Dok adaptor proteins. Dok-6 displays a high degree of similarity to Dok-4 and Dok-5 over the regions comprising the PH and PTB domains and all three genes have a similar genomic structure (53). In addition, all members of this p62Dok subfamily contain a short conserved motif, LPRSAYWHHIT in Dok-6, which Ret is not thought to be required for proper development.

The expression of Dok-6 overlaps with that of Ret in several locations in the nervous system including sensory ganglia (trigeminal, dorsal root, node), cranial parasympathetic ganglia (sphenopalatine), sympathetic chain ganglia, and in the ureteric buds of the embryonic kidney. Gene-targeting studies involving the deletion of Ret, GFLs, or the GFRα co-receptors have established critical developmental and/or postnatal roles for the Ret signaling system in these locations (2–4). For example, ablation of Ret, GDNF, or GFRα1 results in dramatic developmental deficits, resulting in mild to severe losses in cell numbers in affected ganglia, aberrant cell migration, impaired axonal outgrowth and target innervation, and kidney agenesis (6, 42, 66–68). In contrast to the expression pattern reported here for Dok-6, Dok-4 and Dok-5 are potentially co-expressed with Ret in dorsal root, trigeminal, and geniculate ganglia and in the neural tube (20). Whereas Dok-4 expression does occur in the kidney, it appears to be restricted to the endothelia, not the ureteric buds where Ret and Dok-6 are expressed. Thus, while other Dok family members are co-expressed with Ret in some tissues, the expression pattern data presented here suggest that Dok-6 may participate in transducing signals through the Ret cascade in more locations than any previously characterized Dok protein. In addition, Dok-6 is expressed at moderate levels in the testis, raising the possibility that Dok-6 may cooperate with Ret in regulating spermatogonial cell fate and lineage determination. Dok-6 may also function in tissues in which Ret is not thought to be required for proper development.

DISCUSSION

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**Fig. 5.** Dok-6 binds to Ret via the phosphorylated Ret Tyr1062 residue. A. Dok-6 associates with ligand-activated Ret. Neuro2A cells stably transfected with an expression vector for GFRα1 (N2A-a1) were deprived of serum for 2 h, then treated with (+) or without (−) GDNF (30 ng/ml) for 30 min. Cleared cellular extracts (−1 mg of protein/assay) were subjected to pull-down assays using immobilized GST fusion proteins containing the PTB domains of Dok-5 (amino acid residues 110–240), Dok-6 (amino acid residues 110–240), or GST as a control. Cellular protein complexes bound to the fusion proteins were eluted and subjected to SDS-PAGE followed by immunoblotting with Ret and phosphotyrosine (P-Tyr) antibodies. B. Dok-6 PTB domain binding to Ret requires the Ret Tyr1062 residue. CHP126 cells stably expressing wild type Ret9 or Ret51 isoforms (Ret9 and Ret51, respectively), isoforms containing tyrosine 1062 to phenylalanine (Y1062F) point mutations, or a kinase inactive Ret51 (Ret51 K756M) were treated and used in pull-down assays as described in A. above. Wild-type CHP126 cells (CHP) do not express endogenous Ret. The experiments in A and B were performed three times each with similar results. P-Ret, phospho-Tyr905 Ret; IB, immunoblotting.
or maintenance. Dok-6 is robustly expressed in the embryonic brain and in the adult central nervous system, suggesting that Dok-6 may have roles in brain development and/or maintenance functions. Crucial functions for individual adaptor molecules in central nervous system development are not unprecedented. For example, targeted disruption of Disabled-1, a PTB domain containing adaptor protein involved in the Reelin signaling pathway, disturbs neuronal layering in the cerebral cortex, hippocampus, and cerebellum (69–72). Ablation of the Dok-6 gene will be necessary to delineate the in vivo roles of Dok-6 in Ret, and non-Ret, expressing tissues.

The ligand-dependent binding of the Dok-6 PTB domain to the Ret receptor complex required phosphorylated Ret Tyr1062. Several lines of evidence suggest that this association is due to a direct binding of Dok-6 to Ret Tyr1062. Ret Tyr1062 is a bone fide Ret autophosphorylation site, and this tyrosine residue falls within the optimal NXL(p)Y peptide recognition sequence previously defined for the Dok-1 PTB domain (24, 26, 43, 55). All previously characterized p62Dok family members, Dok-1–5, are capable of binding to Ret, suggesting that Dok proteins interact with specific effector molecules through a conserved mechanism (20, 21). For Dok-1 and Dok-5, this interaction with Ret has been shown to require the Dok PTB domains and occurs via Ret Tyr1062 (20, 21). Furthermore, the binding of Dok-5 to Ret Tyr1062 is direct as assessed by far Western analysis (20). Thus, the association of Dok-6 with activated Ret is likely due to the Dok-6 PTB domain directly binding to the recognition sequence surrounding the phosphorylated Ret Tyr1062 residue.

Dok-6 becomes tyrosine-phosphorylated in response to Ret activation. Previously characterized Dok proteins serve as substrates for a variety of RTKs as well as non-receptor tyrosine kinases including Abl and the Src family kinases (30, 33, 64, 73). Recently, we demonstrated that Src associates with the
Ret receptor complex upon GFL activation of Ret and that Src activity is essential for maximal downstream signal transduction events and Ret-mediated cellular responses (47, 48). In this report, we present evidence that the Ret-mediated tyrosine phosphorylation of Dok-6 is dependent upon Src or a Src-like kinase and that Ret-dependent Dok-6 phosphorylation occurs within the unique C-terminal region of Dok-6. Whereas previous studies involving mutational analyses of Dok-1–3 have shown that the unique C termini of these proteins harbor tyrosine residues that serve as phosphorylation sites by RTKs or non-receptor tyrosine kinases (21, 30, 35, 55, 58, 59), the location and mechanisms of tyrosine phosphorylation relevant to Dok-4 and Dok-5 proteins have not been examined. Our results indicate that Ret-dependent tyrosine phosphorylation of Dok-6 is localized exclusively to one or more tyrosine residues contained within the Dok-6 C terminus: Tyr268, Tyr282, Tyr303, Tyr321, and Tyr325. Of these tyrosine residues, only Tyr321 is predicted to act as a suitable phosphorylation site for tyrosine kinases (NetPhos program, (74)). Future studies involving mutational analysis of the Dok-6 C terminus will be

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![Fig. 7. Dok-6 promotes Ret-mediated neurite outgrowth. N2A-α1 cells were infected with lentiviruses to permit the stable expression of HA-tagged Dok-5, Dok-6, C-terminally truncated Dok-6 (Dok-6ΔC), or an empty expression cassette (Control). Virus infected cells selected by FACS against the Venus reporter gene were used in neurite outgrowth assays. A, expression of Dok proteins in lentivirus-infected N2A-α1 cells. Cell extracts were immunoprecipitated using anti-HA antibodies. Immunoprecipitates were subjected to SDS-PAGE followed by HA immunoblotting (bottom panel). Ret activation is not affected by the expression of the Dok proteins used in this study (top panel). B, N2A-α1 cells expressing the indicated Dok gene construct or empty cassette were placed in low serum medium (0.5% FBS), treated with (+) or without (−) GDNF (30 ng/ml) for 24 h, and photographed. C and D, quantification of neurite outgrowth. In C, cells were placed in low serum medium (0.5% FBS) and treated with the indicated concentration of GDNF for 24 h. Neurite outgrowth was assessed by evaluating cells in randomly chosen fields and calculating the percentage of neurite-bearing cells. Expression of Dok-6 significantly enhanced neurite outgrowth compared with N2A-α1 cells expressing Dok-6ΔC or an empty expression cassette (p < 0.0001). There was no significant difference in neurite outgrowth induced by 5 ng/ml versus 30 ng/ml GDNF treatments within any given cell line, between Dok-6- and Dok-5-expressing N2A-α1 cells, or between empty expression cassette and Dok-6ΔC-expressing N2A-α1 cells. In D, neurite lengths were measured in each cell line treated with 30 ng/ml GDNF for 24 h. Average neurite length in Dok-6-expressing N2A-α1 cells was significantly greater than that of N2A-α1 cells expressing Dok-6ΔC or an empty expression cassette (p < 0.002). Results in C and D were obtained from 3–5 independent experiments each. P-Ret, phospho-Tyr905 Ret; IP, immunoprecipitation; IB, immunoblotting.](http://www.jbc.org/content/42079/1/42079/F1.large.jpg)
necessary to identify specific tyrosine residue(s) that become phosphorylated in response to Ret activation.

We demonstrated that Dok-6, as well as Dok-5, potentiates Ret-mediated neurite outgrowth in a neuronal cell line. For these assays, we assessed the functional activity of Dok-6 with respect to Ret in a neuronal cell line that expresses endogenous Ret activated by GFRA1-GDNF. Using this system, we evaluated the in vitro function of both wild-type Dok-6 and a mutant form of Dok-6 lacking the unique Dok-6 C-terminal residues on Ret-mediated neurite outgrowth. We found that Dok-6 could promote Ret-mediated neurite outgrowth and that this activity required the C terminus of Dok-6. Consistent with our results concerning Dok-6, Grimm et al. (20) employing a panel of epidermal growth factor receptor (EGFR/Ret and EGFR/Ret/Dok receptor chimera, reported that both Dok-4 and Dok-5 can promote Ret-dependent neurite outgrowth in vitro and that these activities also require the intact C termini of these Dok family members. These results suggest that other members of the Dok-4/5/6 subfamily perform similar, or overlapping, cellular functions.

Promotion of Ret-dependent neurite outgrowth by Dok-6 required the intact C terminus of Dok-6, indicating that this region is essential for coupling to effectors and mediating the biological activities of Dok-6. In addition, we demonstrate that the Dok-6 C terminus is the sole region of Ret-dependent Dok-6 tyrosine phosphorylation, suggesting that specific phosphorylated tyrosine(s) residing within the C terminus of Dok-6 may serve as binding sites for effector molecules that, in turn, promote the physiologic functions of Dok-6. Consistent with our data, previous studies reported that the biological activities of other Dok family proteins require interactions mediated through their unique C termini (20, 59, 63, 64). For Dok-1–3 generally act as suppressors of the MAP kinase pathway, Dok-4 and Dok-5 may function as positive regulators of MAP kinase activity. Our initial attempts to examine the effects of Dok-6 on proximal events in Ret signal transduction revealed that Dok-6 expression produced little, if any, effect on MAP kinase activation (data not shown). Exactly how Dok-6 functions to potentiate Ret-mediated neurite outgrowth remains to be determined.

In summary, we have identified and provided an initial characterization of Dok-6, a novel member of the recently defined Dok-4/5/6 subclass of the Dok family of intracellular adaptor molecules. The expression of Dok-6 overlapped with that of the Ret tyrosine kinase in several locations and Dok-6 promoted Ret-mediated neurite outgrowth in vitro, suggesting that Dok-6 may function to regulate Ret-dependent processes in vivo. The development of genetic models will be essential to elucidate the physiologic roles of the Dok-4/5/6 subfamily of proteins.

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