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Enhanced Phosphatidylinositol 3-Kinase Activity and High Phosphorylation State of Its Downstream Signalling Molecules Mediated by Ret with the MEN 2B Mutation

Hideki Murakami, Toshihide Iwashita, Naoya Asai, Yohei Shimono, Yosuke Iwata, Kumi Kawai, and Masahide Takahashi[1]
Department of Pathology, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan

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We compared the intracellular signalling pathways through Ret tyrosine kinase activated by glial cell line-derived neurotrophic factor (GDNF), multiple endocrine neoplasia (MEN) 2A, or MEN 2B mutation. Tyrosine phosphorylation of Grb2-associated binder-1 (Gab1) and activation of phosphatidylinositol 3-kinase (PI 3-kinase) were induced at higher levels by GDNF stimulation or the MEN 2B mutation than by the MEN 2A mutation. Tyrosine-phosphorylated Gab1 was a major component that interacted with the active PI 3-kinase *in vivo*. In addition, we found that p62Dok and PKB/Akt were phosphorylated in a PI 3-kinase-dependent manner and the levels of their phosphorylation were significantly higher in the MEN 2B transfectant than in the MEN 2A transfectant. Tyrosine phosphorylation of p62Dok resulted in its complex formation with the Ras GTPase-activating protein (RasGAP) and the Nck adaptor protein. These findings thus suggested that high levels of activation of PI 3-kinase and of phosphorylation of its downstream signalling molecules may be associated with the clinical phenotype of MEN 2B.

Ret is a receptor tyrosine kinase that plays a crucial role in the development of the enteric nervous system and kidney (1, 2). The targeted disruption of *Ret* caused intestinal aganglionosis and renal agenesis or dysgenesis in mice (3) whereas its loss-of-function mutations in human lead to the development of Hirschsprung's disease associated with the absence of intrinsic ganglion cells in the gastrointestinal tract (4–9). Recently, it turned out that members of the glial cell line-derived neurotrophic factor (GDNF) protein family including GDNF, neurturin, persephin and artemin are Ret ligands, although these factors do not bind to Ret with high affinity but glycosyl-phosphatidylinositol (GPI)-linked cell surface proteins (designated GFRα) as ligand-binding components are required for its activation (10–19).

Germline mutations of *RET* are responsible for the development of multiple endocrine neoplasia (MEN) types 2A and 2B that share the clinical feature of medullary thyroid carcinoma and pheochromocytoma (20–24). MEN 2B is distinguished from MEN 2A by a more complex phenotype including mucosal neuroma, hyperganglionosis of the gastrointestinal tract and marfanoid habitus. The MEN 2A mutations always involve cysteine residues present in the Ret extracellular domain whereas the MEN 2B mutations were detected in alanine 883 or methionine 918 in the tyrosine kinase domain (20–24). We and others demonstrated that the MEN 2A mutations induce ligand-independent Ret dimerization on the cell surface, leading to constitutive activation of its intrinsic tyrosine kinase (25–28). On the other hand, the MEN 2B mutations appear to enhance Ret catalytic activity without dimerization and alter its substrate specificity (26–29).

It has been reported that several signalling molecules such as Shc, phospholipase C-γ, Crk, Grb7, Grb10 and Enigma associate with activated Ret (30–39). Among these, Shc binding was crucial for the transforming activity of Ret with the MEN 2A or MEN 2B mutation because a mutation at tyrosine 1062 in Ret that represents a Shc binding site drastically decreased its transforming activity (30). Shc phosphorylation induced the formation of a signal transducing complex consisting of Ret, Shc and Grb2 that could be responsible for the activation of Ras-mitogen activated protein kinase (MAPK) signalling pathway (31).

To understand the mechanism of development of different clinical phenotypes induced by the MEN 2A or MEN 2B mutation, we further investigated intracellular signalling pathways through activated Ret.

[1] To whom correspondence should be addressed. Fax: (+81) 52-744-2098. E-mail: mtakaha@med.nagoya-u.ac.jp.

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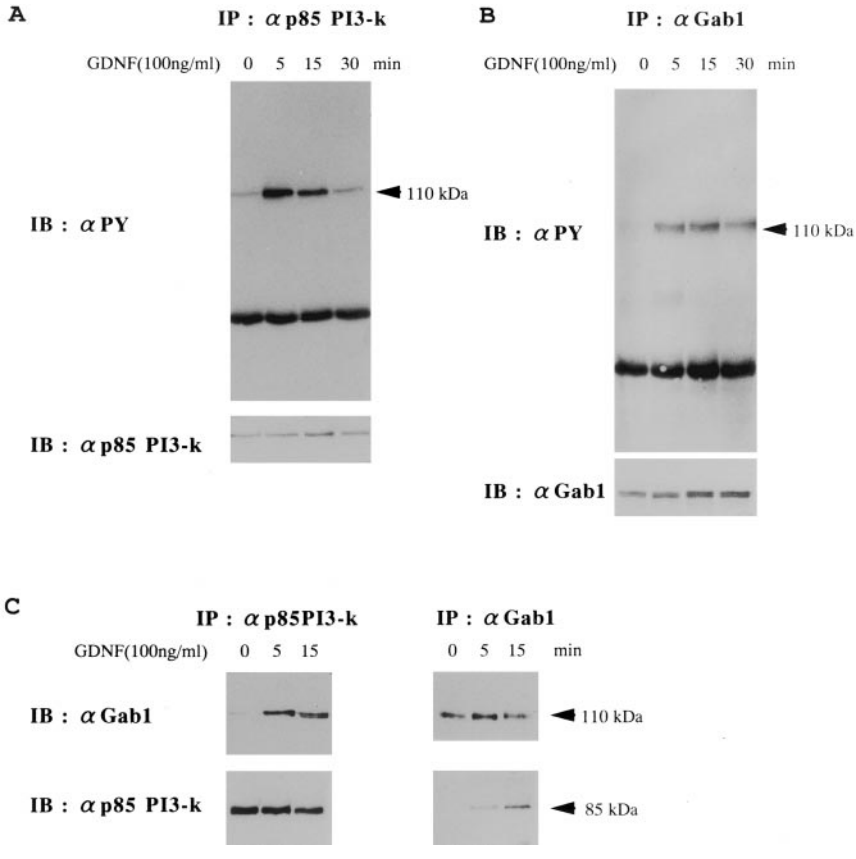

FIG. 1. Gab1 tyrosine phosphorylation and its association with p85 subunit of PI 3-kinase induced by GDNF stimulation. (A) Detection of a 110 kDa tyrosine phosphorylated band coprecipitated with p85 subunit of PI 3-kinase. The lysates from untreated or GDNF-treated TGW human neuroblastoma cells were immunoprecipitated with anti-p85 antibody, followed by immunoblotting with anti-phosphotyrosine or anti-p85 antibody. (B) Gab1 tyrosine phosphorylation in GDNF-treated neuroblastoma cells. The lysates from untreated or GDNF-treated TGW human neuroblastoma cells were immunoprecipitated with anti-Gab1 antibody, followed by immunoblotting with anti-phosphotyrosine or anti-Gab1 antibody. (C) Association between Gab1 and p85 subunit of PI 3-kinase. The lysates from untreated or GDNF-treated TGW cells were immunoprecipitated with anti-p85 antibody, followed by immunoblotting with anti-Gab1 or anti-p85 antibody (left panel). Similarly, the lysates were immunoprecipitated with anti-Gab1 antibody, followed by immunoblotting with anti-Gab1 or anti-p85 antibody (right panel).

Interestingly, Grb2-associated binder-1 (Gab1) is more highly phosphorylated on tyrosine by the MEN 2B mutation than by the MEN 2A mutation, resulting in high levels of activation of PI 3-kinase. In addition, it turned out that phosphorylation of p62Dok and PKB/Akt is induced in a PI 3-kinase-dependent manner. These results suggested that high activation of intracellular signalling through PI 3-kinase may be associated with the development of MEN 2B phenotype.

MATERIALS AND METHODS

Cell culture. A human neuroblastoma cell line, TGW, was grown in RPMI medium supplemented with 10% fetal calf serum. NIH 3T3 cells expressing Ret with a MEN 2A (Cys634 → Arg) or MEN 2B (Met918 → Thr) mutation were described previously (27).

Antibodies. Anti-Gab1, anti-Nck and anti-p85 PI 3-kinase polyclonal antibodies were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-p62Dok polyclonal antibodies and anti-phosphotyrosine and anti-RasGAP monoclonal antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-Akt and anti-phospho-Akt polyclonal antibodies were from New England Biolabs Inc. (Beverly, MA). Anti-Ret antibodies were developed as described previously (25).

Immunoprecipitation and immunoblotting. TGW cells were grown subconfluently in 100-mm dishes and serum-starved for 12 h. Then the cells were stimulated with GDNF (100 ng/ml) for 5–60 min, washed once with ice-cold phosphate-buffered saline (PBS) and lysed in radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris-HCl, pH7.5, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P(NP)-40) containing 1 mM phenylmethylsulfonyl fluoride, 0.5 mM sodium orthovanadate, 0.1 μM aprotinin and 1 μM leupeptin. The cell lysates were centrifuged at 15,000 rpm for 30 min to remove cell debris and incubated with 2 μg of antibodies for 3 h at 4°C. The resulting immunocomplexes were collected with Protein G sepharose (Sigma, St. Louis, MO) and washed four times with RIPA buffer. The complexes were eluted in sodium dodecyl sulfate (SDS) sample buffer (20 mM Tris-HCl, pH6.8, 2 mM EDTA, 2% SDS, 10% sucrose, 20 μg/ml bromophenol blue) by boiling for 5 min and subjected to SDS-polyacrylamide gel electrophoresis. Separated proteins were transferred to polyvinylidene difluoride membranes and reacted with antibodies. The reaction was examined by enhanced chemiluminescence (Amersham Corp, United Kingdom).

Assay of PI 3-kinase. PI 3-kinase was assayed as described previously (36). The lysates from GDNF-treated TGW cells or NIH 3T3 transfectants (10^6 cells) were immunoprecipitated with anti-Gab1 or anti-phosphotyrosine antibody and washed with the following buffers containing (I) 20 mM Tris-HCl, pH8.0, 150 mM NaCl and 1% NP-40, (II) 100 mM Tris-HCl, pH7.4 and 500 mM LiCl and (III) 10 mM Tris-HCl, pH7.4 and 100 mM NaCl. The resulting immunoprecipitates were suspended in 50 μl of reaction buffer (20 mM Tris-HCl, pH7.4, 100 mM NaCl, 10 mM $MgCl_2$, 0.5 mM EGTA) containing 100 μM phosphatidylinositol, 100 μM phosphatidylserine, 120 μM adenosine, 50 μM ATP and 10 μM [γ-^{32}P]ATP (3,000Ci/mmol) and incubated for 10 min at 30°C. The reaction was terminated by adding 200 μl of 1 M HCl. After extraction with 80 μl of chloroform-methanol (1:1; vol/vol), 30 μl of a lower phase of the extract was separated on a Silica Gel 60 thin-layer chromatography plate (Merck; Darmstadt, Germany) in chloroform/methanol/25%NH_4OH/water (43:38:5:7; vol/vol) for 2 h. Labeled PI 3-phosphate was visualized by autoradiography and quantitated by using a BAS2000 image analyzer (Fuji Photo Film Co., Japan).

RESULTS

Tyrosine Phosphorylation of Gab1 by GDNF Stimulation or by Ret with the MEN 2 Mutations

We recently reported that Ret activation by GDNF resulted in 4-fold increase of PI 3-kinase activity in human TGW neuroblastoma cells although direct interaction between Ret and PI 3-kinase was not observed (36). To investigate signalling molecules that associate with PI 3-kinase *in vivo,* the lysate from GDNF-treated TGW human neuroblastoma cells was immunoprecipitated with anti-p85 subunit of PI 3-kinase antibody and then immunoblotted with anti-phosphotyrosine antibody. As shown in Fig. 1A, a tyrosine phosphorylated band of 110 kDa was clearly detected at 5–15 min after GDNF stimulation. As judged from molecular mass, Grb2- associated binder-1 (Gab1) (40–42) was a candidate for this tyrosine-phosphorylated band. In fact, it turned out that Gab1 was tyrosine-phosphorylated in GDNF-treated neuroblastoma cells (Fig. 1B).

When the lysate from GDNF-treated neuroblastoma cells immunoprecipitated with anti-p85 antibody was analyzed by immunoblotting with anti-Gab1 antibody, Gab1 was detected in this immunoprecipitate (Fig. 1C). Similarly, p85 subunit of PI 3-kinase was present in the Gab1 immunoprecipitate (Fig. 1C). Thus, these results indicated that Gab1 was tyrosine-phosphorylated and directly associated with p85 subunit of PI 3-kinase in response to GDNF stimulation.

We next examined Gab1 phosphorylation and its interaction with PI 3-kinase in NIH 3T3 transfectants expressing Ret with a MEN 2A (Cys634 → Arg) or MEN 2B (Met918 → Thr) mutation (designated MEN 2A or MEN 2B transfectant). Interestingly, the level of Gab1 tyrosine phosphorylation in the MEN 2B transfectant was significantly higher than that in the MEN 2A transfectant (Fig. 2B) although the levels of expression of mutant Ret proteins and of their tyrosine phosphorylation were comparable in the two transfectants (Fig. 2A). Consistent with this finding, the amount of

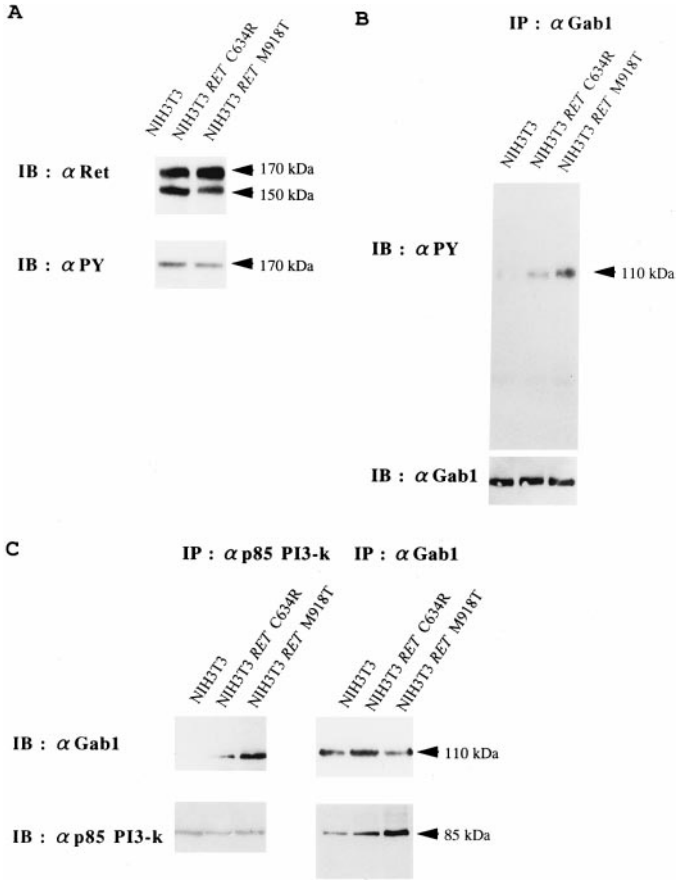

FIG. 2. Gab1 tyrosine phosphorylation and its association with p85 subunit of PI 3-kinase in the MEN 2A and MEN 2B transfectants. (A) Ret expression and tyrosine phosphorylation in the transfectants. The lysates from NIH 3T3 cells, MEN 2A (C634R) and MEN 2B (M918T) transfectants were immunoblotted with anti-Ret or anti-phosphotyrosine antibody. 170 kDa and 150 kDa Ret proteins are indicated. (B) Gab1 tyrosine phosphorylation in MEN 2A and MEN 2B transfectants. The lysates from NIH 3T3 cells, MEN 2A and MEN 2B transfectants were immunoprecipitated with anti-Gab1 antibody, followed by immunoblotting with anti-phosphotyrosine or anti-Gab1 antibody. (C) Association between Gab1 and p85 subunit of PI3-kinase in the transfectants. The lysates from NIH 3T3 cells, MEN 2A and MEN 2B transfectants were immunoprecipitated with anti-p85 antibody, followed by immunoblotting with anti-Gab1 or anti-p85 antibody (left panel). Similarly, the lysates were immunoprecipitated with anti-Gab1 antibody, followed by immunoblotting with anti-Gab1 or anti-p85 antibody (right panel).

Gab1 and p85 subunit of PI 3-kinase coprecipitated from the MEN 2B transfectant was more than that coprecipitated from the MEN 2A transfectant (Fig. 2C). However, the direct interaction of Ret with Gab1 was undetectable in these transfectants as well as in neuroblastoma cells treated with GDNF under our experimental conditions (data not shown).

Comparison of PI 3-Kinase Activity in the MEN 2A and MEN 2B Transfectants

The activity of PI 3-kinase was analyzed by thin layer chromatography in the samples immunoprecipi-

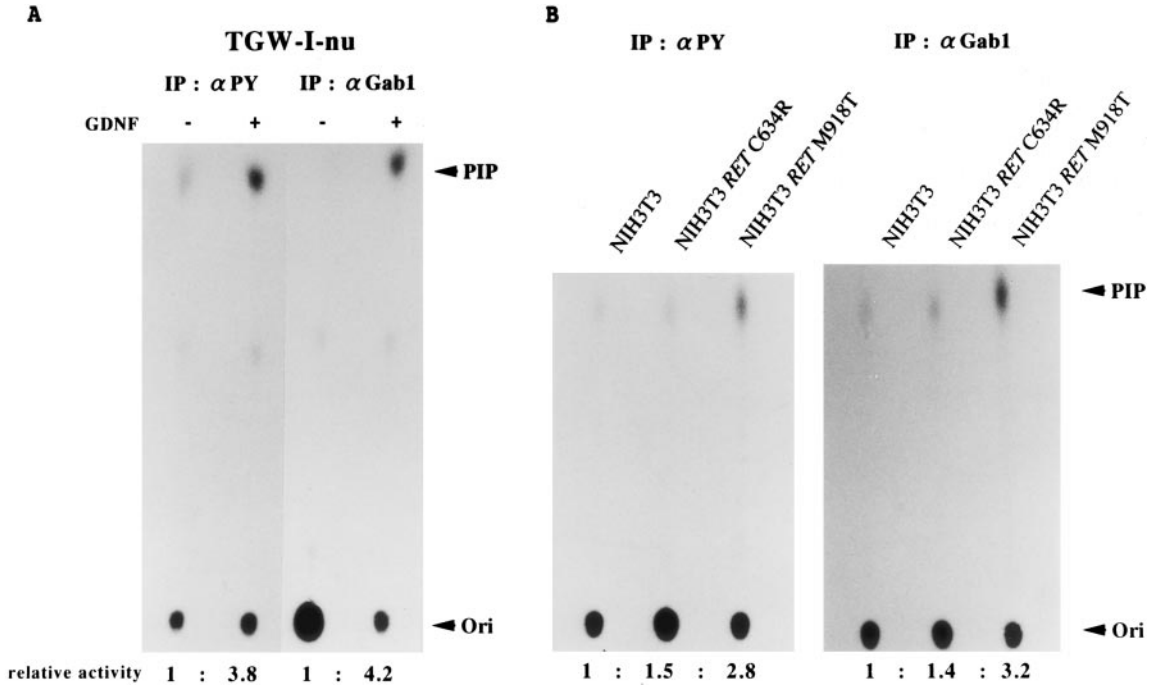

FIG. 3. Activation of PI 3-kinase by GDNF, MEN 2A or MEN 2B mutation. The lysates from untreated or GDNF-treated TGW cells (A) or from NIH 3T3 cells, MEN 2A and MEN 2B transfectants (B) were immunoprecipitated with anti-phosphotyrosine or anti-Gab1 antibody and the activity of PI 3-kinase was analyzed by thin layer chromatography. Relative activity of PI 3-kinase is indicated at the bottom. PIP, phosphatidylinositol phosphate.

tated with anti-phosphotyrosine or anti-Gab1 antibody. Its activity showed approximately 4-fold increase in both immunoprecipitates from GDNF-treated neuroblastoma cells (Fig. 3A), suggesting that tyrosine-phosphorylated Gab1 is a major component that interacts with PI 3-kinase *in vivo*.

The levels of PI 3-kinase activity in the MEN 2A and MEN 2B transfectants were approximately 1.5 and 3-fold higher than that in NIH 3T3 cells, respectively (Fig. 3B). Thus, it appeared that the activity of PI 3-kinase correlates with the level of Gab1 phosphorylation in these transfectants.

PI 3-Kinase-Dependent Tyrosine Phosphorylation of p62Dok and PKB/Akt

Our recent study showed that GDNF stimulation induces tyrosine phosphorylation of focal adhesion kinase (FAK), paxillin and p130Cas in a PI 3-kinase-dependent manner (36). We further searched signalling molecules downstream of PI 3-kinase and found that GDNF induced phosphorylation of p62Dok and PKB/Akt both of which contain the pleckstrin-homology (PH) domain that is known to interact with phosphoinositides (43, 44). Phosphorylation of these proteins occurred at 15 min after GDNF stimulation at maximal levels and was inhibited by wortmannin or LY294002, specific inhibitors of PI 3-kinase, indicating that their phosphorylation is PI 3-kinase-dependent (Fig. 4). Since it was reported that tyrosine-phosphorylated p62Dok forms a complex with the Ras GTPase-activating protein (RasGAP) and Nck (45–47), we also investigated whether this complex formation is induced by GDNF. As shown in Fig. 4C, RasGAP and Nck were clearly detected in the p62Dok immunoprecipitate.

Phosphorylation of p62Dok and PKB/Akt was detected at higher levels in the MEN 2B transfectant than in the MEN 2A transfectant (Fig. 5A and 5C). In addition, the complex formation of p62Dok/RasGAP or p62Dok/Nck was prominent in the MEN 2B transfectant (Fig. 5B). These findings suggested that the signalling through PI 3-kinase was efficiently activated by Ret with the MEN 2B mutation.

DISCUSSION

MEN 2A and MEN 2B are autosomal dominant cancer syndromes characterized by the development of medullary thyroid carcinoma and pheochromocytoma. In addition to these tumors, parathyroid hyperplasia develops in 10–30% of MEN 2A patients, whereas nearly 100% of MEN 2B show mucosal neuroma and/or skeletal abnormality. To understand the mechanism of development of different clinical phenotypes in MEN 2A and MEN 2B, we compared the intracellular signalling through Ret activated by GDNF, MEN 2A or MEN

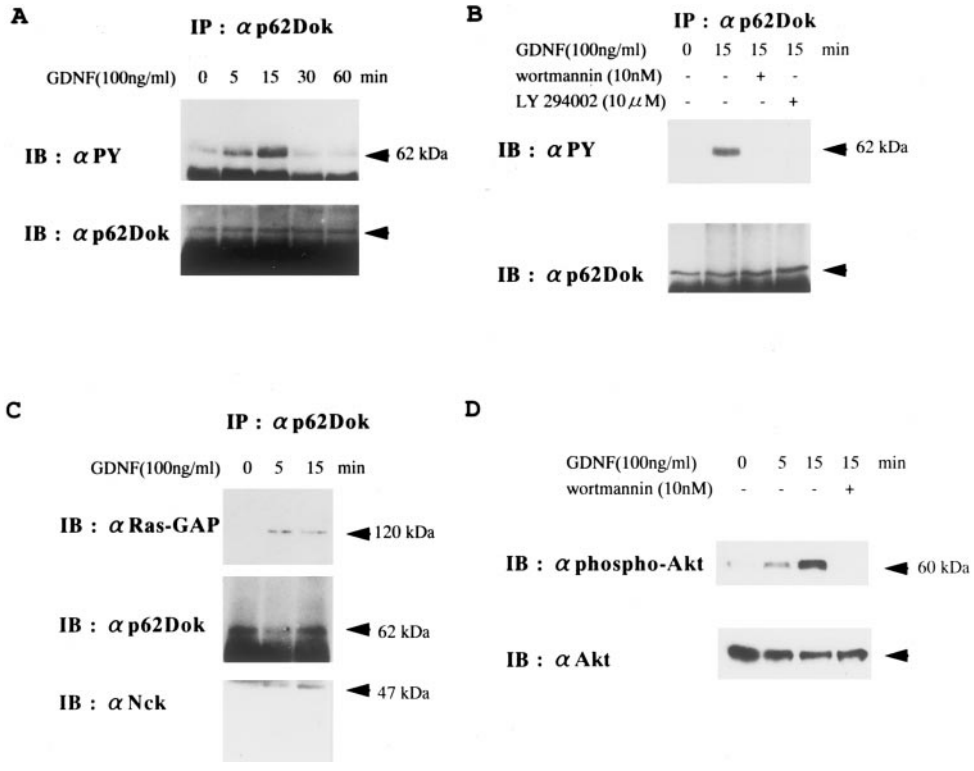

FIG. 4. PI 3-kinase-dependent phosphorylation of p62Dok and PKB/Akt and the complex formation of p62Dok with RasGAP and Nck. (A) Tyrosine phosphorylation of p62Dok in GDNF-treated TGW neuroblastoma cells. The lysates from untreated or GDNF-treated neuroblastoma cells were immunoprecipitated with anti-p62Dok antibody, followed by immunoblotting with anti-phosphotyorisine or anti-p62Dok antibody. (B) Quiescent TGW cells untreated or treated with 10 nM wortmannin or 10 μM LY294002 were incubated with GDNF and tyrosine phosphorylation of p62Dok was analyzed. (C) The lysates from untreated or GDNF-treated TGW cells were immunoprecipitated with anti-p62Dok antibody, followed by immunoblotting with anti-RasGAP anti-p62Dok or anti-Nck antibody. (D) Quiescent TGW cells untreated or treated with 10 nM wortmannin were incubated with GDNF and immunoblotted with anti-phospho-Akt or anti-Akt antibody.

2B mutation. First, we identified tyrosine phosphorylation of Gab1 docking proteins in neuroblastoma cells stimulated by GDNF as well as in NIH 3T3 cells expressing Ret with the MEN 2A or MEN 2B mutation. Interestingly, Gab1 was more highly phosphorylated on tyrosine in the MEN 2B transfectant than in the MEN 2A transfectant.

It is known that Gab1 is expressed ubiquitously and involved in signalling mediated by several growth factors including epidermal growth factor (EGF), insulin, nerve growth factor (NGF) and hepatocyte growth factor (HGF) (40–42). In this study, Gab1 phosphorylation was also detected at high levels in GDNF-treated neuroblastoma cells. Among receptors for growth factors examined, the Met tyrosine kinase that is a receptor for HGF was reported to interact strongly with Gab1 (42). As observed for other receptor tyrosine kinases such as EGF receptor and insulin receptor (42), however, Ret does not seem to bind to Gab1 with high affinity *in vivo* (data not shown) and another molecules may be required for Gab1 phosphorylation mediated by Ret kinase.

Reciprocal coprecipitation experiments revealed that phosphorylated Gab1 was strongly associated with p85 subunit of PI 3-kinase in neuroblastoma cells treated with GDNF as well as in the MEN 2 transfectants, resulting in activation of PI 3-kinase. The level of tyrosine phosphorylation of Gab1 correlated with that of association of these two molecules in the MEN 2 transfectants. As a result, the activity of PI 3-kinase in the MEN 2B transfectant was approximately 2-fold higher than that in the MEN 2A transfectant. It was reported that the PI 3-kinase signalling pathway mediated by GDNF was involved in lamellipodia formation that was required for neuritegenesis (48). Thus, high levels of constitutive activation of PI 3-kinase in neuronal cells might lead to the abnormalities such as mucosal neuroma and hyperganglionosis of the enteric nervous system found in MEN 2B.

Recently, concomitant activation of Grb2 and PI 3-kinase signalling pathway was shown to be required for metastatic ability of cells transformed by the Met oncogene (49). A Met mutant designed to obtain preferential coupling with Grb2 increased the transforming activity of Met but was impaired in causing invasion and metastasis. On the other hand, a Met mutant optimized for binding both p85 subunit of PI 3-kinase and Grb2 induced transformation, invasion and metas-

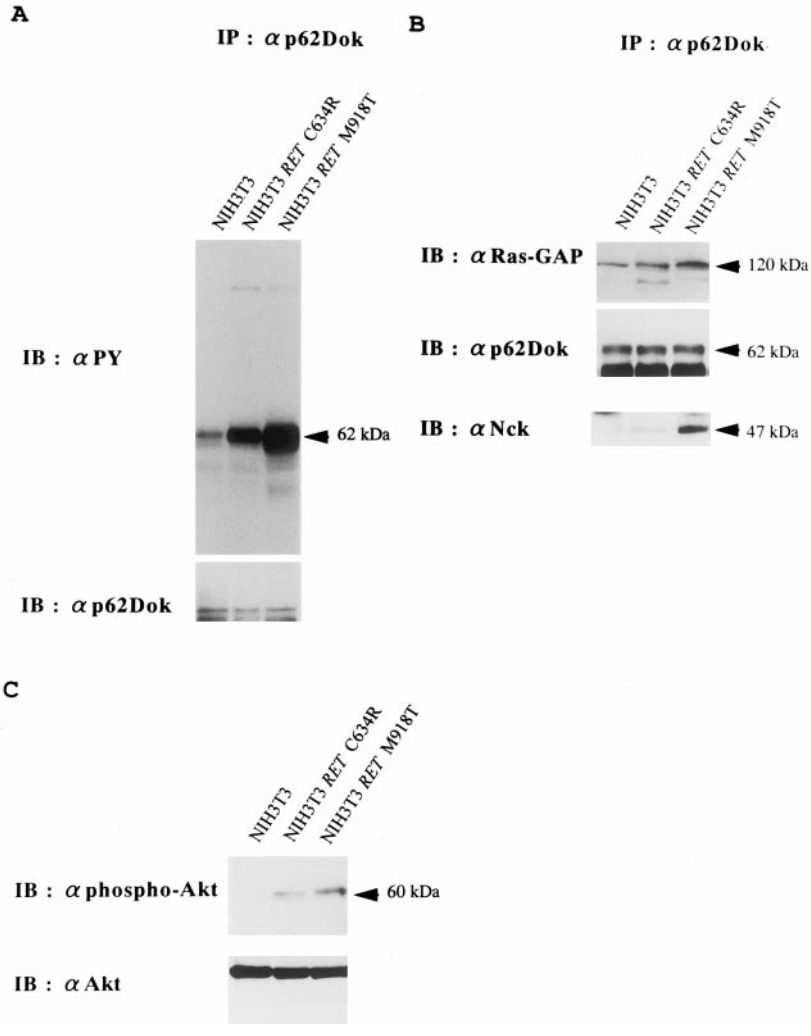

FIG. 5. Phosphorylation of p62Dok and PKB/Akt and the complex formation of p62Dok with RasGAP and Nck in MEN 2 transfectants. (A) Tyrosine phosphorylation of p62Dok in the MEN 2 transfectants. The lysates from NIH 3T3 cells, MEN 2A or MEN 2B transfectants were immunoprecipitated with anti-p62Dok antibody, followed by immunoblotting with anti-phosphotyrosine or anti-p62Dok antibody. (B) The lysates from the designated cells were immunoprecipitated with anti-p62Dok antibody, followed by immunoblotting with anti-RasGAP anti-p62Dok or anti-Nck antibody. (C) The lysates were immunoblotted with anti-phospho-Akt or anti-Akt antibody.

tasis, suggesting an important role of PI 3-kinase in Met-mediated metastasis. We previously demonstrated that the complex formation of Ret-Shc-Grb2 was induced at similar levels in the MEN 2A and MEN 2B transfectants and was crucial for transforming activity of Ret with the MEN 2A or MEN 2B mutation (30, 31). Thus, Shc-Grb2 signalling appears to be responsible for Ret-mediated transformation (Fig. 6). It is interesting to note that medullary thyroid carcinoma in MEN 2B is more aggressive than that in MEN 2A. Marshall *et al.* (50) reported that expression of Ret with the MEN 2B mutation in neuroblastoma cells enhanced their metastatic behavior *in vivo,* although they did not examined the PI 3-kinase activity in the transfectants. Taken together with the study on the Met oncogene mentioned above, it is also possible that high activation of PI 3-kinase by Ret with the MEN 2B mutation may be associated with the ability of invasion and metastasis of medullary thyroid carcinoma developed in MEN 2B.

PI 3-kinase phosphorylates phosphoinositides at the 3 position of the inositol ring. The resulting phosphorylated lipids specifically interact with the pleckstrin homology (PH) domain found in several signalling molecules such as PKB/Akt (43, 44). As expected, PKB/Akt was phosphorylated in GDNF-treated neuroblastoma cells as well as in the MEN 2A and MEN 2B transfectants. In addition, we found that another docking protein, p62Dok that contains the PH domain was highly phosphorylated on tyrosine in these cells. Interestingly, phosphorylation of p62Dok was also PI 3-kinase-dependent and induced the complex formation with RasGAP and Nck, suggesting that p62Dok plays a role as an effector of PI 3-kinase and that its association

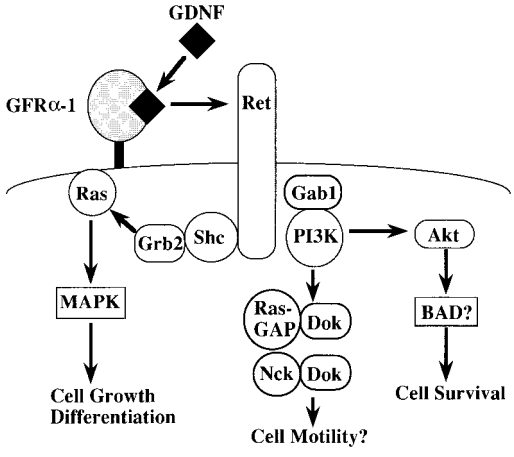

FIG. 6. Schematic illustration of the intracellular signalling pathway through activated Ret.

with RasGAP and Nck could be important for downstream signalling of PI 3-kinase (Fig. 6). Interestingly, Noguchi *et al.* (51) reported that p62Dok could mediate enhancement of insulin-induced cell migration through its interaction with RasGAP or Nck. The fact that phosphorylation of PKB/Akt and p62Dok and the complex formation consisting of p62Dok, RasGAP and Nck were induced at higher levels in the MEN 2B transfectant than in the MEN 2A transfectant suggested that enhanced PI 3-kinase signalling may be involved in the development of the aggressive phenotype of MEN 2B.

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