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Biochemical and biological responses induced by coupling of Gab1 to phosphatidylinositol 3-kinase in RET-expressing cells

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Abstract

Grb2-associated binder-1 (Gab1) is a docking protein closely related to insulin receptor substrates. We previously reported that tyrosine 1062 in RET receptor tyrosine kinase activated by glial cell line-derived neurotrophic factor (GDNF) represents a binding site for the Shc-Grb2-Gab1 complex, and that the p85 subunit of phosphatidylinositol 3-kinase (PI3K) and SHP2 tyrosine phosphatase is associated with Gab1 in GDNF-treated cells. In the present study, we further analyzed the physiological roles of Gab1 downstream of RET, using Gab1 mutants that lack the binding sites for PI3K (Gab1 PI3K-m) or SHP-2 (Gab1 SHP2-m). Expression of Gab1 PI3K-m in SK-N-MC human primitive neuroectodermal tumor cells expressing wild-type RET markedly impaired Akt phosphorylation, Rac1 activation, and lamellipodia formation that were induced by GDNF whereas expression of Gab1 SHP2-m partially impaired Erk activation. Furthermore, expression of Gab1 PI3K-m, but not Gab1 SHP2-m, in TT human medullary thyroid carcinoma cells expressing RET with a multiple endocrine neoplasia 2A mutation enhanced cytochrome *c* release, and apoptosis induced by etoposide, suggesting that PI3K is involved in survival of TT cells via a mitochondrial pathway. These findings demonstrated that coupling of Gab1 to PI3K is important for biological responses in RET-expressing cells.

Keywords: Gab1; RET tyrosine kinase; GDNF; Lamellipodia; Apoptosis

The *RET* proto-oncogene encodes a tyrosine kinase receptor, the ligands of which are members of the glial cell line-derived neurotrophic factor (GDNF) protein family, including GDNF, neurturin, artemin, and persephin [1,2]. The activation of *RET* by these neurotrophic factors is mediated by their binding to the glycosylphosphatidylinositol-anchored co-receptors termed GDNF family receptor α1–4 (GFRα1–4) [1]. GDNF, neurturin, artemin, and persephin use GFRα1, GFRα2, GFRα3, and GFRα4 as their preferred receptors, respectively, and play specific roles in vivo through complex formation with RET. Gene knock-out studies have revealed that the GDNF/RET signaling plays an important role in the development of the enteric nervous system and the kidney [3–6].

RET mutations are responsible for the development of several human diseases including Hirschsprung's disease, multiple endocrine neoplasia (MEN) types 2A and 2B, familial medullary thyroid carcinoma (FMTC), and papillary thyroid carcinoma (PTC) [2,7]. Loss-of-function mutations of *RET* lead to the development of Hirschsprung's disease, which is a congenital malformation associated with the absence of enteric neurons [2,7,8].

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On the other hand, gain-of-function mutations of *RET* contribute to the development of human neoplastic diseases including MEN 2A, MEN 2B, FMTC, and PTC [2,9–11]. MEN 2A, MEN 2B, and FMTC are caused by germ-line point mutations of *RET*, and PTC is caused by its somatic rearrangement. MEN2A and MEN 2B share the clinical features of medullary thyroid carcinoma (MTC) and pheochromocytoma, and FMTC is characterized by the development of MTC alone. In addition, 10–30% of MEN 2A patients develop parathyroid hyperplasia, whereas MEN 2B patients show a more complex phenotype that includes ganglioneuromatosis of the gastrointestinal tract, mucosal neuroma, and marfanoid habitus.

Activated RET recruits a variety of signaling molecules including Grb2, Grb7, Grb10, Shc, Enigma, SNT/Frs2, Dok, insulin receptor substrate-1 (IRS-1), and phospholipase C-γ [12–24]. Intriguingly, several signaling molecules including Shc, SNT/Frs2, Dok, and IRS-1 bind to phosphorylated tyrosine 1062 in RET, through which the Ras/Erk, PI3-K/Akt, p38MAPK, c-Jun amino-terminal kinase (JNK), and Erk5 pathways are activated [17,18,21,25–29]. When Shc binds to tyrosine 1062 in RET, it further complexes with Grb2-Gab1/2 complex, resulting in activation of the Ras/Erk and PI3-K/Akt signaling pathways [25,26].

Grb2-associated binder-1 (Gab1) is a so-called scaffolding or docking protein closely related to IRS-1, IRS-2, and IRS-3 [30]. Gab1 interacts with multiple signaling molecules, including the p85 subunit of PI3K, SHP2 tyrosine phosphatase, phospholipase C-γ, and Crk [31–33]. We and other investigators previously reported that Grb1/2 complex was associated with Shc bound to tyrosine 1062 in RET in GDNF-treated cells [25,26], leading to the interaction of Gab1 with the p85 subunit of PI3K and SHP2 [18,34]. In the present study, we further analyzed physiological roles of Gab1 downstream of RET.

Materials and methods

Plasmid constructs. Full-length human wild-type or mutant *Gab1* cDNAs were amplified by PCR using primers with appropriate restriction enzyme sites. The resulting cDNA fragments were inserted into the pcDNA3.1-V5HisA expression vector (Invitrogen), thereby fusing the *Gab1* cDNAs with the V5 sequence. All *Gab1* mutations were introduced into double-stranded DNA by using the QuikChange site-directed mutagenesis kit (Stratagene) according to the instructions of the manufacturer. The sequences of all constructs were confirmed by DNA sequencing.

Cell lines. NIH 3T3 mouse fibroblast and SK-N-MC human primitive neuroectodermal tumor cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 8% calf serum. TT cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum. NIH 3T3 and SK-N-MC cell lines transfected with the expression plasmids carrying wild-type *RET* or mutant *RET* cDNA were described previously [14,26].

Retroviral vector. The retroviral vectors pHBGAP and pCGCGP were constructed as follows [35]. Briefly, for the construction of pHBGAP, a CMV early promoter was inserted into a pLRNL retrovirus vector [36] cleaved with *Eco*RI and *Sac*I, resulting in pCLRNL. pCLNRL was digested with *Hin*dIII and self-ligated to generate pCLNL. The *Bst*BI–*Cla*I fragment of the GAPDH promoter was inserted into pCLNL cleaved with *Bst*BI and *Cla*I, resulting in pCLNGAP. The replication origin of pCLNGAP was replaced with that of the pBluescript KS(+) vector (Stratagene) in order to make a high-copy plasmid, and a T3–T7 fragment including the multi-cloning site of pBluescript KS(+) vector was inserted in the blunted *Cla*I site. The neomycin resistance gene was replaced with the blasticidin S deaminase coding region from pUCSV-BSD (Kaken, Urayasu, Chiba, Japan), resulting in the pHBGAP vector. For the construction of pCGCGP, an EagI fragment of pHCMV-G, including a CMV early promoter and vesicular stomatitis virus envelope glycoprotein (VSV-G), was inserted into pHCMV-GP [37,38] cleaved with *Not*I. Full-length human wild-type or mutant *Gab1* cDNAs were introduced into the pHBGAP retroviral plasmid vector. Transducing retroviral vectors were obtained by cotransfection of HEK293FT packaging cells with 3 μg of plasmid vectors (*pHBGAP-Gab1s*) and 3 μg of *VSV-G*/*Gag*/*pol*-encoding plasmid pCGCGP using the FuGene 6 (Roche Diagnostics). After overnight incubation at 37 °C in a 5% CO_2 humidified atmosphere, the transfection cocktail was washed out and replaced with fresh medium. The viral particle-containing supernatants were collected 24, 48, and 72 h after transfection and centrifuged at 28,000*g* for 2 h. Viral pellet was resuspended in PBS and used to infect SK-N-MC and TT cells in the presence of polybrene (8 μg/ml). The viral titer of all vector transfectants was estimated to be more than 2×10^6 provirus forming units/ml by assessment of the amount of blasticidin S (Invitrogen)-resistance transfer on 3T3 mouse fibroblasts in the presence of 10 μg/ml blasticidin S. The transduced cells were maintained in growth medium supplemented with blasticidin S for 1 week in SK-N-MC cells and for 2 weeks in TT cells. Polyclonal cell populations were selected and subjected to experiments.

Antibodies. Anti-Gab1, anti-phosphotyrosine (clone 4G10), anti-Shc, anti-Rac1, and anti-p85 PI3K antibodies were purchased from Upstate Biotechnology. Anti-Grb2 antibody was purchased from Transduction Laboratories. Anti-Akt, anti-phospho-Akt (Ser473), anti-p44/42 Erk, and anti-phospho-p44/42 Erk antibodies were purchased from Cell Signaling Technology. Anti-V5 antibody was purchased from Invitrogen. Anti-cytochrome *c* antibody (6H2.B4) was obtained from BD Pharmingen. Anti-SHP2 antibody was obtained from Santa Cruz Biotechnology. Anti-RET antibody was developed as described previously [18].

Immunoprecipitation and immunoblotting. Cells were grown subconfluently in 100-mm dishes and were serum-starved for 12 h. Then they were lysed in lysis buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, and 1 mM sodium orthovanadate) supplemented with one tablet of complete protease inhibitor mixture (Roche Diagnostics) per 50 ml and 1 mM phenylmethylsulfonyl fluoride (PMSF). The lysates were clarified by centrifugation (15,000*g*) for 30 min, and the supernatants were incubated with 5 μg of antibodies for 3 h at 4 °C. The resulting immunocomplexes were collected with protein A– or protein G–Sepharose (Sigma) and washed four times with lysis buffer. The complexes were eluted in sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris–HCl, pH 6.8, 5 mM EDTA, 2% SDS 10% glycerol, and 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 (Nihon Millipore Kogyo) and reacted with the antibodies. The reaction was examined by the enhanced chemiluminescence detection kit (ECL, Amersham Biosciences) according to the instructions of the manufacturer.

GST-CRIB pull-down assay. Rac activation was evaluated using the GST-CRIB (Cdc42/Rac interacting binding) pull-down assay [39]. In brief, SK-N-MC cells expressing wild-type or mutant Gab1 were

stimulated with GDNF and lysed on ice in lysis buffer (50 mM Tris–HCl, pH 7.4, 1% Nonidet P-40, 100 mM NaCl, 10% glycerol, and 5 mM $MgCl_2$) supplemented with one tablet of complete protease inhibitor mixture/50 ml and 1 mM PMSF. The lysates were centrifuged at 15,000*g* for 10 min at 4 °C, and the resulting supernatants were incubated with GST-CRIB (Upstate Biochemicals) for 30 min at 4 °C. Protein complexes were washed three times with lysis buffer, boiled in SDS-sample buffer, and analyzed by immunoblotting with anti-Rac1 antibody.

Immunofluorescence. SK-N-MC cells expressing wild-type or mutant Gab1 were grown overnight and serum-starved for 24 h. Then they were incubated with GDNF, fixed for 10 min in PBS containing 10% formalin, and permeabilized with 0.05% Triton X-100. After incubation in PBS containing 1% bovine serum albumin for 15 min, the cells were reacted with Alexa594-phalloidin for 20 min.

To evaluate the subcellular distribution of cytochrome *c*, TT cells expressing wild-type or mutant Gab1 were grown overnight and treated with etoposide (25 μM) (Sigma) for 48 h, fixed for 10 min in PBS containing 10% formalin, and permeabilized with 0.05% Triton X-100. After incubation in 10% goat serum for 60 min, the cells were reacted with anti-cytochrome *c* monoclonal antibody for 1 h at room temperature, followed by incubation with Alexa488-conjugated anti-mouse IgG antibody (Molecular Probe) and TO-PRO-3 iodide (Molecular Probe). The staining was analyzed using a confocal microscope (Bio-Rad).

Determination of apoptotic cell death with a fluorescence-activated cell sorter (FACS). TT cells expressing wild-type or mutant Gab1 and control TT cells (5×10^5 cells) were plated in 60 mm dishes and incubated for 20–24 h. Then, cells were treated with etoposide (25 μM) or equal amount of DMSO and incubated for 24 or 48 h. Apoptotic cells were evaluated with a FACS Calibur (BD Biosciences), using the annexin-V-fluorescein and propidium iodine (PI) double staining method (Annexin-V-FLUOS staining kit, Roche).

Results

Regulation of Akt and Erk activation by Gab1 in the GDNF/RET signaling pathway

We reported that Gab1 is associated with the p85 subunit of PI3K and SHP2 tyrosine phosphatase in response to GDNF stimulation [18,26]. To analyze the biological roles of the molecules that bind to Gab1, we generated mutant Gab1 to which the p85 subunit of PI3K or SHP2 cannot bind. As shown in Fig. 1A, tyrosine residues in the consensus biding sequences for p85 or SHP-2 were replaced with phenylalanine. Gab1 Y447/474/589F and Gab1 Y627/659F were designated Gab1 PI3K-m and Gab1 SHP2-m, respectively.

Wild-type or mutant Gab1 cDNA inserted into the pcDNA3.1-V5HisA expression plasmid was transiently transfected into NIH3T3 cells expressing RET with a *MEN2B* mutation (designated NIH(RET-MEN2B) cells) and their cell lysates were immunoprecipitated with anti-V5 antibody, followed by immunoblotting with an anti-RET, anti-PI3K or anti-SHP2 antibody. Although wild-type Gab1 was co-immunoprecipitated with SHP2 and the p85 subunit of PI3K, Gab1 PI3K-m, and SHP2-m did not bind to p85 and SHP2, respectively (Fig. 1B). Co-immunoprecipitation of RET with Gab1 was not observed as previously reported [18].

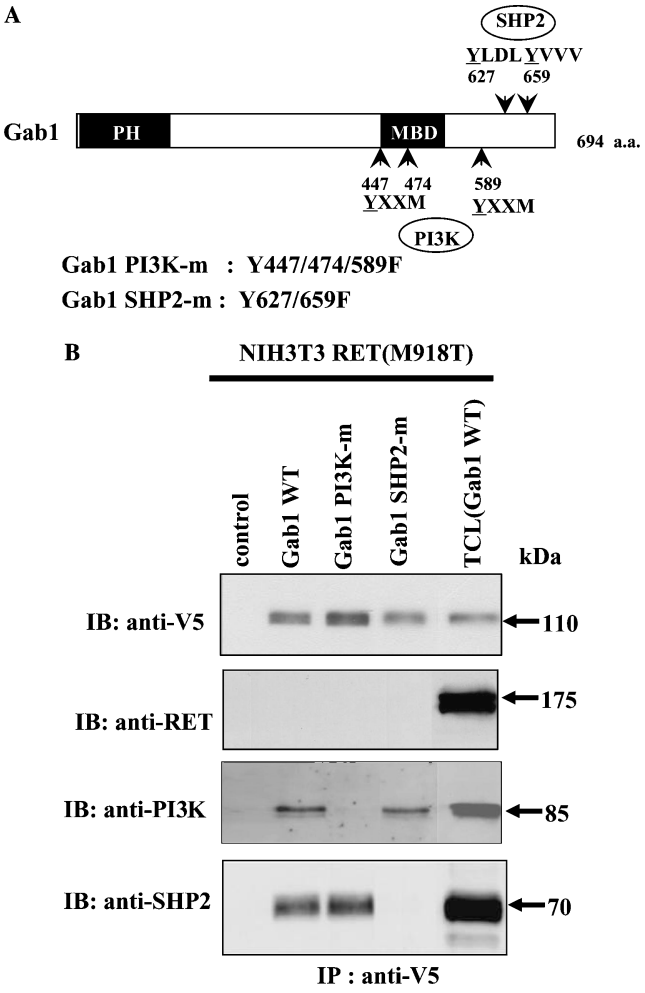

Fig. 1. Generation of Gab1 mutants that lacked the ability to bind with p85 of PI3K and SHP2. (A) Schematic illustration of the Gab1 protein. Gab1 has multiple tyrosine residues that interact with p85 of PI3K and SHP2 tyrosine phosphatase. The indicated tyrosine residues that represent the binding sites for p85 of PI3K or SHP2 were replaced by phenylalanine. Each Gab1 mutant cDNA was designated Gab1 PI3K-m or Gab1 SHP2-m. PH: pleckstrin homology domain. MBD: c-Met binding domain. (B) Binding of wild-type and mutant Gab1 with p85 of PI3K and SHP2. NIH(RET-MEN2B) cells were transiently transfected with wild-type or mutant Gab1 and their lysates were immunoprecipitated with anti-V5 antibody, followed by immunoblotting with an anti-V5, anti-RET, anti-PI3K or anti-SHP2 antibody.

To next investigate the role of Gab1 in the GDNF/RET signaling pathway, we transfected V5-tagged wild-type Gab1, Gab1 PI3K-m, and Gab1 SHP2-m into SK-N-MC human primitive neuroectodermal tumor cells expressing wild-type RET (designated MC(RET) cells) and compared the levels of Akt and Erk1/2 phosphorylation among the transfectants. Expression of wild-type Gab1 and Gab1 SHP2-m somewhat increased the Akt phosphorylation induced by GDNF, whereas expression of Gab1 PI3K-m markedly impaired its phosphorylation (Figs. 2A and B). This finding confirmed that the binding of p85 of PI3K to Gab1 is critical for Akt activation in response to GDNF. On the

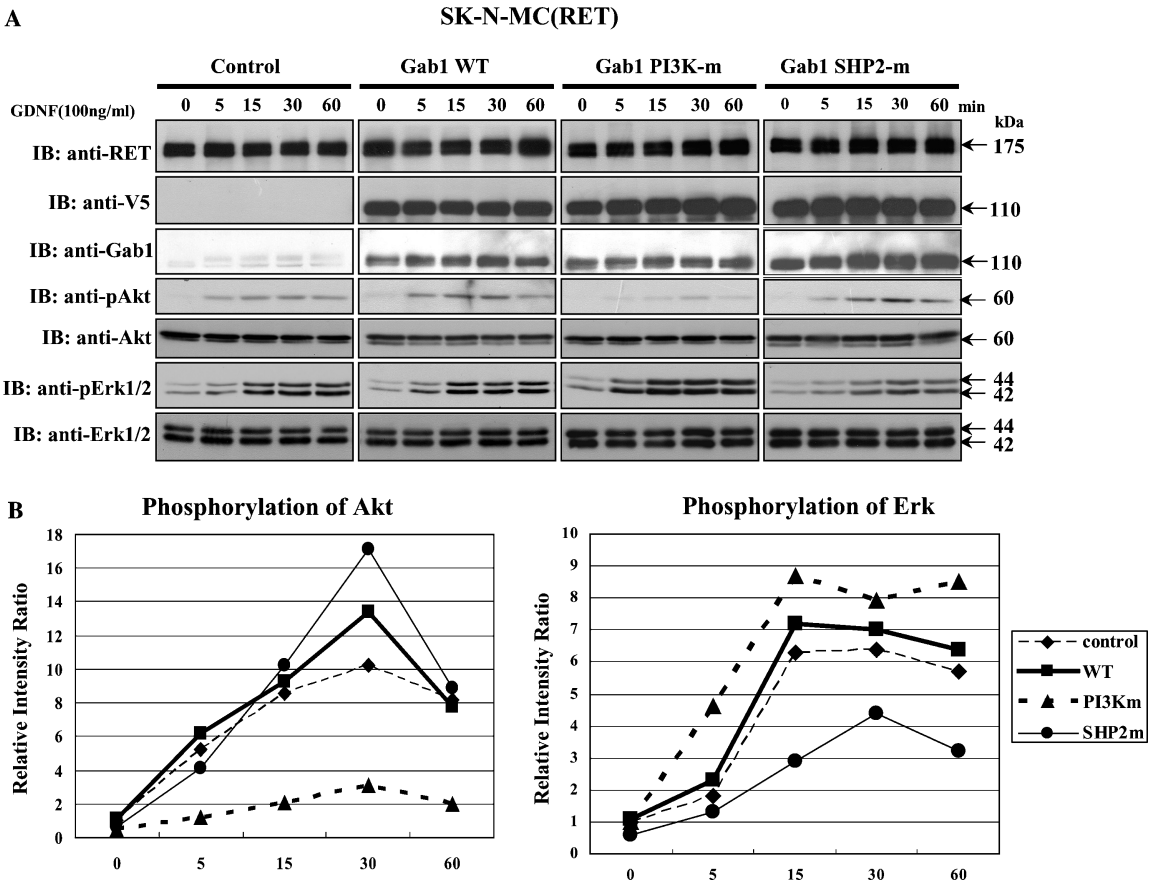

Fig. 2. Effect of wild-type and mutant Gab1 expression on RET-mediated Akt and Erk activation. (A) SK-N-MC cells expressing wild-type RET cells (MC(RET) cells) were transiently transfected with wild-type or mutant Gab1. After 48 h, they were serum-starved for 3 h and treated with GDNF (100 ng/ml). The cell lysates (30 μg of protein) suspended in SDS-sample buffer were subjected to immunoblotting with anti-RET, anti-V5, anti-Gab1, anti-phosphoAkt (pAkt), anti-Akt, anti-phosphoErk1/2 (pErk1/2), or anti-Erk1/2 (Erk) antibody. (B) Phosphorylation levels of Akt and Erk1/2 were quantified by densitometry. The levels in GDNF-untreated cells were set at 1.0.

other hand, Gab1 wild-type and Gab1 PI3K-m slightly increased the Erk1/2 phosphorylation, whereas Gab1 SHP2-m impaired it, suggesting that SHP2 binding to Gab1 plays a role in Erk activation.

Impairment of Rac1 activity and lamellipodia formation by expression of Gab1 PI3K-m

Because activation of PI3K is also required for Rac activation and lamellipodia formation in MC(RET) cells [40,41], we investigated the effects of Gab1 mutants on RET-induced Rac1 activation. MC (RET) cells were infected with a retroviral vector carrying wild-type Gab1 or one of its mutants, and used for the GST-CRIB (Cdc42/Rac interacting binding) pull-down assay. The amount of the activated form of Rac1 (GTP-bound Rac1) was analyzed by immunoblotting with an anti-Rac1 antibody. In the MC (RET) cells, stimulation with GDNF resulted in an increase of a Rac1-GTP form after 5 min stimulation (Fig. 3). Furthermore, the increase of a Rac1-GTP form was observed in the wild-type Gab1 and SHP2-m transfectant, whereas Rac1 activation was not observed in the Gab1 PI3K-m transfectant stimulated with GDNF (Fig. 3).

To elucidate the role of Gab1 in RET-mediated lamellipodia formation, MC(RET) cells expressing wild-type or mutant Gab1 were treated with GDNF (100 ng/ml) for 30 min, followed by staining with Alexa-594-phalloidin (Fig. 4A). Lamellipodia formation was induced in MC(RET) cells as well as in the cells expressing wild-type Gab1 and SHP2-m by GDNF treatment at similar levels (21–27% of the cells). In contrast, lamellipodia formation was almost completely inhibited in the MC(RET) cells expressing Gab1 PI3K-m (Figs. 4A and B), demonstrating that coupling of Gab1 to PI3K is required for lamellipodia formation in GDNF-treated MC(RET) cells.

Effects of Gab1 mutants on Akt activation and apoptosis in TT human medullary thyroid carcinoma cells expressing RET-MEN2A protein

It is known that the PI3K/Akt pathway inhibits apoptosis by a variety of apoptotic stimuli [42]. Therefore, we investigated the effect of a Gab1 mutant on

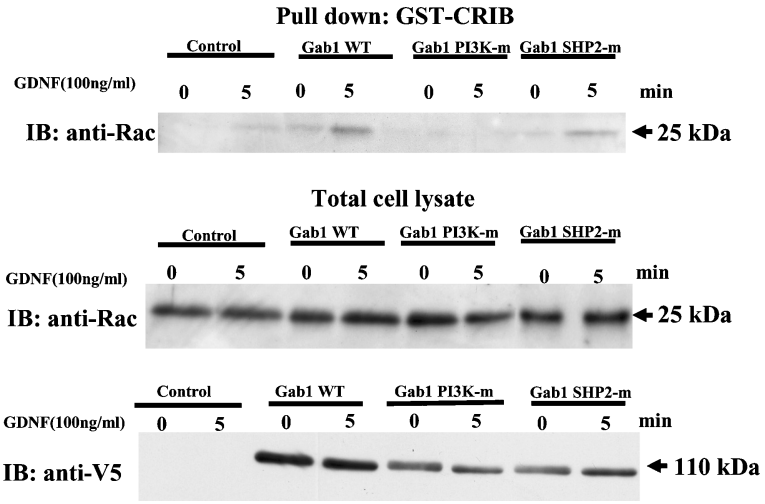

Fig. 3. Effect of wild-type and mutant Gab1 expression on Rac1 activity. MC(RET) cells were infected with a retroviral vector carrying wild-type or mutant Gab1 cDNA and blasticidin S (10 μg/ml)-resistant colonies were grown. Established cell lines expressing wild-type or mutant Gab1 were serum-starved overnight and treated with GDNF (100 ng/ml) for 5 min. The cell lysates were pulled down by the GST-CRIB fusion protein. The GTP form of Rac1 was detected by immunoblotting with an anti-Rac1 antibody (upper panel). Total cell lysates were also immunoblotted with an anti-Rac1 and an anti-V5 antibody (middle and lower panels).

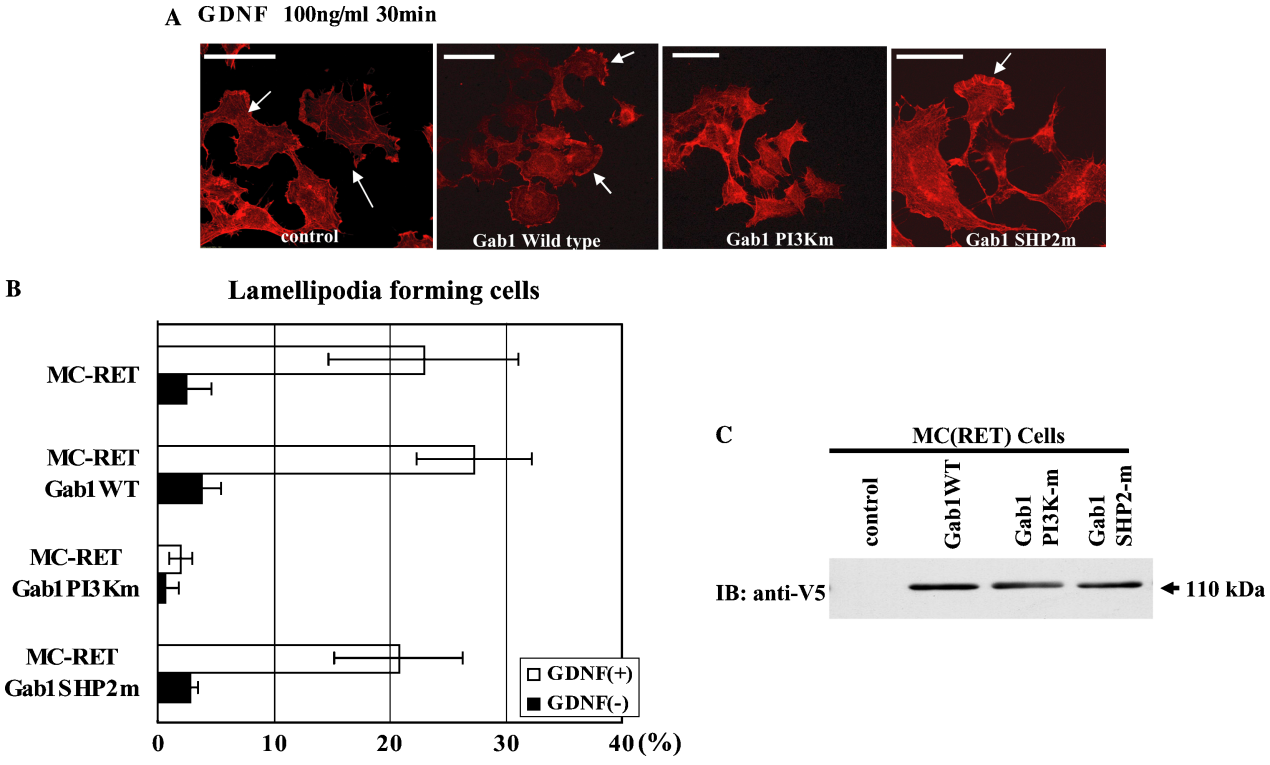

Fig. 4. Lamellipodia formation in MC(RET) cells expressing wild-type or mutant Gab1. (A) Inhibition of lamellipodia formation by expression of Gab1 PI3k-m. MC(RET) cells expressing wild-type or mutant Gab1 were serum-starved overnight, treated with GDNF (100 ng/ml) for 30 min, and stained with Alexa-594-phalloidin. Arrows indicate lamellipodia formation. Bars indicate 50 μm. (B) Quantitative analysis of lamellipodia formation. Results represent averages from three independent experiments, and bars indicate the standard errors. (C) MC(RET) cell lines expressing wild type or mutant Gab1 were immunoblotted with an anti-V5 antibody.

Akt activation and apoptosis in TT human medullary thyroid carcinoma cells expressing RET with a *MEN2A* mutation. Expression of wild-type Gab1 and SHP2-m slightly increased Akt phosphorylation, whereas expression of PI3K-m did not affect it (Fig. 5). In addition, effects of expression of wild-type Gab1, PI3K-m, and

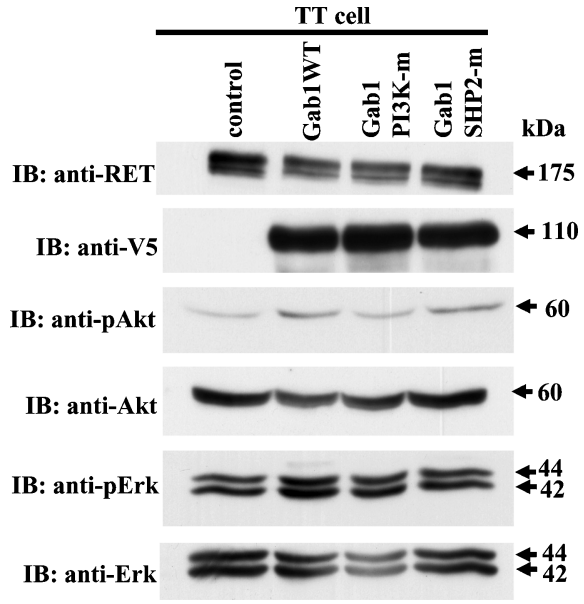

Fig. 5. Effect of wild-type or mutant Gab1 on Akt and Erk activation in TT cells. TT cells were infected with retroviral vector carrying wild-type or mutant Gab1 cDNA and blasticidin S (10 μg/ml)-resistant colonies were grown for establishment of cell lines. The cell lysates (50 μg of protein) suspended in SDS-sample buffer were subjected to immunoblotting with anti-RET, anti-V5, anti-phosphoAkt (pAkt), anti-Akt anti-phosphoErk1/2 (pErk1/2), and anti-Erk1/2 antibody.

SHP2-m on Erk activation were not apparent (Fig. 5). Because a constitutively active form of RET (RET-MEN2A) is expressed in TT cells, additional expression of wild-type Gab1, PI3K-m, and SHP2-m may not significantly affect the activation of Akt and Erk.

We next investigated the effect of Gab1 on apoptosis of TT cells. TT cells infected with a retroviral vector carrying wild-type Gab1 or mutant cDNA were incubated in etoposide (25 μM) or DMSO for 24–48 h to induce apoptosis. The cells were collected and stained with annexin-V-fluorescein and propidium iodide (PI). The proportion of apoptotic cells was evaluated with a FACS Calibur (BD Biosciences). Apoptotic cells (annexin-V-fluorescein positive and PI negative) were located in R1 (red dots in Fig. 6A). In TT cells, apoptotic cells were 6.96% at 24 h and 8.32% at 48 h after etoposide treatment, whereas in TT cells expressing Gab1 PI3K-m, apoptotic cells were 11.08% at 24 h and 20.60% at 48 h (Figs. 6A and B). The proportions of apoptotic cells in TT cells expressing wild-type Gab1 and SHP2-m were 8.88% and 7.72% at 48 h after etoposide treatment, respectively. These findings indicated that apoptosis induced by etoposide was enhanced by expression of Gab1 PI3K-m.

Because mitochondrial cytochrome *c* release is associated with apoptosis, the distribution pattern of cytochrome *c* was examined in TT cells expressing wild-type Gab1 or its mutants. They were incubated in etoposide (25 μM) for 48 h, followed by staining with an anti-cytochrome *c* antibody (green) and TO-PRO-3 iodide (red) (Fig. 7A). The distribution pattern of cytochrome *c* was observed under fluorescence microscopy. Cytochrome *c*

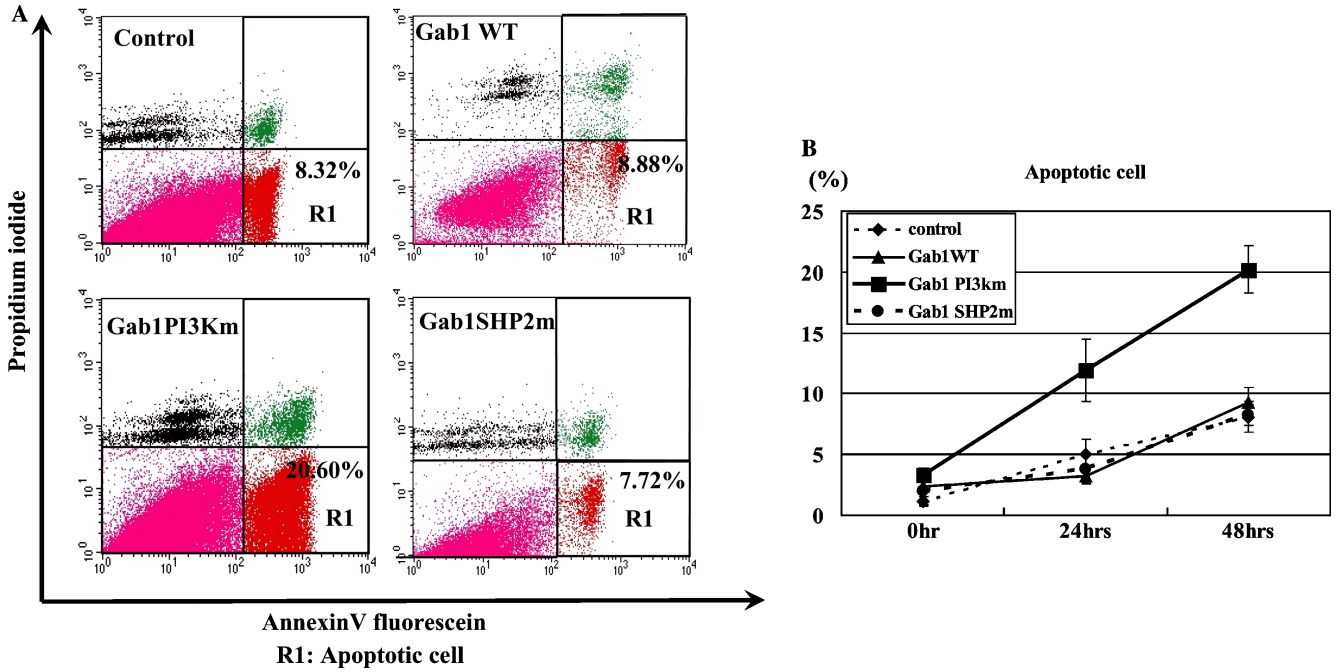

Fig. 6. Gab1 PI3K-m expression enhanced apoptosis of TT cells induced by etoposide. (A) Annexin-V-fluorescein/PI double staining of etoposide-treated TT cells. TT cells expressing wild-type or mutant Gab1 (5×10^5 cells) were plated on a 60 mm dish and incubated in the presence of etoposide (25 μM). After 48 h, the cells were incubated with annexin V-fluorescein and propidium iodide. Apoptosis was evaluated with a FACS Calibur (BD Biosciences, Software: CellQuest). Control, original TT cells. (B) Quantitative analysis of apoptosis of etoposide-treated TT cells. Results represent averages from at least three independent experiments, and bars indicate the standard errors.

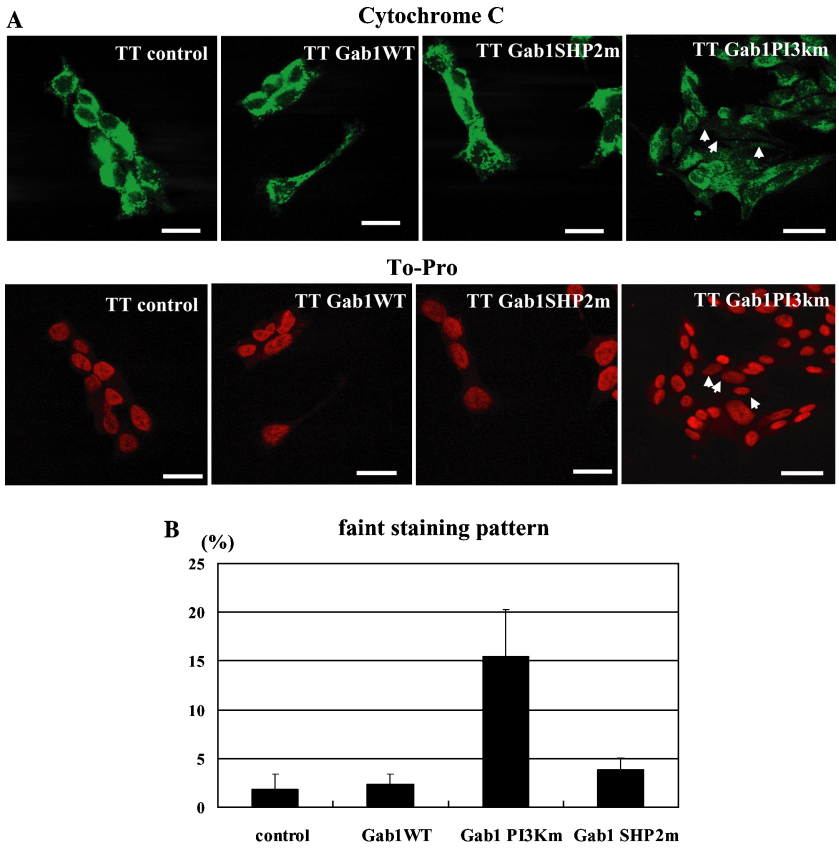

Fig. 7. Cytochrome *c* release in TT cells expressing Gab1 PI3K-m. (A) TT cells expressing wild-type or mutant Gab1 were treated with etoposide (25 μM). After 48 h, the cells were fixed and stained with anti-cytochrome *c* antibody (green) and TO-PRO-3 iodide (red). Arrows indicate the cells with diffuse and faint staining of cytochrome *c*. Bars indicate 20 μm. (B) Quantitative analysis of the faint staining pattern of cytochrome *c*. Results represent averages from three independent experiments, and bars indicate the standard errors.

was distributed mainly in a punctate pattern in untransfected TT cells as well as in TT cells expressing wild-type Gab1 and SHP2-m (more than 95%). On the other hand, in about 15% of TT cells expressing Gab1 PI3k-m, cytochrome *c* was distributed in a diffuse or faint staining pattern (Figs. 7A and B), which was evaluated for the release of cytochrome *c* from mitochondria [43]. In addition, the cells with a diffuse or faint staining pattern of cytochrome *c* sometimes showed fragmented nuclei (data not shown).

Discussion

Gab1 can interact with the p85 subunit of PI3K and SHP2 tyrosine phosphatase in response to GDNF stimulation [18,34]. Analysis with Gab1 mutants that lacked the ability to bind with p85 or SHP2 (designated Gab1 PI3K-m or SHP2-m) suggested that Gab1 plays a more important role in the PI3K-Akt signaling pathway than the Ras-Erk pathway. Expression of Gab1 PI3K-m markedly impaired Akt activation in MC(RET) cells stimulated by GDNF, whereas the effect of SHP2-m expression on Erk activation was moderate. Besset et al. [25] previously reported that Grb2-Gab1/2 complex is indirectly (via Shc) and directly associated with tyrosine 1062 and 1096 in RET, respectively. The present results demonstrated that coupling of Gab1 to p85 of PI3K is essential for activation of the PI3K-Akt signaling pathway downstream of RET (Fig. 8).

On the contrary, Gab1 appeared to play a limited role for activation of the Ras-Erk pathway by GDNF. It is well known that there are several pathways that lead to activation of Ras and Erk. For example, when Shc or Frs2 binds to phosphorylated tyrosine 1062 in RET, the Grb2-Sos complex is recruited to these docking proteins, resulting in Ras activation [26,44]. In addition, Frs2 can bind SHP2 like Gab1, thereby leading to Erk activation [44]. Thus, the finding showing that SHP2-m partially impaired the Erk activation is consistent with the fact that GDNF can activate these alternative Erk pathways (Fig. 8).

We further investigated the biological roles of Gab1 in the *RET* signaling pathway. Rac activity is regulated via activation of PI3K by a variety of receptor tyrosine kinases, and GDNF treatment induces lamellipodia formation in MC(RET) cells [40,41]. In addition, we found that GDNF treatment promotes Rac activation and

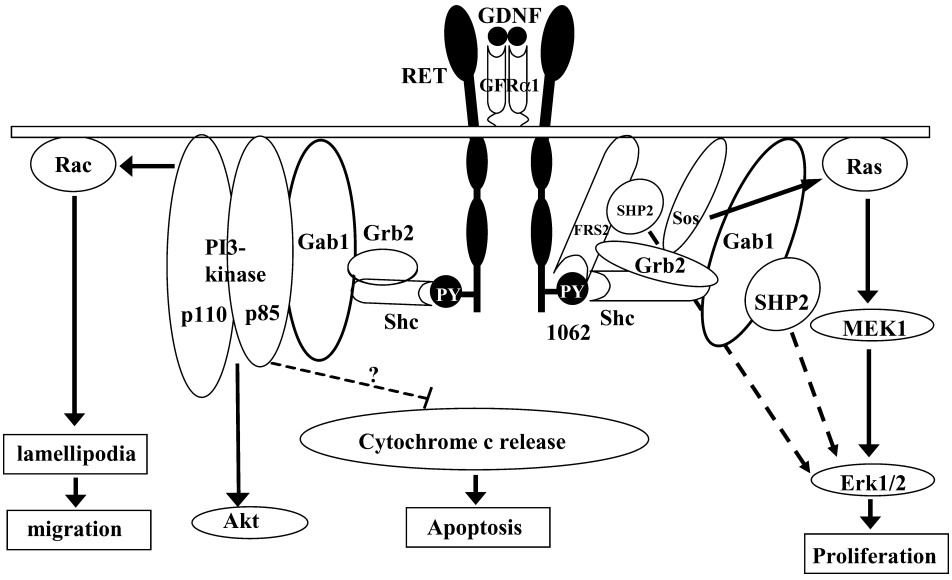

Fig. 8. Schematic illustration of the intracellular signaling pathways downstream of RET tyrosine kinase.

lamellipodia formation in MC(RET) cells in a protein kinase A (PKA)-dependent manner [40], suggesting that both PI3K and PKA pathways cooperate for Rac activation in GDNF-treated cells. In this study, we found that overexpression of Gab1 PI3K-m protein effectively inhibited Rac activation as well as lamellipodia formation in MC(RET) cells induced by GDNF. These results revealed that interaction between Gab1 and p85 of PI3K is also crucial for Rac activation and lamellipodia formation in the RET signaling pathway (Fig. 8).

It is well known that the PI3K-Akt pathway plays an important role in cell survival. Akt can promote cell survival through several pathways, such as phosphorylation of BAD, forkhead-related transcription factors, and caspase-9, and activation of NFκB [42,45,46]. It has been reported that activated RET can also promote cell survival in neuronal and tumor cells [47–50], although the anti-apoptotic effect of activated RET has not been clearly demonstrated. Previous studies have shown that the kinase activity of RET, activation of the PI3K-Akt pathway, and NFκB activation are indispensable for cell survival mediated by RET [47–49]. Here we showed that overexpression of Gab1 PI3K-m, but not wild-type Gab1 and SHP2-m, increased apoptosis in TT cells expressing RET with a *MEN2A* mutation, and this accompanied increased cytochrome *c* release. Because expression of Gab1 wild-type and SHP2-m slightly increased Akt phosphorylation in TT cells, this suggests that the Akt pathway may play a role in the survival of TT cells. However, expression of wild-type Gab1 and SHP2-m did not inhibit apoptosis of TT cells induced by etoposide. Because an active form of RET (RET-MEN2A) is expressed in TT cells, additional expression of wild-type Gab1 and SHP2-m may not result in a significant increase of the anti-apoptotic effect. Alternatively, it is possible that the Akt pathway is not involved in apoptosis of TT cells induced by etoposide. This latter view is supported by the fact that expression of Gab1 PI3K-m did not affect Akt phosphorylation in TT cells, despite significant increase of apoptotic cells after treatment with etoposide.

The finding that cytochrome *c* release increased in TT cells expressing Gab1 PI3K-m suggested that apoptosis was induced via a mitochondrial pathway, although we could not detect a difference in the expression level of p53 and Bcl-2, the phosphorylation level of Ser-136 of Bad, and caspase-3 activation among TT cells expressing wild-type or mutant Gab1 (data not shown). Because apoptotic cells were about 7–20% of etoposide-treated TT transfectants, it may be difficult to detect the differences in expression or phosphorylation levels of these molecules by immunoblotting. In addition, c-Jun phosphorylation was compared among transfectants because the JNK pathway is activated through tyrosine 1062 of RET [26] and was reported to participate in the cell death pathway activated by GDNF withdrawal [50]. However, we could not detect a difference in the phosphorylation level of c-Jun serine 63 among TT transfectants (data not shown). Thus, further investigation is necessary to elucidate apoptotic signals via a mitochondrial pathway downstream of PI3K that are activated by etoposide in TT cells.

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