Novel Mechanism of Regulation of Rac Activity and Lamellipodia Formation by RET Tyrosine Kinase*

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Rac activation in neuronal cells plays an important role in lamellipodia formation that is a critical event for neuritogenesis. It is well known that the Rac activity is regulated via activation of phosphatidylinositol 3-kinase (PI3K) by a variety of receptor tyrosine kinases. Here we show that increased serine phosphorylation on RET receptor tyrosine kinase following cAMP elevation promotes lamellipodia formation of neuronal cells induced by glial cell line-derived neurotrophic factor (GDNF). We identified serine 696 in RET as a putative phosphorylation site by protein kinase A and found that mutation of this serine almost completely inhibited lamellipodia formation by GDNF without affecting activation of the PI3K/AKT signaling pathway. Mutation of tyrosine 1062 in RET, whose phosphorylation is crucial for activation of PI3K, also inhibited lamellipodia formation by GDNF. Inhibition of lamellipodia formation by mutation of either serine 696 or tyrosine 1062 was associated with decrease of the Rac1-guanine nucleotide exchange factor (GEF) activity, suggesting that this activity is regulated by two different signaling pathways via serine 696 and tyrosine 1062 in RET. Moreover, in the presence of serine 696 mutation, lamellipodia formation was rescued by replacing tyrosine 687 with phenylalanine. These findings propose a novel mechanism that receptor tyrosine kinase modulates actin dynamics in neuronal cells via its cAMP-dependent phosphorylation.

The RET proto-oncogene encodes a receptor tyrosine kinase the ligands of which are members of the glial cell line-derived neurotrophic factor (GDNF) family protein, including GDNF, neurturin, artemin, and persephin (1, 2). These neurotrophic factors signal through multisubunit receptor complexes consisting of RET and glycosylphosphatidylinositol-anchored coreceptor called GDNF family receptor α1–4 (GFRα1–4). It turned out that the GDNF/RET signaling plays an important role in survival or differentiation of various neurons as well as kidney organogenesis (3–6). In addition, RET mutations are responsible for development of several human diseases such as papillary thyroid carcinoma, multiple endocrine neoplasia types 2A and 2B, and Hirschsprung’s disease (1, 2).

RET can activate a variety of intracellular signaling pathways, including RAS/ERK, phosphatidylinositol 3-kinase (PI3K)/AKT, and phospholipase Cγ pathways (1, 2). As is the case for other receptor tyrosine kinases, phosphorylated tyrosine residues in RET represent docking sites for several adapter and effector molecules. For example, tyrosines at codons 905, 1015, 1062, and 1096 were identified as docking sites for Grb7, Grb10, phospholipase Cγ1, Shc/Enigma/Frs2/IRS-1/Dok, and Grb2, respectively (7–17). In particular, phosphorylation of tyrosine 1062 is crucial for activation of major intracellular signaling pathways, including the RAS/ERK, PI3K/AKT, JNK, p38 MAPK, and ERK5 pathways (15, 18–21). RET can also activate Rho family GTPases, including Rho, Rac, and Cdc42 that are involved in reorganization of the actin cytoskeleton responsible for cell motility and morphology (22–25). It is well known that Rho, Rac, and Cdc42 induce stress fiber, lamellipodia, and filopodia, respectively, as a result of actin rearrangements (26). Neurite outgrowth and growth cone response to neurotrophic factors appear to be affected by the activation levels of these small G-proteins (27–29).

Recently, it was demonstrated that cAMP functions as a key regulator for neuronal survival, regeneration, and growth cone remodeling mediated by neurotrophic factors (30–33). The increase of intracellular cAMP level results in the activation of protein kinase A (PKA) that affects a variety of biochemical and biological events in neuronal cells. In this study, we ask if cAMP elevation can modulate RET function and cytoskeletal rearrangement in neuronal cells induced by GDNF. Our experiments revealed that increased phosphorylation of serine 696 in RET by forskolin treatment promotes lamellipodia formation induced by GDNF and that mutation of this serine almost abolished its formation. Because mutation of tyrosine 1062 in RET that impairs the PI3K signaling also inhibited lamellipodia formation, these findings suggested that two different signaling pathways via serine 696 and tyrosine 1062 are involved in lamellipodia formation by GDNF, resulting from Rac1 activation. This represents the first demonstration that cytoskeletal rearrangement by activation of a receptor tyrosine kinase is regulated by its serine phosphorylation probably via cAMP-dependent mechanism.
EXPERIMENTAL PROCEDURES

Antibodies—A synthetic phosphopeptide (amino acids 691–701 in RET), including phosphoserine 696, was prepared. Rabbits were immunized with 500 μg of the peptide using the RIBI adjuvant system (RIBI ImmunoChem Research Inc.), and phosphorylation state-specific antibody was purified by immunofinity chromatography. Anti-RET and anti-phospho-RET (tyrosine 1062)-specific polyclonal antibodies were developed as described previously (34). Anti-phosphotyrosine monoclonal antibody was purchased from Upstate Biotechnology Inc., and anti-phospho-ERK and anti-phospho-AKT polyclonal antibodies were purchased from New England BioLabs. Anti-PKA-RII and anti-AKAP79 polyclonal antibodies were purchased from Santa Cruz Biotechnology.

Physical Construction and Transfection—Human RET cDNA was inserted into the pcDNA3.1/Zeo plasmid vector (Invitrogen). Point mutations were generated by using a QuikChange site-directed mutagenesis kit (Stratagene). Transfections were performed by the calcium phosphate precipitation method using a Mammalian Transfection kit (Stratagene). To obtain stable transfectants, colonies were selected in the presence of zeocin (150 μg/ml, Invitrogen).

Western Blotting—Culture cells were lysed at SDS sample buffer (20 mM Tris-HCl, pH 6.8, 2% SDS, 2% DTT, 10% glycerol, 50 mM PMSF, 1 mM leupeptin, 1 mM PMSF). The lysates were centrifuged at 15,000 × g for 10 min at 4 °C, and the remaining supernatants were used for SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes (Millipore). Membranes were blocked for 30 min at room temperature in 3% albumin (in TBS) and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were analyzed by Western blotting.

In Vitro Phosphorylation—A portion of RET juxtamembrane region containing amino acids 662–723 was cloned into pGEX-2T vector (Amersham Biosciences, Inc.) and expressed in Escherichia coli as a glutathione S-transferase (GST) fusion protein. Proteins were purified on glutathione-Sepharose beads. In vitro phosphorylation of the fusion protein was overexpressed in 25 μl of phosphorylation buffer (50 mM Tris-HCl, pH 7.5, 7.5 mM MgCl₂, 20 mM NaCl, 0.1 mM EDTA, 1 mM DTT, and 460 kBq of [γ-32P]ATP (Amersham Biosciences, Inc.) for 30 min at 30 °C. MgCl₂ was then added (25 μM at a final concentration) to stabilize [γ-32P]ATP bound to GST-Rac1. Cell lysates (500 μg/300 μl) were added to the resulting [32P]-labeled GST-Rac1 in the presence of 2 mM cold GTP and 10 mM MgCl₂ at room temperature. Samples (30 μl) were removed at the indicated time and diluted with ice-cold termination buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 20 mM MgCl₂, 0.1 mM EDTA) and treated with the secondary antibody conjugated to horseradish peroxidase (swine anti-rabbit IgG-horseradish peroxidase, Dako) for 1 h at room temperature. The reaction was examined by an enhanced chemiluminescence detection kit (ECL, Amersham Biosciences, Inc.) according to the directions of the supplier.

RESULTS

Effects of cAMP Elevation on RET Phosphorylation and RET-mediated Lamellipodia Formation

Given the results of our previous experiments showing that RET contains a consensus phosphorylation motif and is a target of PKA (34), we investigated the role of RET tyrosine phosphorylation in the induction of lamellipodia formation by GDNF. For the first time, we observed that RET tyrosine phosphorylation, the activation of ERK and AKT phosphorylation, and the formation of lamellipodia were all significantly reduced by forskolin treatment (Fig. 1B). These results suggested that RET tyrosine phosphorylation, the activation of ERK and AKT, and lamellipodia formation were all affected by forskolin.

We next examined whether other cAMP-elevated cells also show similar results. We found that RET tyrosine phosphorylation and the activation of ERK and AKT were all significantly reduced by forskolin treatment (Fig. 1B). These results suggested that RET tyrosine phosphorylation, the activation of ERK and AKT, and lamellipodia formation were all affected by forskolin.

To confirm these observations, similar experiments were performed by using SK-N-MC human primitive neuroectodermal tumor cells transfected with human RET gene (designated MC/RET) cells. Although SK-N-MC cells endogenously express GFRα1 but not RET, lamellipodia formation was not induced by GDNF in them. When MC/RET cells were used, the formation of lamellipodia was accelerated by forskolin and impaired by KT5720 as observed for SH-SY5Y cells (Fig. 2B). These results showed that lamellipodia formation was regulated by both cAMP and cAMP-dependent mechanisms.

Identification of a Putative Phosphorylation Site on RET by GST Pull-down Assay—Rac activation was evaluated using the GST-CRIB pull-down assay (35). In brief, cells 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, 5 mM MgCl₂, 1 μM leupeptin, 1 mM PMSF). The lysates were centrifuged at 15,000 × g for 10 min at 4 °C, and the remaining supernatants were incubated for 30 min at 4 °C with GST-CRIB (Upstate Biochemicals). Protein complexes were washed three times with lysis buffer, boiled in SDS-sample buffer, and analyzed by Western blotting with anti-RET antibody (Sigma Chemical Co.) and then centrifuged at 5000 × g.

GDNF and lysed in 1 ml of lysis buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10 mM MgCl₂, 200 mM sucrose, 0.1 mM EDTA, 1 mM DTT, 0.5 mM sodium vanadate, 1 μM leupeptin, 1 mM PMSF). The cleared cell lysates were assayed for GEF activity. GST-Rac1 (1 μg) bound to glutathione-Sepharose beads were incubated with 460 kBq of [γ-32P]GTP (Amersham Biosciences, Inc.) for 30 min at 30 °C. MgCl₂ was then added (25 μM at a final concentration) to stabilize [γ-32P]GTP bound to GST-Rac1. Cell lysates (500 μg/300 μl) were added to the resulting [32P]-labeled GST-Rac1 in the presence of 2 mM cold GTP and 10 mM MgCl₂ at room temperature. Samples (30 μl) were removed at the indicated time and diluted with ice-cold termination buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 20 mM MgCl₂, 0.1 mM EDTA) and treated with the secondary antibody conjugated to horseradish peroxidase (swine anti-rabbit IgG-horseradish peroxidase, Dako) for 1 h at room temperature. The reaction was examined by an enhanced chemiluminescence detection kit (ECL, Amersham Biosciences, Inc.) according to the directions of the supplier.

Immunoprecipitation—Cells were lysed in radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and the protease inhibitors (1 μg/ml leupeptin, 1 μg/ml apro- tinin, 5 μg/ml benzamidine, and 1 μg/ml pepstatin). The lysate was centrifuged at 15,000 × g for 20 min at 4 °C. The protein concentration of the supernatant was measured using the BCA protein assay kit (Pierce). The lysate was preincubated with protein A-Sepharose beads (Sigma Chemical Co.) and then centrifuged at 5000 × g for 5 min to remove the beads. The supernatant was incubated with 2 μg of antibody for 1 h at 4 °C and mixed with protein A-Sepharose beads (Sigma). The mixture was incubated for 1 h at 4 °C and centrifuged at 5000 × g for 1 min at 4 °C. After washing three times in lysis buffer and twice in high salt buffer, the sample was suspended in SDS-sample buffer and boiled for 5 min. Then the protein A-Sepharose beads were removed by centrifugation, and the proteins were separated by SDS-8% PAGE and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were analyzed by Western blotting.

In Vitro Phosphorylation—A portion of RET juxtamembrane region containing amino acids 662–723 was cloned into pGEX-2T vector (Amersham Biosciences, Inc.) and expressed in Escherichia coli as a glutathione S-transferase (GST) fusion protein. Proteins were purified on glutathione-Sepharose beads. In vitro phosphorylation of the fusion protein was performed by PKA (10 units, catalytic subunit, Promega) was carried out overexpressed in 25 μl of phosphorylation buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM DTT, and 460 kBq of [γ-32P]ATP (Amersham Biosciences, Inc.) for 30 min at 30 °C. The reaction was terminated by adding SDS-sample buffer. Products were boiled and subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. The identification of a putative phosphorylation site on RET by GST pull-down assay confirmed that RET tyrosine phosphorylation, the activation of ERK and AKT phosphorylation, and the formation of lamellipodia were all affected by forskolin.
The phosphorylation of the mutant peptide was observed (Fig. 3B). When serine 696 was replaced with alanine, no phosphorylation occurred. This indicates that this peptide is phosphorylated by PKA, which was confirmed by an in vitro assay using a GST-fused peptide containing amino acids 662–723 of RET.

To elucidate whether serine 696 is phosphorylated, we developed a polyclonal antibody that recognizes phosphorylated serine 696 (designated anti-RET(pS696) antibody). We further investigated whether RET is associated with PKA and/or PKA-anchoring protein, AKAP79, which is known to link to the plasma membrane (36). The cell lysates from untreated or treated SK-N-MC cells were coimmunoprecipitated with RET from the lysates of MC(RET) and S696A cells. Simultaneously, we developed a polyclonal antibody that recognizes phosphorylated tyrosine 1062 (anti-RET(pY1062)) (34) showed that phosphorylation of tyrosine 1062 was also increased, suggesting that the association of RET with AKAP79 and PKA is present in a GDNF-independent manner and is not affected by the S696A mutation. In addition, lamellipodia formation by forskolin and db-cAMP was mediated by direct phosphorylation of this serine by PKA. To investigate whether serine 696 represents a phosphorylation site for PKA, we carried out an in vitro phosphorylation assay, using a GST-fused peptide containing amino acids 662–723 of RET. The assay showed that serine 696 is phosphorylated by PKA (Fig. 3B). When serine 696 was replaced with alanine, no phosphorylation of the mutant peptide was observed (Fig. 3B).

When the SH-SY5Y cells were transfected with forskolin or db-cAMP–stimulated lamellipodia formation was observed (data not shown). These results thus suggested the possibility that PKA could be positioned in close proximity to RET through binding to AKAP79.

Inhibition of Lamellipodia Formation by Mutation of Serine 696 or Tyrosine 1062—Surprisingly, lamellipodia formation was hardly induced by GDNF in the S696A cells (Fig. 4, A and B). We confirm this phenomenon using three independent transfectants expressing RET with the S696A mutation. Unlike in MC(RET) cells, the level of RET tyrosine phosphorylation did not significantly changed in S696A cells after forskolin treatment (Fig. 4C). Because it is well known that the representative intracellular signaling pathways, including the RAS/ERK and PI3K/AKT pathways, are activated mainly through tyrosine 1062 present in the carboxyl-terminal tail of RET (Fig. 3A) (18–20), phosphorylation levels of tyrosine 1062 were compared between MC(RET) cells and S696A cells. Western blot analysis with the antibody that specifically recognizes phosphorylated tyrosine 1062 (anti-RET(pY1062)) (34) showed that phosphorylation of tyrosine 1062 was significantly increased (Fig. 4C), suggesting that the association of RET with AKAP79 and PKA is present in a GDNF-independent manner and is not affected by the S696A mutation. In addition, lamellipodia formation by forskolin and db-cAMP was mediated by direct phosphorylation of this serine by PKA. To investigate whether serine 696 represents a phosphorylation site for PKA, we carried out an in vitro phosphorylation assay, using a GST-fused peptide containing amino acids 662–723 of RET. The assay showed that serine 696 is phosphorylated by PKA (Fig. 3B). When serine 696 was replaced with alanine, no phosphorylation of the mutant peptide was observed (Fig. 3B).

When the SH-SY5Y cells were transfected with forskolin or db-cAMP, the levels of RET phosphorylation recognized by this antibody significantly increased (Fig. 3C). In contrast, a phosphorylated band was detected neither in the lysate from KT5720-treated SH-SY5Y cells nor in the lysate from forskolin-treated S696A cells (Fig. 3C), suggesting that serine 696 represents a putative phosphorylation site by PKA in vivo.

We further investigated whether RET is associated with PKA and/or PKA-anchoring protein, AKAP79, which is known to link to the plasma membrane (36). The cell lysates from MC(RET) and S696A cells were immunoprecipitated with anti-RET antibody, followed by immunoblotting with anti-PKA or anti-AKAP79 antibody. Interestingly, both PKA and AKAP79 were coimmunoprecipitated with RET from the lysates of GDNF-untreated or treated MC(RET) and S696A cells to similar degree (Fig. 3D), suggesting that the association of RET with AKAP79 and PKA is present in a GDNF-independent manner and is not affected by the S696A mutation. In addition, lamellipodia formation by forskolin and db-cAMP was mediated by direct phosphorylation of this serine by PKA. To investigate whether serine 696 represents a phosphorylation site for PKA, we carried out an in vitro phosphorylation assay, using a GST-fused peptide containing amino acids 662–723 of RET. The assay showed that serine 696 is phosphorylated by PKA (Fig. 3B). When serine 696 was replaced with alanine, no phosphorylation of the mutant peptide was observed (Fig. 3B).

To elucidate that serine 696 is phosphorylated in vivo, we transfected RET cDNA with the S696A mutation into SK-N-MC cells and established the cell lines expressing the mutant protein (designated S696A cells). Simultaneously, we developed a polyclonal antibody that recognizes phosphorylated serine 696 in RET (designated anti-RET(pS696) antibody). When the SH-SY5Y cells were treated with forskolin or db-cAMP, the levels of RET phosphorylation recognized by this antibody significantly increased (Fig. 3C). In contrast, a phosphorylated band was detected neither in the lysate from KT5720-treated SH-SY5Y cells nor in the lysate from forskolin-treated S696A cells (Fig. 3C), suggesting that serine 696 represents a putative phosphorylation site by PKA in vivo.

When the SH-SY5Y cells were transfected with forskolin or db-cAMP, the levels of RET phosphorylation recognized by this antibody significantly increased (Fig. 3C). In contrast, a phosphorylated band was detected neither in the lysate from KT5720-treated SH-SY5Y cells nor in the lysate from forskolin-treated S696A cells (Fig. 3C), suggesting that serine 696 represents a putative phosphorylation site by PKA in vivo.
lysates from SH-SY5Y cells untreated or treated with forskolin (100 nM). Phosphorylation of the designated peptide by PKA is shown (left panel). C, in vivo phosphorylation of serine 696. The lysates from SH-SY5Y cells untreated or treated with forskolin (100 nM) were immunoprecipitated with anti-RET antibody, followed by immunoblotting with anti-RET(pS696) or anti-RET antibody (left panel). Similarly, the lysates from forskolin-treated MC(RET) or S696A cells were analyzed (right panel). S696A cells represent SK-N-MC cells expressing RET with the S696A mutation. D, association of RET with PKA and AKAP79. The lysates from MC(RET) and S696A cells were immunoprecipitated by anti-RET antibody, followed by immunoblotting with anti-AKAP79, anti-PKA-RII, or anti-RET antibody. Anti-PKA-RII represents the antibody generated against the type II regulatory subunit (RII) of PKA. 79-kDa AKAP79, 52-kDa type II regulatory subunit of PKA, and 150- and 170-kDa RET are indicated by arrows.

Identification of a putative phosphorylation site on RET by PKA. A, a schematic illustration of RET protein. The locations of serine 696 and tyrosine 1062 are shown. TM, transmembrane domain; TK, tyrosine kinase domain. B, in vitro phosphorylation of serine 696 by PKA. GST-fused peptides containing amino acids 662–723 of RET with or without the S696A mutation were produced and stained with Coomassie Brilliant Blue (left panel). Phosphorylation of the designated peptide by PKA is shown (right panel). C, in vivo phosphorylation of serine 696. The lysates from SH-SY5Y cells untreated or treated with forskolin (100 nM) were immunoprecipitated with anti-RET antibody, followed by immunoblotting with anti-RET(pS696) or anti-RET antibody (left panel). Similarly, the lysates from forskolin-treated MC(RET) or S696A cells were analyzed (right panel). S696A cells represent SK-N-MC cells expressing RET with the S696A mutation. D, association of RET with PKA and AKAP79. The lysates from MC(RET) and S696A cells were immunoprecipitated by anti-RET antibody, followed by immunoblotting with anti-AKAP79, anti-PKA-RII, or anti-RET antibody. Anti-PKA-RII represents the antibody generated against the type II regulatory subunit (RII) of PKA. 79-kDa AKAP79, 52-kDa type II regulatory subunit of PKA, and 150- and 170-kDa RET are indicated by arrows.

To confirm the importance of Rac1 activity for lamellipodia formation, we transiently transfected dominant-active Rac1 tagged with hemagglutinin A (HA) into MC(RET), S696A (Fig. 5B), or Y1062F (data not shown) cells, followed by staining with FITC-phalloidin and anti-HA antibody. Expression of dominant-active Rac1 induced lamellipodia formation in these transfectants without GDNF stimulation (Fig. 5B). In contrast, when HA-tagged dominant-negative Rac1 was transiently transfected, lamellipodia formation was inhibited in the transfectants treated with GDNF (Fig. 5B). These observations demonstrated that Rac1 activity is essential for lamellipodia formation induced by GDNF and that the S696A and Y1062F mutations strongly impaired its activity.

The functions of small G-proteins in response to extracellular signals are regulated by activities of guanine nucleotide exchange factor (GEF) or GTPase-activating proteins (GAP). Thus, we investigated whether impairment of Rac1 activity in S696A and Y1062F cells resulted from alteration of Rac1-GEF or Rac1-GAP activity. GST-Rac1 preincubated with [γ-32P]GTP was added to the lysates from MC(RET), S696A, and Y1062F cells in the presence of cold GTP, and GEF activity was determined by measuring the amount of [γ-32P]GTP retained on GST-Rac1. After GDNF stimulation, a significant increase in Rac1-GEF activity was observed in the lysate from MC(RET) cells but not in the lysate from the S696A and Y1062F cells (Fig. 5C). On the other hand, when GST-Rac1 preincubated with [γ-32P]GTP was subjected to Rac1-GAP assay, there was no significant difference in Rac1-GAP activity among MC(RET), S696A, and Y1062F cells treated with GDNF (Fig. 5C), suggesting that impairment of Rac1 activity by S696A or Y1062F mutation is mainly due to the decrease of Rac1-GEF activity. In addition,

**Fig. 3.** Identification of a putative phosphorylation site on RET by PKA. A, a schematic illustration of RET protein. The locations of serine 696 and tyrosine 1062 are shown. TM, transmembrane domain; TK, tyrosine kinase domain. B, in vitro phosphorylation of serine 696 by PKA. GST-fused peptides containing amino acids 662–723 of RET with or without the S696A mutation were produced and stained with Coomassie Brilliant Blue (left panel). Phosphorylation of the designated peptide by PKA is shown (right panel). C, in vivo phosphorylation of serine 696. The lysates from SH-SY5Y cells untreated or treated with forskolin (100 nM) were immunoprecipitated with anti-RET antibody, followed by immunoblotting with anti-RET(pS696) or anti-RET antibody (left panel). Similarly, the lysates from forskolin-treated MC(RET) or S696A cells were analyzed (right panel). S696A cells represent SK-N-MC cells expressing RET with the S696A mutation. D, association of RET with PKA and AKAP79. The lysates from MC(RET) and S696A cells were immunoprecipitated by anti-RET antibody, followed by immunoblotting with anti-AKAP79, anti-PKA-RII, or anti-RET antibody. Anti-PKA-RII represents the antibody generated against the type II regulatory subunit (RII) of PKA. 79-kDa AKAP79, 52-kDa type II regulatory subunit of PKA, and 150- and 170-kDa RET are indicated by arrows.
we found that the Rac1-GEF activity was strongly impaired in the KT5720-treated MC(RET) cells stimulated with GDNF (data not shown).

**A Y687F Mutation Rescues Lamellipodia Formation in S696A Cells**—The finding that forskolin and db-cAMP treatment decreased RET tyrosine phosphorylation (Fig. 1, A and B) suggested that a few tyrosine residues in RET may be dephosphorylated as a result of serine 696 phosphorylation responsible for acceleration of lamellipodia formation. Because tyrosine 687 is present near serine 696 and was reported to be auto-phosphorylated in activated RET (37), we replaced this tyrosine with phenylalanine (Y687F) to see the importance of its phosphorylation to actin rearrangement. The Y687F mutation was introduced into RET cDNA with or without the S696A mutation, and the resulting mutant cDNAs were transiently transfected into SK-N-MC cells and stimulated with GDNF. Interestingly, lamellipodia formation was induced in ∼30% of the S696A/Y687F mutant cells as well as the Y687F cells in repeated experiments (Fig. 6). This suggested the possibility that a signal that negatively regulates the Rac1 activity could be transduced through tyrosine 687 (Fig. 7A).

**DISCUSSION**

The present study demonstrated that phosphorylation at serine 696 in RET is required for activation of Rac1-GEF as well as lamellipodia formation by GDNF. We identified serine 696 as a putative phosphorylation site by PKA, although it is possible that this phosphorylation is also induced by other kinases in a cAMP-dependent fashion (Fig. 7A). Increased levels of serine 696 phosphorylation by forskolin and db-cAMP accelerated lamellipodia formation by GDNF whereas inhibition of this phosphorylation by KT5720 almost abolished it. To our knowledge, this is the first report showing that lamellipodia formation induced by activation of receptor tyrosine kinase is regulated by its serine phosphorylation probably via a cAMP-dependent mechanism. It could be possible that other members of receptor tyrosine kinases also contain cAMP-dependent phosphorylation sites that play important roles in their biological activities. In addition, the results may suggest cross-talk between receptor tyrosine kinases and other receptors such as G-protein-coupled receptors that are known to activate PKA in neuronal cells (Fig. 7A) (38, 39). Although GDNF stimulation itself was reported to elevate intracellular cAMP levels to some degree (32), the level of serine 696 phosphorylation in RET did not significantly increase in GDNF-treated SH-SY5Y cells as compared with untreated cells (data not shown), suggesting the importance of a basal cAMP-dependent kinase activity for lamellipodia formation.

We also demonstrated that RET is associated with AKAP79 that is known to be a PKA-anchoring protein (40). It was reported that the carboxyl-terminal region of AKAP79 contains a high affinity binding site for the type II regulatory subunit (RII) of PKA and that AKAP79 and RII are coenriched and colocalized in neurons that utilize the PKA signaling pathway (36, 41). Because AKAP79 appears to be associated with the plasma membrane through lipid-protein interactions (36), PKA could be also positioned in close proximity to the plasma mem-

**FIG. 4. Inhibition of lamellipodia formation by a mutation at serine 696 or tyrosine 1062.** A, inhibition of lamellipodia formation by S696A or Y1062F mutation. The designated cells were untreated or treated with forskolin (100 μM) for 120 min, followed by stimulation with GDNF, and stained with FITC-phalloidin. Lamellipodia formation was almost undetectable in S696A and Y1062F cells. Y1062F cells represent the SK-N-MC cells expressing RET in which tyrosine 1062 was replaced with phenylalanine. B, quantitative analysis of lamellipodia formation. Results represent averages from three independent experiments. C, phosphorylation of tyrosine 1062 and activation of ERK and AKT by GDNF in S696A cells. The MC(RET), S696A, and Y1062F cells untreated or treated with forskolin (100 μM) for 120 min were stimulated with GDNF (150 ng/ml) for 5 min. The resulting cell lysates were analyzed with the designated antibody.
brane through the AKAP79-PKA complex formation, facilitating its access to the juxtamembrane region of RET. Although it remains unknown whether the association of AKAP79-PKA complex with RET is direct or indirect, this finding suggested that the membrane targeting of PKA through this complex formation in the cells may be a crucial step for RET phosphorylation (Fig. 7A).

It has been well established that activation of PI3K by receptor tyrosine kinases is important for Rac activity, leading to lamellipodia formation (42). In the case of RET, PI3K is activated mainly via phosphorylated tyrosine 1062 that is a binding site for Shc adaptor proteins (15, 18–20). Shc bound to tyrosine 1062 further mediates the complex formation of Gab1/2 and p85 subunit of PI3K, resulting in the activation of PI3K (18, 19, 43). Consistent with this finding, our current results showed that the Y1062F mutation almost completely inhibited the Rac1-GEF activity and lamellipodia formation induced by GDNF. In addition, van Weering and Bos (22) reported that RET-mediated lamellipodia formation in SK-N-MC cells was inhibited by treatment with PI3K inhibitors, wortmannin or LY294002, confirming a crucial role of PI3K in its formation (Fig. 7A). Thus, the Rac1-GEF activity could be regulated by PI3K activation via the Shc-Gab1/2 association on tyrosine 1062.

Although it is possible that other cAMP-dependent phosphorylation sites are present in the RET intracellular domain, our results revealed that phosphorylation of serine 696 plays a crucial role in lamellipodia formation by GDNF. A proposed model for regulation of lamellipodia formation mediated by serine 696 phosphorylation is shown in Fig. 7. As judged from the results of the experiments using S696A and Y687F mutant cells, phosphorylation of serine 696 and tyrosine 687 appeared to induce opposite effects on lamellipodia formation (Fig. 7, A and B). Increased levels of serine 696 phosphorylation by forskolin and db-cAMP treatment appeared to inhibit Rac1 activity, whereas the S696A mutation impaired the Rac1-GEF activity and lamellipodia formation without affecting phosphorylation of tyrosine 1062 and activation of the PI3K/AKT signaling pathway. More in-
Interestingly, lamellipodia formation by GDNF was recovered in S696A/Y687F double mutant cells (Fig. 7B). Although tyrosine 687 in RET was previously identified as an autophosphorylation site by phosphopeptide mapping (37), its role in the signal transduction has not been elucidated so far. We speculate that phosphorylation of serine 696 may induce a conformational change of the juxtamembrane region of RET and inhibit phosphorylation of tyrosine 687 that is suggested to negatively regulate the Rac1-GEF activity (Fig. 7A). The change of conformation or electric charge in the juxtamembrane region induced by phosphorylation of either serine 696 or tyrosine 687 may be involved in the regulation of the Rac1-GEF activity responsible for lamellipodia formation. Taken together, our results strongly supported the view that the Rac1-GEF activity is regulated by two different signaling pathways via serine 696/tyrosine 687 and tyrosine 1062 in RET (Fig. 7).

To further elucidate the role of tyrosine 687 phosphorylation in lamellipodia formation, it will be necessary to investigate the change of its phosphorylation levels by forskolin treatment or change of the juxtamembrane region of RET and inhibit phosphorylation of tyrosine 687 that is suggested to negatively regulate the Rac1-GEF activity (Fig. 7A). The change of conformation or electric charge in the juxtamembrane region induced by phosphorylation of either serine 696 or tyrosine 687 may be involved in the regulation of the Rac1-GEF activity responsible for lamellipodia formation. Taken together, our results strongly supported the view that the Rac1-GEF activity is regulated by two different signaling pathways via serine 696/tyrosine 687 and tyrosine 1062 in RET (Fig. 7).

Fig. 7. A model for lamellipodia formation mediated by GDNF/RET signaling. A, a deduced mechanism of Rac-GEF activation by GDNF. B, modulation of Rac-GEF activity by S696A and Y687F mutations.
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