Regulation of Protein Kinase B Tyrosine Phosphorylation by Thyroid-Specific Oncogenic RET/PTC Kinases

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Papillary thyroid carcinoma (PTC) is a heterogenous disorder characterized by unique gene rearrangements and gene mutations that activate signaling pathways responsible for cellular transformation, survival, and antiapoptosis. Activation of protein kinase B (PKB) and its downstream signaling pathways appears to be an important event in thyroid tumorigenesis. In this study, we found that the thyroid-specific oncogenic RET/PTC tyrosine kinase is able to phosphorylate PKB in vitro and in vivo. RET/PTC-transfected cells showed tyrosine phosphorylation of endogenous and exogenous PKB, which was independent of phosphorylation of T308 and S473 regulated by the upstream kinases phosphoinositide-dependent kinase-1 and -2, respectively. The PKB Y315 residue, which is known to be phosphorylated by Src tyrosine kinase, was also a major site of phosphorylation by RET/PTC. RET/PTC-mediated tyrosine phosphorylation results in the activation of PKB kinase activity. The activation of PKB by RET/PTC blocked the activity of the forkhead transcription factor, FKHR1, but a Y315F mutant of PKB failed to inhibit FKHR1 activity. In summary, these observations suggest that RET/PTC is able to phosphorylate the Y315 residue of PKB, an event that results in maximal activation of PKB for RET/PTC-induced thyroid tumorigenesis. (Molecular Endocrinology 19: 2748–2759, 2005)

Papillary thyroid carcinoma (PTC) is a heterogenous disorder characterized by unique gene rearrangements and gene mutations that activate signaling pathways responsible for cellular transformation, survival, and antiapoptosis. These effectors in turn mediate the effects of PKB on cell growth, proliferation, protection from proapoptotic stimuli, and migration. The full activation of PKB is a multistep process and several proteins responsible for each step have been identified and characterized. PI3-K regulates the intracellular levels of PtdIns(3,4,5)P3 and PtdIns(3,4)P2, which are needed for the activation of PKB by phosphorylation (11–13). PKB has multiple phosphorylation sites in its serine/threonine and tyrosine residues. T308 and S473 are phosphorylated by phosphoinositide-dependent kinase (PDK)-1 and -2, respectively, when cells are stimulated by a PI3-K activator (14, 15); the phosphorylation of these two sites is necessary for the full activation of PKB (15). Recently, two groups reported that PKB is also activated through the phosphorylation of tyrosine residues (16, 17). PKB Y315 and Y26 were identified as sites apparently required for activation by EGF (16), and Y474 in the hydrophobic motif was found to be phosphorylated after IGF-1 stimulation (17).

Protein tyrosine kinases are important regulators of intracellular signal transduction pathways and their activity is tightly controlled and regulated in normal cells. Perturbation of tyrosine kinase signaling by mutations and other genetic alterations results in deregulation of several downstream effectors, such as nuclear factor-κB (6), mammalian target of rapamycin (mTOR) (7), Forkhead transcription factors (8), glycogen synthase kinase-3 (9), and murine double minute-2 (10).
ulated kinase activity. The BCR/ABL (breakpoint cluster region/v-abl Abelson murine leukemia viral oncogene homolog 1) oncogenic tyrosine kinase is able to activate PKB and this phenomenon is essential for leukemogenesis (18). TEL-JAK2 (ets variant gene 6/Janus kinase), a chimeric protein tyrosine kinase found in the subset of acute leukemia constitutively activates PKB (18). The Src homology 2 domains in BCR/ABL and TEL-JAK2 interact with the regulatory p85 subunit of PI3-K to initiate the activation of PKB (19).

Gene rearrangements of the RET receptor tyrosine kinase are specifically associated with thyroid cancer (1). RET mediates the cellular responses to glial cell-derived neurotrophic factor family ligands, including glial cell-derived neurotrophic factor, artemin, neuturin, and persephin (20, 21), and activates several intracellular signaling cascades, which regulate cell survival (22), differentiation (22), proliferation (23), migration (24), chemotaxis (24), branching morphogenesis (25), and neurite outgrowth (26). Point mutations and gene rearrangements convert RET into a dominantly acting transforming gene, leading to endocrine tumors. The point mutations MEN-2A and MEN-2B and the RET/PTC rearrangement are three major oncogenic mutations of RET that result in constitutive receptor activation and catalytic activity (27–29). It has been reported that RET proteins carrying the MEN-2A mutation or RET/PTC rearrangement activate PI3-K and PKB (30–32). Interactions of adapters or the regulatory subunits of PI3-K with the C-terminal region of RET have been suggested to provide the molecular basis for activation of PI3-K and PKB (32). Src and RET/PTC have been reported to share substrates, and are able to phosphorylate the same tyrosine residues in signal transducer and activator of transcription (STAT) 1 and STAT3 (33–35). In addition, they also activate PDK1 by phosphorylating specific tyrosine residues in PDK1 (35). In this study, we show that PKB is another such shared substrate. RET/PTC activates PKB through tyrosine phosphorylation and reduces the activity of the forkhead transcription factor, FKHRL1.

RESULTS

RET/PTC Tyrosine Kinase Phosphorylates PKB in Vitro and in Vivo

Previous studies have been showed that Src and RET/PTC tyrosine kinases share substrates in the transformation of the cells (33–35). To test whether PKB might be one of these common substrates, we examined the tyrosine phosphorylation of PKB by Src and RET/PTC. PKB obtained by immunoprecipitation from cells transfected with a Src and RET/PTC, PKB obtained by immunoprecipitation from cells transfected with a Src and RET/PTC expression vector showed tyrosine phosphorylation (Fig. 1A). These observations indicate that PKB may be one of the common targets of Src and RET/PTC signaling pathways.

We next examined whether RET/PTC3 is able to phosphorylate PKB in vitro, using recombinant PKB

Fig. 1. RET/PTC Tyrosine Kinase Phosphorylates PKB in Vitro and in Vivo and Requires for Its Kinase Activity

A, NIH3T3 cells were cultured in 6-cm dishes until 80% confluence, and transfected with RET/PTC3 (1 μg/dish) or Flag-Src (1 μg/dish) and HA-PKB (1 μg/dish). Twenty-four hours after transfection, the cells were lysed, and the cell lysates were subjected to immunoprecipitation (IP) as described in Materials and Methods. B, In vitro phosphorylation of PKB by RET/PTC3. Recombinant RET/PTC3 and PKB were purified from insect cells as described in Materials and Methods. The reaction was stopped by the addition of 3× SDS sample buffer. Sample aliquots were subjected to SDS-PAGE, transferred to Immobilon-P membranes, and probed with antiphosphotyrosine antibody (4G10). The loading of equal amounts of PKB was verified by staining the SDS-PAGE gels with Coomassie blue. WB, Western blot.
and purified RET/PTC3 protein obtained from baculovirus (Fig. 1B). The levels of active phosphorylated RET/PTC3 in the reaction mixture gradually increased due to autophosphorylation. PKB incubated without RET/PTC3 (Fig. 1B, lane 2) did not show phosphorylation, but the phosphorylation of PKB increased along with the amount of active RET/PTC3 (Fig. 1B), suggesting that RET/PTC is able to phosphorylate PKB directly.

RET/PTC isoforms share a common kinase domain, but they have different N-terminal coiled-coil domains, which originate from different genes by genomic rearrangement (36). RET/PTC3 and RET/PTC1 are the rearranged forms of RET/PTC most frequently found in patients with papillary thyroid cancers (37). We cotransfected RET/PTC1 or RET/PTC3 with hemagglutinin (HA)-PKB and observed the tyrosine phosphorylation of the immunoprecipitated PKB (Fig. 2A). RET/PTC3 and RET/PTC1 produced the same level of tyrosine phosphorylation of PKB (Fig. 2A, lane 3 vs. lane 4), indicating that the N-terminal domains of RET/PTC1 and RET/PTC3 (H4 and ELE1, respectively) do not influence the tyrosine phosphorylation of PKB. PKB is activated upon binding of PI3K lipid products to its pleckstrin homology domain and by phosphorylation of its T308 and S473 residues by PDK1 and PDK2, respectively. We therefore tested whether PKB was activated in cells expressing RET/PTC1 and RET/PTC3. As shown in Fig. 2A, PKB became phosphorylated on T308 in cells expressing RET/PTC1 and RET/PTC3.

The kinase domain and a specific tyrosine residue of RET or RET/PTC are important in the signaling and transformation processes (38–40). The kinase activity is related to tyrosine phosphorylations of PDK1 and STATs and is associated with tumor formation in the thyroid gland of a transgenic animal model (33–35). In addition, Y1062 in RET (correspondent to Y588 in RET/PTC3) is a critical residue for the interaction of adapters and involved in transformation (38, 40). To identify the role of kinase domain and Y588 in the tyrosine phosphorylation of PKB, we examined whether the expression of wild-type RET/PTC3, kinase-deficient RET/PTC3 K284M, or RET/PTC3 Y588F affected the level of tyrosine-phosphorylated PKB in human embryonic kidney (HEK) 293 cells (Fig. 2B). The RET/PTC3 wild-type and Y588F induced tyrosine phosphorylations of PKB to the same extent. However, the kinase-deficient RET/PTC3 K284M failed to induce significant tyrosine phosphorylation of PKB (Fig. 2B). Interestingly, T308 phosphorylation of PKB was increased by expression of RET/PTC3; however, the kinase-deficient RET/PTC3 and RET/PTC3 Y588F mutants did not induce T308 phosphorylation of PKB. The kinase-deficient RET/PTC3 K284M mutant is unable to autophosphorylate tyrosine residues, especially the Y588 residue, which is known to be critical for PI3K-dependent PKB activation. These observations suggest that RET tyrosine kinase activity and intactness of the Y588 residue for PI3K activation are both important for full activation of PKB.

Human thyroid papillary carcinoma (TPC1) cells are derived from papillary thyroid cancer and harbor an endogenous, constitutively active RET/PTC1 rearrangement. As shown in 2C, endogenous PKB immunoprecipitated from TPC1 cells was tyrosine phosphorylated. In contrast, PKB immunoprecipitated from ARO cells, an anaplastic RET/PTC-negative thyroid
cancer cell line, did not show tyrosine phosphorylation. The indoline compound, SU11248, is a highly active inhibitor of RET/PTC tyrosine kinase (Chung H. S., and M. Shong, manuscript in preparation). Treatment of TPC1 cells with SU11248 decreased tyrosine phosphorylation of PKB. These findings suggest that RET/PTC1-positive thyroid cancer cells phosphorylate endogenous PKB.

RET/PTC3 Interacts with PKB

The above observations suggest that RET/PTC tyrosine kinase is able to phosphorylate PKB. To test whether RET/PTC3 and PKB interact directly, we performed immunoprecipitation experiments and found that RET/PTC3 coimmunoprecipitated with PKB (Fig. 3A). Immunocytochemistry and confocal microscopy of NIH3T3 cells showed that RET/PTC3 is mainly confined to the cytoplasm, whereas PKB is distributed in the cytoplasm and nucleus. The merged image in Fig. 3B suggests that RET/PTC3 and PKB colocalize in the cytoplasm.

RET/PTC3-Mediated Phosphorylation of PKB Is Independent from PI3-K and PDK1

The phosphorylation sites, T308 and S473 in PKB regulate kinase activity by generating an active conformation (41). To examine the roles of the T308 and S473 residues of PKB in activation by RET/PTC3, we compared the ability of RET/PTC3 to tyrosine phosphorylate wild-type PKB and two PKB mutants, T308A and S473A. As shown in Fig. 4A, the level of PKB tyrosine phosphorylation was the same in RET/PTC-transfected cells containing the wild-type PKB or either of the two mutants. The phosphorylation of the T308 residue of exogenous wild-type and mutant PKB S473A were observed after coexpression of RET/PTC3 (Fig. 4A). These data indicate that the T308 and S473 residues are not involved in RET/PTC-mediated tyrosine phosphorylation of PKB.

We also observed tyrosine phosphorylation of PKB in RET/PTC3-transfected HEK293 cells in the presence of inhibitors of PI3-K, MAPK kinase kinase, mTOR, and Src (Fig. 4B). RET/PTC3-induced phosphorylation of the T308 residue of PKB was inhibited by PI3K inhibitors, wortmannin and LY294002. However, the addition of these inhibitors did not significantly hamper the tyrosine phosphorylation of PKB by RET/PTC.

As shown in Fig. 1, because Src is able to phosphorylate PKB, the role of Src in RET/PTC-mediated tyrosine phosphorylation of PDK1 was investigated. We employed c-Src-deficient mouse embryo fibroblasts (MEFs) from c-Src null mice to determine the involvement of Src in RET/PTC3-induced tyrosine phosphorylation of PKB. Wild-type Src (+/+ ) MEFs and Src (−/− ) MEFs were cotransfected with RET/PTC3 and HA-PKB, and tyrosine phosphorylation of PKB was observed. RET/PTC3 induced Y9 phosphorylation in both Src (+/+ ) (data not shown) and Src (−/− ) cells (Fig. 4C, lane 4). These results and the finding that the Src inhibitor, PP1, did not affect RET/PTC3-induced tyrosine phosphorylation of PKB (Fig. 4B, lane 7) support the hypothesis that tyrosine phosphorylation of PKB by RET/PTC3 results from direct

![Fig. 3. RET/PTC3 Interact with PKB and Colocalize in Cytoplasm](image-url)

A. CHO cells were cultured in 6-cm dish until 80% confluence and transfected with RET/PTC3 (1 μg/dish) and HA-PKB (1 μg/dish). Twenty-four hours after transfection, whole-cell lysates were immunoprecipitated with an anti-HA and blotted with an anti-RET antibody. B. NIH 3T3 cells were cultured in a six-well dish until 80% confluence and transiently transfected with RET/PTC3 (0.5 μg/dish) and GFP-PKB (0.5 μg/dish). Twenty-four hours after transfection, cells were fixed in 3.7% formaldehyde for 40 min. Fixed cells were mounted onto glass slides with PBS and observed with a laser-scanning confocal microscope. GFP-fused wild-type PKB was detected by autofluorescence and RET/PTC3 was detected by staining with primary anti-RET antibody and rhodamine-conjugated secondary antibody. The yellow stain in the merged image depicted colocalization of RET/PTC3 and PKB. Similar results were obtained in three independent experiments.
phosphorylation events that do not require endogenous Src tyrosine kinase activity.

**RET/PTC3 Induces Y315 Phosphorylation in PKB**

Previous studies have shown that the proline-rich motif of PKB that includes the C terminus is essential for the PKB activity induced by Src (42). Interaction of the SH3 domain of Src with the proline-rich motif of PKB induced tyrosine phosphorylation on Y315 and Y326 residues of PKB but not on Y340 (16), although all three of these residues have been highly conserved during evolution (16). In addition, Y474, which is located in the regulatory domain of PKB, is directly phosphorylated by IGF and pervanadate (17). To verify whether these tyrosine residues are important for the PKB phosphorylation induced by RET/PTC3, we replaced them with phenylalanines, which does not change the conformation of the kinase domain (16).

We then examined HEK293 cells expressing the PKB constructs and wild-type RET/PTC3 using immunoprecipitation with an anti-HA antibody. We showed that the mutation of Y315 abolished PKB phosphorylation and mutation of Y326 decreased PKB phosphorylation slightly compared with the wild-type PKB.
The double mutation of Y315/326 also abolished RET/PTC3-induced PKB phosphorylation, but the Y340 and Y474 mutations had no effect on the level of phosphorylation (Fig. 5B). We next examined whether RET/PTC3 is able to phosphorylate PKB Y315F in vitro, using recombinant PKB Y315F and purified recombinant RET/PTC3 protein obtained from a recombinant baculovirus (Fig. 5C). PKB Y315F incubated with recombinant RET/PTC3 did not show phosphorylation, whereas PKB incubated without RET/PTC3 showed phosphorylation, suggesting that the Y315 residue of PKB is directly phosphorylated by RET/PTC3.

**RET/PTC3 Increases Tyrosine Phosphorylation-Dependent PKB Activity and Regulates Forkhead Transcription Factor**

To test whether RET/PTC3-induced tyrosine phosphorylation of PKB affect its activity, we measured PKB kinase activity with an immune complex kinase assay. PKB activity was higher in HEK293 cells co-transfected with RET/PTC3 and PKB than in cells transfected with PKB alone (Fig. 6). Mutation of Y315 abolished the RET/PTC3-induced increase in PKB activity. The double mutation of Y315 and Y326 strongly decreased the activation of PKB by RET/PTC3, but single mutation of Y340 or Y474 did not affect RET/PTC3-induced activation of PKB. These results suggest that phosphorylation of Y315 by RET/PTC3 is necessary for the full activation of PKB.

The human forkhead transcription factors FKHRL1, AFX, and FKHR are directly phosphorylated by activated PKB (43–45). FKHRL1 has three potential PKB phosphorylation sites [RXXRXX(S/T)]: T32 (RPRSCS32), S253 (RRRAVSS253), and S315 (RSRTNSS315). When cells are stimulated with serum or growth factors, FKHRL1 is phosphorylated by activated PKB and is exported from the nucleus to the cytoplasm, resulting in the down-regulation of target gene transcription (43–45). We examined the effects of RET/PTC and PKB expression on the ability of FKHRL1 to activate a promoter containing the FKHR binding motif (FRE) fused to a luciferase reporter gene (FRE-Luc). As shown in Fig. 7A, the reporter activities of FRE-Luc increased about 3.2-fold after cotransfection of the FKHRL1 expression plasmid. However, coexpression of RET/PTC3 or PKB decreased the FRE-Luc activity and simultaneous cotransfections of RET/PTC3 and PKB showed additive inhibition of FRE-Luc activity (Fig. 7A). To determine whether the RET/PTC3-mediated suppression of FRE-Luc activity requires intact kinase activity, we cotransfected the kinase-deficient RET/PTC3 K284M. RET/PTC3 K284M did not suppress FRE-Luc activity. In addition, the RET/PTC3 Y588F mutant inhibited FKHRL1 transcriptional activity but to a lesser degree than wild-type RET/PTC3 (Fig. 7B).

To determine whether the tyrosine kinase activity results in the repression of the transcriptional activities of FKHRL1, we measured FRE-Luc activity after transfection with wild-type and mutant RET/PTC3 (Fig. 7B). Again, the expression of PKB resulted in the suppression of the transcriptional activities of FKHRL1, and this repressive effect was markedly enhanced by cotransfection with wild-type RET/PTC3. However, kinase-deficient RET/PTC3 K284M failed to show suppression of FKHRL1-mediated transactivation. Interestingly, RET/PTC3 Y588F, which is known to interact with adapters for PI3-K activation, was also able to repress FKHRL1 activity.

To determine whether mutation of the PKB phosphorylation site affects the transcriptional activity of FKHRL1, we cotransfected RET/PTC3, FKHRL1, and
mutant PKB constructs into Chinese hamster ovary (CHO) cells (Fig. 7B). RET/PTC3-mediated repression of FKHRL1 activity was not evident in PKB Y315F-transfected cells. However, other PKB mutants, such as PKB Y326F, PKB Y315F/Y326F, PKB Y340F, and Y474F were as competent as wild-type PKB for RET/PTC3-induced repression of FKHRL1 transcription (Fig. 7C).

To determine whether RET/PTC3-mediated suppression of FRE-Luc activity was influenced by PI3-K, we observed FRE-Luc activity in cells transfected with RET/PTC3 in the presence or absence of PI3-K, Src and mTOR inhibitor. None of the inhibitors fully reversed the suppression of FRE-Luc activity in the cells transfected with RET/PTC3 (Fig. 7D).

**DISCUSSION**

RET/PTC rearrangements are observed specifically in thyroid gland, and may play an important role in the initiation and progression of specific types of thyroid cancer (30–32). Several studies have suggested that the activating RET mutants MEN2A, MEN2B, and RETPTC are able to activate PI3-K and PKB, an effect that is known to be critical for cellular transformation (30, 32). RET mutants are thought to activate PKB by recruiting adapters or the p85 regulatory subunit to a specific phosphotyrosine residue, Y1062, located in the C-terminal region of intracellular domain (32). It is possible that activated MEN2A and MEN2B recruits PI3-K in the membrane, where these kinases allow the generation of active phosphatidylinositol products, such as phosphatidyl inositol phosphate 3. This would lead to further activation by translocating kinases containing pleckstrin homology domain, such as PDK1 and PKB.

Unlike MEN2A and MEN2B, which are located in the plasma membrane, RET/PTC is mainly distributed in the cytoplasm (35, 46). Because the tyrosine residue in RET/PTC that corresponds to Y1062 of MEN2A and MEN2B is phosphorylated, it may recruit the p85 regulatory subunit of PI3-K. However, the generation of phosphatidyl inositol phosphate 3 products by RET/PTC-associated PI3-K is unlikely because most RET/PTC is distributed in cytoplasm. Therefore, RET/PTC may activate PKB through a different mechanism than MEN2A and MEN2B. A recent study demonstrated that RET/PTC induces tyrosine phosphorylation of PDK1, a pivotal kinase that activates PKB in the PI3-K signaling pathway (35). RET/PTC causes specific phosphorylation of the Y9 residue of PDK1, which causes further activation of PDK1 and regulates PKB and downstream signaling pathways. In this study, we add additional evidence that RET/PTC is able to activate PKB through the phosphorylation of a specific tyrosine residue that is known to be a target site of Src.

RET/PTC is able to phosphorylate several target proteins, including PDK1 and STAT3 that are also known to be phosphorylated by Src kinase (33–35).
Although RET/PTC shares substrates with Src kinase, the phosphorylation motifs do not completely match. Three tyrosine phosphorylation sites of PDK1 (Y9 and Y373/376) were identified using in vivo labeling and mass spectrometry (47). These residues can be phosphorylated by v-Src tyrosine kinase in vitro, and co-expression of v-Src leads to tyrosine phosphorylation and activation of PDK1. In contrast, RET/PTC leads to phosphorylation of Y9 of PDK1, but not of Y373/376 (35). Chen et al. (16) identified two tyrosine residues of PKB, Y315, and Y326, that can be phosphorylated by Src both in vivo and in vitro. The tyrosine residues in PDK1 and PKB that are phosphorylated by RET/PTC have no peptide similarities in their primary structure. PKB Y315, which is phosphorylated by RET/PTC, lies in the activation loop. Phosphorylation of this residue, like phosphorylation of T308, may change the conformation of the kinase domain to open up the activation loop.

In this study, tyrosine phosphorylation of PKB by RET/PTC results in the activation of the kinase. It is difficult to clarify how tyrosine phosphorylation of PKB affects the kinase activity. RET/PTC-induced phosphorylation of T308 and S473 of PKB may affect translocation to the plasma membrane. However, the subcellular localizations of PKB is not ap-
parently changed by coexpression of RET/PTC and tyrosine phosphorylation is independent from serine/threonine phosphorylation because PKB T308A and S473A are also tyrosine phosphorylated. Our data suggest that, in addition to phosphorylation of Thr308 and Ser473, tyrosine phosphorylation of PKB by RET/PTC may be required for the full activation of its biological functions.

To verify whether PKB tyrosine phosphorylation induced by RET/PTC3 modulates the biological functions of PKB, we tested the effects of RET/PTC3 and PKB on the transcriptional activity of FKHR1, which is inhibited by PKB. RET/PTC3 suppresses endogenous FKHR1 transcriptional activity and the suppression of FKHR1 transcriptional activity is more prominent upon coexpression with PKB. Moreover, RET/PTC3-mediated suppression of FKHR1 transcriptional activity is not reversed by treatment with the PI-3K inhibitors wortmannin and LY294002, the MAPK kinase inhibitor PD98059, the Src inhibitor PP1, and the mTOR inhibitor rapamycin. These observations support the hypothesis that RET/PTC3-induced PKB tyrosine phosphorylation controls the function of FKHR1 in vivo. All of these findings support the notion that RET/PTC rearrangements result in maximal activation of PKB during thyroid tumorigenesis.

MATERIALS AND METHODS

Materials

Media, cell culture reagents, and materials were purchased from Life Technologies, Inc. (Gaithersburg, MD), Sigma (St. Louis, MO), Fisher Scientific (Fairlawn, NJ), Corning, Inc. (Corning, NY), and Hyclone Laboratories, Inc. (Logan, UT). Wortmannin, PP1, and PD98059 were purchased from Calbiochem (La Jolla, CA). LY294002 was obtained from New England Biolabs (Beverly, MA). Antibody for RET was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), antiphosphotyrosine antibody (4G10) from Upstate Biotechnology Inc. (Lake Placid, NY), antiphosphospecific antibodies (Thr308 and Ser473) from Cell Signaling Inc. (Beverly, MA). Rapamycin was purchased from Sigma.

Plasmids

pCDNA3-RET/PTC1 (iso 9) and pCDNA3-RET/PTC3 (iso 9) were made by PCR (33–35). The pCDNA3-RET/PTC3 K284M and pCDNA3-RET/PTC3 Y588F mutants were constructed by site-directed mutagenesis using PCR with TaKaRa Ex-Taq polymerase in a 9700 thermocycler (PerkinElmer, Boston, MA). The amplified full-length fragment was digested with HindIII and cloned into a pCMV6 expression vector. HA-PKB Y474F mutants was constructed by mutagenesis using PCR with TaKaRa Ex-Taq polymerase in a PerkinElmer 9700 thermocycler. The primers used were: PKB forward, 5'-aggccgtagctagctggaggt-3'; PKB reverse, 5'-tgtggctagctagctggaggt-3'; and PKB Y474F reverse, 5'-agtctcctctcaagcagtgctgaggt-3'. Right and left fragments were employed in a further PCR with the PKB forward and PKB reverse primers. The amplified full-length fragment was digested with KpnI HindIII and cloned into a pcMV6 expression vector.

HA-tagged PKB constructs were used. pCMV6-HA-PKB was constructed by PCR. pCMV6-HA-PKB S473A, T308A, Y315, Y326, Y340, and Y315/326F were provided by Dr. Yun Qiu (University of Minnesota, Minneapolis, MN), pCMV6-HA-PKB Y474F mutants was constructed by mutagenesis using PCR with TaKaRa Ex-Taq polymerase in a PerkinElmer 9700 thermocycler. The primers used were: PKB forward, 5'-aggccgtagctagctggaggt-3'; PKB reverse, 5'-tgtggctagctagctggaggt-3'; and PKB Y474F reverse, 5'-agtctcctctcaagcagtgctgaggt-3'. Right and left fragments were employed in a further PCR with the PKB forward and PKB reverse primers. The amplified full-length fragment was digested with KpnI HindIII and cloned into a pcMV6 expression vector. pGL3-FRE luc and pELE-FKHRL1 were supplied by Dr. H.-S. Choi (Chonnam National University, Gwangju, Korea), pEGFP-N1-PKB construct was provided by Dr. J. Chung (Korea Advanced Institute of Science and Technology, Chungnam National University School of Medicine, Daejeon, Korea).

Cell Culture

The CHO cell line was maintained in Hams F-12 medium (Sigma) containing 10% fetal bovine serum (Invitrogen Life Technologies, Carlsbad, CA), 1% penicillin/streptomycin (Invitrogen Life Technologies), 1% nonessential amino acid and L-glutamate in an atmosphere of 5% CO2 at 37 C. NIH3T3 cell and HEK293 cell were cultured in DMEM with 10% fetal bovine serum and 1% penicillin/streptomycin. Human thyroid carcinoma-derived cell lines, anaplastic thyroid carcinoma cell and TPC1 were cultured in RPMI supplemented with 10% fetal calf serum and 2 mM L-glutamine.

Immunoprecipitation and the PKB Kinase Assay

For immunoprecipitation, the transfected cells were lysed in the RIPA buffer containing protease inhibitors (20 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml chymostatin, 2 μg/ml aprotinin, 1 μM phenylmethylsulfonyl fluoride). Insoluble material was removed by centrifugation, and the lysates were incubated with primary antibodies overnight at 4 C. The antibodies were collected with protein A/agarose, and the protein complexes were washed three times with cold lysis buffer. Immunoprecipitates were divided equally into two aliquots: one for kinase assay and the other for immunoblotting. For the measurement of PKB activity in vivo, anti-HA immunoprecipitates were washed twice with 1× lysis buffer and 0.5% NaCl, once with 1× lysis buffer, and once with reaction buffer [50 mM Tris-HCl (pH 7.5) and 0.1% β-mercaptoethanol]. The immune complex was then incubated at 30 C for 30 min in 50 μL of the reaction buffer with 300 μM peptide (GRPRTSSFAEG) as substrate, 100 μM MgCl2, 10 μM protease, 1 μM phosphatase inhibitor (PKA inhibitor, 500 μM ATP, and 103-P-ATP). The reaction was stopped by the addition of 20 μL EDTA. After spotting the reaction mixtures on p81 filter paper, the filters were washed with 0.1% phosphoric acid and rinsed with acetone to assist filter drying. γ32-P incorporation was measured using a scintillation counter (Hewlett-Packard, Palo Alto, CA).

Immunoblotting

Cells were lysed by adding sodium dodecyl sulfate (SDS) sample buffer [62.5 mM Tris-HCl (pH 6.8), 6% (wt/vol) SDS, 30% glycerol, 125 mM dithiothreitol (DTT), 0.03% (wt/vol) bromophenol blue]. Total cell lysates were denatured by boiling for 5 min, resolved in SDS-polyacrylamide gel, and trans-
ferred to nitrocellulose membranes. The membranes were blocked in TBS containing 5% (wt/vol) milk and 0.1% Tween for 1 h at room temperature, and incubated with primary antibody overnight at 4 C. The blot was developed using a horseradish peroxidase-conjugated secondary antibody (Phototope-HRP Western Blot Detection Kit, New England Biolabs).

**Purification of Recombinant RET/PTC3 and PKB from Insect Cells and the RET/PTC Kinase Assay**

The transfer vectors pBacPAK9-RET/PTC3, pBacPAK9-PKB and pBacPAK9-PKB Y315F were created by cloning corresponding PKBa cDNAs into the EcoRI site of pBacPAK9 (CLONTECH, Palo Alto, CA). Recombinant baculoviruses were generated by Lipofectin-mediated transfection of Sf21 cells with Bsu36I-digested BacPAK6 viral DNA (CLONTECH) and the appropriate transfer vector. Recombinant baculovirus clones were then isolated and the viral isolate was amplified. Sf21 cells were grown at 27 C in IPL-41 media supplemented with 10% heat-inactivated fetal calf serum, 0.1% pluronic F-68, 50 U penicillin/ml, and 50 g of streptomycin/ml. Sf21 cells were infected with recombinant baculoviruses at a defined multiplicity of infection and then harvested (10-min centrifugation, 3000 x g) at various times after infection. Cell pellets were washed in cold PBS (pH 7.4), and then frozen and stored at -80 C. Typically, 4 x 10^6 cells were solubilized by resuspension and incubation in 1 ml of lysis buffer [1% Triton X-100, 5 mm EDTA, 50 mm NaCl, 30 mm sodium pyrophosphate, 50 mm sodium fluoride, 0.2 mm sodium orthovanadate, 1 mm phenylmethylsulfonyl fluoride, 5 g/ml aprotinin, 1 g/ml pepstatin A, 2 g/ml leupeptin, 10 mm Tris-Cl (pH 7.6)] for 1 h at 4 C. Nuclei and insoluble material were removed by centrifugation for 10 min at 3000 x g. The lysate was incubated with 0.1% volume of 10% protein A-Sepharose CL-4B (Pharmacia Biotech Inc., Piscataway, NJ) at 1 h at 4 C and centrifuged 15 min at 18,000 x g. The supernatants were then removed and transferred to new vials. For the RET/PTC3 kinase assay, separate reactions mixtures were made for RET/PTC3 and PKB. The RET/PTC3 enzyme mixture was made with kinase buffer [20 mm HEPES (pH 7.5), 150 mm NaCl, 1% glycerol, 0.1% Triton X-100, 15 mm MgCl2, and 15 mm MnCl2] plus 2 mm DTT and various concentrations of RET/PTC3. The PKB substrate mixture was made with kinase buffer plus 2 mm DTT, 200 mm Na2VO4, 10 mm ATP and PKB (1 mg reaction). The substrate and enzyme mixtures were mixed on ice and incubated at 30 C for 10 min in a water bath. The reaction was stopped by the addition of 3 x SDS sample buffer.

**Confocal Microscopy**

NIH3T3 Microscopy were grown on coverslips and transfected with pEGFP-PKB and pcDNA3-RET/PTC3 by the LipofectAmine method (Life Technologies, Inc., Gaithersburg, MD). Twenty-four hours after transfection, the cells were washed three times with cold PBS and fixed with 3.7% formaldehyde for 40 min. Fixed cells were mounted on glass sides with PBS and observed with a laser-scanning confocal microscope (Olympus, Shinjuku-ku, Tokyo, Japan). For detection of pcDNA3-RET/PTC3, cells mounted on glass slides were permeabilized with 2 ml PBS containing 0.1% Triton X-100 and 0.1% glycine at room temperature, incubated for 15 min, washed three times with PBS and blocked with 3% (wt/vol) BSA in PBS for 10 min at room temperature. Cells were incubated with primary anti-RET antibody (Santa Cruz Biotechnology, Inc.) for 1 h and a rhodamine-conjugated antirabbit secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at 37 C. The pEGFP-PKB construct was detected by the autofluorescence of the green fluorescent protein (GFP).

**Luciferase Assay**

A plasmid containing a FKHR promoter and the firefly luciferase gene, FRE-luc, pELE-FKHR1, pcDNA3-RET/PTC3, and pcMV6-PKB were transfected into CHO cells with 100 ng of pRL-SV40 plasmid encoding Renilla luciferase (Promega, Madison WI). After transfection, the cells were allowed to recover for 24 h. The cells were then washed with 1 x PBS and lysed with 200 ml 1 x lysis buffer containing 40 mm tricine (pH 7.8), 50 mm NaCl, 2 mm EDTA, 1 mm MgSO4, 5 mm DTT, and 1% Triton X-100. Light intensity was measured using a luminometer (Berthold, Bad Wildbad, Germany). The fluorescence intensity was measured using a luminometer (Berthold). The firefly and Renilla luciferase activities were measured with a Dual-Luciferase Reporter assay system (Promega). Luciferase activity was integrated over a 10-sec period. Firefly luciferase values were standardized to Renilla values.

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