Regulation of p27Kip1 Protein Levels Contributes to Mitogenic Effects of the RET/PTC Kinase in Thyroid Carcinoma Cells

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ABSTRACT

We show that treatment of a panel of thyroid carcinoma cell lines naturally harboring the RET/PTC1 oncogene, with the RET kinase inhibitors PPI and ZD6474, results in reversible G1 arrest. This is accompanied by interruption of Shc and mitogen-activated protein kinase (MAPK) phosphorylation, reduced levels of G1 cyclins, and increased levels of the cyclin-dependent kinase inhibitor p27Kip1 because of a reduced protein turnover. MAP/extracellular signal-regulated kinase 1/2 inhibition by U0126 caused G1 cyclins down-regulation and p27Kip1 up-regulation as well. Forced expression of RET/PTC in normal thyroid follicular cells caused a MAPK- and proteasome-dependent down-regulation of p27Kip1. Reduction of p27Kip1 protein levels by antisense oligonucleotides abrogated the G1 arrest induced by RET/PTC blockade. Therefore, in thyroid cancer, RET/PTC-mediated MAPK activation contributes to p27Kip1 deregulation. This pathway is implicated in cell cycle progression and in response to small molecule kinase inhibitors.

INTRODUCTION

Rearrangements of the RET receptor tyrosine kinase (TK), caused by chromosomal inversions or translocations, are hallmarks of PTC. These rearrangements mediate fusion of the TK-encoding domain of RET with heterologous genes, leading to the generation of chimeric RET/PTC oncogenes. RET/PTC1 (the H4-RET fusion) and RET/PTC3 (the RFG-RET fusion) are the most prevalent variants (1). RET fusion partners code for dimerization-motif containing proteins, which mediate ligand-independent activation of RET/PTC kinases (1). It is known that phosphorylation of RET tyrosine 1062 is crucial to recruit, through Shc and other docking proteins, Grb2-Sos complexes, leading to Ras-dependent activation of the mitogen-activated protein kinase (MAPK) pathway (2). Once activated, MAPK can phosphorylate several nuclear transcription factors and regulate cell cycle progression (3).

Targeting TKs’ enzymatic activity by small molecule inhibitors is a promising strategy in human cancer therapy (4). Several molecules are being successfully used in clinical trials, and one of them, STI571 (imatinib mesylate or Gleevec), is now a standard for the treatment of BCR-ABL-positive chronic myeloid leukemia (5). BHP2-7, BHP5-16, BHP7-13, BHP10-3, BHP14-9, and BHP17-10 cell lines were established from six different PTC patients as described elsewhere (9). All human cell lines were grown in RPMI medium supplemented with 10% FCS (Life Technologies, Inc., Paisley, PA) and antibiotics. PC Cl 3s are normal rat thyroid follicular cells and were grown in Coon’s modified Ham’s F-12 medium supplemented with 5% FCS and a mixture of hormones as described previously (10). PC-RET/PTC1 and RET/PTC1(Y1062F) are mass populations of PC Cl 3 cells transfected with RET/PTC1 or its Y1062F mutant (11). The P5 primary culture of normal human thyroid follicular cells was a kind gift of Francesco Curcio.

The P1 probes used for hybridization analysis were synthesized in a 50-wt% agarose/formaldehyde gel and transferred to Nylon filters (Hybond-N; Amersham Pharmacia Biotech). A [32P]dATP-labeled full-length human p27Kip1 mRNA, RNA (10 μg) was denatured and resolved by electrophoresis in a 1% (w/v) agarose/formaldehyde gel and transferred to Nylon filters (Hybond-N, Amersham Pharmacia Biotech). The amplifiers for RET/PTC1 were as follows: 5′-ATTGTGATCTTCCGTCCGTC-3′ (forward, nucleotides 145–162) and 5′-CTTCCAGCATCTTCTCAA-3′ (reverse, nucleotides 434–451). To establish the expression levels of p27Kip1 mRNA, RNA (10 μg) was denatured and resolved by electrophoresis in a 1% (w/v) agarose/formaldehyde gel and transferred to Nylon filters (Hybond-N, Amersham Pharmacia Biotech). A [α-32P]dATP-labeled full-length human p27Kip1 cDNA (11) was used as a probe. Equal loading was verified by ethidium bromide staining of 18S and 28S rRNA.

Fluorescence in Situ Hybridization Analysis. The P1 probes used for dual color fluorescence in situ hybridization analysis were RMC10P013,
corresponding to the RET gene, and 29F6, corresponding to the H4/D10S170 gene, as described previously (8). The RET probe was labeled with SpectrumGreen-dUTP and the H4 probe with SpectrumRed-dUTP (Vysis, Inc., Richmond, United Kingdom) using the Nick Translation kit (Vysis, Inc.). In addition, an α-centromeric probe specific for chromosome 10 (CEP-10; Vysis, Inc.) was used.

Western Blot Analysis. Lysates containing the same amount of proteins, estimated by a modified Bradford assay (Bio-Rad, Munich, Germany), were immunoprecipitated with the required antibody or immunoblotted. Immune complexes were detected by enhanced chemiluminescence kit (Amersham Pharmacia Biotech). Anti-RET is a polyclonal antibody raised against the TK protein fragment of human RET, anti-pY1062, anti-pY1015, and anti-pY905 are affinity-purified polyclonal antibodies raised against RET peptides containing phosphorylated Y1062, Y1015, or Y905 (6, 7). Anti-phosphotyrosine (4G10) is a mouse monoclonal antibody from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-MAPK (no. 9101) and anti-phospho-MAPK (no. 9102) were from New England Biolabs (Beverly, MA). Polyclonal and monoclonal antibodies anti-cyclin E (M-20), anti-p21Waf1 (C-19), anti-cyclin D1 (A-12), and anti-Shc (H-108) were from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-α-tubulin was from Sigma Chemical Co., and monoclonal anti-p27Kip1 was from Cell Signaling (Beverly, MA). Secondary antibodies coupled to horseradish peroxidase were from Amersham Pharmacia Biotech.

Antisense p27Kip1 Oligonucleotides. p27Kip1 antisense and mismatch control oligonucleotides (Ref. 11; final concentration, 200 nM) were heated for 5 min at 65°C in serum-free medium, then mixed with oligofectamine reagent (Invitrogen Corporation, Carlsbad, CA) and added to the cells according to manufacturer’s instructions. After 4 h, fresh medium was added to each dish, and the cells were incubated in the absence or presence of RET/PTC kinase inhibitors for 48 additional hours before harvesting proteins or measuring bromodeoxyuridine (BrdUrd) incorporation.

BrdUrd Incorporation. DNA synthesis was measured by the 5′-bromo-3′-deoxyuridine Labeling and Detection kit from Boehringer Mannheim. Cells were seeded on glass coverslips and treated or not with the compounds for 24 h. Then, cells were incubated for 1 h with BrdUrd (final concentration of 10 µM), fixed with paraformaldehyde (4%), and permeabilized with Triton X-100 (0.2%). Coverslips were incubated with anti-BrdUrd mouse monoclonal antibody and with a Texas red-conjugated antimouse antibody (Jackson Immunoresearch Laboratories, Philadelphia, PA). All coverslips were counterstained in PBS containing Hoechst 33258 (1 mM; Sigma Chemical Co.), rinsed in PBS, and mounted in Moviol on glass slides. The fluorescent signal was visualized with an epifluorescent microscope (Axiovert 2, Zeiss; equipped with a ×100 lens) interfaced with the image analyzer software KS300 (Zeiss).

Cell Cycle Analysis. Cells were grown to subconfluence and subjected or not to RET kinase inhibitors treatment for extra 12 or 36 h. After harvesting, cells were fixed in cold 70% ethanol in PBS, then washed with PBS. Propidium iodide (50 µg/ml) was added to the cells in the dark, and samples were analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA) interfaced with a Hewlett Packard computer (Palo Alto, CA).

Luciferase Assay. Luciferase assay was performed as described previously (12). The reporter plasmid was the −1745CD1LUC, containing an 1882-bp PvuII fragment of the human cyclin D1 promoter subcloned into the vector pA3,LUC (13). A total of 5 × 10⁴ cells was plated 48 h before transfection in 60-mm tissue culture dishes. The medium was changed to DMEM (Life Technologies, Inc.) containing 5% FCS. Three h later, calcium phosphate DNA precipitates were incubated with the cells for 1 h. DNA precipitates were removed, and cells were washed with serum-free DMEM and incubated with 15% glycerol in HEPES-buffered saline for 2 min. Finally, cells were washed with DMEM and incubated in complete medium; luciferase activity was determined 48 h after transfection by using an Autolumat LB 953 (EG&G, Berthold, Bad Wildbad, Germany). ZD6474 or U0126 treatment was performed 24 h before harvesting. Activity was reported as fold change with respect to untreated cells or cells transfected with the empty vector; results were the average ± SD of three independent experiments performed in duplicate.

RESULTS

RET/PTC1 Rearrangement in BHP2-7, 7-13, and 10-3 Cell Lines. We analyzed by reverse transcription-PCR BHP2-7, BHP7-13, BHP10-3, BHP5-16, BHP14-9, and BHP17-10 PTC cell lines for the presence of RET/PTC rearrangements. As control, we used TPC1 and FB-2, harboring the RET/PTC1 (H4-RET) oncogene, and NPA, negative for RET/PTC. Fig. 1A shows that BHP2-7, BHP7-13, and BHP10-3 cell lines expressed RET/PTC1. We digested genomic DNA from the various cell lines with EcoRI or BamHI and used as a probe a 1-kbp BglII-BamHI DNA fragment mapping in RET intron 11 (8). BHP2-7, BHP7-13, and BHP 10-3 cells showed extra bands corresponding to rearranged RET alleles (asterisks in Fig. 1B). Finally, dual-color fluorescence in situ hybridization analysis showed fusion of H4 and RET signals consistent with H4-RET recombination events in BHP2-7, BHP7-13, and BHP10-3 (Fig. 1C and data not shown).

Cytostatic Effects of RET/PTC1 Kinase Inhibition. We used the above-characterized cell lines to investigate the biological effects of RET/PTC1 kinase blockade. All of the experiments described thereafter have been performed on at least three of the five RET/PTC1-positive cancer cell lines. Because the different cell lines gave identical results, data obtained with representative cells are shown, unless specified otherwise.

Treatment with two different RET kinase inhibitors (PP1 and
ZD6474 elicited a strong reduction of DNA synthesis rate, measured by BrdUrd incorporation, in the five RET/PTC1-positive cell lines (Fig. 2A and data not shown). These effects were specific because they were neither observed in BHP17-10 cells nor in normal thyroid follicular PC Cl 3 cells. Accordingly, flow cytometry analysis showed a sharp increase of the G0-G1 fraction and a marked reduction of the S fraction upon ZD6474 treatment of the five RET/PTC1-positive cell lines but not of BHP17-10 cells (Fig. 2B and data not shown).

**Up-Regulation of p27Kip1 Is Implicated in ZD6474-Mediated Growth Arrest.** To study the mechanism of cell cycle arrest upon RET/PTC blockade, RET/PTC1-positive and control cells were treated with 5 μM ZD6474 or PP1 for 24 h, proteins were harvested, and expression levels of cell cycle regulatory proteins were determined by immunoblot. Upon treatment, a remarkable increase (4 ± 0.5 folds) in the expression levels of p27Kip1 was observed in all of the RET/PTC-positive cancer cells but not in RET/PTC-negative cells, whereas p21WAF1 levels did not change significantly (Fig. 3A and data not shown). ZD6474 treatment was also followed by reduced levels of G1 cyclins D1 and E (Fig. 3A). Identical findings were obtained upon PP1 treatment (data not shown). These findings indicate a critical role played by p27Kip1 in the G1 arrest that follows RET/PTC1 kinase blockade.

**Interruption of RET/PTC1 Signaling to MAPK by ZD6474 and PP1.** Protein lysates were immunoblotted with antibodies specific for phosphorylated tyrosines 905, 1015, and 1062, three major autophosphorylation sites in RET (2). All of the five RET/PTC1-positive cancer cell lines expressed p57 and p62 RET/PTC1 protein products constitutively phosphorylated on the three tyrosines (Fig. 4A, left panel, and data not shown). In RET-transfected cells, phosphorylated tyrosine 1062 binds Shc, a docking protein implicated in triggering the Ras/MAPK cascade (14). We immunoprecipitated cell lysates with anti-Shc and blotted the immunocomplexes with anti-phosphotyrosine antibodies. Shc products were constitutively phosphorylated in all cell
lines harboring RET/PTC but not in RET/PTC-negative cells (Fig. 4B, left panel, and data not shown). Finally, MAPK (extracellular signal-regulated kinases 1 and 2) were constitutively phosphorylated in PTC cell lines but not in P5, a primary culture of normal human thyroid follicular cells (Fig. 4C, left panel). In RET/PTC-negative cancer cell lines (see, for example, BHP17-10 in Fig. 4C), constitutive phosphorylation of MAPK is likely explained by the expression of an oncogenically activated B-raf (V599E) allele. Indeed, activating point mutations in B-raf have been identified in a large fraction of human papillary thyroid carcinomas and cell lines. When mutated, the B-raf kinase activates MAPK. No overlap was observed between carcinomas harboring RET/PTC and B-raf mutations, suggesting that B-raf acts along the RET/PTC-B-raf-MAPK pathway in thyroid carcinoma cells (15).

Importantly, inhibition of the RET/PTC kinase by ZD6474 or PP1 abrogated detectable levels of RET/PTC, She, and MAPK phosphorylation as early as after 1 h of treatment in all of the five RET/PTC-positive cell lines (Fig. 4, right panels, and data not shown).

Interruption of RET/PTC1 Signaling Increases p27Kip1 Protein Stability. Ras/MAPK signaling has been implicated in the regulation of p27Kip1 protein levels, directly and indirectly, by up-regulating cyclin D1/cdk4 and cyclin E/cdk2 complexes. We treated TPC1, BHP10-3, and BHP2-7 cells with the MEK1/2 inhibitor U0126. Parallel to reduction of MAPK phosphorylation and accumulation of cells in G1 (data not shown), p27Kip1 protein levels increased, although cyclin D1 and E levels decreased (Fig. 5A and data not shown), pointing to a role for MAPK in regulation of cell cycle downstream RET/PTC. U0126 treatment resulted in increased levels of p27Kip1 and reduced levels of cyclin D1 also in BHP17-10 cells (Fig. 5A). This was consistent with the constitutive activation of MAPK, probably sustained by oncogenic activation of B-raf, also in BHP cell lines negative for RET/PTC.

Cyclin D1 transcription was determined by a luciferase assay using the −1745CD1LUC reporter. Treatment with ZD6474 caused a roughly 3-fold reduction of cyclin D1 transcription; this occurred selectively in BHP cells carrying the RET/PTC rearrangement. In contrast, according to a general activation of MAPK in thyroid papillary carcinoma cell lines, U0126 treatment caused reduced cyclin D1 promoter activity in BHP2-7 as well as BHP17-10 cells (Fig. 5B).

To understand level of p27Kip1 regulation, RNA was extracted from untreated and ZD6474- and U0126-treated cells and probed with a p27Kip1 cDNA. p27Kip1 mRNA levels were not changed upon ZD6474 (Fig. 5C) or U0126 (data not shown) treatment, suggesting a

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posttranscriptional level of regulation. To analyze whether changes in protein stability accounted for p27kip1 regulation, we used the protein synthesis inhibitor cycloheximide. Treatment with cycloheximide revealed a half-life for p27kip1 of ~6 h in untreated BHP2-7 cells. In sharp contrast, p27kip1 did not undergo degradation in cells treated with ZD6474 or U0126, even after 24 h of cycloheximide treatment, suggesting that chemical blockade of the RET/PTC-MAPK pathway increased p27kip1 protein stability (Fig. 5D).

To further address the mechanisms of RET/PTC-MAPK-dependent degradation of p27kip1, we treated BHP2-7 cells with ZD6474 (Fig. 6A) or U0126 (Fig. 6B) for 24 h to induce p27kip1 accumulation. Starting after 6 h of RET or MEK inhibitors withdrawal, a progressive reduction of p27kip1 levels was observed. Such down-regulation was strongly slowed down by the addition of MG132, an inhibitor of the ubiquitin-proteasome pathway, indicating that, in BHP2-7 cells, p27kip1 protein levels are kept under check by proteasome-mediated degradation. Taken together, these results indicate that in RET/PTC-positive thyroid cancer cells, MAPK decreases p27kip1 expression by increasing the p27kip1 protein turnover exerted by the 26S proteasome.

**Forced Expression of RET/PTC Caused Proteasome-Dependent p27kip1 Down-Regulation.** To confirm these findings in another model system, we transfected normal thyroid follicular PC Cl 3 cells with RET/PTC1 or with a mutant harboring the substitution of tyrosine 1062 with phenylalanine (Y1062F). Such Y1062F mutant is unable to recruit Shc and to stimulate MAPK (10). Cells were kept in low serum (0.5% for 48 h) to minimize the effects of serum-derived growth factors; proteins were harvested from mass populations, and cyclin D1, cyclin E, and p27kip1 protein levels were determined by immunoblot. Forced expression of wild-type RET/PTC1, but not of its mutant, caused increased levels of cyclin D1 and E and reduced levels of p27kip1 with respect to parental cells (Fig. 7A). Transient expression of RET/PTC1, but not its Y1062F mutant, caused a significant up-regulation of the activity of the cyclin D1 promoter in PC Cl 3 cells, confirming that RET/PTC effects on cyclin D1 are mainly at the transcriptional level (Fig. 7B). Restoration of p27kip1 protein levels was observed when PC-RET/PTC1 cells were treated with MG132 or U0126 (Fig. 7C), confirming that RET/PTC is able to promote proteasome-dependent p27kip1 down-regulation through MAPK.

**DISCUSSION**

ZD6474 and PP1 efficiently inhibit RET/PTC kinase (6, 7), thus providing a promising therapeutic approach for thyroid cancer as well as an useful tool to study RET/PTC-driven cellular effects. By using a panel of newly characterized human cell lines harboring naturally occurred RET/PTC1, here we show that sustained RET/PTC signaling is implicated in G1-S progression. Progression through the cell cycle involves the sequential activation of cdks. Cdk’s are regulated by association with cyclins, phosphorylation events, and interaction with cdk inhibitors (16). D-Type cyclins are crucial for G1-S transition in various thyroid epithelial cell systems where they are under growth factors control (17, 18). PP1 and ZD6474 treatment caused down-regulation of G1 cyclins and up-regulation of p27kip1 in RET/PTC-positive cancer cells. Accordingly, opposite expression changes were observed in normal follicular cells when RET/PTC1 was introduced. The use of antisense oligonucleotides confirmed that p27kip1 plays a key role in growth arrest induced by ZD6474 and PP1. An increased half-life of p27kip1 protein was detected upon RET/
PTC inhibition, whereas adoptive RET/PTC expression in normal thyrocytes caused a reduced half-life of p27Kip1 that was reversed by proteasome block. PTC cell lines exerted a RET/PTC-dependent constitutive phosphorylation of Shc and activation of MAPK. Upon RET/PTC blockade, this pathway was rapidly turned off. Furthermore, p27Kip1 accumulated parallel to MEK1/2 inhibition. Finally, expression of a RET/PTC mutant unable to stimulate MAPK in normal thyrocytes failed to cause p27Kip1 degradation. All together, these data indicate that MAPK triggering by RET/PTC accounts for p27Kip1 down-regulation. Activation of the MAPK pathway is a common finding in most PTC cell lines (Fig. 4C). Indeed, also those carcinomas that are negative for RET/PTC rearrangements often feature activating mutations in B-raf (15), a direct upstream regulator of MEK. Thus, the MAPK-p27Kip1 pathway could be a promising therapeutic target to achieve cytostatic effects in PTCs initiated by different (RET/PTC or B-raf) oncogenic events.

p27Kip1 protein degradation is a three-step process that requires phosphorylation at threonine 187, recognition by the F-box protein SKP2, ubiquitination, and degradation by the 26S proteasome (19). MAPK can affect p27Kip1 protein levels primarily by triggering cyclin E-Cdk2 that, in turn, phosphorylates p27Kip1 on T187 and promotes its degradation (19–23). Up-regulation of cyclin D1/cdk4 secondary to MAPK-triggering by stimulating cyclin E transcription may contribute to p27Kip1 obstruction as well. In addition, MAPK activation, according to some studies (24, 25), could reduce p27Kip1 protein stability by directly phosphorylating it. Therefore, we speculate that the increase of p27Kip1 induced by RET/PTC kinase inhibitors could be secondary to: (a) abrogation of MAPK-mediated destabilization of p27Kip1; and (b) reduction of cyclin D1/cdk2 and cyclin E/cdk2 levels.

Our findings indicate that reduction of the levels of p27Kip1 protein is necessary, but they do not prove that it is sufficient for obstruction of p27Kip1 in thyroid cancer cells. Subcellular localization is another important level of regulation of p27Kip1. Up-regulated cyclin D/cdk complexes may sequester p27Kip1. Furthermore, interaction of p27Kip1 with JAB1/CNS5 or phosphorylation at serine 10 (26, 27) promotes its export from the nucleus. Finally, phosphorylation of threonine 157 by PKB/AKT impairs p27Kip1 nuclear import (28–30). It is feasible that RET/PTC triggers at least some of these additional pathways that could therefore synergistically obstruct p27Kip1 by cooperating with MAPK-dependent down-regulation.

In conclusion, p27Kip1 down-regulation mediates subversion of the G1-S checkpoint in RET/PTC-positive thyroid carcinoma cell lines, the reversal of this phenomenon being critical for growth arrest mediated by RET/PTC kinase inhibitors. We hypothesize that failure in retrieving p27Kip1 expression or block of p27Kip1 activity at different levels such as for instance delocalization can cause escape of thyroid cancer cells from the therapeutic effects of RET/PTC chemical blockade.

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