PP1 Inhibitor Induces Degradation of RETMEN2A and RETMEN2B Oncoproteins through Proteosomal Targeting

Cristiana Carniti, Carla Perego, Piera Mondellini, Marco Alessandro Pierotti, and Italia Bongarzone

ABSTRACT

RET tyrosine kinase oncoproteins are potential targets for anticancer therapy. We show here that along with the inhibition of RET tyrosine phosphorylation, the pyrazolo-pyrimidine inhibitor PP1 induces RETMEN2A and RETMEN2B oncprotein destruction. In fact, as a consequence of PP1 treatment, RET oncoproteins translocate from the outer limiting membrane to inner cellular compartments and are rapidly addressed to the degradative pathway. The cleavage of RET oncoproteins is associated with an impairment of RET mitogenic signaling pathways that causes a reversion of the oncogenic transformation and establishes a long-term cytostatic effect. By using specific inhibitors of both the proteosome and the lysosome, we assessed that PP1 targets RET oncoproteins to proteosomal, rather than lysosomal, degradation. In this context of studies, we interestingly demonstrated that RETMEN2A and RETMEN2B receptors are constitutively ubiquitinated and interact with the ubiquitin ligase CHIP. Moreover, PP1 does not modify these interactions, although it indeed causes RET dephosphorylation. Therefore, even if the degradative pathway stimulated by the inhibitor appears to be mediated by the proteosome, PP1 does not seem to enhance nor promote receptor ubiquitination. These observations lead us to favor two models for PP1-induced RET oncoprotein degradation: either PP1-mediated RET dephosphorylation per se targets the oncoproteins for destruction or alternatively, PP1 insertion in the RET ATP-binding pocket promotes a mechanism for fast stress-induced degradation. The use of PP1, which therefore acts as a degradation-inducing factor, may represent a promising new strategy to selectively target RET oncogenic products for destruction and holds promise for future mediulary thyroid cancer therapy.

INTRODUCTION

The RET proto-oncogene encodes a RTK whose ligands are members of the GDNF protein family, including GDNF, neurturin, artemin, and persephin (1, 2). These neurotrophic factors signal through multisubunit receptor complexes consisting of RET and a glycosphosphatidylinositol-anchored coreceptor called GDNF family receptor. The GDNF/RET signaling pathway plays an important role in survival and differentiation of various neurons, as well as in kidney organogenesis (3–6). In addition, different RET mutant forms are responsible for the development of several human diseases such as PTC, the inherited cancer syndrome multiple endocrine neoplasia types 2A and 2B (MEN2A and MEN2B) and Hirschsprung’s disease (1, 2, 7–9). In virtually all MEN2A and in several familial MTC cases, there are germ-line substitutions of cysteines of the RET extracellular domain (10) with the C634R being the most common mutation associated with MEN2A, whereas most MEN2B cases are caused by the M918T mutation in the RET tyrosine kinase domain (9, 11). The M918T substitution is also found in sporadic MTCs, with M918T mutation-positive tumors often displaying a more aggressive phenotype. Rearrangements of the RET tyrosine kinase receptor with different genes are frequently found in PTCs (12–14) and, in particular, in radiation-induced childhood PTC (15, 16). RET/PTC and RETMEN2A oncoproteins display constitutive kinase activity because of ligand-independent dimerization (17, 18). The M918T mutation modifies the structure of the kinase, thereby switching on the enzymatic function and altering the substrate specificity of RETMEN2B (18–20).

The knowledge of the availability of inhibitors specific for RET oncoproteins could provide new tools to highlight the physiologically and pathologically activated pathways involved and help in developing new therapeutic strategies for RET-associated diseases. The inhibition of tyrosine kinase activity by small cell-permeable molecules is a promising approach to target oncoproteins and has already reached clinical application for ErbB/HER subgroup of receptors (21, 22). Carломagno et al. (23) have very recently demonstrated that the pyrazolo-pyrimidine PP1 blocks tumorigenesis induced by RET/PTC cytoplasmic oncoproteins because it can inhibit RET enzymatic activity and its transforming effects. We here additionally explored the mechanism of action of the inhibitor specifically showing that PP1 abrogates the cellular morphological modifications induced by the expression of different cytoplasmic or membrane-bound RET oncoproteins in NIH3T3 cells, restoring the characteristic fibroblast actin organization and focal adhesion formation. We also present evidence that along with the inhibition of tyrosine phosphorylation, PP1 induces proteosomal destruction of the activated receptors. In fact, in the presence of the inhibitor, the receptors are rapidly targeted to the degradative pathway, and the intracellular destruction of ubiquitinated RET oncoproteins results accelerated. Taken together, our results suggest that PP1 treatment would represent a promising new strategy to selectively target RET oncogenic products for destruction and could be used to develop new therapeutic techniques for medullary thyroid cancer therapy whose tumors respond very poorly to chemotherapy agents.

MATERIALS AND METHODS

Plasmids, Antibodies, and other Reagents. The human cDNA coding the short isoform of RET was cloned into the pAlter vector (Promega) and site-directed mutagenesis Cys634→Arg (TGC→GCC) and Met918→Thr (ATG→ACG) was performed. The wild-type (WT) as well as the other RET constructs were cloned in the pReCMV expression vector (Invitrogen, Groningen, The Netherlands). The point mutation of the desired codon was confirmed by DNA sequencing.

Anti-RET polyclonal antibody (C-19) raised against a peptide corresponding to an amino acid sequence mapping at the COOH terminus of RET was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The following antibodies were also used: mouse antiphosphotyrosine (4G10; Upstate Biotechnology, Inc., Lake Placid, NY); Vinculin (V-4505; Sigma Chemical Co., St. Louis, MO) monoclonal antibody; Tubulin polyclonal antibody (T-3526;
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Sigsigu Co.); TRK polyclonal antibody (Santa Cruz Biotechnology); EGFR polyclonal antibody (sc-03, Santa Cruz Biotechnology, CA); ubiquitin (MMS-258; Babco) monoclonal antibody for Western blots; and ubiquitin monoclonal antibody (FK1, which recognizes only polyubiquitin-protein conjugates; Affiniti BioReagents, Inc.); Cbl (C-15 Santa Cruz Biotechnology) polyclonal antibody; and clathrin (MA1-065, Affiniti BioReagents, Inc.) monoclonal antibody. FITC-labeled phalloidin (Sigma Chemical Co.) was used to detect filamentous actin (final concentration 125 ng/ml). Secondary antibodies were from Santa Cruz Biotechnology.

PP1 inhibitor [4-aminoo-5-(4-methylphenyl)-7-[(2-buty1)pyrazolo]-3,4-pyrimidine] was obtained from BioMol Research Laboratories, dissolved in DMSO, and diluted into culture medium at the time of experimentation to a final concentration of 1 μM. PP2, PP3, Genistein, and Herbimycin from Calbiochem (Novacalbiochem Corporation, La Jolla, CA) were dissolved in DMSO and, respectively, diluted into the culture medium to final concentrations of PP2 (1 μM), PP3 (1 μM), Genistein (100 μM), or Herbimycin (0.1 μM).

Lactacystin was purchased from Sigma Chemical Co., dissolved in DMSO, and diluted in culture medium to a final concentration of 2 μM. MG132 was purchased from Calbiochem (Novacalbiochem Corporation), dissolved in DMSO, and then diluted in culture medium to a final concentration of 10 μM. Chloroquine, from Sigma Chemical Co., was dissolved in water and diluted in culture medium to a final concentration of 100 μM. The human recombinant epidermal growth factor was from Life Technologies, Inc. (13247-051) and was diluted into the culture medium to a final concentration of 20 ng/ml.

Cell Culture. Mouse fibroblast NIH3T3-untransfected cells were grown in DMEM containing 10% calf serum. Cells were transfected as previously described (13) with pDNA3 plasmids carrying RET/CreA, RET/M918T, and RET/PTC3 inserts. Transformation foci were selected in DMEM with 5% calf serum. E25-427 cells, NIH3T3 cells overexpressing the human TRK proto-oncogene, were kindly provided by Dr. Mariano Barbacid and grown in DMEM supplemented with 10% calf serum. HEK 293T cells were grown in DMEM supplemented with 10% FCS and transiently transfected with calcium phosphate precipitation. The human TT cell line was cultured in Ham’s F-12 (Life Technologies, Inc.) supplemented with 15% FCS. The MDA-MB-231 cell line was cultured in RPMI supplemented with 10% FCS.

Immunofluorescence and Confocal Laser Microscopy. Cells were seeded at low confluence on glass coverslips and cultured for 24 h. Cells were fixed in 4% paraformaldehyde and 2% sucrose and then permeabilized with 0.5% Triton X-100 in PBS. Immunostaining with primary antibodies was followed by incubation with rhodamine-conjugated antirabbit (Dako), FITC-conjugated anti-IgG from Jackson Immunoresearch (West Grove, PA). The confocal images were obtained using a Bio-Rad MRC-1024 confocal microscope.

Cell Motility Assay. Migration of RET-expressing cells was determined using an in vitro model of wound repair as described previously (24). The monolayer cells were wounded with a 10-μm sterile plastic micropipette tip, and cellular debris was removed by washing with serum-free DMEM. The monolayers were incubated in DMEM with or without 1 μM PP1 for 12 h. Cell migration was observed using a phase contrast microscope under x400 magnification. Each condition was examined in duplicate, and the experiments were repeated at least three times.

Immunoprecipitation and Western Blot Analysis. Protein samples were prepared as previously reported (25) and immunoprecipitated with the specific antisera. Protein concentration was estimated by a modified Bradford assay (Bio-Rad, Munich, Germany). Immune complexes were separated by electrophoresis on 8% SDS-PAGEs, transferred onto nitrocellulose membranes (Millipore Co., Bedford, MA), and analyzed by immunoblotting with the appropriate antibodies. For Western blots, cells were lysed in SDS lysis buffer [62.5 mM Tris-HCl (pH 6.8) and 2% SDS]. Total extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes by electroblotting. Membrane nitrocellulose bands were visualized using horseradish peroxidase-conjugated antirabbit or antimouse antisera (Santa Cruz Biotechnology) and detected with the enhanced chemiluminescence kit (enhanced chemiluminescence detection reagents; Amersham Pharmacia Biotech, Little Chalfont, United Kingdom).

RET Kinase Activity. To determine the effect of PP1 on the receptor kinase activity, 293T cells were transiently transfected to express RET/CreA, RET/M918T, PT3C, TRK, and wild-type RET proteins with the calcium-phosphate method. Two days after transfection, cells were lysed in NP40 buffer. Their kinase activity was assessed in vitro by their ability to autophosphorylate and phosphorylate myelin basic protein (MBP; Sigma). Protein extracts were immunoprecipitated with anti-RET or anti-TRK antibodies washed twice with lysis buffer and once with incubation buffer [50 mM HEPES (pH 7.2), 20 mM MgCl2, and 5 mM phenylmethylsulfonyl fluoride] and subjected to kinase assay with MBP as an exogenous substrate [γ-32P]ATP and PP1 (1 μM) or PP2 (1 μM), where indicated. After a 15-min incubation at +4°C in 20 μl of the same buffer containing 4 μCi [γ-32P]ATP diluted with unlabeled ATP to a final concentration of 26 pmol ATP/sample and 50 μM MBP, the reactions were stopped by the addition of reduced Laemmli buffer, and the products were subjected to SDS-PAGE. The intensity of the bands corresponding to RET immunoprecipitates was determined photometrically. The intensity of the bands corresponding to RET was quantified by PhosphorImager (Molecular Dynamics) analysis.

RESULTS

PP1 Reverts the Transformed Morphology and Affects Motility of Cells Expressing RET/MEN2 Oncoproteins. In agreement with what has been described by Carlomagno et al. (23), PP1 exerts an effect on the morphology of fibroblasts expressing RET carrying either the C634R or M918T substitutions. When examined by phase-contrast microscopy (Fig. 1A), these cells exhibited a bright, rounded morphology with elongated processes very different from the typical phenotype of parental NIH3T3 cells. However, in the presence of PP1 at 1 μM for 12 h, their morphology reverted, becoming similar to the one of untransformed NIH3T3 fibroblasts. As expected, PP1 exerted clear effects on RET/PTC3 cells morphology as well. As a control, parental NIH3T3 cells and NIH3T3-overexpressing TRKA were also examined by phase-contrast microscopy and did not show any significant change upon PP1 treatment (Fig. 1A).

To additionally characterize the cytoskeletal rearrangements associated with the morphological changes observed in RET/C634R, RET/M918T-, and RET/PTC3-expressing cells upon PP1 treatment, immunofluorescence analysis was performed. Cells were double stained with RET and FITC-labeled phalloidin to examine filamentous actin (F-actin; Fig. 1B). When compared with untreated cells, those expressing RET/C634R, RET/M918T, and RET/PTC3 and treated with 1 μM PP1, exhibited a markedly greater number of actin stress fibers that were well organized with a pattern similar to that seen for RET-negative NIH3T3 cells (Fig. 1B). In contrast, parental NIH3T3 cells and NIH3T3-overexpressing TRKA did not show any significant morphological change upon PP1 treatment (Fig. 1B). Moreover, PP1 addition also drastically changed subcellular localization of vinculin in RET/C634R-, RET/M918T-, and RET/PTC3-transformed fibroblasts (Fig. 1C). In fact, although in untreated cells we found a consistent cytoplasmic pool of vinculin primarily in the perinuclear region, in PP1-treated cells a significant fraction of vinculin was seen as punctate structures at the cell periphery, reminiscent of focal adhesion plaques (Fig. 1C). Therefore, PP1 addition induces actin stabilization and formation of enlarged vinculin-positive focal adhesions similar to the ones observed in parental NIH3T3 cells (Fig. 1C), thus reverting the transformed phenotype of RET/C634R, RET/M918T, and RET/PTC3 fibroblasts. PP1 has no effect on vinculin distribution in TRKA-expressing cells.

An in vitro wound repair model (24) was used to assess whether the architectural changes induced by PP1 treatment of RET transformed cells could also affect cell motility. Cells were seeded and cultured to 50% confluence, wounded, and the cell culture medium removed. After 12 h from wounding, RET/C634R- and RET/M918T-expressing
cells demonstrated extensive migration into the denuded area (Fig. 2). In contrast, NIH3T3-untransfected cells showed no significant migratory potential (Fig. 2). When the same experiments were repeated with cells cultured with PP1, we observed that PP1 treatment affected the ability of the RETC634R- and RETM918T-expressing cells to migrate, whereas the motility of parental NIH3T3 cells was not affected (Fig. 2). This result provided new information on the effects of PP1 treatment on RET-transformed cells.

Inhibition of RET Oncoprotein in Vitro Kinase Activity by PP1 and PP2. To evaluate RET in vitro kinase activity in the presence of PP1 inhibitor, RET wild-type and the RETC634R, RETM918T, and RET/PTC3 mutants were transiently transfected into 293T cells, and the synthesis of the correctly sized proteins was verified (data not shown). In vitro RET kinase activity was dramatically reduced when the assay was performed in the presence of PP1 inhibitor at a concentration of 1 μM. As depicted (Fig. 3), both RET autophosphorylation activity and transphosphorylation of MBP was impaired. As a control, 293T cells were also transiently transfected with a construct coding for TRKA wild-type protein and the enzymatic activity evaluated. In this case, no significant differences were found when the assay was performed in the presence or absence of PP1 inhibitor (Fig. 3). These results clearly demonstrated that PP1 was able to strongly reduce the ability of RET to both auto- and trans-phosphorylate substrates probably by specifically competing for the ATP binding site as already reported (23). To further verify that PP1 could fit in the RET ATP binding domain, another inhibitor PP2, which has a chemical structure very similar to that of PP1, was used. PP2 could mimic the results obtained with PP1 reducing both RET autophosphorylation and the phosphorylation of MBP (data not shown). In contrast, other tyrosine kinase inhibitors such as Genistein or Herbamycin, only partially affected RET enzymatic activity (data not shown and Ref. 23). Taken together, our results additionally suggest that PP1 and PP2 have structures that specifically fit in the ATP binding domain of RET, thus preventing ATP binding.

PP1 Treatment of RETMEN2A and RETMEN2B-expressing Cells not only Affects RET Autophosphorylation but Induces RET Oncoprotein Degradation. To assess the effects of PP1 in living cells, RETC634R- and RETM918T-expressing cells were tested
for RET expression and phosphorylation at various times. Parental and TRKA-overexpressing NIH3T3 cells were used as controls. Cells were incubated in the absence (−) or the presence of 1 μM PP1 for 0, 3, 6, 12, 24, 48, and 72 h before cell lysis. Tyrosine-phosphorylated proteins were detected by Western blotting with anti-pTyr antibodies. As expected, PP1 could dramatically reduce the amount of phosphorylated bands in anti-RET immunocomplexes or WCLs when visualized on a gel. RET phosphorylation already appeared reduced 3 h after the addition of PP1 and was observed to remain very low after 72 h (Fig. 4A). Surprisingly, when we checked for equivalent loading of proteins by reprobing the same blots with anti-RET antibodies, we discovered that even the entire RET protein content of the cell decreased along the time course for both RETC634R and RETM918T (Fig. 4A), whereas tubulin levels remained unchanged. Therefore, PP1 was not only able to reduce RET phosphorylation but, unexpectedly, it was also able to cause a progressive loss of RET expression. The effect is specific for RET because PP1 treatment was ineffective on TRKA-overexpressing fibroblasts (Fig. 4B). A progressive loss of RET expression was also observed for RET/PTC3 as shown in Fig. 4B, although the protein level appears clearly reduced only after 18 h. RET protein disappearance was also observed in Western blotting experiments when RETC634R and RETM918T cells were treated with PP2 but not when the same cells were cultured in the presence of PP3 (a negative control of PP2 that is able to inhibit the activity of EGFR kinase) or in the presence of other tyrosine kinase inhibitors such as Genistein or Herbamycin (Fig. 4C). Interestingly, a significant decrease in the amount of RET protein along with a decrease in its phosphorylation was detected even when proteins extracted from TT cells (a human MTC cell line) were immunoprecipitated with anti-RET antibodies, and Western blot analysis was performed with anti-RET and anti-pTyr antibodies (Fig. 4D). As expected, in this case, the rate of RET protein degradation was slower than in NIH3T3 cells overexpressing the oncoproteins.

Consistent with a progressive loss of RET expression upon PP1 treatment, immunofluorescence analysis of RETC634R -expressing cells stained for RET showed that the cellular distribution of the receptor changed during the period of observation. In fact, whereas in untreated cells, RET localized both to the cell surface and to intracellular compartments, in PP1-treated cells RET staining was mainly visible in intracellular compartments. Consistent with this, the staining of the cells with anti-RET and anti-clathrin antibodies indicated that RET colocalizes with clathrin upon 20 min of PP1 treatment, indicating that PP1 stimulates a translocation of the receptor from the membrane to intracellular clathrin-positive structures (Fig. 4E). RET relocalization was observed even when immunofluorescence analysis for RET was performed on RETM918T-transfected cells treated with PP1 (data not shown).

To highlight any significant defect in RET oncoprotein biosynthesis in the presence of PP1, we followed the fate of metabolically labeled mutant RET receptors expressed in NIH3T3 cells. Subconfluent cul-
**Fig. 4. Time course of PP1 treatment.**

*Panel A.* The indicated cell lines were incubated in the absence (-) or presence of 1 μM PP1 for the indicated time, before cells lysis. Anti-Ret immunocomplexes (Ip) or WCLs for RET<sup>C634R</sup> or RET<sup>M918T</sup>-expressing fibroblasts were separated on reducing 7% SDS-PAGE and transferred to nitrocellulose membranes. Upon incubation with anti-pTyr antibodies, tyrosine-phosphorylated proteins were detected by Western blotting. After stripping, the amount of proteins was verified by reprobing the same blots with anti-tubulin antibody. *B.* For TRKA-overexpressing cells, anti-TRK immunocomplexes (Ip) or WCLs (data not shown) were also separated on reducing 7% SDS-PAGE and transferred to nitrocellulose membranes. Upon incubation with anti-pTyr antibodies, tyrosine-phosphorylated proteins were detected by Western blotting. Anti-TRK antibody was used to verify the amount of proteins on the gel after stripping. For RET/PTC3, anti-RET immunocomplexes (Ip) or WCLs (data not shown) were also analyzed for RET phosphorylation and expression. *C.* The effects of other tyrosine kinase inhibitors were also analyzed. Anti-RET immunocomplexes for RET<sup>C634R</sup>-expressing cells treated with PP1 (1 μM), PP2 (1 μM), PP3 (1 μM), Genistein (100 μM), or Herbamycin (0.1 μM) for 12 h were separated on reducing 7% SDS-PAGE and transferred to nitrocellulose membranes. Upon incubation with anti-pTyr antibodies, tyrosine-phosphorylated proteins were detected by Western blotting. After stripping, the amount of proteins was verified by reprobing the same blots with anti-RET. *D.* TT cells (a human MTC cell line) were immunoprecipitated with anti-RET, and Western blot analysis with anti-RET and anti-pTyr was performed. Equal protein loading was confirmed by Western blotting on the same TT-cell extracts. *E.* PP1 induces RET redistribution in RET<sup>C634R</sup>-expressing cells. Where indicated, the transfected cells were plated in the presence of 1 μM PP1 and, after 20 min from plating, fixed and double-stained with RET (green) and clathrin (red) antibodies. The yellow staining in the merged images indicates colocalization of RET and clathrin mainly after PP1 treatment (details in the inset). Images were analyzed using confocal microscopy. *Inset:* ×4 magnification of normal image; bar: 15 μm.
tures were labeled with [35S]methionine-cysteine for 20 min and subsequently chased for varying times. Cells were previously grown for 12 h in the presence or absence of PP1 and then metabolic pulse-chased experiments performed as for untreated cells. As already demonstrated by Bongarzone et al. (26), the amount of RET$^{C634R}$ and RET$^{M918T}$ increased significantly within the first hour of the chase. In PP1-treated cells, the fate of both RET$^{C634R}$ and RET$^{M918T}$ receptors was comparable with that found in untreated cells during the period of observation, indicating that PP1 did not affect protein biosynthesis (data not shown).

In addition, we examined the cellular proliferation patterns of RET$^{C634R}$-, RET$^{M918T}$-, and RET/PTC3-expressing cells in the absence or presence of the inhibitor. One μM PP1 exerted a remarkable inhibitory effect on the growth of RET$^{C634R}$-, RET$^{M918T}$-, and RET/PTC3-expressing cells, as already demonstrated by Carlomagno et al. (23). Notably, we observed that the cytostatic effect only started after 48 h. In fact, RET-expressing cells resembled the parental cells for the first 2 days, but at later times, no increase in the number of living cells in the presence of PP1 could be detected (Fig. 5). This is consistent with the fact that after 48 h after PP1 addition, cells had almost completely lost the expression of the RET oncoproteins, suggesting that RET depletion induces growth arrest. No apoptotic effect was observed as demonstrated by terminal deoxynucleotidyl transferase-mediated nick end labeling analysis when performed on cells treated with PP1 or PP2 (data not shown). This result was confirmed by Western blot analysis using anti-poly(ADP-ribose) polymerase antibodies and performed on whole cell extracts from cells treated with PP1 or PP2 for various times. In fact, no cleavage of poly(ADP-ribose) polymerase, which facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis, was detected (data not shown). The proliferation rate of parental- and TRKA-overexpressing NIH3T3 cells was only partially slowed down in the presence of PP1 (Fig. 5).

**PP1 Treatment Induces Ubiquitinated RET Oncoprotein Degradation via Proteosomal Targeting.** We examined whether the PP1 inhibitor could induce RET degradation. Incubation of NIH3T3 fibroblasts expressing RET$^{C634R}$ and RET$^{M918T}$ receptors with both PP1 and the proteosome inhibitor, lactacystin, abolished RET destruction as shown by Western blot detection of the RET protein using an anti-RET specific antibody (Fig. 6A). Lactacystin alone did not significantly modify the amount of RET proteins. RET degradation was also substantially reduced upon incubating cells with another proteosome inhibitor, MG132 (data not shown). Moreover, RET tyrosine-phosphorylated proteins were detected by Western blotting with anti-
pTyr antibodies (Fig. 6A). The bands corresponding to the RET mutants were not phosphorylated upon treatment with PP1 and lactacystin together, again confirming that PP1 exerts its normal effect by reducing RET phosphorylation but that the proteosomal inhibition was preventing the destruction of the unphosphorylated protein. Taken together, these results strongly indicate that PP1-induced RET degradation is because of proteosomal targeting as the inhibition of the proteosome substantially attenuates the decrease in RET protein levels. Consistent with these observations, chloroquine, a weak base that alkalinizes the lysosome, was ineffective in inhibiting RET degradation (Fig. 6A), thus confirming that PP1 treatment most probably targets RET oncoproteins to proteosomal, rather than lysosomal, degradation and whether PP1 treatment could modify RET oncoprotein degradation and whether PP1 treatment could modify RET oncoprotein interaction with this ubiquitin ligase, Western blot analysis of anti-Cbl immunoprecipitates with anti-RET antibodies from cells treated or untreated with PP1, indicated that the RETC634R and RETM918T oncoproteins could associate with c-Cbl (Fig. 6B). Moreover, c-Cbl binds to the RET oncoproteins irrespective of PP1 treatment and of RET phosphorylation status. Western blot analysis of both anti-Cbl immunoprecipitates with anti-RET antibodies and anti-RET immunoprecipitates with anti-Cbl antibodies to detect RET expression, RET phosphorylation, c-Cbl expression, and phosphorylation in anti-RET immunoprecipitates. Proteins were also extracted from untransfected NIH3T3 fibroblasts treated with PP1 (1 μM for 6 and 12 h as indicated), and c-Cbl immunoprecipitates were subjected to Western blot analysis anti-Cbl and anti-pTyr antibodies to detect c-Cbl expression and phosphorylation in anti-Cbl immunoprecipitates.
oncoproteins were ubiquitinated. As proteosome inhibition leads to a depletion of free ubiquitin (28–30), the signal corresponding to ubiquitinated RETC634R oncoproteins was more intense when cells were cultured in the presence of lactacystin, although ubiquitinated RETC634R cells treated with PP1 alone (Fig. 7A). The same results were obtained when analyzing the ubiquitination pattern of RETM918T oncoproteins (data not shown). Additional immunofluorescence studies were performed to confirm that the RETC634R oncoproteins can be ubiquitinated. RETC634R-expressing cells were double stained with anti-RET polyclonal antibodies and monoclonal antipolyubiquitin antibodies (FK1) to detect ubiquitinated RET mutants. Even in these studies, ubiquitinated RETC634R oncoproteins could be observed in untreated cells (Fig. 7B, top panel), as well as in PP1-treated cells (Fig. 7B, bottom panel), thus suggesting that PP1 does not promote ubiquitination. Thus, RET oncoproteins are constitutively ubiquitinated, and PP1 treatment does not induce nor affect protein ubiquitination. Taken together, these findings indicate that RET oncoproteins can bind to
c-Cbl, can be ubiquitinated, and therefore targeted to proteasomal degradation irrespective of PP1 treatment, but proteasomal degradation is strongly accelerated when cells are treated with PP1.

**DISCUSSION**

In recent years, several efforts have been made to develop therapeutic strategies targeting oncoproteins. Cancer therapy directed at specific molecular alterations in the signaling pathways of cancer cells has been validated, especially for the treatment of advanced breast cancer and gastrointestinal stromal tumors. For ErbB proteins, a group of drugs are already in advanced stages of clinical testing (31, 32). These tyrosine kinase inhibitors are very selective, and as a consequence of blocking the kinase activity, most of the downstream signaling pathways are inhibited, leading to growth arrest of tumors in which the proliferation depends on ErbB signaling.

It would be of great interest to identify inhibitors for RET tyrosine kinase and to develop new therapeutic strategies for RET-associated diseases and, in particular, for MTC, which is the common clinical manifestation of MEN2 syndromes. MTC responds very poorly to chemotherapy, and at present, the only cure involves a total thyroidectomy. Recently, Carломagno et al. (23) have suggested that PP1 could be used to inhibit RET/PTC-induced tumorigenesis.

A key finding made in the course of the present study, which was aimed to detail the effects of PP1 on RET<sup>C634R</sup>, and RET<sup>M918T</sup>, expressing cells, was the unexpected ability of both the two related tyrosine kinase inhibitors, PP1 and PP2, to induce proteosomal destruction of the RET oncoproteins. In fact, although PP1 was shown to inhibit RET oncoprotein kinase activity (our data and Ref. 23) and to affect RET/PTC3-dependent mitogen-activated protein kinase phosphorylation as early as 2 h after exposure (23), the analysis of the long-term effects of PP1 treatment showed that there was a remarkable decrease in the levels of RET oncoproteins after 12 h (Fig. 4A), and a clear reversion of the transformed phenotype was observed (Fig. 1). Moreover, cells expressing RET oncoproteins treated with PP1 could proliferate as the untreated ones for the first 2 days after PP1 addition, but at later times, a clear cytostatic effect was detectable (Fig. 5), and no RET oncoprotein expression was found (Fig. 4A).

Therefore, our observations extend previous reports on a linkage between the inhibition of RET phosphorylation and cell growth (23) and suggest that drug-induced receptor destruction clearly contributes to the cytostatic effect of tyrosine kinase inhibitors. Moreover, it is tempting to speculate that a stable phenotypical reversion of RET-transformed cells and growth arrest might be because of the initial dephosphorylation of RET and its consequent depletion.

No studies have been performed to elucidate the way RET receptor becomes physiologically degraded in cells, and no data are available on constitutively activated receptor down-regulation. For ErbB proteins, two major inducible pathways that control their degradation have been well highlighted: ligand-induced rapid endocytosis of ErbB-1 (33); and antibody- and oncogenic mutation-induced destruction of ErbB-2 (34), pathways that are both mediated, at least in part, by the c-Cbl ubiquitin ligase. Cbl proteins have a key role in sorting active RTKs into invaginating pits and function as ubiquitin protein ligases that direct multiubiquitination and down-regulation of RTKs. Cbl-mediated ubiquitination accelerates EGFR endocytosis and its delivery to lysosomes for degradation (33). Thus, the sorting of RTKs to be internalized is controlled by two types of reversible modification, phosphorylation, and ubiquitination.

The RET mutants associated with MEN2A and MEN2B used in this study are constitutively activated and therefore constitutively phosphorylated. Unexpectedly, consistent with this constitutive phosphorylation status of the RET oncoproteins, we reported that a high proportion of these oncogenic proteins are ubiquitinated and associate with c-Cbl and ubiquitin. Moreover, the treatment of the cells with PP1 does not promote nor modify these processes. In particular, the binding of c-Cbl and RET, which had yet to be demonstrated but has now been shown, is an early event for these oncogenic products that is not affected by PP1-mediated RET dephosphorylation. This is in line with our data showing that none of the RET tyrosine residues phosphorylated and/or relevant for RET-mediated mitogenesis seem to be directly involved in binding c-Cbl (data not shown). Therefore, the ability of RET to bind c-Cbl can be separated from its ability to drive mitogenic signaling and probably none of the RET tyrosine residues whose phosphorylation is impaired by the inhibitor are involved in c-Cbl binding. PP1 might then have a role in inhibiting the RET-mitogenic signaling cascade but not the pathways that control its degradation. Taken together, our results also give rise to intriguing questions on how the fate of RET oncoproteins is determined because their phosphorylation is constitutive as it is their binding to c-Cbl and their ubiquitination.

Interestingly, Citri et al. (35) indicated that the tyrosine kinase inhibitor CI-1033 directed ErbB-2 to a degradative fate mediated by the chaperone destructive system, which is functionally and structurally distinct from the c-Cbl-mediated pathway. The Cbl-mediated pathway requires kinase activity to induce degradation, whereas the chaperone-mediated route is recruited to the kinase domain mainly upon structural perturbation of the ATP-binding pocket of the oncoprotein. PP1 could act on RET oncoproteins through the activation of similar pathways and the hypothesis that PP1 could target the receptor to destruction by inducing such structural perturbation and thus recruiting the stress-inducible machinery is under current study.

In conclusion, we have shown here that the previously reported results on PP1 activity on cytoplasmic RET fusion proteins can be convincingly extended to membrane-bound RET mutants associated with MEN2A and MEN2B syndromes and to sporadic MTCs, emphasizing the potential therapeutic role of this compound. In fact, after PP1-mediated dephosphorylation, RET oncoproteins are rapidly targeted to proteasomal destruction. The interaction with the inhibitor can therefore either block most of the downstream mitogenic signaling, apart from the one driven by c-Cbl so that recycling is prevented and invagination and degradation accelerated, or it can misfold the protein structure, which is then targeted to stress-induced rapid destruction.

**ACKNOWLEDGMENTS**

We thank Dr. Aaron Cranston for critical reviewing of the manuscript, Dr. Manuela Campiglio for the gift of the MDA-MB-231 cell line and for the EGFR antibody, Simona Polo for the gift of the clathrin antibody and for helpful discussions, and Cristina Mazzadi and Vittoria Volpe for secretarial help.

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