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Original Research Communication

Osmotic Stress-Mediated Activation of RET Kinases Involves Intracellular Disulfide-Bonded Dimer Formation

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ABSTRACT

We showed that osmotic stress induces activation of c-RET and second-set activation of constitutively activated RET-MEN2B. A few percentage of RET proteins normally formed disulfide-bonded dimers in the cell, and osmotic stress promoted formation of these dimers. The disulfide-bonded dimers displayed higher levels of autophosphorylation and catalytic activity per molecule than did monomers. Osmotic stress also promoted activation and disulfide-bonded dimerization of the extracellular domain-depleted mutant RET (RET-PTC-1), suggesting that the target amino acid(s) for dimerization is located intracellularly rather than in the cysteine-rich region of the extracellular domain. In the mutant c-RET and RET-PTC-1 in which Cys^{987} of c-RET or Cys^{376} of RET-PTC-1 was replaced with Ala, the levels of intrinsic kinase activity were greatly reduced and barely increased in response to osmotic stress. Correspondingly, the Cys^{376}-defective RET-PTC-1 did not form any demonstrable levels of dimers even after exposure to osmotic stress. In contrast, another RET-PTC-1 mutant that had a replacement of Cys^{365} with Ala mostly behaved like parental RET-PTC-1. These results suggest that Cys^{987} of c-RET or Cys^{376} of RET-PTC-1 plays a crucial role in maintenance and promotion of dimerization and activation of the RET kinases. Antioxid. Redox Signal. 3, 473–482.

INTRODUCTION

THE C-RET PROTO-ONCOGENE encodes a receptor-type tyrosine kinase (23), which is an essential signaling component for renal organogenesis and enteric neurogenesis (19, 22). c-RET can be physiologically activated by binding of an appropriate glial cell-derived neurotrophic factor to the receptor (9). The RET kinase has also been shown to be activated by point mutations or rearrangement of the coding gene (21). Mutation in c-RET can cause hereditary neoplastic disorders such as multiple endocrine neoplasia type 2A (MEN2A) and type 2B (MEN2B) (7, 14), and rearrangement of this gene is frequently found in human papillary thyroid carcinoma (PTC) (6, 8). A point mutation of RET-MEN2A is found in cysteine in the cysteine-rich region of the extracelullar domain, and this mutation induces the activation of RET through the formation of disulfide bonds between unpaired cysteine residues in the extracellular domains of two RET molecules (2, 18). It has also been reported that activation of RET-MEN2B results from an altered conformation of the kinase domain (18). Therefore, multiple events, either extracellular or intracellular domain of RET protein-oriented,

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seem to be involved in control of the kinase activity.

Osmotic stress has been reported to induce the activation of both receptor-type and non–receptor-type protein tyrosine kinases (PTKs) such as epidermal growth factor receptor and Fyn and Syk (1, 15, 17). The mechanism of osmotic stress-mediated PTK activation is not known, but recent studies have suggested that cell shrinkage, rather than high osmolarity, regulates the PTK activity through inducing aggregation of cell surface receptors potentially as a result of macromolecular crowding (1, 13, 15, 17). The PTK (Syk) activation was further shown to be elicited by reactive oxygen species (ROS) that had been produced downstream of receptor aggregation in response to osmotic stress (16). Very little, however, is known about the molecular target of cell shrinkage and ROS for the osmotic stress-mediated activation of either receptor-type or non–receptor-type PTKs. In this study, we analyzed the molecular basis of the osmotic stress-induced activation of a receptor-type PTK, RET, and its non–receptor-type mutant, and we found that a conserved cysteine residue in the C-terminus of the kinase domain is crucial for both disulfide-bonded dimerization and activation of both types of RET kinases in response to osmotic stress.

MATERIALS AND METHODS

Plasmid construction

A cDNA clone containing the sequence of the human c-RET gene was inserted into an APtag-1 vector containing the Moloney murine leukemia virus long terminal repeat (4; kindly provided by Dr. P. Leder, Harvard Medical School, Cambridge, MA, U.S.A.). Mutation was induced by a polymerase chain reaction. In brief, the primer containing the mutation was synthesized and used for amplification of a c-RET sequence of ~100–150 bp. The corresponding sequence of the c-RET gene was replaced with the amplified fragment containing the mutation. The amplified fragment was sequenced to confirm that the proper mutation had been introduced.

Transfection and cell culture

Each recombinant plasmid (0.1 μg) was transfected into NIH3T3 cells (5×10^5 cells in a 60-mm diameter dish) with 10 μg of DNA, as described previously (2). Cells were cultured in Dulbecco's modified Eagle minimal essential medium (DMEM; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 8% bovine calf serum (HyClone Laboratories, Inc., Logan, UT, U.S.A.), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified 5% CO_2 atmosphere. For experimental use, near-confluent cells were starved by incubation in serum-free DMEM for 1 h. Stress was achieved by the addition of 0.4 *M* NaCl solution, 0.8 *M* sorbitol solution, or urea for 5–30 min. Cells were stimulated at 37°C.

Preparation of cell lines expressing mutant RET proteins

The c-RET cDNA with a MEN2B mutation (RET-MEN2B, $Met^{918} \rightarrow Thr$; M918T), rearranged RET-PTC-1 cDNA (8), c-RET whose Cys^{987} in the C-terminal end of the kinase domain was replaced with alanine (c-RET-C987A, $Cys^{987} \rightarrow Ala$), and RET-PTC-1 whose Cys^{365} or Cys^{376} in the C-terminal end of the kinase domain was replaced with alanine (RET-PTC-1-C365A, $Cys^{365} \rightarrow Ala$; RET-PTC-1-C376A, $Cys^{376} \rightarrow Ala$) were prepared and inserted into the expression vector (Fig. 1). They were transfected into NIH3T3 cells. More than two independent cell lines were analyzed for each RET form in the basic experiments for both immunoblot and *in vitro* kinase assay with consistent results, and only representative data are presented.

Immunoblot and antibodies

Western blotting (immunoblot) was performed according to the method described previously (11). Cells were lysed in sample buffer [62.5 m*M* Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerin] with 5% 2-mercaptoethanol (2ME) (reducing) except when otherwise noted, where 2ME was omitted (nonreducing). The lysates (30 μg/lane) were subjected to SDS–polyacrylamide gel (5–10%) electrophoresis (SDS-PAGE) and transferred to

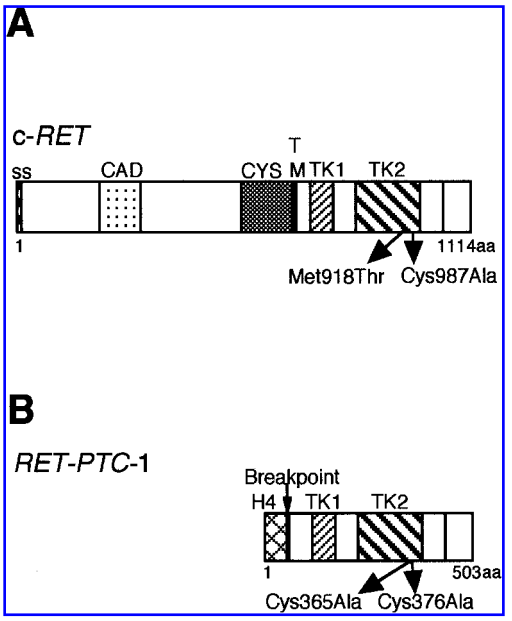

FIG. 1. Schematic illustration of mutant *RET* cDNA constructs. (**A**) *RET* cDNA in which methionine at codon 918 was replaced with threonine (M918T; *RET*-MEN2B) or cysteine at codon 987 was replaced with alanine (c-*RET*-C987A). (**B**) Mutant *RET* cDNA in which the extracellular domain was deleted (*RET*-PTC-1), and *RET*-PTC-1 in which cysteine at codon 365 or 376 was replaced with alanine (*RET*-PTC-1-C365A, *RET*-PTC-1-C376A). ss, signal sequence; CAD, cadherin-like domain; CYS, cysteine-rich region; TM, transmembrane domain; TK1, tyrosine kinase domain 1; TK2, tyrosine kinase domain 2; aa, amino acids.

polyvinylidene difluoride membranes (Nihon Millipore Kogyo KK, Yonezawa, Japan). After the membranes had been reacted with the first antibody (antibody specific to RET or phosphotyrosine), the reaction was examined by western blot chemiluminescence reagent (DuPont NEN, Boston, MA, U.S.A.). Anti-RET rabbit polyclonal antibody was produced as described previously (24). An anti-phosphotyrosine polyclonal antibody was obtained from Transduction Laboratories (Lexington, KY, U.S.A.).

Immunoprecipitation and kinase assay

Immunoprecipitation was performed as described previously (11). In brief, stimulated cells were lysed in lysis buffer (10 m*M* Tris-HCl, pH 8.0, 1% Triton X-100, 150 m*M* NaCl, 1 m*M* EDTA, 0.5 m*M* Na_3VO_4). The lysates were centrifuged at 13,000 rpm for 30 min at 4°C, and the anti-RET polyclonal antibody was added to the supernatant. The immunoprecipitates were collected by incubating with protein A-Sepharose beads. The immunoprecipitated RET proteins were washed three times with lysis buffer (10 m*M* Tris-HCl, pH 8.0, 1% Triton X-100, 150 m*M* NaCl, 1 m*M* EDTA, 0.5 m*M* Na_3VO_4) for either immunoblot or *in vitro* kinase assay. The *in vitro* RET kinase assay was performed as described previously (2, 11). In brief, the immunoprecipitated RET proteins were washed three times with kinase buffer (30 m*M* Tris-HCl, pH 7.4, 5 m*M* $MgCl_2$), suspended in the kinase buffer with 2.0 μg of myelin basic protein (MBP; Sigma Chemical Co.) as an exogenous substrate, and radiolabeled [γ-^{32}P]ATP (370 kBq; NEN, Wilmington, DE, U.S.A.). The kinase reaction was carried out for 20 min in a 30°C water bath and was terminated by adding sample buffer with (reducing) or without (nonreducing) 2ME. The immunoprecipitates were then boiled for 4 min and loaded on 5–13% SDS–polyacrylamide gels. The gels were dried and exposed to Fuji x-ray film at −80°C for autoradiography. The molecular sizes of the developed proteins were estimated by comparison with protein molecular weight standards (GIBCO, Gaithersburg, MD, U.S.A.).

RESULTS

Osmotic stress induces activation of RET kinase

By increasing the extracellular NaCl concentration with the addition of 0.4 *M* NaCl (osmotic stress), the level of tyrosine phosphorylation of cellular proteins was increased in NIH3T3 cells that had been transfected with c-*RET*, and less extensively in nontransfected control NIH3T3 (data not shown). This suggested that the transfected RET kinase is activated in response to osmotic stress. The tyrosine phosphorylation of cellular proteins was also induced by 0.8 *M* sorbitol, another high-osmolarity producer, but not by 0.8 *M* urea (data not shown). Urea is known to be a rapidly permeating solution that does not affect cell volume (13). This result suggested that hypertonicity itself is not sufficient to increase the level of tyrosine phosphorylation in cells and confirmed earlier observations that membrane

deformation due to a decrease in cell volume, rather than the change in extra- and/or intracellular tonicity, is responsible for osmotic stress-induced tyrosine phosphorylation promotion (5, 10, 13).

Before directly studying the effect of osmotic stress on RET kinase activity, we measured the levels of kinase activity of RET proteins in cell lines expressing c-RET or RET-MEN2B. RET proteins were isolated from cell lysates by immunoprecipitation and were subjected to western blotting with anti-RET antibodies and *in vitro* kinase assay. As shown in Fig. 2A, the relative level of catalytic activity of RET-MEN2B (lane 2) was higher for both autophosphorylation and phosphorylation of MBP as an exogenous substrate than that of the c-RET (lane 1), whereas the levels of overall protein expression of c-RET and RET-MEN2B in cells were comparable (Fig. 2B). These results confirmed that RET kinase is constitutively activated by MEN2B mutation.

We next examined the effects of hypertonic treatment of NIH3T3 cells that had been transfected with c-*RET* or *RET*-MEN2B on the levels of tyrosine phosphorylation and kinase activity of RET. RET proteins were isolated from cell lysates by immunoprecipitation and were subjected to western blotting with anti-RET and anti-phosphotyrosine antibodies. Figure 2C (left panel) shows that phosphotyrosine accumulation was detected in c-RET obtained from hypertonically challenged cells. In further experiments, we investigated whether hypertonic treatment could actually cause a detectable change in the catalytic activity of this kinase. As shown in Fig. 2D (left panel), high osmolar exposure for 5 min markedly up-regulated the kinase activity of c-RET, which was demonstrated for both autophosphorylation and MBP phosphorylation. Interestingly, the levels of tyrosine phosphorylation (Fig. 2C, right) and kinase activity (Fig. 2D, right) of constitutively activated RET-MEN2B were also elevated further by the hypertonic treatment.

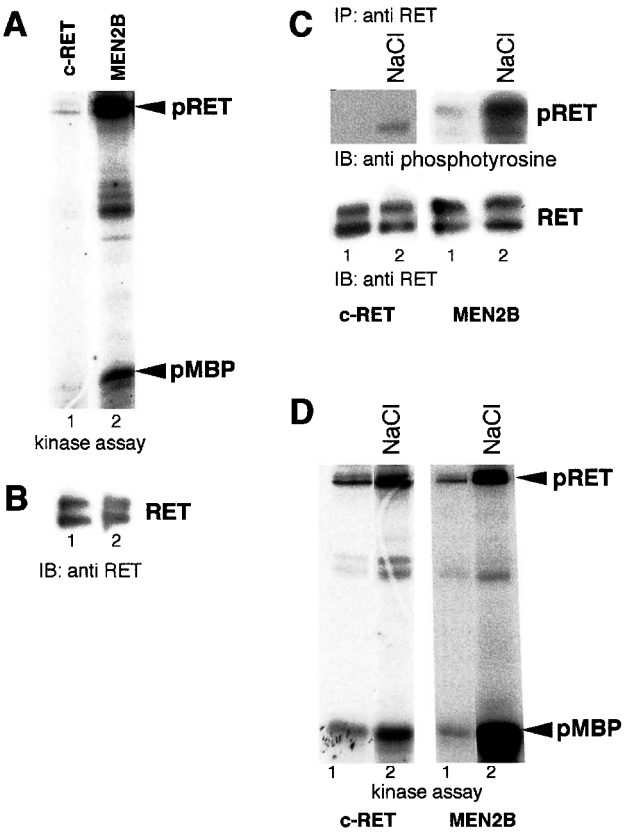

FIG. 2. Osmotic stress activates c-RET and RET-MEN2B. (**A** and **B**) Lysates from NIH3T3 cells transfected with c-*RET* (lane 1) or *RET*-MEN2B (lane 2) were immunoprecipitated with anti-RET antibody for either *in vitro* kinase assay (A) or western blotting (IB) with anti-RET antibody (B). Although data are not shown in the figure, no band developed at the position of RET or pRET in the gel for control NIH3T3 cells that did not carry RET. (**C** and **D**) Lysates from NIH3T3 cells transfected with c-*RET* (**left**) or *RET*-MEN2B (**right**) after incubation in isotonic solution (lane 1) or additional 0.4 *M* NaCl (lane 2) solution for 5 min were immunoprecipitated with anti-RET antibody for western blotting with anti-phosphotyrosine antibody (top in C) or anti-RET antibody (bottom in C) or for *in vitro* kinase assay (D). For direct comparison of kinase activities of c-RET and RET-MEN2B, see panel A, because panel D for c-RET was obtained after longer exposure of dried gel to x-ray films than that for RET-MEN2B. SDS-PAGE was done in 8% (B and C) or 13% (A and D) polyacrylamide gel. We performed three independent experiments for A–D with basically the same results, and only the representative data are shown. RET, RET proteins forming doublet (175-kDa and 155-kDa bands); pRET, autophosphorylated RET; pMBP, phosphorylated MBP. In addition to the indicated specific protein bands, a few nonspecific bands developed between pRET and pMBP in A and D.

Osmotic stress promotes disulfide-bonded dimerization of RET proteins for activation

Earlier studies showed that promotion of the dimerization of RET by a ligand (3) or MEN2A-type point mutation (2, 18) leads to RET kinase activation. As shown in Fig. 3A (lane 3), a small amount of c-RET was shown to be located at the position of disulfide-bonded dimers from

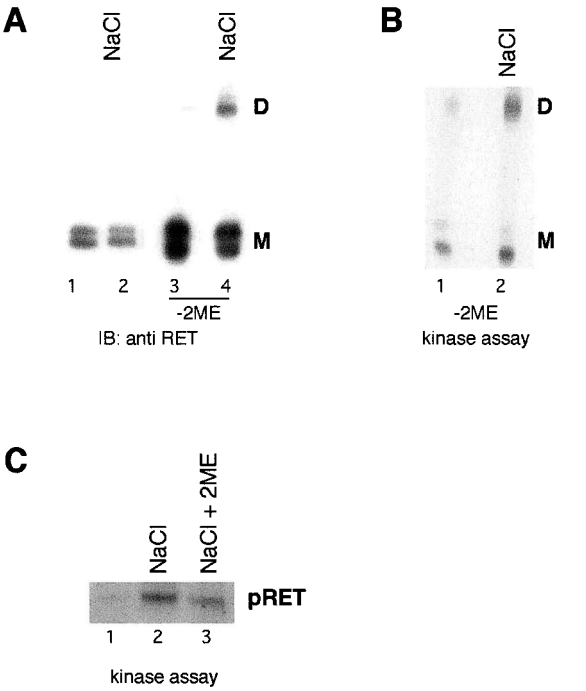

FIG. 3. Osmotic stress promotes disulfide-bonded dimerization of c-RET in association with promotion of the catalytic activity. (**A**) Lysates from NIH3T3 cells transfected with c-*RET* after incubation in isotonic solution (lanes 1 and 3) or additional 0.4 *M* NaCl (lanes 2 and 4) solution for 5 min were subjected to western blotting with anti-RET. SDS-PAGE was done in 5% polyacrylamide gel under ordinary reducing (lanes 1 and 2) or nonreducing (without 2ME; lanes 3 and 4) conditions. (**B**) Lysates from NIH3T3 cells transfected with c-*RET* after incubation in isotonic solution (lane 1) or additional 0.4 *M* NaCl (lane 2) solution for 5 min were subjected to *in vitro* kinase assay after immunoprecipitation with anti-RET antibody. SDS-PAGE was done in 5% polyacrylamide gel under a nonreducing condition. (**C**) Lysates from the NIH3T3 cells transfected with c-*RET* after incubation in isotonic solution (lane 1) or additional 0.4 *M* NaCl (lanes 2 and 3) solution for 5 min were immunoprecipitated with anti-RET antibody. The immunoprecipitated RET proteins were treated (lane 3) or not treated (lane 2) with 5% 2ME for 30 min, then washed five times to remove 2ME, and subjected to *in vitro* kinase assay. SDS-PAGE was done in 5% polyacrylamide gel. We performed three independent experiments for (**A–C**) with basically the same results, and only representative data are shown. M, monomer RET (175 kDa/155 kDa, estimated by comparison with protein molecular mass standards that are not shown in the figure); D, dimer RET (~310–350 kDa); pRET, autophosphorylated RET.

the molecular weight in the gel when analyzed by SDS-PAGE under a nonreducing condition. High osmolar exposure to cells promoted this dimerization of c-RET (lane 4). Interestingly, the dimerized c-RET displayed a greater than three times higher level of autophosphorylation per molecule in the kinase assay than did monomer c-RET (lane 2 in Fig. 3B), by comparable densitometric analyses of protein bands in Fig. 3A and B. A small amount of RET-MEN2B also formed disulfide-bonded dimers in cells before treatment, and high osmolar exposure of cells promoted dimerization of RET-MEN2B, in close association with the promotion of RET autophosphorylation (data not shown).

The up-regulated kinase activity of c-RET from cells that had been treated with hypertonic solution was clearly reduced by treating the isolated RET with 2ME as a disulfide bond-dissociating reagent before the *in vitro* kinase assay (Fig. 3C). Correlated with this effect, 2ME actually inhibited the RET dimerization (data not shown). This result supported the view that chemical modification of RET proteins for disulfide-bonded dimerization, which was induced by osmotic stress, is involved in the mechanism of up-regulation of their kinase activity.

Osmotic stress can affect the intracellular domain of RET

The major question for us at this stage was whether, like MEN2A (2, 18), the target amino acids for disulfide-bonded dimerization of c-RET or RET-MEN2B are located in the cysteine-rich region of the extracellular domain. To answer this question, we used RET-PTC-1 with deletion of the extracellular domain as the possible target of osmotic stress. As shown in Fig. 4A, the exposure of cells to hyperosmolarity increased both autophosphorylation and MBP phosphorylation levels. These results show that the extracellular domain of RET kinase is not essential for the osmotic stress-induced activation of the kinase. We then examined whether high osmolar exposure could promote disulfide-bonded dimerization of RET-PTC-1. A small amount of RET-PTC-1 was found to be dimerized in cells before treatment under nonreducing conditions (lane 3 in Fig. 4B), and exposure of the cells to hyperosmolarity promoted dimerization of RET-PTC-1 (lane 4). The dimerized RET-PTC-1 (lane 2 in Fig. 4C) displayed a 20 times higher level of autophosphorylation per molecule in the kinase assay than did monomer

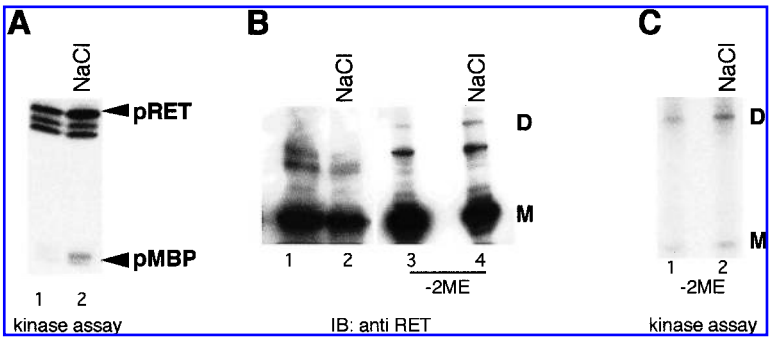

FIG. 4. Osmotic stress promotes dimerization-linked activation of extracellular domain-deleted RET-PTC-1. (**A** and **C**) Lysates from NIH3T3 cells transfected with *RET*-PTC-1 after incubation in isotonic solution (lane 1) or additional 0.4 *M* NaCl (lane 2) solution for 5 min were immunoprecipitated with anti-RET antibody for either *in vitro* kinase assay with (A) or without (C) addition of MBP. SDS-PAGE was done in 13% polyacrylamide gel under ordinary reducing conditions (A) or in 8% polyacrylamide gel under non-reducing conditions (C). (**B**) Lysates from NIH3T3 cells transfected with *RET*-PTC-1 after incubation in isotonic solution (lanes 1 and 3) or additional 0.4 *M* NaCl (lanes 2 and 4) solution for 5 minutes were subjected to western blotting with anti-RET. SDS-PAGE was done under ordinary reducing (lanes 1 and 2) or nonreducing conditions (lanes 3 and 4) in 8% polyacrylamide gel. We performed three independent experiments for A–C with basically the same results, and only representative data are shown. pRET, autophosphorylated RET; pMBP, phosphorylated MBP; M, monomer RET; D, dimer RET. In addition to the indicated specific bands, a few nonspecific bands developed between pRET and pMBP in A. Nonspecific bands also developed between D and M in B, and these bands were common to pictures obtained under reducing (lanes 1 and 2) and nonreducing (lanes 3 and 4) conditions and did not correspond to any of the autophosphorylated protein bands in C.

RET-PTC-1, as determined by comparative densitometric analyses of protein bands in Fig. 4B and C. These results suggested that the major target amino acid(s) of osmotic stress for dimerization and activation of RET kinase are located in their intracellular domain.

Partial identification of the target amino acid for osmotic stress-induced dimerization-linked activation of RET kinases

We performed further analyses to partially identify the possible target amino acid(s) of osmotic stress in RET kinase proteins for dimerization and activation. We focused our study on a few cysteines in the C-terminus of the kinase domain that are highly conserved among many protein tyrosine kinases (23, 25). We prepared cDNAs of c-RET-C987A (a mutant in which Cys^{987} is replaced with alanine), RET-PTC-1-C376A and RET-PTC-1-C365A, and we produced three to five clones of transfectants for each of the three mutant RET genes.

Due to an as-yet-unidentified mechanism, protein expression levels of c-RET-C987A and RET-PTC-1-C376A were 20–50% of those of original c-RET or RET-PTC-1 (Fig. 5B, lane 2, compared with lane 1 as a control; Fig. 5D, lane 3, compared with lane 1), whereas the expression level of RET-PTC-1-C365A was not much different from that of original RET-PTC-1 (Fig. 5D, lane 2, compared with lane 1). The RET-PTC-1-C365A displayed slightly less basic catalytic activity (Fig. 5C, lane 2 compared with lane 1 as a control) and responded to osmotic stress for increase in the catalytic activity as well as original RET-PTC-1 did (Fig. 5F compared with Fig. 4A as original). In contrast, c-RET-C987A and RET-PTC-1-C376A showed greatly reduced basic catalytic activities, as measured by the levels of RET autophosphorylation and phosphorylation of MBP (Fig. 5A, lane 2, compared with lane 1; Fig. 5C, lane 3, compared with lane 1). The extents of reduction in the catalytic activity of these mutant RET proteins ($<5\%$ by densitometric analysis) were greater than expected from the decrease in the protein expression level (20–50%; Fig. 5B, lane 2, and Fig. 5D, lane 3), and in the case of RET-PTC-1-C376A, only long-term film exposure resulted in development of detectable bands for RET autophosphorylation and MBP phosphorylation (Fig. 5G, lane 1). These results suggested that Cys^{987} of c-RET and Cys^{365} of RET-PTC-1 play crucial roles in the maintenance of basic catalytic activities of the RET ki-

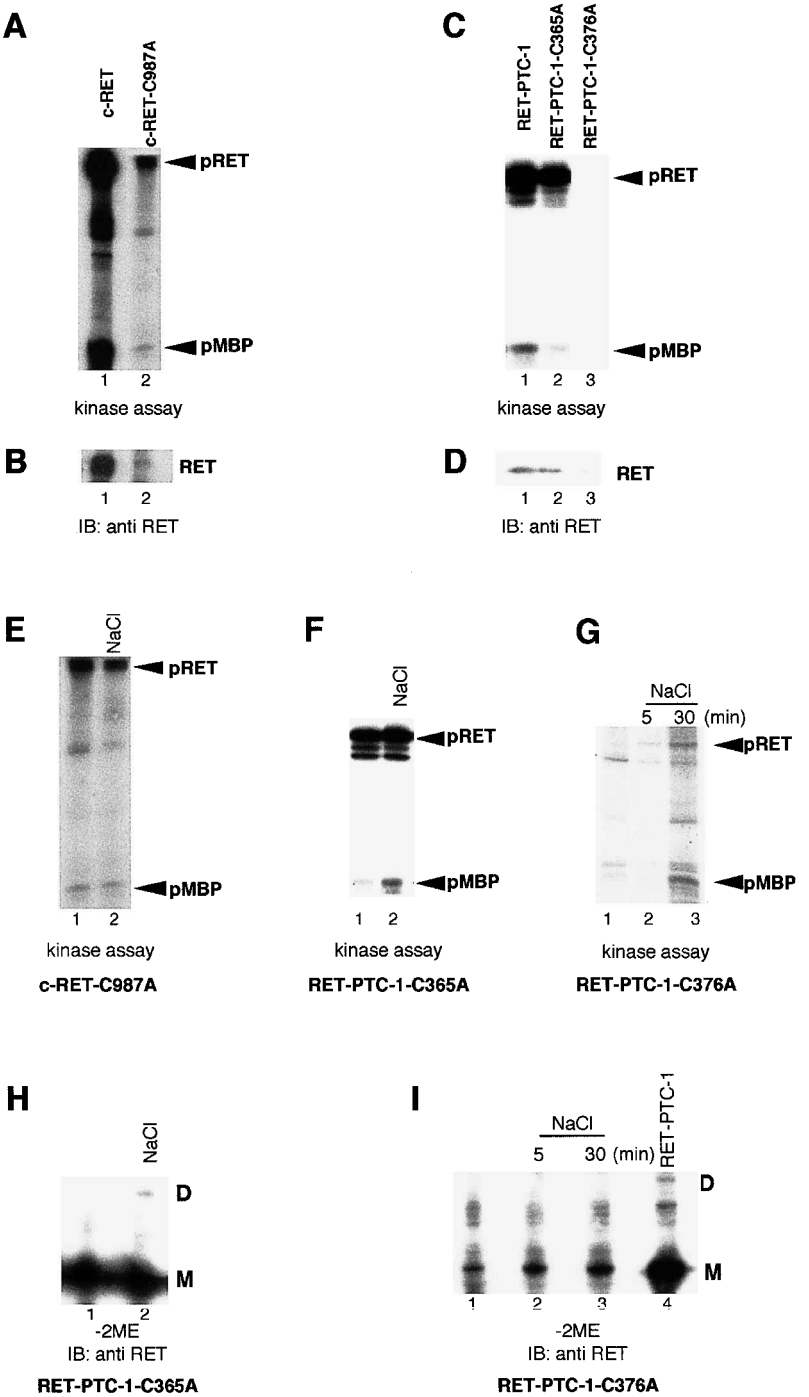

FIG. 5. Osmotic stress promotes dimerization-linked activation of RET-PTC-1-C365A, but not of c-RET-C987A or RET-PTC-1-C376A. (**A** and **B**) Lysates from NIH3T3 cells transfected with c-*RET* (lane 1) or c-*RET*-C987A (lane 2) were subjected to *in vitro* kinase assay after immunoprecipitation with anti-RET antibody (A) or to western blotting with anti-RET antibody (B). SDS-PAGE was done in 13% (A) or 5% (B) polyacrylamide gel. (**C** and **D**) Lysates from NIH3T3 cells transfected with *RET*-PTC-1 (lane 1), *RET*-PTC-1-C365A (lane 2), or *RET*-PTC-1-C376A (lane 3) were subjected to *in vitro* kinase assay after immunoprecipitation with anti-RET antibody (C) or to western blotting with anti-RET antibody (D). SDS-PAGE was done in 13% (C) or 8% (D) polyacrylamide gel. (**E**) Lysates from NIH3T3 cells transfected with c-RET-C987A after incubation in isotonic solution (lane 1) or additional 0.4 *M* NaCl solution for 5 min (lane 2) were subjected to *in vitro* kinase assay after immunoprecipitation with anti-RET antibody. SDS-PAGE was done in 13% polyacrylamide gel. (**F–I**) Lysates from NIH3T3 cells transfected with *RET*-PTC-1-C365A (F and H) or *RET*-PTC-1-C376A (G and I) after incubation in isotonic solution (lane 1) or additional 0.4 *M* NaCl solution for 5 min (lane 2) or 30 min (lane 3) were subjected to *in vitro* kinase assay after immunoprecipitation with anti-RET antibody (F and G) or to western blotting with anti-RET antibody (H and I). SDS-PAGE was done in 13% polyacrylamide gel under ordinary reducing conditions (F and G) or in 8% polyacrylamide gel under nonreducing conditions (H and I). We performed three or four independent experiments for (A–I) with basically the same results, and only representative data are shown. pRET, autophosphorylated RET; pMBP, phosphorylated MBP; M, monomer RET; D, dimer RET. In addition to the indicated specific bands, a few nonspecific bands developed between pRET and pMBP in A, C, E, F, and G as in Figs. 2D and 4A, and between D and M in H and I as in Fig. 4B.

nases. More intriguingly, c-RET-C987A and RET-PTC-1-C376A had lost their activities to respond quickly to osmotic stress for activation promotion in 5 min (Fig. 5E and G, lane 2, compared with lane 1 as a control with no osmotic stress), which was regularly seen in the cases of original c-RET (Fig. 2D), original RET-PTC-1 (Fig. 4A), and RET-PTC-1-C365A (Fig. 5F). A slight increase in the catalytic activity, however, was observed at a late time after exposure of RET-PTC-1-C376A to osmotic stress (Fig. 5G, lane 3, compared with lane 1 as a control). These results suggested that Cys^{987} of c-RET and Cys^{376} of RET-PTC-1 play important roles in normal quick response to osmotic response for kinase activation promotion in 5 min. Corresponding to these results, no detectable amounts of RET-PTC-1-C376A (Fig. 5I, lane 1, compared with lane 4 as an original control) or c-RET-C987A (data not shown) were located at the position of dimers, and hyperosmotic treatment never promoted dimer formation (Fig. 5I, lanes 2 and 3, compared with lane 1 as a control with no osmotic stress and with lane 4 as an original control). The properties of quick response to osmotic stress for disulfide-bonded dimerization-linked kinase activation promotion were shown to be shared by each of the three to five clones of transfectants of c-RET-C987A, RET-PTC-1-C376A, and RET-PTC-1-C365A (data not shown). These results suggested that Cys^{987} of c-RET and Cys^{376} of RET-PTC-1 in the C-terminus of the kinase domain are critical molecular targets of osmotic stress for the promotion of disulfide-bonded dimerization-linked activation of RET kinases.

DISCUSSION

The present study has demonstrated for the first time that osmotic stress-mediated RET kinase activation is linked to promotion of disulfide-bonded dimerization of RET proteins at a conserved cysteine residue in their kinase domain.

First, we showed that hyperosmotic treatment of cells expressing c-RET or RET-MEN2B promoted 2ME-sensitive and, thereby, disulfide-bonded dimerization of RET proteins that was linked to activation of RET kinases. Further experiments, testing the effect of hyperosmotic stress on extracellular domain-deleted mutant RET-PTC-1, revealed that the intracellular domain of RET can be the primary target of osmotic stress for inducing disulfide-bonded dimerization and activation of the kinase proteins. We prepared c-RET-C987A, RET-PTC-1-C365A, and RET-PTC-1-C376A in which Cys^{987} of c-RET, Cys^{376} of RET-PTC-1, or Cys^{365} of RET-PTC-1 in the kinase domain was replaced with alanine. The results of our study using these mutant RET proteins demonstrated that Cys^{987} of c-RET and Cys^{376} of RET-PTC-1 play crucial roles in maintenance of basic kinase activity of RET and its promotion in response to osmotic stress. This observation reveals a further molecular mechanism of activation of receptor-type and non–receptor-type tyrosine kinases by osmotic stress, potentially following the previously reported receptor clustering and superoxide production (16, 17).

Protein dimerization is expected to occur when each of two molecules carries a single reactive site, whereas protein polymerization requires two or more reactive sites per molecule. Therefore, Cys^{376} of RET-PTC-1, which was shown to be essential for RET-PTC-1 dimerization (Fig. 5I), is probably the only amino acid among the multiple cysteine residues in the kinase domain of RET proteins that is responsible for their dimer formation.

This conclusion does not, however, rule out the possibility that oxidation of other cysteine residues on RET proteins, including Cys^{365} of RET-PTC-1 or some molecules that regulate the catalytic activity of the kinase, additionally modulates the kinase activity. We showed that RET-PTC-1-C376A, which had lost its normal quick response to osmotic stress for dimerization-linked kinase activation promotion, displayed a delayed response to a low-grade kinase activity increase (Fig. 5G, lane 3). Another experiment performed in parallel with this study also demonstrated poor response of the C376A mutant of RET-PTC-1 to ultraviolet light (UV) irradiation for RET kinase activation (12). However, we failed to detect such a delayed response of the mutant to UV as observed to osmotic stress. This suggested that, although the basic Cys^{376}-dependent mechanism is shared by both osmotic stress and UV irradiation for

RET kinase activation, additional Cys^{376}-independent mechanism operates in osmotic stress-mediated, but not UV irradiation-induced, RET kinase activation. It might be that Cys^{365} of RET-PTC-1, which remained in RET-PTC-1-C376A, is involved in the slow response to osmotic stress. However, this seems unlikely because, in a supplemental experiment, RET-PTC-1-C365+376A, an additionally prepared double mutant in which both Cys^{365} and Cys^{376} were replaced with alanine, also showed a slow response to osmotic stress (Takeda *et al.*, unpublished observation). We therefore speculate that, specifically during osmotic stress, in addition to Cys^{376} of RET-PTC-1 (=Cys^{987} of c-RET)-dependent quick response for dimerization-linked kinase activation promotion, a supplemental mechanism independently works to regulate the RET kinase activity, potentially affecting some kinase activity-regulatory molecules such as protein tyrosine phosphatases that carry a sulfhydryl group in the active site of the catalytic domain to be inactivated by oxidation (20).

Perspectives

The intracellular environment is known to be maintained in a reducing state because of the presence of a large amount of glutathione (GSH) and a small amount of glutathione disulfide (GS-SG) in equilibrium. It is therefore expected that a small portion of protein cysteine SH groups in cells exist as an oxidized or disulfide-bonded form. Dimerization might spontaneously occur after cell lysis, but the premium dimerization that was demonstrated in the sample from cells exposed to osmotic stress must reflect an *in vivo* effect. Taken together, the results suggest that ROS, which is possibly produced by cell shrinkage (16), promotes production of disulfide-bonded dimers of RET proteins by shifting the GSH ⇄ GS-SG equilibrium to the right and that the disulfide-bonded dimerization causes a conformational change in the kinase proteins for activation. The same mechanism was suggested to work also for RET-MEN2B (Fig. 2D), which is constitutively activated due to a mutation-linked conformational change (Fig. 2A). This result suggests that the demonstrated mechanism of osmotic stress-mediated kinase activation is independent of conformational change due to MEN2B mutation.

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ABBREVIATIONS

DMEM, Dulbecco's modified Eagle minimal essential medium; GSH, glutathione; GS-SG, glutathione disulfide; MBP, myelin basic protein; 2ME, 2-mercaptoethanol; MEN2A, multiple endocrine neoplasia type 2A; MEN2B, multiple endocrine neoplasia type 2B; PAGE, polyacrylamide gel electrophoresis; PTC, papillary thyroid carcinoma; PTK, protein tyrosine kinase; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; UV, ultraviolet light.

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