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A Redox-Linked Novel Pathway for Arsenic-Mediated RET Tyrosine Kinase Activation

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ABSTRACT

We examined the biochemical effects of arsenic on the activities of RET proto-oncogene (c-RET protein tyrosine kinases) and RET oncogene (RET-MEN2A and RET-PTC1 protein tyrosine kinases) products. Arsenic activated c-RET kinase with promotion of disulfide bond-mediated dimerization of c-RET protein. Arsenic further activated RET-MEN2A kinase, which was already 3- to 10-fold augmented by genetic mutation compared with c-RET kinase activity, with promotion of disulfide bond-mediated dimerization of RET-MEN2A protein (superactivation). Arsenic also increased extracellular domain-deleted RET-PTC1 kinase activity with promotion of disulfide bond-mediated dimerization of RET-PTC1 protein. Arsenic increased RET-PTC1 kinase activity with cysteine 365 (C365) replaced by alanine with promotion of dimer formation but not with cysteine 376 (C376) replaced by alanine. Our results suggest that arsenic-mediated regulation of RET kinase activity is dependent on conformational change of RET protein through modulation of a special cysteine sited at the intracellular domain in RET protein (relevant cysteine of C376 in RET-PTC1 protein). Moreover, arsenic enhanced the activity of immunoprecipitated RET protein with increase in thiol-dependent dimer formation. As arsenic (14.2 μM) was detected in the cells cultured with arsenic (100 μM), direct association between arsenic and RET in the cells might modulate dimer formation. Thus, we demonstrated a novel redox-linked mechanism of activation of arsenic-mediated RET proto-oncogene and oncogene products. J. Cell. Biochem. 110: 399–407, 2010.

KEY WORDS: ARSENIC; RET TYROSINE KINASE; ACTIVATION; CANCER; REDOX; PROTO-ONCOGENE; ONCOGENE

Arsenic has been reported to induce chromosomal abnormalities and instability [Liu et al., 2003]. It has also been shown that arsenic modulates DNA repair and methylation patterns [Kitchin, 2001; Singh and DuMond, 2007]. Furthermore, arsenic has an effect on the function of telomerase [Zhang et al., 2003]. Thus, in most previous studies, the mechanism of arsenic-mediated development of cancer was investigated with focus on the genotoxicity of arsenic [Huang et al., 2004; Shi et al., 2004]. However, there have been studies aimed at clarifying the mechanism of arsenic-mediated activation of oncogene products that directly regulate cancer development.

The c-*RET* proto-oncogene encodes a receptor-type tyrosine kinase [Takahashi, 2001]. The ligand of c-RET is glial cell line-derived neurotrophic factor (GDNF), and its signaling is essential for

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renal organogenesis and enteric neurogenesis [Takahashi, 2001; Asai et al., 2006]. The c-RET kinase is also activated by point mutations or gene rearrangement [Takahashi, 2001; Kato et al., 2002]. Germ line mutations of the c-*RET* proto-oncogene are associated with the development of multiple endocrine neoplasia type 2A (MEN2A). In RET proteins with MEN2A mutation (RET-MEN2A), dimerization is promoted through the formation of disulfide bonds between unpaired cysteine residues in the extracellular domains of two molecules, and their levels of autophosphorylation and tyrosine kinase activity are elevated in parallel [Asai et al., 1995; Santoro et al., 1995]. In addition, augmentation of RET-MEN2A kinase activity through autophosphorylation of tyrosine 905 (Y905) and tyrosine 1062 (Y1062) is crucially important for transformation [Kato et al., 2002]. On the other hand, RET-PTC1 is developed by rearrangement of c-*RET* and has no extracellular domain, and it is one of causal genes in human papillary thyroid carcinoma [Takeda et al., 2006].

In this study, we focused on the biochemical effects of arsenic (10–1,000 μM) on activities of RET proto-oncogene and oncogene products, and we propose a redox-linked novel mechanism of arsenic-mediated activation of RET tyrosine kinases.

MATERIALS AND METHODS

PLASMID CONSTRUCTION, TRANSFECTION, CULTURE AND PURIFICATION

A cDNA clone containing the sequence of the human *RET* (c-*RET* or *RET-PTC1*) gene was inserted into the APtag-1 vector containing the Moloney murine leukemia virus long terminal repeat as described previously [Kato et al., 2002]. Mutation was introduced according to the method described previously [Kato and Wickner, 2003]. In brief, a primer containing the mutation was synthesized and used for amplification of a *RET* sequence of 100–150 bp. The corresponding sequence of the *RET* was replaced by the amplified fragment containing the mutation. The amplified fragment was sequenced to confirm that the proper mutation had been introduced. Each recombinant plasmid (0.1 μg) was transfected into NIH 3T3 cells (5×10^5 cells in a 60-mm-diameter dish) with 10 μg of NIH 3T3 DNA, as described previously [Asai et al., 1995].

Parental NIH3T3 cells and RET-transfected NIH3T3 cells were cultured in Dulbecco's modified Eagle's minimal essential medium supplemented with 8% bovine calf serum (BCS; HyClone Laboratories, Inc., UT). TGW cells were cultured in RPMI supplemented with 8% BCS.

ARSENIC TREATMENT

Arsenic ($NaAsO_2$) was purchased from Wako Pure Chemical Industries, Ltd. After 70–80% confluent cells had been cultured for 24 h in DMEM or RPMI supplemented with 0.5% BCS, the cells were again cultured for 90 min in DMEM or RPMI without bovine calf serum (starvation). In the experiment for which results are shown in Figures 1–4, arsenic and GDNF were added to the starved culture media and the cells were incubated for 90 and 10 mins, respectively. Treatment with 10–100 μM of arsenic for 90 min did not affect cell number. However, treatment with 1,000 μM of arsenic for 90 min resulted in a 20% decrease in cell number. In the experiment for which results are shown in Figure 5, arsenic was added to immunoprecipitated RET-PTC1 protein, and the protein was subjected to immunoblot analysis after incubation for 20 min at 30°C or subjected to kinase assay.

IMMUNOBLOT ANALYSIS

Immunoblotting was performed according to the method described previously [Kato et al., 2007]. Lysates (50 μg/lane) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene difluoride membranes. After treatment with antibodies, the reactions were examined by using Western Blot Chemiluminescence Reagent (DuPont NEN, Boston, MA). An anti-RET rabbit polyclonal antibody was purchased from IBL (Takasaki, Japan). An anti-phosphotyrosine polyclonal antibody was purchased from Transduction Laboratories (Lexington, KY). Polyclonal antibodies for detection of phosphorylated tyrosine 1062 (anti-Y1062-P; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and tyrosine 905 (anti-Y905-P; Cell Signaling Technology, Inc, Danvers, MA) in RET protein were purchased. c-RET and RET-MEN2A developed two bands of 175 (a mature glycosylated form) and 155 kDa (an immature glycosylated form) under reducing conditions; they also formed a doublet band depending on gel conditions. A previous study revealed that dimerized RET bands under unreducing conditions were composed of 175 and 155 kDa of RET proteins [Asai et al., 1995]. RET-PTC1, which consisted of a cytoplasmic domain only, developed only one band under reducing conditions.

IMMUNOPRECIPITATION AND KINASE ASSAY

Immunoprecipitation was performed as described previously [Kato et al., 1999]. The immunoprecipitated RET proteins were washed three times with lysis buffer (30 mM Tris–HCl pH 8.0, 1% Triton-X-100, 150 mM NaCl, 1 mM EDTA, and 0.5 mM Na_3VO_4) for either immunoblot or in vitro kinase assay. In vitro RET kinase assay was performed as described previously [Kato et al., 1998]. Briefly, the immunoprecipitated RET proteins were washed three times with kinase buffer (10 mM Tris–HCl pH 7.4 and 5 mM $MgCl_2$), suspended in the kinase buffer with 2.0 μg myelin basic protein (MBP; Sigma–Aldrich Co.) as an exogenous substrate, and radiolabeled with [γ-32P]ATP (370 kBq; NEN, Wilmington, DE). The kinase reaction was carried out for 20 min in a 30°C water bath and was terminated by adding sample buffer with or without 2ME. The immunoprecipitates were then boiled for 3 min and loaded on SDS–PAGE. The gels were dried and exposed to X-ray film at −80°C for autoradiography. As reported previously [Kato et al., 2002], in both immunoblot and kinase assays, c-RET and RET-MEN2A developed two bands of 175 (a mature glycosylated form) and 155 kDa (an immature glycosylated form) under reducing conditions; they also formed a doublet band depending on gel conditions. However, RET-PTC1, which consisted of a cytoplasmic domain only, developed only one band under reducing conditions.

MEASUREMENT OF ARSENIC CONCENTRATION IN CELLS

After c-RET-transfected NIH3T3 cells had been treated with 0–1,000 μM of arsenic for 90 min, the cells were washed 3–4 times with PBS. Intracellular concentration of arsenic was measured by hydride generation atomic absorption spectrometry using an AA-6200 atomic absorption spectrophotometer and an HVG-1

hydride vapor generator (Shimadzu Co., Kyoto, Japan) according to the previously described method [Hinwood et al., 2003].

RESULTS

ARSENIC-MEDIATED c-RET KINASE ACTIVATION WITH PROMOTION OF DIMER FORMATION

Arsenic (10–1,000 μM) dose-dependently promoted both 175 and 155 kDa of c-RET tyrosine kinase activity in c-RET-transfected NIH3T3 cells (lanes 1–4 in Fig. 1A). Arsenic (100 μM) increased the phosphorylation levels of both tyrosines 905 (Y905) and 1062 (Y1062) of the c-RET molecule (Fig. 1B), which are important for tyrosine kinase activation and transforming activity [Kato et al., 2002]. Enhancement of both autophosphorylation and MBP phosphorylation by 100 μM of arsenic was confirmed by the in vitro kinase assay (Fig. 1C). Arsenic (100 μM) promoted 175 kDa of intrinsic c-RET kinase activity in TGW cells (lanes 1–3 in Fig. 1D), whereas the protein expression levels of 175 kDa of c-RET were lower than those of 155 kDa of c-RET (lanes 4–6 in Fig. 1D). These results suggest that arsenic activates not only transfected c-RET kinase but also intrinsic c-RET kinase.

Previous studies suggested that promotion of the dimerization of RET by a ligand [Chiariello et al., 1998] or MEN2A-type point mutation [Asai et al., 1995; Santoro et al., 1995] is closely associated with the promotion of RET kinase activation. As detection of dimerized c-RET protein in TGW cells was harder than that in RET-transfected NIH3T3 cells, we next examined whether arsenic-mediated c-RET kinase activation depended on promotion of the dimerization of c-RET proteins in RET-transfected NIH3T3 cells. As shown in Figure 1E, dimerized c-RET protein was dose-dependently increased by 10–1,000 μM of arsenic under unreducing conditions, whereas monomer c-RET was dose-dependently decreased. The

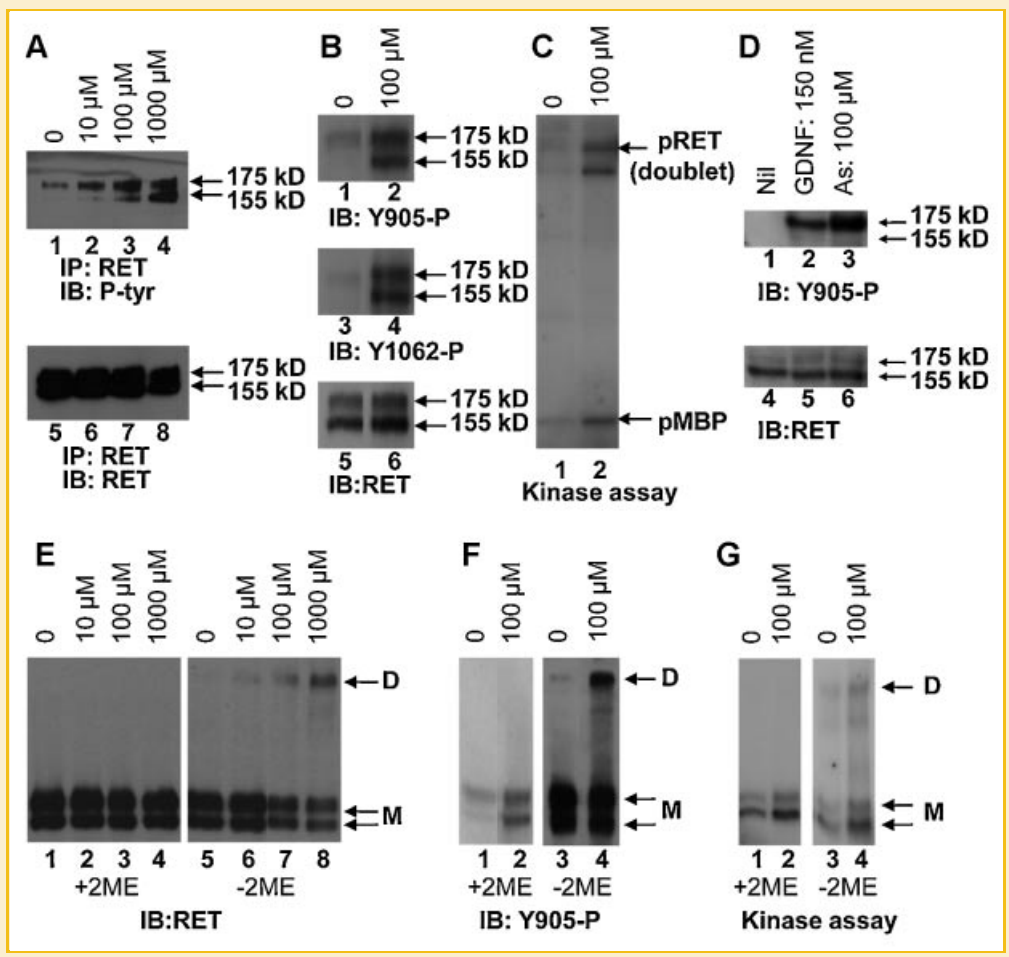

Fig. 1. Arsenic promotes c-RET kinase activity with promotion of the dimerization of c-RET protein. Lysates from c-RET-transfected NIH3T3 cells in the presence or absence of arsenic were subjected to immunoblotting with anti-phosphotyrosine (lanes 1–4 in A) or anti-RET antibody (lanes 5–8 in A) or to in vitro kinase assay (C,G) after immunoprecipitation with anti-RET antibody. The lysates were also subjected to simple immunoblotting with anti-Y905-P (lanes 1 and 2 in B,F), anti-Y1062-P (lanes 3 and 4 in B) or anti-RET antibody (lanes 5 and 6 in B,E). Lysates from TGW cells stimulated with GDNF (150 nM) or arsenic (100 μM) were subjected to simple immunoblotting with anti-Y905-P (lanes 1–3 in D) or anti-RET antibody (lanes 4–6 in D). SDS–PAGE was performed under reducing (A–D, lanes 1–4 in E, lanes 1 and 2 in F and G) or unreducing (lanes 5–8 in E, lanes 3 and 4 in F and G) conditions in 6% (E–G), 10% (A,B,D) or 13% (C) gel. 175 and 155 kDa of c-RET proteins formed a doublet band in the 13% gel condition (C). Loading controls for lanes 5–8 corresponded to lanes 1–4 (E), respectively. IP, immunoprecipitation; IB, immunoblotting; pRET (doublet band), autophosphorylated c-RET; pMBP, phosphorylated myelin basic protein; M, monomer RET; D, dimer RET.

enhanced dimerized c-RET protein levels were decreased by treatment with a reducing reagent (2ME), suggesting that the dimerization occurred in a thiol-dependent manner. Correspondingly, the levels of Y905 phosphorylation (Fig. 1F) and autophosphorylation (Fig. 1G) in the dimerized position were highly enhanced by arsenic (100 μM). The augmented Y905 phosphorylation (lanes 3 and 4 in Fig. 1F) and autophosphorylation (lanes 3 and 4 in Fig. 1G) levels in the dimerized position were decreased under reducing conditions (lanes 1 and 2 in Fig. 1F,G). These results suggest that arsenic induces activation of c-RET kinase by promoting dimerization through a redox mechanism.

ARSENIC-MEDIATED RET-MEN2A KINASE SUPERACTIVATION BY FURTHER PROMOTION OF DIMERIZATION

Constitutive kinase activity of RET-MEN2A was 5- to 10-fold activated by genetic mutation compared with c-RET kinase activity (calculated by densitometric analysis of the bands in Fig. 2A). This is because the percentage of dimerized RET-MEN2A protein (around 30%; Fig. 2E, lane 3) is much higher than that of c-RET protein (<1%; lane 5 in Fig. 1E). The levels of autophosphorylation (Fig. 2B,D) and MBP phosphorylation (Fig. 2D) were further 3- to 10-fold enhanced by 100 μM of arsenic. Phosphorylation of Y905 and Y1062 of RET-MEN2A kinase was also 3- to 10-fold upregulated by 100 μM of arsenic (Fig. 2C). These results suggest that arsenic further activates RET-MEN2A kinase, which is already activated by genetic mutation (superactivation). More than 50% of RET-MEN2A protein was in the dimerized position by stimulation with 100 μM of arsenic under unreducing conditions (lane 4 in Fig. 2E). Correspondingly, the dimerized position of RET-MEN2A was highly activated by 100 μM (Fig. 2F,G) of arsenic under unreducing conditions. As shown for c-RET (Fig. 1), protein and activity levels of

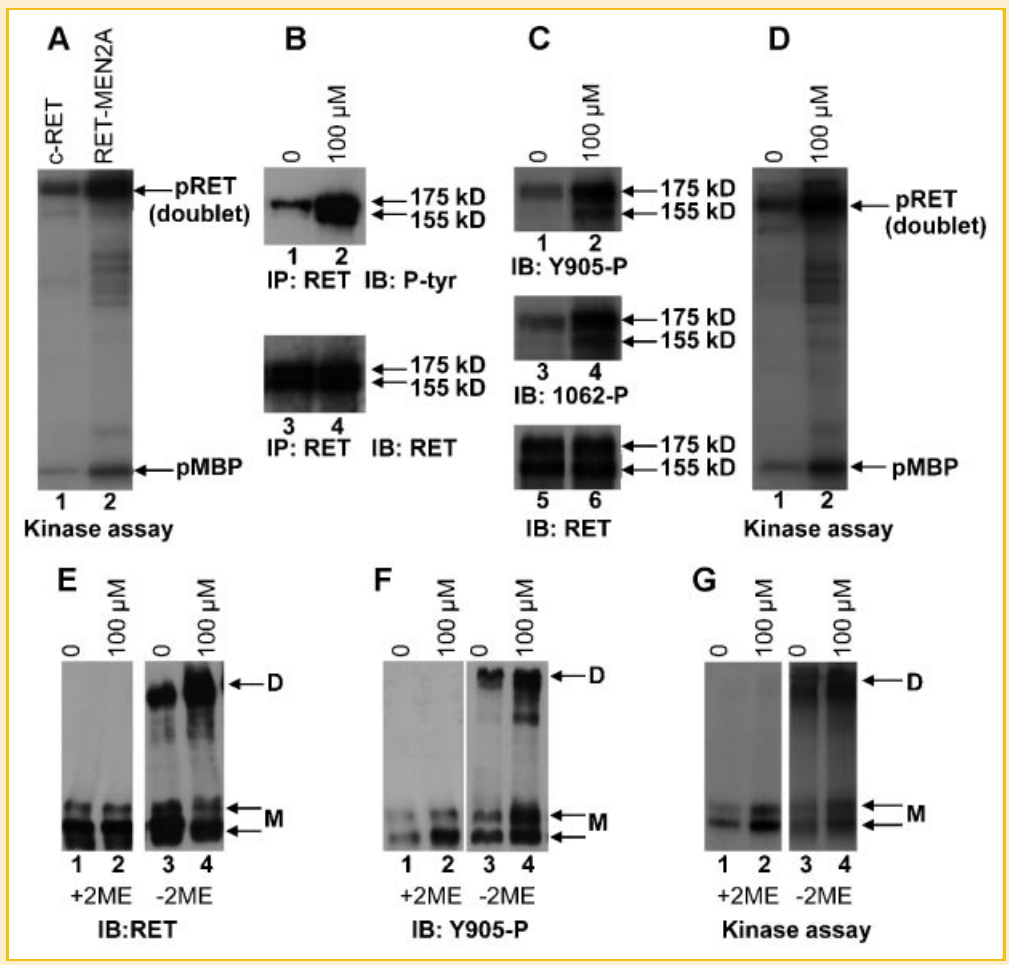

Fig. 2. Arsenic superactivates RET-MEN2A kinase activity, which was already activated by genetic mutation, with further promotion of the dimerization of RET-MEN2A protein. Lysates from RET-MEN2A-transfected NIH3T3 cells in the presence (100 μM) or absence of arsenic were subjected to immunoblotting with anti-phosphotyrosine (lanes 1 and 2 in B) or anti-RET antibody (lanes 3 and 4 in B) or to in vitro kinase assay (A,D,G) after immunoprecipitation with anti-RET antibody. The lysates were also subjected to simple immunoblotting with anti-Y905-P (lanes 1 and 2 in C,F), anti-Y1062-P (lanes 3 and 4 in C), or anti-RET antibody (lanes 5 and 6 in C,E). SDS-PAGE was performed under reducing (A–D, lanes 1 and 2 in E–G) or unreducing (lanes 3 and 4 in E–G) conditions in 6% (E–G), 10% (B,C), or 13% (A,D) gel. 175 and 155 kDa of RET-MEN2A proteins formed a doublet band in the 13% gel condition (A,D). Loading controls for lanes 3 and 4 corresponded to lanes 1 and 2, respectively (E). IP, immunoprecipitation; IB, immunoblotting; pRET (doublet band), autophosphorylated RET-MEN2A; pMBP, phosphorylated myelin basic protein; M, monomer RET; D, dimer RET.

RET-MEN2A in the dimerized position were decreased under reducing conditions (lanes 1 and 2 in Fig. 2E–G). These results suggest that arsenic superactivates RET-MEN2A kinase through the promotion of disulfide bond-mediated dimerization.

SUBMOLECULAR TARGET FOR ARSENIC-MEDIATED RET KINASE ACTIVATION

We next examined the arsenic-reactive domain of RET protein. As shown in Figure 3A–C, arsenic (10 and/or 100 μM) upregulated the kinase activity of extracellular domain-deleted PET-PTC1. Arsenic (10 and/or 100 μM) also enhanced the levels of protein (Fig. 3D) and activity (Fig. 3E,F) in the dimerized position in RET–PTC1. RET–PTC1 protein level (Fig. 3D) and activity level (Fig. 3E,F) in the dimerized position were decreased under reducing conditions. These results suggest that arsenic at least reacts with the cysteine in the intracellular domain of RET protein, promotes disulfide bond formation and induces kinase activation.

TARGET AMINO ACID IN ARSENIC-MEDIATED RET-PTC1 KINASE ACTIVATION

We next examined the target cysteine of RET-PTC1. C376 and C365 are well conserved compared with other tyrosine kinases [Kawamoto et al., 2004]. Therefore, we established two kinds of mutants (RET-PTC1-C365A and RET-PTC1-C376A), in which C376 and C365 were replaced by alanine, respectively. Although there was no difference between protein expression levels of RET-PTC1-C365 and RET-PTC1-C376 cells (lanes 1 and 2 in Fig. 4A), kinase activity level of the former was much higher than that of the latter (lanes 3 and 4 in Fig. 4A). Arsenic enhanced the activity of RET-PTC1-C365A kinase with promotion of dimer formation under unreducing conditions (lanes 1 and 2 in Fig. 4B, lanes 3 and 4 in Fig. 4C, lanes 3 and 4 in Fig. 4D). However, 100 and 1,000 μM of arsenic did not enhance the activity of RET-PTC1-C376A kinase (lanes 3–5 in Fig. 4B). The levels of protein (lanes 8–10 in Fig. 4C) and activity (lanes 7 and 8 in Fig. 4D) in the dimerized position in RET-PTC1-C376A under unreducing conditions were not changed by stimulation with arsenic. These results suggest that C376 is one

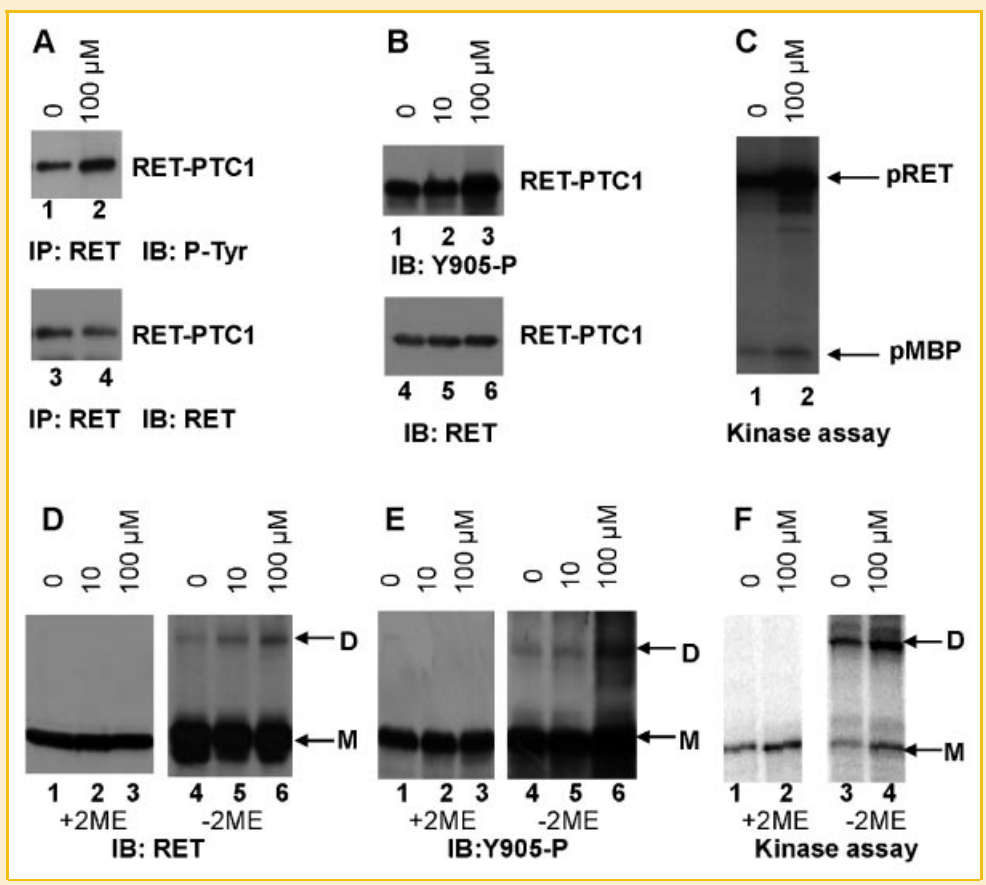

Fig. 3. Arsenic promotes activation and dimerization of RET without an extracellular domain. Lysates from RET-PTC1-transfected NIH3T3 cells in the presence (10–100 μM) or absence of arsenic were subjected to immunoblotting with anti-phosphotyrosine (lanes 1 and 2 in A) or anti-RET antibody (lanes 3 and 4 in A) or to in vitro kinase assay (C,F) after immunoprecipitation with anti-RET antibody. The lysates were also subjected to simple immunoblotting with anti-Y905-P (lanes 1–3 in B,E) or anti-RET antibody (lanes 4–6 in B,D). SDS–PAGE was performed under reducing (A–C, lanes 1–3 in D and E, lanes 1 and 2 in F) or unreducing (lanes 4–6 in D and E, lanes 3 and 4 in F) conditions in 10% (A,B,D,E,F) or 13% (C) gel. Loading controls for lanes 4–6 corresponded to lanes 1–3, respectively (D). IP, immunoprecipitation; IB, immunoblotting; pRET, autophosphorylated RET-PTC1; pMBP, phosphorylated myelin basic protein; M, monomer RET; D, dimer RET.

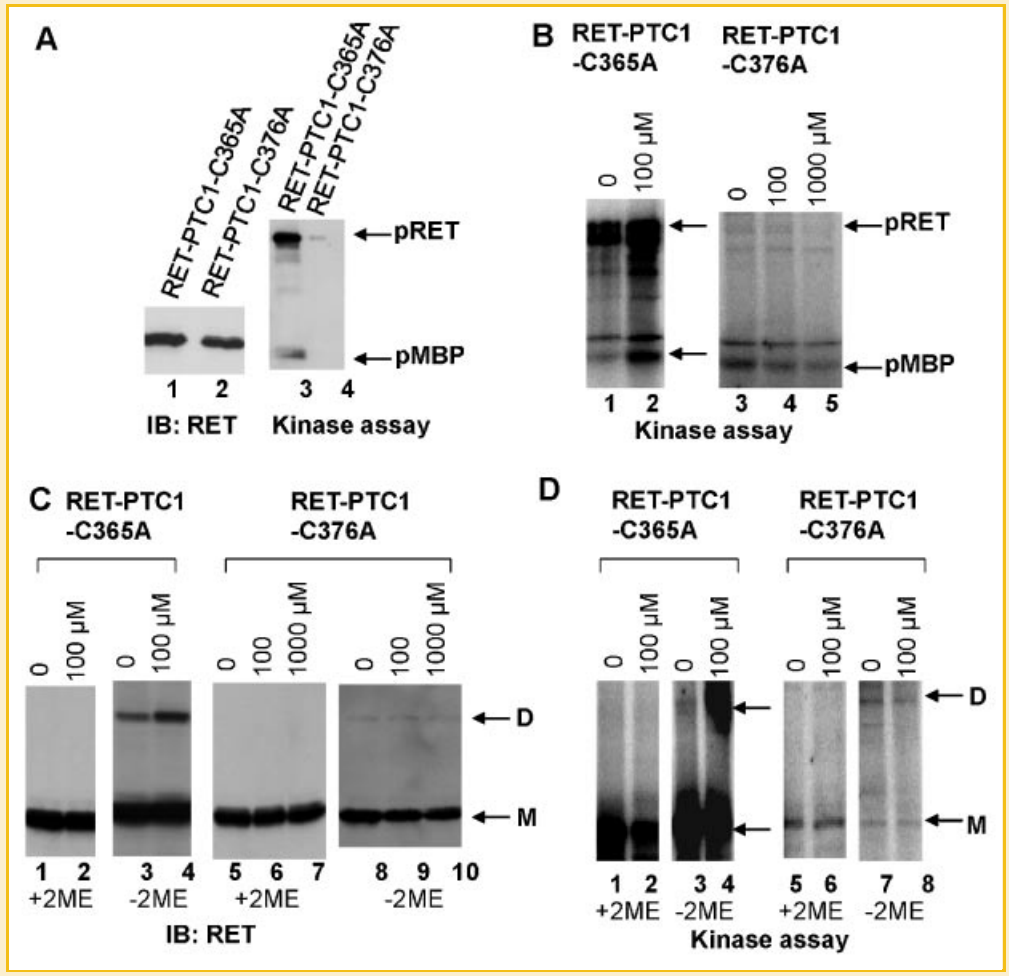

Fig. 4. Arsenic does not promote activation and dimerization of extracellular domain-deleted RET whose cysteine 376 was replaced by alanine. Lysates from RET-PTC1-C365A or RET-PTC1-C37A-transfected NIH3T3 cells in the presence (100–1,000 μM) or absence of arsenic were subjected to in vitro kinase assay (lanes 3 and 4 in A,B,D) after immunoprecipitation with anti-RET antibody. The lysates were also subjected to simple immunoblotting with anti-RET antibody (lanes 1 and 2 in A,C). SDS–PAGE was performed under reducing (A,B, lanes 1, 2, 5, 6, and 7 in C, lanes 1, 2, 5, and 6 in D) or unreducing (lanes 3, 4, 8, 9, and 10 in C, lanes 3, 4, 7, and 8 in D) conditions in 10% (lanes 1 and 2 in A and B,CD) or 13% (lanes 3 and 4 in A, lanes 3–5 in B) gel. In RET-PTC1-C365A, loading controls for lanes 3 and 4 corresponded to lanes 1 and 2, respectively (C). In RET-PTC1-C376A, loading controls for lanes 8–10 corresponded to lanes 5–7, respectively (C). IP, immunoprecipitation; IB, immunoblotting; pRET, autophosphorylated RET-PTC1; pMBP, phosphorylated myelin basic protein; M, monomer RET; D, dimer RET.

of the major target amino acids for arsenic-mediated dimer formation and kinase activation.

ARSENIC-MEDIATED KINASE ACTIVATION OF IMMUNOPRECIPITATED RET PROTEIN

We finally examined whether arsenic modulates the activity of immunoprecipitated RET protein with increase in thiol-dependent dimer formation. As shown in Figure 5A, arsenic increased the kinase activity of immunoprecipitated RET-PTC1. Kinase activity of immunoprecipitated RET-PTC1 in the dimerized position was enhanced by 100 nM (lanes 3 and 4 in Fig. 5B) and 1 μM (data not shown) of arsenic under unreducing conditions. Kinase activity of RET-PTC1 in the dimerized position was decreased under reducing conditions (lanes 1 and 2 in Fig. 5B). Most of immunoprecipitated RET-PTC1 proteins were in the dimer and polymer position under unreducing conditions (lane 3 in Fig. 5C). Dimer and polymer formation of immunoprecipitated RET–PTC1 protein was further increased by arsenic (100 nM) treatment under unreducing conditions (lane 4 in Fig. 5C). In contrast, the monomer of immunoprecipitated RET-PTC1 protein was decreased (lane 4 in Fig. 5C). Most of the dimerized and polymerized RET-PTC1 proteins were decreased under reducing conditions (lanes 1 and 2 in Fig. 5C), suggesting that this was a thiol-dependent reaction. In addition, atomic absorption spectrophotometer analysis revealed the presence of 4.2, 14.2, and 31.8 μM of arsenic in c-RET-transfected NIH3T3 cells treated with 10, 100, and 1,000 μM of arsenic, respectively, while no arsenic was detected in cells cultured without arsenic. These results suggest that there is a sufficiently high concentration of arsenic in the cells to promote intracellular direct association between arsenic and RET.

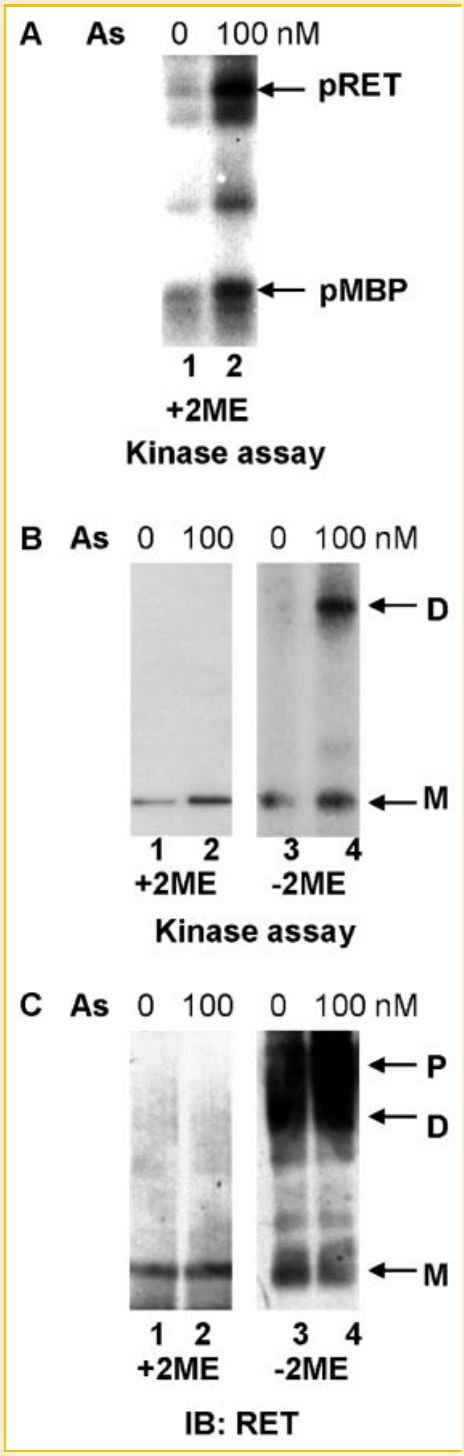

Fig. 5. Arsenic promotes activation and dimerization of immunoprecipitated RET–PTC1 protein. Immunoprecipitated RET–PTC1 protein with anti-RET antibody was subjected to in vitro kinase assay (A,B) or to immunoblotting with anti-RET antibody (C) in the presence (100 nM) or absence of arsenic. SDS–PAGE was performed under reducing (lanes 1 and 2 in A–C) or unreducing (lanes 3 and 4 in B and C) conditions in 10% (B,C) or 13% (A) gel. Loading controls for lanes 3 and 4 corresponded to lanes 1 and 2, respectively (C). IP, immunoprecipitation; IB, immunoblotting; pRET; autophosphorylated RET-PTC1; pMBP, phosphorylated myelin basic protein; M, monomer RET; D, dimer RET; P, polymer RET.

DISCUSSION

In this study, we demonstrated that 10–1,000 μM of arsenic promoted both transfected (Fig. 1A–C) and intrinsic (Fig. 1D) c-RET kinase activity. We next examined the biochemical mechanism of arsenic-mediated c-RET kinase activation. Arsenic (10–1,000 μM) dose-dependently promoted c-RET kinase activity through disulfide bond linked-dimer formation of c-RET protein (Fig. 1E–G). These results suggest that a redox-linked pathway of arsenic contributes to regulation of the proto-oncogene product activity.

We next examined effects of arsenic (100 μM) on an oncogene product (RET-MEN2A). Arsenic synergistically 3- to 10-fold upregulated the genetically 3- to 10-fold enhanced RET-MEN2A kinase activity (Fig. 2A–D). The synergistic effect of genetic mutation and arsenic stimulation induces 9- to 100-fold enhancement of RET-MEN2A kinase activity compared with constitutive c-RET kinase activity. As ultraviolet irradiation further enhanced genetically increased oncogenic RET (RFP-RET) activity in melanocytic benign tumors from RFP-RET-transgenic mice (line 192) and promoted cutaneous malignant melanoma development in the mice [Kato et al., 2000], these results suggest that arsenic-mediated synergistic enhancement of oncogenic RET kinase (superactivation) further promotes the crisis for cancer development in oncogene-carrying patients with arsenicosis. Arsenic-mediated RET-MEN2A kinase activation is induced by further increased disulfide bond linked-dimer formation (Fig. 2E–G). These results suggest that the redox-linked pathway of arsenic also contributes to regulation of oncogene product activity.

We then tried to identify the arsenic-reactive domain of RET protein. Arsenic again upregulated the kinase activity of extracellular-domain-deleted RET (RET-PTC1) in the cells with increase in its disulfide bond-dependent dimer formation (Fig. 3). As cysteine is the only amino acid that has a thiol group, the result suggests that the target cysteine(s) of arsenic in RET protein is in the intracellular domain. We sequentially examined the arsenic-reactive cysteine(s) in the intracellular domain of RET protein. Because C365 and C376 in RET-PTC1 are well conserved in the protein tyrosine kinases [Kawamoto et al., 2004], we established RET-PTC1-C365A and RET-PTC1-C376A (Fig. 4). Promotion of arsenic-mediated activation of RET-PTC1-C365A kinase but not that of RET-PTC1-C376A kinase was observed in this study (Fig. 4). These results suggest that a candidate of target cysteines for arsenic is cysteine 376 in RET-PTC1 protein. Taken together with the finding that cysteine 376 in RET-PTC1 protein is highly conserved in other protein tyrosine kinases [Kawamoto et al., 2004], the cysteine might be a good target in the future for therapy of arsenic-induced cancer through regulation of oncogene product activity.

We finally examined whether thiol-dependent conformational change of RET protein is caused by intracellular direct association between arsenic and RET protein or an arsenic-mediated second messenger such as reactive oxygen species (ROS). As shown in Figure 5, arsenic promoted immunoprecipitated RET kinase activity with regulation of thiol-dependent dimer and polymer formation of immunoprecipitated RET protein. We also showed that there is a sufficiently high concentration of arsenic in the cells to promote

kinase activation via conformational change of RET protein. In future, we need to examine intracellular association between RET and arsenic.

Many tube wells throughout the world contain more than 750 μg/L (10 μM) of arsenic have been reported [Chen et al., 2006]. Urinary arsenic concentrations of more than 750 μg/L (10 μM) have been reported in samples obtained from people living in arsenic-contaminated areas [Vahter et al., 2006]. Arsenic concentrations in nail (around 100,000 μg/kg) and hair (around 10,000 μg/kg) samples were more than 10- to 100-fold higher than those in urinary samples in people living in arsenic-contaminated areas [Hinwood et al., 2003; Gault et al., 2008]. Furthermore, arsenic concentrations of more than 7,500 μg/L (=100 μM) have been reported in urinary samples from patients with acute promyelocytic leukemia who were treated with arsenic [Wang et al., 2005]. These reports indicate the importance of discussion of the biological significance of RET kinase activation mediated by a high concentration of arsenic (10- to 1,000 μM). Previous studies have suggested that arsenic is a strong carcinogen in humans [Smith et al., 1992; Schwartz, 1997; Mandal et al., 2001]. RET proto-oncogene [Iwahashi et al., 2002; Garnis et al., 2005; Esseghir et al., 2007; Zeng et al., 2008] and oncogene [Takahashi, 2001; Kato et al., 2002; Asai et al., 2006] products were also reported to be involved in the pathogenesis of various kinds of human cancer. Therefore, our observation of arsenic-mediated activation of proto-oncogenic (Fig. 1) and oncogenic (Figs. 2 and 3) products may be associated with cancer development. In contrast, arsenic can induce apoptosis in many cancer cells [Liu et al., 2003; Zhang et al., 2003] and is effective for treatment of acute promyelocytic leukemia [Leu and Mohassel, 2009]. RET proto-oncogene product also promotes apoptosis [Bordeaux et al., 2000]. In fact, activation of the Ret/Pit-1/p53 pathway by retroviral introduction of Ret blocked tumor growth in vivo [Cañibano et al., 2007]. Therefore, our observation of arsenic-mediated activation of oncogenic (Fig. 1) and proto-oncogenic (Figs. 2 and 3) products may also be associated with anti-cancer effects. In any case, further study is needed to address the biological significance of arsenic-mediated RET kinase activation thorough a redox-linked mechanism.

In summary, we demonstrated that arsenic activates c-RET kinase and superactivates genetically activated RET-MEN2A kinase. We also showed that arsenic-mediated control of kinase activity is involved in the disulfide bond-linked conformational change of RET protein, and we revealed a potential target amino acid for arsenic in a novel pathway for arsenic-mediated activation of RET proto-oncogene and oncogene products.

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