Ultraviolet Light Induces Redox Reaction–mediated Dimerization and Superactivation of Oncogenic Ret Tyrosine Kinases

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The c-RET proto-oncogene encodes a receptor-type tyrosine kinase, and its mutations in the germ line are responsible for the inheritance of multiple endocrine neoplasia type 2A (MEN2A) and 2B (MEN2B). Ret kinases are constitutively activated as a result of MEN2A mutations (Ret-MEN2A) or MEN2B mutations (Ret-MEN2B). Here we demonstrate that UV light (UV) irradiation induces superactivation of the constitutively activated Ret-MEN2A and Ret-MEN2B as well as activation of c-Ret. Before UV irradiation, small percentages of c-Ret (3–4%) and Ret-MEN2B (1–2%) and large percentages of Ret-MEN2A (30–40%) were dimerized through disulfide bonds. These dimerized Ret proteins were preferentially autophosphorylated, suggesting a close relation between up-regulated kinase activity and disulfide bond–mediated dimerization of Ret proteins. We found that UV irradiation promotes the disulfide bond–mediated dimerization of the Ret proteins, in close association with activation and superactivation of Ret kinases. UV irradiation also induced dimerization and activation of the extracellular domain–deleted mutant Ret (Ret-PTC-1). Interestingly, the levels of basic kinase activity and dimerization of Ret-PTC-1-C376A, in which cysteine 376 in the tyrosine kinase domain of Ret-PTC-1 was replaced by alanine, were low and were not increased by UV irradiation. These results suggest that Ret-PTC-1 cysteine 376 is one of possibly multiple critical target amino acids of UV for Ret kinase activation. Overexpression of Cu/Zn superoxide dismutase in cells as a result of gene transfection prevented both the UV-mediated promotion of dimerization and the superactivation of Ret-MEN2A kinase. These results suggest that the UV-induced free radicals in cells attack intracellular domains of Ret to dimerize the kinase proteins for superactivation.

INTRODUCTION

The c-RET proto-oncogene encodes a receptor-type tyrosine kinase with a cadherin-like motif in the extracellular domain, and this kinase is an essential signaling component for renal organogenesis and enteric neurogenesis (Schuchardt et al., 1994; Takahashi, 1995, 1997). The Ret kinase has been shown to be activated by point mutations or gene rearrangement (Takahashi, 1995). Germ line mutations of the c-RET proto-oncogene are associated with the development of multiple endocrine neoplasia type 2A (MEN2A) and 2B (MEN2B), and rearrangement of this gene is frequently found in human papillary thyroid carcinoma (PTC) (Grieco et al., 1990; Ishizaka et al., 1990; Donis-Keller et al., 1993; Mulligan et al., 1993; Carlson et al., 1994; Hofstra et al., 1994). Ret proteins with the MEN2A mutation (Ret-MEN2A) are dimerized 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). It has been suggested that activation of Ret proteins with the MEN2B mutation

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(Ret-MEN2B) results from an altered conformation of the kinase domain that also leads to altered substrate specificity (Asai et al., 1995; Santoro et al., 1995). A number of earlier studies showed that, in addition to the known action to damage DNA, UV light (UV) irradiation induces activation of transcriptional factors such as an NF-kB and AP-1 for cell proliferation or cytokine production. Evidence has been provided that this UV-mediated signal transduction is started at the cell surface–oriented area, initially promoting autophosphorylation/activation of receptor-type and non-receptor-type protein tyrosine kinases, including EGF receptor (EGFR), insulin receptor, c-Src, and ZAP-70 (Devery et al., 1992; Schieven et al., 1994; Warmuth et al., 1994; Cofer et al., 1995). Little is known, however, about the mechanism of the UV-mediated initiation of the cell surface–oriented events, and the primary target of UV may (Cofer et al., 1995; Rosette and Karin, 1996) or may not (Knebel et al., 1996) be the kinase protein itself. No earlier reports concerned the potential action of UV irradiation on the oncogenic protein tyrosine kinases whose structures have been genetically modulated for constitutive activation. In this study, we have found that UV irradiation induces not only activation of c-Ret but also superactivation of constitutively activated Ret-MEN2A and Ret-MEN2B. Further study has revealed that, independent of the change attributable to genetic mutations, UV promotes dimerization of the Ret kinase proteins through the formation of intracellular disulfide bonds.

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 (kindly provided by P. Leder, Harvard Medical School, Cambridge, MA) (Flanagan and Leder, 1990; Ishizaka et al., 1990). Mutation was induced by a PCR. In brief, the primer containing the mutation was synthesized and used for amplification of a c-RET sequence of ~100–150 base pairs. The corresponding sequence of the c-RET gene was replaced by the amplified fragment containing the mutation. The amplified fragment was sequenced to confirm that the proper mutation was introduced. A CDNA clone containing the entire sequence of the human Cu/Zn superoxide dismutase (SOD1) gene tagged with the influenza hemagglutinin (HA) in the N terminus was also introduced into the same vector.

Transfection and Cell Culture

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 DNA, as described previously (Asai et al., 1995). Cells were cultured in DMEM (Nissui Pharmaceutical, Tokyo Japan) supplemented with 8% bovine calf serum (Hyclone Laboratories, Logan, UT).

Preparation of Cell Lines Expressing Mutant Ret Proteins

The c-RET cDNA encoding a long (1114 amino acids) isoform with a MEN2A mutation (RET-MEN2A; C634R), a MEN2B mutation (RET-MEN2B; M918T) (Figure 1A), rearranged RET-PTC-1 cDNA (Ishizaka et al., 1990), and RET-PTC-1 whose cysteine 365 or 376 in the C-terminal end of the kinase domain was replaced by alanine (RET-PTC-1-C365A and RET-PTC-1-C376A) (Figure 1B) was prepared and inserted into the expression vector. It was transfected into NIH 3T3 cells. For some experiments, HA-tagged SOD1 (HA-SOD1) cDNA was also inserted into the same expression vector and transfected together with RET-MEN2A cDNA into NIH 3T3 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, Immunohistochemistry, and Antibodies

Western blotting was performed according to the method described previously (Kato et al., 1998a). The lysates (30 μg/lane) were subjected to SDS-PAGE (5–10% polyacrylamide gels) and transferred to polyvinylidene difluoride membranes (Nihon Millipore Kogyo, Yonezawa, Japan). After the membranes were reacted with the first antibody (antibody specific to Ret, HA, phosphotyrosine, or any signal-transducing molecules), the reaction was examined by Western blot chemiluminescence reagent (DuPont–New England Nuclear, Boston, MA). Anti-Ret rabbit polyclonal antibody was produced as described previously (Takahashi et al., 1993). Anti-HA probe (Y-11), anti-Jun N-terminal kinase (JNK) polyclonal antibody, and anti-p38 polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-phosphotyrosine polyclonal antibody, anti-extracellular signal–regulated kinase (ERK) anti-ERK, and anti-JNK polyclonal antibodies were from Transduction Laboratories (Lexington, KY). Expression of phospho-ERK, phospho-JNK, phospho-p38, and phospho-c-Jun was examined by the Phosphoplus Immunoblot, Immunohistochemistry, and Antibodies

Immunoprecipitation and Kinase Assay

Immunoprecipitation was performed as described previously (Kato et al., 1998a). 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, 0.5 mM Na_2VO_4 for either

Figure 1. Scheme of mutant RET cDNA constructs. (A) RET cDNA encoding a long (1114 amino acids) isoform in which the cysteine at codon 634 was replaced by arginine (C634R, RET-MEN2A) or the methionine at codon 918 was replaced by threonine (M918T, RET-MEN2B). (B) Mutant RET cDNA encoding a long isoform in which the extracellular domain was deleted (RET-PTC-1). In RET-PTC-1, the cysteine at codon 365 or 376 was replaced by alanine (RET-PTC-1-C365A, RET-PTC-1–C376A). SS, signal sequence; CAD, cadherin-like domain; CY5, cysteine-rich region; TM, transmembrane domain; TK1, tyrosine kinase domain 1; TK2, tyrosine kinase domain 2; aa, amino acids.

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immunoblot or in vitro kinase assay. In vitro Ret kinase assay was performed as described previously (Asai et al., 1995; Kato et al., 1998b). Briefly, the immunoprecipitated Ret proteins were washed three times with kinase buffer (10 mM Tris-HCl, pH 7.4, 5 mM MgCl₂), suspended in the kinase buffer with 2.0 µg of myelin basic protein (MBP) (Sigma Chemical, St. Louis, MO) as an exogenous substrate, and radiolabeled with [γ-32P]ATP (370 kBP) (New England Nuclear, Wilmington, DE). The kinase reaction was carried out for 20 min in a 30°C water bath and was terminated by adding sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerin) with (reducing) or without (unreducing) 2-mercaptoethanol (2ME). The immunoprecipitates were then boiled for 3 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 mass standards (Life Technologies, Gaithersburg, MD).

As reported by Asai et al. (1995) previously, in both immunoblot and kinase assays, c-Ret, Ret-MEN2A, and Ret-MEN2B developed two bands of 175 kDa (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-PTC-1, which consisted of a cytoplasmic domain only, developed only one band under reducing conditions.

**UV Irradiation**

After 70–80% confluent cells were cultured in DMEM supplemented with 0.5% bovine calf serum overnight, UV irradiation (UV-B from the UV lamp, model FL20S.E-30/DMR; peak wave, 305 nm; Toshiba Medical Supply, Tokyo, Japan) was performed on the cells according to the method of Dhanwada et al. (1995). UV irradiation was carried out in a chamber safe for UV-B exposure. The UV dose was quantified in joules per square meter with the use of a microvolt ammeter (UVR-3036/S, Topcon, Tokyo, Japan).

**RESULTS**

**Genetic Mechanisms of Ret Kinase Activation**

We first measured the levels of tyrosine phosphorylation and kinase activity of Ret proteins in cell lines expressing c-Ret, Ret-MEN2A, and Ret-MEN2B. Ret proteins were isolated from cell lysates by immunoprecipitation and were subjected to Western blotting with anti-Ret and anti-phosphotyrosine antibodies. As shown in Figure 2A, the levels of tyrosine phosphorylation of the 175- and 155-kDa Ret-MEN2A and Ret-MEN2B were much higher than that of c-Ret, whereas the levels of overall protein expression of c-Ret, Ret-MEN2A, and Ret-MEN2B in cells were comparable with each other, although the expression level of the 175-kDa c-Ret (top band) was slightly less than that of the 175-kDa Ret-MEN2A and Ret-MEN2B (Figure 2B). The demonstration of the extent of tyrosine phosphorylation of Ret proteins seemed to reflect the extent of their autophosphorylation in vivo, because the cells expressing a mutant Ret defective in the tyrosine required for the Ret kinase activity was not susceptible to UV irradiation for increased tyrosine phosphorylation (our unpublished results). The immunoprecipitated Ret proteins were also subjected to in vitro kinase assay. The results are shown in Figure 2C. The relative levels of catalytic activity of Ret-MEN2A (lane 3) and Ret-MEN2B (lane 4) (calculated by densitometric analyses of the bands in Figure 2, B and C) were 5–7 and 3–4 times higher, respectively, for autophosphorylation and 10–12 and 7–8 times higher, respectively, for phosphorylation of MBP as an exogenous substrate than those of the c-Ret (lane 2). These results confirmed that Ret kinase is activated by MEN2A and MEN2B mutation.

**UV Irradiation Induces Superactivation of Mutant Ret Kinases**

We next examined the effect of UV irradiation of NIH 3T3 cells that had been transfected with c-RET, RET-MEN2A, or RET-MEN2B on the levels of tyrosine phosphorylation and kinase activity of Ret. The level of tyrosine phosphorylation of c-Ret in cells was increased 5–7 times 5 min after UV-B irradiation (600 J/m²) (Figure 3A). UV-B actually up-regulated the kinase activity of c-Ret, which was demonstrated by an in vitro kinase assay (Figure 3C) for both autophosphorylation and MBP phosphorylation (for MBP, 3–4 times greater than the background level in three independent experiments). Interestingly, UV-B irradiation (600 J/m²) further up-regulated the levels of tyrosine phosphorylation in cells (for Ret-MEN2A, 5–7 times greater than control [Figure...
3D); for Ret-MEN2B, 3–4 times greater than control [Figure 3G]) and kinase activity measured by in vitro kinase assay (for Ret-MEN2A, 4–6 times greater than control [Figure 3F]; for Ret-MEN2B, 4–6 times greater than control [Figure 3I]) of Ret-MEN2A and Ret-MEN2B that already had been elevated by point mutation of DNA. UV-B irradiation, however, barely changed the expression level of c-Ret, Ret-MEN2A, and Ret-MEN2B protein in the culture cells under the conditions examined (Figure 3, B, E, and H). These results suggest that UV irradiation induces the second-step activation (superactivation) of the constitutively activated mutant Ret kinases in cells.

**Analyses of Signaling Elements Potentially Downstream of Ret Kinase**

To further examine the relation between genetic activation and UV-induced superactivation of Ret kinase, we measured the expression and activation levels of signal-transduction elements potentially downstream of Ret kinases in nontransfected control, c-Ret-transfected, and Ret-MEN2A-transfected NIH 3T3 cells before and after UV irradiation. The results are shown in Figure 4. Protein expression levels in NIH 3T3 cells of ERK, JNK, p38, and c-Jun were more or less up-regulated by transfection of c-Ret or Ret-MEN2A, compared with those in nontransfected control cells, and the effect of MEN2A mutation over c-Ret was seen for more extensive up-regulation of expression of ERK and c-Jun. The levels of phosphorylated (activated) ERK, JNK, p38, and c-Jun in c-Ret transfectant were almost parallel to these protein expression levels, demonstrating no c-Ret–mediated premium activation of these signal-transducing elements. In Ret-MEN2A transfectant, however, the levels of phosphorylated JNK, p38, and c-Jun but not ERK were more extensively up-regulated than expected from the low-grade increase in protein expression levels compared with the levels in c-Ret transfectant. These results suggest that MEN2A mutation primarily affected JNK, p38, and c-Jun for activation and ERK for protein expression promotion. We next examined the effects of UV irradiation of Ret-MEN2A transfectant on phosphorylation of ERK, JNK, p38, and c-Jun. As
expected, UV irradiation of cells did not cause any change in protein expression levels of these signal transduction elements in a brief (10-min) incubation of cells after irradiation (our unpublished results). UV irradiation, however, caused further prompt (in 10 min) increases in the levels of phosphorylation of ERK, JNK, p38, and c-Jun in the Ret-MEN2A transfectant, which had been constitutively high as a result of the action of Ret-MEN2A (Figure 4, I–L). This suggested that superactivation of Ret-MEN2A involves a second-step activation of the potentially downstream signal transduction elements. The UV irradiation–mediated activation, however, was not specific to Ret kinase, because the originally low levels of phosphorylation of signal delivery elements in nontransfected control NIH 3T3 cells were also up-regulated by UV irradiation proportionally (our unpublished results). All of these results suggest the two steps of up-regulation of ERK, JNK, p38, and c-Jun by oncogenic mutation of Ret and UV irradiation.

**UV Irradiation Promotes Dimerization of Ret**

Earlier reports suggested that promoting the dimerization of Ret by ligand (Chiariello et al., 1998) or MEN2A-type point mutation (Asai et al., 1995; Santoro et al., 1995) activates its kinase activity. We examined whether UV would promote dimerization of Ret proteins as a potential mechanism for its activation and superactivation. As shown in Figure 5, a small amount (3–4%) of c-Ret formed dimers under unreducing conditions (Figure 5B, lane 1), and 40% of the total autophosphorylated Ret protein was in this dimerized state (Figure 5D, lane 1). From these data, the relative autophosphorylation level for dimers (level of phosphorylation/protein amount) was calculated as ~30 times higher than that for monomers. This demonstrates a close relationship between dimerization and autophosphorylation (activation) of c-Ret kinases at their background levels. UV irradiation promoted dimerization of c-Ret up to 8–9% (two- to threefold increase; Figure 5B, lane 2), in close association with the promotion of Ret autophosphorylation (Figure 5, C and D, lanes 2). We next examined whether the disulfide bond–mediated dimerization of Ret proteins would be needed for the up-regulation of the kinase activity. The up-regulated kinase activity of c-Ret from UV-irradiated cells (Figure 5E, lane 1) was decreased significantly by treating the Ret with 2ME as a reducing reagent (Figure 5E, lane 2) before the in vitro kinase assay, which almost fully inhibited the Ret dimerization (our unpublished results). This result suggests a close connection between the activation of c-Ret kinase and its dimerization by disulfide bonds. Corresponding to the earlier result, a greater portion (30–40%) of Ret-MEN2A formed dimers as a result of disulfide bond–mediated cross-linkage of Ret proteins in their extracellular domain, with a mutation at cysteine 634 (Figure 6B, lane 1), and 60–70% of the total autophosphorylated Ret protein was in this dimerized state (Figure 6D, lane 1). Interestingly, UV irradiation further promoted dimerization of Ret-MEN2A, up to 50–60% (1.5- to 2-fold increase; Figure 6B, lane 2), in close association with the promotion of Ret autophosphorylation (Figure 6D, lane 2). Dimerized bands of Ret-MEN2A were cut out from the gel in Figure 6D and separated under reducing conditions (Figure 6E). It was found by this experiment that UV affected both 175- and 155-kDa Ret for dimerization and activation. A close association between the enzyme activities for the autophosphorylation and dimerization of the kinase protein, and their coordinated promotion by UV irradiation, was also observed with Ret-MEN2B, although only a small amount (1–2%) of Ret-MEN2B formed dimers before UV irradiation (Figure 6F, lane 1). This increased to 3–4% after UV irradiation (Figure 6F, lane 2). These results suggest that UV induces superactivation of Ret-MEN2A and Ret-MEN2B as well as activation of c-Ret kinase by promoting their dimerization through a redox mechanism.

**Intracellular Domain of Ret Can Be the Submolecular Target of UV**

We next tried to partially determine the submolecular target of UV in promoting dimerization and activation of Ret. As shown in Figure 7A, UV irradiation increased both autophosphorylation (9–10 times) and MBP phosphorylation (8–9 times) levels of Ret-PTC-1 with deletion of the extra-
cellular domain. These results show that the extracellular domain of Ret kinase is unnecessary for the UV-induced activation of the kinase. We then examined whether UV irradiation could promote dimerization of Ret-PTC-1. Only a small amount (1–2%) of Ret-PTC-1 was dimerized before UV irradiation (Figure 7C, lane 1), but 80% of the total autophosphorylated Ret resided in this dimerized position (Figure 7E, lane 1). The relative autophosphorylation level (phosphorylation level/protein amount) of dimers was thus calculated to be 50 times greater than that of monomers. UV irradiation promoted dimerization of Ret-PTC-1 up to 5–8% (three- to fivefold increase; Figure 7C, lane 2), in close association with the increase in Ret autophosphorylation (Figure 7, D and E, lanes 2). These results suggest that the intracellular domain of Ret may be the major submolecular target of UV for the promotion of dimerization and activation of the Ret kinase.

**Cysteine 376 of Ret Can Be the Target Amino Acid of UV**

We next examined whether the target amino acid(s) of UV, we focused our study on two cysteines in the kinase domain 2 (cysteines 365 and 376 of Ret-PTC-1) and prepared RET-PTC-1–C365A and RET-PTC-1–C376A transfectants (Figure 1B). There was not much difference in the background kinase activity between original Ret-PTC-1 and Ret-PTC-1–C365A, whereas the background Ret kinase activity of Ret-PTC-1–C365A was much lower than that of Ret-PTC-1–C365A or original Ret-PTC-1, particularly for autophosphorylation. For RET-PTC-1–C365A, UV irradiation increased both autophosphorylation (3–5 times) and MBP phosphorylation (6–8 times) levels (Figure 8A) in a time-dependent manner, as it did for original Ret-PTC-1 (Figure 7A). In contrast to this observation, neither autophosphorylation nor MBP phosphorylation level was significantly increased by UV irradiation for Ret-PTC-1–C376A (Figure 8B). Corresponding to the normal sensitivity to UV of Ret-PTC-1–C365A for up-regulation of the Ret kinase activity, the background level of dimerization of Ret-PTC-1–C365A proteins under unreducing conditions and the sensitivity to UV for its promotion were similar to those of

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**Figure 6.** UV promotes dimerization of Ret-MEN2A and Ret-MEN2B. Lysates from NIH 3T3 cells transfected with RET-MEN2A (A–E) or RET-MEN2B (F–I) after sham or UV irradiation were subjected to either Western blotting with anti-Ret antibody (A, B, F, and G) or in vitro kinase assay (C, D, E, H, and I) after immunoprecipitation with anti-Ret antibody. SDS-PAGE was done under reducing (A, C, E, F, and H) or unreducing (B, D, G, and I) conditions in 5% polyacrylamide gels. (E) The dimerized bands in D were cut from the gel and separated under reducing conditions. Lanes 1, sham irradiation; lanes 2, 5 min after UV-B irradiation (600 J/m²). M, monomer Ret; D, dimer Ret. For direct comparison of kinase activity between Ret-MEN2A and Ret-MEN2B, see Figure 2. All measurements were repeated three times with basically the same results. Representative results are provided.

**Figure 7.** UV promotes activation and dimerization of extracellular domain–deleted Ret. Lysates from NIH 3T3 cells transfected with RET-PTC-1 after sham or UV irradiation were analyzed by either Western blotting with anti-Ret antibody (B and C) or in vitro kinase assay after immunoprecipitation with anti-Ret antibody (A, D, and E). SDS-PAGE was done under reducing (A, B, and D) or unreducing (C and E) conditions in 7% (B–E) or 13% (A) polyacrylamide gels. Lanes 1, sham irradiation; lanes 2, 5 min after UV-B irradiation (600 J/m²). M, monomer Ret; D, dimer Ret. All measurements were repeated three times with basically the same results. Representative results are provided.
original Ret-PTC-1 (Figure 8D). In contrast, no detectable amounts of Ret-PTC-1–C376A proteins were located at the positions of dimers, and UV irradiation never promoted the formation of dimers (Figure 8E). The bands observed at the positions over dimers for both Ret-PTC-1–C376A and original Ret-PTC-1 were invariable in density by UV irradiation, and no kinase activity was detectable with this band for Ret-PTC-1–C376A in kinase assay (our unpublished results). All of these results suggest that Ret-PTC-1 cysteine 376 is the major amino acid target of UV for both promotion of dimerization and kinase activation of Ret-PTC-1.

**SOD1 Prevents UV-mediated Ret Superactivation**

All of the results described so far suggest that UV induces activation and superactivation of the Ret kinases by changing the cellular redox level to oxidative, thereby promoting disulfide bond–mediated dimerization of Ret. To confirm this, we examined the effect of the overexpression of SOD1 in cells on the UV-mediated superactivation of the Ret-MEN2A. As shown in Figure 9, A and B, the SOD1 protein expression level in Ret-MEN2A + SOD1–transfected (HA-SOD1+) NIH 3T3 cells was much greater than that in HA-SOD1− cells transfected with Ret-MEN2A alone, whereas there was no difference in Ret protein expression levels between these two types of cells. Interestingly, UV irradiation induced superactivation of Ret-MEN2A in HA-SOD1− cells (Figure 9C) but not in HA-SOD1+ cells (Figure 9D). Correspondingly, UV further promoted the formation of Ret-MEN2A dimers in HA-SOD1− cells (Figure 6B) but not in HA-SOD1+ cells (Figure 9E), which already had been promoted extensively by MEN2A mutation in both types of cells before UV irradiation. These results suggest that SOD1, acting to scavenge superoxides, counteracts UV in the mechanism for the redox reaction–mediated dimer formation and activation of Ret.

**DISCUSSION**

In this study, we demonstrated that UV irradiation induces not only activation of c-Ret tyrosine kinase as a proto-oncogene product but also the second-step activation of consti-
tutively activated Ret-MEN2A and Ret-MEN2B kinases coded by active oncogenes. This was demonstrated by two-step promotion of levels of both tyrosine phosphorylation in vivo and catalytic activity measured by in vitro kinase assay of Ret proteins, by oncogenic mutation of DNA (step 1) and UV irradiation (step 2). The levels of catalytic activity of Ret-MEN2A and Ret-MEN2B, e.g., as measured for MBP phosphorylation in the kinase assay, were originally 10–12 times or 7–8 times, respectively, greater than those of c-Ret. After UV irradiation, these levels were calculated as 40–72 (10–12 × 4–6) times and 28–48 (7–8 × 4–6) times greater than those of sham-irradiated c-Ret. We called this step 2 activation of the oncogenic mutant Ret kinases by UV “superactivation.”

Earlier studies on the effect of UV on EGFR (Sachsenmaier et al. 1994) and ZAP-70 (Schieven et al. 1994) suggested that the UV-mediated signal delivery pathway correlates the pathway mediated by the receptor cross-linkage with natural ligands. In our experiment, the cascade of signaling by superactivation of Ret was also shown to be basically similar to that by activation linked to oncogene mutation, although the two cascades involved signal transduction through ERK and JNK/p38, ending in the regulation of the activity of AP-1 (c-Jun/c-Fos) as a transcriptional factor in slightly different manners. Therefore, oncogene mutation and UV irradiation probably should affect the MAPK/AP-1 signal transduction pathway additively or cooperatively.

Finally, we demonstrated that overexpression of SOD1 in cells prevents UV-mediated promotion of dimerization and superactivation of Ret-MEN2A. This result suggests that UV irradiation of cells promotes the production of free radicals to be scavenged by SOD1, which would otherwise affect the sulfhydryl groups of cysteines in the intracellular domain of Ret for the promotion of dimerization and activation. UV irradiation is known to damage DNA and bring about stepwise oncogenesis. Based on the results obtained, we would propose that UV also affects proteins as oncogene products for superactivation, which might be involved in the mechanism of stepwise oncogenesis. Partially supporting this view, we have observed that repeated UV irradiation of otherwise benign melanocytic tumors developed in RET-transgenic mice (Iwamoto et al., 1991; Kato et al., 1999) induced their malignant transformation, accompanying extensive superactivation of Ret (our unpublished results), although the exact cause-and-effect relation of these two events remains to be proved.

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