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Mechanism of Activation of the *ret* Proto-oncogene by Multiple Endocrine Neoplasia 2A Mutations

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Transforming activity of the c-*ret* proto-oncogene with multiple endocrine neoplasia (MEN) 2A mutations was investigated by transfection of NIH 3T3 cells. Mutant c-*ret* genes driven by the simian virus 40 or cytomegalovirus promoter induced transformation with high efficiencies. The 170-kDa Ret protein present on the cell surface of transformed cells was highly phosphorylated on tyrosine and formed disulfide-linked homodimers. This result indicated that MEN 2A mutations induced ligand-independent dimerization of the c-Ret protein on the cell surface, leading to activation of its intrinsic tyrosine kinase. In addition to the MEN 2A mutations, we further introduced a mutation (lysine for asparaginic acid at codon 300 [D300K]) in a putative Ca^{2+}-binding site of the cadherin-like domain. When c-*ret* cDNA with both MEN 2A and D300K mutations was transfected into NIH 3T3 cells, transforming activity drastically decreased. Western blot (immunoblot) analysis revealed that very little of the 170-kDa Ret protein with the D300K mutation was expressed in transfectants while expression of the 150-kDa Ret protein retained in the endoplasmic reticulum was not affected. This result also demonstrated that transport of the Ret protein to the plasma membrane is required for its transforming activity.

The c-*ret* proto-oncogene encodes a transmembrane tyrosine kinase that contains a cadherin-like structure in the extracellular domain (9, 10, 19, 22, 23). Its expression was detected at high levels in the peripheral nervous systems such as the enteric and autonomic nervous systems as well as in the excretory system during embryogenesis (1a, 15, 25). In addition, it is expressed preferentially in human tumors such as neuroblastoma, pheochromocytoma, and thyroid medullary carcinoma (8, 18, 24). Since the peripheral nervous systems and tumors mentioned above derive from neural crest cells, the physiological function of the c-*ret* proto-oncogene appears related to their normal growth and differentiation.

Recent studies revealed that germ line mutations in the c-*ret* proto-oncogene are associated with the development of four different neural crest disorders (neurocristopathies): multiple endocrine neoplasia (MEN) 2A and 2B, familial medullary thyroid carcinoma, and Hirschsprung's disease (2, 4, 5, 7, 13, 16). MEN 2A and MEN 2B are autosomal dominant cancer syndromes characterized by the development of medullary thyroid carcinoma and pheochromocytoma. MEN 2B is distinguished from MEN 2A by a more complex phenotype including mucosal neuroma, hyperganglionosis of the gastrointestinal tract, and marfanoid habitus. MEN 2A and familial medullary thyroid carcinoma mutations always involve cysteine residues present in the extracellular domain of the c-*ret* proto-oncogene (4, 12, 13). These cysteine residues are conserved in both human and mouse c-*ret* proto-oncogenes, suggesting that they are important for normal conformation of the c-Ret protein (9, 22, 23). On the other hand, a single point mutation in exon 16 of the tyrosine kinase domain has been found in 95% of patients with MEN 2B (6). This difference of the mutation sites may account for different phenotypes of MEN 2A and MEN 2B. Alternatively, it is possible that the diverse phenotypes observed in MEN 2A and MEN 2B are due to mutations in other modifier genes.

Hirschsprung's disease is a developmental disorder of the enteric nervous system, inherited in an autosomal dominant manner with incomplete penetrance and variant expressivity. Several mutations have been found in different domains of the c-*ret* proto-oncogene, including the extracellular and tyrosine kinase domains (5, 16). Since mice homozygous for c-*ret* disruption showed phenotypes similar to Hirschsprung's disease (20), it is likely that the abnormalities observed in Hirschsprung's disease are caused by inactivation of the c-Ret function. On the other hand, MEN 2A and MEN 2B mutations might represent gain-of-function mutations.

To elucidate the mechanism of development of MEN 2A syndrome, we introduced MEN 2A mutations in the extracellular domain of the c-*ret* proto-oncogene and analyzed their functions. Biochemical analysis of the Ret protein with MEN 2A mutations indicated that it is activated by ligand-independent dimerization on the cell surface. In addition, we showed that a mutation in a putative Ca^{2+}-binding site of the cadherin-like domain significantly decreased the transforming activity of the Ret protein with MEN 2A mutations by inhibiting its transport to the cell surface, suggesting a unique function of the cadherin-like domain.

MATERIALS AND METHODS

Plasmid construction. A cDNA clone containing the entire coding sequence (for 1,072 amino acids) of the human c-*ret* gene was inserted between the *Hin*dIII and *Sma*I sites of the pSV2neo plasmid or between the *Hin*dIII and *Eco*RI sites of the Rc/CMV plasmid (Invitrogen, San Diego, Calif.). Each mutation (arginine for cysteine at codon 634 [C634R], C634Y, or D300K) was introduced by PCR. In brief, primers containing these mutations were synthesized and used for amplification of c-*ret* sequences of approximately 200 to 300 bp. The corresponding sequences of the c-*ret* gene were replaced with the amplified fragments with the mutations. The amplified fragments were sequenced to confirm that proper mutations were introduced.

Transfection. Each recombinant plasmid (0.2 to 0.5 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 (23). Transformed foci were scored on day 14 after transfection. Then foci were picked up and grown into cell lines.

To isolate cell lines expressing the wild-type c-Ret protein, two μg of pSV2c-

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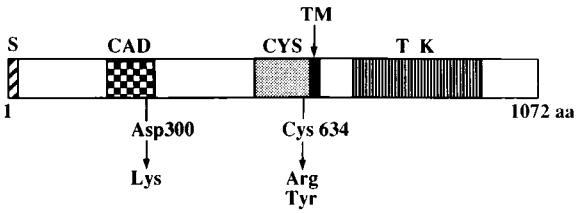

FIG. 1. Schematic illustration of the c-Ret protein. S, signal sequence; CAD, cadherin-like domain; CYS, cysteine-rich region; TM, transmembrane domain; TK, tyrosine kinase domain; aa, amino acids. Mutant cDNAs in which cysteine at codon 634 was replaced by arginine or tyrosine were used in this study. cDNA that substituted lysine for asparaginic acid at codon 300 was also constructed.

ret was cotransfected with 100 ng of pSV2neo plasmid and 10 μg NIH 3T3 DNA. After 24 h, cells were split 1 to 5 and fed with Dulbecco modified Eagle medium, 8% calf serum, and 0.5 mg of G418 per ml. G418-resistant colonies expressing the wild-type c-Ret protein were selected after 2 weeks.

Western blotting (immunoblotting). Total cell lysates were prepared from NIH 3T3 cells and transfectants as described previously (24). The lysates were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Nihon Millipore Kogyo, KK, Tokyo, Japan). After membranes were reacted with anti-Ret antibody against the carboxy-terminal 19 amino acids of the c-Ret protein (21) or antiphosphotyrosine antibody (Zymed Laboratories, Inc., South San Francisco, Calif.), the reaction was examined by the avidin-biotin complex immunoperoxidase method (24) or ^{125}I-protein A (ICN, Irvine, Calif.).

Cell labeling. Cells were labeled for 4 to 5 h in methionine-free RPMI medium supplemented with 10% dialyzed fetal calf serum and [^{35}S]methionine (500 μCi/ml; Amersham, Aylesbury, England). Similarly, they were labeled in phosphate-free RPMI medium containing $^{32}P_i$ (500 μCi/ml; ICN). After washing with phosphate-buffered saline (pH 7.2), cells were lysed in radioimmunoprecipitation assay buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 2 mM EDTA, 1% Triton X-100) containing 1 mM phenylmethylsulfonyl fluoride, 0.5 mM sodium orthovanadate, and 10 mM iodoacetamide. The lysates were clarified by centrifugation (100,000 × *g*) for 1 h and used for immunoprecipitation.

Immunoprecipitation. Cell lysates were incubated with Sepharose beads conjugated with antibodies at 4°C overnight and washed with radioimmunoprecipitation assay buffer four times. The resulting antigen-antibody complex was suspended in SDS sample buffer (20 mM Tris-HCl [pH 6.8], 2 mM EDTA, 2% SDS, 10% sucrose, 20 μg of bromophenol blue per ml) in the presence or absence of 80 mM dithiothreitol and boiled for 3 min. The samples were subjected to SDS-polyacrylamide gel electrophoresis.

Phosphoamino acid analysis. Phosphorylated c-Ret proteins were cut out from gels and eluted in 1.5 ml of 50 mM NH_4HCO_3. The proteins were digested with 50 μg of proteinase K per ml at 37°C overnight. After lyophilization, the samples were subjected to acid hydrolysis in 50 μl of 6 N HCl for 2 h at 110°C. Then they were suspended in 1 ml of distilled water and lyophilized three times. Phosphoamino acids were resolved by two-dimensional electrophoresis at pH 1.9 and 3.5 on cellulose thin-layer plates.

Biotinylation of cells. Cells (3×10^6) were washed with biotinylation buffer (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 8.8], 150 mM NaCl) three times. Biotin *N*-hydroxysuccinimide ester (Vector Laboratories, Inc., Burlingame, Calif.) was dissolved in dimethyl sulfoxide at a concentration of 10 mg/ml and added to cells at a final concentration of 150 μg/ml in 1 ml of the biotinylation buffer. After incubation for 15 min at 20°C, the reaction was terminated by addition of Tris-HCl (pH 7.4) to a final concentration of 50 mM. Cells were washed with 10 mM Tris-HCl (pH 7.4) containing 150 mM NaCl and lysed in radioimmunoprecipitation assay buffer. The resulting lysate was used for immunoprecipitation.

RESULTS

Transforming activity of c-Ret protein with MEN 2A mutations. Mutations identified in MEN 2A affected one of five cysteine residues (codons 609, 611, 618, and 620 in exon 10 and codon 634 in exon 11) encoded in the extracellular domain of the c-*ret* proto-oncogene (12). The most frequent mutation was cysteine to arginine at codon 634, and the second most frequent was cysteine to tyrosine at the same codon (Fig. 1). These mutations account for 54 and 11% of all mutations, respectively. Thus, we introduced these two mutations (C634R and C634Y) into c-*ret* cDNA encoding a protein of 1,072 amino acids. The cDNA with each mutation was inserted into expression vectors containing the simian virus 40 or cytomegalovirus promoter. When these constructs were transfected into NIH 3T3 cells, both mutations showed similar transforming activities although the constructs driven by the simian virus 40 promoter appeared to be more active than those driven by the cytomegalovirus promoter in this assay (Table 1). In contrast, the constructs containing the wild-type c-*ret* cDNA (pSV2c-ret and CMVc-ret) induced no foci.

TABLE 1. Transfection of NIH 3T3 cells with *ret* constructs

DNA	Focus-forming activity (foci/μg of DNA)	No. of scid mice with tumor formation/ total no. of scid mice[a]	Latency (days)
pSV2c-ret	<0.2	2/5	34–38
CMVc-ret	<0.2	ND	
pSV2ret(C634R)	100–200	3/3	6–8
pSV2ret(C634Y)	100–200	ND	
CMVret(C634R)	30–60	3/3	6–8
CMVret(C634Y)	30–60	3/3	6–8
pSV2ret(D300K)	<0.2	1/3	53
pSV2ret(C634R, D300K)	5–10	3/3	23–27
NIH 3T3	<0.02	2/6	37–41

[a] Cells (3×10^6) expressing each construct were subcutaneously injected in female scid mice. Mice were checked for tumor formation until 60 days after injection. ND, not done.

We established cell lines expressing the wild-type or mutant c-Ret protein. Each cell line expressed 150- and 170-kDa c-Ret proteins at high levels (Fig. 2). Cells expressing the wild-type c-Ret protein were morphologically flat (Fig. 3A), while cells transformed by the mutant c-Ret protein were spindle shaped and highly refractile (Fig. 3B). When transformed cells (3×10^6) were injected subcutaneously into female scid mice (6 to 8 weeks old), they gave rise to visible tumors with a short latency after injection (6 to 8 days) (Table 1). On the other hand, NIH 3T3 cells and nontransformed cells expressing the wild-type c-Ret protein induced tumors with a long latency (34 to 41 days) or no tumors by 60 days after injection.

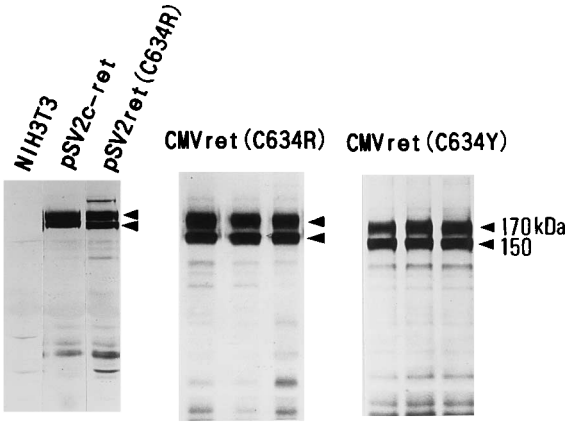

FIG. 2. Expression of the c-Ret protein in transfectants. Total cell lysates were prepared from the designated transfectants of NIH 3T3 cells. pSV2c-ret is a cell line expressing the wild-type c-Ret protein. pSV2ret(C634R), CMVret(C634R), and CMVret(C634Y) represent independent cell lines expressing the mutant c-Ret proteins that were established from transformed foci. The lysates containing 20 μg of proteins per ml were separated on SDS–8% polyacrylamide gels under reducing conditions and reacted with anti-Ret antibody. The reaction was detected by the avidin-biotin complex immunoperoxidase method. The 150- and 170-kDa Ret proteins are indicated.

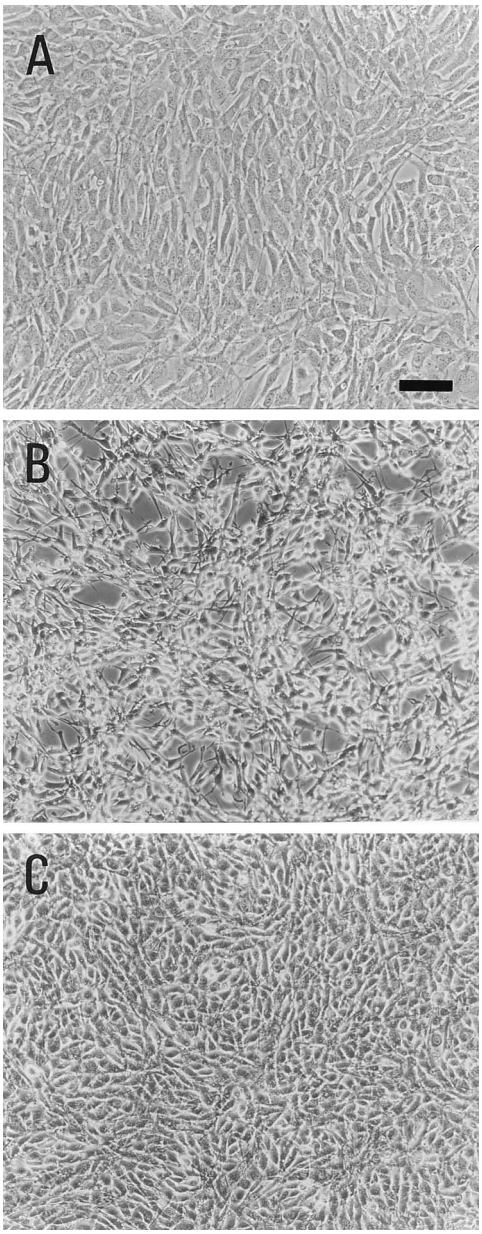

FIG. 3. Phase-contrast micrographs of cell lines expressing the wild-type or mutant c-Ret protein. Cells expressing the wild-type c-Ret protein (A) or mutant c-Ret protein with the C634R mutation (B) or with C634R and D300K mutations (C) were photographed. The scale bar represents 100 μm.

Increased tyrosine phosphorylation of the 170-kDa Ret protein on the cell surface. We next examined tyrosine phosphorylation in the established cell lines by Western blotting with antiphosphotyrosine antibody. Figure 4A shows that the antibody detected 150- and 170-kDa tyrosine phosphorylated bands in cell lysates expressing the mutant c-Ret protein. The level of tyrosine phosphorylation of the 170-kDa band was approximately 10-fold higher than that of the 150-kDa band. In the case of cells expressing the wild-type c-Ret protein, both 150- and 170-kDa proteins were weakly phosphorylated on tyrosine. In addition, a 60-kDa phosphorylated band was detected specifically in MEN 2A transfectants.

To investigate whether these phosphorylated bands represent the c-Ret protein, the samples immunoprecipitated with antiphosphotyrosine antibody were subjected to Western blotting with anti-Ret antibody. As a consequence, the highly phosphorylated 170-kDa band in the immunoprecipitate prepared from a MEN 2A transfectant was found to be the c-Ret protein (Fig. 4B, lane 1). Similarly, small amounts of the 150- and 170-kDa tyrosine phosphorylated proteins were detected by anti-Ret antibody in the immunoprecipitate prepared from cells expressing the wild-type c-Ret protein (lane 3). These results thus demonstrated that the 170-kDa Ret protein was phosphorylated on tyrosine at high levels in the MEN 2A transfectants.

Phosphoamino acid analysis of the mutant c-Ret proteins was performed after the MEN 2A transfectants were metabolically labeled with $^{32}P_i$. As shown in Fig. 4C and D, the 150- and 170-kDa Ret proteins were phosphorylated in transfectants and contained all phosphoserine, phosphothreonine, and phosphotyrosine. However, the level of phosphotyrosine detected in the 170-kDa protein was much higher than that in the 150-kDa protein (Fig. 4D), confirming the result of Western blotting.

In the previous study, it was suggested that the 170-kDa Ret protein represents a mature glycosylated form present on the cell surface and the 150-kDa protein is an immature glycosylated form in the cytoplasm (21). To directly show this, cell surface proteins of the transfectants were biotin labeled and their cell lysates were precipitated with anti-Ret antibody. As a consequence, the 170-kDa protein but not the 150-kDa protein was biotinylated (Fig. 5, lanes 1 and 3), indicating that the 170-kDa protein is a cell surface protein. On the other hand, the 150-kDa protein seems to be a glycoprotein carrying high-mannose oligosaccharides that is present in the endoplasmic reticulum, because experiments of digestion with endoglycosidase H showed that the electrophoretic mobility of the 150-kDa protein but not the 170-kDa protein was largely affected by endoglycosidase H treatment (data not shown). Thus, the present data revealed that transformation induced by MEN 2A mutations was associated with high levels of tyrosine phosphorylation of the 170-kDa Ret protein on the cell surface.

Mechanism of transformation by MEN 2A mutations. To investigate further the mechanism of transformation by MEN 2A mutations, cells expressing the wild-type or mutant c-Ret protein were metabolically labeled with [^{35}S]methionine. The c-Ret proteins were immunoprecipitated from each transfectant and analyzed on SDS–6% polyacrylamide gels. As expected, radiolabeled 150- and 170-kDa c-Ret proteins were precipitated under reducing conditions (Fig. 6A). In addition to these proteins, a 340-kDa band was detected specifically in the immunoprecipitates of MEN 2A transfectants under nonreducing conditions (Fig. 6B). Since an apparent molecular mass of 340 kDa is consistent with that of a dimer form of the 170-kDa Ret protein, the 340-kDa band was cut out from the gel and separated under reducing conditions. As shown in Fig. 6C, we found a single band (lane 3) that showed the same electrophoretic mobility as that of the 170-kDa Ret protein (lane 2), indicating that the 340-kDa band represents disulfide-linked homodimers of the 170-kDa Ret protein.

Western blot analysis with antiphosphotyrosine antibody under nonreducing conditions revealed that a highly tyrosine phosphorylated band of 340 kDa was detected only in the MEN 2A transfectant (Fig. 7). This band was also confirmed to represent the c-Ret protein by subjecting the sample immunoprecipitated with antiphosphotyrosine antibody to Western blotting with anti-Ret antibody (data not shown). In addition, the level of tyrosine phosphorylation of the 170-kDa protein in the MEN 2A transfectant was almost the same as that in cells expressing the wild-type c-Ret protein (Fig. 7). These findings

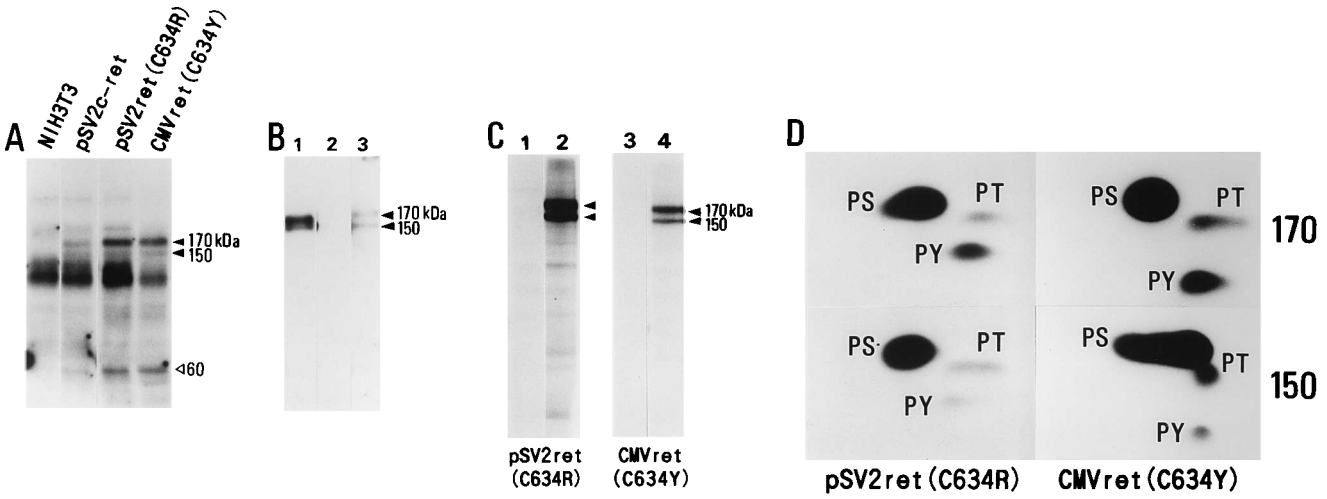

FIG. 4. Analysis of tyrosine phosphorylation of the c-Ret protein in transfectants. (A) Total cell lysates of the designated cell lines were separated on SDS–8% polyacrylamide gels under reducing conditions and reacted with antiphosphotyrosine antibody. Binding of the antibody was detected with ^{125}I-protein A. (B) The lysates prepared from pSV2ret(C634) (lane 1) and pSV2c-ret (lanes 2 and 3) transfectants were immunoprecipitated with antiphosphotyrosine antibody and subjected to Western blotting with anti-Ret antibody. Autoradiographic exposures were 16 h for lanes 1 and 2 and 5 days for lane 3. (C) Cell lines were metabolically labeled with $^{32}P_i$, and their lysates were immunoprecipitated with normal rabbit immunoglobulin G (lanes 1 and 3) or anti-Ret antibody (lanes 2 and 4). The 150- and 170-kDa phosphorylated Ret proteins are indicated. (D) Phosphoamino acid analysis of the phosphorylated Ret proteins detected in panel C. The 170-kDa (upper panel) and 150-kDa (lower panel) Ret proteins were analyzed. PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine.

strongly suggested that high levels of tyrosine phosphorylation of the 170-kDa Ret protein observed under reducing conditions (Fig. 4A) were mainly due to its disulfide-linked homodimerization that could be responsible for transformation of NIH 3T3 cells.

A mutation in the cadherin-like domain impairs transforming activity of c-Ret protein with MEN 2A mutations. The structure of the extracellular domain in the c-Ret protein is unique because of the presence of a cadherin-like sequence. To examine whether this sequence is required for transforming activity, we introduced a mutation (D300K) in a putative Ca^{2+}-binding site identified in the cadherins (Fig. 1) (9, 14).

When c-*ret* cDNA with both C634R and D300K mutations under the control of the simian virus 40 promoter was transfected into NIH 3T3 cells, transforming activity drastically decreased (Table 1). To establish cell lines expressing the c-Ret protein with the D300K mutation, the constructs were cotransfected with pSV2neo plasmid. After selection in G418, we isolated cell lines expressing the c-Ret protein either with the D300K mutation or with the C634R and D300K mutations. Cells expressing the c-Ret protein with C634R and D300K mutations showed a partially transformed phenotype (Fig. 3C), while cells expressing the c-Ret protein with only the D300K mutation were morphologically flat. Consistent with these phenotypes, subcutaneous injection of partially transformed cells formed tumors in scid mice with a relatively long latency (23 to 27 days) as compared with the MEN 2A transfectants (Table 1). Cells expressing the c-Ret protein with the D300K mutation induced a tumor on day 53 or no tumor by 60 days after injection.

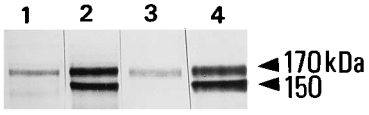

FIG. 5. Biotinylation of the 170-kDa Ret protein present on the cell surface. Cells expressing the wild-type (lanes 1 and 2) or mutant (C634R) (lanes 3 and 4) Ret protein were labeled with biotin as described in Materials and Methods and immunoprecipitated with anti-Ret antibody. The samples were separated on SDS-polyacrylamide gels and reacted with streptavidin-biotin peroxidase complex (lanes 1 and 3). To identify the locations of the 150- and 170-kDa Ret proteins, half of the immunoprecipitated samples were also reacted with anti-Ret antibody (lanes 2 and 4).

Very little of the 170-kDa Ret protein was detected in all established cell lines expressing the c-Ret protein either with the D300K mutation or with C634R and D300K mutations, while expression of the 150-kDa Ret protein was not affected (Fig. 8A). In addition, the 150- and 170-kDa tyrosine phosphorylated bands were undetectable or very faint in the lysates of these transfectants (Fig. 8B). These results suggested that the D300K mutation inhibited transport of the c-Ret protein to the cell surface. Again, cell lines were metabolically labeled with [^{35}S]methionine and their lysates were immunoprecipitated with anti-Ret antibody. As shown in Fig. 9, a band representing homodimers of the 170-kDa protein was undetectable in the immunoprecipitate of cells expressing the c-Ret protein with C634R and D300K mutations, confirming that a significant amount of homodimers of the 170-kDa protein is required for full transforming activity.

DISCUSSION

In the present study, we investigated the mechanism of activation of the c-*ret* proto-oncogene with MEN 2A mutations. The c-*ret* cDNA with MEN 2A mutations showed a high level of transforming activity by transfection of NIH 3T3 cells. Since NIH 3T3 cells do not express the endogenous c-*ret* gene, this result supported the view that the MEN 2A mutations represent dominant activating changes rather than dominant negative changes. In this respect, the c-*ret* proto-oncogene is unique because most genes responsible for hereditary cancer syndromes are believed to be tumor suppressor genes.

Biochemical analysis of the c-Ret protein with MEN 2A mutation demonstrated that it is activated by ligand-independent homodimerization. All mutations identified in MEN 2A patients involve cysteine residues present in the extracellular domain of the c-Ret protein (4, 11, 12). Amino acid sequencing revealed that 27 of 28 cysteine residues in the extracellular

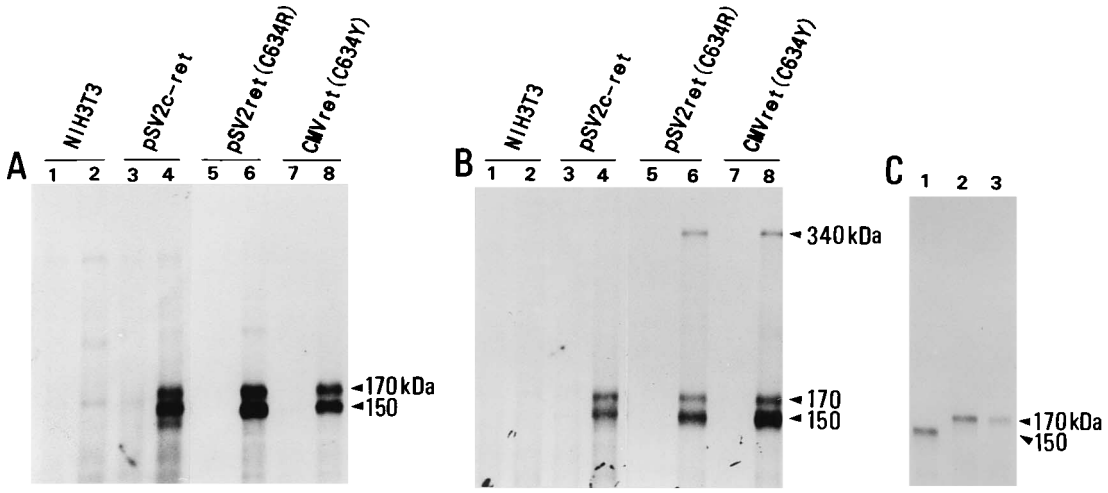

FIG. 6. Immunoprecipitation of the c-Ret protein in transfectants. Cells were metabolically labeled with [^{35}S]methionine, and their lysates were immunoprecipitated with normal rabbit immunoglobulin G (lanes 1, 3, 5, and 7) or anti-Ret antibody (lanes 2, 4, 6, and 8). The immunoprecipitates were separated on SDS–6% polyacrylamide gels under reducing (A) or nonreducing (B) conditions. (C) The 150-kDa (lane 1), 170-kDa (lane 2), and 340-kDa (lane 3) bands detected in lane 8 of panel B were cut out from the gel and separated under reducing conditions.

domain are conserved in the human and mouse c-Ret proteins, suggesting that most of these cysteine residues could be involved in the formation of intrachain disulfide bonds that are crucial for appropriate tertiary structure of the c-Ret protein (9, 22, 23). As a result of the MEN 2A mutations in which one cysteine residue is lost, it is possible that another cysteine is unpaired and involved in the formation of aberrant intra- or intermolecular disulfide bonds in the mutant protein. Indeed, we found homodimerization of the 170-kDa c-Ret protein present on the cell surface of the MEN 2A transfectant by immunoprecipitation under nonreducing conditions, while dimerization did not occur in cells expressing the wild-type c-Ret protein. In addition, homodimers of the 170-kDa c-Ret protein in MEN 2A transfectants were phosphorylated on tyrosine at high levels, showing activation of its intrinsic tyrosine kinase.

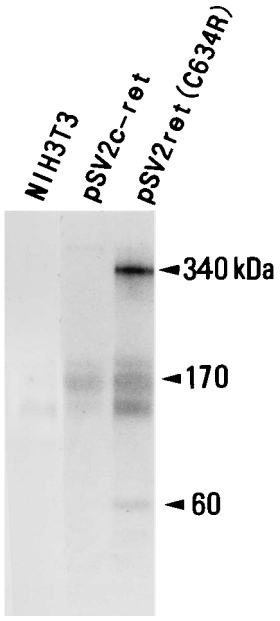

FIG. 7. Tyrosine phosphorylation of homodimers of the 170-kDa protein. Total cell lysates prepared from the designated cells were separated on SDS–7% polyacrylamide gels under nonreducing conditions and reacted with antiphosphotyrosine antibody. The 340-, 170-, and 60-kDa tyrosine phosphorylated bands are indicated.

On the other hand, we could not detect dimerization of the 150-kDa Ret protein in the MEN 2A transfectant. Since the 150-kDa Ret protein represents an immature glycosylated form present in the endoplasmic reticulum that is sensitive to endoglycosidase H treatment (1), this suggested that dimerization occurs after the protein passes through the Golgi complex, where the N-linked oligosaccharides of the protein are processed to complex carbohydrate chains, and that cell surface expression of the 170-kDa Ret protein is required for its homodimerization.

Activation by a point mutation in the extracellular domain was also reported in the *fms* and *trk* receptor tyrosine kinases (3, 17, 27). Substitution of serine for leucine at codon 301 was detected in the *fms* gene, resulting in activation of its transforming potential. In the case of the *trk* proto-oncogene, cysteine at codon 345 was replaced by serine. It is possible that this change also caused an aberrant intermolecular disulfide bond in the *trk* gene product, although it was not directly demonstrated. In addition, the erythropoietin receptor that does not have a tyrosine kinase domain was activated by a point mutation of the extracellular domain where arginine residue at codon 129 was replaced by cysteine (28). Cells expressing the mutant erythropoietin receptor acquired the ability to induce hormone-independent growth and tumorigenicity. Homodimerization of the mutant receptor, probably through the mutated cysteine residue, was demonstrated by biochemical and mutagenesis studies (26). Although activation of the c-Ret protein by MEN 2A mutations is reminiscent of that of these genes, this type of mutation of the c-Ret protein might represent the first example for the mechanism of development of human neoplastic disease.

In contrast to MEN 2A mutations, a mutation that substitutes threonine for methionine at codon 918 in the tyrosine kinase domain was identified in 95% of MEN 2B patients (6, 7). It is therefore expected that different mechanisms are involved in activation of the c-*ret* gene between the MEN 2A and

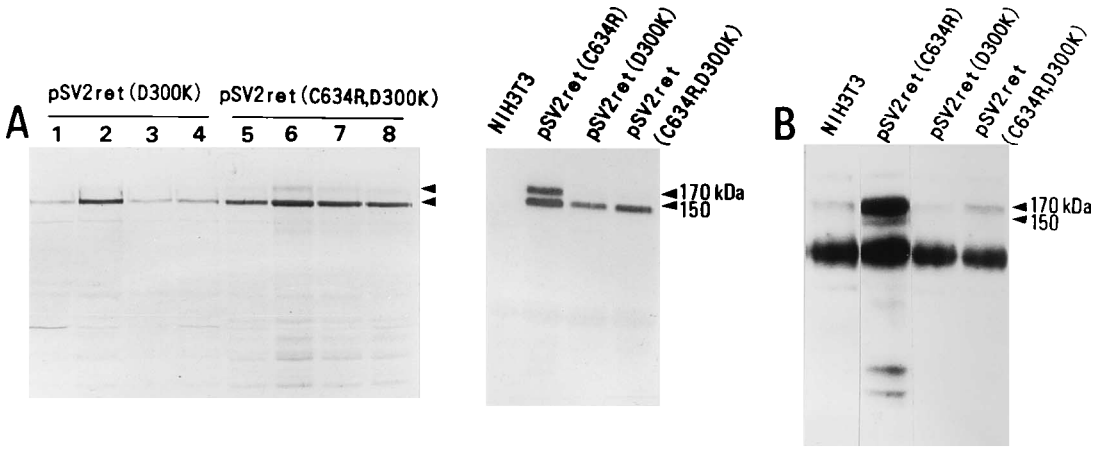

FIG. 8. Analysis of the c-Ret protein with the D300K mutation by Western blotting. (A, left panel) Cell lysates (20 μg of proteins) prepared from four independent lines expressing the c-Ret protein with D300K mutation (lanes 1 to 4) or with both C634R and D300K mutations (lanes 5 to 8) were reacted with anti-Ret antibody. (A, right panel) The expression of the c-Ret protein was compared among the designated cell lines. pSV2ret(D300K) and pSV2ret(C634R, D300K) correspond to the cell lines of lanes 2 and 7 in the left panel, respectively. The reaction was detected by the avidin-biotin complex immunoperoxidase method. (B) The lysates from the designated cells were reacted with antiphosphotyrosine antibody and probed with ^{125}I-protein A.

MEN 2B mutations, leading to diverse phenotypes observed in patients. Our preliminary data indicated that unlike c-Ret with the MEN 2A mutation, the c-Ret protein with a MEN 2B mutation does not form homodimers in transfectants (9a).

The role of the cadherin-like structure in the c-Ret protein is unknown. In the previous study, we reported that unlike cadherins, the c-Ret protein did not have homophilic binding activity when the c-Ret protein was expressed in mouse L cells (21). In order to examine whether this structure is necessary for transforming activity, we introduced a mutation (D300K) in a putative Ca^{2+}-binding site of this structure. In the case of cadherins, this type of mutation almost abolished their homophilic binding activity (14). Interestingly, the D300K mutation largely impaired the transforming activity of the c-Ret protein with MEN 2A mutations. As a result of the mutation, expression of the 170-kDa Ret protein present on the cell surface drastically decreased although expression of the 150-kDa Ret protein present in the endoplasmic reticulum was not affected. A partially transformed phenotype observed in cells expressing the c-Ret protein with MEN 2A and D300K mutations might be due to expression of a small amount of homodimers of the 170-kDa Ret protein. These findings also indicated that the cell surface expression is necessary for transforming activity of the mutant Ret protein and that the D300K mutation impairs intracellular transport of the immature c-Ret glycoprotein to the plasma membrane or the posttranslational processing. Thus, the cadherin-like domain may contain a sequence necessary for transport through the Golgi complex or influence the processing of oligosaccharides. Alternatively, it is possible that inhibition of transport to the cell surface results simply from incorrect folding of the Ret protein with the D300K mutation.

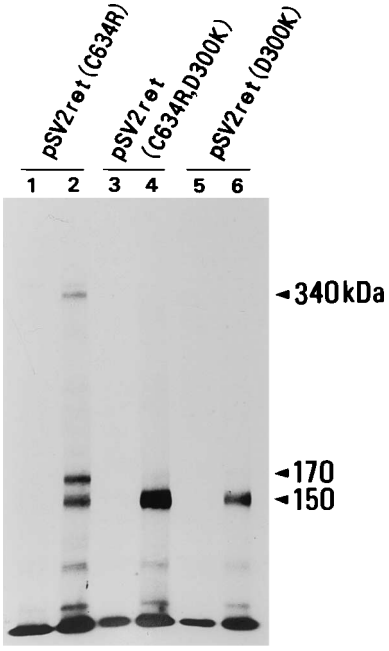

FIG. 9. Immunoprecipitation of the c-Ret protein with the D300K mutation. Cells were metabolically labeled with [^{35}S]methionine, and their lysates were immunoprecipitated with normal rabbit immunoglobulin G (lanes 1, 3, and 5) or anti-Ret antibody (lanes 2, 4, and 6). The resulting immunoprecipitates were separated on SDS–6% polyacrylamide gels under nonreducing conditions.

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REFERENCES

1. **Asai, N., et al.** Unpublished results.
1a. **Avantaggiato, V., N. A. Dathan, M. Grieco, N. Fabien, D. Lazzaro, A. Fusco, A. Simeone, and M. Santoro.** 1994. Developmental expression of the *RET* protooncogene. Cell Growth Differ. **5:**305–311.
2. **Carlson, K. M., S. Dou, D. Chi, N. Scavarda, K. Toshima, C. E. Jackson, S. A. Wells, P. J. Goodfellow, and H. Donis-Keller.** 1994. Single missense mutation in the tyrosine kinase catalytic domain of the *RET* protooncogene is associated with multiple endocrine neoplasia type 2B. Proc. Natl. Acad. Sci. USA **91:**1579–1583.
3. **Coulier, F., R. Kumar, M. Ernst, R. Klein, D. Martin-Zanca, and M. Barbacid.** 1990. Human *trk* oncogenes activated by point mutation, in-frame deletion, and duplication of the tyrosine kinase domain. Mol. Cell. Biol. **10:**4202–4210.
4. **Donis-Keller, H., S. Dou, D. Chi, K. M. Carlson, K. Toshima, T. C. Lair-**

more, J. R. Howe, J. F. Moley, P. Goodfellow, and S. A. Wells. 1993. Mutations in the RET proto-oncogene are associated with MEN 2A and FMTC. Hum. Mol. Genet. **2:**851–856.

5. **Edery, P., S. Lyonnet, L. M. Mulligan, A. Pelet, E. Dow, L. Abel, S. Holder, C. Nihoul-Fekete, B. A. J. Ponder, and A. Munnich.** 1994. Mutations of the *RET* proto-oncogene in Hirschsprung's disease. Nature (London) **367:**378–380.
6. **Eng, C., D. P. Smith, L. M. Mulligan, M. A. Nagai, C. S. Healey, M. A. Ponder, E. Gardner, G. F. W. Scheumann, C. E. Jackson, A. Tunnacliffe, and B. A. J. Ponder.** 1994. Point mutation within the tyrosine kinase domain of the *RET* proto-oncogene in multiple endocrine neoplasia type 2B and related sporadic tumours. Hum. Mol. Genet. **3:**237–241.
7. **Hofstra, R. M. W., R. M. Landsvater, I. Ceccherini, R. P. Stulp, T. Stelwagen, Y. Luo, B. Pasini, J. W. M. Hoppener, H. K. P. van Amstel, G. Romeo, C. J. M. Lips, and C. H. C. M. Buys.** 1994. A mutation in the *RET* proto-oncogene associated with multiple endocrine neoplasia type 2B and sporadic medullary thyroid carcinoma. Nature (London) **367:**375–376.
8. **Ikeda, I., Y. Ishizaka, T. Tahira, T. Suzuki, M. Onda, T. Sugimura, and M. Nagao.** 1990. Specific expression of the *ret* proto-oncogene in human neuroblastoma cell lines. Oncogene **5:**1291–1296.
9. **Iwamoto, T., M. Taniguchi, N. Asai, K. Ohkusu, I. Nakashima, and M. Takahashi.** 1993. cDNA cloning of mouse *ret* proto-oncogene and its sequence similarity to the cadherin superfamily. Oncogene **8:**1087–1091.

9a. **Iwashita, T., et al.** Unpublished data.

10. **Kuma, K., N. Iwabe, and T. Miyata.** 1993. Motifs of cadherin- and fibronectin type III-related sequences and evolution of the receptor tyrosine kinases: sequence similarity between proto-oncogene *ret* and cadherin family. Mol. Biol. Evol. **10:**539–551.
11. **Maruyama, S., T. Iwashita, T. Imai, H. Funahashi, I. Ceccherini, Y. Luo, G. Romeo, S. Matsuo, M. Matsuyama, and M. Takahashi.** 1994. Germ line mutations of the *ret* proto-oncogene in Japanese patients with multiple endocrine neoplasia type 2A and type 2B. Jpn. J. Cancer Res. **85:**879–882.
12. **Mulligan, L. M., C. Eng, C. S. Healey, D. Clayton, J. B. J. Kwok, E. Gardner, M. A. Ponder, A. Frilling, C. E. Jackson, H. Lehnert, H. P. H. Neumann, S. N. Thibodeau, and B. A. J. Ponder.** 1994. Specific mutations of the *RET* proto-oncogene are related to disease phenotype in MEN 2A and FMTC. Nature Genet. **6:**70–74.
13. **Mulligan, L. M., J. B. J. Kwok, C. S. Healey, M. J. Elsdon, C. Eng, E. Gardner, D. R. Love, S. E. Mole, J. K. Moore, L. Papi, M. A. Ponder, H. Telenius, A. Tunnacliffe, and B. A. J. Ponder.** 1993. Germ-line mutations of the *RET* proto-oncogene in multiple endocrine neoplasia type 2A. Nature (London) **363:**458–460.
14. **Ozawa, M., J. Engel, and R. Kemler.** 1990. Single amino acid substitutions in one Ca^{2+} binding site of uvomorulin abolish the adhesive function. Cell **63:**1033–1038.
15. **Pachnis, V., B. Mankoo, and F. Costantini.** 1993. Expression of the *ret* proto-oncogene during mouse embryogenesis. Development **119:**1005–1017.
16. **Romeo, G., P. Ronchetto, Y. Luo, V. Barone, M. Seri, I. Ceccherini, B. Pasini, R. Bocciardi, M. Lerone, H. Kaariainen, and G. Martucciello.** 1994. Point mutations affecting the tyrosine kinase domain of the *RET* proto-oncogene in Hirschsprung's disease. Nature (London) **367:**377–378.
17. **Roussel, M. F., J. R. Downing, C. W. Rettenmier, and C. J. Sherr.** 1988. A point mutation in the extracellular domain of the human CSF-1 receptor (c-fms proto-oncogene product) activates its transforming potential. Cell **55:**979–988.
18. **Santoro, M., R. Rosati, M. Grieco, M. T. Berlingieri, G. L.-C. D'Amato, V. de Franciscis, and A. Fusco.** 1990. The *ret* proto-oncogene is consistently expressed in human pheochromocytomas and thyroid medullary carcinomas. Oncogene **5:**1595–1598.
19. **Schneider, R.** 1992. The human protooncogene *ret*: a communicative cadherin? Trends Biochem. Sci. **17:**468–469.
20. **Schuchardt, A., V. D'Agati, L. Larsson-Blomberg, F. Constantini, and V. Pachnis.** 1994. Defect in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. Nature (London) **367:**380–383.
21. **Takahashi, M., N. Asai, T. Iwashita, T. Isomura, K. Miyazaki, and M. Matsuyama.** 1993. Characterization of the *ret* proto-oncogene products expressed in mouse L cells. Oncogene **8:**2925–2929.
22. **Takahashi, M., Y. Buma, and H. Hiai.** 1989. Isolation of *ret* proto-oncogene cDNA with an amino-terminal signal sequence. Oncogene **4:**805–806.
23. **Takahashi, M., Y. Buma, T. Iwamoto, Y. Inaguma, H. Ikeda, and H. Hiai.** 1988. Cloning and expression of the *ret* proto-oncogene encoding a tyrosine kinase with two potential transmembrane domains. Oncogene **3:**571–578.
24. **Takahashi, M., Y. Buma, and M. Taniguchi.** 1991. Identification of the *ret* proto-oncogene products in neuroblastoma and leukemia cells. Oncogene **6:**297–301.
25. **Tsuzuki, T., M. Takahashi, N. Asai, T. Iwashita, M. Matsuyama, and J. Asai.** 1995. Spatial and temporal expression of the *ret* proto-oncogene product in embryonic, infant and adult rat tissues. Oncogene **10:**191–198.
26. **Watowich, S. S., A. Yoshimura, G. D. Longmore, D. J. Hilton, Y. Yoshimura, and H. F. Lodish.** 1992. Homodimerization and constitutive activation of the erythropoietin receptor. Proc. Natl. Acad. Sci. USA **89:**2140–2144.
27. **Woolford, J., A. McAuliffe, and L. R. Rohrshneider.** 1988. Activation of the feline c-*fms* proto-oncogene: multiple alterations are required to generate a fully transformed phenotype. Cell **55:**965–977.
28. **Yoshimura, A., G. Longmore, and H. F. Lodish.** 1990. Point mutation in the exoplasmic domain of the erythropoietin receptor resulting in hormone-independent activation and tumorigenicity. Nature (London) **348:**647–649.