Mechanism of Ret dysfunction by Hirschsprung mutations affecting its extracellular domain

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Hirschsprung disease (HSCR) is a congenital disorder associated with the absence of intrinsic ganglion cells in the distal gastrointestinal tract. Recently, many missense, nonsense and frameshift mutations of the ret proto-oncogene were found in familial and sporadic cases of HSCR. Consistent with the view that the HSCR phenotype is the result of inactivation of Ret, the missense mutations detected in the tyrosine kinase domain were demonstrated to result in a marked decrease of the kinase activity of Ret. However, the effects of missense mutations found in the extracellular domain remain unknown. We now report that five mutations in the extracellular domain examined inhibit transport of the Ret protein to the plasma membrane. As a consequence, they significantly decreased the transforming activity of Ret with multiple endocrine neoplasia (MEN) 2A mutation for which cell surface expression is required. Our results also demonstrated that long segment HSCR mutations more severely impair transport of Ret to the plasma membrane than a short segment HSCR mutation, suggesting that the level of its cell surface expression may correlate to the HSCR phenotype.

INTRODUCTION

Hirschsprung disease (HSCR) is a common congenital malformation (1/5000 live births) associated with the absence of intrinsic ganglion cells in the distal gastrointestinal tract. Based on the length of the aganglionic segment, it is classified into two groups: short segment HSCR and long segment HSCR. Genetic analyses demonstrated that two different loci on chromosomes 10 and 13 are tightly linked to HSCR (1–3). Recently, it turned out that mutations of the ret proto-oncogene on chromosome 10q11.2 account for 50% of familial and 15–20% of sporadic cases of HSCR, most of which (~75%) were associated with long segment HSCR, a severe form of the disease (4–9). In addition, a missense mutation in the endothelin-B receptor (EDNRB) gene on chromosome 13q22 was found in a large inbred Mennonite kindred affected by HSCR (10). Shortly after this discovery, several missense mutations in EDNRB and its deletion were reported in sporadic cases of short segment HSCR but not long segment HSCR (11–14).

Since mice homozygous for c-ret or EDNRB disruption showed phenotypes similar to HSCR, it seems likely that the mutations of these genes in HSCR represent loss-of-function mutations (15–17). Consistent with this view, Pasini et al. reported that the missense mutations detected in the tyrosine kinase domain of Ret result in severe impairment of the kinase activity of Ret/PTC2, a rearranged form of Ret found in human papillary thyroid carcinoma (18). In the present study, to elucidate the effects of the mutations in the extracellular domain of Ret, we introduced five HSCR mutations into the extracellular domain of ret cDNA with or without multiple endocrine neoplasia (MEN) 2A mutation. We report that the mutations examined inhibit transport of the Ret protein to the plasma membrane and significantly decrease the transforming activity of Ret with the MEN 2A mutation. Interestingly, we also found that long segment HSCR mutations of ret more severely impaired transport of Ret to the plasma membrane than a short segment HSCR mutation.

RESULTS

Five HSCR mutations detected in the extracellular domain of the c-ret proto-oncogene were introduced into its cDNA encoding a protein of 1114 amino acids (long isoform of Ret) (19–21) (Fig. 1a). Four mutations (Ser32-Leu, Leu40-Pro, Arg330-Gln, Phe393-Leu) were identified in long segment HSCR and one (Pro64-Leu) in short segment HSCR (6,8). The cDNA with each mutation was inserted into an expression vector containing Moloney murine leukemia virus LTR and transfected into NIH 3T3 cells. After selection in G418, we established the cell lines expressing each mutant protein. The wild-type c-Ret proteins of 1114 amino acids (long isoform of Ret) (19–21) (Fig. 1a). Four mutations (Ser32-Leu, Leu40-Pro, Arg330-Gln, Phe393-Leu) were identified in long segment HSCR and one (Pro64-Leu) in short segment HSCR (6,8). The cDNA with each mutation was inserted into an expression vector containing Moloney murine leukemia virus LTR and transfected into NIH 3T3 cells. After selection in G418, we established the cell lines expressing each mutant protein. The wild-type c-Ret proteins were expressed as 155 and 175 kDa proteins in the transfected (Fig. 1b). As we have already reported, the 175 kDa Ret protein represents a mature glycosylated form on the cell surface and the 155 kDa protein is an immature glycosylated form in the endoplasmic reticulum (22,23). Interestingly, the five HSCR mutations resulted in a striking decrease of expression of the 175 kDa Ret protein in the transfectants while expression of the 155 kDa protein was not affected (Fig. 1b). In particular, the 175 kDa Ret protein with the long segment HSCR mutations was almost undetectable. In the case of the cell line expressing Ret with the short segment HSCR mutation (P64L), the level of the 175 kDa Ret protein was reduced by ~70% in comparison with the cell line expressing the wild type c-Ret protein. These results

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thus suggested that the HSCR mutations analyzed inhibit the transport of the Ret protein to the plasma membrane.

MEN 2A is an autosomal dominant cancer syndrome characterized by the development of medullary thyroid carcinoma and pheochromocytoma. MEN 2A mutations were detected in one of the characterized by the development of medullary thyroid carcinoma and pheochromocytoma. MEN 2A mutations were detected in one of the five cysteine residues in the Ret extracellular domain (24–26).

As shown in Table 1, all HSCR mutations significantly decreased the transforming activity of Ret with the MEN 2A mutation (designated MEN2A-Ret). The transforming activity of wild-type MEN2A-Ret was 6–10-fold higher than that of MEN2A-Ret with the long segment HSCR mutations and 2-fold higher than that of MEN2A-Ret with the short segment HSCR mutation. Again, we established the cell lines expressing each mutant protein and analyzed the level of Ret expression by Western blotting. The results indicated that the expression of the 175 kDa Ret proteins is indicated.

**DISCUSSION**

In Hirschsprung disease, a variety of mutations including frameshift, nonsense and missense mutations were identified in different domains of the ret proto-oncogene (4–9). Pasini et al. (18) reported that three missense mutations detected in the tyrosine kinase domain abolished the transforming activity of Ret/PTC2, a rearranged form of Ret found in papillary thyroid carcinoma, by impairing its tyrosine kinase activity. Since many missense mutations were also reported to be present in the extracellular domain, we investigated the biological and biochemical effects of these mutations by introducing them into c-Ret and MEN2A-Ret proteins. Our results clearly demonstrated that five mutations examined inhibit the transport of the Ret protein to the plasma membrane and significantly decrease the transforming activity of MEN2A-Ret for which its cell surface expression is required (22,27,28). Interestingly, the mutations found in long segment HSCR impaired transport of Ret to the plasma membrane and the transforming activity of MEN2A-Ret more severely than the short segment HSCR mutation. This represents the first report indicating that the expression level of Ret on the cell surface may correlate to the Hirschsprung phenotype, although incomplete penetrance, variable expressivity...
and sex-dependent effect of ret mutations still suggest the presence of modifier genes and/or the involvement of microenvironmental factors.

The HSCR mutations examined in this study did not completely abolish the transforming activity of MEN2A-Ret. This is in contrast with the results reported by Pasini et al. that the Ret/Ptc2 protein carrying HSCR mutations detected in the kinase domain lost any transforming potential. The low level of transforming activity of the MEN2A-Ret protein with HSCR mutations in the extracellular domain might be due to expression of a small amount of the 175 kDa Ret protein on the cell surface. In fact, when we performed longer exposure of Western blot film, a faint band of each 175 kDa mutant protein was detected. Alternatively, it is possible that the 155 kDa mutant proteins contain weak transforming activity.

The fact that ret heterozygous deletions and its truncation were detected in both familial and sporadic HSCR patients (4–8) implies that haploinsufficiency for ret is crucial for development of HSCR.

Our data presented here suggests that, in most cases of missense mutations in the Ret extracellular domain, a haploinsufficiency effect might also be postulated because cell surface expression of the Ret proteins with long segment HSCR mutations was extremely low. HSCR is thought to be caused by the premature arrest of the cranio-caudal migration or differentiation of enteric neuroblasts which highly express the c-Ret protein during embryogenesis (30,31). Thus, it seems likely that sufficient levels of the Ret expression on the cell surface are required for their migration towards the distal portion of the colon or full differentiation.

It was recently reported that glial cell line-derived neurotrophic factor (GDNF) deficient mice lacked the enteric nervous system and kidney as observed in ret-deficient mice (32). In addition, it turned out that GDNF induces the formation of a physical complex between GDNF receptor and Ret, resulting in tyrosine phosphorylation of Ret (33,34). When human and mouse neuroblastoma cells were treated with GDNF, the 175 kDa Ret protein but not the 155 kDa Ret protein was highly phosphorylated on tyrosine (33,34). These findings thus suggested that GDNF, GDNF receptor and Ret can form a complex on the cell surface that plays a crucial role in the differentiation of enteric neurons. Establishment of cell lines expressing GDNF receptor and Ret with or without HSCR mutations might provide a useful system for analysis of an intracellular signalling pathway via this multicomponent receptor.

**MATERIALS AND METHODS**

**Plasmid construction**

A cDNA clone containing the entire coding sequence (for 114 amino acids) of the human c-ret gene was inserted into the APag-1 vector containing Moloney murine leukemia virus LTR, kindly provided by Dr P. Leder (Harvard Medical School) (35). Each HSCR or MEN 2A mutation was introduced by polymerase chain reaction (PCR). In brief, primers containing the mutations were synthesized and used for amplification of c-ret sequences of ~100–150 base pairs. 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 (0.1 µg) was transfected into NIH 3T3 cells (5 × 10^5 cells in a 60 mm diameter dish) with 10 µg NIH 3T3 DNA as described previously (19). Transformed foci were scored on day 12 after transfection. Then foci were picked up and grown into cell lines.

To isolate cell lines expressing the c-Ret protein with HSCR mutations, NIH 3T3 cells co-transfected with the mutant ret genes and pSV2neo plasmid were fed with Dulbecco’s modified Eagle’s medium, 8% calf serum and 0.5 mg/ml G418. G418-resistant colonies were selected after 2 weeks.

**Western blotting**

Total cell lysates were prepared from transfectants as described previously (36). The lysates were subjected to SDS–PAGE and transferred to polyvinylidene difluoride membranes (Nihon Millipore Kogyo KK, Tokyo, Japan). After membranes were reacted with anti-Ret antibody against the tyrosine kinase domain of the c-Ret protein (36), the reaction was examined by 125I-protein A (ICN, Irvine, CA).

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**ABBREVIATIONS**

HSCR, Hirschsprung disease; MEN, multiple endocrine neoplasia; EDNRB, endothelin-B receptor gene.

**REFERENCES**


