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Original

A Novel Missense Mutation in the Thyroid Peroxidase Gene, R175Q, Resulting in Insufficient Cell Surface Enzyme in Two Siblings

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Abstract. Thyroid peroxidase (TPO) abnormality is one of the causes of congenital hypothyroidism. Two missense mutations were found as a compound heterozygous mutation in two siblings with congenital goitrous hypothyroidism. One of these mutations, G614A (R175Q), was a novel mutation. Characterization of the novel mutation and a cotransfection experiment with two mutated TPO mRNAs were carried out. G614A-mRNA introduced into CHO-K1 cells expressed TPO protein with the same molecular weight as that of wild-type mRNA. The R175Q-TPO was thought to possess enzyme activity. In terms of localization, a very small amount of mutated TPO was expressed on the plasma membrane of CHO-K1 cells. This plasma membrane expression of R175Q-TPO was insufficient to perform thyroid hormone synthesis, but was markedly different from R665W-TPO. When G614A- and C2083T-mRNAs were cotransfected, cell surface TPO-positive cells were only 13.1% in contrast to 54.4% for wild-type mRNA. The low positivity and intensity of cell surface TPO suggested that in the patients' thyroids thyroid hormone synthesis was hardly performed. The congenital hypothyroidism of the patients was thought to be a result of the mutations of the TPO gene (G614A/C2083T).

Key words: congenital hypothyroidism, goiter, TPO gene, missense mutation

Introduction

Thyroid peroxidase (TPO) is an essential enzyme in the synthesis of thyroid hormone. TPO catalyses the iodination of tyrosine residues and the coupling of iodotyrosines on thyroglobulin to form thyroxine (T_4) and 3, 3', 5-tri-iodothyronine (T_3). These functions of iodination and coupling are mainly catalysed by TPO located on the apical membrane surface of thyrocytes (1). Human TPO protein consists of 933 amino acids (2) and is encoded by the gene locating to 2p25 (3), which spans at least 150 kbp and contains 17 exons (4).

Most cases of congenital hypothyroidism are a

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result of thyroid dysgenesis, with about 15–20% caused by defective thyroid hormone synthesis (5). The most common cause of congenital defects in thyroid hormone synthesis is believed to be TPO deficiency (6). About 30 mutations in the human TPO gene that cause iodide organification defect have been reported (7–17). These are classified as frameshift mutations, missense mutations, mutations affecting splicing, nonsense mutations and gene deletion. Because of the molecular size and complex function of TPO protein, more mutations are likely to be reported.

In this report, we describe two mutations of the TPO gene in sibling patients with congenital goitrous hypothyroidism. Although one of these mutations has been previously characterized (15), the other was novel. This novel mutation was characterized and compared with the other mutation in respect to cell surface expression.

Subjects and Methods

Patients

Brother: 3 yr old. In the TSH mass screening test, TSH was 30 mIU/l of blood (reference value, 0.5~4.3 mIU/l) at 5 d after birth, and increased to 90 mIU/l of blood at 10 d after birth. In the re-examination at 14 d after birth, TSH was 264 mIU/l of blood, and free T_4 was 5.5 pmol/l of serum (reference value, 11.6~23.2 pmol/l). He had been treated with thyroid hormone under the diagnosis of congenital hypothyroidism. Enlarged thyroid gland and other signs such as cretinism were not found, but, when his sister was examined with ultrasound, his thyroid gland also showed diffuse goiter.

Sister: 10 mo old. In the TSH mass screening test at 5 d after birth, TSH was 100 mIU/l of blood. In the re-examination at 11 d after birth, TSH was 183 mIU/l of blood, free T_4 was 6.1 pmol/l of serum, and free T_3 was 4.4 pmol/l of serum (reference value, 3.8~6.5 pmol/l). Ultrasound examination showed an enlarged thyroid gland with a maximal transverse diameter of 45 mm (reference value, 16~25 mm) (18). No other sign suggesting cretinism was found. She had been treated immediately with thyroid hormone under the diagnosis of congenital hypothyroidism.

While undergoing medication, these two patients were suspected of having TPO abnormality as a cause of congenital hypothyroidism, and a molecular genetic study was indicated. Informed consent for these examinations was obtained from the parents, and approval was received from the Miyazaki Medical College ethics committee.

The parents were not consanguineous and had no common ancestors. Both had normal thyroid function. In the father, TSH was 0.8 mIU/l of serum, free T_4 15.5 pmol/l of serum, and free T_3 4.4 pmol/l of serum. In the mother, TSH was 2.2 mIU/l of serum, free T_4 14.2 pmol/l of serum, and free T_3 3.5 pmol/l of serum. They had no children other than the patients.

DNA isolation and sequencing

Genomic DNA was isolated from peripheral blood white blood cells, with GenTLE (Takara Bio, Otsu, Japan). PCR primer sets and the conditions for sequencing have been previously described (11, 15). The nucleotide sequence of TPO is according to Kimura *et al.* (4).

Introduction of G614A to TPO cDNA

With the use of wild-type human (h) TPO-1 cDNA (2) as a template, PCR products were amplified with the following primer sets: set A, 5'-CAG AAG AGT TAC AGC CGT GA-3' (F0024) and 5'-CAT AGA CTG GAG GGA GCC ATT GTG CA-3' (G614A-R); set B, 5'-CTG GCA CAA TGG CTC CCT CCA GTC TAT-3' (G614A-F) and 5'-GAC GCG TCC AGG AAC GAG G-3' (R1070). As a 25-nucleotide sequence of the 3'-end of the set A product and 5'-end of the set B product was the same, the F0024/R1070 fragment was produced by 10 cycles of PCR amplification without primers. The fragment was further amplified by PCR reaction with F0024 and R1070 primers for 25 cycles. The PCR product was

Table 1 Mutations and single nucleotide polymorphism (SNPs) of the TPO gene found in the patients and their parents

	Position		Brother	Sister	Father	Mother	Normal subjects		Reference
Mutations	Exon 6	614	A*/G	A*/G	G	A*/G	G (100%)		
	Exon 11	2083	T*/C	T*/C	T*/C	C	C (100%)		
SNPs	Promoter	−95	G/T	G/T	G/T	G	G (58%)	T (42%)	15
	Promoter	−35	G	G	G	G	A (18%)	G (82%)	9
	Exon 1	11	G/A	G/A	G/A	G/A	G (68%)	A (32%)	4
	Exon 2	102	C	C	C/G	C	C (76%)	G (24%)	12
	Exon 7	859	G	G	G	G	G (76%)	T (24%)	9
	Exon 8	1207	T	T	G/T	T	G (74%)	T (26%)	7
	Exon 8	1283	C	C	G/C	C	G (26%)	C (74%)	7
	Exon 10	1818	G	G	G	G	G (87%)	A (13%)	9
	Exon 11	2088	C/T	C/T	T	C	C (42%)	T (58%)	9
	Exon 12	2235	C/T	C/T	T	C	C (47%)	T (53%)	9
	Exon 12	2263	A/C	A/C	C	A	A (50%)	C (50%)	9
	Exon 15	2630	C	C	C	C	T (32%)	C (68%)	12
	Exon 17	2973	C	C	C	C	G (47%)	C(53%)	9

The nucleotide sequence of TPO is according to Kimura *et al.* (4). Allelic frequencies of the TPO gene are based on direct sequencing of genomic DNA from 19 normal subjects. Asterisks indicate mutations.

digested with *Bgl* II and *Mlu* I, and inserted into the same restriction enzyme sites of hTPO-1/pUC9. Introduction of the G614A mutation was confirmed by sequencing. Preparation of mRNA and its transfection into CHO-K1 cells have been described (11, 15).

Other methods of functional analysis used to characterize mutated TPOs

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), western blot analysis, guaiacol oxidation assay, peroxidase activity staining, indirect immunofluorescence study, immunoelectron microscopy and flow cytometry were performed as previously described (10, 11, 15).

Results

Two missense mutations in the patients' TPO gene

All exons and the promoter region in the TPO gene were directly sequenced with genomic DNA prepared from peripheral blood white blood cells. As shown in Table 1, both patients showed identical single nucleotide polymorphisms and two mutations, indicating that the patients had the same pair of alleles. The two mutations were G614A (R175Q) and C2083T (R665W). The former was a novel mutation, whereas the latter was previously reported (15). As G614A was found in their mother's DNA as heterozygous and C2083T was detected in DNA from their father as heterozygous, the two mutations were compound heterozygous.

To examine the importance of R175 for human TPO protein, the neighboring amino acids were compared in the peroxidase superfamily (Fig. 1). R175 was well conserved in the peroxidase superfamily. Further analysis of this mutation with hydrophilicity/hydrophobicity plot by Hopp and Woods' algorithm (19) showed a mild change in this region: R175Q changed a weakly hydrophobic region to a more hydrophobic one

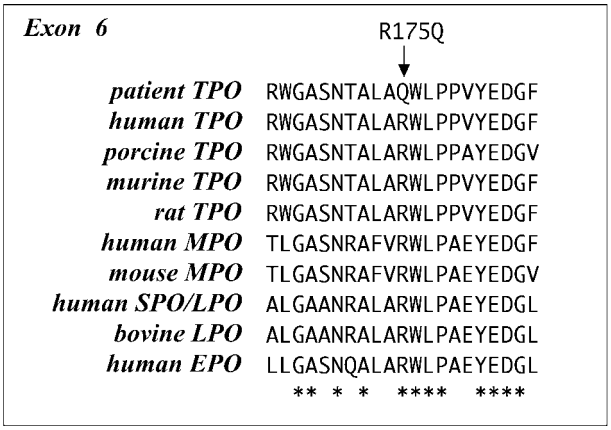

Fig. 1 Comparison of the amino acid sequence among various peroxidases neighboring the mutation. Arrow indicates the position of amino acid substitution R175Q. Asterisks indicate completely conserved amino acids. The amino acid sequences are based on the GenBank/EMBL/DDBJ DNA data base: human TPO, Kimura *et al.* (J02969); porcine TPO, Magnusson *et al.* (X04645); mouse TPO, Kotani *et al.* (X60703); rat TPO, Derwahl *et al.* (X17396); human MPO, Morishita *et al.* (J02694); mouse MPO, Venturelli *et al.* (X15313); human SPO/LPO, Kise *et al.* (U39573) and Dull *et al.* (M58151); bovine LPO, Dull *et al.* (M58150); human EPO, Ten *et al.* (X14346).

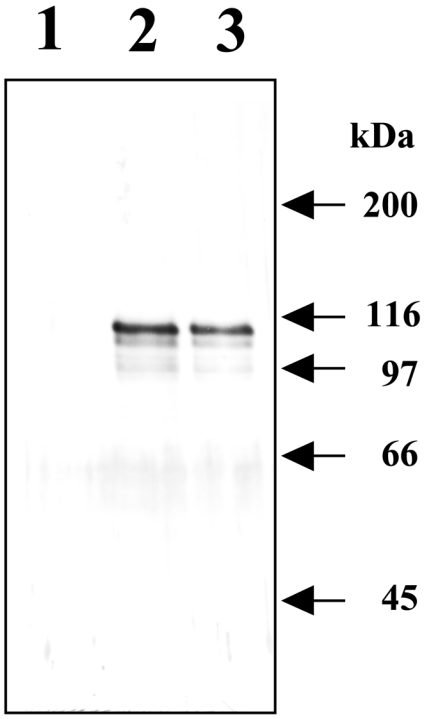

Fig. 2 Western blot analysis of CHO-K1 cells transfected with 30 μg of mRNA. Lane 1, without mRNA (negative control); lane 2, wild-type TPO-1 mRNA; lane 3, G614A-mRNA.

(data not shown).

Functional analyses of R175Q-TPO

To analyse the function of mutated TPO, mRNA containing G614A was transfected into CHO-K1 cells. Western blot analysis was performed to determine mutated TPO protein expression by using whole-cell lysates prepared from cells transfected with G614A-mRNA. As shown in Fig. 2, G614A-mRNA expressed a 107-kDa TPO protein as well as wild-type mRNA. To examine the amount of TPO protein expressed after mRNA transfection, CHO-K1 cells were stained with polyclonal anti-TPO antibodies at 24 and 34 h after mRNA transfection. At 24 and 34 h after transfection TPO-positive cells in the G614A-mRNA transfection were almost the same in percentage terms as in wild-type mRNA (Table 2).

As the next investigation, CHO-K1 cells transfected with mRNA were fractionated into membrane and supernatant fractions to examine the membrane-bound and enzyme activity of mutated TPO protein. R175Q-TPO protein was detected in the membrane fraction, as was wild-type TPO-1 protein, by western blot analysis (data not shown). The guaiacol oxidation assay used to measure TPO activity showed 5.5 mU/mg protein in the membrane fraction of cells transfected with wild-type mRNA, but activity was not detectable in the membrane and supernatant fractions of cells transfected with G614A-mRNA (data not shown). As the lower limit for the guaiacol oxidation assay is 2 mU/mg protein, it was suspected that the

Table 2 TPO-positive cells after mRNA transfection

mRNA	TPO-positive cells (%)	
	24 h	34 h
(−)	0.0	NT*
Wild-type	78.0	70.2
G614A	73.8	69.8

CHO-K1 cells were transfected with mRNA by electropolation. After 24- and 34-h culture, they were subjected to anti-TPO antibody staining. About 200 cells stained with antibody were counted. *, not tested.

Table 3 Indirect immunofluorescence study

mRNA	Non-permeabilized	Permeabilized
(−)	0.8%	1.6%
Wild-type	61.9%	68.5%
G614A	5.2%	53.6%

CHO-K1 cells were transfected with 30 μg of mRNA. After 24-h culture, they were subjected to indirect immunofluorescence study. About 200 cells were observed.

amount of protein expressed in G614A-mRNA transfection was insufficient for TPO activity to be detected by this assay. Activity staining with 3,3'-diaminobenzidine (DAB) as a substrate was carried out. CHO-K1 cells transfected with G614A-mRNA showed weak oxidating activity (data not shown).

To examine the localization of mutated TPO expression, immunofluorescence studies were carried out. In the nonpermeabilized condition, cells transfected with wild-type mRNA showed fluorescence on the plasma membrane at a rate of 61.9%, whereas G614A-mRNA-transfected cells exhibited fluorescence at a rate of only 5.2%. Permeabilized cells transfected with these types of mRNA showed fluorescence at the respective rates of 68.5 and 53.6% (Table 3). Confocal laser-scanning microscopic images are shown in Fig. 3. Some nonpermeabilized cells transfected with G614A-mRNA showed faint fluorescence on their plasma membrane (Fig. 3b'). To confirm faint plasma membrane surface localization for R175Q-TPO, immunoelectron microscopy was performed. In wild-type mRNA transfection, dense deposits were observed on the plasma membrane, endoplasmic reticulum (ER) and nuclear envelope (Fig. 4a). Cells transfected with G614A-mRNA exhibited small plasma membrane deposits in addition to dense deposits on the ER and nuclear envelope (Fig. 4b). These studies on TPO localization revealed that a very small amount of R175Q-TPO was located on the plasma membrane surface.

Cotransfection experiment

In a previous study, the C2083T (R665W) missense mutation resulted in a localization defect for R665W-TPO such that it completely lost the ability for cell surface expression (15). But, R175Q-TPO encoded by the novel missense mutation was able to translocate onto the cell surface, although the amount was very small. Therefore, we attempted to compare the flow cytometric profiles of the two mutations by performing a cotransfection experiment, to estimate the apical surface expression of TPO on the thyrocytes of the patients. As shown in Fig. 5, CHO-K1 cells transfected with 30 μg or 60 μg of C2083T-mRNA showed 5.9% and 8.9% in cell surface TPO positivity, respectively (e, d), although mock transfection exhibited only 2.1% (a). But with 30 μg or 60 μg of G614A-mRNA, cell surface TPO-positive cells increased 9.8% and 17.6% (g, h). Nevertheless, this increase in TPO positivity was far less than with wild-type mRNA-transfcted cells (c, b). As a further finding, the increase in TPO positive cells with G614A-mRNA transfection was due to the low intensity of TPO-bearing cells when compared with cells transfected with wild-type mRNA (g, h, c, b). In the cotransfection of 30 μg of C2083T-mRNA and 30 μg of G614A-mRNA, cell

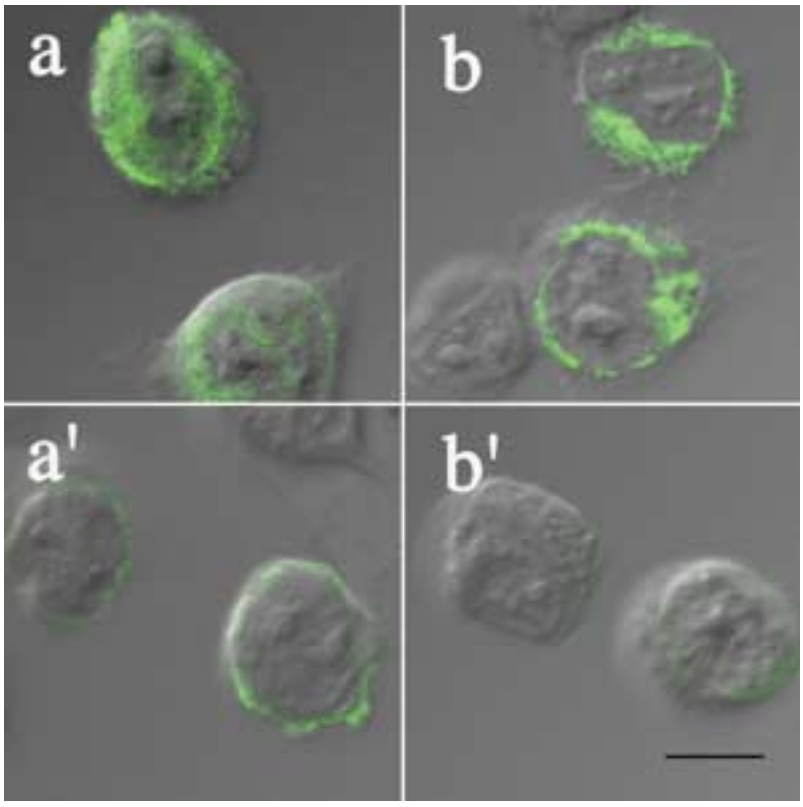

Fig. 3 Immunofluorescence studies by confocal laser-scanning microscope. Permeabilized CHO-K1 cells expressing wild-type TPO-1 protein show fluorescence on the plasma membrane and in intracellular structures like the nuclear envelope and ER (a), and non-permeabilized cells expressing wild-type TPO-1 exhibit fluorescence on the plasma membrane (a'). CHO-K1 cells transfected with G614A-mRNA show the same pattern as those transfected with wild-type mRNA (b). Some non-permeabilized CHO-K1 cells transfected with G614A-mRNA exhibit faint fluorescence on the plasma membrane (b'). Overlays of Nomarski and fluorescence images. Bar: 10 μm.

surface TPO positive cells were only 13.1% (f). This percentage was far less than with 60 μg wild-type mRNA-transfected cells (b).

Discussion

The present patients were suspected of having congenital thyroid dyshormonogenesis, because of severe hypothyroidism and goiter. Thyroid dyshormonogenesis should be differentiated by tests such as iodide uptake of the thyroid gland, perchlorate discharge test, and TRH test. But, these tests were not done, because the patients' parents did not agree to the use of radioisotopes because of the children's young age. As congenital goitrous hypothyroidism is thought to be due to some extent to TPO deficiency (6), the TPO genes of the patients were screened. Their TPO genes

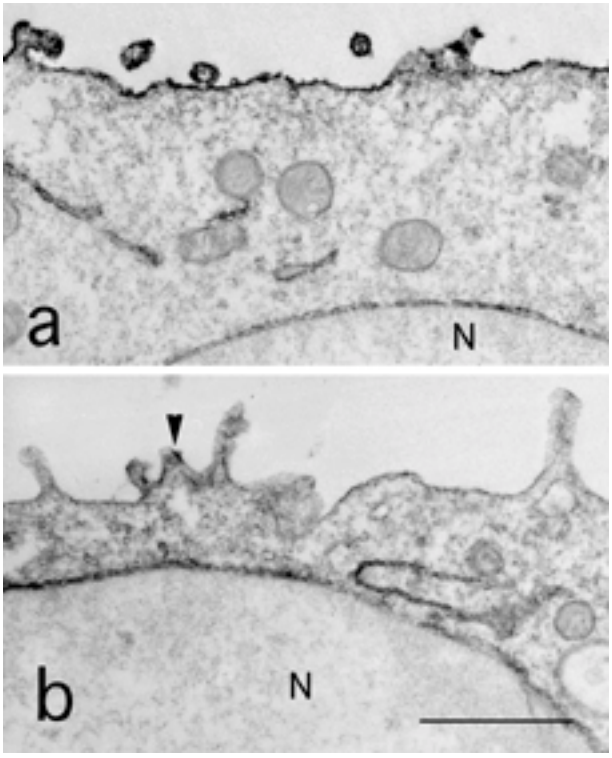

Fig. 4 Immunoelectron micrographs of CHO-K1 cells expressing mutated TPO. Wild-type TPO-1 is located on the plasma membrane, ER, and nuclear envelope (a). A very small amount of R175Q-TPO localizes on the plasma membrane (arrowhead) in addition to localization on the ER and nuclear envelope (b). N, nucleus; bar, 1 μm.

were found to contain two mutations, G614A (exon 6) and C2083T (exon 11). The former nucleotide substitution did not correspond to any mutations or polymorphisms reported to date, whereas the latter substitution was the same as that reported previously (15). Sequencing of the parents' DNA showed that G614A was derived from the mother and C2083T from the father.

G614A and C2083T resulted in R175Q and R665W, respectively. A previous study showed that R665 is well conserved not only in other species' TPOs but also in myeloperoxidase (MPO) and salivary peroxidase (SPO) / lactoperoxidase (LPO). R665W-TPO protein could still have weak TPO activity. The mutated TPO expressed in CHO-K1 cells was located on the ER and perinuclear membrane but was not on the plasma membrane (15).

In this study R175 was also well conserved in the peroxidase superfamily, suggesting that this amino acid is important for TPO structure or function. CHO-K1 cells transfected with G614A-mRNA expressed TPO protein with the same molecular weight as that of wild-type TPO, and the rate of G614A-mRNA expression was almost the same as that of wild-type mRNA. The R175Q-TPO protein was thought to possess the nature of a type-I membrane enzyme of wild-type TPO and to have TPO enzyme activity. In the localization studies on R175Q-TPO, a very small amount of mutated TPO was located on the plasma membrane of CHO-K1 cells, in addition to large amounts of TPO on the ER and nuclear envelope. Because the iodination of tyrosine residues and coupling of iodotyrosines on thyroglobulin are catalysed by TPO mainly on the apical membrane surface of thyrocytes (20), R175Q-TPO could not perform sufficient synthesis of thyroid hormone in the thyroid.

A cotransfection experiment was performed to estimate apical surface TPO expression on the thyrocytes of the patients. Experiments with 30 or 60 μg of each mRNA, in which 30 μg was the initial saturating amount determined in the preliminary experiment for wild-type mRNA, G614A-mRNA increased cell surface TPO-positive cells in a dose-dependent fashion, although TPO intensity on the cell surface was low. In contrast, C2083T-mRNA did not increase cell surface TPO-positive cells in spite of the higher amount of mRNA. Mixed mRNAs (30 μg of G614A-mRNA and 30 μg of C2083T-mRNA) showed an intermediate value in TPO positivity between 60 μg of G614A-mRNA and 60 μg of C2083T-mRNA. In terms of cell surface TPO positivity, the percentage of mixed mRNA was about a quarter of that exhibited by 60 μg of wild-type mRNA, but considering TPO intensity on the cell surface, the expression of mixed mRNAs was far less than that of wild-type mRNA. A very small

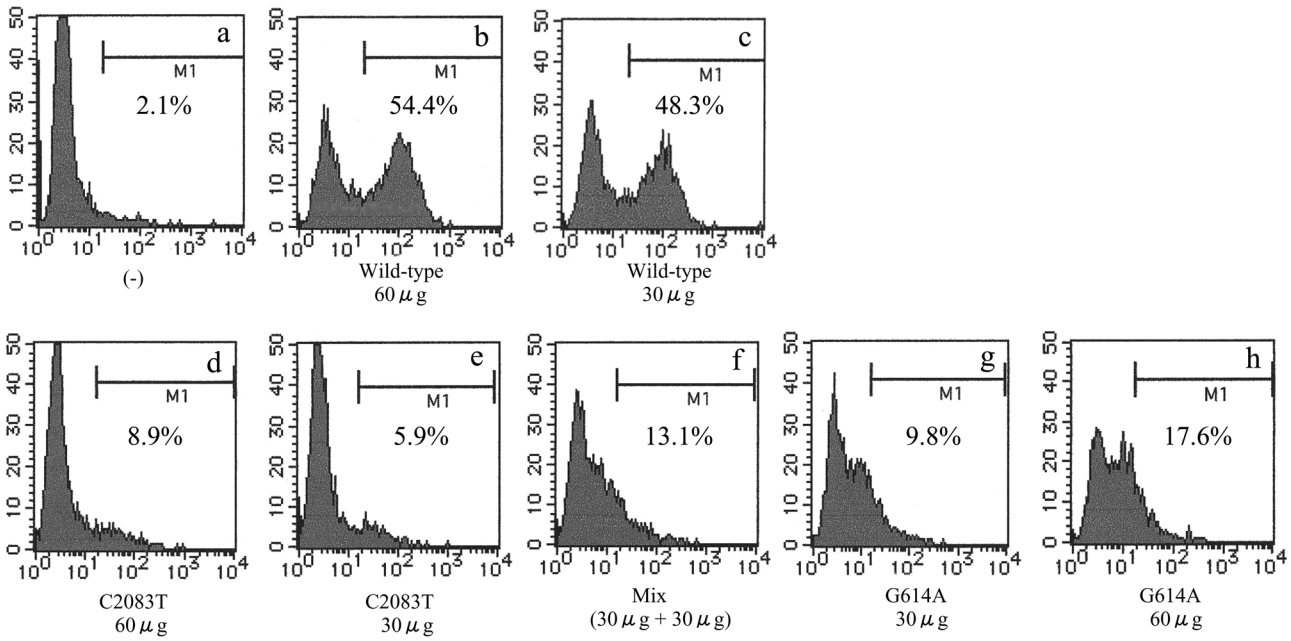

Fig. 5 Flow cytometric analysis of cotransfection experiment. Thirty micrograms, which is the initial saturating amount in cell surface TPO expression for wild-type mRNA, or 60 μg of mRNA was transfected into CHO-K1 cells. After 24 h culture, cells detached from the plastic surface with mild trypsinization were subjected to flow cytometry to measure TPO cell surface expression. a, mock transfection; b, 60 μg of wild-type mRNA; c, 30 μg of wild-type mRNA; d, 60 μg of C2083T-mRNA; e, 30 μg of C2083T-mRNA; f, 30 μg of C2083T-mRNA plus 30 μg of G614A-mRNA; g, 30 μg of G614A-mRNA; h, 60 μg of G614A-mRNA. Vertical and transverse axes in each figure express the cell count and fluorescence intensity, respectively.

amount of TPO protein could be detected on the apical membrane surface of the patients' thyrocytes, although it was insufficient to carry out the synthesis of thyroid hormone.

The translocation onto the plasma membrane of two mutated TPO proteins, R175Q- and R665W-TPOs, is thought to be obstructed owing to their protein misfolding. Nevertheless, a remarkable difference was found between the two mutated proteins. R665W-TPO could not translocate at all onto the plasma membrane from the ER, whereas a small amount of R175Q-TPO was able to migrate onto the plasma membrane. Why did this difference occur in respect to cell surface expression? The position of amino acid substitution is different in R175Q- and R665W-TPOs, and these changes may alter the general structure of TPO protein in addition to the regional structure. The extent of such general conformational change may be reflected in the absence of TPO protein translocation. Although the calnexin cycle and following retrograde translocation mechanism for misfolding protein have been clarified to some extent (21), mechanisms by which to recognize misfolding have not been established.

The present two siblings with goiter and hypothyroidism had a compound heterozygous mutation, G614A/ C2083T, of the TPO gene. From the expression studies, one of the two mutated TPOs, R175Q-TPO, could be expressed on the plasma membrane, although the amount was most inadequate for thyroid hormone synthesis to be performed. Because of the patients' young age, we

could not conduct the perchlorate discharge test. If the test were done, its value would show total iodide organification defect in both siblings. The patients were finally diagnosed as having congenital hypothyroidism caused by TPO gene mutations.

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