CLINICAL STUDY

Demonstration of reduced *in vivo* surface expression of activating mutant thyrotrophin receptors in thyroid sections

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Abstract

Objective: Thyroid function and growth are controlled by TSH. Hyperthyroidism can be due to Graves' Disease (GD), in which thyroid-stimulating antibodies mimic TSH, or gain-of-function mutations in the TSH receptor (TSHR). These activating mutations have poor surface expression when assessed in non-thyroidal cells *in vitro* but nothing is known of their *in vivo* behaviour. Several TSHR antibodies have been produced but none has been applied to thyroid paraffin sections. This study aimed to develop a technique suitable for use on paraffin sections and apply it to investigate TSHR expression in thyroids harbouring activating TSHR germline mutations compared with normal and GD thyroids. **Design and methods:** Immunocytochemistry coupled with antigen retrieval, using a spectrum of antibodies to the TSHR, was applied to paraffin sections of GD thyroid tissue. Subsequently, TSHR immunoreactivity was examined in three normal thyroids, three patients with GD and three patients with familial hyperthyroidism, due to different gain-of-function TSHR germline mutations, using the optimised protocol.

Results: Two antibodies, A10 and T3-495, to the extracellular domain (ECD) and membrane spanning region (MSR) of the TSHR respectively, produced specific basolateral staining of thyroid follicular cells. In normal and GD thyroids, basolateral staining with T3-495 was generally more intense than with A10, suggesting a possible surfeit of MSR over ECD. Graves' Disease thyroids have more abundant TSHR than normal glands. In contrast, thyroids harbouring gain-of-function mutations have the lowest expression *in vivo*, mirroring *in vitro* findings.

Conclusions: The development of an immunocytochemical method applicable to paraffin sections has demonstrated that different molecular mechanisms causing hyperthyroidism result in the lowest (mutation) and highest (autoimmunity) levels of receptor at the thyrocyte surface.

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Introduction

The growth and function of the thyroid gland are controlled by thyrotrophin (TSH), acting via its G protein coupled receptor (TSHR), to increase intracellular levels of cAMP (1). The TSHR shares homology with the receptors for the other glycoprotein hormones, luteinising hormone (LH) and follicle-stimulating hormone (FSH). All three are characterised by a large extracellular domain (ECD), responsible for high affinity ligand binding, and a seven membrane spanning region (MSR) which connects with the signal transduction machinery of the cell (2).

The low physiological levels of TSHR expression have hampered *ex vivo* examination of its structure. Several investigators have studied TSHR expression in various thyroid pathologies, mainly using RT-PCR and other transcript-based methods (3–7) but very little information is available evaluating receptor protein levels (8) due to the lack of suitable probes.

Since the cloning of the TSHR, several different approaches have been applied to the generation of polyclonal and monoclonal antibodies (mabs), including immunisation with synthetic peptides, receptor fragments or ECD (9–11) produced in bacteria or insect cells and genetic immunisation (12). A range of valuable reagents for use in Western blot, flow cytometry and immunoprecipitation has been produced. Some of the mabs have been used to visualise the receptor in intact thyroid glands by immunocytochemistry. The majority of reports have studied cryostat sections of snap-frozen thyroid tissue (10, 13) and not formalin fixed paraffin sections. As a consequence, the wealth of information in archival material has not been available.

There is some evidence that the TSHR may exist as two subunits at the cell surface. Analysis of thyroid membranes suggests that the MSR is more abundant than the ECD, particularly in the presence of TSH, leading to the proposal that shedding of the ECD may be a

mechanism of receptor activation (10, 14). Whether this mechanism is in operation in pathological conditions, in which cAMP levels are elevated without the physiological interaction of TSH with the TSHR resulting in hyperthyroidism, has not been investigated.

Depending on the iodide intake of a given population, the most common cause of hyperthyroidism is Graves' Disease (GD), in which thyroid-stimulating antibodies (TSab) mimic the action of TSH (15). More recently, a second thyrotoxic mechanism has been defined at the molecular level. Point mutations in the TSHR, resulting in a single amino acid change, were shown to produce constitutive activity in the receptor, i.e. permanent activation of the adenylate cyclase (which produces cAMP) even in the absence of TSH. This was first described as somatic mutation occurring in single toxic adenomas (16) but has since also been found in some toxic multinodular goitre lesions (17). Germline mutations in the TSHR have also been identified in familial non-auto-immune hyperthyroidism, in which it displays autosomal dominant inheritance, and in cases of sporadic neonatal thyrotoxicosis, which may become familial in time (18, 19). The age of onset of symptoms can vary widely, even in the same family (20).

Currently, in excess of 25 different gain-of-function mutations, chiefly located in the MSR of the receptor implicated in signal transduction, have been described. Both the plethora of mutations (21) and the high prevalence of GD (0.2% in men, 2% in women) attests to the extreme lability of the TSHR, a feature which is not shared by the other glycoprotein hormone receptors (22).

A common feature of activating mutant forms of TSHR is their reduced surface expression compared with the wild type, when analysed by flow cytometry of transfected non-thyroidal cells (23, 24). Whether this reflects the situation *in vivo* has not been possible to test in the absence of relevant frozen thyroid tissue or an immunocytochemical method suitable for fixed tissue.

In this study we report the development of an immunocytochemical method for the TSHR suitable for use on archival paraffin-embedded sections, using a microwave heat antigen retrieval technique, and its application to the investigation of TSHR expression in hyperthyroidism, as a consequence of GD and activating TSHR mutations, compared with normal thyroid glands.

Methods

Case and tissue material

Tissue samples Formalin-fixed, paraffin-embedded pathological specimens, from nine patients comprising three with GD, three having a normal thyroid lobe and three with an activating TSHR germline mutation were retrieved from the pathology archives of the

University Hospital of Wales, Cardiff, the Universities of Leipzig and Freiburg in Germany. The three patients with a normal thyroid histopathology were operated for a completion total thyroidectomy having previously undergone a lobectomy for a follicular carcinoma. The initial histological sections stained with haematoxylin and eosin were reviewed to confirm normal morphology in these three patients and the relevant pathology in the others. Details of the histopathology are listed in Table 1.

Patient characteristics Details of the patient's age at surgery, sex, thyroid status at diagnosis, immediate pre-operative thyroid function tests, TSab assay, previous relevant anti-thyroid therapy, immediate pre-operative preparation, diagnosis and histopathology are listed in Table 1. The characteristics of the TSHR germline mutant forms studied are summarised in Table 2.

Antibodies used in the study

A range of TSHR polyclonal and monoclonal antibodies were evaluated; their details are given in Table 3.

Immunocytochemistry

In pilot experiments, as a screening procedure the monoclonal antibodies were used at a 1:20 dilution and the polyclonal antibodies at a 1:500 dilution on archival formalin-fixed paraffin-embedded GD sections. A minor modification of an antigen retrieval method (25) developed and refined in the Department of Pathology was utilised as standard procedure for all the experiments. This consisted of heating the sections in a microwave at full power for 25 min, in a 1 mmol/l EDTA solution at pH 8.0.

The antibodies selected for further study were then titrated in doubling dilutions. Optimal concentration of primary antibody was determined at a dilution where only specific staining of follicular cells was visible and the background stroma and connective tissue was negative.

Antibody specificity was also established, using the optimal concentrations of the two antibodies selected for further study, on a range of paraffin-embedded tissue controls after both antigen retrieval and endogenous biotin blockade (see below). These included sections from the testis, appendix, liver, fallopian tube, skin, prostate, gall bladder, uterus and lymph nodes.

Immunohistochemical analysis Utilising the optimised immunocytochemical protocol, TSHR expression in the three subsets of patients was studied by staining with the two monoclonal antibodies selected, i.e. A10 and T3-495. A10 was used at a 1:320 and T3-495 at a 1:400 dilution. Diluent without any antibody was utilised as negative control. The test was repeated

Table 1 Clinical characteristics of the patients studied. Abbreviations used: T₄, serum free thyroxine; TSH, serum TSH; TSab, TSHR-stimulating antibody assay; Tg, thyroglobulin; TPO, thyroperoxidase; CTT, completion total thyroidectomy; STT, subtotal thyroidectomy; NTT, near-total thyroidectomy; NAH, non-autoimmune hyperthyroidism.

No.	Age at surgery	Sex	Thyroid status at diagnosis	Pre-op. T ₄ (normal: 8–26 pmol/l)	Pre-op. TSH (normal: <5 mU/l)	TSab assay (normal: < 10.0%)	Anti-Tg/ TPO Abs	Pre-op. treatment	Surgery/indication for surgery	Hemotoxylin and eosin
1	20	F	Euthyroid	16	1.7	N/A	N/A	Nil	CTT/Follicular Ca	Normal thyroid tissue
2	70	F	Euthyroid	19	1.4	N/A	N/A	Nil	CTT/Follicular Ca	Normal thyroid tissue
3	35	M	Euthyroid	14	1.8	N/A	N/A	Nil	CTT/Follicular Ca	Normal thyroid tissue
4	69	F	Hyperthyroid	59.2	0.02	82.6%	Anti-TPO positive	Carbimazole and thyroxine	STT/GD	Combined thyroiditis and Graves' (hashitoxicosis), oncocytic change and nodularity of thyroid. GD and thyroiditis
5	44	F	Hyperthyroid	10.2	0.10	88.3%	Anti-TPO positive	Carbimazole and thyroxine	STT/GD	Consistent with Graves', mild to moderate atypia, chronic inflammatory cells. GD
6	28	F	Hyperthyroid	14.9	0.34	66.6%	Both positive	Carbimazole and thyroxine	STT/GD	Consistent with Graves', prominent lymphoid aggregates. GD
7	11	M	Hyperthyroid	14.3	2.7	Negative	Negative	Methimazole and thyroxine	NTT/NAH (mutation L629F)	Mild to moderate degree of nodular hyperplasia with variable-sized colloid-filled follicular profile
8	2	M	Hyperthyroid	76.8	<0.04	Negative	Negative	PTU/Lugol's iodine	NTT/NAH (mutation S505N)	Mild to moderate degree of nodular hyperplasia with variable-sized colloid-filled follicular profile
9	26	F	Hyperthyroid	17.0	0.04	3.8%	Positive	Carbimazole	STT/NAH (mutation M463V)	Mild atypia of follicular epithelium consistent with carbimazole therapy

Table 2 Characterisation of mutants compared with the wild-type receptor. Thyroid glands harbouring three different activating germline mutations (leucine 629 replaced by phenylalanine; serine 505 replaced by asparagine; methionine 463 replaced by valine) have been investigated. All three mutants have been compared previously with the wild type (WT) receptor, for basal cAMP levels and cell surface expression, by transient transfection studies in non-thyroidal (mostly COS) cells. Flow cytometry (% of WT) refers to the surface expression of the mutant receptor, taking the WT as 100% and using either the BA8 or 2C11 TSHR monoclonal as defined. cAMP (% of basal) increase refers to the basal cAMP produced by transient transfection with the mutant receptor compared with the WT, which is 100%. The cAMP levels have either been measured directly in a RIA or indirectly using a cAMP-responsive luciferase reporter as described in the relevant references.

Mutant	Flow Cytometry (% of WT)	Antibody used in flow cytometry	cAMP (% of basal)		Reference
			cAMP assay	Luminescent assay	
L629F	63	2C11	~600	~170	24
S505N	79	2C11	~600		30
M463V	50	BA8		~200	29

in duplicate and results reported are the mean of the two readings. Two of the authors (MS and BJ), who were blinded to the diagnosis, independently scored each section and results from both assessors were pooled to arrive at the final score. Any discrepancies between the two authors were resolved by a joint assessment of the sections.

The streptavidin–biotin complex immunoperoxidase procedure was, in brief, as follows: the sections were de-paraffinised, then incubated in methanol (98.4%) and hydrogen peroxide (1.6%) to block endogenous peroxidase activity and then subjected to antigen retrieval. Antigen retrieval was performed by microwaving at full power for 25 min in 1 mmol/l EDTA solution. Endogenous biotin activity was blocked using a commercially prepared avidin/biotin blocking kit (Vector Labs, Peterborough, UK) by incubating the slides for 15 min each in two or three drops per slide of Avidin followed by Biotin. The slides were thoroughly washed in PBS between each step and prior to application of the primary antibody. The sections were then incubated overnight at 4°C with the primary antibody. Multi-Link biotinylated anti-IgG (BioGenex, San Ramon, CA, USA) and streptavidin peroxidase label (BioGenex) were utilised at a 1:40 dilution, each for 30 min at room temperature. PBS was utilised to wash sections between each step of the procedure. The chromogenic substrate utilised was diaminobenzidine tetrahydrochloride (DAB) made up in PBS with two drops of hydrogen peroxide. The sections were counterstained with Harris haematoxylin.

Sections were analysed as to the homogeneity and distribution of staining, pattern of cytoplasmic and

cell membrane staining, intensity of staining and staining of plasma and blood elements. Intensity of staining was scored based on the power of the lens needed to clearly demarcate stained (brown) cells from unstained (blue) background as follows: $\times 4$ – strong staining (+++), $\times 10$ – moderate staining (++), $\times 25$ – weak staining (+), $\times 40$ – virtually absent ($\pm$), and no staining whatsoever at any power – absent (–).

Results

Epitope retrieval permits immunostaining of the TSHR on thyroid paraffin sections

Figure 1A shows the basolateral staining obtained in a normal thyroid gland following immunocytochemistry using A10 and T3-495 in the optimised protocol. Overall, the intensity of staining seen with A10 was stronger than that observed with T3-495, although the latter produced more abundant membrane-associated staining. This staining was specific to the thyroid and none of the other tissues studied, including testis, appendix, liver, fallopian tube, skin, prostate, gall bladder, uterus and lymph nodes, demonstrated any significant staining with either antibody (data not shown).

TSHR expression levels vary in hyperthyroid glands having different pathogenesis

The nine sections from normal thyroids and hyperthyroid glands from GD and activating germline TSHR mutations were analysed in parallel. Wide variations

Table 3 Antibodies used in this study and their characteristics.

Antibody	How produced	Subclass	Epitope	Reference
BA8	Genetic immunization	IgG2a	Conformational	12
3G4	Genetic immunization	IgG2a	ECD 354-359	12
A10	ECD from insect cells	IgG2b	ECD 22-35	11
T3-495	MSR 604-764 in bacteria	IgG1	MSR 604-764	10
59N	Peptide antibody in rabbits	N/A	ECD 326-345	9
60N	Peptide antibody in rabbits	N/A	ECD 370-389	9

Table 4 Immunocytochemical staining of normal, Graves' and mutant receptor thyroids with monoclonal antibodies A10 and T3-495. Distribution: scored as a percentage of the entire section showing the staining. C – Cytoplasmic, M – membranous. Intensity of staining: the results were scored based on the power of the lens needed to clearly demarcate stained (brown) cells from unstained (blue) background as follows: $\times 4$ – strong staining (+++), $\times 10$ – moderate staining (++) , $\times 25$ – weak staining (+), $\times 40$ – virtually absent ($\pm$) and no staining whatsoever at any power – absent (–). Pattern: D – diffuse, MF – multifocal, F – focal, VF – very focal, PZ – peripheral (subcapsular) zonation. PMN – peripheral white blood elements.

No.	A10							T3-495						
	Distribution (%)		Intensity		Pattern		Remarks	Distribution		Intensity		Pattern		Remarks
	C	M	C	M	C	M		C	M	C	M	C	M	
Normal														
1	80–90	10–25	+/++	+	D	MF	Plasma +++. Patchy membrane expression.	0	30–40	–	+/++	–	MF	Plasma–. Entire basolateral.
2	90	50	++	++	D	MF	Plasma +++. Patchy membrane expression.	10–20	80	–	+/++	–	D	Plasma–. Entire basolateral.
3	90	5–10	±/+	±/+	D	F	Plasma +. Patchy membrane expression.	20–30	30–40	±/+	±/+	MF	MF	Plasma–. Patchy membrane expression.
Graves'														
4	>95	5–10	+/++	+	D	VF/PZ	Plasma +++. Speckled cytoplasm.	>90	5–10	+ / ±	+/++	D	VF/PZ	Plasma and PMN–. Speckled cytoplasm.
5	>95	50	++	+/++	D	MF	Plasma ++. Speckled cytoplasm.	90	25–50	± to ++	+++	D	PZ	Plasma and PMN–. Speckled cytoplasm.
6	50–75	25–40	+	± to ++	D	MF	PMN–. Fine granular cytoplasm.	15–20	25–40	± to +	+/++	D	F/PZ	Plasma and PMN–. Fine granular cytoplasm.
Mutant receptor														
7	90	5–10	±/+	–	D	VF	Plasma ++	80–90	5–10	+	±/+	D	VF	
8	90	<1	±/+	±	D	VF/PZ	PMNs –	0	0	–	–	–	–	
9	40–50	0	+/++	–	MF	–	Plasma ±	0	0	–	–	–	–

in the staining intensity and patterns of staining were observed. On the whole, the most intense staining was present in the GD thyroids (moderate to occasional strong staining) and least intense on the three thyroids harbouring germline TSHR mutations (very weak to weak staining) with normal thyroids weaker than patients with GD.

In normal thyroids, staining with A10 was more cytoplasmic than membranous which, if present, was patchy and discontinuous. The intensity of staining ranged from weak to moderate. Consistent, moderate to strong plasma and occasional colloid staining was noted in this and all other thyroid tissues studied with this antibody. Staining of peripheral white blood elements was difficult to comment on because of adherence of plasma to these cells and the absence of these elements in some of the other thyroid pathologies.

Staining of normal thyroids with T3-495 was predominantly associated with the membrane in two of the three patients and covered the entire basolateral surface. In contrast, in case 3, even though the membrane-associated staining was more abundant than the cytoplasmic, it was discontinuous and not over the entire basolateral surface as in cases 1 and 2. No plasma or colloid-associated staining was observed in this or any other type of tissues studied when applying this antibody.

In the Graves' group as a whole, the intensity of membrane-associated staining noted was moderate to strong although case 4, who displayed the weakest staining, was still hyperthyroid at the time of surgery. When compared with normal thyroid tissue, more cytoplasmic than membranous staining was evident with both A10 and T3-495, with over 90% of the section displaying cytoplasmic staining with T3-495, in two of three cases. In common with normal thyroid tissue, membrane-associated staining was more intense with T3-495 than with A10.

In the thyroids harbouring mutant TSHR, the picture was dissimilar to the above two scenarios. Most staining, if present, was in the cytoplasmic compartment, where it could reach the intensity obtained in normal thyroids. In contrast, weak membranous staining was demonstrated only in the thyroid of patient 7 and

was confined to the hyperplastic areas within the section. There was a complete absence of staining for T3-495 in patients 8 and 9, the former being hyperthyroid at the time of surgery and the only patient treated pre-operatively with Lugol's iodine. Overall, the intensity of staining ranged from absent to weak.

Representative sections stained for A10 and T3-495 of normal thyroid, Graves' thyroid and all three mutant thyroids are shown in Fig. 1A–J. The results are summarised in Table 4.

Discussion

Our results indicate that it is possible to assess TSHR protein expression in paraffin-embedded sections of formalin-fixed thyroid tissue, using an antigen retrieval protocol which has previously been successfully applied to study oestrogen and progesterone receptors and a wide variety of other antigens in archival tissue (26).

Not all antibodies evaluated were susceptible to the method but the two selected, A10 (11) and T3-495 (10) produced basolateral staining of most of the thyroid specimens examined, consistent with the known location of the TSHR. Of interest, one of the T3 family of antibodies was used in an earlier study of receptor expression in paraffin sections of thymus, adrenal and kidney, without recourse to an antigen retrieval method (27). Unfortunately, the staining obtained on paraffin sections of thyroid tissue was not shown or discussed so it is difficult to evaluate the work in the context of the present study in which specific receptor staining was restricted to the thyroid gland. Previously we have applied A10 to cryostat sections of Graves' thyroid (28), and found that the intensity of membrane staining in the present study is comparable to that obtained in the earlier work. In contrast, cytoplasmic staining with A10 is more abundant on paraffin than on cryostat tissue sections. We also examined frozen orbital fat tissues and demonstrated marked A10 immunoreactivity in samples from patients with thyroid eye disease but only faint staining in normal orbital fat samples (28). Parallel investigation of cryostat sections of abdominal fat showed even less staining (not

Figure 1 (Opposite) Representative sections of normal (patient 3), Graves' (patient 5) and all three mutant thyroids (patients 7–9) stained with A10 and T3-495 (magnification $\times 100$). (A) Normal thyroid stained with A10 showing membranous (see the magnified image in inset 1) and diffuse cytoplasmic (see the magnified image in inset 2) staining. (B) Normal thyroid stained with T3-495 showing patchy and almost exclusively membrane staining (see the magnified image in the inset). The intensity of staining is equal to that seen in (A), in which the membrane staining is largely masked by the cytoplasmic staining. (C) Graves' thyroid stained with A10 showing more intense but discontinuous membrane and weaker cytoplasmic staining (see the magnified image in the inset) than in (A) or (B). L = lumen. (D) Graves' thyroid stained with T3-495 showing strong continuous membrane staining (see the magnified image in the inset) which is more complete and stronger than that seen in (C). (E) Mutant L629F showing weak diffuse cytoplasmic staining (see the magnified image in the inset) with A10. (F) Mutant L629F showing weak cytoplasmic staining (see the magnified image in the inset) and focal hyperplastic regions with minor amounts of membrane staining with T3-495. (G) Mutant S505N showing diffuse weak cytoplasmic staining (see the magnified image in the inset) with A10. (H) Mutant S505N showing apparent absence of any cytoplasmic or membranous staining (see the magnified image in the inset) with T3-495. (I) Mutant M463V showing moderate cytoplasmic staining (see the magnified image in the inset) with A10. (J) Mutant M463V showing apparent absence of any cytoplasmic or membranous staining (see the magnified image in the inset) with T3-495.

reported) and in the current study, fat tissue present in the non-thyroidal specimens examined were negative. This suggests that the antigen retrieval protocol is less sensitive than immunostaining of cryostat sections but it provides a means of comparing TSHR expression in archive material from varying thyroid pathologies, including hyperthyroidism as the result of gain-of-function TSHR mutation.

It is possible that antibodies raised against the wild-type receptor will fail to recognise mutated TSHR. A number of studies have applied a variety of TSHR antibodies in flow cytometry analysis of surface expression of various mutant TSHR, including the mutants examined in the present work (23, 24, 29, 30). The antibodies used have been predominantly BA8 and 3G4/2C11, the former recognises conformation and the latter two a linear epitope in the ECD of the TSHR. To our knowledge, to date only one mutant, Ile167Asn, resulting in loss of TSHR function (31), has failed to be detected in flow cytometry using BA8, whose conformational epitopes might be more susceptible to disruption by mutation than those recognising linear epitopes, such as the antibodies amenable to use on paraffin sections, A10 and T3-495. The obvious exception is T3-495 applied to sample 7, which harbours a L629F mutation falling within the epitope recognised by this antibody. As can be seen from the results, sample 7 was the only 'mutant thyroid' in which some staining, albeit weak, was obtained with this antibody. The staining was predominantly cytoplasmic, suggesting that the receptor is indeed detected by the antibody but fails to reach the cell surface.

In the normal and Graves' thyroids, membrane staining was more intense using the T3-495 antibody which detects the MSR, compared with A10 which binds the amino terminus of the ECD. In contrast, staining of the plasma was observed with A10 but not T3-495. Whether the apparent excess of MSR is further support for the shedding of the ECD from the thyrocyte surface, or merely reflects the relative affinities of the antibodies used, remains open to debate.

Immunoreactivity in the Graves' thyroids is modified both by the autoimmune process and treatment for hyperthyroidism, yet we found the highest receptor protein levels in these glands. This is in agreement with a previous study comparing TSHR expression in cryostat sections of different thyroid pathologies, in which GD demonstrated the most intensive staining (13) and is supported by ongoing quantitative real-time PCR analysis of TSHR transcript expression (manuscript in preparation). It also confirms the preference for thyroid follicular cells obtained from GD patients in early assays developed to measure TSab (32). The increased receptor expression in GD is probably not due to treatment, since patients with a germline receptor mutation had undergone similar regimens and their cell surface TSHR expression is dramatically reduced. So is the increase the consequence or part of the cause of the

autoimmune response to the TSHR? This would be difficult to resolve, for practical and ethical reasons, but may be possible by quantifying TSHR transcripts in fine needle aspirates of first-degree relatives of GD patients.

Perhaps the most interesting aspect of the study is the finding that surface expression of receptors with a gain-of-function mutation is greatly reduced *in vivo*, even when compared with normal thyroids. *In vitro* comparison of mutant and wild-type TSHR surface expression in non-thyroidal cells has shown that this is a feature of the majority of the activating mutants studied. Indeed it had been suggested that the true biological activity of a particular mutant form should take into account its low level of surface expression (23). Our demonstration of little or no basolateral membrane staining, with either A10 or T3-495, implies that the physiological intracellular trafficking of these activating mutations is impaired, as proposed for other mutated thyroid-specific proteins assessed in non-thyroidal cells (33).

In conclusion, the development and application of an immunocytochemical method for the TSHR has revealed wide variations in the level of receptor protein expression in normal and hyperthyroid glands. Different molecular mechanisms to generate the hyperthyroid state result in the lowest (mutation) and highest (autoimmunity) levels of receptor at the thyrocyte surface, the pathophysiological consequences of which merit further investigation.

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