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WESTERN BLOT ANALYSIS OF THYROTROPIN RECEPTOR EXPRESSION IN HUMAN THYROID TUMOURS AND CORRELATION WITH TSH-BINDING

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Expression of thyrotropin receptor (TSHr) was analyzed in normal human thyroids and differentiated thyroid tumours by Northern blotting of total RNA and by Western blotting of detergent-solubilized membrane proteins with an antibody specific for the extracellular domain of TSHr. Under nonreducing conditions TSHr stained as a 90-kDa protein in normal thyroid tissue. Expression varied in differentiated carcinomas from normal to not detectable in parallel to steady state TSHr mRNA levels and TSH binding in a TSH binding assay. The negative expression of TSHr in some patients with differentiated carcinomas may have consequences for the TSH suppressive treatments of these patients.

Thyrotropin (TSH) is the main regulator for thyrocyte differentiation and growth (1) and exerts its effects by binding to the extracellular domain of the TSH receptor (TSHr) leading to activation of cAMP-dependent pathways and in humans of the PIP_2 cascade (2). The recent cloning of the TSHr revealed that the protein belongs to the family of G-protein-coupled receptors with seven transmembrane domains and a deduced molecular weight of the encoded mature protein of 84 kDa (3-6). The availability of TSHr gene probes has allowed the examination of TSHr mRNA expression in human thyroid tissues. It was recently found that steady state levels of TSHr mRNA were variably decreased in thyroid tumours and

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Abbreviations: TSH=Thyrotropin; TSHr=Thyrotropin receptor; nt=normal thyroid; ptc=papillary carcinoma; ftc=follicular carcinoma; go=goitre.

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inversely related to the degree of dedifferentiation (7-9). When compared to mRNA levels of other thyroid differentiation markers the loss of TSHr mRNA expression occurred late in the process of dedifferentiation. This is in agreement with *in vitro* obtained data showing the robustness of the expression of this receptor (10). These studies are also in agreement with earlier results of indirect measurements of TSH-receptor function like TSH-binding and adenylate cyclase responsiveness in thyroid tumours (11,12). However, it remained unclear if the TSHr mRNA data could be interpreted in terms of TSHr expression at the protein level. Using a recently generated polyclonal TSHr antibody (13) we compared in the present study the expression levels of the TSHr in human thyroid tumours by Western blotting with steady state TSHr mRNA expression and TSH-binding.

Materials and Methods

Materials Thyroid tissues were obtained from patients undergoing surgery either for thyroid carcinoma or euthyroid goitre. Histological diagnosis was performed by the local Department of Pathology. The tissue samples were immediately frozen in liquid nitrogen and stored at -80°C until use. The protocol was approved by the local Committes on Medical Ethics and all patients gave their written consent.

RNA analysis Isolation of total RNA from tissue fragments and Northern blot analysis with equal RNA amounts (8 μg/lane) was performed as previously described (7,14). TSHr-transcripts were detected by hybridization to a [^{32}P]-labeled human TSHr-probe obtained by random prime labeling of a 2.4 kbp Bam HI-Xho I insert of a pSVL-construct (3) as described (7).

Thyroid membrane preparation and TSHr solubilization 0.2 to 0.4 mg frozen tissue was homogenized in a micro dismembrator (Braun, Melsungen, Germany). The frozen material was transferred in a precooled glass/teflon Potter homogenizer and homogenized in a 1:5 weight:volume ratio in ice-cold buffer H (50 mM NaCl, 5 mM EDTA, 10 mM NaP_i, 20% glycerol pH 7.5 supplemented with protease inhibitors (benzamidine, pepstatin, antipain, 3 μg/ml each and 1 mM phenylmethylsulfonylfluoride)) with 10 strokes in a motor driven device. The suspension was centrifuged for 1 h at 120000 ×g at 4°C. Sedimented thyroid membranes were homogenized again in 2 ml buffer H and centrifuged as before. Extraction was performed by homogenizing the membranes with ice-cold 2% Triton X100 in buffer H in a 1:2 tissue fresh weight:volume ratio. Following 10 minutes incubation on ice the suspension was recentrifuged and the supernatant containing solubilized membrane proteins stored in aliquots at -80°C. Protein concentrations were determined using bovine serum albumin as reference protein (15).

TSH radio-receptor assay For determination of [^{125}I]-TSH binding solubilized thyroid membranes (40 μg protein in 50 μl extraction buffer) and 150 μl PBS containing bovine [^{125}I]-labeled TSH (30000 cpm; spec. activity 50 μCi/μg, Henning, Berlin, Germany) and 5% human serum from euthyroid patients were incubated for 2 h at room temperature. Then 0.5 ml 1% skimmed milk and 1 ml cold 1 M NaCl, 30% polyethylene glycol 6000 pH 7.4 were added. After centrifugation at 3000×g for 15 min the radioactivity of the pellet was counted. Incubations of all samples were performed simultaneously and with less than 1% difference between duplicates. Specific binding was obtained by subtraction of unspecific binding from total binding. Unspecific binding estimated either by addition of 70 mU bovine TSH (Sigma) or by omission of extracted protein was smaller than 4% of added radioactivity.

Affinity chromatography of TSHr 10 U of bovine TSH (Sigma) were coupled to N-hydroxy succinimide activated Sepharose (1 ml HiTrap column, Pharmacia) with 0.5 M NaCl 0.2 M $NaHCO_3$ pH 8.3 as coupling buffer. After extensive washings the column was equilibrated with 0.5% Triton X100 in buffer H. Solubilized thyroid membranes proteins (1.5 mg protein in the same buffer) were applied and unbound proteins removed by washing with 0.05% Triton X100 in buffer H. Bound proteins were eluted with 0.05% Triton X100 in 10 mM HCl in 0.3 ml fractions. After neutralization by addition of 5 μl 1.5 M Tris HCl pH 8.8 fractions were stored at -20°C. Aliquots were analyzed by Western blotting and [^{125}I]-TSH binding.

SDS-PAGE and Western blotting Solubilized membrane proteins were mixed with an equal volume of double concentrated sample buffer without reducing agent (16). After 10 minutes incubation on ice equal amounts of protein (10 μg) were loaded onto 8.5% SDS-polyacrylamide gels. For reduction samples were incubated with 5% 2-mercaptoethanol under the same conditions prior to electrophoresis. After separation proteins were electrophoretically transferred on PVDF-membranes (Immobilon P, Millipore) (17). Membranes were blocked for 1 h at room temperature with 5% skimmed milk in PBST (50 mM NaCl, 0.05% Tween-20, 10 mM NaP_i pH 7.4). The blots were incubated overnight at room temperature with anti-TSHr-antiserum diluted 1:2000 with PBST including 0.5% skimmed milk. The antiserum was raised in rabbits against a recombinant fusionprotein consisting of maltose binding protein and the extracellular domain of human TSHr produced in *E.coli* (13). The membranes were washed five times for 5 min each with PBST and then incubated for 1 hour with an alkaline phosphatase coupled donkey anti-rabbit antibody (Dianova, Hamburg, Germany) diluted with 0.5% skimmed milk in PBST. After washing immunoreactive bands were detected by chemoluminescence using AMPPD-substrate and Emerald-Enhancer (Oncogene Science, Uniondale, NY).

Results and Discussion

As shown in Tab. 1 we examined TSHr expression on steady state mRNA and protein level as well as binding of labeled TSH in 21 papillary carcinomas (ptc), 2 follicular carcinomas (ftc) and compared this to normal thyroid tissue (nt;n=3) obtained from patients operated for a parathyroid adenoma and to tissue from patients with diffuse and multinodular goitre (go).

In Western blots from normal thyroid tissues 2 main bands were detected running at positions of 150 kDa and 90 kDa under non-reducing conditions (Fig. 2, lane 1-3). Using reducing conditions immunostaining of the 90 kDa band disappeared and a diffuse band of 50-55 kDa was detected (Fig. 1, lane 2). This band was occasionally present also in minor amounts in some unreduced samples. Using human liver extracts as a control no 90 kDa band was detected indicating thyroid specific expression of the 90 kDa protein (data not shown). Specificity was further tested by subjecting the solubilized thyroid membrane proteins to affinity chromatography on TSH-Sepharose. Western blotting of eluted fractions showed a single band at 90 kDa (Fig. 1, lane 1) and only fractions containing the 90 kDa band revealed binding of labeled TSH in the binding assay (data not shown).

Table 1: Steady state levels of TSHr mRNA, TSHr protein levels and TSH-binding in human thyroid tissues

Patient	Age/Sex	Histology	TSHr mRNA	TSHr 90 kDa band	% TSH binding
1	57/F	nt	+++	+++	
2	76/F	nt	++	++	100 ± 30
3	27/F	nt	+++	+++	
4	55/M	ptc	0	0	0
5	47/F	ptc	0	0	0
6	79/F	ptc	+	+	0
7	82/F	ptc	0	0	0
8	66/F	ptc	0	0	0
9	74/F	ptc	+	0	0
10	20/M	ptc	+	++	135
11	26/F	ptc	+	++	110
12	51/F	ptc	+++	+++	135
13	31/F	ptc	++	++	115
14	72/F	ptc	++	++	65
15	65/F	ptc	+	+	65
16	27/F	ptc	+	+	35
17	77/F	ptc	+	+++	110
18	63/F	goitre	++	++	95
19	13/F	goitre	+	++	60
20	49/M	goitre	++	++	75
21	43/M	ftc	+	0	5
22	55/F	ftc	++	++	75
23	74/M	ptc	+++	++	80
24	70/F	ptc	+	+	35
25	76/F	ptc	+	+	65
26	50/F	ptc	+	+	85
27	57/F	ptc	0	+	55
28	42/M	ptc	+	+	35
29	74/F	ptc	+	+	45

Subjective classification of signals from Northern and Western blots: 0 no signal; + weak signal; ++ medium signal; +++ strong signal. %TSH binding is given relative to the mean of binding found in normal thyroids.

Several arguments indicate the identity of this band with the TSHr. First, the size of the TSHr amino acid backbone is deduced from molecular cloning to be 84 kDa and 6 potential N-glycosylation sites within the sequence could contribute to a higher molecular weight (3-6). Recent deglycosylation studies with TSHr preparations revealed the glycoprotein nature of the TSHr (18-20). The observed TSHr size of 90 kDa is in agreement with 90 kDa immunopurified TSHr from human thyroid membranes detected by Western blotting with monoclonal antibodies (18) and with a reported 93 kDa TSHr detected by Western blotting of human thyroid membranes with an antiidiotypic monoclonal TSHr antibody (22). The size difference to a reported 104 kDa protein of CHO cells transfected with human TSHr (21)

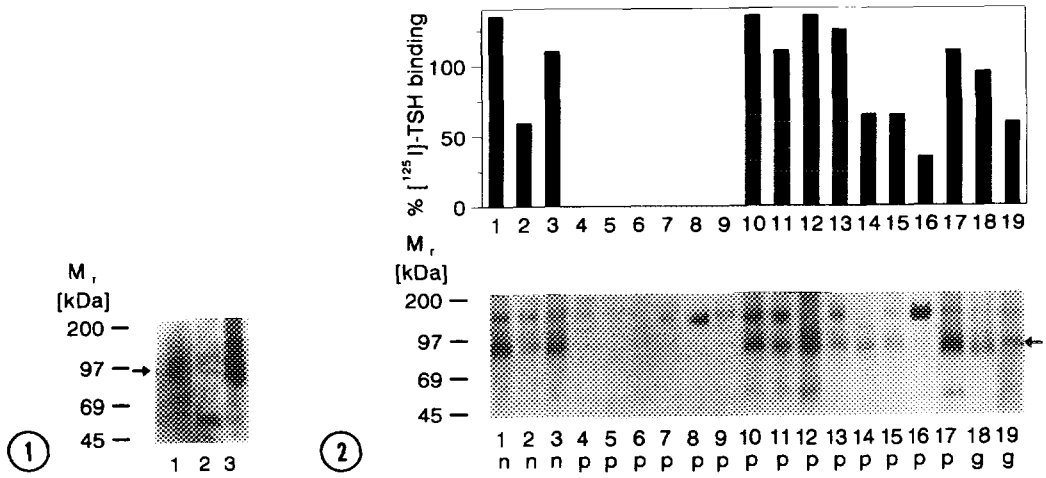

Fig. 1. Western blot analysis with an anti TSHR antibody directed to the extracellular domain using detergent-solubilized membrane proteins of normal human thyroid after SDS-PAGE under reducing conditions with 2-mercaptoethanol (lane 2) and under nonreducing conditions (lane 3). Lane 1 shows the immunostaining of a sample after SDS-PAGE under nonreducing conditions following affinity chromatography on TSH-Sepharose and elution with 10 mM HCl. Sizes of molecular weight marker proteins are given on the left, the arrow indicates the 90-kDa TSHr band.

Fig. 2. TSHr expression and TSH-binding in human thyroid tissues.
Upper panel: Relative binding of labeled TSH to 40 μg solubilized thyroid membrane proteins (see Table 1). Lower Panel: Western blot analysis of solubilized thyroid membrane proteins (10μg/lane) after SDS-PAGE under nonreducing conditions probed with the TSHr antibody. Sizes of molecular weight marker proteins are given on the left, the arrow indicates the 90-kDa TSHr band (tissues: n=nt; g=goitre; p=ptc).
Numbers in both panels correspond to patient numbers in Table 1.

could be due to different processing events in these cells. Under reducing conditions a 50-55 kDa band appears and the 90 kDa band disappears. This protein corresponds presumably to the TSH binding α-subunit of the TSHr and was also detected by others (13, 18). It remains however unclear whether the cleavage observed by us is due to a thiol activated proteolytic activity present in the extracts or due to cleavage of TSHr disulfide brigdes (18, 21). Second, TSH binding ability and the relative levels of TSHr mRNA closely correlate with the 90 kDa band in the Western analysis. Third, when membrane extracts where bound to TSH-Sepharose the eluted fractions revealed TSH binding and contained only the 90 kDa protein band.

In the differentiated thyroid carcinomas a comparable pattern was found in Western analysis. However, the intensity of the 90 kDa band varied considerably from normal to nearly absent immunoreactivity when compared to nt or go (Fig. 2). Binding experiments using radiolabeled bovine TSH and identical amounts of extracted proteins demonstrated

parallelity of TSH binding and expression of the 90 kDa band in all tissues (Tab. 1, Fig. 2). In contrast, the staining intensity of the 150 kDa band did not match the TSH binding with strong immunostaining and low TSH binding (Fig. 2, lanes 8, 16) or with low immunostaining but strong TSH binding (Fig. 2, lanes 12, 14) suggesting that the crossreactive 150 kDa protein band does not correspond to a functional TSHr. Analysis of steady state levels of TSHr mRNA compared well with the data of TSH binding and the intensity of the 90 kDa band in immunostaining. However, in 5 of the 24 tumours steady state TSHr mRNA levels appeared to be lower than the 90 kDa band (Tab. 1, Fig. 2). As RNA is particularily sensitive to preparation artefacts such discrepancies could be expected. The positive and more robust results obtained by Western blotting and hormone binding should therefore given more credit.

In summary, the present results show good parallelism between total TSHr steady state mRNA and protein expression as determined by Western blotting. Some of the differentiated tumours showed no detectable levels of TSHr by both analytical approaches and this was paralleled by undetectable binding in our simplified radioreceptor assay. The variability of TSHr expression obtained on the protein level in all other tumours confirms the results previously reported on TSHr mRNA expression (7-9). If the observed parallelism between TSHr protein expression in the Western analysis and TSH binding is substantiated in a larger group of patients the simplified binding assay presented here may serve as a means to identify patients with tumours lacking TSHr expression (and TSH binding) and thus avoid the potentially negative effects of subclinical hyperthyroidism induced by standard TSH suppressive therapy with thyroxine in these patients.

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