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Sequential Cleavage and Excision of a Segment of the Thyrotropin Receptor Ectodomain*

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The thyrotropin (TSH) receptor belongs to a subfamily of G protein-coupled receptors, which also includes luteinizing hormone and follicle-stimulating hormone receptors. The TSH receptor (TSHR) differs from the latter by the presence of an additional specific segment in the C-terminal part of its ectodomain. We show here that this insertion is excised in the majority of receptor molecules. Preparation of specific monoclonal antibodies to this region, microsequencing, enzyme-linked immunosorbent assay, and immunoblot studies have provided insight into the mechanisms of this excision. In the human thyroid gland, N termini of the transmembrane receptor β subunit were found to be phenylalanine 366 and leucines 370 and 378. In transfected L cells a variety of other more proximal N termini were found, probably corresponding to incomplete excisions. The most extreme N terminus was observed to lie at Ser-314. These observations suggest that after initial cleavage at Ser-314 the inserted fragment of TSHR is progressively clipped out by a series of cleavage reactions progressing up to amino acids 366–378. The impossibility of recovering the excised fragment from purified receptor, cell membranes, or culture medium supports this interpretation. The cleavage enzyme has previously been shown to be inhibited by BB-2116, an inhibitor of matrix metalloproteases. However, we show here that it is unaffected by tissue inhibitors of metalloproteases. The cleavage enzyme is very similar to TACE (tumor necrosis factor α-converting enzyme) in both these characteristics. However, incubation of the TSH receptor with the purified recombinant catalytic domain of TACE, cotransfection of cells with TACE and TSHR expression vectors, and the use of mutated Chinese hamster ovary cells in which TACE is inactive suggested that the TSHR cleavage enzyme is different from TACE. TACE and TSHR cleavage enzyme may thus possibly be related but different members of the adamalysin family of metzincin metalloproteases.

The thyrotropin receptor (TSHR)[1] plays a key role in thyroid growth and function (reviewed in Refs. 1 and 2). This receptor is the target of stimulating or blocking autoantibodies produced in patients with autoimmune diseases (reviewed in Refs. 3 and 4).

The TSHR was initially cloned by cross-hybridization with the luteinizing hormone receptor (5), or by polymerase chain reaction using degenerate primers (6–8). Expression of the cloned receptor in *Escherichia coli* allowed its use as an immunogen to prepare monoclonal antibodies. These were used for immunoblotting and immunoprecipitation experiments which showed that the TSH receptor in human thyroid membranes underwent a post-translational cleavage event yielding two subunits: a ~53-kDa α extracellular subunit and a ~38-kDa broad β membrane spanning subunit. The subunits are held together by disulfide bridges (9). This maturation is unique among G protein-coupled receptors.

In human thyroids, cleavage of the TSHR is almost complete. By contrast, in heterologous transfected cells monomeric uncleaved precursors may also be observed. They consist either of the mature ~120-kDa uncleaved receptor present on the cell surface or of a ~95-kDa mannose-rich precursor which can be identified by its sensitivity to specific endoglycosidases (10). The latter form accumulates in the endoplasmic reticulum.

The cleavage of the TSHR has been disputed by different authors (11–16). Indeed, due to the low concentration of the TSHR in thyroid tissue, nearly all studies have been performed in transfected cells where the monomeric precursors and specially the mannose-rich form have in many cases been mistaken for the mature receptor. Recently, however, a consensus has emerged and most authors now agree on the existence of a physiological cleavage of the TSH receptor (17, 18).

Very recently the group of Rapoport reported the existence of two cleavage sites in the TSHR extracellular region, suggesting the existence of a third polypeptide fragment ("C peptide" by homology with insulin) released during intramolecular cleavage of the receptor into two subunits (19–21).

In this work we show that no third fragment of the TSHR is produced during its maturation, but rather that cleavage occurs initially at a first site, followed by the processive digestion and excision of a whole region of the receptor ectodomain. The region which is deleted is located in an additional segment specific to the TSHR which shows no homology with the gonadotropin receptors (5). In addition, besides the similarities that we had previously observed between pro-TNFα (pro-tumor necrosis factor α) and TSHR convertases (22), we provide here new infor-

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[1] The abbreviations used are: TSH, thyrotropin; TSHR, TSH receptor; Ecto-Ab, Endo-Ab, and Del-Ab, antibodies recognizing, respectively, receptor ectodomain, endodomain, and the putative deleted fragment; MMP, matrix metalloprotease; TIMP, tissue inhibitor of metalloproteases; TNF, tumor necrosis factor; TACE, TNFα converting enzyme; TGF, transforming growth factor; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; CHO, Chinese hamster ovary; HA, hemagglutinin.

mation concerning the protease involved in the maturation of the TSHR and show that it may correspond to a novel enzyme.

EXPERIMENTAL PROCEDURES

Materials—Phorbol 12-myristate 13-acetate was purchased from Sigma. Anti-hemagglutinin (HA) monoclonal (12CA5) antibody and the "Complete" protease inhibitor mixture tablets were obtained from Boehringer (Mannheim, Germany). Human recombinant tissue inhibitor of metalloproteases 1 (TIMP-1) was purchased from Valbiotech (Paris, France). Human recombinant tissue inhibitor of metalloproteases 2 (TIMP-2) was a gift from Dr Agnès Noël (University of Liège, Belgium).

The wild-type and mutant Chinese hamster ovary (CHO) cell lines defective in the shedding of several membrane proteins (23, 24) were kindly provided by Dr. Joaquin Arribas (Val d'Hebron General Hospital, Barcelona, Spain). The recombinant catalytic domain of tumor necrosis factor α enzyme (TACE) and the murine TACE expression vectors (25) were gifts from Dr. Roy Black (Immunex Corp., Seattle, WA). BB-2116 (26) was a gift from British Biotech Co. (Oxford, United Kingdom). The synthetic peptides used for the localization of Del-Ab (antibodies recognizing the putative deleted fragment of TSHR ectodomain) epitope(s) and the study of the action of TACE were purchased from the microchemistry laboratory of Institut Gustave Roussy (Villejuif, France).

Anti-TSHR Monoclonal Antibodies—The preparation of Ecto-Ab (T5–317, an antibody recognizing the TSHR ectodomain) and Endo-Ab (T3–365, an antibody recognizing the TSHR endodomain) has been previously described (9).

For the preparation of Del-Ab, a cDNA fragment encoding amino acids 19–389 of the human TSHR was introduced into the polylinker of the vector pNMHUB. A fusion protein of TSHR with ubiquitin and polyhistidine was produced in *E. coli*. The protein was purified from inclusion bodies using chelate chromatography on nickel-agarose in denaturing conditions. Immunization of BALB/c mice, preparation and screening of hybridomas, production of ascites, and purification of antibodies were performed as described previously (9).

L Cell Line—The L cell line stably expressing the human TSH receptor has been previously described (10).

Immunopurification and Immunoblotting—The TSH receptor was immunopurified and Western blotting performed as described (10), except that the secondary antibody used during the Western blots was a mouse anti-Ig coupled to horseradish peroxidase. Revelation was performed with the ECL detection reagent (Amersham Corp., Buckinghamshire, United Kingdom). Ecto-Ab, Endo-Ab, or Del-Ab were used for immunomatrix preparation and Western blot detection. For the immunopurification of the shed α subunit from the cell culture medium, the medium was first concentrated approximately 15-fold using a Minitan apparatus (Millipore, Bedford, MA) equipped with a filter having a 1,000 Da molecular mass cut-off.

Immunological Assays—Enzyme-linked immunosorbent assays (ELISA) were performed as described previously (9). The concentration of the TSH receptor molecules was measured by reference to known concentrations of TSHR fragments expressed in *E. coli* (hTSHR 19–389 when Ecto-Ab or Del-Ab were used as primary antibodies, hTSHR 640–764 when Endo-Ab was used as primary antibody). The primary antibodies were used at saturating concentrations (5 μg/ml for Ecto-Ab or Endo-Ab, 1 μg/ml for Del-Ab) in order to bypass differences in antibody affinities. It was also verified that the secondary polyclonal antibody had the same affinity for the three primary antibodies. The immunoradiometric assay of the TSH receptor and of its α subunit have been previously described (22). For the assay of the α subunit, the cell culture medium of wild-type and mutant CHO cells transfected with TSHR cDNA was concentrated 13-fold using Centriprep-10 concentrators (Amicon, Beverly, MA).

Preparation of TSHR Enriched in Monomeric Receptor Species and Incubation with TACE—L cells expressing the human TSH receptor were incubated for 2 h at 4 °C with 5 ml/g of phosphate-buffered saline, 100 mM dithiothreitol, and protease inhibitors. This procedure provokes the reduction of disulfide bonds and the release of α subunits (22). The cells were washed three times with phosphate-buffered saline. TSHR was then extracted as described (10) and immunopurified on an immunomatrix containing Ecto-Ab. This procedure leads to the purification of uncleaved monomeric TSHR since α subunits have previously been released. The recombinant catalytic domain of TACE (10,000 units) was preincubated for 30 min at 37 °C in the presence or absence of the BB-2116 inhibitor. The TSH receptor enriched in monomeric forms was then added and incubation was continued for 2 h at 37 °C. The samples were then subjected to electrophoresis and Western blots were performed using various monoclonal antibodies.

Co-transfection of Expression Vectors Encoding TACE and TSHR—COS-7 cells ($\sim 10^6$ cells per Petri dish) were seeded 24 h before transfection. TACE expression vector (5 μg) was then transfected with 5 μg of either human TSHR expression vector or herring sperm DNA (Promega, Madison, WI). The Superfect reagent (Qiagen Inc., Hilden, Germany) was used according to the manufacturer's protocol.

N-terminal Microsequencing of the TSH Receptor β Subunits—The human TSH receptor was immunopurified with either Endo-Ab or Del-Ab. Endo-Ab immunopurification and microsequencing were performed twice using two different euthyroid goiters (yielding ~1 pmol of TSHR/g of tissue) or four times with different pools of stably transfected L cells (yielding ~50 pmol of TSHR/g of cells). Del-Ab was used to enrich receptor preparation in incompletely processed β subunits present in lower amounts in transfected L cells. A microsequencing experiment was performed on this material.

After immunopurification, the TSH receptor (~200–500 pmol) was electrophoresed and transferred onto a polyvinylidene difluoride membrane (ProBlott, Applied Biosystems, Foster City, CA). Proteins were colored by Coomassie Blue. Bands of interest were localized by reference to immunoblots, separately cut and sequenced following Edman's method using a multi-sample chemical microsequencer (Procise 494–610A, Applied Biosystems). The sequences observed were at least 12 amino acids long and the yield of the different fragments varied from 0.5 to 8 pmol. In repeated experiments identical results were obtained.

Culture and Transfection of the Wild-type and Mutant CHO Cell Lines—These cell lines were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 600 μg/ml geneticin (all reagents were from Life Technologies, Inc., Grand Island, NY). The DEAE-dextran method was used to transfect the PSG5-TSHR expression vector.

Processing of Pro-TGFα in Wild-type and Mutant CHO Cells—Exponentially growing wild-type and mutant CHO cells ($\sim 2 \times 10^6$ cells) expressing HA-tagged pro-TGFα (pro-transforming growth factor α) were incubated for 30 min at 37 °C with labeling medium (cysteine-free modified Eagle's medium, 20 mM Hepes pH 7.5, 1 mg/ml bovine serum albumin (BSA)). [^{35}S]Cysteine (500 μCi/ml) (NEN Life Science Products, Boston, MA) was then added to the labeling medium and cells were further incubated for 1 h at 37 °C. The cells were then washed twice in Dulbecco's modified Eagle's medium without $NaHCO_3$, 20 mM Hepes pH 7.5, 2 mg/ml BSA and incubated for 45 min in chase buffer (Dulbecco's modified Eagle's medium without $NaHCO_3$, 20 mM Hepes pH 7.5, 10 mg/ml BSA) with or without 1 μM phorbol 12-myristate 13-acetate. Protease inhibitors were added to the collected cell culture medium. The cells were washed twice with cold chase buffer and once with cold phosphate-buffered saline. The cells were then gently scraped and harvested in lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 0.2% BSA, and protease inhibitors). The cells were lysed for 30 min at 4 °C on a rotating wheel. The lysed cells were centrifugated for 30 min at 100,000 $\times$ *g* at 4 °C and the supernatant collected. Anti-HA monoclonal antibody was added to the cell culture media and the cell lysates for 1 h. The immune complexes were incubated with protein A-Sepharose (Pharmacia Biotech, Uppsala, Sweden) for 1 h at 4 °C, washed twice with lysis buffer, twice with 20 mM Tris pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 5 mM EDTA, 0.1% sodium dodecyl sulfate, 0.2% BSA, twice with 20 mM Tris pH 7.5, 500 mM NaCl, 0.5% Triton X-100, 0.2% BSA and twice with 50 mM Tris pH 7.5. The samples were then boiled for 10 min in Laemmli buffer and analyzed on a 14% polyacrylamide gel for the lysate samples and on a 16% polyacrylamide gel for the medium samples.

RESULTS

A Fragment Is Excised from the TSHR Ectodomain

Monoclonal Antibodies Recognizing the Excised TSHR Fragment: Detection of Extended Heterogeneous TSHR β Subunit Precursors—A TSH receptor fragment (amino acids 19–389) was expressed in *E. coli* as a fusion protein with ubiquitin. Immunization of mice with this antigen allowed the preparation of several monoclonal antibodies. The study of one group of these monoclonal antibodies (called here Del-Ab) gave puzzling results. When used for Western blot analysis of TSHR from human thyroids they reacted neither with the α nor with the β subunits but recognized a group of proteins (~39–44 kDa) larger than the most abundant β subunit (~38 kDa) (Fig. 1*A*).

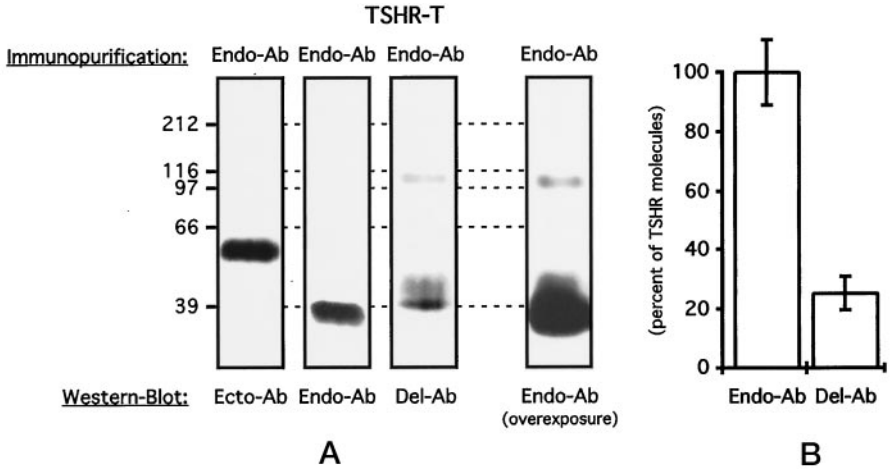

FIG. 1. **Some anti-TSHR monoclonal antibodies recognize an epitope excised from the majority of receptor molecules: studies with receptor purified from human thyroid glands.** *A,* Western blot. TSH receptor was immunopurified from human thyroid glands (*TSHR-T*) using an antibody which recognizes the receptor endodomain. Western blots were performed using antibodies which recognize the receptor ectodomain (Ecto-Ab), endodomain (Endo-Ab), or the putative deleted fragment (Del-Ab). Overexposure of the Endo-Ab immunoblot showed that the receptor species recognized by Del-Ab represented a minor fraction of those recognized by Endo-Ab. Molecular size standards (in kilodaltons) are indicated on the *left. B,* ELISA. The purified receptor was absorbed onto ELISA plates and incubated with antibodies recognizing either the receptor endodomain (Endo-Ab) or the putative deleted epitope (Del-Ab). Results are expressed as the mean ± S.E. (n = 3).

The same proteins were recognized by anti-endodomain antibodies (Endo-Ab), as seen after overexposure of the corresponding immunoblot (Fig. 1*A*). These proteins were thus extended β subunits. The Del-Ab gave no reaction with the α subunit nor with any protein larger than this subunit. Traces of the mannose-rich precursor of TSHR present in human thyroid glands and observed in overexposed immunoblots (10) were also detected by these antibodies. The same results were obtained when five different thyroid samples were studied.

These results are compatible with the possibility that the epitope recognized by Del-Ab antibodies was cleaved-off during maturation of the TSHR in human thyroids. However, this epitope remained covalently attached to a minority of β subunits ("extended" β subunits). There were no extended α subunits carrying the epitope.

We undertook the localization of the epitope recognized by this group of antibodies. Using bacterially expressed proteins encoding various segments of the TSHR ectodomain (amino acids 19–389, 19–246, and 246–389) and chemically synthesized peptides corresponding to residues 332–369, 332–356, and 357–369 we localized the epitope to amino acids 357–369. We also observed that the antibodies we had prepared, and which gave the pattern described in Fig. 1, were all non-additive and thus probably recognized the same epitope or very closely positioned epitopes (data not shown).

Western blot experiments thus suggested that only a minority of human thyroid TSHR molecules had conserved the epitope recognized by Del-Ab. We used a quantitative method to measure this population of receptors more precisely. ELISA tests were performed with purified human thyroid TSHR, using an antibody which recognizes either the receptor endodomain (Endo-Ab) or the segment which was cleaved-off (Del-Ab). As shown in Fig. 1*B*, about 75% of receptor molecules were devoid of this segment.

Since human thyroid tissue is difficult to obtain, and since many studies of the TSH receptor are performed using transfected cells, we repeated the immunoblot studies using TSHR purified from L cells stably expressing the receptor. Previous studies (10) have shown that in transfected cells there is accumulation of a mannose-rich intracellular precursor (~95 kDa) and incomplete receptor cleavage with persistence of various amounts of uncleaved full-length mature receptor (~120 kDa). Furthermore, in transfected cells the β subunit has been shown

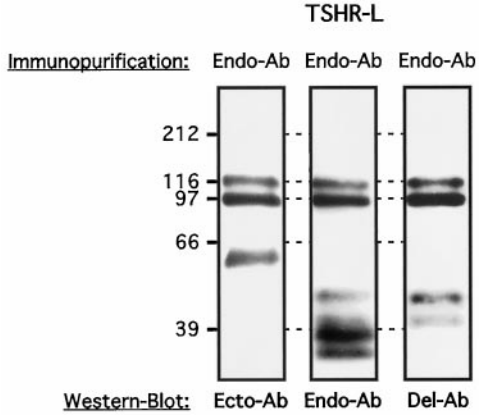

FIG. 2. **Some anti-TSHR monoclonal antibodies recognize an epitope excised from the majority of receptor molecules: studies with TSHR purified from transfected L cells.** The TSH receptor was purified from stably transfected L cells (*TSHR-L*) using an antibody which recognizes the receptor endodomain. Western blots were performed using antibodies directed against the receptor ectodomain (Ecto-Ab), endodomain (Endo-Ab), or the putative deleted fragment (Del-Ab). Molecular size standards (in kilodaltons) are indicated on the *left*.

to be heterogeneous (10). In the receptor prepared from transfected L cells, Del-Ab antibody reacted as expected with the 2 uncleaved forms of the receptor (Fig. 2). It also reacted with the largest forms of the β subunit (extended β subunits). Again no reaction was seen with the α subunit and there was no indication of larger forms of the α subunit carrying the Del-Ab epitope.

Cleaved-off TSHR Fragment Cannot be Recovered from the Cell Culture Medium or from the Cell Membrane and Does Not Remain Associated with the Receptor—The preceding experiments raised the question of the fate of the excised receptor fragment. Theoretically, it could have remained bound to the receptor by non-covalent interactions (constituting a third subunit of the receptor), or it could have remained attached to the cell membrane, or it could have been released from the cells.

We examined the latter possibility by searching for the fragment in the cell culture medium of L cells expressing the TSH receptor. As shown in Fig. 3 no antigen able to bind Del-Ab could be detected in the cell culture medium, whereas the shed α subunit (27) was detected by anti-ectodomain antibodies.

We then prepared a human thyroid membrane Triton X-100 extract and subjected it to chromatography on a Del-Ab immunomatrix. Immunoblots (Fig. 4*A*) with Del-Ab only showed the extended forms of β subunit previously described (see Fig. 1*A*). There was no evidence of a fragment of ~6.5 kDa which could correspond to the excised fragment (not shown). This experiment thus suggested that the receptor fragment which was cleaved-off did not remain attached to the membrane either in a free form or bound to the receptor.

Another line of evidence also showed that the excised fragment did not constitute a third subunit of the receptor. Indeed, if this had been the case immunopurification using Del-Ab would have yielded all 3 fragments of the receptor. This was not the case: no normal (non-extended) β subunit of ~38 kDa was purified. Only the larger β subunits of ~39–44 kDa, to which the epitope for Del-Ab remains covalently attached due to incomplete cleavage, were purified. The existence of these extended β subunits to which α subunits remain bound would explain why some of the latter are also retained during chromatography on the Del-Ab immunomatrix.

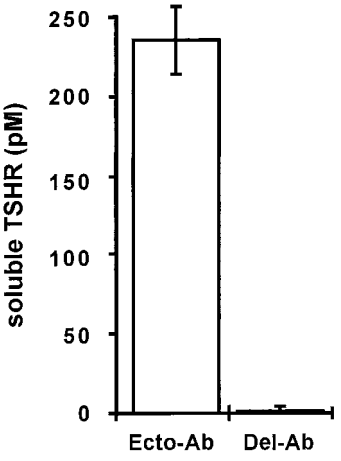

FIG. 3. **The TSHR fragment cleaved off the receptor ectodomain is not present in cell culture medium.** L cells stably expressing the TSHR were cultured for 24 h in Dulbecco's modified Eagle's medium supplemented with 1% fetal calf serum. The cell culture medium was concentrated approximately 15-fold and immunopurified using Ecto-Ab or Del-Ab as indicated. Quantification of purified TSHR molecules containing the Ecto-Ab or Del-Ab epitopes was performed by ELISA using the corresponding monoclonal antibody. Results are expressed as the mean ± S.E. (n = 3).

Furthermore, when the human thyroid membrane extract was chromatographed on two successive immunomatrices, first Del-Ab then Endo-Ab, the latter retained the majority of β subunits (of ~38 kDa) and the majority of α subunits (not shown). This experiment shows that there are two populations of receptors: a majority of α-β dimers devoid of the Del-Ab epitope and a minority of α-"extended β" dimers. The latter carry the Del-Ab epitope.

We also used the Del-Ab immunomatrix to purify a Triton X-100 extract from membranes of L cells expressing the TSHR (Fig. 4*B*). The results were similar to those obtained with human thyroid TSHR: absence of a third fragment and purification of only the extended, largest, forms of the β subunit.

Determination of Cleavage Sites in the Receptor Ectodomain by Microsequencing—The inability to recover the excised fragment from the receptor, membrane extracts, and cell culture medium suggested that it was either immediately destroyed or initially excised in small pieces. Microsequencing experiments suggested that the latter explanation was the most probable.

The TSHR was immunopurified from human thyroid glands using an anti-endodomain antibody. After electrophoresis and transfer to a polyvinylidene difluoride membrane the region corresponding to the β subunits was excised in successive slices and submitted to microsequencing (Fig. 5). Three N-terminal TSHR amino acid sequences were observed in approximately equimolar amounts, originating at amino acids phenylalanine 366, leucine 370, and leucine 378.

We have previously observed that the cleavage of TSHR is incomplete in transfected cells (10). Furthermore, we have shown here that immunochromatography with Del-Ab allows receptor preparations to be enriched with large incompletely cleaved β subunits (see Fig. 4). We thus immunopurified the receptor from TSHR-expressing L cells, using either Endo-Ab or Del-Ab. Microsequencing of β subunits from such preparations showed a variety of species extending upstream from those observed in the human thyroid, up to Ser-314 (Fig. 5). The most abundant species started at leucine 370 and threonine 388.

These observations suggested that the excised fragment of the receptor could not be isolated because it was not produced as a single polypeptide, but that multiple cleavages occurred which yielded fragments too small to be detected. Furthermore, the initial cleavage probably occurred in the N terminus of this

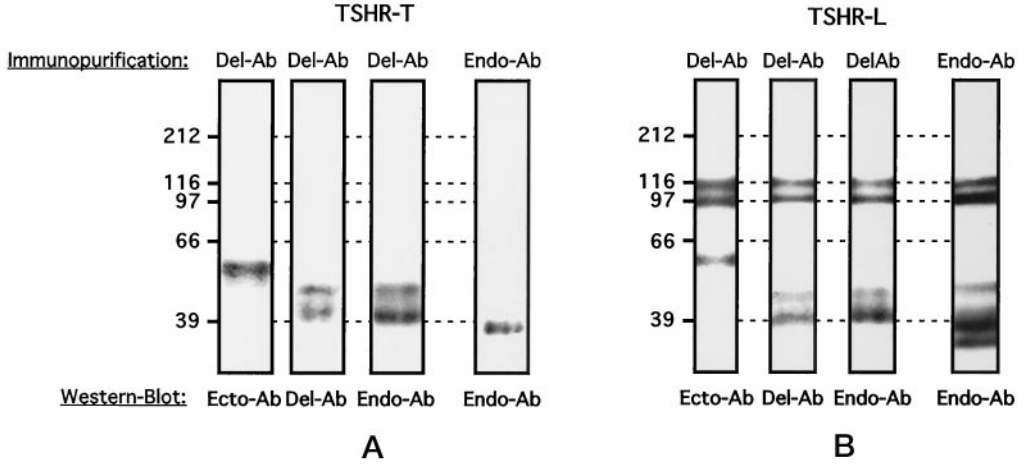

FIG. 4. **The TSHR fragment cleaved off the receptor ectodomain does not remain attached to the receptor.** Membrane fractions were prepared from human thyroid glands (*TSHR-T, panel A*) or from L cells stably expressing TSHR (*TSHR-L, panel B*). After treatment with Triton X-100, the extracts were subjected to chromatography on an immunomatrix containing Del-Ab. Western blots of the immunopurified receptor were performed using antibodies directed against the receptor ectodomain (Ecto-Ab), endodomain (Endo-Ab), or excised fragment (Del-Ab). On the *right* of each panel is shown an immunoblot (with Endo-Ab) of receptor purified on an immunomatrix containing Endo-Ab. Molecular size standards (in kilodaltons) are indicated on the *left* of each panel.

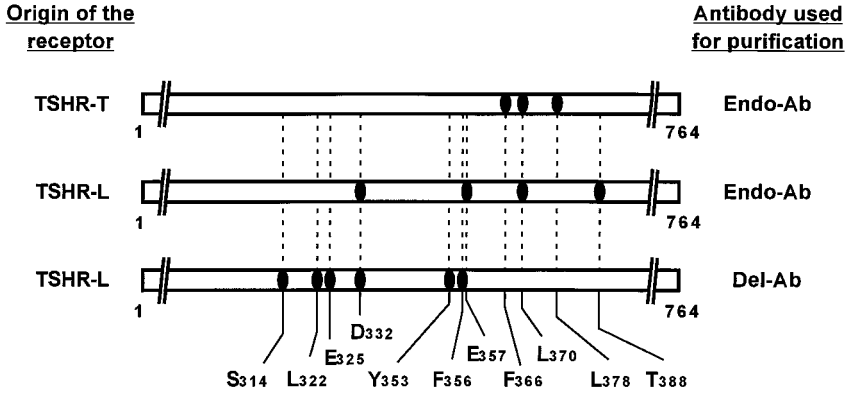

FIG. 5. **N-terminal microsequencing of TSHR β subunits.** The TSHR was immunopurified from human thyroids (*TSHR-T*) or from transfected L cells (*TSHR-L*) using either an anti-endodomain antibody (Endo-Ab) or an antibody which recognizes the excised fragment (Del-Ab). Purified receptors were subjected to electrophoresis in denaturing conditions and transferred onto a polyvinylidene difluoride membrane. The region of the membrane containing the β subunits was determined by comparison with Western blots and cut into sections which were used for microsequencing. The N-terminal amino acids thus determined are shown in the figure.

region around Ser-314. Successive cleavages would then progress toward the C terminus up to phenylalanine 366 and leucines 370 and 378. This conclusion is based on the fact that the epitope recognized by Del-Ab remains in some molecules associated with the β subunit, but never in α subunits. Furthermore, whereas the β subunit is known to be of heterogeneous size, especially in transfected cells, the α subunit has a discrete size (more conveniently observed after deglycosylation (10)).

Characterization of the TSHR Cleavage Enzyme

Absence of Inhibition by TIMPs—We have previously shown that cleavage of the TSHR, and the ensuing shedding of its α subunit, were inhibited by BB-2116, a potent specific inhibitor of matrix metalloproteases (22). Recent studies have shown that the majority of these enzymes are inhibited by the natural tissue inhibitors of metalloproteases called TIMPs, whereas some, including TACE (tumor necrosis factor α-converting enzyme), are insensitive to these compounds (28).

We thus studied TSHR α subunit shedding in the absence or presence of either TIMP-1 or TIMP-2. As shown in Fig. 6 there was no significant inhibition of shedding in the presence of either of these compounds. This experiment thus suggested that the TSHR cleavage enzyme could be a matrix metalloprotease-like enzyme, identical or similar to TACE.

The TSHR Cleavage Enzyme Is Distinct from TACE—To study the effect of TACE on the TSHR, we initially used the purified enzyme. The TSHR was enriched in monomeric uncleaved forms as described above and was incubated with the purified recombinant catalytic domain of TACE (see Fig. 7). Some cleavage was observed, but this yielded a fragment markedly smaller than the physiological α subunit. BB-2116 inhibited this cleavage.

It is thus probable that this cleavage is non-physiological. It is likely that the very high concentrations of purified TACE used in this experiment provoked cleavage of the TSHR at a different site to that recognized *in vivo*.

However, it is possible that TACE can only act specifically on its substrate in the context of a membrane. To examine this hypothesis, we co-transfected cells with expression vectors encoding TSHR and TACE. In these conditions we found a fragment larger than the physiological α subunit released from the cells and present in the cell culture medium (Fig. 8). Immunopurification of the receptor from cells co-transfected with TACE showed that there was a decrease in receptor concentration (probably reflecting nonspecific proteolysis) but no increased concentration of α subunits.

Finally, to further approach this problem we used a mutant CHO cell line obtained by Dr. Joaquin Arribas (23), which has been shown to be deficient in the cleavage and shedding of a variety of membrane protein ectodomains. The most extensively studied reaction to date has been the inability of these

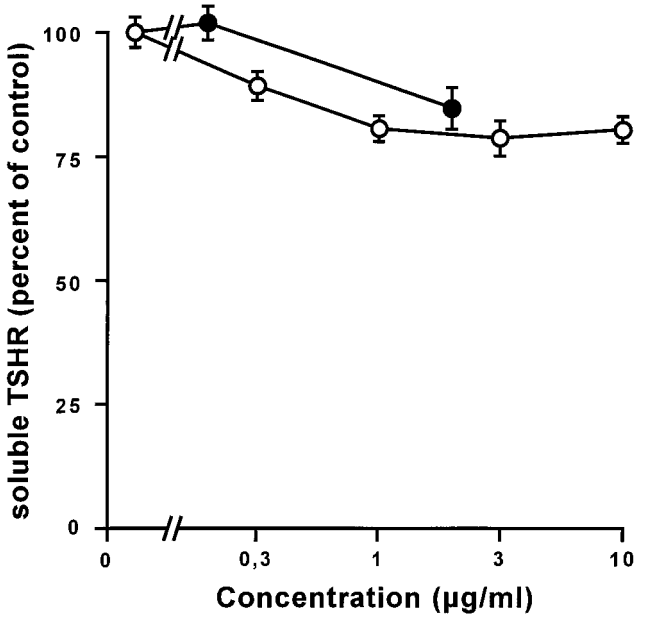

FIG. 6. **TIMP-1 and TIMP-2 do not inhibit shedding of the TSHR α subunit.** L cells stably expressing the TSH receptor were incubated for 24 h in the presence of various concentrations of human recombinant TIMP-1 (*closed circles*) or TIMP-2 (*open circles*). The amount of shed α subunit in the culture medium was assayed, and is expressed as a percentage compared with that in the absence of either TIMP. Results shown are the mean ± S.E. (n = 3).

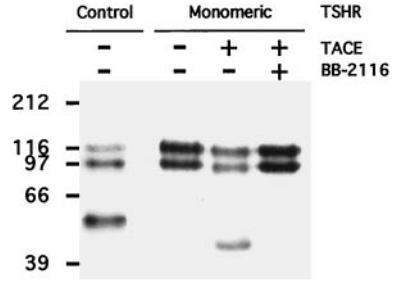

FIG. 7. **Cleavage of TSHR by purified TACE.** A TSHR preparation enriched in monomeric precursors (see "Experimental Procedures") was incubated for 2 h at 37 °C with (+) or without (−) 10,000 units of the recombinant human catalytic domain of TACE in the presence (+) or absence (−) of BB-2116. A control receptor preparation (not enriched in monomers) is shown on the *left*. Western blots were performed using an antibody directed against the receptor ectodomain. Molecular size standards (in kilodaltons) are indicated on the *left*.

cells to convert pro-TGFα into TGFα. Recently, this cell line has also been shown not to express active TACE (29). We thus transfected wild-type and mutant CHO cells with TSHR. As shown in Fig. 9, *A* and *B*, cleavage of the TSHR occurred in the

mutant cells, and shedding of the α subunit was identical to that observed in wild-type CHO cells. We verified that the cells we have used were indeed unable to process pro-TGFα into TGFα (Fig. 9*C*).

DISCUSSION

A group of anti-TSHR monoclonal antibodies were obtained which recognized an epitope cleaved-off from the majority of molecules of the mature receptor. This epitope was localized in a specific segment of the TSHR ectodomain characterized by its non-homology with gonadotropin receptors. The excised TSHR fragment was not shed into the cell culture medium, and did not remain associated with the cell membrane or the mature receptor. These observations suggest that the excision produced multiple small undetectable fragments.

This hypothesis was confirmed by the determination of TSHR cleavage sites. Mature β subunits were isolated from human thyroid glands and from transfected L cells, and extended β subunits (incompletely processed) were purified from transfected L cells. In transfected cells cleavage of the receptor has previously been shown to be incomplete (10). Multiple cleavage sites located within the excised region were detected. The most N-terminal site observed was located at Ser-314 while a group of C-terminal sites recognized in thyroid tissue were located at Phe-366, Leu-370 and -378. Furthermore, the existence of a single discrete α subunit in the thyroid, or in transfected L cells, in contrast to the heterogeneity of the β subunits strongly suggested sequential cleavages. A first N-terminal cleavage reaction probably occurs around Ser-314, followed by a series of downstream cleavages up to amino acids 366–378.

It has previously been proposed that two cleavage sites in the TSHR extracellular domain release a C peptide by a mechanism similar to the processing of pro-insulin (19). We show here that no C peptide is actually produced, but that a series of sequential cleavage reactions lead to the excision of this segment of the TSHR ectodomain. The exact location of the TSHR cleavage sites could not be determined by mutagenesis (20, 21). This concords with the hypothesis that several sites, in close proximity to each other, can be cleaved. These could thus be used alternatively if one of them was mutated. The cleavage of β amyloid protein precursor has also been shown to occur at several sites in a region of the precursor close to the cell surface (30). A sequential proteolysis of the tyrosine kinase ErbB4 receptor has been described (31). However, sequential cleavages of the ErbB4 receptor are due both to an exofacial metalloprotease, inhibited by hydroxamic acids, and to the cytoplasmic proteasome (31).

The cleavage sites within the THSR do not exhibit primary sequence similarity. It is possible that their location is dictated by their position relative to the cell membrane. Indeed, for several membrane proteins cleavage occurs at a site located a fixed distance from the membrane (32–36). Furthermore, mutational analysis has shown a lack of strict sequence specificity for the cleavage of β amyloid precursor protein (32), the TNFα receptor (33), the interleukin-6 receptor (37), L selectin (35), and pro-TNFα (36). In addition, a mechanism involving a recognition of secondary structure in the juxtamembrane region has been suggested by the domain swap experiments of J. Arribas (38).

The precise determination of the most N-terminal cleavage site of the TSHR would necessitate the determination of the C-terminal sequence of the α subunit. We have submitted the purified α subunit to cyanogen bromide digestion, chromatographic separation of peptides, and microsequencing. However, the amounts of protein which could be purified have proved insufficient to date to give meaningful results.

The TSHR β subunits produced by receptor cleavage are very heterogeneous in size. This is especially true in transfected cells where cleavage is incomplete. But even in human thyroid

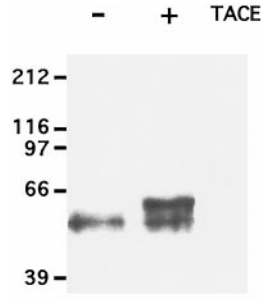

FIG. 8. **Cleavage of the TSHR in cells co-transfected with an expression vector encoding TACE.** Cos-7 cells were co-transfected with expression vectors encoding the human TSHR and either herring sperm DNA (−) or murine TACE (+). The shed α subunit was then immunopurified using an antibody directed against the extracellular domain of the TSH receptor. Molecular size standards (in kilodaltons) are indicated on the *left*.

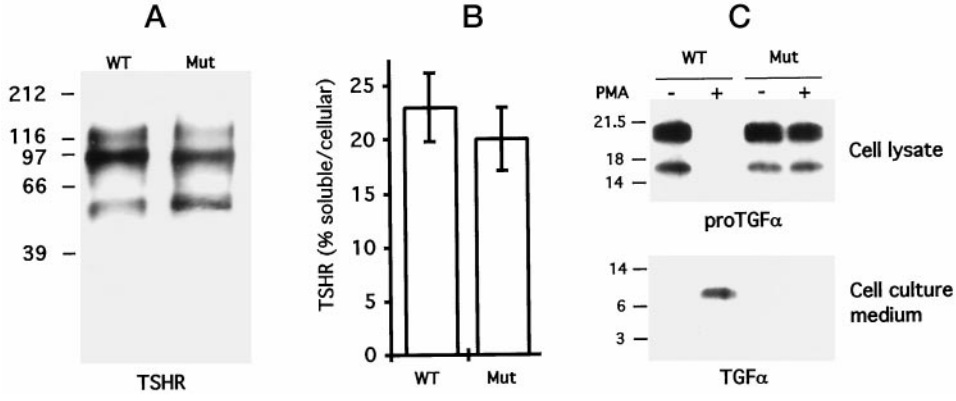

FIG. 9. **The TSH receptor is cleaved in mutant CHO cells.** Wild-type (*WT*) and mutant (*Mut*) CHO cell lines were transiently transfected with the TSH receptor expression vector. *A,* the receptor was immunopurified using an antibody directed against its intracellular domain and analyzed on a Western blot using an antibody recognizing the ectodomain. Molecular size standards (in kilodaltons) are indicated on the *left. B,* the amount of TSHR ectodomain in cell membrane extracts and cell culture medium was quantified by immunoradiometric assays. Results are expressed as percent of TSHR ectodomain shed into the cell culture medium (mean ± S.E., $n = 3$). *C,* as a control, wild-type or mutant CHO cells transfected with HA-tagged pro-TGFα, were metabolically labeled with ^{35}S-cysteine and then chased for 45 min in the presence or absence of phorbol 12-myristate 13-acetate (phorbol 12-myristate 13-acetate (*PMA*) activates pro-TGFα cleavage). Cell lysates and medium samples were immunoprecipitated with anti-HA monoclonal antibody and analyzed by polyacrylamide gel electrophoresis and autoradiography. Molecular size standards (in kilodaltons) are indicated on the *left*.

glands, 25% of receptor molecules undergo incomplete cleavage and are of larger molecular weight. In the majority of receptor molecules the N termini of β subunits cluster from amino acids 366 to 378.

We have shown that TSHR cleavage is inhibited by BB-2116 (22), a synthetic hydroxamic acid which was initially claimed to inhibit specifically matrix metalloproteases (MMP) (26). This compound also inhibits the cleavage of pro-TNFα. This led to the conclusion that the enzymes involved in the maturation of TNFα (TACE) and TSHR belong to the MMP family. However, pro-TNFα (28) and also interleukin 6 receptor (39) and TSHR (present study) convertases are not inhibited by the natural physiological inhibitors of MMPs, *i.e.* TIMPs, which are secreted by cells to control MMP action (40). These observations suggest that pro-TNFα and TSHR convertases are not bona fide MMPs. Furthermore, TACE has recently been cloned (25, 41) and shown to be a new member of the adamalysin family of the metzincin metalloproteases (or ADAMS). These enzymes are characterized by the presence of a metalloprotease domain and of a disintegrin domain. The latter may interact with integrins and in some mammalian adamalysins may mediate cell fusion (42).

The recombinant catalytic domain of TACE failed to cleave immunopurified TSHR at the physiological site. It could be that the correct action of this enzyme depends on its location, and that of its substrate, in the cell membrane. This has proven to be the case for the angiotensin-converting enzyme secretase (43). To examine this possibility we performed co-transfection experiments with TACE and TSHR expression vectors. However, this did not result in cleavage of the TSHR at the physiological site.

Another argument suggesting that TACE and TSHR convertase are different enzymes was provided by the use of a mutant CHO cell line. This cell line is defective in the shedding of several membrane proteins (23, 24) including TNFα (29). It is not known if the defect is due to the alteration of a single protease that acts on all these proteins, or if it is due to an alteration of a common regulatory mechanism necessary for the activation of several proteases. In either case, these cells do not express active TACE but they do properly cleave the TSHR.

We conclude that TACE and TSHR convertases are very likely to be distinct enzymes, although related to each other as shown by their common location at the cell surface, their inhibition by hydroxamic-based metalloprotease inhibitors and their lack of inhibition by TIMPs. Further experiments will be needed to establish the functional role of the cleavage of the TSHR, its possible involvement in receptor autoimmunity, and to identify precisely the corresponding protease.

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