Cell Surface Protein Disulfide-Isomerase Is Involved in the Shedding of Human Thyrotropin Receptor Ectodomain†

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ABSTRACT: In human thyroid glands the TSH receptor undergoes a cleavage reaction which yields to an extracellular α subunit and a membrane spanning β subunit linked together by disulfide bridges. A similar reaction is observed in transfected L cells although some uncleaved monomers persist in these cells. We have recently shown that the α subunit of the TSH receptor undergoes partial shedding in human thyroid cells and heterologous cells permanently transfected with an expression vector encoding the receptor. This shedding is a two-step process. The first step consists in the cleavage of the proreceptor at the cell surface probably by a matrix metalloprotease and the second step in the reduction of the disulfide bridge(s) (Couet, J., Sar, S., Jolivet, A., Vu Hai, M. T., Milgrom, E., & Misrahi, M. 1996, *J. Biol. Chem. 271,* 4545−4552). We have used the transfected L cells to study the second step involved in sTSHR shedding. The membrane impermeant sulfhydryl reagent DTNB (5,5′-dithiobis(2-nitrobenzoic acid)) allowed us to confirm that the reduction of the TSH receptor disulfide bonds occurred at the cell surface. The antibiotic bacitracin even at low concentrations also elicited a marked inhibition of TSH receptor shedding. This led us to implicate the enzyme protein disulfide isomerase (PDI, EC 5.3.4.1) in this process. We thus tested the inhibitory activity of specific monoclonal antibodies raised against PDI. All antibodies elicited a marked inhibition of sTSHR shedding. This confirmed that cell surface PDI is involved in the shedding of the TSH receptor ectodomain. The shed α subunit may be at the origin of circulating TSH receptor ectodomain detected in human blood.

The growth and function of the thyroid gland are controlled by thyrotropin (TSH)[1] via the G-protein coupled thyrotropin receptor (reviews in: Vassart & Dumont, 1992; Misrahi et al., 1994a). The TSH receptor (TSHR) is unusual in that it is the target of relatively frequent autoimmune diseases (Rees Smith et al., 1988; Weetman & Mcgregor, 1994). In Graves' disease, stimulating autoantibodies directed against the receptor lead to hyperthyroidism. Blocking autoantibodies are found in some cases of myxedema.

Precise information on the structure of the human TSH receptor came initially from the cloning of its cDNA (Libert et al., 1989; Nagayama et al., 1989; Misrahi et al., 1990). A preprotein with a calculated molecular weight of ∼84.5 kDa was deduced from the sequence. This receptor has seven transmembrane segments, a characteristic of G-protein coupled receptors, and a large extracellular domain involved in high affinity hormone binding (Nagayama et al., 1991). We have prepared monoclonal antibodies against the various domains of the receptor expressed in *Escherichia coli* (Loosfelt et al., 1992). This allowed us to study by immunoblot and immunopurification the structure of the TSH receptor in human thyroid glands. A cleavage of the polypeptide chain was observed, and the structure of the receptor consisted in an extracellular α subunit of ∼53 kDa and a membrane-spanning β subunit of ∼38 kDa. Both subunits were shown to be held together by disulfide bridge(s) (Loosfelt et al., 1992; Misrahi et al., 1994b). The two-subunit structure of the TSH receptor fits one of the several models which were proposed for its structure before its cDNA was cloned (Furmaniak et al., 1987). Such a cleavage seems to be unique among G-protein coupled receptors (review in: Strosberg, 1991). The TSH receptor was also cleaved in a L cell line stably transfected with the human TSHR cDNA, however less efficiently, some monomeric receptor precursors being also observed (Misrahi et al., 1994b). A cleaved form of the TSH receptor has also been observed in CHO cells (Chazenbalk & Rapoport, 1994).

In human thyroid membranes a precise quantification of each subunit of the TSH receptor by specific ELISA tests demonstrated an ∼2.5-fold excess of β over α subunits (Loosfelt et al., 1992). This observation led us to postulate that the α subunit might be shed from cell membranes and released into the extracellular space or bloodstream. We thus studied cultured human thyrocytes and L cells stably trans-

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[1] Abbreviations: TSH: thyroid stimulating hormone; TSHR: thyroid stimulating hormone receptor; hTSHR: human thyroid stimulating hormone receptor; sTSHR: soluble thyroid stimulating homone receptor; DTNB: 5,5′-dithiobis(2-nitrobenzoic acid); PDI: protein disulfide isomerase.

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fected with TSH receptor. Indeed, we observed in both cell types the release of a soluble form of the TSH receptor α subunit (Couët et al., 1996).

We also established that the shedding process involved two steps: the cleavage of the receptor and the reduction of the disulfide bonds bridging the two subunits. We found that the first step very probably involved a matrix metalloprotease acting at the cell surface (Couët et al., 1996).

This led us to ask the question whether the reduction of the disulfide bonds was also enzymatically catalyzed. The use of various inhibitors, including specific antibodies, allows us to conclude here that cell surface protein disulfide-isomerase (PDI, EC 5.3.4.1) is involved in the second step leading to the shedding of TSH receptor ectodomain.

MATERIALS AND METHODS

Materials. 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was purchased from Sigma (St. Louis, MO), bacitracin and TSH were from Calbiochem (La Jolla, CA). The monoclonal antibodies against human PDI (HP13) and rat PDI (RL90 and RL77) were kindly provided by Dr. Charlotte S. Kaetzel (University of Kentucky). Monoclonal antibodies were purified from ascites fluid by protein G affinity chromatography (Mab Trap G kit from Pharmacia LKB). BB 2116 was kindly provided by British Biotech Co.

Culture of an L Cell Line Permanently Expressing the Human TSH Receptor. This cell line was established and cultured as previously described (Misrahi et al., 1994b).

Primary Culture of Human Thyrocytes. The human thyroid tissue was obtained by surgery from patients with benign thyroid diseases. Primary cultures of human thyrocytes were prepared as described (Misrahi et al., 1994b) and cultured for 72 h.

Immunoradiometric Assay of the TSH Receptor. The double-determinant ("sandwich" type) radioimmunoassay developed for the specific quantification of the TSH receptor α subunit has been previously described (Couët et al., 1996). This assay uses two additive monoclonal antibodies specific for the extracellular domain of the TSHR (T5−317 and T5−51) (Loosfelt et al., 1992; Couët et al., 1996).T5−317 was used as the capture antibody and biotinylated T5−51 in conjunction with ^{125}I-streptavidin as the "reporter" antibody. Samples were quantified relatively to a standard curve of TSHR immunopurified from the stably transfected L cell line (Misrahi et al., 1994b). This cellular TSHR had been previously assayed comparatively to a standard hTSHR (the receptor amino acids 19−243) fused to β-galactosidase expressed in *E. coli* and purified (Loosfelt et al., 1992). This assay allowed us to detect routinely as little as 20 fmol/mL sTSHR. We also developped a second assay for the measurement of the concentration of the full length TSHR by replacing T5−317 with an antibody specific for the intracellular part of the TSHR (T3−365) (Misrahi et al., 1994b). This assay measures both heterodimeric (cleaved) and monomeric (uncleaved) forms of the full length TSHR.

TSHR Determinations in Culture Medium and Cellular Extracts. Cells were grown in 1% serum (Couët et al., 1996) for 24 h. The culture medium was removed, centrifuged at 1500*g* for 5 min, and stored at −20 °C for the assay of soluble TSH receptor. Cell membranes were extracted with 1.2% Triton X-100 as previously described (Misrahi et al., 1994b). An aliquot of the extract was used for the assay of cellular TSHR (cTSHR). The protein concentration was measured with the Pierce BCA reagent kit (Rockford, IL).

Immunopurification of TSHR. Cells were grown for 24 h in the absence or in the presence of bacitracin or BB 2116. Cell membranes were prepared and solubilized with Triton X-100 as previously described (Misrahi et al., 1994b). The receptor was immunopurified as described using a T3−365 Affigel 10 immunomatrix (Misrahi et al., 1994b) saturated with a monoclonal antibody specific for the intracellular part of the receptor.

Immunoblotting of Purified Human TSH Receptor. This assay was performed as described (Misrahi et al., 1994b) using monoclonal antibody T5−317 directed against the extracellular part of the receptor (Couët et al., 1996).

Statistical Analysis. Statistical differences between groups were analyzed by the one-way analysis of variance (ANOVA) followed by Fisher's least significant difference test for multiple comparisons. $P<0.05$ was considered significant. Statview computer program (Abacus Concepts Inc., Berkeley, CA) was used for the calculations.

RESULTS

The Membrane-Impermeant Thiol Reagent DTNB Inhibits the Shedding of TSH Receptor Ectodomain. Cleavage of the TSH receptor occurs at the cell surface (Couët et al., 1996). Disulfide bridge reduction necessary for the shedding of receptor ectodomain may thus either precede the cleavage or also occur at the cell surface. To answer this question, we used the membrane-impermeant sulfhydryl reagent 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (Czech, 1976).

L cells stably transfected with the TSH receptor were grown in the absence or in the presence of increasing concentrations of DTNB. The latter was devoid of cytotoxic effects up to a concentration of 100 μM. It was also verified that DNTB did not modify the cell number or the total concentration of cellular receptor (not shown). The concentration of shed α subunits was assayed in the culture medium using a double-determinant (sandwich type) immunoradiometric assay (Couët et al., 1996). As shown in Figure 1, there was a strong dose-dependent inhibition of soluble TSHR (sTSHR) shedding by DTNB. A maximal inhibition of 70% was observed, and the half-maximal effect was obtained at ∼15 μM.

These results thus confirmed that the reduction of cleaved TSHR disulfide bonds occurred by a thiol−disulfide exchange reaction at the cell surface. Furthermore, the very low concentrations at which DTNB exerted its inhibitory activity evoked the possibility of an enzyme-catalyzed reaction (Mandel et al., 1993; Ryser et al., 1994).

Three enzymes are known to catalyze disulfide oxidoreduction reactions in mammalian cells: protein disulfide-isomerase (PDI) (review in: Freedman et al., 1994), thioredoxin (review in: Holmgren, 1985), and glutaredoxin (review in: Holmgren, 1989). The latter has been cloned from calf thymus but has never been described to be present on the cell surface of mammalian cells (Holmgren, 1989). Protein disulfide-isomerase and thioredoxin have both been shown to be present at the cell surface (Mandel et al., 1993; Holmgren, 1985; Terada et al., 1995). It is possible to distinguish reactions catalyzed by either of these enzymes by the use of bacitracin. This antibiotic inhibits PDI (Roth,

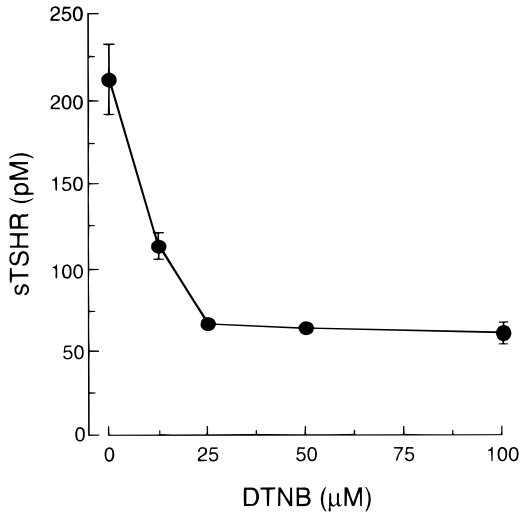

FIGURE 1: Effect of DTNB, a membrane-impermeant sulfhydryl reagent, on the shedding of TSHR ectodomain. L cells expressing TSH receptor were cultured for 24 h in the presence of increasing concentrations of DTNB. Soluble TSH receptor ectodomain (sTSHR) concentration in the culture medium was assayed as described in Materials and Methods. Results are expressed in pM as the mean ± SEM ($n = 3$).

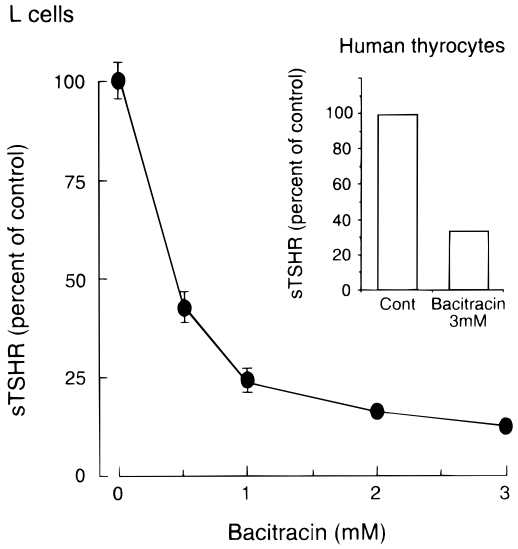

FIGURE 2: Effect of bacitracin on the shedding of TSHR ectodomain. L cells expressing the TSH receptor were cultured for 24 h in the presence of increasing concentrations of bacitracin. sTSHR concentration in the culture medium was assayed as described in Materials and Methods. Results are expressed as percent of control, as the mean ± SEM ($n = 3$). The effect of bacitracin on the shedding of sTSHR from primary culture of human thyrocytes is represented in the inset. Cont: Control cells not treated with bacitracin.

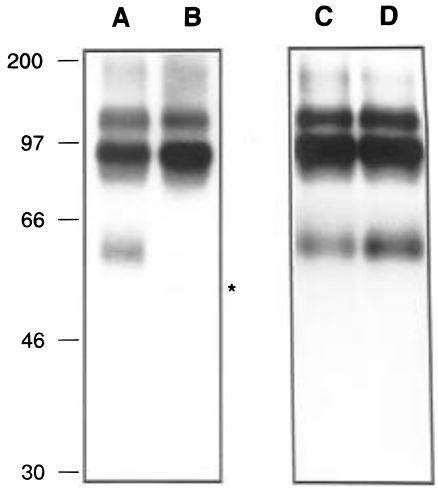

FIGURE 3: Comparison of the effects of bacitracin and BB 2116 on TSHR cleavage. L cells were incubated without treatment (A and C) or in the presence of 100 μg/mL BB 2116 (B) or 3 mM bacitracin (D). Cell membranes were prepared and solubilized with Triton X-100 as described (Misrahi et al., 1994b). The TSHR was immunopurified using antibody T3−365 raised against the intracellular domain of the TSHR, and western blots were performed using antibody T5−317 raised against the ectodomain of the TSHR. * indicates a band corresponding to immunoglobulins leaking from the immunomatrix.

1981; Mizunaga et al., 1990) but not thioredoxin (Mandel et al., 1993).

Bacitracin Inhibits the Shedding of TSH Receptor Ectodomain. The antibiotic bacitracin has been shown to inhibit both the reductive (Roth, 1981) and the oxidative (Mizunaga et al., 1990) functions of PDI. Bacitracin is transported poorly into mammalian cells and thus probably inhibits PDI at the surface of target cells (Mandel et al., 1993).

L cells were grown for 24 h with increasing concentrations of bacitracin. Cell viability, assessed by trypan blue cell exclusion test, was unaffected. A slight (<10%) increase in cell number and in the cellular receptor concentration was observed in bacitracin-treated cells when compared to control cells. (The observed sTSHR shedding was thus corrected for this difference.) As shown in Figure 2, there was a dose-dependent inhibition of sTSHR accumulation in L cell medium when cells were incubated with bacitracin. At 0.5 mM bacitracin a ~60% inhibition was already observed while at 3 mM bacitracin the inhibition reached almost ~90%. This is consistent with the concentrations of bacitracin known to inhibit the reduction of disulfide bonds by PDI (Mandel et al., 1993).

We also analyzed the effect of bacitracin in human thyroid cells in culture. As shown in Figure 2 inset, bacitracin markedly reduced the shedding of TSH receptor ectodomain from these cells. This result suggested that the enzymatic mechanisms involved in the shedding were similar in L and thyroid cells.

Besides being an inhibitor of PDI, bacitracin is also known to be a protease inhibitor (Makinen, 1972; Mizunaga et al., 1990). Its effect on TSH receptor ectodomain shedding thus could have occurred through impairment of the receptor cleavage reaction and not through inhibition of disulfide bridge reduction. We thus examined the effect of bacitracin on TSH receptor species present on the cell membrane. We compared the effect of bacitracin with that of BB 2116, the matrix metalloprotease inhibitor, which has previously been shown to inhibit receptor ectodomain shedding (Couët et al., 1996). Immunoblot was initially performed in control cells, nontreated with inhibitors (Figure 3A,C). A monoclonal antibody was used which interacts with receptor ectodomain. As previously described, three receptor species were detected: the ~120 kDa species which is the mature non-cleaved receptor, the ~95 kDa species which is the intracellular receptor precursor containing mannose-rich carbohydrates, and the ~60 kDa α subunit corresponding to the cleaved mature receptor (Misrahi et al., 1994b; Couët et al., 1996). Treatment with BB 2116 nearly completely suppressed the α subunit (Figure 3B), confirming that the

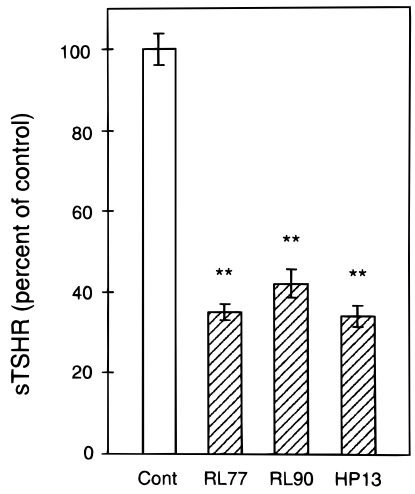

FIGURE 4: Inhibition by monoclonal anti-PDI antibodies of TSHR ectodomain shedding. L cells expressing TSH receptor were grown for 24 h in the presence of 200 μg/mL of affinity purified irrelevant IgG (Cont) (Legrain et al., 1983) or anti-PDI monoclonal antibodies (RL 77, RL 90, and HP13) (Kaetzel et al., 1987). sTSHR concentration in the cell medium was measured as described in Materials and Methods. Results are expressed as the mean ± SEM ($n = 3$). (**) $P < 0.01$ vs control with an irrelevant IgG.

inhibition occurred on receptor cleavage. On the contrary treatment with bacitracin did not decrease receptor cleavage (Figure 3D), thus confirming that the inhibition was very probably acting on the reduction of disulfide bonds.

Anti-PDI Monoclonal Antibodies Inhibit the Shedding of TSH Receptor Ectodomain. Inhibition by DTNB and bacitracin suggested that PDI is involved in the shedding of TSH receptor ectodomain. Monoclonal antibodies have been prepared against this enzyme, and three of these antibodies have been shown to be inhibitory of its activity (Kaetzel et al., 1987). Moreover, these antibodies have been shown to cross-react with PDI of other species (Kaetzel et al., 1987). We thus tested the activity of the three inhibitory antibodies (RL77 and RL90 raised against rat PDI and HP13 against human PDI) on the shedding of TSH receptor ectodomain. Compared to a control nonrelated antibody, all anti-PDI antibodies markedly inhibited the shedding of TSH receptor ectodomain (Figure 4).

DISCUSSION

The TSH receptor is initially synthetized as an ~95 kDa precursor containing mannose-rich carbohydrates. During its progression through the Golgi complex the latter are replaced by complex carbohydrates yielding a ~120 kDa monomeric glycoprotein (Misrahi et al., 1994b). Other authors also confirm the presence of cleaved and monomeric TSH receptors in CHO cells (Chazenbalk & Rapoport, 1994). On the cell surface the receptor is cleaved probably by a matrix metalloprotease into two subunits linked by disulfide bridge(s) (Couët et al., 1996). The α subunit is extracellular, and the β subunit spans the membrane. Part of the α subunit is shed from the cells (Couët et al., 1996). In the present study we have shown that the cell membrane-associated protein disulfide-isomerase (PDI) is involved in this reduction of disulfide bridge(s) leading to the shedding of the α subunit.

PDI and thioredoxin are two enzymes which are known to be involved in the reduction of disulfide bonds in mammalian cells and to be expressed at the cell membrane (Mandel et al., 1993; Holmgren, 1985; Terada et al., 1995). They perform electron transfers in the reversible oxidation of vicinal SH groups present in proteins (Freedman et al., 1994; Holmgren, 1985). These enzymes thus catalyze the formation and reduction of disulfide bridges. Thioredoxin is a short polypeptide of ~100 aminoacids and contains one reactive sequence, while PDI is a homodimer of a ~57 kDa protein and contains four homologous reactive sequences per molecule. PDI has also been called thiol protein disulfide-oxidoreductase (EC 1.8.4.2) or glutathione-insulin transhydrogenase. PDI is found in many organs and is highly conserved between species. It is a major intracellular protein corresponding to 0.4% (Freedman et al., 1994) or 0.7% (Terada et al., 1995) of the total cellular protein content. Most of PDI is localized in the endoplasmic reticulum where it catalyzes disulfide bridge formation, isomerization, or reduction (reviews in: Freedman, 1989; Noiva & Lennarz, 1992; Wang & Tsou, 1993). These reactions are necessary for the correct folding of newly synthesized proteins. PDI also catalyzes the reduction of disulfide bonds of insulin as the first step in the degradation of this hormone (Roth, 1981). Besides, PDI was identified as a subunit of prolyl hydroxylase and of the microsome triglyceride transfer protein complex (review in Noiva & Lennarz, 1992). It has been suggested that PDI also serves as a molecular chaperone (Noiva & Lennarz, 1992; Wang & Tsou, 1993).

Recently, PDI has been detected at the surface of mammalian cells and implicated in a variety of mechanisms. The membrane PDI catalyzes the cleavage of disulfide bonds occurring during the earliest stages of endocytosis of exogenous macromolecules (Feener et al., 1990; Mandel et al., 1993). Membrane PDI is also involved in the action of diphtheria toxin. This toxin is a disulfide-linked heterodimer that binds to a specific cell surface receptor. Chain separation then occurs, catalyzed by PDI, and free chains become biologically active (Mandel et al., 1993). PDI has been implicated in the infection of human lymphoid cells by membrane bound human immunodeficiency virus (Ryser et al., 1994). Cloning and sequencing have revealed that PDI is identical to a membrane T3-binding protein which has been independently studied (Cheng et al., 1987). It may also be noted that a thioredoxin-like activity has been described for FSH and LH (Boniface & Reichert, 1990), hormones closely related to TSH. We have previously shown that in transfected L cells TSH elicited a ~30% increase in sTSHR shedding (Couët et al., 1996). This limited effect did not allow us to establish precisely the mechanisms involved (increase in the cleavage or in the reduction of disulfide bonds).

Shedding of extracellular domains has been described for a variety of membrane proteins (reviews in: Tedder, 1991; Ehlers & Riordan, 1991): receptors for interleukins, transferrin, insulin, growth hormone, prolactin, tumor necrosis factor, colony-stimulating factor I, c-erb B-2 (Mori et al., 1990), nerve growth factor, receptor ligands, cell adhesion molecules, leukocyte antigen, ectoenzymes, and amyloid precursor protein. Different regulatory mechanisms have been observed: in some cases, the shedding is induced by ligand binding or by kinase C activation (Tedder, 1991; Ehlers & Riordan, 1991; Pandiella & Massagué, 1991; Mui et al., 1992). It has been suggested that this may be due to the activation of a cell membrane-associated protease. Such a mechanism has been described for leukocyte adhesion molecule (L-selectin), the colony-stimulating factor 1 recep-

tor, the tumor necrosis factor receptor, the interleukin receptors, the membrane-anchored growth factors, and the LAR tyrosine phosphatase. The latter protein is expressed as a complex of two noncovalently associated subunits, and the shedding is the consequence of a proteolytic cleavage at a second furin-like site of the ectodomain of the protein (Serra-Pages et al., 1994). In other cases, the shedding is spontaneous and occurs after cleavage of the protein at the cell membrane. This is the case for the polymeric IgA receptor, the angiotensin-converting enzyme, the class I major histocompatibility complex proteins, and the amyloid precursor protein (reviews in: Tedder, 1991; Ehlers & Riordan, 1991). In some cases (transferrin and NGF receptors), the shedding requires cycling through an endosomal compartment where proteolysis occurs (Rutledge et al., 1994; Zupan & Johnson, 1991).

The role of the reduction of disulfide bonds in the shedding of membrane proteins has not been previously studied. In the case of the TSH receptor, the shedding results from two steps: cleavage of the protein by a matrix metalloprotease and reduction of disulfide bonds by PDI. Both steps occur at the cell membrane (we cannot however eliminate the possibility that in a small fraction of receptor molecules no disulfide bonds are formed and that the cleavage alone is sufficient for the shedding to occur). PDI involvement in the shedding of other membrane proteins is currently unknown.

The role of receptor cleavage reaction and of the shed extracellular domain of the TSHR is presently unclear. We have previously shown that this sTSHR is able to bind TSH with an affinity similar to that of the full length receptor. Other soluble receptors have been shown to act as activators or inhibitors of receptor function (Tedder, 1991; Ehlers & Riordan, 1991; Mosley et al., 1989; Vuhai-Luuthi et al., 1992). In membranes of human thyroid glands we have shown that there is a large excess of β subunits compared to α subunits (Loosfelt et al., 1992). It is thus possible that ectodomain shedding is a mechanism of down regulation of hormone action. It has proven very difficult to analyze such physiological regulations in transfected cells, due to the lower efficiency of the cleavage and shedding reactions (Couët et al., 1996). We are currently trying to determine the site of cleavage of the receptor, in order to mutate the corresponding amino acids and analyze the effect of such mutagenesis on receptor function.

We have detected the presence of a soluble form of the TSH receptor in human serum (Couët et al., 1996). This circulating receptor may correspond to the shed ectodomain of the TSH receptor. Immunopurification of this soluble receptor from human serum will allow its structure to be studied comparatively with the thyroid TSH receptor. Also the identification of the cleavage site of the receptor will allow to determine whether the soluble TSH receptor is generated by shedding. The role of this soluble TSH receptor as a TSH binding protein and its involvement in the development of autoimmune thyroid diseases are currently under study.

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