Isolation of radiochemically pure ^{125}I -labeled human thyrotropin receptor and its use for the detection of pathological autoantibodies in sera from Graves' patients

W B Minich and U Loos

Department of Internal Medicine I, University of Ulm, D-89070 Ulm, Germany

(Requests for offprints should be addressed to U Loos, Abteilung Innere Medizin I, Medizinische Klinik und Poliklinik, Universitaet Ulm, Robert-Koch-Str 8, D-89070 Ulm, Germany)

Abstract

We report a method for the purification and radioactive labeling of human TSH receptor (TSHR). The method is based on the construction of a fusion TSHR (TSHR-Xa-BIO) which consists of the N-terminal 725 amino acids of human TSHR linked to the 4-amino acid Xa protease cleavage site and the 87-amino acid C-terminal domain of the biotin carboxyl carrier protein subunit of *Escherichia coli* acetyl-CoA carboxylase (the C-terminal domain directs the efficient posttranslational biotinylation of the protein). TSHR-Xa-BIO was produced in HeLa cells using recombinant vaccinia virus. The expressed protein was

fully functional and was biotinylated with an efficiency of about 90%. Streptavidin-agarose-immobilized TSHR-Xa-BIO was labeled with ^{125}I using the chloramine T oxidation procedure and specifically eluted from the solid phase after cleavage with protease Xa. Isolated native radiochemically pure ^{125}I -labeled TSHR specifically interacted with pathological autoantibodies in the sera of patients with Graves' disease, and thus could be useful for the detection of these autoantibodies by immunoprecipitation analysis.

Journal of Endocrinology (1999) **160**, 239–245

Introduction

The thyroid-stimulating hormone receptor (TSHR) plays a pivotal role in the function and growth of thyroid cells. It is a member of a subfamily of G-protein-coupled glycoprotein receptors with a large N-terminal extracellular domain which is essential for ligand binding and has been shown to be involved in the process of signal transduction. Signal transduction by TSHR is predominantly mediated by the activation of adenylate cyclase with a subsequent increase in intracellular cAMP (Kohn *et al.* 1995, Refetoff *et al.* 1996, Tonacchera *et al.* 1996, Misrahi & Milgrom 1997).

TSHR has been the subject of great interest because of its role as a primary autoantigen in Graves' disease autoimmune hyperthyroidism, one of the most common human autoimmune diseases (Kohn *et al.* 1995, Refetoff *et al.* 1996, Tonacchera *et al.* 1996, Misrahi & Milgrom 1997). It is present on the thyroid cell surface in very small amounts (in the region of 1000–10 000 sites per cell) (Furmaniak & Ress Smith 1991). It has been expressed in a variety of systems, but functional TSHR can be produced only in mammalian cells, both transiently and as a stable line (Perret *et al.* 1990, Chazenbalk *et al.* 1990, Ban *et al.* 1992, Misrahi *et al.* 1994, Loos *et al.* 1995), and also using adenovirus

(Okamoto *et al.* 1995) and vaccinia virus (Minich *et al.* 1997, 1998) expression systems. Because of the importance of TSHR as an autoantigen, great efforts have been made over the past few years to purify it. Despite many attempts, there are no convincing data that functional TSHR has ever been isolated in amounts satisfactory for pathological autoantibody binding and detection. Purification has been hampered by the relatively small number of TSHR molecules expressed in mammalian cells and the functional instability of the protein. An important factor contributing to this difficulty is that TSHR autoantibodies predominantly recognize highly conformational epitopes (which are not necessarily identical with those that bind TSH) (Nagayama *et al.* 1991, Tahara *et al.* 1991).

The lack of labeled TSHR antigen has made it impossible to develop a direct immunoprecipitation clinical assay for pathological TSHR autoantibodies. The only clinical assay used at present is the indirect assay of ^{125}I -TSH-binding inhibition using porcine TSHR from thyroid glands solubilized with detergent. It is clear that a direct immunoprecipitation assay would be a great advantage in Graves' disease diagnosis and investigation, because it may be capable of detecting all autoantibodies to the TSHR, particularly those that bind but do not inhibit the hormone-receptor interaction.

In the present study we report a method for the purification and radioactive labeling of human TSHR expressed in HeLa cells using recombinant vaccinia virus. Isolated ^{125}I -labeled TSHR could be useful for the direct immunoprecipitation analysis of pathological autoantibodies in unfractionated sera of patients with Graves' disease.

Materials and Methods

Materials

Bovine ^{125}I -TSH (56 Ci/g) and TRAK assay kits were gifts from Brahms Diagnostica, Berlin, Germany. Streptavidin-agarose was purchased from Sigma, Diesenhofen, Germany. Protease Xa and N-glycosidase F were obtained from Boehringer-Mannheim, Mannheim, Germany. Na ^{125}I (1000 Ci/mmol) and the ECL Western blotting detection kit were purchased from Amersham, Braunschweig, Germany. NCL-TSHR mouse monoclonal antibodies against the extracellular domain of TSHR were obtained from Novocastra Laboratories (Newcastle, UK). DNA primers, P1 (5'-GTCGGTT ACCATGAAGGCCGCATGGAAGCGCCAGCAGCA G-3') and P2 (5'-CGCGGATCCTTATTCGATAACA ACAAGCGGTTTC-3') were purchased from Interactiva (Uem, Germany).

Cell culture

HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1 mM biotin. The cells were cultivated in a 5% CO $_2$ atmosphere at 37 °C.

Addition of Xa protease site sequence to TSHR-BIO cDNA

The plasmid p7.5K131-TSHR-BIO contains the sequence for the N-terminal 725 amino acids of human TSHR linked to the 87-amino acid C-terminal domain of the biotin carboxyl carrier protein (BCCP) subunit of *Escherichia coli* acetyl-CoA carboxylase (Minich *et al.* 1998). The BCCP sequence was amplified by PCR using primer P1 (containing the BstEII restriction site and the sequence for the Xa protease site) and primer P2 (containing the stop codon and the BamHI restriction site) giving the BstEII/BamHI PCR fragment. The p7.5K131-TSHR-BIO plasmid (Minich *et al.* 1998) was digested with BstEII and BamHI; the BstEII/BamHI fragment was removed and replaced with the BstEII/BamHI PCR fragment giving vaccinia recombination plasmid p7.5 K131-TSHR-Xa-BIO.

Generation of vaccinia virus recombinants

The TSHR-Xa-BIO cDNA (from p7.5K131-TSHR-Xa-BIO plasmid) was incorporated into the genome

of wild-type vaccinia virus strain Copenhagen by homologous recombination into the thymidine kinase gene as described in detail by Metzger *et al.* (1994) and Minich *et al.* (1997).

Preparation of TSHR-Xa-BIO extract

Confluent HeLa cells grown in a 75 cm 2 plate were infected with 1 plaque forming unit of recombinant vaccinia virus per cell and incubated for 24 h at 37 °C. Infected cells were resuspended by scraping into PBS and washed four times in the same buffer by centrifugation at 2500 r.p.m. The resulting cells were lysed in 0.3 ml buffer A (20 mM Hepes-KOH, pH 7.5, 50 mM NaCl, 1% Tween 20, 10% glycerol) by freeze/thawing. The suspension was centrifuged at 30 000 *g* for 1 h, and the supernatant (~8 mg/ml total protein) was collected and dialyzed overnight against buffer A to remove free biotin.

Endoglycosidase-F digestion

The HeLa cells infected with recombinant vaccinia virus (see above) were lysed in 0.15 ml 1% Nonidet P40 in PBS by freeze/thawing, insoluble material being removed by centrifugation at 30 000 *g* for 1 h. The supernatant was diluted with an equal volume of 0.1% SDS in PBS. Then 2 units of N-glycosidase F was added to 20 μl extract, and the mixture was incubated at 37 °C for 16 h and analyzed by Western blotting.

Immunoprecipitation analysis

For immunoprecipitation, 40 μl ^{125}I -TSHR (~16 000 c.p.m.) was mixed with 10 μl serum (or mixture of sera) and incubated for 2 h at room temperature. Then 20 μl 50% suspension of Protein A-agarose in buffer A was added and the mixture was incubated for 1 h at room temperature with shaking. After removal of the supernatant, the resin was washed with 0.3 ml buffer A by centrifugation, and γ radiation in the pellet was counted. Results were expressed as c.p.m. bound. For SDS-PAGE, the pellet was resuspended in 20 μl SDS sample buffer and analyzed in an 8% polyacrylamide gel.

Binding assay for the ^{125}I -TSH and Graves' autoantibodies

Binding of ^{125}I -TSH and patient autoantibodies to solubilized porcine TSHR (or solubilized human TSHR) was measured using the TRAK assay kit according to the manufacturer's instructions. The assay was performed in a total volume of 200 μl , containing 50 μl TSHR solution, 50 μl serum and 100 μl ^{125}I -TSH (~20 000 c.p.m.). Incubation was for 2 h at room temperature; the ^{125}I -TSH-TSHR complexes were precipitated by adding 2 ml polyethylene glycol and centrifugation at 2000 *g* for 10 min, and γ radiation in the pellet was counted. Results

were expressed as c.p.m. bound or as an inhibition index (%) calculated as: $1 - (\text{c.p.m. in the presence of test serum}) / (\text{c.p.m. in the presence of zero standard serum})$.

Non-specific interaction was determined in the presence of 10^{-7} M TSH; it was less than 10% of total binding.

In the case of immobilized TSHR, the ^{125}I -TSH-binding assay was performed as described above, except that 50 μl 20% suspension of TSHR bound to streptavidin-agarose was used. The mixture was incubated for 2 h at room temperature with continuous agitation. After removal of the supernatant, the resin was washed with 0.3 ml buffer A by centrifugation, and γ radiation in the pellet was counted. Non-specific interaction was determined in the presence of 10^{-7} M TSH; it was less than 10% of total binding.

Western blotting

Proteins were electrophoresed in SDS-8% polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were probed overnight at 4 °C with NCL-TSHR monoclonal antibody to the extracellular domain of TSHR, diluted 1:20. Bound antibodies were detected using the ECL Western blotting detection kit.

Results

Construction of fusion TSHR and its expression in HeLa cells

We fused the DNA sequence encoding the N-terminal 725 amino acids of human TSHR (complete protein has 764 amino acids) to the sequence encoding the 4-amino acid Xa protease cleavage site and the 87-amino acid C-terminal domain of the BCCP subunit of *E. coli* acetyl-CoA carboxylase. The fusion protein (TSHR-Xa-BIO) was produced using the vaccinia virus expression system. Figure 1 (lane a) shows a Western blot analysis of HeLa cell extract infected with recombinant vaccinia virus containing the TSHR-Xa-BIO cDNA insert. The major band has a molecular mass of about 100 kDa, which is in a good agreement with the expected molecular mass of TSHR-Xa-BIO. No receptor protein was detected in cells infected with wild-type vaccinia virus (Fig. 1, lane d). The efficiency of TSHR-Xa-BIO biotinylation in infected HeLa cells was determined by chromatography on streptavidin-agarose. The amount of TSHR-Xa-BIO in the HeLa cell extract was diminished by more than 90% after incubation of the extract with streptavidin-agarose (Fig. 1, lanes a and b). In control experiments, the amount of TSHR-Xa-BIO in the cell extract was not decreased after incubation with streptavidin-agarose that had been pretreated with excess biotin (Fig. 1, lane c). These data show that more than 90% of TSHR-Xa-BIO in the HeLa

Figure 1 TSHR-Xa-BIO expression in recombinant vaccinia virus-infected HeLa cells. Lane a, extract of HeLa cells infected with recombinant virus; lane b, extract of HeLa cells infected with recombinant virus after incubation of 50 μl extract with 20 μl streptavidin-agarose for 2 h at 4 °C; lane c, same as lane b but streptavidin-agarose was pretreated with 10 mM biotin; lane d, extract of HeLa cells infected with wild-type virus; lane e, extract of HeLa cells infected with recombinant virus after treatment with N-glycosidase F; lane f, same as lane e but N-glycosidase F was omitted. Proteins present in 5 μl extract were separated by SDS-PAGE, and TSHR-Xa-BIO was detected by Western blot analysis using antibody to the extracellular domain of TSHR. Molecular size markers are given in kDa.

cell extract was biotinylated. Treatment of cell extract with N-glycosidase F decreased the molecular size of TSHR-Xa-BIO from 100 kDa to about 80 kDa (Fig. 1, lanes e and f). This experiment shows that TSHR expressed by vaccinia virus is N-glycosylated.

The expression parameters and features of TSHR-Xa-BIO were practically identical with those of biotinylated TSHR (TSHR-BIO), the expression of which in the vaccinia virus system has been described in detail previously (Minich *et al.* 1998). HeLa cells infected with recombinant virus were found to express approximately 120 000 molecules of TSHR-Xa-BIO per cell. The receptor was fully functional, interacting with TSH and coupling with the cellular cAMP second-messenger cascade (data not shown).

The protease Xa site in the TSHR-Xa-BIO chimera was completely susceptible to cleavage by the Xa factor (Fig. 2). The biotinylated TSHR was bound to streptavidin-agarose, washed free of contaminating proteins and the amount of immobilized receptor estimated by measurement of its ^{125}I -TSH-binding activity. Figure 2 shows that Xa treatment completely removed TSHR-Xa-BIO from streptavidin-agarose. At the same time biotinylated TSHR without the factor Xa cleavage site (TSHR-BIO) was insensitive to Xa digestion over a wide range of protease amounts.

Figure 2 Effect of Xa protease on streptavidin-agarose-immobilized TSHR-BIO (○) and TSHR-Xa-BIO (●). First 50 µl TSHR extract were incubated with 10 µl streptavidin-agarose. The resin was then washed with buffer B, and immobilized TSHR was incubated with different amounts of protease Xa for 12 h at 4 °C in a final volume of 35 µl. At the end of the incubation time, ¹²⁵I-TSH-binding activity of the rest of the immobilized TSHR was measured as explained in the text. Results are expressed as a percentage of maximal binding (2400 c.p.m. and 2600 c.p.m. for TSHR-BIO and TSHR-Xa-BIO respectively). The total amount of added ¹²⁵I-TSH was ~20 000 c.p.m.; non-specific binding was in the region of 100 c.p.m. Each point represents the mean of closely agreeing duplicate determinations.

Purification and labeling of TSHR

TSHR was isolated from HeLa cells infected with recombinant vaccinia virus. The concentration of expressed protein was similar in different cell extract preparations, and was about 1 nM according to Satchard analysis (data not shown). TSHR was purified by a three-step procedure. We describe in the following a representative experiment out of a total of five protein isolation experiments. All operations except those indicated were performed at 4 °C. The amount of TSHR was estimated from its ¹²⁵I-TSH-binding activity.

Step 1: immobilization of biotinylated TSHR-Xa-BIO on streptavidin-agarose TSHR-Xa-BIO HeLa cell extract (5 ml) was passed through a 0.5 ml streptavidin-agarose column, equilibrated with buffer A. The column was washed with 100 ml buffer A and 5 ml 50 mM sodium phosphate buffer (pH 7.5) to remove contaminating proteins. With this ratio of extract to resin, about 50% of TSHR-Xa-BIO was bound to streptavidin-agarose. ¹²⁵I-TSH-binding capacity of the original cell extract and flow through fraction was ~120 000 and ~55 000 c.p.m. per 5 ml respectively, i.e. approximately 65 000 c.p.m. of ‘¹²⁵I-TSH-binding activity’ was immobilized on streptavidin-agarose.

Table 1 Effect of chloramine T on the ¹²⁵I-TSH-binding activity of TSHR. Each point represents the mean of triplicate determinations

Chloramine T (mg/ml)	Presence of dithiothreitol	¹²⁵ I-TSH bound (c.p.m.)
—	—	2512
—	+	2492
0.005	+	2537
0.01	+	2424
0.1	+	1410
1.0	+	776

TSHR (50 µl) extract was incubated with 10 µl streptavidin-agarose. The resin with bound receptor was washed with buffer A and 50 mM sodium phosphate buffer (pH 7.5). The immobilized TSHR was incubated with different amounts of chloramine T, followed by the addition of NaI (1 µM) and dithiothreitol (2 µg/ml) in a final volume of 20 µl. At the end of the incubation time, the resin was washed with buffer A, and the ¹²⁵I-TSH-binding activity of the immobilized TSHR was measured as explained in the text. The total amount of added ¹²⁵I-TSH was ~20 000 c.p.m.; non-specific binding was in the region of 100 c.p.m.

Step 2: ¹²⁵I-labeling of streptavidin-agarose-bound TSHR-Xa-BIO Streptavidin-agarose with immobilized TSHR-Xa-BIO was transferred to an Eppendorf tube, and 1 mCi Na¹²⁵I and chloramine T (to final concentration of 0.005 mg/ml) were added. Control experiments demonstrated that this amount of chloramine T did not affect the TSH-binding activity of immobilized TSHR (Table 1). The mixture in a total volume of 1 ml was incubated, with shaking, for 1 min at room temperature. The reaction was terminated by the addition of 2 µg dithiothreitol. The resin was transferred to a column, which was washed with 50 mM sodium phosphate buffer (pH 7.5) and 100 ml of buffer A to remove unbound radioactive material.

Step 3: elution of ¹²⁵I-labeled TSHR from streptavidin-agarose using Xa protease The streptavidin-agarose with immobilized protein was washed with 3 ml buffer B (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM CaCl₂, 1% Triton X-100, 10% glycerol, 3 mg/ml BSA). ¹²⁵I-labeled TSHR was eluted by incubation, with constant agitation, of the column matrix with 1.25 ml buffer B containing 3 µg protease Xa for 12 h at 4 °C. The suspension was centrifuged at 14 000 r.p.m. for 10 min, and the supernatant containing ¹²⁵I-TSHR was collected. The ¹²⁵I-labeled TSHR was purified from low molecular mass ¹²⁵I-containing material (probably Na¹²⁵I) using spin filtration on Sephadex G-25. For this purpose, 1.25 ml of the protein solution was applied to the 7 ml Sephadex G-25 column equilibrated with buffer B. The column was centrifuged at 2000 r.p.m. for 5 min. The flowthrough fraction (1.25 ml) containing the ¹²⁵I-TSHR preparation was aliquoted and stored at -70 °C.

Finally, we isolated ~0.6 × 10⁶ c.p.m. ¹²⁵I-labeled TSHR. Control experiments (performed under identical

Figure 3 SDS-PAGE of isolated ^{125}I -labeled TSHR. Lane a, autoradiography of protein present in $3\ \mu\text{l}$ ^{125}I -TSHR preparation. Lane b, Western blot analysis of protein present in $3\ \mu\text{l}$ ^{125}I -TSHR preparation using antibody against extracellular domain of TSHR. Lane c, autoradiography of ^{125}I -TSHR precipitated by a mixture of five normal sera (the first five normal sera shown in Fig. 4 were used). Lane d, autoradiography of ^{125}I -TSHR immunoprecipitated by a mixture of five Graves' disease sera (the last five Graves' disease sera shown in Fig. 4 were used). Protein was separated on 8% polyacrylamide gels. Molecular size markers are given in kDa.

conditions but with non-radioactive NaI) showed that isolated receptor was active, binding $\sim 40\ 000$ c.p.m. ^{125}I -TSH per $1.25\ \text{ml}$ TSHR preparation. Non-specific interaction was determined in the presence of $10^{-7}\ \text{M}$ TSH; it was less than 10% of total binding. As assessed by measuring the TSH-binding activity, the yield of TSHR was $\sim 30\%$ of that in the original cell extract and $\sim 60\%$ of the streptavidin-agarose-immobilized TSHR. Taking into account the amount of TSHR in $5\ \text{ml}$ of cell extract (see above) and the yield of TSHR, we can calculate the approximate amount and specific activity of isolated ^{125}I -TSHR. They should be in the region of $0.2\ \mu\text{g}$ and $1.5\ \text{Ci/g}$ ($1.5 \times 10^5\ \text{Ci/mol}$) TSHR respectively. On the basis of the specific activity of Na^{125}I , we can calculate that $\sim 20\%$ of TSHR molecules in the receptor preparation are labeled.

SDS-PAGE and autoradiography of ^{125}I -labeled TSHR gave one major band with a molecular mass of about $90\ \text{kDa}$, which is in a good agreement with the expected molecular mass of cleaved TSHR (Fig. 3, lane a). A band of the same size was detected by Western blot analysis of the ^{125}I -TSHR preparation using antibodies against the extracellular domain of TSHR (Fig. 3, lane b). Moreover, Fig. 3 (lanes c and d) shows that the $90\ \text{kDa}$ protein can be effectively immunoprecipitated using a mixture of five highly positive Graves' disease sera, but not by a mixture of five normal sera (in this experiment a mixture of the last five Graves' disease sera and a mixture of the first five normal sera shown in Fig. 4 were used).

Detection of Graves' autoantibodies in patient sera using ^{125}I -labeled TSHR

^{125}I -labeled TSHR was used for the detection of pathological autoantibodies in sera of patients with Graves' disease by immunoprecipitation analysis. The reaction mixture contained test serum, ^{125}I -labeled TSHR and Protein A-agarose. At the end of the incubation period, antibodies-Protein A-agarose-bound radioactivity was pelleted, and γ radiation in the pellet was counted. The amount of TSHR autoantibodies in the patient sera is proportional to the measured radioactivity. Control studies, which included the binding of patient autoantibodies and ^{125}I -TSH to solubilized porcine TSHR, were performed using a TRAK assay kit. In this case, autoantibodies were assayed by their ability to inhibit ^{125}I -TSH binding to the TSHR. There was a clear positive correlation ($r=0.9$; $P<0.001$) between the result obtained in the immunoprecipitation assay and the TRAK assay in 25 sera of patients with different degree of Graves' disease and 25 normal sera (Fig. 4). All Graves' disease sera tested immunoprecipitated ^{125}I -TSHR; binding was in the range $2000\text{--}4000$ c.p.m. and up to 25% of added radioactivity was bound. The serum concentration used in the reaction represents the linear part of the binding curve generated using the most positive Graves' serum, with an inhibition index of 75%. The normal sera bound ^{125}I -TSHR with lower efficiency, in the region of $400\text{--}1000$ c.p.m. The level of non-specific binding of ^{125}I -TSHR by Protein A-agarose in the absence of serum was about of 300 c.p.m. Experiments with autoimmune control groups have shown that ^{125}I -TSHR was precipitated with low efficiency by sera obtained from 10 patients with Hashimoto's disease and 10 patients with insulin-dependent diabetes mellitus. The ^{125}I -TSHR-precipitating activities were in this case comparable to those of the control sera, showing binding values in the range $300\text{--}900$ c.p.m. (data not shown). All of these sera were also unable to inhibit ^{125}I -TSH binding to porcine TSHR; their inhibition indexes were $0.5\text{--}8\%$.

Discussion

We have previously described the expression of biotinylated TSHR, TSHR-BIO, in HeLa cells using recombinant vaccinia virus (Minich *et al.* 1998). In the present work we prepared a biotinylated TSHR, TSHR-Xa-BIO, containing a site for blood coagulation protease factor Xa cleavage at the junction between TSHR and the biotin acceptor domain. TSHR-Xa-BIO was produced in HeLa cells using the vaccinia virus expression system. This system has been shown to be well suited for the production of functional, correctly processed and glycosylated TSHR (Minich *et al.* 1997, 1998). The vaccinia virus-infected HeLa cells were used for the isolation of radiochemically pure ^{125}I -labeled TSHR, capable of

Figure 4 Comparison of the ability of normal and Graves' disease sera to immuno-precipitate ^{125}I -TSHR with the ability of these sera to inhibit ^{125}I -TSH binding to porcine TSHR. The immunoprecipitating activities of the sera (solid bars) are expressed as c.p.m. bound, and the inhibiting activities (hatched bars) as the inhibition index, as explained in the text. The dotted line shows the level of non-specific binding of ^{125}I -TSHR by Protein A-agarose in the absence of serum. Each value represents the mean $\pm$ S.D. of duplicate measurements in three separate experiments.

binding TSH and Graves' autoantibodies. The isolation method is based on the use of two highly selective purification stages: (a) immobilization of TSHR-Xa-BIO on streptavidin-agarose, and (b) selective elution of bound TSHR-Xa-BIO by protease Xa cleavage.

The binding of biotinylated TSHR to streptavidin-agarose is very effective, because streptavidin has an exceedingly high affinity for biotin, with a K_d of 10^{-15} M, one of the strongest non-covalent bonds known between a protein and a ligand. The immobilized protein was radioiodinated using the chloramine T oxidation procedure directly on the solid phase. Solid phase labeling allowed quick and efficient (i.e. without loss of protein) removal of unreacted radioiodide from the reaction mixture. Moreover, after this stage, the ^{125}I -TSHR could be kept and eluted later in the presence of BSA and another agent to stabilize the receptor activity.

It is well known that the extremely tight binding of biotinylated proteins to avidin or streptavidin cannot be reversed under non-denaturing conditions. Thus ^{125}I -labeled TSHR was selectively eluted (freed of the biotinylation segment) from streptavidin-agarose by specific factor Xa cleavage. Factor Xa is a serine protease which recognizes and cleaves the peptide bond following the tetrapeptide sequence -Ile-Glu-Gly-Arg- with a high degree of specificity. This protease has often been used in the processing of recombinant fusion proteins (Nagai & Thogersen 1987). The method described can be very useful for isolation/radiolabeling of different proteins. Moreover, proteins can also be isolated in a non-radioactive form, using commercially available biotinylated Xa factor, which can easily be removed from the reaction

mixture by chromatography on streptavidin-agarose. A similar strategy was described by Osuga *et al.* (1998) for isolation of the ectodomain of human TSHR. They used a fusion between the TSHR ectodomain and a single-transmembrane domain of CD8 connected by a thrombin-cleavage site. The isolated protein was able to bind TSH and neutralize the stimulatory effects of Graves' antibodies on cAMP production.

The interest in labeled TSHR antigen is based on the possibility of its use for the detection of pathological autoantibodies. Previously, *in vitro* translated ^{35}S -labeled TSHR has been claimed to interact with Graves' autoantibodies (Morgenthaler *et al.* 1996). However, others were unable to detect any specific binding between *in vitro* translated ^{35}S -TSHR and Graves' autoantibodies or TSH (Prentice *et al.* 1997). We have now shown that ^{125}I -labeled TSHR prepared by our method could be useful for the immunoprecipitation analysis of pathological autoantibodies in unfractionated sera of patients with Graves' disease. All Graves' disease sera tested were positive in the immunoprecipitation assay, as they bound ^{125}I -TSHR more effectively than normal sera, and there was a clear positive correlation between the immunoprecipitating activity and the TSH-binding-inhibiting activity of different sera. The methodology described in this paper could be used for future development of a direct immunoprecipitation assay for TSHR autoantibodies. This assay would be capable of detecting all pathological autoantibodies to TSHR, not only those that inhibit hormone-receptor interaction, and thus could be very useful in the diagnosis and investigation of Graves' disease.

Acknowledgements

The authors wish to thank A Lüske (University of Ulm) for expert help in the preparation of recombinant vaccinia viruses. We express appreciation to Dr A Bergmann, Dr N Morgenthaler and T Chen (Brahms Diagnostica, Berlin) for useful discussions and assistance with experiments. We thank Dr D B Ramsden (University of Birmingham, UK) and Dr T Schmitt (University of Ulm) for critical reading of the manuscript. We thank J D Weymayer and J Siegmund (University of Ulm) for help in preparation of the manuscript. This work was supported by the Forschungsförderung of the University of Ulm (P 260) and Brahms Diagnostica (Berlin).

References

- Ban T, Kosugi S & Kohn LD 1992 Specific antibody to the thyrotropin receptor identifies multiple receptors forms in membranes of cells transfected with wild-type receptor complementary deoxyribonucleic acid: characterization of their relevance to receptor synthesis, processing, structure, and function. *Endocrinology* **131** 815–829.
- Chazenbalk GD, Nagayama Y, Russo D, Wadsworth HL & Rapoport B 1990 Functional analysis of the cytoplasmic domains of the human thyrotropin receptor by site-directed mutagenesis. *Journal of Biological Chemistry* **265** 20970–20975.
- Furmaniak J & Rees Smith B 1991 Structure of the TSH receptor. *Experimental and Clinical Endocrinology* **97** 160–164.
- Kohn LD, Shimura H, Shimura Y, Hidaka, Giulia C, Napolitano G, Ohmor M, Laglia G & Saji M 1995 The thyrotropin receptor. *Vitamins and Hormones* **50** 287–384.
- Loos U, Hagner S, Bohr URM, Bogatkewitsch GS, Jakobs KH & Van Koppen C 1995 Enhanced cAMP accumulation by the human thyrotropin receptor variant with the Pro52Thr substitution in the extracellular domain. *European Journal of Biochemistry* **232** 62–65.
- Metzger C, Michel D, Lüske A, Schlicht HJ & Mertens T 1994 Human cytomegalovirus UL97 kinase confers ganciclovir susceptibility to recombinant vaccinia virus. *Journal of Virology* **68** 8423–8427.
- Minich WB, Behr M & Loos U 1997 Expression of a functional tagged human thyrotropin receptor in HeLa cells using recombinant vaccinia virus. *Experimental and Clinical Endocrinology and Diabetes* **5** 282–290.
- Minich WB, Weymayer JD & Loos U 1998 Expression of a biotinylated human thyrotropin receptor in HeLa cells using recombinant vaccinia virus and its application for the detection of Graves' autoantibodies. *Thyroid* **8** 3–7.
- Misrahi M & Milgrom E 1997 The TSH receptor. In *Pharmacotherapeutics of the Thyroid Gland*, pp 33–73. Eds AP Weetman & A Grossman. Heidelberg/New York: Springer Verlag.
- Misrahi M, Ghinea N, Sar S, Saunier B, Jolivet A, Loosfelt H, Cerruti M, Devauchelle G & Milgrom E 1994 Processing of the precursors of the human TSH receptor in various eukaryotic cells (human thyrocytes, transfected L cells and baculovirus infected insect cells). *European Journal of Biochemistry* **222** 711–719.
- Morgenthaler N, Tremble J, Huang G, Scherbaum WA, McGregor AM & Banga J 1996 Binding of antithyrotropin receptor auto-antibodies in Graves' disease serum to nascent, *in vitro* translated thyrotropin receptor: ability to map epitopes recognized by antibodies. *Journal of Clinical Endocrinology and Metabolism* **81** 700–706.
- Nagai K & Thogersen HC 1987 Synthesis and sequence-specific proteolysis of hybrid proteins produced in *Escherichia coli*. *Methods in Enzymology* **153** 461–469.
- Nagayama Y, Wadsworth HL, Chazenbalk GD, Russo D, Seto P & Rapoport B 1991 Thyrotropin–luteinizing hormone/chorionic gonadotropin receptor extracellular domain chimeras as probes for TSH receptor function. *Proceedings of the National Academy of Sciences of the USA* **88** 902–905.
- Okamoto Y, Hayashi Y & DeGroot LJ 1995 Expression of human thyrotropin receptor by recombinant adenovirus. *Thyroid* **5** 51–53.
- Osuga Y, Liang SG, Dallas JS, Wang C & Hsuen AJW 1998 Soluble ecto-domain mutant of thyrotropin (TSH) receptor incapable of binding TSH neutralizes the action of thyroid-stimulating antibodies from Graves' patients. *Endocrinology* **139** 671–676.
- Perret J, Ludgate M, Libert F, Gerard C, Dumont JE, Vassart G & Prabhakar BS 1990 Stable expression of the human TSH receptor in CHO cells and characterization of differentially expressing clones. *Biochemical and Biophysical Research Communications* **171** 1044–1050.
- Prentice L, Sanders JF, Perez M, Kato R, Sawicka J, Oda Y, Jaskolski D, Furmaniak J & Rees Smith B 1997 Thyrotropin (TSH) receptor autoantibodies do not appear to bind to the TSH receptor produced in an *in vitro* transcription/translation system. *Journal of Clinical Endocrinology and Metabolism* **82** 1288–1292.
- Refetoff S, Sunthornthepvarakul T, Gottschalk ME & Hayashi Y 1996 Resistance to thyrotropin and other abnormalities of the thyrotropin receptor. *Recent Progress in Hormone Research* **51** 97–122.
- Tahara K, Ban T, Minegishi T & Kohn LD 1991 Immunoglobulins from Graves' disease patients interact with different sites on TSH receptor/LH-CG receptor chimeras than either TSH or immunoglobulins from idiopathic myxedema patients. *Biochemical and Biophysical Research Communications* **179** 70–77.
- Tonacchera M, Van Sande J, Parma J, Duprez L, Cetani F, Costagliola S, Dumont JE & Vassart G 1996 TSH receptor and disease. *Clinical Endocrinology* **44** 621–633.

Received 9 April 1998

Revised manuscript received 15 September 1998

Accepted 24 September 1998