Overexpression of the extracellular domain of the thyrotrophin receptor in bacteria; production of thyrotrophin-binding inhibiting immunoglobulins

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ABSTRACT
The availability of high affinity antibodies to the human TSH receptor (TSHR) would help in defining its functional domains, but this requires the production of pure receptor as immunogen. We have expressed the extracellular domain (ECD) of the TSHR (residues 21–414) as a fusion protein with maltose-binding protein (MBP) in Escherichia coli, using the pMAL-cR1 vector. The major protein in an electrophoretically separated, crude bacterial lysate had a molecular mass of 89 kDa, in agreement with the size predicted for the MBP-ECD fusion product. Its identity was confirmed by Western blotting in which it was recognized by two polyclonal antibodies to synthetic peptides of the TSHR and an anti-MBP. Following purification on an amylose column, 15 mg pure MBP-ECD per litre of culture were produced, which was 5% of the total bacterial protein. Following extensive dialysis in a buffer which produces slight denaturation, MBP-ECD was cleaved with factor Xa. The identity of each protein was confirmed by Western blotting.

To investigate the possibility of using the fusion protein as an immunogen we produced rabbit polyclonal antibodies to the ECD which were able to produce immunofluorescent staining of Chinese hamster ovary cells that expressed the TSHR, and revealed a protein of 95 kDa in Western blots of the same cells, in addition to a protein of 55 kDa. Only the protein of 55 kDa was detected in Western blots of human thyroid membranes. Subsequently, immunoglobulins from mice immunized with MBP-ECD were shown to contain TSH-binding inhibiting activity and to inhibit TSH-mediated cyclic AMP production; these mice had a lower serum thyroxine level when compared with mice immunized with the MBP-β galactosidase fusion protein MBP-GAL.

The study shows the feasibility of using recombinant TSHR expressed in E. coli (i) to produce antibodies which recognize the native receptor and thus could be applied to studies of TSHR expression (e.g. in thyroid tumours), (ii) to establish animal models of autoimmune hypothyroidism and (iii) as the starting material in denaturation and refolding experiments which may help in defining structure–function relationships.

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INTRODUCTION
The growth and differentiated function of the thyroid are controlled by thyrotrophin (TSH) which, on binding to its receptor, activates primarily the adenylyl cyclase–cyclic AMP (cAMP) cascade (Vassart & Dumont, 1992). The TSH receptor (TSHR), like those of the other glycoprotein hormones, luteinizing hormone (LH) and follicle-stimulating hormone, is a G protein-coupled receptor which is predicted to span the cell membrane seven times (Libert et al. 1991b). The TSHR thus has three extracellular loops and an extensive extracellular amino terminus which together constitute the ligand-binding site. The membrane-spanning domains with the three intracellular loops plus the carboxyl terminus are responsible for G protein coupling and signal transduction (Parmentier et al. 1989; Vassart et al. 1991). In addition to its natural ligand, the TSHR is the target of thyroid-stimulating autoantibodies (TSAbs) (Manley et al. 1982), which result in the hyperthyroidism characteristic of Graves’ disease (Rees Smith et al. 1988), or autoantibodies which
may bind and interfere with TSH binding, TSH-blocking antibodies (TBAbs), resulting in hypothyroidism in some cases of idiopathic myxoedema (Orgiazzi et al. 1976; Matsuura et al. 1993).

Since its cloning, many diverse strategies have been employed to try to define the binding sites of TSH/TSAbs/TBAbs and to elucidate how these large ligands do or do not affect signal transduction (Nagayama & Rapoport, 1992; Baker, 1993). The use of LHR/TSHR chimeras and screening a TSHR epitope library (Libert et al. 1991a) have revealed that TSH-/TSAb-/TBAb-binding sites are conformational and are thus composed of discontinuous segments throughout the extracellular domains (ECDs). These findings are difficult to reconcile with studies making use of synthetic peptides of the receptor, either to produce antibodies which have biological activity or to measure directly the binding of autoantibodies (Ohmori et al. 1991; Sakata et al. 1992). Another approach has been to produce full-length or truncated receptors in eukaryotic or prokaryotic expression systems; this has resulted in the establishment of binding (Costagliola et al. 1992) and bioassays (Ludgate et al. 1990) for TSAbs/TBAbs, and the demonstration that high affinity binding of TSH requires all of the extracellular part of the receptor, unlike autoantibodies whose binding seems not to require the loops (Harfst et al. 1992).

Some previous studies have produced monoclonal antibodies to the TSHR (Costagliola et al. 1991; Marion et al. 1992). The availability of large amounts of pure receptor as immunogen would constitute an obvious advantage both for antibody production and for establishment of animal models of autoimmunity. Here we report the production of milligram quantities of the amino-terminal ECD of the TSHR, fused to the maltose-binding protein (MBP) (di Guan et al. 1988), in bacteria, and the production and characterization of rabbit and mouse antibodies to the protein.

MATERIALS AND METHODS

Preparation of plasmid for expression studies

The 1.2 kb EcoRI–Xba I CDNA fragment coding for the ECD of human TSHR (ECD: amino acids 20–414, numbered as in Libert et al. 1989) was produced by PCR with the human TSHR in pSVL as template (Saiki et al. 1988). The fragment was introduced into the polylinker of vector pMAL-cR1 (Maina et al. 1988), downstream from the malE gene, which encodes MBP and results in the expression of an MBP fusion protein upon induction with isopropylthiogalactoside (IPTG). Transcription is controlled by the tac promoter.

Production of fusion proteins

*Escherichia coli* (TOPP 1 strain; Stratagene, La Jolla, CA, U.S.A.) was transformed with pMAL-cR1 or pMAL-cR1-ECD and transformants were selected with 100 μg ampicillin/ml, the presence of cDNA for pMAL and pMAL-ECD being verified by restriction analysis. Rich broth (1 litre; 10% tryptone, 5% yeast extract, 5% NaCl, 2% glucose, 100 μg ampicillin/ml) was inoculated with 10 ml of an overnight culture of cells containing the fusion plasmid or pMAL alone (note that the pMAL plasmid results in the production of an MBP–β galactosidase fusion protein referred to as MBP-GAL throughout), and maintained at 30 °C until an optical density (OD) at 600 nm of 0.3 was reached. Expression of the MBP-GAL and MBP-ECD fusion proteins was induced by growing the bacteria for a further 4 h in the presence of IPTG (50 μM). After this time, the cells were harvested by centrifugation (4000 g for 10 min), the supernatants discarded and the pellets resuspended in 10 ml lysis buffer (20 mM Tris, pH 7.4, 1 mM EDTA, 200 mM NaCl, 10 mM β-mercaptoethanol and lysozyme (1 mg/ml)) and saved for 30 min on ice. These suspensions were frozen overnight at −20 °C, thawed in cold water and sonicated for 2 × 1 min. After centrifugation at 9000 g for 20 min, the supernatants (CE1) were decanted and saved on ice.

Purification of fusion protein; factor Xa cleavage

The MBP-GAL and MBP-ECD CE1 fractions each obtained from 1 litre of culture were loaded onto an amylose column prewashed with a solution of 20 mM Tris, pH 7.4, 1 mM EDTA, 200 mM NaCl, 10 mM β-mercaptoethanol. The MBP-GAL or MBP-ECD fusion protein was eluted with column buffer containing 10 mM maltose. Fractions were collected and protein concentration measured by the Bradford method (Bradford, 1976). The purified MBP-GAL and MBP-ECD were dialysed against 50 mM Tris–Cl, pH 8, 100 mM NaCl, 1 mM CaCl₂, 0.5 M urea and the latter treated with 2 units of factor Xa (Boehringer, Mannheim, Germany)/50 μg fusion protein for 5 h at 4 °C.

Production of anti-human TSHR peptide antibodies and anti-MBP-ECD antibodies

Peptides were synthesized according to the deduced amino acid sequence of human TSHR (Libert et al.
1989), using an automated peptide synthesizer. The sequences of the TSHR-derived peptides were as follows: peptide 59 (residues 306–325), MQSLRQRKSVSLNSPLHQC (p59); peptide 60 (residues 350–369), AHYYVFEEQENIIQFG (p60). The peptides were conjugated to carrier keyhole limpet haemocyanin (Maloy et al. 1992) and rabbits were immunized by intradermal injection of 3 mg peptide conjugate complex. Three injections of 3 mg were given 2 weeks apart (the first with complete Freund’s adjuvant, the two others with incomplete Freund’s adjuvant). Blood was collected 2 weeks later. A similar protocol was used for immunization with MBP-ECD protein except that it was not coupled to a carrier and 500 μg fusion protein were used for each intradermal injection, in complete or incomplete adjuvant as described above.

**Purification of IgG from immune serum (IS) to MBP-ECD**

Immunoglobulins (IgGs) from IS or preimmune serum (preIS) were prepared by caprylic acid–ammonium sulphate precipitation (McKinney & Parkinson, 1987). Anti-MBP antibodies were eliminated by passing the IS through a column containing MBP-GAL coupled to CNBr-Sepharose (this resulted in IS-MBP<sup>−</sup>). Anti-ECD IgGs were then purified by binding the IS-MBP<sup>−</sup> to a column containing MBP-ECD coupled to CNBr-Sepharose and then eluting with HCl–glycine buffer, 0·2 m, pH 2·5.

**SDS-PAGE and Western blotting**

Induced and non-induced cell samples (100 μl) or the extract samples and purified proteins (50 μl) (approximately 20 μg/lane) were loaded onto 10% SDS-polyacrylamide gels (Laemmli, 1970), after reduction with β-mercaptoethanol at 100°C for 2 min, and electrophoresed. Proteins were then stained with Coomassie blue or electrophoretically transferred to nitrocellulose membranes (Towbin et al. 1979). Blots were incubated for 30 min with blocking solution (TBSN+milk: 10 mM Tris, pH 8, 150 mM NaCl, 0·05% Nonidet, 5% milk) at room temperature, and then stained with p59 and p60 antibodies or anti-MBP antibodies, followed by alkaline phosphatase-labelled anti-rabbit IgG and finally NBT-BCIP (nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl-phosphate) as substrate, both from Promega Corporation (Madison, WI, U.S.A.).

The same protocol was used for Western blots of Chinese hamster ovary (CHO) cells expressing the recombinant human TSHR (Perret et al. 1990) or human TSHR from thyroid membranes probed with purified anti-MBP-ECD antibodies. The 40 000 g membrane fraction from 10<sup>5</sup> CHO cells transfected with the human TSHR gene (JP09) or with the neomycin resistance gene only (JP02) was resuspended in 1 ml 3 mM Tris–Cl, pH 7·4, 1 mM MgCl<sub>2</sub>, 1 mM EGTA, 250 mM sucrose (buffer A). Purified plasma membranes were obtained by discontinuous sucrose gradient centrifugation. The 40 000 g membrane pellet was resuspended in 3·5 ml 3 mM Tris–Cl, pH 7·4, 1 mM EGTA (buffer B) enriched with 50% (w/w) sucrose (density=1·23 g/ml) to obtain a concentration of 2 mg protein/ml. This solution was placed in 1·35 ml tubes and overlaid by sucrose solutions of 41% (2·2 ml; density=1·18 g/ml), 37% (3·2 ml; density=1·16 g/ml) and 26% (2·9 ml; density=1·10 g/ml) (Carayon et al. 1979). Centrifugation was performed in a Beckman ultracentrifuge in rotor SW 27 at 25 000 r.p.m. and 4°C for 3 h. Membranes were harvested from the interfaces, diluted with 10 ml buffer B and centrifuged at 40 000 g for 10 min. The fraction recovered at the interface between 37 and 26% sucrose was washed twice with cold acetone/water/ethanol, dried, resuspended in electrophoresis sample buffer (2% SDS, 6 mM Tris–Cl, pH 6·8, 10% glycerol) containing 5% β-mercaptoethanol and boiled for 1 min; 100 μg were loaded for 10% SDS-PAGE.

Human thyroid tissue was obtained, with informed consent, from patients with Graves’ disease undergoing thyroidectomy. The fresh tissue was transported to the laboratory on ice, and the crude thyroid membranes were prepared at 4°C, as previously described (Carayon et al. 1979). Briefly, the thyroid tissue was homogenized in buffer A. After differential centrifugation between 300 and 30 000 g, the fraction obtained was resuspended in the homogenization buffer A and stored at −80°C. Purified plasma membranes were obtained by discontinuous sucrose gradient as described for CHO cells and 100 μg were loaded for 10% SDS-PAGE after reduction with β-mercaptoethanol at 100°C for 1 min.

**Protein determination**

Protein concentrations were determined by the colorimetric method of Bradford using BSA as standard (Bradford, 1976).

**Enzyme-linked immunosorbent assay (ELISA)**

Purified fusion protein MBP-ECD diluted in 0·1 M sodium carbonate–bicarbonate buffer, pH 9·6 (5 μg/ml, 100 μl/well), was incubated in microwell plates
overnight at 4 °C. After washing and saturation with 10 mM sodium phosphate, 150 mM NaCl, pH 7.3 (PBS), containing 0.1% Tween and 5% BSA, 100 µl rabbit serum samples containing anti-TSHR, anti-p59 or anti-p60 diluted in blocking buffer were added for 2 h at room temperature. Subsequently, the plates were washed and alkaline phosphatase-labelled goat anti-rabbit antibodies were added. After 1 h of incubation at room temperature, wells were washed and substrate added (1 mg p-nitrophenyl phosphate/ml in 10% diethanolamine buffer, 1 mM MgCl₂, pH 9.8). All determinations were performed in duplicate and the absorbance was read at 405 nm in a spectrophotometer. The same method was used with polyclonal antibody to the ECD fusion protein (IS), the preIS and the IS-MBP-fraction on ELISA plates coated with MBP-ECD fusion protein (as above) or MBP alone (5 µg/ml, 100 µl/well).

**Immunofluorescence**

CHO cells, JP09 and JP02, were cultured on 13 mm coverslips in 1 ml Ham’s F12 medium+10% fetal calf serum in 24-well culture plates at 37 °C overnight. The medium was aspirated and the cells were washed three times in PBS. They were incubated for 1 h at room temperature with 100 µl/coverslip of preIS, IS (both diluted 1:20 in PBS) or neat affinity-purified anti-ECD IgGs or
without a first antibody. Following three washes with PBS, coverslips were incubated with 100 µl sheep anti-rabbit IgG conjugated to fluorescein isothiocyanate (Amersham International plc, Amersham, Bucks, U.K.) diluted 1:30 in PBS, for 1 h at room temperature in the dark. The coverslips were washed three times in PBS, rinsed in cold 5% acetic acid/95% ethanol, mounted in Gelvatol (20–30 polyvinyl alcohol; Monsanto Polymers and Petrochemicals Co., St Louis, MO, U.S.A.) and viewed by oil immersion on a fluorescence microscope.

Immunization of mice

Two groups of ten 6-week-old male Balb c × Jico mice were immunized on day 0 with 100 µg MBP-GAL or MBP-ECD in 200 µl containing 10 µl Maalox (Rhone Poulenc Rover, Paris, France) and 50 µl Vaxicoq (Institut Merieux, Lyon, France) in 0.9% NaCl. On days 15 and 35 they were injected with the same preparations but containing 50 µg MBP or MBP-ECD.

Blood was obtained via the retro-ocular capillaries on days 0 and 49. Immunoglobulins were prepared by caprylic acid–ammonium sulphate precipitation as previously described. Immunoglobulins from 100 µl serum were resuspended in 100 µl PBS.

TSH-binding inhibiting immunoglobulin (TBI1) activity was measured on intact CHO cells expressing the human TSHR (JP09; Costagliola et al. 1991). Briefly, 10^6 cells in 24-well plates were incubated in 0.3 ml modified Hanks' buffer without NaCl with isotonicity maintained with 280 mM sucrose, supplemented with 0.2% BSA, 125I-labelled TSH (10 000 c.p.m.; the kind gift of Henning Tempelhof, Berlin, Germany) and 10 µl of mouse immunoglobulins for 2 h at 37 °C. At the end of the incubation period, the cells were rapidly rinsed with the same ice-cold buffer and solubilized with 1 ml 1 M NaOH, and radioactivity was measured in a γ counter. Results are expressed as c.p.m. 125I-labelled TSH bound.

Thyroid-stimulating and -blocking antibody activities were measured using CHO cells (JP26; Ludgate et al. 1990). Briefly 5 × 10^4 cells/well in 96-well plates were incubated in 5.4 mM KCl, 0.44 mM KH₂PO₄, 0.47 mM MgSO₄, 0.35 mM Na₂HPO₄, 0.9 mM CaCl₂, 0.1% glucose, 2 mM isobutyl methylxanthine, 20 mM Hepes and 0.3% BSA containing 10 µl immunoglobulins in PBS to a total volume of 100 µl per well. Incubation was for 4 h at 37 °C; CAMP released into the medium was measured using an RIA (Amersham International plc). TSAbs were measured in the basal conditions described above and TBAbs in identical conditions but with the addition of bovine TSH (1 mU/ml final concentration; Thytopar; Rorer Pharmaceutical Corp., Fort Washington, PA, U.S.A.). Results are expressed as pmol cAMP/well.

Serum free thyroxine (T₄) levels were measured by Gammacoat RIA (Travenol-Genentech Diagnostics, Cambridge, MA, U.S.A.). Results are expressed as µg/dl.

Statistical analysis

Results are presented as means ± s.d. and compared by Student’s t-test; a value of P<0.05 was considered significant.
RESULTS

Protein expression

An 89 kDa species, representing the major protein of the total bacterial lysate, appeared on Coomassie blue-stained SDS-polyacrylamide gels for E. coli transformed with pMAL-cR1-ECD and induced with 50 μM IPTG (Fig. 1, lanes 2–5), in agreement with the predicted molecular mass of the MBP-ECD fusion protein. From 1 litre of culture, we were able to obtain 15 mg of this fusion protein after purification on an amylose column (Fig. 2, lane 2). In addition, a significant proportion of the protein remained in the unpurified fraction as insoluble inclusion bodies.

Western blot analysis of fusion proteins

The identity of the 89 kDa protein as an MBP-ECD fusion protein was confirmed in Western blots using two anti-TSHR peptide antibodies, anti-p59 and anti-p60, each of which revealed a band of 89 kDa for induced E. coli transformed with pMAL-cR1-ECD, but not for non-induced bacteria or control E. coli transformed with pMAL-cR1 (Fig. 3a).

Cleavage of purified MBP-ECD with factor Xa

Extensively dialysed MBP-ECD (1 mg) was cleaved with 40 μg factor Xa and subjected to SDS-PAGE (Fig. 2). Lane 3 shows the digestion of MBP-ECD with factor Xa. After 5 h at 4 °C with the enzyme, the amount of 89 kDa protein was reduced and two new proteins of lower molecular mass appeared at approximately 47 kDa (ECD) and 42 kDa (MBP). Within experimental error, the sum of the sizes of cleaved bands equals that of the original uncleaved band. Western blotting (Fig. 3b) clearly demonstrated that the band at 47 kDa was reactive with anti-p59, while the lower band of 42 kDa was recognized by the anti-MBP serum only. As
expected, some uncleaved material remaining in the sample was recognized by both antisera.

**Reactivity of anti-TSHR peptides with purified MBP-ECD**

When affinity-purified MBP-ECD protein was coated onto ELISA plates, anti-MBP, anti-p59 and anti-p60 bound with high affinity, but this was not the case with normal rabbit serum or preIS (Fig. 4). To verify the specificity of the binding of the anti-TSHR peptides to MBP-ECD, we preincubated the sera with 5 µg each of p59 or p60, MBP-ECD protein or MBP alone. For each serum, only the original peptide used for immunization or the MBP-ECD protein was able to inhibit this binding, as shown in Fig. 5.

**Production and characterization of rabbit IS**

Purified MBP-ECD was used to immunize two rabbits to produce polyclonal antibodies to the TSHR. Results from the two rabbits were identical; only one is reported. The resulting IS bound with high affinity to the MBP-ECD in an ELISA (Fig. 6) and we were able to demonstrate specificities for the ECD part of the fusion protein by testing the IS, depleted of anti-MBP, after passage through an MBP-CNBr-Sepharose column. This IS-MBP⁻ fraction showed the same level of reactivity as the IS on MBP-ECD-coated plates but the signal was greatly decreased on plates coated with MBP alone, as shown in Fig. 7. This result was further confirmed using affinity-purified anti-ECD, generated by binding the IS-MBP⁻ on an MBP-ECD-CNBr-Sepharose column and elution at acid pH.

**Western blotting of JP09, JP02 and human thyroid membranes**

In Western blots of a purified plasma membrane preparation from CHO cells expressing the human TSHR (JP09), purified anti-ECD IgGs revealed...
two broad bands, at about 95 and 55 kDa, which had the characteristic appearance of glycosylated proteins (Fig. 8a). These bands were absent in blots of JP02 or in blots of JP09 probed with the preIS or anti-ECD IgGs preincubated with MBP-ECD fusion protein. In contrast, repeated attempts using various human thyroid preparations probed with anti-ECD IgGs revealed only a protein band at about 55 kDa (Fig. 8b), which was absent from blots probed with the preIS or anti-ECD IgGs preincubated with MBP-ECD fusion protein.

**Immunofluorescence on JP09 and JP02**

As shown in Fig. 9, when affinity-purified anti-ECD IgGs or the IS were applied to unfixed JP09 cells grown on coverslips, bright speckled immunofluorescence was observed. This was absent on JP02 cells or on JP09 cells stained with preIS or IS preincubated with MBP-ECD. Depending on the field, individual JP09 cells appeared variably fluorescent, but on adjusting the focus all cells were seen to be positively stained.

**Characterization of mouse IS**

Because of the difficulty in obtaining large serum samples by retro-ocular bleeding, samples from the ten mice in each group were pooled. As shown in Table 1, the preIS from both groups contained essentially identical amounts of T₄. Following immunization, the T₄ levels were reduced by 19% in the MBP-treated group and by 31% in the MBP-ECD-treated mice; the difference was significant (P<0.02). There was no difference in the binding of ¹²⁵I-labelled TSH to the human TSHR in the presence of immunoglobulins from preimmune and immune MBP-treated mice and this result was confirmed by TSH-mediated cAMP production. Conversely, immune immunoglobulins from the MBP-ECD-treated animals inhibited the binding of ¹²⁵I-labelled TSH to the receptor and also reduced the cAMP produced in response to 1 mU bovine TSH/ml (P<0.005). None of the immunoglobulins contained TSAb activity (data not shown).

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FIGURE 9. Immunofluorescence of (a) CHO cells expressing the human TSHR and (b) control CHO cells. In both cases, cells were non-fixed and were stained with affinity-purified anti-ECD immunoglobulins.

TABLE 1. Characteristics of immunoglobulins and sera of MBP-GAL- and MBP-ECD-treated mice. Values are means ± s.d. of duplicate estimations, each performed at least twice.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>MBP-GAL-treated</th>
<th>MBP-ECD-treated</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Preimmune</td>
<td>Immune</td>
</tr>
<tr>
<td>Serum T₄ (µg/dl)</td>
<td>4.3 ± 0.37</td>
<td>3.5 ± 0.1</td>
</tr>
<tr>
<td>TBIIs (c.p.m. ¹²⁵I-labelled TSH bound)</td>
<td>3233 ± 35</td>
<td>3151 ± 31</td>
</tr>
<tr>
<td>TBAbs (pmol cAMP/well)</td>
<td>122 ± 8</td>
<td>139 ± 3</td>
</tr>
<tr>
<td>(1 mU/ml TSH)</td>
<td></td>
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</tr>
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*P<0.02, **P<0.005 compared with MBP-GAL-treated mice.

TBIIs = TSH-binding inhibiting immunoglobulins.
TBAbs = TSH-blocking antibodies measured in the presence of 1 mU TSH/ml.

DISCUSSION

In the present work we describe the production of milligram quantities of a fusion protein containing the ECD of the human TSHR. In previous studies of this type either much lower quantities of protein were produced or (Huang et al. 1992, 1993), where sufficient protein to be visualized by Coomassie blue staining was expressed, it was insoluble and thus impossible to purify from the other bacterial proteins (Harfst et al. 1992). We also encountered problems of solubility, as shown by the quantity of protein remaining in the unpurified fraction, but were still able to produce useful quantities of pure material. We can confirm the limited biological activity of the recombinant protein (Huang et al. 1992, 1993), since we were unable to demonstrate any binding of TSH to the fusion protein, either directly or by displacement, even after slow renaturation in the presence of a glutathione protein disulphide isomerase cocktail.
We attributed this lack of binding to improper folding of the ECD in the presence of MBP. Extensive dialysis in 0.5 M urea enabled us to cleave MBP from the ECD with factor Xa (Nagai & Thogersen, 1984). Further denaturation and refolding experiments are underway and will be the subject of future study.

One of the aims of producing large amounts of receptor was to use this as an immunogen to generate monoclonal antibodies of potential interest, e.g. antibodies which inhibit the binding of TSH to its receptor. In view of the small quantities of MBP and ECD produced after factor Xa cleavage we used the intact fusion protein to produce rabbit polyclonal antibodies. These antibodies clearly contained specificities capable of recognizing the native receptor, as demonstrated by the positive immunofluorescence of CHO cells expressing the human TSHR. We tested the biological activity of our anti-TSHR peptides and the anti-rabbit MBP-ECD. No stimulating or TSH-binding inhibiting activity in any of these polyclonal antibodies could be found when compared with their respective preIS. However, many of them, both immune and preimmune, had a non-specific stimulating effect on cAMP accumulation when measured using CHO cells expressing the TSHR. This indicates the need for careful controls when claiming bioactivity for antibodies to synthetic peptides of TSHR (Ohmori et al. 1991; Sakata et al. 1992).

Unlike other authors (Russo et al. 1991; Endo et al. 1992), we experienced considerable difficulty in obtaining a satisfactory Western blot of the TSHR. We were obliged to enrich our receptor preparation on sucrose gradients, even when using cells with 100 times the number of receptors expressed on a thyrocyte, and to use an affinity-purified antibody. In these conditions we were able to demonstrate a protein of 95 kDa, whose appearance on the blot suggests a glycosylated protein, in CHO cells expressing the TSHR. The staining was specific, since it was not present with control CHO cells and could be eliminated by preincubation of the antibody with fusion protein (data not shown). From this we calculated that 10-15 kDa were contributed by glycosylation of the TSHR; this is in agreement with the value calculated for the ECD expressed in eukaryotic cells (Harfst et al. 1992). When using sucrose-enriched receptors from several human thyroid preparations we could demonstrate a protein of 55 kDa. Despite the use of protease inhibitors, it seems difficult to make a choice between the single subunit (Russo et al. 1991) or two subunit (Loosfelt et al. 1992) model of the TSHR when deciding which form is present on thyrocytes. Some, who favour the two subunit model for the receptor (Loosfelt et al. 1992), interpret the result in CHO cells as being due to incomplete processing of the receptor overproduced in these non-thyroidal cells. The production of high affinity monoclonal antibodies which can be applied to Western blots of normal thyrocytes with no need for sucrose enrichment may solve this controversy.

Finally, we have immunized mice with MBP and the MBP-ECD protein. Our preliminary results indicate the presence of TBI and TBAb activities in the immunoglobulins of the receptor-immunized mice when compared either with their preimmune immunoglobulins or with immunoglobulins from mice treated with MBP alone. These activities were accompanied by a reduction in circulating $T_4$ levels which is highly suggestive of an induced autoimmune hypothyroidism. Details of these experiments, and their extension to mice of different genetic backgrounds, will be reported separately.

In conclusion, the present study demonstrates the potential of using the TSHR expressed in bacteria (i) to generate antibodies capable of recognizing the native receptor, which in the future will allow the investigation of receptor expression in thyroid tumours and in extra-thyroidal tissues containing receptor mRNA transcripts, (ii) as an immunogen to establish animal models of autoimmune diseases involving the receptor and (iii) to produce large quantities of receptor for denaturation and refolding experiments which are prerequisites for detailed structural analyses.

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REFERENCES


**REVISED MANUSCRIPT RECEIVED 18 November 1993**