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PROKARYOTIC EXPRESSION OF THE THYROTROPIN RECEPTOR AND IDENTIFICATION OF AN IMMUNOGENIC REGION OF THE PROTEIN USING SYNTHETIC PEPTIDES

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Graves' disease is characterized by hypersecretion of thyroid hormones due to binding of autoantibodies to the thyrotropin receptor (TSHR). In order to study immunological aspects of the TSHR we expressed the extracellular domain of the rat TSHR (ETSHR) as a fusion protein with β-galactosidase in a prokaryotic system. The identity of this ETSHR-fusion protein was confirmed by Western blot, using antibodies to synthetic peptides derived from TSHR. Patients' sera reacted to a significantly greater extent with the affinity purified ETSHR relative to control sera. Similarly, sera from patients with Graves' disease displayed significant reactivity with only one of five peptides, RH2 (residues 352-366), when compared with normal sera. These data, together with the predicted hydrophilicity of the peptide RH2, suggest that amino acids 352-366 which lie within one of the unique regions of the extracellular domain of the TSHR may be important for antibody binding.

Graves' disease is an autoimmune disease characterized by hypersecretion of thyroid hormones and enlargement of the thyroid gland (1). Autoantibodies directed at the thyrotropin receptor (TSHR) are able to stimulate the gland, like TSH, and are central to the pathogenesis of Graves' disease (2). Very little is known about underlying mechanisms of the pathogenesis of the disease. Until recently, not much was known about the structure of the receptor because of the difficulty in purifying TSHR. (3). However, the recent cloning of cDNAs for this receptor has revealed several aspects of the nature of the TSHR (3-7). The rat cDNA encodes a protein of 764 amino acids containing a large extracellular domain of approximately 400 residues and seven hyrophobic membrane spanning regions. Transfection of TSHR cDNA into non-thyroidal cells confers TSH binding properties to these cells and allows transfected cells to respond to TSH, as assessed by cAMP production.

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Expression of relatively pure protein is essential to understand the structure-function relationships of the TSHR and its role in the autoimmune process. In this report, we describe successful expression and purification of the rat ETSHR in a prokaryotic system. This receptor was derived from FRTL-5 cells, which are known to respond to thyroid stimulating antibodies in 90-100% of Graves' patients. FRTL-5 cells are currently the most used biodetection system for such antibodies 2. We describe the reactivity of the expression product with sera from patients with Graves' disease.

The TSHR belongs to a group of G protein-coupled receptors, and bears strong homology to the luteinizing hormone/chorionic gonadotropin (LH/CG) and follicle stimulating hormone (FSH) receptors (8). However, the cDNA for TSHR encodes two segments, amino acids 38-45 and 317-366, unique to the extracellular domain of the TSHR (9). We have used synthetic peptides from in and around one of these unique areas (residues 217-366) and antibodies to these peptides to map an area of the extracellular domain that possesses high immunoreactivity and may prove to be of importance in the pathogenesis of Graves' disease.

MATERIALS AND METHODS

Construction of plasmid for expression studies: The 1.2 kb EcoRI-EcoRV cDNA fragment encoding all but the terminal five amino acids of the extracellular domain of the rat TSHR was purified from pSG5-TSHR (4), blunt ended, ligated (T4 DNA ligase, BRL, Gaithersburg, MD) to NcoI linker and then subjected to digestion with NcoI (Figure 1) (10). This

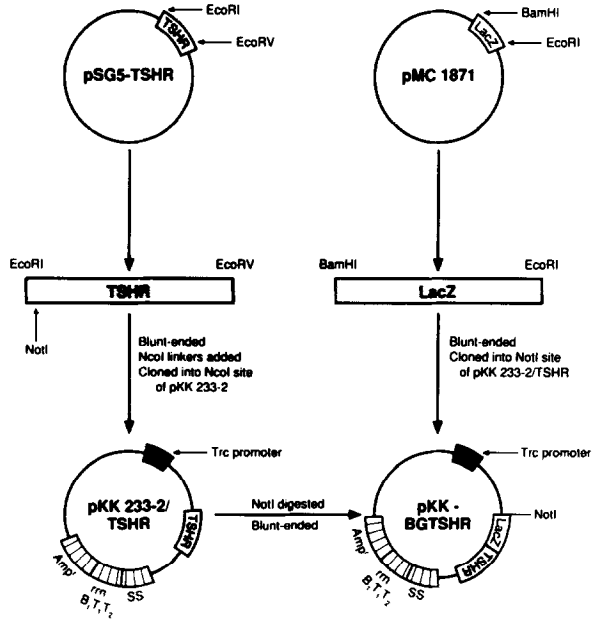

Figure 1. Construction of expression plasmid for β-gal-ETSHR fusion protein.

fragment was cloned into the NcoI site of the plasmid pKK233-2 (Pharmacia). The resulting ligated product was digested with NotI and blunt ended (pKK-TSHR). A BamHI-EcoRI fragment coding for β galactosidase was purified from pMC1871 (Pharmacia, Piscataway, NJ), blunt ended and ligated to pKK-TSHR to yield pKK-BGTSHR. Correct orientation of cDNA for TSHR and β-galactosidase was confirmed by restriction analysis.

Production and purification of fusion protein: JM105 *E. coli* were transformed with pKK-BGTSHR (10). Transformants were selected by growing bacteria in the presence of 100 μg/ml ampicillin. The presence of pKK-BGTSHR was confirmed in the selected colony by hybridization, and correct orientation of cDNA for β-galactosidase and TSHR was verified by restriction analysis. Transformed JM105 *E. coli* were cultured in LB media with 100 μg/ml ampicillin and allowed to grow at 37°C overnight. Fifty μl of this overnight culture was added to 5 ml of LB medium and cultured for 2 hrs. The TSHR synthesis was induced by growing bacteria for 3 hrs in the presence of isopropyl β-D-thiogalactoside (IPTG) (1 mM). Bacterial pellet obtained from 200 ml of culture was suspended in two ml of TEP buffer (100 mM Tris-HCl pH 7.4, 10 mM EDTA, 1 mM phenyl methyl sulfonyl fluoride). This suspension was sonicated and subjected to two freeze-thaw cycles, and then centrifuged. The supernatant was applied to an immunoaffinity column containing 2 ml of ProtoSorb lacZ HA, an adsorbent containing monoclonal antibodies to β-galactosidase (Promega, Madison, WI). The column was washed with a solution of 50 mM Tris-HCl, pH 7.3 and 0.2% NP-40. Elution was with 3 ml of 0.1M $NaHCO_3/Na_2CO_3$ (pH 10.8).

SDS-PAGE and Western Blot: Bacteria from 100 μl of culture were subjected to SDS-PAGE (Novex, San Diego, CA). Proteins were electrophoretically transferred (S&S Mini-Profile System, Keene, NH) to nitrocellulose membranes (S & S). Blots were incubated for 1 hr with blocking solution (1% bovine serum albumin in phosphate buffered saline, pH 7.2) at room temperature, and then stained using anti-peptide antibodies, peroxidase labeled anti-rabbit IgG (H and L) (Kirkegaard and Perry Labs, Gaithersburg, MD), and 4-chloro-1-naphthol (Fluka, Switzerland) as described earlier (11).

Generation of anti-TSHR Peptide Antibodies: Overlapping TSHR derived peptides were selected based on their position in and around one of the unique regions of the TSHR and potential for immunogenicity (Antigen program, IntelliGenetics, Inc., Mountain View, CA) (Table 1). Peptides were synthesized according to the deduced amino acid sequence of TSHR using an automated peptide synthesizer, as previously described (11). The peptides were conjugated to super carrier EDC (Pierce, Rockford, IL), and rabbits were immunized by intradermal injection of 0.5 mg of the peptide-conjugate complex. Two booster injections of 0.5 mg were given 2 weeks apart. Blood was obtained from the ear vein 2 weeks later and separated to give serum containing anti-TSHR peptide antibodies.

TABLE 1. SEQUENCE OF THE TSHR-DERIVED PEPTIDES

Peptide Designation	Residue Numbers	Amino Acid Sequence
RH2	352-366	YYVFFEEQEDEIIGF
RH3A	357-372	EEQEDEIIGFGQELKN
RH3B	373-388	PQEETLQAFDSHYDYT
H4	377-397	TLQAFDSHYDYTICGDSKDMV
R4	377-397	TLQAFDSHYDYTVCGDNEDMV

R refers to peptide sequence derived from the rat TSHR, H to that derived from the human TSHR, and RH implies that the predicted amino acid sequences are identical in the rat and the human TSHR. Underlined residues in H4 and R4 depict the differences between these two peptides.

ELISA: Immulon 2 microtiter plates (Dynatech, VA) were coated with 1 μg of TSHR derived peptides in 100 μl of 0.1 M sodium carbonate-bicarbonate buffer, pH 9.6. Wells were washed with 0.05% Tween 20 in PBS, pH 7.3. The following were then added in succession, with washing between each step: 100 μl of blocking buffer (0.05% Tween 20 and 0.1% gelatin in PBS, pH 7.3) for 30 minutes at room temperature, 100 μl of serum samples diluted with blocking buffer at room temperature for 1 hr, 100 μl of 1:500 peroxidase labeled affinity purified antibody to rabbit IgG at room temperature for 1 hr, and 100 μl of substrate solution (o-phenylenediamine and hydrogen peroxide in water) at room temperature for 20 minutes. The enzymatic reaction was terminated by the addition of 2N sulfuric acid, and absorbance read at 488 nm in a microELISA reader.

Serological testing of the ETSHR fusion protein was done in a similar manner. One hundred μl of buffer containing 100 ng of the affinity purified ETSHR was used to coat each well of a 96 well plate. Sera from newly diagnosed, untreated patients with Graves' disease and normal subjects were diluted 1:160 in PBS containing 0.1% fetal bovine serum. Six μl of a 1 mg/ml β-galactosidase (BRL) solution and 30 μl of a *E. coli* lysate solution were added to 1 ml of the diluted sera. This sample was incubated at room temperature for one hour, and then centrifuged. One hundred μl of the supernatant was added to each well and incubated for 2 hrs at room temperature. Wells were then washed, followed by the addition of 100 μl of 0.5 μg/ml solution of a Gammabind G peroxidase conjugate (Genex, Gaithersburg, MD). The detection of antibody binding was as described above.

RESULTS

Protein Expression: No unique bands were visible in JM105 *E. coli* transformed with pKK-BGTSHR in comparison to untransformed bacteria, on Coomassie blue staining of IPTG induced proteins (data not shown). Bacterial proteins were affinity purified using anti-β galactosidase antibodies and then separated on SDS-PAGE. Coomassie blue staining revealed two major proteins, one of molecular weight 106 kDa (representing β galactosidase), which was present in both transformed and non-transformed bacteria, and a unique band of 162 kDa was noted only in transformed bacteria (data not shown). The identity of this protein as a ETSHR fusion protein was confirmed by its detection by a cocktail of anti-TSHR peptide antibodies on Western blot (Figure 2). The apparent MW of the ETSHR produced in this prokaryotic expression system was calculated to be 56 kDa: this is in agreement with the predicted size.

Reactivity of patients' sera: The affinity purified fusion protein was coated on ELISA plates. Sera from patients with active Graves' disease and normal subjects were tested for reactivity with the ETSHR. Sera were preadsorbed extensively with *E. coli* extract. Sera from Graves' patients displayed significantly greater reactivity with the ETSHR than did those from normal control subjects (Figure 3). Approximately 60% of sera from Graves' patients, gave readings significantly greater than those found with sera from normal subjects.

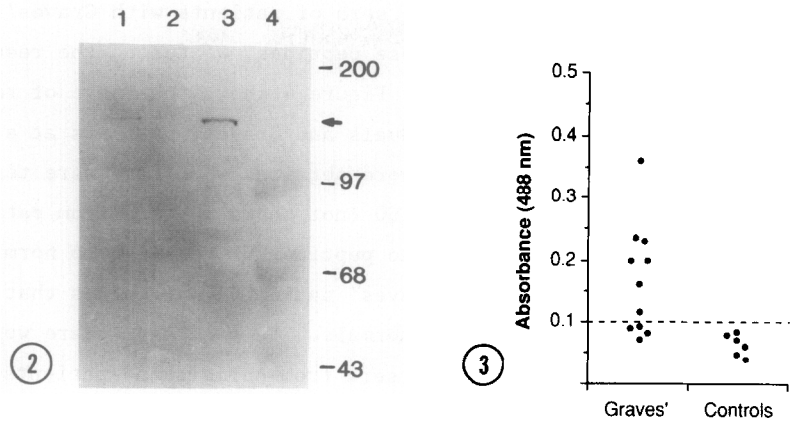

Figure 2. Expression of β-gal-ETSHR fusion protein in transformed JM105 *E. coli*. Western blot analysis of proteins. Lanes 1 and 2: Transformed and non-transformed *E. coli* lysate respectively; purified on an anti-β-galactosidase column. Lane 3: transformed *E. coli* lysate. Lane 4: Molecular weight markers. The arrow demonstrates a unique band in lanes 1 and 3, representing β gal ETSHR-fusion protein, in transformed bacteria. A lower molecular weight band seen in Lane 1 is perhaps a cross-reacting protein present in transformed bacteria and disappears on affinity purification (Lane 3).

Figure 3. Reactivity of human sera at a dilution of 1:160 with purified β-galactosidase TSHR fusion protein. Dotted line indicates mean ± 2SD for control group.

Studies with Synthetic Peptides: To start to define the epitopes on the TSHR with which autoantibodies react, sera from Graves' patients were tested with a number of synthetic peptides derived from the rat and human TSHR sequences. These peptides were chosen in part on the basis of predicted potential immunogenicity. The sequences are shown in Table

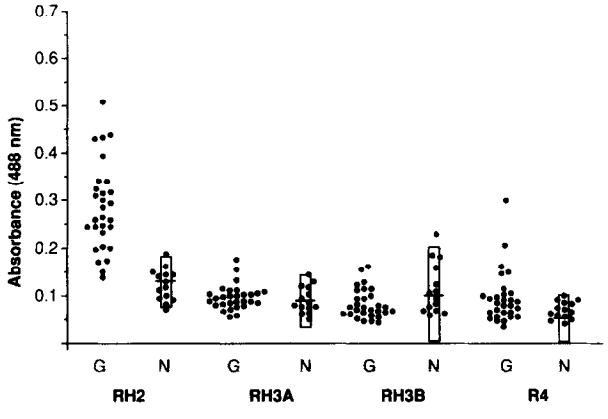

Figure 4. Reactivity of human sera with indicated TSHR derived peptides shown at a serum dilution of 1:40. Essentially similar results were obtained over the range of 1:20 - 1:160 dilution. Boxed areas depict mean ± 2SD for normal sera against each peptide. Sera were also tested against peptide H4 and yielded results similar to that shown for R4.

1. To see whether antibodies in the sera of patients with Graves' diseases could be detected using these peptides, we tested the reactivity of human sera against peptides. Figure 4 shows the range of reactivity of sera from normal individuals and Graves' patients at a dilution of 1:40. Similar results were obtained when sera were tested at dilutions ranging from 1:20 - 1:160 (not shown). Sera from patients showed considerably higher binding to peptide RH2 relative to normals. Over 85 percent of the sera from Graves' patients gave values that were greater than the mean + 2SD of the normals. In contrast, there were no significant differences between the sera from normals and patients in their binding to petides RH3A, RH3B, and R4. Results obtained with peptide H4 were essentially similar to that obtained with peptide R4 (not shown).

DISCUSSION

We have expressed the extracellular domain of the TSHR as a fusion protein in a prokaryotic system. Studies with a number of receptor proteins (for example, CD4, and acetylcholine receptor) had strongly indicated that the critical binding sites for the ligand and antibodies reside in the extracellular domain (12,13). Moreover, in an attempt to reduce the toxicity of membrane proteins to bacteria (14), and to aid in purification, only the soluble portion of the TSHR was expressed, purified, and used for serological testing. As predicted, the presence of immuno-reactive epitopes in the extracellular domain of the TSHR was confirmed by the finding that sera from a majority of patients with Graves' disease, but none of the normals showed significant reaction with purified ETSHR fusion protein.

In order to identify immunogenic regions on the TSHR that might be relevant to the Graves' disease, we used synthetic peptides. The use of synthetic peptides has proven very useful in studies of myelin basic protein and the acetylcholine receptor (AChR) (15,16). A potentially important area on the TSHR lies between residues 317-366. This area is unique to the TSHR, when compared to LH/CG-R (9). Moreover, amino acid residues 339-367 are the most hydrophilic residues of the extracellular domain, and therefore have a very high probability of being immunogenic. Using peptides derived from this region, we have mapped an area of high immunogenicity represented by residues 352-366 (peptide RH2). This area is different from those reported in previous studies using synthetic peptides (17-19). Our conclusion is supported by the findings that peptide RH2 was one of the most immunogenic in rabbits, and mice (not

shown); and that over 85% of sera from patients with Graves' disease reacted with RH2. Peptide RH3A (357-372) has 10 amino acid residues overlapping those of peptide RH2 (352-366). Despite this similarity, RH3A peptide showed no significant reactivity when tested with Graves' sera. Similarly, Graves' sera did not react with two other peptides (residues 347-361 and 362-376) (not shown). These differences may be due to differences in composition, charge or conformation between these peptides, or indicate that amino acid residues 352-356 may play a critical role in determining the immunoreactivity of sera from patients with the Graves' disease.

Availability of purified TSHR, peptides, and antipeptide antibodies should facilitate studies to further delineate the structure and function relationship of the receptor. Moreover, this will also aid in understanding the pathogenesis and pathology of Graves' disease.

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