Production of bioactive amino-terminal domain of the thyrotropin receptor via insertion in the plasma membrane by a glycosylphosphatidylinositol anchor

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Abstract A chimeric cDNA construct encoding the extracellular amino-terminal domain (ECD) of the thyrotropin receptor fused to the signal for addition of glycosylphosphatidylinositol from the Thy-1 gene directs efficient expression of the ECD at the plasma membrane of transfected CHO cells. A cell line (GT14) expressing over 10^6 receptors/cell was isolated, which allows direct detection, by flow cytometry, of autoantibodies from the majority of patients with Graves' disease or autoimmune idiopathic myxedema. Treatment of GT14 cells with a glycosylphosphatidylinositol-specific phospholipase C (PI-PLC) releases a soluble 80 kDa molecule which neutralizes the autoantibodies from Graves patients. Whereas it does not bind TSH when released from the cells by PI-PLC in free form, the soluble ECD displays clear TSH binding activity when it is released as a complex with a monoclonal antibody recognizing a conformational epitope of the ECD. Our results allow production of bioactive ECD of the thyrotropin receptor in high yield, with possible applications in structural analyses.

Key words: Glycoprotein hormone receptor; Graves' disease; Autoimmunity; Autoantibody

1. Introduction

Together with the other glycoprotein hormone receptors, the thyrotropin receptor (TSHr) displays a bipartite structure, with a large amino-terminal extracellular domain (ECD) responsible for high affinity binding of the hormone and a serpentine carboxy-terminal portion, characteristic of the opsin family of G protein-coupled receptors [1–3]. Attempts to understand the interplay between both domains in the mechanism of activation of the receptor have justified numerous studies, mainly involving site-directed mutagenesis (for references, see [4–6]). A logical approach to such studies would be the production of the two portions of the receptor in isolation. In particular, availability of the amino-terminal domain in soluble form would constitute an invaluable tool to define precisely its interaction with TSH or autoantibodies from patients with Graves' disease. Whereas this was achieved relatively easily for the LH/CG receptor [7], production of the bioactive (i.e. capable of binding TSH) amino-terminal portion of the TSHr has proved a very difficult task.

Most available expression systems have been tried: *Escherichia coli* [7–10], acellular translation [11,12], the baculovirus system [13–16] and mammalian cells [10,17–21]. Only recently has it been possible to obtain soluble ECD preparations that efficiently bind autoantibodies, by making truncated constructs in CHO cells [21], or a ECD-CD8 chimera with a thrombin site engineered at the junction of the chimera [19,20]. With one notable recent exception [19], preparations capable of binding TSH are still lacking.

In the present study we have succeeded in expressing high levels of bioactive ECD of the TSHr at the surface of CHO cells, via anchoring by a glycosylphosphatidylinositol (GPI) moiety [22,23]. The ECD can be released from the cell surface by a GPI-specific phospolipase-C (PI-PLC), in a form capable of binding Graves' autoantibodies, but also TSH, provided it is stabilized by a monoclonal antibody recognizing a conformational epitope of the receptor.

2. Materials and methods

2.1. Reagents

The BA8 and 3G4 monoclonal antibodies recognize a conformational epitope [24] and a linear epitope (VFFEEQE, residues 355–362, unpublished) of the ECD, respectively. PI-PLC was purchased from Boehringer, Mannheim. Iodinated bovine TSH (bTSH) and TRAK assay reagents were kind gifts from Brahms Diagnostica (Berlin).

2.2. Construction of TSHr ECD with a GPI anchor

A 170 bp fragment encoding the signal peptide for GPI addition at the C-terminus of mouse Thy-1 cDNA and contained in plasmid pTM813 [23] was PCR-amplified using the upstream primer 5′-TTAGAATTCAGCTCCAATAAAAGTATCAGTGTGTA-3′ and the downstream primer 5′-ATTGGATCCTCACAGAGAAATGAAGTCTAGG-3′. The PCR product was digested with *Eco*RI and *Bam*HI and inserted into the prokaryotic vector SK^+. The resulting construct was digested with *Eco*RV and *Eco*RI, and an oligonucleotide adapter encoding DIMGYKEF (see Fig. 1) was inserted (upper strand: 5′-ATCATGGGCTACAAGG-3′; lower strand: 5′-AATTCCTTGTAGCCCATGAT-3′). An *Eco*RV site was engineered in position 1230 of the ECD of human TSHr. The resulting construct, in SK^+, was excised with *Xho*I/*Eco*RV and fused upstream of the segment encoding the signal for GPI addition. The TSHr-ECD construct with its signal for GPI attachment is illustrated in Fig. 1. This construct was subcloned in pEFIN, a bicistronic vector developed at Euroscreen (Brussels, Belgium), and transfected in CHO-K1 cells. Stable cell lines were selected by resistance to geneticin [25].

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Abbreviations: TSH, thyrotropin; TSHr/LHCGr/FSHr, thyrotropin/lutropin/follitropin receptors; TSHr-ECD, extracellular amino-terminal domain of the TSHr; GPI, glycosylphosphatidylinositol; PI-PLC, GPI-specific phospholipase-C; TSAb, thyroid stimulating antibody; TBII, TSH binding inhibiting immunoglobulins

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2.3. Flow cytofluorometry with BA8 or 3G4 antibodies or autoimmune sera

The experiments were performed as described previously [24]. Incubation with antibodies was for 30 min at room temperature with 100 μl PBS-BSA 0.1% containing 10 μl culture supernatant from BA8 or 3G4 hybridoma, or 10 μl of autoimmune patient serum.

2.4. Extraction of GPI-anchored TSHr-ECD with Triton X-100

Confluent cells were detached from the plates with PBS-EDTA/EGTA, centrifuged at 500×*g* for 3 min and washed twice with PBS. 5×10^6 cells were resuspended in 100 μl of 1% (v/v) Triton X-100 in PBS containing protease inhibitors (*Complete*, Boehringer, Mannheim) and incubated on ice for 30 min. The samples were centrifuged at 13 000×*g* for 10 min and the supernatants were collected for Western blotting with the 3G4 antibody [24] (chemiluminescence kit RPN2108, Amersham Pharmacia Biotech, Gent, Belgium), or binding experiments.

2.5. Release of TSHr-ECD from its GPI anchor by PI-PLC

For binding with radiolabelled TSH, cells grown in 96-well plates (2×10^4 cells/well) were washed twice with DMEM without serum and incubated for 1, 2 or 3 h at 37°C with various amounts of PI-PLC in the same medium containing protease inhibitors (see above). Cells were washed twice with binding buffer and radiolabelled TSH was added. Binding was performed as described below.

For binding experiments with polyethylene glycol (PEG) precipitation, cells were removed from two 9 cm dishes with PBS-EDTA/EGTA, washed with PBS and incubated with 0.5 U PI-PLC in 100 μl of binding buffer with protease inhibitors for 1 h at 37°C. Cells were centrifuged at 13 000×*g* for 10 min and the supernatant stored at −20°C.

For FACS analysis, 300 000 cells in 3 cm dishes were washed twice with DMEM with protease inhibitors, and incubated with 0.5 U PI-PLC in DMEM, at 37°C for 1 h. Cells were then washed twice with PBS and FACS analysis performed as described above.

For deglycosylation and Western blotting, cells from one 9 cm Petri dish were washed twice with PBS and incubated for 1 h at 37°C with PI-PLC (1 U/ml) in DMEM with protease inhibitors. The medium was removed and concentrated with Ultrafree (NMWL 5000, Millipore) to a final volume of 500 μl. Cells were washed twice with PBS, scraped from the dish and centrifuged at 13 000×*g* for 10 min at 4°C. The pellet was solubilized for 30 min on ice in 500 μl of PBS containing 1% Triton X-100. The insoluble material was removed by centrifugation at 13 000×*g* for 15 min.

2.6. Removal of carbohydrates

30 μl of the concentrated medium containing the material released from cells by PI-PLC, or 30 μl of the material solubilized by 1% Triton X-100 from PI-PLC treated cells were denatured by boiling for 10 min in SDS 0.5%, β-mercaptoethanol 1%. After centrifugation (10 min, 13 000×*g*), 10 μl samples of the supernatants were treated for 16 h at 37°C with 2 U of *N*-glycosidase F, or 50 mU endoglycosidase H in a final volume of 50 μl containing 1% Triton X-100 and 50 mM phosphate buffer pH 7.5, or 50 mM citrate buffer pH 5.5. Control samples were incubated under identical conditions in the absence of enzymes. After addition of 15 μl of 5×SDS-PAGE loading buffer and boiling for 10 min, 20 μl samples were loaded on 8% polyacrylamide gels, and Western blotting was performed as described above.

2.7. TSH binding to GPI-anchored TSHr-ECD in intact cells

Thyrotropin binding was measured on GT14 or JP19 cells as previously described with minor modifications [26]. Briefly 2×10^4 cells/well in 96 well plates were incubated with [^{125}I]TSH (30 000 cpm) for 4 h at room temperature in 0.1 ml modified Hanks' buffer without NaCl (isotonicity maintained with 280 mM sucrose), supplemented with 5% BSA. After rapid rinsing with the same buffer at 0°C and solubilization with 0.2 ml 1 N NaOH, radioactivity was measured in a gamma counter. All experiments were done in triplicate and results are expressed as cpm bound.

2.8. Adsorption of TBII activity on GPI-anchored TSHr-ECD released with PI-PLC

TBII activity in the sera from patients with autoimmune thyroid diseases was measured with a commercial TRAK assay (Brahms, Berlin), in the presence or absence of TSHr-ECD released from its GPI anchor on GT14 cells by PI-PLC.

Briefly, 50 μl of autoimmune sera were incubated for 20 min at 20°C with 50 μl of solubilized porcine thyroid membrane, in the presence of 10 μl of soluble TSHr-ECD released from GT14 cells by PI-PLC (see above). Labeled [^{125}I]bTSH was added and incubation was for 2 h at 20°C. 2 ml of the precipitating solution (PEG) was added, the tubes were centrifuged 15 min at 2500×*g* and the radioactivity in the pellet counted. Results were calculated against the standard curve provided in the TRAK assay. For some patients with very high TRAK values (>405 IU), the same test was performed after dilution of the sera (see Section 3).

2.9. Binding of radioiodinated TSH to GPI-anchored TSHr-ECD released by PI-PLC

GT14 or JP02 control cells were detached with PBS-EGTA/EDTA, and washed twice with PBS. 10^6 cells were incubated with 500 ng of native BA8 antibody (or BA8 antibody previously denatured by a 10 min incubation at 100°C) in 100 μl of binding buffer for 1 h on ice. Cells were treated with Triton X-100 or PI-PLC as described above. After centrifugation at 13 000×*g* for 10 min, 90 μl of soluble material was transferred to a 5 ml tube, 100 μl of [^{125}I]bTSH (20 000 cpm) was added and the sample was incubated for 4 h at 4°C, in the presence or absence of 100 mU/ml cold bTSH. 50 μl of bovine γ-globulins (10 mg/ml) were added followed by 2 ml of ice-cold PEG 4000 15% in NaCl 1 M. After centrifugation for 30 min at 1500×*g*, the radioactivity in the pellet was measured.

3. Results and discussion

3.1. A GPI-anchored ECD of the TSHr is efficiently expressed at the plasma membrane

The 53 amino acid signal peptide for GPI anchor addition from mouse Thy-1 was fused at position 415 of the TSH receptor (see Section 2 and Fig. 1). This peptide includes two putative *N*-glycosylation sites, respectively before (NKS) and after (NTS) the site for cleavage and GPI addition (Fig.

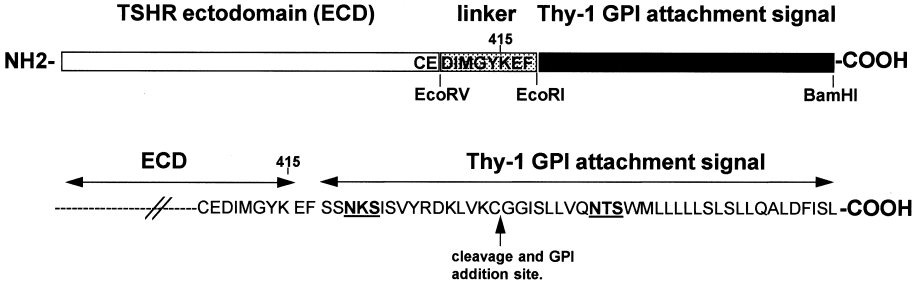

Fig. 1. Schematic representation of the GPI-anchored TSHR-ECD chimeric protein. The C-terminal part of the chimeric Thy-1 TSHr-ECD construct is shown; fusion is realized after position 415 of the TSHr, with two intervening amino acids (EF) preceding 53 residues of the mouse Thy-1 molecule responsible for GPI addition. The two potential sites of *N*-glycosylation (NKS and NTS) introduced with the Thy-1 GPI attachment signal are underlined.

1). The construct was transfected in CHO cells and one cell line (GT14) expressing particularly high levels of ECD at the cell surface was selected by flow cytometry, using BA8, a monoclonal antibody recognizing a conformational epitope of the ECD (Fig. 2A) [24]. The immunoreactive ECD was effectively anchored by a GPI moiety, since it could be removed from the cells by treatment with PI-PLC (Fig. 2B). The GPI-anchored ECD at the surface of GT14 cells was also probed with the monoclonal antibody 3G4 (Fig. 2B, black bars). This monoclonal recognizes a linear epitope (see Section 2) present in a 'connecting' peptide which has been shown to be removed from a fraction of holoreceptors expressed at the surface of transfected cells [27,28]. It is similar to the 2C11 antibody described previously [29]. The ratio between the 3G4 and BA8 signals for GT14 and JP19 cells, expressing the GPI-ECD and holoreceptor, respectively, was about 2.5-fold higher for GT14 cells (Fig. 2C). This suggests that the proportion of receptor molecules with the connecting peptide removed (if any) is lower in GT14 than in JP19 cells.

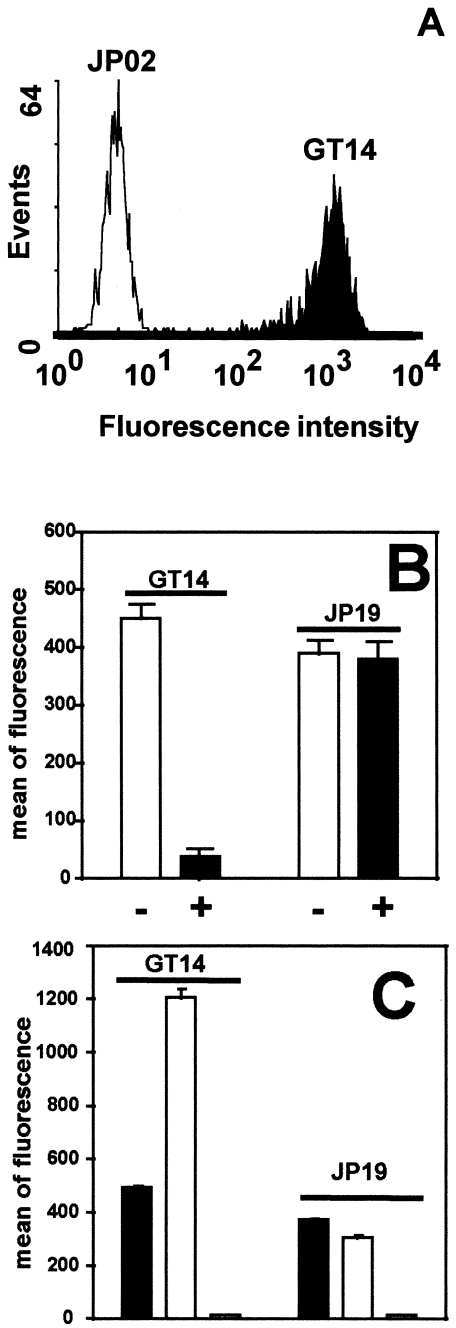

Fig. 2. Analysis of cell surface expression of GPI-anchored TSHr by flow cytofluorometry. A: Histograms showing binding of the BA8 antibody to a CHO line expressing the GPI-anchored TSHr-ECD (GT14) or no receptor (JP02). B: CHO cells expressing the GPI-anchored TSHr-ECD (GT14) or the full-length TSHr (JP19) were incubated with (black bars) or without (white bars) PI-PLC as described in Section 2. Binding of the BA8 antibody was assayed by flow cytofluorometry and quantitated as mean fluorescence. C: Same as in B except that no PI-PLC was used and that binding was assayed for BA8 (black bars), 3G4 (white bars) or an irrelevant antibody (gray bars).

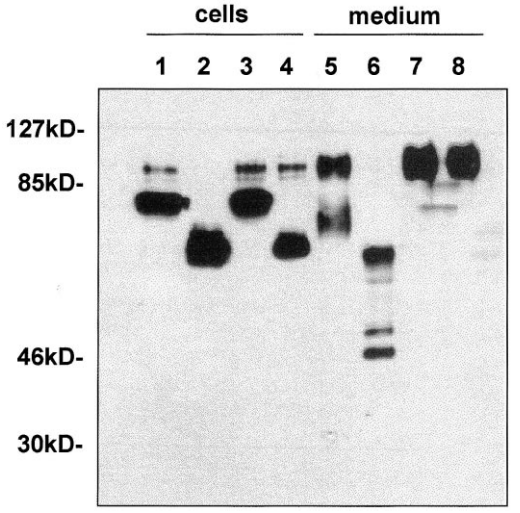

Fig. 3. Analysis of GPI-anchored TSHr-ECD by immunoblotting with the 3G4 antibody. GT14 cells were treated with PI-PLC (see Section 2). Lanes 1–4, Triton X-100 extracts from cells after PI-PLC treatment; lanes 5–8, material released from the cells by PI-PLC; lanes 1 and 5, overnight control incubation in phosphate buffer; lanes 2 and 6, overnight incubation in phosphate buffer containing *N*-glycosidase F; lanes 3 and 7, overnight control incubation in citrate buffer; lanes 4 and 8, overnight incubation in citrate buffer containing endoglycosidase H.

On Western blots using the 3G4 monoclonal antibody, the material released from the cells by PI-PLC migrates as a 100 kDa band (Fig. 3, lanes 5 and 7). This material is converted to a 62 kDa species by treatment with *N*-glycosidase F (Fig. 3, lanes 6 versus 5). It is completely resistant to endoglycosidase H (lanes 8 versus 7), suggesting that it corresponds to a mature protein with complex carbohydrate chains. A soluble ECD with a lower apparent molecular weight of 82 kDa was recently produced from a construct with an engineered thrombin cleavage site [19]. It is likely that this difference in size is accounted for by glycosylation of the GPI-ECD at the level of the first site for *N*-glycosylation which is present in the signal peptide for GPI addition (NKS, see Fig. 1).

Triton X-100 extraction of the material remaining in cells after PI-PLC treatment releases a major band of 80 kDa, in addition to residual amounts of 100 kDa material (Fig. 3, lanes 1 and 3). This 80 kDa species is sensitive to both *N*-glycosidase F (lanes 2 versus 1) and endoglycosidase H (lanes 4 versus 3), suggesting that it corresponds to intracellular precursors of the GPI-ECD with immature high-mannose carbohydrates.

It must be stressed that the ECD molecules identified here

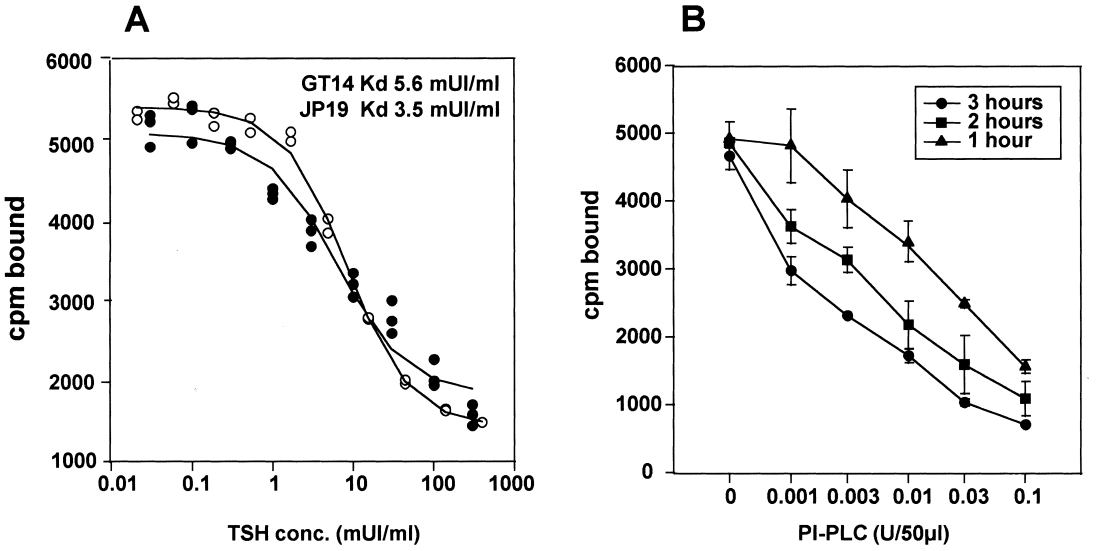

Fig. 4. TSH binding to GPI-anchored TSHr-ECD in intact cells. A: CHO lines expressing the GPI-anchored TSHr-ECD (GT14, solid circles) or full length TSHr (JP19, open circles) were incubated with 30 000 cpm of [^{125}I]bTSH and varying concentrations of cold bTSH. Each point was tested in triplicate and the results are expressed as bound radioactivity. B: GT14 cells expressing the GPI-anchored TSHr-ECD were treated for various time periods with increasing concentrations of PI-PLC and binding of [^{125}I]bTSH to the cells was measured (see Section 2).

all contain the 3G4 epitope, which is included in the peptide connecting the two subunits of the TSHr in its precursor [27,28]. As the apparent molecular weights of the molecules released from the cells by SDS, Triton X-100 or PI-PLC were the same whether or not the samples were reduced before electrophoresis (not shown), we conclude that the ECD molecules identified here are made of a monomeric polypeptide chain.

3.2. *TSH binds with high affinity to the GPI-anchored ECD at the surface of GT14 cells*

Intact GT14 cells bind [^{125}I]bTSH with an affinity comparable to that of JP19 cells [25] expressing the full-length holoreceptor (K_d, 5.6 vs. 3.5 mIU/ml, for GT14 and JP19 cells, respectively) (Fig. 4A). The binding was completely lost after pretreatment of the cells with PI-PLC (Fig. 4B), which was without effect on the binding to JP19 cells (not shown). Esti-

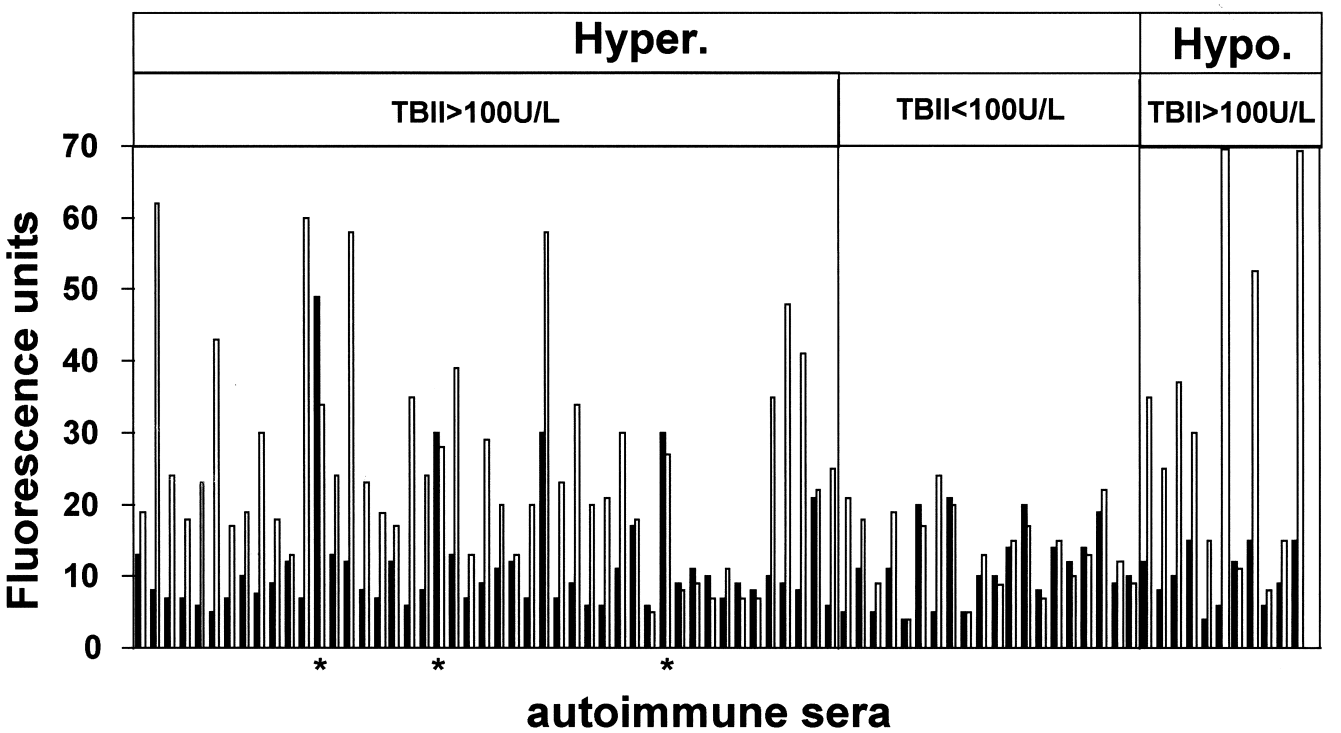

Fig. 5. Flow cytofluorometry of autoantibody binding to CHO cells expressing the GPI-anchored TSHr-ECD (GT14, open bars) or no receptor (JP02, solid bars). Cells were incubated with sera (1:10) from patients with autoimmune hypothyroidism ('hypo') or Graves' disease ('hyper'). Fluorescence was analyzed as described in Section 2. Some sera (indicated with asterisks) exhibited elevated fluorescence on the two cell lines.

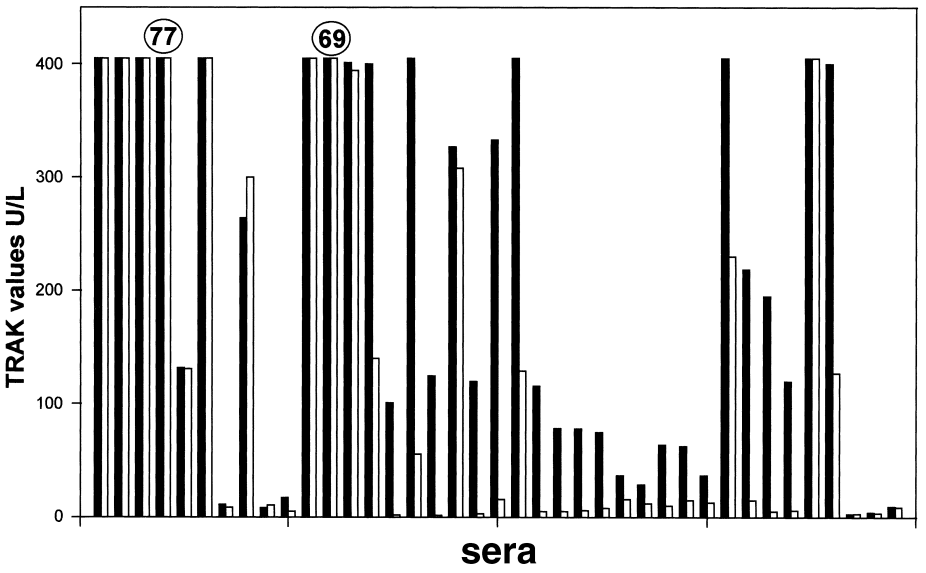

Fig. 6. Neutralization with soluble TSHr-ECD of autoantibodies to the TSHr present in the serum of patients with autoimmune hypothyroidism or Graves disease. The TRAK assay (Section 2) was modified by preincubating sera (with TBII levels ranging from 0 to >405 U/l) with the soluble material released from GT14 cells by PI-PLC (open bars) or control buffer (solid bars). Results are expressed in U/l. Sera 69 and 77 were used after dilution in a further experiment (see Fig. 7).

mation of the number of TSHr/cell from analysis of the binding curves [30] gave numbers in excess of 10^6.

Expression of ECD of the TSHr in a form capable of binding TSH with high affinity has been unexpectedly difficult. The present results demonstrate that high affinity binding of TSH to the TSHr does not require interaction with, or the presence of the serpentine portion of the receptor. They agree with recent data showing binding to ECD anchored to the cell by a CD8 transmembrane segment [19]. This does not exclude that the extracellular loops of the serpentine portion of the receptor may interact with the hormone, as suggested by some site-directed mutagenesis experiments [31,32]. It indicates, however, that, as for the other glycoprotein hormone receptors, the major high affinity interaction is between the hormone and the ECD.

3.3. *The GPI-anchored TSHr-ECD allows direct detection of autoantibodies by flow cytometry*

Direct detection of the interaction between autoantibodies and the TSH receptor has been achieved in rare instances, and only with a handful of potent antisera [33,34]. It was concluded from these observations that TSAbs must be present in very low concentrations in the serum of patients with Graves' disease [33,34]. Incubation of intact GT14 cells with a panel of sera from patients with Graves' disease or autoimmune hypothyroidism yielded clearly positive signals in flow cytometry for the majority of them (Fig. 5). The need for stringent controls in such type of experiments is illustrated by a series of false positives, giving as strong signals with the control JP02 cells as with GT14 cells (see e.g. Fig. 5, asterisk). A rough direct relation was observed between the level of TBII in the serum and the fluorescence signal (compare results from serum with TRAK > 100 U/l and < 100 U/l, Fig. 5). Also, sera from patients with autoimmune hypothyroidism tended to give stronger fluorescent signals than those from Graves patients ('Hypo' vs. 'Hyper', Fig. 5). This observation suggests that blocking antibodies have a higher titer than stimulating antibodies. Whether the positive results obtained

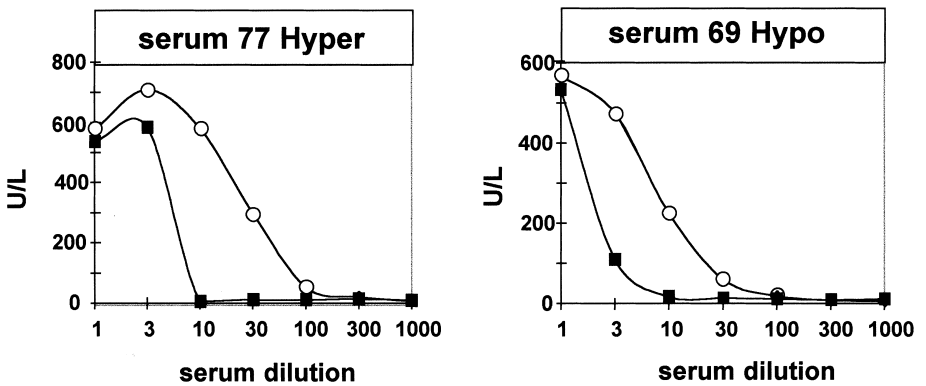

Fig. 7. Neutralization with soluble TSHr-ECD of autoantibodies to the TSHr present in the serum of patients with autoimmune hypothyroidism (serum 69), or Graves' disease (serum 77) exhibiting very high TBII levels (>405 U/l). Sera were diluted in normal human serum and incubated with soluble TSHr-ECD (solid squares) or control buffer (open circles) before performing the TRAK assay (see legend to Fig. 6).

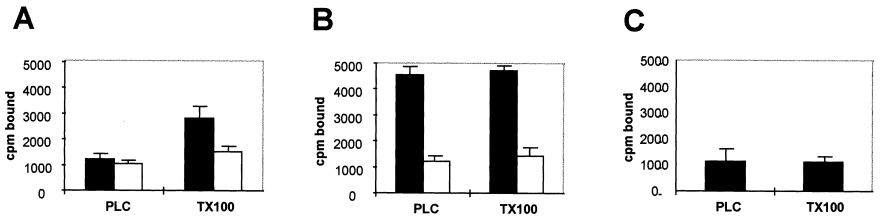

Fig. 8. Binding of [^{125}I]bTSH to the ECD released from GT14 cells by PI-PLC and stabilized by the BA8 antibody. GT14 cells (A and B) or JP02 cells (C) were exposed to native (B and C) or heat-denatured BA8 (A), under conditions where the intact monoclonal binds efficiently to the GPI-ECD at the cell surface (see Fig. 2). The cells were treated with PI-PLC (PLC) or Triton X-100 (TX100) and [^{125}I]TSH binding in the presence (open bars) or absence (solid bars) of an excess of cold bTSH (100 mU/ml) was assayed by precipitation with PEG (see Section 2).

with the GPI-ECD are explained by the higher amounts of antigens at the surface of GT14 cells, as compared to those expressing the holoreceptor [33,34], or to a structural peculiarity of the GPI-ECD antigen remains to be determined. The GPI-TSHr at the surface of GT14 cells constitutes a novel reagent, with promising characteristics for the development of new direct assays of autoantibodies against the TSHr.

3.4. *The soluble TSHr-ECD released by PI-PLC binds autoantibodies but not TSH*

A batch of 10^6 GT14 cells was treated with PI-PLC and the material released from the cells was assayed for its ability to bind [^{125}I]bTSH by precipitation with PEG. No binding was detected (see below). This characteristic of the soluble ECD, severed from the GPI moiety, was exploited to test whether it would compete for binding of autoantibodies, with the porcine TSHr present in the TRAK assay system (see Section 2). A series of sera with a spectrum of TBII values were tested (Fig. 6). The soluble ECD competed efficiently with the porcine receptor for most of the sera with an intermediate or low TBII value. With a few exceptions, sera with a very strong TBII value tended to resist competition. To investigate if the absence of competition for the strongly positive sera could reflect a quantitative problem due to an excess of autoantibodies capable of saturating both the porcine receptor (of the TRAK) and the GPI-ECD, the same experiments were performed with a selection of such sera, after serial dilution. Fig. 7 illustrates the results obtained with two sera with a particularly high TBII value (serum 77 and 69 in Fig. 6, TRAK $>$ 400 U/l). While competition between GPI-ECD and the porcine TSHr of the TRAK was not observed with undiluted serum, it was readily detected as soon as the amounts of immunoglobulins in the assay were reduced. Together with the flow cytometry experiments, this indicates that the GPI-ECD material, be it soluble or anchored in the plasma membrane, contains a large proportion of the epitopes recognized by autoantibodies from patients. It also suggests that the concentration of autoantibodies recognizing the receptor may be relatively high in some of the samples.

These results agree with observations from many groups that soluble or solubilized ECD preparations of the TSHr that are unable to bind TSH display efficient binding of autoantibodies [14,16,19,21,35]. In some studies involving truncated versions of the ECD [19,21], the differential recognition may be due to the absence of a segment contributing to the surface of interaction with TSH; in others, including the present one, using complete ECD constructs, it is likely that misfolding, abnormal glycosylation or partial denaturation of the ECD was responsible for the lack of TSH binding [14,16,35].

3.5. *When complexed with BA8, the soluble TSHr-ECD released by PI-PLC binds TSH*

The loss of the capability to bind TSH, when the GPI-ECD is released from the membrane by PI-PLC does not seem to be due to gross proteolysis: Western blotting indicates that the material released migrates as a single major band (Fig. 3). The possibility was explored that this loss of activity was due to an intrinsic instability of the ECD, when it is severed from the serpentine domain and moved away from its natural membrane environment. The BA8 monoclonal antibody, which recognizes a conformational epitope of the human TSHr and does not interfere with binding of TSH or autoantibodies [24], was tested as a potential stabilizer of the ECD structure. GT14 cells were exposed to native or heat-denatured BA8, under conditions where the intact monoclonal binds efficiently to the GPI-ECD at the cell surface (see Fig. 2). The cells were then treated with PI-PLC and the presence of [^{125}I]TSH binding activity released in the incubation medium was assayed by precipitation with PEG. A clear TSH binding activity was released by PI-PLC in the medium from cells pre-exposed to native BA8 (Fig. 8B). Absence of BA8 (not shown) or prior heat denaturation of the monoclonal (Fig. 8A) yielded inactive material. Binding of TSH to the ECD from the Triton X-100 extract was also enhanced by BA8. These results confirm recent observations [19,27] demonstrating that the ECD alone is enough for interaction with TSH.

4. Conclusion

Until very recently, constructs encoding the complete ECD alone did not yield bioactive material capable of binding TSH with high affinity (see [21] for references). Together, our results and those of Osuga et al. [19] suggest that the complete ECD of the TSH receptor requires additional 'signal' sequences to be correctly targeted to the plasma membrane in a native form. In the holoreceptor, this signaling is probably carried out by the serpentine portion of the receptor itself. The first two transmembrane segments are not effective, as the correspondingly truncated versions of the receptor remain trapped intracellularly (our unpublished experiments). It is likely that the GPI-anchoring peptide (the present study) or the CD8 transmembrane segment [19] provide adequate substitutes for this targeting, allowing a significant proportion of the ECD molecules to undergo normal glycosylation and maturation, during their journey through the membrane system of the cell.

Another characteristic of the ECD of the TSHr, when it is separated from its normal environment, seems to be its susceptibility to partial denaturation. Using a wide diversity of experimental systems, a series of laboratories succeeded in making ECD material that is efficiently recognized by autoantibodies but fails to bind TSH. Complex formation between a protein of interest and a natural or artificial ligand recognizing its native structure has been used to stabilize labile proteins with a view to structure determination by X-ray crystallography [36,37]. Our results with the BA8 monoclonal antibody indicate that this approach constitutes a promising means to prepare large amounts of the ECD of the TSHr, opening the way to a direct determination of its three-dimensional structure.

When the present study was completed, a similar approach was published by Da Costa and Johnstone [38]. Results are in good general agreement with ours, but no indication was given that the soluble ECD could bind TSH.

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