Interaction of autoantibodies to thyrotropin receptor with a hydrophilic subunit of the thyrotropin receptor

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Reduction of human thyroid membranes with dithiothreitol caused the release of a water-soluble glycoprotein which neutralized the thyrotropin (TSH) receptor-binding and thyroid-stimulating activities of Graves' serum. Analysis of the protein by gel filtration and sucrose density gradient centrifugation allowed estimates of 3.45 nm for the Stokes' radius, 3.6S for the $s_{20,w}$ and $47\,000 \pm 5\,000$ (mean $\pm$ S.D.; $n = 4$) for the M_r . The material released by dithiothreitol treatment could be crosslinked to ^{125}I -labelled TSH coupled to *N*-hydroxysuccinimidyl 4-azidobenzoate (^{125}I -HSAB-TSH), suggesting that it contained a component of the TSH receptor. Furthermore, analysis of the crosslinked material by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis indicated that it contained the TSH receptor A subunit (M_r 50000). Several factors suggested therefore that the glycoprotein released by dithiothreitol treatment of human thyroid membranes was the TSH receptor A subunit. In particular, (a) both preparations were hydrophilic and were released from membranes by reduction, (b) they had similar M_r values and (c) both preparations crosslinked to ^{125}I -HSAB-TSH. Material similar to the TSH receptor A subunit was released from thyroid membranes by treatment with papain, probably as a result of cleavage of the receptor A subunit at a site close to the interchain disulphide bridge. A similar mechanism, involving thyroid proteinases, was probably involved in release of material with similar properties to the TSH receptor A subunit during freezing and thawing of human thyroid homogenates.

Recent studies have shown that freezing and thawing of human thyroid homogenates releases a water-soluble component of the TSH receptor with binding sites for both TSH and TSH receptor antibodies (Davies Jones & Rees Smith, 1984). This water soluble component (originally referred to as long-acting thyroid-stimulator absorbing activity, or LAA) is conveniently detected by its ability to neutralize TSH receptor antibody activity.

In the present paper we describe a study of the mechanism of LAA release from thyroid membranes and a more detailed investigation of its relationship to the TSH receptor.

Abbreviations used: TSH, thyrotropin; HSAB, *N*-hydroxysuccinimidyl 4-azidobenzoate; SDS, sodium dodecyl sulphate; LAA, long-acting thyroid-stimulator absorbing activity.

Materials and methods

Calibration proteins and other reagents

All standard proteins used for calibration, dithiothreitol, cysteine, iodoacetamide, phenylmethanesulphonyl fluoride, papain, bovine pancreatic trypsin and soya bean trypsin inhibitor were purchased from Sigma. Concanavalin A-Sepharose was purchased from Pharmacia. All other reagents were purchased from BDH and were of AnalaR grade.

Serum samples

Normal pool serum was obtained by mixing serum from several normal healthy volunteers. The Graves' sera used were selected on the basis of high levels of TSH receptor antibody activity as measured by the receptor assay (Southgate *et al.*, 1984). All sera were stored in portions at -70°C .

and repeat freezing and thawing was avoided. Gel filtration studies confirmed the presence of the TSH-receptor binding activity in the IgG fraction of the sera.

Preparation of thyroid membranes

Human thyroids were obtained at partial thyroidectomy for Graves' disease, cut into small pieces (approx. 200 mg), frozen in *n*-hexane at -70°C and stored at -70°C . Porcine thyroids were obtained from the local abattoir and frozen within 1 h of slaughter. When required the tissue was thawed, chopped finely with scissors and scalpel, homogenized in 10 vol. of ice-cold 10 mM-Tris/HCl, pH 7.5, and filtered through muslin. A crude membrane preparation was sedimented (20000g, 20 min, 4°C) from the filtered homogenate. The membranes were washed with 2 vol. of 50 mM-NaCl/10 mM-Tris/HCl containing 3 mM- NaN_3 (Tris/NaCl), pH 7.5, resedimented (20000g, 20 min, 4°C) and resuspended in Tris/NaCl, pH 8.2, (1 ml/2 g of starting thyroid tissue unless otherwise specified).

Treatment of thyroid membranes with dithiothreitol and papain

Thyroid membrane suspensions (1 g of starting tissue/ml of Tris/NaCl, pH 8.2) were treated with dithiothreitol (30 min, 22°C) or papain (15 min, 22°C) followed by iodoacetamide (2-fold excess over reducing agent concentration). Cysteine (final concn. 5 mM) was added to the mixtures digested with papain. The mixtures were then centrifuged (100000g 1 h, 4°C) and the supernatants were dialysed overnight against 2×1 litre of Tris/NaCl, pH 7.5, at 0°C . Both pellets and supernatants were assayed for LAA (see below).

Preparation of detergent-solubilized TSH receptors and ^{125}I -labelled TSH

Porcine thyroid membranes were solubilized with 1% Lubrol in Tris/NaCl, pH 7.5, containing 0.1 mM-phenylmethanesulphonyl fluoride (Rees Smith & Hall, 1981). Highly purified bovine pituitary TSH (60–70 units/mg) was prepared by a method based on the procedure of Pierce *et al.* (1971). The hormone was labelled to a specific radioactivity of $50 \mu\text{Ci}/\mu\text{g}$ with ^{125}I (Amersham International) by using the Iodogen method (Fraker & Speck, 1978) and receptor-purified (Rees Smith & Hall, 1981).

Assay of LAA

LAA was measured by its ability to neutralize TSH receptor antibody activity in a standard Graves' serum (Davies Jones & Rees Smith, 1984). One series of samples (0–500 μl) of LAA were incubated with 40 μl of the standard Graves' serum

for 1 h at 37°C , while a second identical series of samples were incubated simultaneously with 40 μl of normal pool serum. All reaction volumes were then adjusted to 540 μl with Tris/NaCl, pH 7.5, triplicate 100 μl samples were then taken, incubated with detergent-solubilized receptors (40 μl , 45 min, 22°C) followed by ^{125}I -TSH (100 μl , 6000 c.p.m., 1 h, 37°C). The reaction volumes were adjusted to 750 μl with Tris/NaCl pH 7.5, containing bovine serum albumin (1 mg/ml) and 750 μl of 30% (w/v) poly(ethylene glycol) in 1 M-NaCl was added. The tubes were mixed and centrifuged (1500g, 30 min, 4°C) and the pellets were counted for receptor-bound ^{125}I -TSH. At the end of the reaction, bound ^{125}I -TSH is associated almost completely with the receptor, due to the rapid dissociation of any LAA- ^{125}I -TSH complex formed. Furthermore, LAA- ^{125}I -TSH complexes are not precipitated by poly(ethylene glycol) under the conditions used (Dawes *et al.*, 1978). LAA was expressed in terms of its ability to reduce the effects of the standard Graves' sera in the receptor assay using the formula $(T/C) \times 100$, where $T = ^{125}\text{I}$ -TSH specifically bound in the presence of LAA and normal pool serum minus ^{125}I -TSH specifically bound in the presence of LAA and Graves' serum, and $C = ^{125}\text{I}$ -TSH specifically bound in the presence of normal pool serum alone minus ^{125}I -TSH specifically bound in the presence of Graves' serum alone. Alternatively, LAA was expressed as a percentage neutralization of Graves' serum using $[1 - (T/C)] \times 100$. Membrane-bound LAA was assayed by using a slight modification to the above method. After the reaction volumes were adjusted to 540 μl with Tris/NaCl, pH 7.5, the membranes were separated by centrifugation (20000g, 15 min, 4°C) and the supernatant assayed for TSH receptor binding activity.

Ultracentrifugation

Sedimentation studies were carried out in a Sorvall OTD 65B centrifuge using a Sorvall TV-865B vertical rotor. Samples (1 ml) were applied to 15.5 ml linear sucrose gradients [5–20% (w/v) sucrose in Tris/NaCl, pH 7.5]. Following centrifugation (60000 rev./min, 4.5 h, 4°C), fractions (650 μl) were extracted by carefully inserting a fine bore capillary tube into the bottom of the tubes and pumping the gradients out. Sedimentation values ($s_{20,w}$) were estimated according to Martin & Ames (1961), and an estimation of M_r was obtained by using the formula:

$$M_r = \frac{s_{20,w} 6\pi\eta aN}{(1 - \bar{v}\rho)}$$

(Le Maire *et al.*, 1980) where η = viscosity, a = Stokes' radius, N = Avogadro's number, $\bar{v}$ = partial specific volume and ρ = density.

Preparation of LAA by freezing and thawing

In some experiments, LAA was released from human thyroid homogenates by freezing and thawing (Dirmikis & Munro, 1973a; Davies Jones & Rees Smith, 1984). Briefly, human (Graves') thyroid homogenates (1 g of starting tissue/ml of Tris/NaCl, pH 7.5) were frozen and thawed, centrifuged (100000g, 4°C, 1 h) and then used for further analysis.

Photoaffinity labelling

TSH was coupled to the photoactive heterobifunctional reagent HSAB and labelled with ^{125}I (^{125}I -HSAB-TSH) as described previously (Buckland *et al.*, 1982; Buckland & Rees Smith, 1984). Aliquots of ^{125}I -HSAB-TSH [(6–10) $\times 10^3$ c.p.s./ml] were incubated (3 h at 20°C) with equal volumes of human thyroid membranes (material from 1 g of starting tissue in 1 ml of Tris/NaCl, pH 7.5) or LAA (released by dithiothreitol treatment of human thyroid membranes and purified by chromatography on Sephadex G-200 as shown in the Results section). Photolysis was then carried out for 20 min at 254 nm.

In the case of membranes, the preparations were washed three times by sedimentation and resuspension in Tris/NaCl and finally suspended in Tris/NaCl (material from 1 g of tissue in 200 μl of Tris/NaCl, pH 8.2). Dithiothreitol was then added to a final concentration of 10 mM and the mixture was incubated for 1 h at 20°C. After centrifugation at 100000g for 1 h at 4°C the supernatant was treated with a 5-fold molar excess of iodoacetamide and run on a 250 ml column of Sephacryl S-300 in Tris/NaCl to separate the TSH receptor A subunit crosslinked to ^{125}I -HSAB-TSH from free ^{125}I -HSAB-TSH and aggregates (Kajita *et al.*, 1985).

In the case of LAA, the photolysed mixture was run directly on the Sephacryl S-300 column.

Column fractions from both series of experiments were concentrated by ultrafiltration and analysed by polyacrylamide-gel electrophoresis in SDS using 8–12% gradient gels. After electrophoresis, the gels were fixed and stained in Coomassie Blue solution, dried and subjected to autoradiography as described previously (Buckland *et al.*, 1982; Buckland & Rees Smith, 1984).

Assay of thyroid-stimulating activity

An assay based on stimulation of cyclic AMP release by cultured thyroid cells was used (Kasagi *et al.*, 1982). Briefly, finely minced porcine thyroid tissue was subjected to digestion with Dispase (50 mg/g of tissue per 10 ml) and the isolated cells were cultured in 96 well plates in Eagle's minimum essential medium containing 10% newborn calf serum. After 2 days, the medium was replaced with

low-sodium Hanks' balanced salt solution containing test material and 2 mM-3-isobutyl-1-methylxanthine (five wells for each test material) and incubation was continued for 2 h. The cyclic AMP content of the medium in each plate was then determined with a radioimmunoassay kit (New England Nuclear). Detectable stimulation of cyclic AMP release into the medium was observed with as little as 1 μunit of bovine TSH (Armour), the mean ($\pm$ s.d.) response in eight separate assays being 1.44 ± 0.25 times basal (balanced salt solution only). The mean response to 10 μunits of bovine TSH was 5.34 ± 1.06 times basal over eight separate assays. The TSH agonist activity of Graves' sera was present in the IgG fraction.

Results

When homogenates of human thyroid membranes were treated with various concentrations of either dithiothreitol or papain followed by centrifugation, a dose-dependent release of LAA into the supernatant was observed (Fig. 1). Treatment of membranes with cysteine (5, 10, 20 or 40 mM) alone did not cause the release of significant amounts of LAA (results not shown).

The release of LAA from the membranes was paralleled by a loss of receptor antibody binding activity from the membrane preparations (Fig. 1).

Reduction of porcine thyroid membrane yielded similar results. Less LAA was released, however, with only 50% neutralization of Graves' serum achieved with 10 mM-dithiothreitol.

The LAA released from human thyroid membranes was analysed by gel filtration (Fig. 2) and the material eluted principally as a peak of activity just before haemoglobin. Using standard proteins and a plot of Stokes' radius versus $(1 - K_{av})$ (Le Maire *et al.*, 1980) the Stokes' radius of the LAA was calculated to be 3.45 nm. The elution characteristics of LAA were unchanged following trypsin treatment (Fig. 2) and were similar to those of LAA released from human thyroid homogenates by freezing and thawing (results not shown). LAA released by freezing and thawing was also resistant to trypsin treatment (results not shown).

Sucrose density gradient analysis showed that LAA prepared by dithiothreitol treatment of human thyroid membranes sedimented at a similar rate to bovine serum albumin (Fig. 3) and from the observed sedimentation behaviour of standard proteins (Fig. 3) an $s_{20,w}$ value of $3.6 \pm 0.3\text{S}$ (mean $\pm$ s.d.; $n = 4$) was estimated. From this value and the Stokes' radius estimate of 3.45 nm it was possible to estimate the M_r (mean $\pm$ s.d.; $n = 4$) of LAA released by dithiothreitol treatment of human thyroid membranes as 47000 ± 5000 . LAA released by dithiothreitol treatment of hu-

Fig. 1. Papain or dithiothreitol treatment of human thyroid membranes

Release of LAA from 1 ml of human thyroid membranes by treatment with papain or dithiothreitol is shown; see the text for experimental details. LAA activity was expressed as percentage neutralization of Graves' serum, i.e. $[1 - (T/C)] \times 100$, as detailed in the text. ●, Released material; ○, residual activity in membranes.

man thyroid membranes was also run on a column of concanavalin A-Sepharose and the LAA was completely absorbed by the column.

An analysis on Sephacryl S-300 of LAA crosslinked to ^{125}I -HSAB-TSH is shown in Fig. 4(a). The formation of an LAA-TSH complex which was eluted ahead of the leading edge of the free TSH peak was evident with active LAA, but this was absent when heat-inactivated LAA or LAA pretreated with unlabelled TSH were used. The poor resolution of the ^{125}I -labelled-HSAB-TSH-

Fig. 2. Gel filtration of LAA released by dithiothreitol (10 mM) treatment of human thyroid membranes (a) LAA (7.5 ml) was run (16 ml/h) on a Sephadex G-200 column (3.2 cm $\times$ 35 cm) in Tris/NaCl at 4°C. Fractions (3.8 ml) were collected and samples (500 μ l) assayed for LAA as detailed in the text. (b) As (a) except that the LAA had been pretreated with trypsin (0.2 mg/ml; 22°C, 30 min). Abbreviations used: V_0 and V_t , void and total volume of the column respectively; Cat, catalase (beef liver); BSA, bovine serum albumin; OSA, ovine serum albumin; TF, transferrin (human); Ald, aldolase (rabbit muscle); Hb, haemoglobin (bovine); RNAase, ribonuclease (bovine pancreas); Cyt c, cytochrome c (bovine heart); Tg, human thyroglobulin.

LAA complex and unbound TSH was probably due to the dissociation of non-crosslinked ^{125}I -labelled HSAB-TSH-LAA complex during the gel filtration. When fractions 45–58 were pooled, concentrated by ultrafiltration and re-run on the same column the complex and free TSH eluted as two distinct peaks (results not shown).

Analysis by SDS/polyacrylamide-gel electrophoresis of LAA crosslinked to ^{125}I -HSAB-TSH is shown in Fig. 4(b). Also shown is SDS/polyacrylamide-gel electrophoretic analysis of the material released by dithiothreitol treatment of thyroid membranes crosslinked to ^{125}I -HSAB-TSH. Both analyses show the presence of major bands at M_r 28000 (TSH) and 78000. Formation of the 78000 band was completely inhibited by preincubating

Fig. 3. Ultracentrifugation of LAA

LAA (1 ml) released from human thyroid membranes with 10 mM-dithiothreitol was sedimented in linear sucrose gradients as detailed in the text. Following centrifugation, 300 μ l aliquots of the tube fractions were assayed for LAA. The sedimentation positions of standard proteins (abbreviations as in Fig. 2) are shown.

membranes with unlabelled TSH (100 munit). Crosslinking studies with LAA showed the presence of bands at 55 000 and 40 000 in addition to the 28 000 and 78 000 bands.

The ability of LAA released by dithiothreitol treatment of human thyroid membranes to neutralize the thyroid-stimulating activity of Graves' sera as measured by the bioassay was also investigated. As shown in Fig. 5, neutralization was completely effective in terms of the Graves' serum, but there was only a small effect of LAA on TSH.

Similar results were obtained in the bioassay using LAA that had been purified by gel filtration on Sephadex G-200 (results not shown). When either crude or G-200-purified LAA were heated at 56°C for 30 min, their ability to neutralize the thyroid-stimulating activities of Graves' sera was abolished.

Discussion

The LAA released from human thyroid membranes by treatment with dithiothreitol showed characteristics similar to those of the water-soluble fragment of the TSH receptor released by freezing and thawing of human thyroid homogenates (Davies Jones & Rees Smith, 1984). In particular, the activities were co-eluted from Sephadex G-200, both were glycoproteins as judged by their ability to bind concanavalin A-Sepharose, both were heat-labile, but neither appeared to be markedly influenced by trypsin treatment (Fig. 2).

LAA was also released from porcine thyroid membranes by dithiothreitol treatment, but in smaller amounts than from human thyroid membranes. This was in agreement with the observation that freezing and thawing of porcine thyroid

Fig. 4. Crosslinking of LAA to 125 I-HSAB-TSH. LAA released by dithiothreitol treatment of human thyroid membranes was purified on Sephadex G-200 (Fig. 2) and used directly, heat inactivated (56°C, 30 min) or incubated (20°C, 5 min) with unlabelled TSH (1 unit). The three preparations of LAA were then crosslinked to 125 I-HSAB-TSH, chromatographed on Sephacryl S-300, and analysed by SDS/polyacrylamide-gel electrophoresis (see the text for full experimental details). (a) S-300 analysis showing (i) untreated and (ii) heat-inactivated LAA. A similar profile to that shown for heat-inactivated LAA was observed when LAA was preincubated with unlabelled TSH. (b) 'LAA': fractions 45–58 from the chromatographic run shown in (a) were pooled, concentrated and re-run on Sephacryl S-300. The LAA- 125 I-HSAB-TSH complex peak was collected, concentrated and analysed by SDS/polyacrylamide-gel electrophoresis. 'A subunit': TSH receptor A subunit cross-linked to 125 I-HSAB-TSH. This was formed by crosslinking TSH receptors in thyroid membranes to 125 I-HSAB-TSH before treatment with dithiothreitol and centrifugation (see the text for experimental details).

Fig. 5. Neutralization of the thyroid-stimulating activity of Graves' serum by LAA

LAA released by dithiothreitol treatment of human thyroid membranes was incubated for 1 h at 37°C with a Graves' serum or normal pool serum (basal) diluted 200-fold in Hanks' balanced salt solution; 200 μ l aliquots were bioassayed. In some experiments, bovine TSH was included in the normal pool serum, so as to give a final concentration of 10 μ unit/well. Values are means $\pm$ S.D.

membranes did not release readily detectable amounts of LAA (Dirmikis & Munro, 1973b). The difference between human and porcine membranes could reflect the considerably higher concentration of TSH receptors in human thyroid membranes (Dawes *et al.*, 1978).

An inhibiting effect of dithiothreitol on the ability of human thyroid membranes to neutralize TSH receptor antibody activity has been reported previously by Shishiba *et al.* (1982) and presumably this was due to the release of LAA from the membranes. The ability of thyroid membrane preparations to bind 125 I-labelled TSH is also inhibited by dithiothreitol treatment (Ozawa *et al.*, 1979; Ginsberg *et al.*, 1982). Cysteine treatment of human thyroid membranes did not release significant amounts of LAA, and this suggested that the disulphide bridge(s) reduced by dithiothreitol were not readily accessible to cysteine.

Gel filtration and sucrose density gradient centrifugation of LAA released by dithiothreitol treatment of human thyroid membranes enabled estimates of an $s_{20,w}$ of 3.6S, a Stokes' radius of 3.45 nm and an M_r of 47 000.

Although LAA neutralized the TSH receptor binding (Figs. 1–3) and thyroid-stimulating activities (Fig. 5) of Graves' serum it did not neutralize TSH very effectively (Fig. 5), in agreement with previous observations (Dawes *et al.*, 1978). This was probably due to the relatively rapid dissociation rate of TSH from its receptor when compared with the receptor antibodies and consequently, demonstration of a direct interaction between LAA and TSH has required special techniques. For example it has been possible to use columns of Sepharose–TSH containing high concentrations of hormone to bind LAA released from thyroid membranes by freezing and thawing (Davies Jones & Rees Smith, 1984). These observations were confirmed in studies of crosslinking LAA (prepared by reduction of thyroid membranes) to 125 I-HSAB-TSH (Fig. 4).

Recent observations have indicated that the human TSH receptor consists of a hydrophilic A subunit, M_r 50 000, linked by a disulphide bridge to an amphiphilic B subunit, M_r 25 000, (Kajita *et al.*, 1985). When TSH receptors in human (or other species) thyroid membranes were crosslinked to 125 I-HSAB-TSH and treated with 10 mM-dithiothreitol, the receptor A subunit crosslinked to 125 I-HSAB-TSH was released into aqueous solution (Fig. 4; Kajita *et al.*, 1985). A band corresponding to the TSH receptor A subunit crosslinked to 125 I-HSAB-TSH (M_r 78 000) was also observed in SDS/polyacrylamide-gel electrophoretic analyses of LAA crosslinked to 125 I-HSAB-TSH (Fig. 4). Bands at lower M_r were also observed and these may have resulted from the effects of thyroid proteinases in the LAA preparation. It is known that the A subunit is susceptible to limited digestion with trypsin (Buckland & Rees Smith, 1984). The observation that trypsin treatment did not markedly diminish the ability of LAA to neutralize TSH receptor antibody activity (Davies Jones & Rees Smith, 1984) would suggest that the digestion products still contain an antibody-binding site.

The human TSH receptor A subunit has an M_r of 50 000 (78 000 minus 28 000 for TSH) as judged by SDS/polyacrylamide-gel electrophoretic analysis and this is close to the value of $47 000 \pm 5000$ obtained by gel filtration and sucrose density gradient centrifugation analysis of LAA released by reduction of human thyroid membranes. Several factors suggest therefore that LAA released by reduction of human thyroid membranes is the A subunit of the human TSH receptor, in particular, (a) both preparations are hydrophilic and are released by reduction, (b) they have similar M_r values, and (c) both preparations crosslink to 125 I-HSAB-TSH.

It would appear therefore that the A subunit of

the TSH receptor contains binding sites for the receptor-binding and thyroid-stimulating activities of Graves' serum as well as for TSH. Furthermore, LAA and therefore the receptor A subunit appear to be glycosylated.

The studies described in this paper provide some indication of the mechanism of LAA release by freezing and thawing of human thyroid membranes. Reduction of the disulphide bridge between the A and B subunits of the receptor did not seem a likely mechanism, as mild reduction with cysteine was ineffective in releasing LAA.

Previous studies have shown that the interchain disulphide bridge is close to one end of the receptor A subunit (Buckland & Rees Smith, 1984) and consequently, limited proteolysis of the A subunit in the region of the disulphide bridge might be expected to have a similar effect to reduction and provide a mechanism for LAA release during freezing and thawing. This possibility was supported by the observation that LAA was released from thyroid membranes by papain digestion. Furthermore, the TSH receptor A subunit is susceptible to cleavage by trypsin (Buckland & Rees Smith, 1984), and papain (Y. Kajita & B. Rees Smith, unpublished work) in the region of the interchain disulphide bridge(s). Consequently, limited proteolysis might be expected to release a fragment of the receptor A subunit only slightly smaller than the intact subunit and with the ability to bind TSH and TSH receptor antibodies.

In conclusion therefore, our studies suggest that LAA released by reduction of human thyroid membranes is the A subunit of the TSH receptor. The receptor A subunit appears to contain the binding sites for TSH and the receptor binding and thyroid stimulating activities of Graves' serum. Freezing and thawing of human thyroid homogenates appears to release a large fragment of the receptor A subunit, probably as a result of enzymic

cleavage in the region of the interchain disulphide bridge(s).

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