Thyrotropin cross-links to the thyrotropin receptor through both the α and β subunits

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We have recently shown that the β subunit of thyrotropin (TSH) can be cross-linked to the TSH receptor [Buckland, Strickland, Pierce & Rees Smith (1985) *Endocrinology* (Baltimore) **116**, 2122–2124; Buckland, Strickland & Rees Smith (1985) *Biochem. Soc. Trans.* **13**, 942–943]. We failed, however, to cross-link the α subunit to the receptor; leaving the role of this subunit in the TSH–TSH-receptor interaction uncertain. We now report the successful cross-linking of the TSH α subunit to the receptor by the use of two different cross-linking reagents. Our studies suggest therefore that both subunits of TSH form part of the hormone's receptor-binding site.

INTRODUCTION

Recently, we have been able to show that TSH coupled to the photoactive cross-linking reagent HSAB (Fig. 1) cross-links to the TSH receptor by way of the hormone's β subunit (Buckland *et al.*, 1985a). This indicated that the β subunit of TSH forms part of its receptor binding site, but it was not possible to ascertain the role of the α subunit. We have now extended our studies using two different photoactive cross-linking reagents, NHS-ASA and ANB-NOS (Fig. 1). These reagents cross-link both the α and β subunits of TSH directly to the TSH receptor.

METHODS

Preparation of TSH and TSH derivatives

Highly purified bovine TSH (70 units/mg) was coupled to the photoactive heterobifunctional reagents ANB-NOS, NHS-ASA and HSAB by using procedures described previously (Buckland *et al.*, 1982, 1985c) to give preparations of ANB-NOS-TSH, NHS-ASA-TSH and HSAB-TSH which contained on average one molecule of reagent on each of the two subunits. The ANB-NOS-TSH, NHS-ASA-TSH and HSAB-TSH were labelled with ^{125}I by the Iodogen method (one atom of ^{125}I /molecule of protein) and purified by column chromatography on Sephadex G-100 (Fraker & Speck, 1978; Rees Smith & Hall, 1981). Approx. 20–30% of the ^{125}I -labelled TSH derivatives could be specifically bound by an excess of detergent-solubilized porcine TSH receptors.

TSH receptor preparations

Crude membranes were prepared from porcine thyroid tissue as described previously (Buckland *et al.*, 1984) and solubilized by treatment with the non-ionic detergent Lubrol-12A9 (Rees Smith & Hall, 1981). The solubilized receptors were then partially purified by Sepharose–TSH affinity chromatography (Rickards *et al.*, 1981).

Fig. 1. Structures of cross-linking reagents used

Antisera

Antisera to RCM-TSH α and RCM-TSH β subunits were generously given by Professor J. G. Pierce, and their characteristics have already been described (Buckland *et al.*, 1985a; Pierce *et al.*, 1976). Anti-RCM-TSH α precipitated 91% of RCM-TSH α and 1.3% of RCM-TSH β . Anti-RCM-TSH β precipitated 92% of RCM-TSH β and 1.8% of RCM-TSH α .

Abbreviations used: TSH, thyrotropin; HSAB, *N*-hydroxysuccinimide 4-azidobenzoate; DSS, disuccinimidyl suberate; NHS-ASA, *N*-hydroxysuccinimide 4-azidosalicylate; ANB-NOS, *N*-hydroxysuccinimide 5-azido-2-nitrobenzoate; RCM, reduced and carboxymethylated.

Fig. 2. Summary of experimental protocol

Cross-linking

^{125}I -ANB-NOS-TSH, ^{125}I -NHS-ASA-TSH or ^{125}I -HSAB-TSH (500 μl , 6×10^6 c.p.m.) were incubated with affinity-purified TSH receptor preparations (500 μl , 50 μg of protein) at room temperature for 3 h and cross-linked by photolysis (Buckland *et al.*, 1982); the efficiency of the cross-linking process can be in the region of 50% (Buckland *et al.*, 1984). Bound and free ^{125}I -labelled TSH analogues were separated by precipitation with poly(ethylene glycol) (Rickards *et al.*, 1981). The pelleted material was redissolved in 6 M-urea in 100 mM-Tris/HCl, pH 7.5, (300 μl) and reduced by addition of 2-mercaptoethanol (50 μl). After incubation at 37 °C overnight, S-carboxymethylation was carried out by addition of 320 μl of iodoacetic acid (2 M; adjusted to pH 8).

After 45 min at room temperature, the samples were desalted on a Sephadex G-25 medium grade column (0.7 cm $\times$ 10 cm) run in 10 mM-sodium phosphate buffer, pH 7.5, containing 1% Triton X-100, 1% sodium deoxycholate and 150 mM-NaCl (Triton/deoxycholate) and the pooled fractions were divided into two. To one sample, anti-RCM-TSH α (5 μl) was added and to the other anti-RCM-TSH β (10 μl) was added and the mixtures were incubated overnight at 5 °C. Protein A (*Staphylococcus aureus* cells; 50 μl of 10% suspension) was added to each sample, followed by incubation at room temperature for 30 min. The protein A was pelleted by centrifugation at 14000 g for 1 min (the supernatants were retained), washed with Triton/deoxycholate, and resuspended in SDS sample buffer (Laemmli, 1970).

The supernatant from the precipitation with anti-RCM-TSH α was incubated for a further 24 h at 5 °C with anti-RCM-TSH β and similarly the supernatant from the precipitation with anti-RCM-TSH β was incubated with anti-RCM-TSH α .

Both samples were incubated with protein A, pelleted by centrifugation and resuspended in SDS sample buffer as above. This experimental protocol is summarized in Fig. 2. Samples were heated at 100 °C for 15 min and pelleted by centrifugation at 14000 g for 20 min. The supernatants were analysed by gradient (8–12%) SDS/polyacrylamide-gel electrophoresis and autoradiography of the dried gels was carried out with the use of Kodak X-OMAT S film with Cronex Lightning Plus enhancing screens (Dupont) for 16 h at –70 °C.

RESULTS

An autoradiograph of analysis by SDS/polyacrylamide-gel electrophoresis of ^{125}I -ANB-NOS-TSH-TSH-receptor cross-linked complexes (reduced and carboxymethylated) precipitated with different antisera is shown in Fig. 3. Individual subunits of TSH (M_r approx. 14000) cannot be distinguished due to 'burning out' of the film. However, it was possible to distinguish the characteristic patterns of TSH α and β (Buckland *et al.*, 1982) by using shorter exposure times (results not shown).

Precipitation of the cross-linked complex with anti-RCM-TSH α (Fig. 3, lane 1) gave bands due to TSH α subunit, intact TSH (internally cross-linked, M_r 28000)

Fig. 3. Analysis by SDS/polyacrylamide-gel electrophoresis and autoradiography of cross-linked ^{125}I -ANB-NOS-TSH-receptor complexes (RCM) after precipitation with anti-RCM-TSH α and anti-RCM-TSH β sera and protein A

Lane 1, cross-linked complex and anti-RCM-TSH α ; lane 2, cross-linked complex and anti-RCM-TSH β ; lane 3, supernatant from lane 1 and anti-RCM-TSH β ; lane 4, supernatant from lane 2 and anti-RCM-TSH α . The data are typical of those from five experiments.

and a further band at M_r 58000 corresponding to the receptor cross-linked to a single subunit of TSH. A less intense band at M_r 74000 was also present, and this corresponded to the receptor cross-linked to intact TSH. Precipitation of the cross-linked complex with anti-RCM-TSH β (Fig. 3, lane 2) gave essentially the same pattern of bands as seen in lane 1. The supernatant from the precipitation with anti-RCM-TSH α , when precipitated with anti-RCM-TSH β , gave a band corresponding to TSH β and a further band at M_r 60000 (Fig. 3, lane 3).

Fig. 4. Analysis by SDS/polyacrylamide-gel electrophoresis and autoradiography of cross-linked ^{125}I -NHS-ASA-TSH-receptor complexes (RCM) after precipitation with anti-RCM-TSH α and anti-RCM-TSH β sera and protein A

Lane 1, cross-linked complex and anti-RCM-TSH α ; lane 2, cross-linked complex and anti-RCM-TSH β ; lane 3, supernatant from lane 1 and anti-RCM-TSH β ; lane 4, supernatant from lane 2 and anti-RCM-TSH α . Data are typical of those from five experiments.

The supernatant from precipitation with anti-RCM-TSH β when precipitated with anti-RCM-TSH α (Fig. 3, lane 4) gave essentially the same pattern of bands as seen in lane 3 (TSH α being present rather than TSH β).

Material at the top of the autoradiograph (high M_r) was probably TSH-TSH-receptor complex undissociated from immunoglobulins.

Heating the protein A-pelleted immunocomplexes for longer than 15 min reduced the intensity of these bands

but also reduced the clarity of the bands at lower M_r . If the protein A-pelleted immunocomplexes were not heated, the only material visible corresponded to this band at high M_r (at the top of the autoradiograph).

An autoradiograph of analysis by SDS/polyacrylamide-gel electrophoresis of ^{125}I -NHS-ASA-TSH-TSH-receptor cross-linked complexes (reduced and carboxymethylated) precipitated with different antisera is shown in Fig. 4. The samples in each lane corresponded to those shown in Fig. 3 and the bands seen were similar. Analysis of ^{125}I -HSAB-TSH-TSH-receptor cross-linked complex by the method used above showed that anti-RCM-TSH α would only precipitate a band at M_r 74000 (in addition to TSH) whereas anti-RCM-TSH β would precipitate bands at M_r 74000 and 59000 as previously described (Buckland *et al.*, 1985a).

DISCUSSION

Our results showed that the reagents ANB-NOS and NHS-ASA, which are similar in structure to HSAB, could both be used to cross-link TSH to its receptor. Experiments using subunit-specific antisera, however, showed significant differences between these new reagents and HSAB, principally that, with cross-linked complexes formed using ANB-NOS or NHS-ASA, anti-RCM-TSH α precipitated a band of M_r 58000 corresponding to a single subunit of TSH cross-linked to the receptor. Anti-RCM-TSH β precipitated a band at M_r 60000, also corresponding to a single subunit of TSH cross-linked to the receptor. This small difference in M_r of approx. 2000 between these two bands may have been due to structural rather than true molecular mass differences (Griffiths, 1972) as suggested previously with cross-linked TSH-TSH-receptor complexes (Buckland *et al.*, 1982). It can be seen from lanes 3 and 4 in both Figs. 3 and 4 that these two bands at M_r 58000 and 60000 were specifically precipitated by the respective antisera only. It is clear, therefore, that both TSH α and TSH β were cross-linking directly to the receptor with similar efficiency. Both reagents also cross-linked the two subunits of TSH to each other, as could be seen from the bands at M_r 28000; in the absence of cross-linking, TSH dissociates into its component M_r 14000 subunits under the conditions used (Sorimachi & Condliffe, 1978). Bands seen at M_r 74000 corresponding to both subunits of TSH cross-linked to

the receptor were relatively faint compared with those produced by using HSAB (Buckland *et al.*, 1985a). This may have been because both subunits of TSH did not cross-link efficiently to the receptor at the same time. The faint bands at M_r 74000 were possibly due, therefore, to intact TSH (internally cross-linked) cross-linking to the receptor through only one of the subunits.

In summary, therefore, our studies indicated that both the α and β subunits of TSH cross-link directly to the TSH receptor. As the effective cross-linking lengths of the reagents used were in the region of 1 nm (Ji & Ji, 1981), this suggests that both subunits of TSH were in close proximity with the receptor and formed part of the hormone's receptor-binding site.

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