A structural comparison of guinea pig thyroid and fat TSH receptors by photoaffinity labelling

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Received 20 October 1983

TSH receptors from guinea pig thyroid and epididymal fat have been covalently crosslinked to ^{125}I-labelled TSH conjugated to *N*-hydroxysuccinimidyl-4-azidobenzoate. Analysis by SDS–PAGE and autoradiography showed bands corresponding to TSH subunits (M_r 14000) and intact TSH (M_r 28000; subunits crosslinked) and two at higher M_r separated by 14000. The latter bands represented one or two subunits of TSH crosslinked to a subunit of the TSH receptor with M_r 57000 (fat) or 60000 (thyroid). These M_r values were reduced by trypsin treatment to 43000 and 50000, respectively. Analysis under non-reducing conditions showed that both fat and thyroid receptors have a second disulphide linked subunit of M_r 30000.

TSH receptor *Photoaffinity labelling* *TSH–TSH receptor complex* *Disulphide-linked subunit* *Partial trypsin digestion* *Guinea pig adipocyte*

1. INTRODUCTION

Receptors for thyroid-stimulating hormone (TSH) are present on the surfaces of thyroid cells and adipocytes [1,2]. Both receptors are functionally linked to adenylate cyclase [1,3–5] and have similar TSH binding characteristics [1,2] but no structural comparisons have yet been made. Consequently we have used affinity labelling [6] with ^{125}I-labelled TSH to analyze both receptors, and our results suggest that they are structurally similar but not identical.

2. MATERIALS AND METHODS

2.1. *Preparation of ^{125}I-labelled HSAB-TSH*

Highly purified bovine TSH (70 MRC units/mg) was coupled to the photoactive heterobifunctional reagent *N*-hydroxysuccinimidyl-4-azidobenzoate (HSAB) as in [7] to give a preparation which contained, on average, one molecule of HSAB on each of the 2 subunits of TSH. The HSAB-TSH retained about 40% of its TSH receptor binding and thyroid stimulating activities. After labelling with ^{125}I using the Iodogen method [8,9] (1 atom of ^{125}I per molecule of HSAB-TSH) 20–30% of the labelled HSAB-TSH could be specifically bound by an excess of detergent solubilised porcine TSH receptors.

2.2. *Preparation and analysis of crosslinked TSH–TSH receptor complexes*

Crude membranes were prepared from guinea pig thyroid and epididymal fat pad tissue as in [2] and solubilised by treatment with the non-ionic detergent Lubrol-12A9 [9].

Equal volumes of [^{125}I]HSAB-TSH and TSH receptors (cell membranes or detergent solubilised) were incubated together, photolysed, and bound and free [^{125}I]HSAB-TSH were separated by precipitation with polyethylene glycol in the case of soluble receptors or centrifugation in the case of cell membranes [7]. About 40% of the TSH specifically bound to the receptor was crosslinked to its receptor as judged by the inability of 2 M NaCl or sodium dodecyl sulphate (SDS) to cause dissociation of the ^{125}I-labelled TSH–TSH receptor complex.

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00145793/84/$3.00

Pelleted material was resuspended in 50 μl NaCl (50 mM) Tris (10 mM), pH 7.5. Trypsin (1%, 0.5 μl) was added to some samples and the mixture incubated at room temperature for 15 min followed by the addition of a 2-fold excess of trypsin inhibitor and a further 10-min incubation; 50 μl of sodium dodecyl sulphate (SDS) solution (4%) containing 2-mercaptoethanol (2%) in some cases was added to trypsin-treated or -untreated samples followed by heating on a boiling water bath for 30 min. A 2-fold molar excess of iodoacetamide was added and heating continued for 5 min. Samples were analyzed by 8–12% gradient SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography of the dried gels carried out using Kodak X-OMAT film with Cronex lightning plus enhancing screens (Dupont) for 16 h at −70°C.

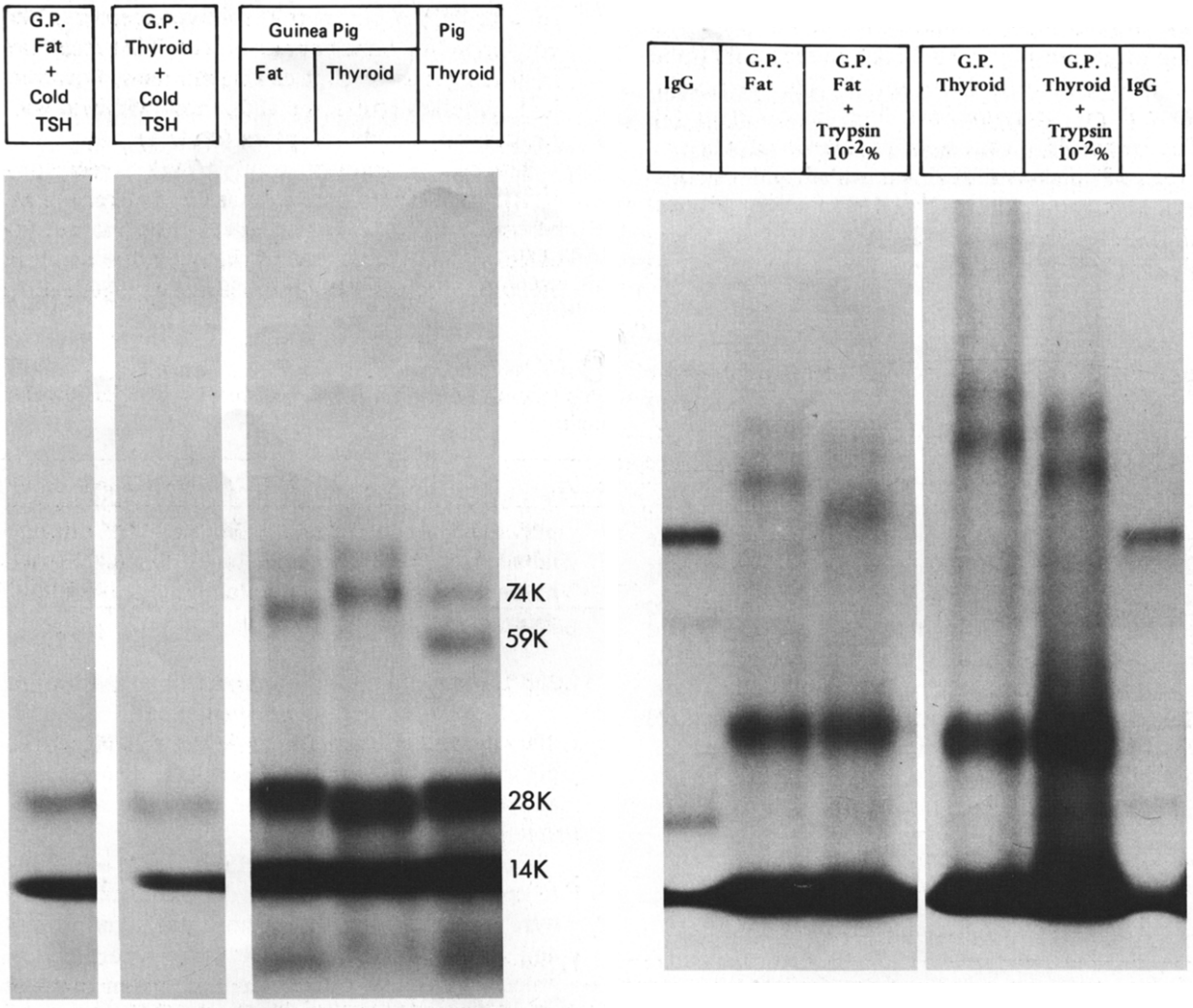

Fig.1. Analysis under reducing conditions of porcine thyroid, guinea pig thyroid and guinea pig epididymal fat TSH receptors cross-linked to ^{125}I-labelled HSAB-TSH. (left) Porcine thyroid, guinea pig thyroid and guinea pig epididymal fat soluble receptors cross-linked to ^{125}I-labelled HSAB-TSH and analyzed under reducing conditions in the absence of trypsin. Addition of an excess of unlabelled TSH to the TSH receptors prior to cross-linking prevents the formation of the cross-linked complex (left). M_r values shown are those of TSH (28000) and its subunits (14000) [11] and cross-linked TSH-porcine TSH receptor (59000 and 74000) [7]. (right) Guinea pig epididymal fat and guinea pig thyroid membrane-bound TSH receptors cross-linked to [^{125}I]HSAB-TSH and analyzed under reducing conditions before and after trypsin treatment.

3. RESULTS

When photolysed mixtures of [^{125}I]HSAB-TSH and TSH receptors were analyzed on SDS–PAGE under reducing conditions the ^{125}I was associated with 4 major bands. In the case of soluble receptors, bands corresponding to TSH subunits (M_r 14000) and intact TSH (M_r 28000) were present in addition to bands at higher M_r (fig.1a). These bands had M_r values of 70500 and 85500 in the case of guinea pig fat and 74000 and 88000 in the case of guinea pig thyroid soluble receptors (table 1). M_r values of 59000 and 74000 for porcine thyroid preparations have been reported in [7]. The higher M_r bands were not observed when an excess of unlabelled TSH was added to the incubation mixture prior to [^{125}I]HSAB-TSH (fig.1a). Similarly these bands were not observed in the absence of photolysis, receptors or cross-linking reagent. Consequently these bands appeared to be components of the TSH–TSH receptor complex.

Analysis of [^{125}I]HSAB-TSH cross-linked to TSH receptors in crude cell membrane preparations showed a similar pattern of bands (fig.1b). In addition to bands due to TSH and its subunits, two bands representing TSH–TSH receptor complexes could be seen. In the case of guinea pig thyroid membranes, these had the same M_r as those obtained with guinea pig thyroid soluble receptors. The corresponding bands obtained with TSH receptors in crude guinea pig fat cell membranes, however, had M_r values 8000 lower than those obtained with fat derived soluble receptors (table 1).

Trypsin treatment of TSH receptor–[^{125}I]HSAB-TSH complexes did not effect the M_r of bands due to TSH and its subunits but had the effect of lowering the M_r of the two other bands in all cases. The M_r of bands obtained with guinea pig

Table 1

Relative M_r values of cross-linked TSH–TSH receptor complexes on SDS–PAGE and reduced M_r of TSH receptor subunits

TSH receptor	TSH–TSH receptor complex			TSH receptor subunit		
	M_r values reduced	M_r unreduced	M_r values reduced + trypsin	Subunit 'A'	Subunit 'B'	'A' subunit after trypsin treatment
Guinea pig[a]	74100 ± 1800 (16)	118700 ± 1000 (11)	66000 ± 900 (8)	60000	30500	51000
Thyroid	88200 ± 1600 (16)		79400 ± 1300 (8)			
Guinea pig fat membrane-bound receptors	62500 ± 700 (13)	109200 ± 1350 (16)	56100 ± 500 (8)	49000	32000	42500
	77200 ± 900 (13)		70600 ± 900 (8)			
Guinea pig fat detergent-solubilised receptors	70500 ± 1900 (6)		58100 ± 1800 (4)	57000		43500
	85500 ± 1500 (6)		71600 ± 1500 (4)			

[a] Values shown are means of those obtained using membrane-bound and detergent-solubilised receptors

M_r values of TSH–TSH receptor complexes are expressed as means ± SD with the number of determinations shown in parentheses. Receptor 'A subunit' M_r values are derived by subtracting the M_r of TSH (28300 [11]) from the highest M_r band observed with TSH–TSH receptor complexes under reducing conditions. Receptor 'B subunit' M_r values are derived by subtracting the M_r value of the highest M_r band observed with TSH–TSH receptor complexes under reducing conditions from the corresponding M_r value obtained under non-reducing conditions. Receptor subunit M_r values are rounded to the nearest 500. Reduced M_r values for guinea pig thyroid were derived from studies with both membrane-bound and detergent-solubilised receptors. Non-reduced values were only obtained using membrane-bound receptors

fat crude cell membranes and soluble receptors were lowered by different amounts but the resulting M_r values were similar (table 1).

When complexes of crude cell membranes and [^{125}I]HSAB-TSH were analyzed in the absence of 2-mercaptoethanol a different pattern of bands was produced (fig.2). In addition to bands due to TSH and its subunits, a single broad and indistinct band was observed at M_r 119000 in the case of guinea pig thyroid and 109000 in the case of guinea pig fat (fig.2, table 1).

Fig.2. Analysis of guinea pig fat and thyroid TSH–TSH receptor cross-linked complexes under non-reducing conditions.

4. DISCUSSION

Previous studies [7] have indicated that the two bands differing in M_r by 14000 which comprise the components of the TSH–TSH receptor complexes observed on SDS–PAGE under reducing conditions represent a single subunit of the TSH receptor (designated the A subunit) cross-linked to (i) one subunit of TSH (lower M_r band) or to (ii) two subunits of TSH (higher M_r band) where the TSH subunits are themselves cross-linked so dissociation cannot occur.

On this basis, this study indicates that the subunit of the receptor observed on the reduced gels (A subunit) has a slightly higher M_r in detergent solubilised preparations of guinea pig thyroid (M_r 60000) than in similar preparations of guinea pig fat (M_r 57000).

Further evidence for differences in the complexes formed between ^{125}I-labelled HSAH-TSH receptors solubilised from guinea pig fat and thyroid was obtained from experiments which involved treating the complexes with trypsin prior to electrophoresis. This resulted in a reduction in M_r of bands corresponding to TSH–TSH receptor complexes as shown in table 1 and fig.1b. However the change in M_r of the detergent-solubilised fat receptor preparation was greater than that observed with the thyroid preparation (table 1).

Trypsin treatment of cross-linked complexes formed between TSH and detergent-solubilised receptors reduced their M_r by 10000 in the case of guinea pig thyroid and 13500 in the case of guinea pig fat. The trypsin cleavage points may have been on TSH but there were several reasons which suggested that the observed cleavage occurred on the receptor:

(i) Cross-linked complexes formed between TSH and fat and thyroid-derived receptors lost different sized peptides on treatment with trypsin. This would not be expected if only TSH was cleaved;

(ii) The difference in M_r of one subunit of TSH (14000) between the two bands was unchanged after trypsin treatment;

(iii) The ^{125}I-labelled HSAB-TSH present which was not cross-linked to the receptor gave essentially the same pattern on the gels in the presence or absence of trypsin.

A comparison was also made between the com-

plexes formed when ^{125}I-labelled HSAB-TSH was cross-linked to membrane-bound and detergent-solubilised TSH receptors. In the case of the thyroid, the same results were obtained with membrane-bound and detergent-solubilised preparations and similar effects were observed with trypsin. Although membrane-bound fat TSH receptors gave the same two-band pattern, in the absence of trypsin, both bands were of lower M_r (fig.1b; table 1) than those obtained with detergent solubilised fat TSH receptor.

The results obtained with fat TSH receptor are consistent with an enzyme present in membrane-bound fat receptor preparations which cleaves a small peptide (M_r 8000) from the receptor A subunit during incubation with ^{125}I-labelled HSAB-TSH. We can then postulate that the enzyme is inactivated in, or absent from the detergent-solubilised preparations.

Trypsin treatment of membrane bound fat TSH receptor–TSH complexes lowered their M_r by 6500 whereas the same treatment lowered the M_r of detergent-solubilised fat TSH receptor–TSH complexes by 13500. However, the fragments produced were of similar M_r in each case and this suggested that trypsin cleaves off a portion of the receptor which contains the site of action of the proposed fat membrane enzyme (fig.3).

It would appear therefore that trypsin treatment

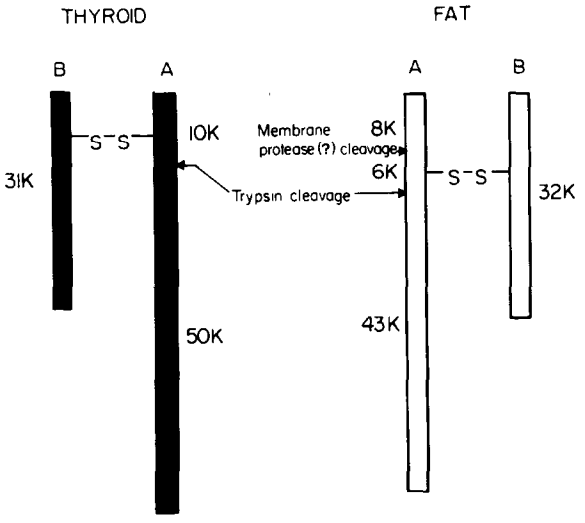

Fig.3. Proposed structure of guinea pig thyroid and fat TSH receptors. M_r values are approximate. Position and number of disulphide bridges is not known.

results in:

(i) cleavage of a 10000 M_r peptide from the guinea pig thyroid TSH receptor (whether it is membrane-bound or solubilised) giving a final M_r of 50000;

(ii) cleavage of a 13500 M_r peptide from the detergent-solubilised guinea pig fat TSH receptor giving a final M_r of 43500;

(iii) cleavage of a 6500 M_r peptide from membrane-bound guinea pig fat TSH receptor giving a final M_r of 42500.

Further information on the structure of the TSH receptor preparations were obtained from SDS–PAGE analysis of TSH–TSH receptor complexes in the absence of reducing agent. Although gels run under non-reducing conditions gave higher backgrounds and more diffuse bands it was possible to demonstrate the presence of ^{125}I-labelled TSH–TSH receptor complexes (fig.2). Broad major bands with approximate M_r values of 120000 and 110000 were observed for cross-linked TSH–TSH receptor complexes prepared from guinea pig thyroid and fat cell membranes, respectively. As TSH does not dissociate readily into its subunits under the conditions used for analysis on non-reducing gels, these complexes would be expected to contain both subunits of TSH [10]. When the complexes were eluted from the gel and re-run under reducing conditions the characteristic 2 bands differing in M_r by 14000 were observed confirming the presence of both TSH subunits in the TSH–TSH receptor complexes observed under non-reducing conditions.

These studies suggest that under non-reducing conditions the guinea pig thyroid TSH receptor has an M_r of about 90000 (after subtraction of the M_r of intact TSH from 120000) which is reduced to 60000 in the presence of mercaptoethanol. Consequently the thyroid receptor appears to contain an additional subunit (designated B subunit) of about M_r 30000 which is not directly cross-linked to TSH but linked to the A subunit by a disulphide bridge. Similarly the fat TSH receptor appears to contain an A subunit of M_r 57000 disulphide bonded to a B subunit of about M_r 30000.

These structures are summarised in fig.3. The bases of the small differences in A subunit M_r values and trypsin action are not clear at present but they could reflect differences in the primary structure of the two receptors.

ACKNOWLEDGEMENTS

This work was supported by grants from the Medical Research Council and the Welsh Office. We are most grateful to Professor R. Hall for helpful advice and encouragement.

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