

## Characterization and partial purification of the human receptor for the heat-stable enterotoxin

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The receptor for the *Escherichia coli* heat-stable enterotoxin has been characterized and partially purified from the T84 human colonic cell line. Using a novel mutant heat-stable enterotoxin peptide as a radioligand (the C-terminal tyrosine residue is replaced by phenylalanine in the mutant), a single class of high-affinity receptor sites was detected in T84 cells, with a  $K_d$  of 0.1 nM, similar in affinity to the receptor described in human intestinal tissue. The receptor was solubilised from T84 cell membranes and affinity cross-linking of the solubilised preparation indicated that a single species of  $M_r$  160000 served as the receptor. Freshly solubilised preparations of the receptor retained heat-stable enterotoxin-activable guanylyl cyclase activity. Purification of the receptor was achieved through sequential affinity chromatography on GTP–epoxy-Sepharose and wheat-germ-agglutinin columns resulting in purification of the receptor by 3000 fold. The heat-stable enterotoxin-binding characteristics of the receptor were unchanged during the purification and silver staining of the purified receptor preparation indicated a band of  $M_r$  160000, which was specifically cross-linked to the  $^{125}\text{I}$ -labeled mutant peptide. The purified receptor retained guanylyl cyclase activity, but the activity was not stimulated on addition of human heat-stable enterotoxin, suggesting that accessory structural factors may be involved in the activation of the guanylyl cyclase/receptor.

The heat-stable enterotoxins (ST) are a family of low-molecular-mass peptide toxins, and are one of the major causes of watery secretory diarrhoea all over the world [1, 2]. Various forms of the toxins, differing in amino acid sequence, are produced by a number of pathogenic bacteria [3, 4], and all these peptides contain a cysteine-rich core essential for full biological activity [5, 6]. ST peptides bind to a receptor on intestinal cells and activate membrane-bound guanylyl cyclase [7, 8]. Increased levels of cyclic GMP (cGMP) within the cell are hypothesised to lead to enhanced  $\text{Cl}^-$  secretion from the intestinal cell by as yet undefined mechanisms, resulting in fluid loss and subsequent diarrhoea [9].

Early biochemical studies had postulated that in rat intestinal membranes the ST-binding and guanylyl cyclase activities were located on separate molecules [10]. However, the cloning and expression of the rat and human intestinal ST receptor [11–13] suggested that the ST receptor was in fact a high-molecular-mass protein and a member of the guanylyl cyclase family of receptors, described earlier for atrial natriuretic factor and the sea urchin egg peptides [14]. These observations implied that the ST-binding and guanylyl cyclase activities were present in the same receptor molecule. How-

ever, there is no biochemical evidence using purified receptor preparations to confirm that the ST receptor does in fact exist in the cell as suggested for the recombinant protein. Hugues et al. reported the purification of the rat ST receptor from intestinal membranes using ST–ligand affinity chromatography and suggested that a protein of  $M_r$  70000 was the receptor [15]. However, the purified protein did not possess guanylyl cyclase activity, and this was attributed to the instability of the guanylyl cyclase activity from rat intestinal membranes during purification procedures. However, the major protein purified in these studies was of a lower molecular mass than that predicted from the DNA sequence of the cloned receptor and hence may not have represented the functional ST receptor [15].

Earlier studies from this laboratory have demonstrated [17] that application of ST and other forms of ST peptides, produced by a variety of bacteria, to T84 cells, a cell line derived from a human colonic carcinoma [18], resulted in enhanced cGMP production by the cells, strongly suggesting that these peptides all bind to the same receptor. Interestingly, the response of T84 cells to various analogues of ST led to the differential production of cGMP [19], suggesting altered interaction of the ST peptides with the human receptor on T84 cells. The T84 cell line thus served as a model system to study ST/human receptor interactions in detail. In this study, the characterization and purification of the ST receptor from T84 cells are described. We report the partial purification of a high-molecular-mass protein that retains both ST-binding and guanylyl cyclase activities, and presumably acts as a functional ST receptor.

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Abbreviations. cGMP, cyclic GMP; ST, heat-stable enterotoxin(s);  $\text{ST}_h$ , human heat-stable enterotoxin(s);  $\text{ST}_p$ , porcine heat-stable enterotoxin; ST Y72F, heat-stable enterotoxin with C-terminal tyrosine residue replaced by phenylalanine.

Enzyme. Guanylyl cyclase (EC 4.6.1.2).

## MATERIALS AND METHODS

### Culture and maintenance of T84 cells

T84 cells were cultured and maintained as described earlier [18]. Cells were either grown in 24-well dishes (Nunc) to a density of  $5 \times 10^5$  cells/well, at which time they were used directly for monitoring the activation of guanylyl cyclase by ST [19], or grown to confluency in bottles and harvested for the preparation of membranes.

### Production of mutant ST peptide

Mutagenesis was performed on the cloned ST gene [16], essentially according to the method of Kunkel [20]. The sequence of the mutagenic primer was 5' CCGGGTGCTTTT-AATAATAT 3', and the sequence of the product mutant gene was confirmed by DNA sequencing [21]. The mutant gene was cloned into the pET7 vector as a *Bam*HI–*Hind*III fragment to yield the vector pARC 0730, which was transformed into the strain BL21 (DE3) as described earlier [16]. The mutant ST peptide was overexpressed under identical conditions described for the human heat-stable enterotoxin (ST<sub>h</sub>) gene [16].

### Purification of ST<sub>h</sub> and mutant ST peptide from the culture fluid of the overexpressing strains

ST<sub>h</sub> peptide was purified according to the method reported earlier [16]. The purification procedure for the ST Y72F mutant peptide (tyrosine at position 72 is replaced by phenylalanine) was similar to that employed for ST<sub>h</sub>, except for a few modifications. Following adsorption of the peptide to Amberlite XAD-2, elution and concentration by lyophilisation of the bound peptide, ST Y72F was purified by reverse-phase HPLC, using an Applied Biosystems 150 series HPLC system on an Aquapore RP 300 column (7- $\mu$ m particle size, 220 mm  $\times$  4.6 mm, Applied Biosystems). Gradient elution of the bound peptide was achieved using a gradient of 20–100% B over 30 min at a flow rate of 1 ml/min. Buffer A consisted of 0.05% trifluoroacetic acid in water, and buffer B consisted of 70% acetonitrile in 0.05% trifluoroacetic acid/water. ST Y72F peptide was eluted with a retention time of 16–17 min in this gradient. Both ST<sub>h</sub> and ST Y72F were purified again through the gradient system described above to ensure homogeneity of the preparations.

Peptides were quantified by amino acid analysis [22], and the analysis confirmed the sequence of the two peptides.

### Radio-iodination of ST Y72F and purification of the radiolabelled peptide

Purified ST Y72F was radiolabelled with carrier-free Na<sup>125</sup>I (ICN Radiochemicals) by the lactoperoxidase method. Lactoperoxidase beads (BioRad) were reacted with 5  $\mu$ g ST Y72F and 1 mCi carrier-free Na<sup>125</sup>I in 50  $\mu$ l 100 mM sodium phosphate, pH 7.5, containing 40  $\mu$ g of  $\beta$ -D-glucose, at 4°C for 1 h. The reaction mixture was applied to a Sep-Pak cartridge (Millipore) previously equilibrated with water. The cartridge was washed with water to remove free Na<sup>125</sup>I and the bound peptide was eluted with 50% acetonitrile in water. The fractions containing radiolabelled peptide were concentrated to remove the acetonitrile, and subjected to reverse-phase HPLC to purify the radiolabelled peptide. Reverse-phase HPLC was performed on a  $\mu$ Bondapak C<sub>18</sub> (column 4- $\mu$ m particle size, 2 mm  $\times$  30 mm, Waters Associates). The

radiolabelled peptide was eluted using a gradient from 10% acetonitrile in water to 30% acetonitrile in water in 90 min at a flow rate of 0.3 ml/min. Fractions were collected every 2 min, and the radiolabelled peptide eluted with a retention time of 70–75 min in this gradient. Under identical conditions, unlabelled ST Y72F eluted with a retention time of 60–65 min. The specific activity of the radiolabelled ST Y72F was 2000 Ci/mmol, equivalent to the specific activity of the Na<sup>125</sup>I. Radiolabelled ST Y72F was stored at 4°C in the presence of 20% acetonitrile, under which conditions it was stable for more than a month.

### Guanylyl cyclase assays on T84 monolayers

Assays were performed as described earlier [19]. Peptides were added in serum-free media and intracellular cGMP was measured by radioimmunoassay after 15 min following application of the toxin. Radioimmunoassay was performed using <sup>125</sup>I-labelled cGMP as reported earlier [19]. All cGMP levels were measured without acetylation of either the sample or the standards.

### Preparation of T84 membranes

Confluent monolayers of T84 cells were harvested by scraping into 50 mM Hepes, pH 7.5, containing 0.25 M sucrose, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml aprotinin, 2 mM phenylmethylsulphonyl fluoride, 100 mM NaCl, 1 mM dithiothreitol and 5 mM EDTA ( $10^8$  cells/10 ml buffer). Cells were homogenised in this buffer and the broken cell suspension was subjected to centrifugation at 100000 *g* for 1 h. The membrane pellet thus obtained was suspended at a concentration of 5–10 mg protein/ml in 50 mM Hepes, pH 7.5, 10  $\mu$ g/ml leupeptin and 10  $\mu$ g/ml aprotinin. Protein was estimated by the method of Bradford [23].

### *In vitro* activation of guanylyl cyclase

*In vitro* guanylyl cyclase assays were performed essentially according to Hugues et al. [15]. T84 membrane fractions, or solubilised or purified preparations of the ST receptor, were suspended in 60 mM Tris/HCl, pH 7.6, containing 10 mM theophylline, 7.5 mM creatine phosphate, and 20  $\mu$ g creatine phosphokinase. ST peptides, at the indicated concentrations were added to the mixture, and the assay was initiated by addition of a Mg-GTP solution to a final concentration of 1 mM GTP and 4 mM MgCl<sub>2</sub>. The assay mixture (final volume 100  $\mu$ l) was incubated at 37°C for 10 min and the reaction was stopped by the addition of 0.4 ml 50 mM sodium acetate, pH 4.0, and boiling of the reaction mixture for 10 min. Samples were centrifuged at 10000 *g* for 10 min and analysed for cGMP by radioimmunoassay. For monitoring guanylyl cyclase activity following the binding of ST to T84 membranes at different pH, ST<sub>h</sub> (1  $\mu$ M) was added to membrane preparations at the pH indicated, and the incubation was continued at 37°C for 10 min. Samples were centrifuged, the membranes suspended in buffer containing components for the *in vitro* guanylyl cyclase assay and the assay was started by the addition of Mg-GTP. The assay was performed at 37°C for 10 min and cGMP was measured as described above.

### Binding assays

Binding to T84 membranes was performed using 20–50  $\mu$ g T84 membrane protein, in 50 mM Hepes, pH 7.5,

4 mM MgCl<sub>2</sub>, 0.1% bovine serum albumin, and 10 µg/ml leupeptin, at 37°C for 60 min. The total assay volume was 100 µl and contained 0.1 nM <sup>125</sup>I-labelled ST Y72F. Non-specific binding was determined in the presence of unlabelled ST Y72F, ST<sub>h</sub>, or atrial natriuretic factor. Following incubation, samples were filtered through GF/B filters (Whatman) and washed three times with 3 ml chilled 10 mM sodium phosphate, pH 7.2, containing 0.9% NaCl and 0.2% bovine serum albumin. Filters were measured for radioactivity using a scintillation counter (LKB Clini Gamma).

Binding assays at different pH were performed in either 50 mM sodium acetate, pH 5.0, or 100 mM sodium carbonate/sodium bicarbonate buffer, pH 10.7. Washing of the filters was performed as above.

Binding assays to solubilised preparations of the receptor or purified fractions were performed under identical conditions in a total volume of 100 µl. However, the separation of bound ligand from free peptide was through precipitation using poly(ethylene glycol) 6000. Following binding, 200 µl 30% poly(ethylene glycol) in 50 mM Tris/HCl, 1 mM EDTA, pH 7.5, and 20 µl 3 mg/ml bovine immunoglobulin G was added to the samples and they were incubated at 4°C for 10 min. Samples were filtered through GF/B filters previously soaked in 0.2% bovine serum albumin, and filters were washed thrice with 3 ml chilled 10% poly(ethylene glycol) in 50 mM Tris/HCl, pH 7.5, containing 1 mM EDTA. Filters were dried and the radioactivity was measured as before.

In all binding assays, non-specific binding contributed to less than 10% of the total binding observed. Equilibrium binding data were analysed by the LUNDON 1 programme supplied by LUNDON Software Inc.

#### Association and dissociation kinetics for the binding of <sup>125</sup>I-labelled ST Y72F

Association kinetics of <sup>125</sup>I-labelled ST Y72F were determined at an ST Y72F concentration of 0.1 nM in the presence or absence of 100 nM unlabelled ST Y72F. Binding was performed as described above and aliquots of the reaction mixture were removed at the times indicated and filtered. For dissociation experiments, membranes were incubated with <sup>125</sup>I-labelled ST Y72F (1 nM) for 1 h followed by centrifugation at 25000 g for 10 min at 4°C. Membranes were suspended in the original binding buffer in the absence of labelled ligand. Aliquots were removed at the times indicated and samples were filtered. The kinetic data for ligand association and dissociation were subjected to the analysis of Weiland and Molinoff [24]. The dissociation rate constant ( $k_{-1}$ ) was determined directly from a first-order plot of ligand dissociation versus time. The rate of ligand association ( $k_i$ ) was determined from the equation  $k_i = k_{obs} ([LR]_e / ([L][LR]_{max}))$ , where [L] is the ligand concentration, [LR]<sub>e</sub> is the concentration of the complex at equilibrium, [LR]<sub>max</sub> is the maximum number of receptors present and  $k_{obs}$  is the slope of the pseudo-first-order plot of  $\ln ([LR]_e / ([LR]_e - [LR]_t))$  versus time.

#### Solubilisation and cross-linking of the ST receptor from T84 membranes

T84 membrane suspensions (5 mg/ml) were adjusted to 0.3% Lubrol PX (Sigma) and 0.5 M NaCl. The suspension was gently homogenised and kept stirring at 4°C for 1 h. The

sample was centrifuged at 100000 g for 1 h and the supernatant was used as the soluble receptor preparation.

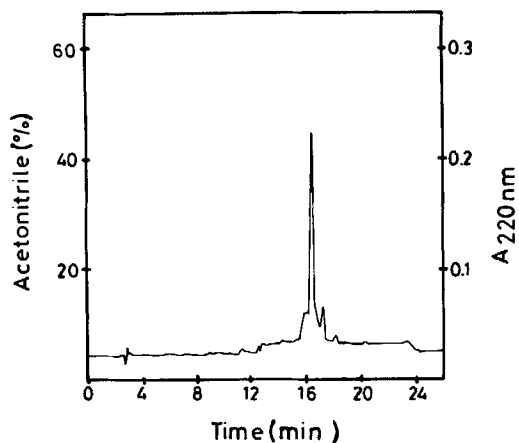
For cross-linking of <sup>125</sup>I-labelled ST Y72F to the receptor, 100 µg solubilised receptor preparation was incubated with 1 nM <sup>125</sup>I-labelled ST Y72F, either in the presence or absence of 100 nM unlabelled ST<sub>h</sub>, at 37°C for 1 h. The cross-linker dithiobis-(succinimidyl propionate), obtained from Pierce, was added to a concentration of 2 mM in a minimal volume of dimethyl sulphoxide, and the incubation was continued at 25°C for 30 min. Samples were subjected to SDS/PAGE according to the method of Laemmli [25]. Samples were boiled either in the presence or absence of 2-mercaptoethanol and electrophoresed in 7.5% acrylamide gels. Following electrophoresis, the gels were dried and subjected to autoradiography.

#### Affinity cross-linking of the ST receptor from human intestinal tissue

Biopsy tissue from regions of the human small intestine were obtained from a local hospital, and scrapings of the mucosal membrane tissue were prepared. The scrapings were suspended in 50 mM Hepes, pH 7.5, containing 1 µg/ml leupeptin, 100 mM NaCl and 2 mM dithiothreitol and homogenised. The suspension was centrifuged at 100000 g for 30 min, and the membrane fraction suspended in 50 mM Hepes, pH 7.5, containing 10 µg/ml leupeptin, 10 µg/ml aprotinin and 4 mM MgCl<sub>2</sub> to a concentration of 20 mg membrane protein/ml. The suspension was adjusted to 0.3% Lubrol PX and 0.5 M NaCl, and stirred at 4°C for 1 h. The supernatant of this suspension, obtained after centrifugation at 100000 g for 1 h, was used for cross-linking analysis in an identical manner to that employed for the T84 cells, except that 500 µg total protein was used for cross-linking.

#### Purification of the ST receptor from T84 cells

Solubilised receptor preparations were dialysed against 50 mM Hepes, pH 7.5, containing 0.1% Lubrol PX, 2 mM Na<sub>3</sub>N, 4 mM MgCl<sub>2</sub>, 1 µg/ml leupeptin, 1 µg/ml aprotinin and 20% glycerol. Removal of NaCl in the solubilised preparations was essential prior to subjecting the preparation to GTP-epoxy-Sepharose affinity chromatography. The GTP-epoxy-Sepharose affinity matrix was prepared by coupling GTP to epoxy-activated Sepharose (Pharmacia) following the manufacturer's instructions, which resulted in the coupling of 8–15 µmol GTP/ml agarose. The dialysed receptor preparation was mixed gently with the affinity matrix at 4°C for 16 h after which the beads were washed with the dialysis buffer to remove unbound proteins. The receptor was eluted with the same buffer without MgCl<sub>2</sub>, but containing 10 mM EDTA. Fractions were collected and tested for their ability to bind radioactive ST Y72F. Fractions containing ST-binding activity were pooled, adjusted to 0.5 M NaCl and directly applied to a column of wheat-germ lectin coupled to Sepharose 6B. Unbound proteins were removed by washing with the buffer used for dialysis containing 0.5 M NaCl and bound proteins were eluted by incorporating 0.5 M *N*-acetylglucosamine in the buffer. Approximately 35% of the ST-binding activity could be recovered in the eluted fractions. Samples were subjected to electrophoresis and silver staining [26]. Cross-linking of the purified receptor preparations and guanylyl cyclase activity were determined as described above.



**Fig. 1. Reverse-phase HPLC profile of purified ST Y72F peptide.** The peptide was purified from the culture supernatants of the strain overexpressing the ST Y72F peptide and chromatographed for a second time using the gradient described in the Materials and Methods section.

## RESULTS

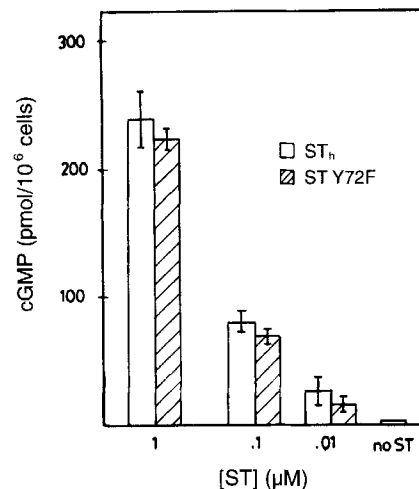
### Production and characterization of mutant ST peptide for radioligand-binding studies

The ST<sub>h</sub> form of the toxin has two tyrosine residues which can incorporate iodine during preparation of a radioligand; the presence of an iodine atom in the C-terminal tyrosine residue prevents binding of the radioligand to intestinal cells in the porcine heat-stable enterotoxin form (ST<sub>p</sub>) of the toxin [27]. We therefore chose to change the tyrosine residue at position 19 of ST<sub>h</sub> to phenylalanine, thereby retaining the hydrophobic nature of the C-terminus but preventing the incorporation of iodine into the C-terminal residue during radioiodination. Incorporation of iodine at position 5 of ST<sub>h</sub> has been shown not to affect the biological activity of the toxin [13].

The mutant ST peptide, ST Y72F, was purified from the culture medium of the overexpressing strain [16], and the purity of the peptide was confirmed by reverse-phase HPLC (Fig. 1). When similar amounts of purified ST<sub>h</sub> and ST Y72F were applied to the T84 monolayer, a comparable activation of guanylyl cyclase was observed, since intracellular concentrations of cGMP were similar at different concentrations of the toxin (Fig. 2). This confirmed the equipotency of ST<sub>h</sub> and ST Y72F in the T84 cell line, and their near identical interaction with the receptor on T84 cells. The *in vivo* activity of ST Y72F was also confirmed by the suckling mouse assay (data not shown), and 10 ng peptide induced fluid accumulation in the intestine of the suckling mouse, which was a concentration identical to that observed for ST<sub>h</sub> [19].

### Binding of <sup>125</sup>I-labelled ST Y72F to T84 membranes

Membranes were prepared from monolayer cultures of T84 cells. On addition of ST<sub>h</sub> or ST Y72F to the membrane preparations of T84 cells, the accumulation of cGMP through activation of membrane-bound guanylyl cyclase was observed (Table 1). Atrial natriuretic factor was unable to induce cGMP accumulation. These membrane preparations were used to monitor the binding of <sup>125</sup>I-labelled ST Y72F to the receptor (Table 2). Specific binding was detected which could be efficiently displaced by 100 nM of either ST<sub>h</sub> or ST Y72F, but not atrial natriuretic factor, confirming the earlier



**Fig. 2. Activation of guanylyl cyclase following application of ST<sub>h</sub> and ST Y72F to T84 monolayers.** Purified ST<sub>h</sub> and ST Y72F were applied to T84 cells at the concentrations indicated and the intracellular concentrations of cGMP were monitored by radioimmunoassay as described earlier [19].

**Table 1. Activation of guanylyl cyclase in T84 membranes.** Peptides were added to T84 membranes and cGMP was measured by radioimmunoassay. The values represent the mean  $\pm$  SE of duplicate experiments. ANF, atrial natriuretic factor.

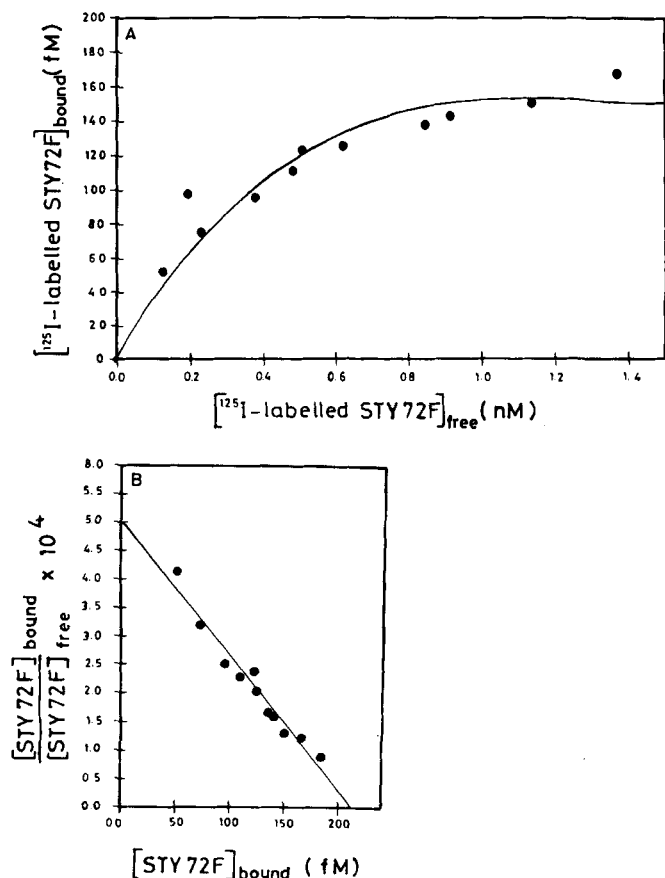
Conditions	Guanylyl cyclase activity
	pmol cGMP/min/mg protein
T84 membranes (basal)	7.9 $\pm$ 1.3
+ST <sub>h</sub> (1 $\mu$ M)	14.9 $\pm$ 2.6
+ANF (1 $\mu$ M)	6.6 $\pm$ 1.2
+ST <sub>h</sub> (1 $\mu$ M) + ANF (1 $\mu$ M)	13.2 $\pm$ 1.7

**Table 2. Specificity of binding of <sup>125</sup>I-labelled ST Y72F analog.** T84 membranes were incubated with <sup>125</sup>I-labelled ST Y72F (0.1 nM) for 1 h at 37°C in the absence or presence of peptides as indicated. Samples were filtered and the radioactivity bound to individual filters was measured. The values represent the mean  $\pm$  SE of duplicate experiments. ANF, atrial natriuretic factor.

Displacing ligand	Amount of bound <sup>125</sup> I-labelled ST Y72F
	cpm/20 $\mu$ g protein
None	8604 $\pm$ 260
ST <sub>h</sub> (0.2 $\mu$ g/ml)	1152 $\pm$ 146
ST Y72F (0.2 $\mu$ g/ml)	1063 $\pm$ 216
ANF (0.2 $\mu$ g/ml)	8312 $\pm$ 320

observation [19] that there was no interaction between atrial natriuretic factor and the ST Y72F receptor.

Scatchard analysis of the equilibrium binding data of <sup>125</sup>I-labelled ST Y72F to T84 membranes (Fig. 3) indicated that a single class of binding sites of affinity 0.37 nM ( $\pm$  0.03) was present on T84 cells at concentrations of 200 fmol/mg membrane protein. A similar affinity was detected when binding of <sup>125</sup>I-labelled ST Y72F to T84 monolayers was studied, indicating no gross changes in affinity during preparation of the membranes (data not shown.) Using a truncated



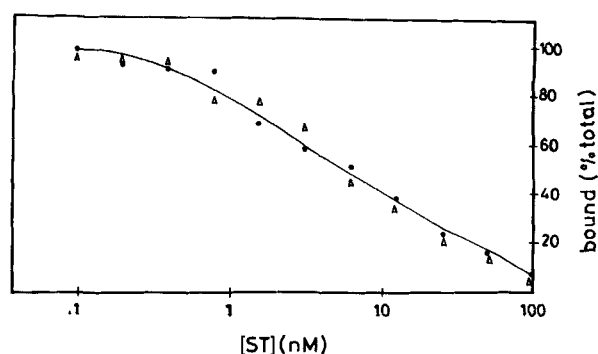
**Fig. 3. Equilibrium binding of ST Y72F to T84 membranes.** (A)  $^{125}\text{I}$ -labelled ST Y72F (0.1 nM) was incubated with 50  $\mu\text{g}$  T84 membrane protein at 37°C for the times indicated, the suspensions were filtered and the radioactivity of the filters was measured. Non-specific binding was determined at each point by incubation in the presence of 100 nM  $\text{ST}_h$ . The values represent the mean of triplicate determinations in two independent experiments. (B) Scatchard analysis of the equilibrium binding data. The data presented in Fig. 3A were subjected to an analysis using the LUNDON 1 programme to determine the  $K_d$  and the  $B_{\text{max}}$  values. The  $K_d$  was found to be 0.3 nM and  $B_{\text{max}}$  was 200 fmol/mg protein.

analog of ST [29], the cloned human receptor showed a  $K_d$  of 0.1 nM, indicating that the radioligand we have used for our studies shows properties similar to  $\text{ST}_h$ . We were unable to detect either the presence of a low-affinity binding site coupled to the activation of guanylyl cyclase, as has been described in rat intestinal membranes, or a second class of high-affinity binding sites, as has been reported for rat intestinal membranes in the presence of NaCl [30].

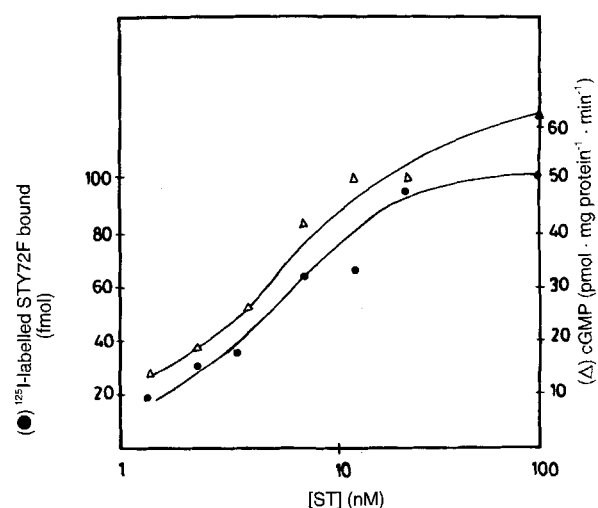
Competition binding analysis was performed using equal concentrations of  $\text{ST}_h$  and ST Y72F to inhibit the binding of  $^{125}\text{I}$ -labelled ST Y72F to T84 membranes (Fig. 4). The  $\text{IC}_{50}$  value of  $\text{ST}_h$  (the concentration giving half-maximal binding) was similar to ST Y72F indicating that the binding affinities of  $\text{ST}_h$  and ST Y72F were not significantly different.

#### Correlation of ST binding and guanylyl cyclase activation

Application of varying concentrations of  $\text{ST}_h$  to T84 membranes exhibited a dose-dependent increase in cGMP production which correlated with an increase in binding of ST to the membranes (Fig. 5). Thus, half-maximal binding and half-maximal activation of guanylyl cyclase occurred at



**Fig. 4. Inhibition of binding of  $^{125}\text{I}$ -labelled ST Y72F by  $\text{ST}_h$  and ST Y72F.** Purified  $\text{ST}_h$  and ST Y72F at the concentrations indicated were incubated with 0.1 nM  $^{125}\text{I}$ -labelled ST Y72F and 50  $\mu\text{g}$  T84 membrane protein for 1 h at 37°C. Samples were filtered and the radioactivity of the filters was measured. The values represent the mean of duplicate determinations of two independent experiments.  $\text{ST}_h$  ( $\bullet$ ); ST Y72F ( $\Delta$ ).



**Fig. 5. Correlation of ST binding and guanylyl cyclase activation.** Increasing concentrations of  $\text{ST}_h$  were added to 50  $\mu\text{g}$  of T84 membrane protein and guanylyl cyclase activity was determined as described in the Materials and Methods section. In a parallel experiment, 0.1 nM  $^{125}\text{I}$ -labelled ST Y72F was incubated with varying concentrations of unlabelled  $\text{ST}_h$  and the amount of  $\text{ST}_h$  bound to the membranes at each concentration was determined from the binding data obtained. The values represent the mean of duplicate determinations of two independent experiments.

5 nM  $\text{ST}_h$ , indicating that the binding of  $\text{ST}_h$  was functionally coupled to the activation of guanylyl cyclase.

The binding of ST to the human receptor showed a marked pH dependency in that the binding of  $^{125}\text{I}$ -labelled ST Y72F was doubled at pH 5 (Table 3). Further analysis revealed no change in the affinity of the receptor to the ligand, but a doubling in the capacity of the binding sites. Similar observations were reported earlier using monolayer cultures of T84 cells [28], and the cloned human receptor expressed in 293 cells [29]. We could show however that the increase in binding at pH 5.0 was correlated with an increase in cGMP production by T84 membranes, indicating a functional coupling of binding and activation at low pH (Table 3). A higher basal activity of guanylyl cyclase was observed following prior incubation at lower pH, but there was a concomitant increase in activation of guanylyl cyclase

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