Presence of TRF immunoreactivity in marginal islet cells in rat pancreas

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Abstract. Isolated rat pancreatic islet extracts were submitted to reverse phase high pressure chromatography (HPLC) and assayed for thyrotrophin releasing factor (TRF), glucagon and insulin in radioimmunoassays (RIA). Our anti-TRF serum was prepared by immunizing rabbits with TRF conjugated to bovine thyroglubulin (bTG) by bisdiazobenzidine. Anti-glucagon and anti-insulin sera were from a commercial source (Novo, Denmark). The concentrations of TRF, glucagon and insulin were 0.05 ± 0.02, 0.06 ± 0.02 and 1.0 ± 0.2 pmol/islet, respectively.

Formalin-fixed pancreatic sections were stained for TRF, glucagon and insulin by peroxidase, antiperoxidase (PAP) complex method. When the adjacent sections were stained for glucagon or insulin, it was observed that TRF and glucagon-specific peroxidase reactions were confined to the marginal islet cells, but insulin reactions to the central cells. The TRF-specific peroxidase reaction was clearly reduced when the anti-TRF serum was adsorbed with TRF-TG-Sepharose, whereas it was unchanged when the antiserum was pre-incubated with synthetic TRF.

The present HPLC results suggest that the islets contain TRF. Immunocytochemical studies show that the TRF-immunoreactive material, either synthesized or bound, is localized in the marginal islet cells.

Three laboratories, including ours, have reported that extracts from rat pancreatic tissue and isolated islets contain TRF-like immunoreactivity which has similar chromatographic and biological characteristics to synthetic TRF (Leppäluoto et al. 1977, 1978; Morley et al. 1977; Martino et al. 1978). There are no reports on the cellular localization of TRF in the pancreas.

The role of pancreatic TRF is not known exactly, although it has been shown that TRF enhances arginine-induced glucagon release from isolated perfused rat pancreas preparations (Morley et al. 1979). In this study we have measured the TRF, glucagon and insulin contents of isolated rat pancreatic islets by RIA after purification by high pressure liquid chromatography and identified the cells containing these hormones by the peroxidase-antiperoxidase (PAP) complex method. Our results suggest that TRF is located in the marginal cells of the islets.

Materials and Methods

Antisera
An immunogen consisting of a TRF-bisdiazobenzidine-bovine thyroglubulin complex (TRF-bTG) was prepared by Dr. Ling, the Salk Institute (USA). The immunogen (0.5–1 mg) was emulsified in Freund’s complete adjuvant and monthly sc injections were given to 8 rabbits. The animals were bled at 1–3 month intervals after the first month.

[¹²⁵I]TRF was prepared as described elsewhere (Ling et al. 1976) and used for binding studies with the antisera at various dilutions (1:100–1:40 000). Six months from the first injections, one of the antisera (a-TRF 1) bound 40% of tracer added, at a dilution of 1:40 000. Three antisera bound about 10% and the rest < 10%, even at 1:100.

The cross-reactivity of some TRF-related amino acids and analogues (provided by Dr. Ling), LRF, somatostatin, thyroid hormones, bTG (Sigma, USA) and highly purified porcine glucagon (Novo, Denmark) was tested...
with a-TRF I (see Table 1). There was slight, or no, cross-reactivity with TRF-analogues with changes in the first and third amino acid positions. On the other hand, changes in position two resulted in some, or total, cross-reactivity. Obviously, antibodies formed to TRF coupled through histidine to the thyroglobulin are not directed against that part of the TRF molecule, rather to the N- and C-termini of TRF. TRF-related amino acids, LRF, somatostatin, glucagon, carrier protein and thyroid hormones and their precursors did not cross-react with TRF (for glucagon, see Fig. 1).

A previously characterized anti-TRF antiserum, a-TRF II, (antibody B, Leppäluoto 1976) was also used in this study. This antiserum was raised against TRF coupled to bovine serum albumin (bSA) and has essentially the same immunological specificity as the a-TRF I, but binds $^{125}$I-TRF 30% at a dilution of 1:2000.

Antiser against porcine glucagon (K 5563, C-terminal specific) and insulin (M 8302) were supplied by Novo. The a-insulin has a 60% cross-reactivity with human proinsulin (Heding, Novo Research Institute, personal communication). The anti-hCG antiserum used in this study will be described elsewhere.

**Table 1.**

Cross-reactivity of some TRF-related and other compounds as a percentage of TRF binding.

<table>
<thead>
<tr>
<th>Compound</th>
<th>a-TRF</th>
<th>Biological potency*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRF (pGlu-His-Pro-NH$_2$)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>p-Glu</td>
<td>&lt; 0.01</td>
<td></td>
</tr>
<tr>
<td>His</td>
<td>&lt; 0.01</td>
<td></td>
</tr>
<tr>
<td>Pro-NH$_2$</td>
<td>&lt; 0.01</td>
<td></td>
</tr>
<tr>
<td>pGlu-His-OMe</td>
<td>&lt; 0.01</td>
<td></td>
</tr>
<tr>
<td>Glu$_1$-TRF</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Pro$_1$-TRF</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>1-Me-His$_2$-TRF</td>
<td>20</td>
<td>0.04</td>
</tr>
<tr>
<td>3-Me-His$_2$-TRF</td>
<td>45</td>
<td>800</td>
</tr>
<tr>
<td>Tyr$_2$-TRF</td>
<td>100</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Ala$_2$-TRF</td>
<td>1</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Lys$_2$-TRF</td>
<td>1</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Orn$_2$-TRF</td>
<td>1</td>
<td>&lt; 0.1</td>
</tr>
<tr>
<td>Thyroxine, triiodothyronine, mono- and diiodothyrosine, bovine thyroglobulin, LRF, somatostatin and glucagon</td>
<td>&lt; 0.01</td>
<td></td>
</tr>
</tbody>
</table>

* Vale & Rivier (1975).

Glucagon- and insulin-RIA. Commercial RIA kits (Novo, containing the above-mentioned antisera) were used.

**Isolation of islets from rat pancreas**

Pancreata from decapitated male Sprague-Dawley rats (200–300 g) were incubated with collagenase (15 mg/organ, type IV, Worthington, USA) at 37°C and the islets collected, after several washes with Hank’s solution, under a preparation microscope (Lacy & Kostianovsky 1967).

The washes, the exocrine part and the islets were separately extracted with ten parts of methanol. After centrifugation, the supernatants were dried under vacuum suction at 40°C and the protein concentrations of the pellets were determined according to Lowry et al. (1951). The dried residue was washed with 2 ml of 0.01 M acetic acid and chloroform and the upper phase purified by cation exchange chromatography as described earlier (Leppäluoto et al. 1978) and assayed for TRF. In some experiments, 80% acid ethanol (pH was adjusted to 3 with HCl) was used for the extraction and the dried residues were used for HPLC-studies. There was no loss of TRF, glucagon and insulin immunoreactivity during the extraction procedure.

**Radioimmunoassays**

**TRF-RIA.** The diluted antiserum (a-TRF I, final dilution 1:40 000) and the standard or the unknown and 8000 CPM of $^{125}$I-TRF (200 µl each) were incubated in duplicates overnight. The assay buffer consisted of PBS (pH 7.4) containing 0.1% human IgG (SPR, Finland). The immunocomplex was precipitated with 2 ml of 2.5 M (NH$_4$)$_2$SO$_4$. After centrifugation (25 min at 3000 r.p.m.) the precipitate was counted for radioactivity. The assay sensitivity was 1 pg.

The TRF (filled circles) and glucagon (open circles) inhibition curves with a-TRF I at a dilution of 1:100. No cross-reactivity is seen. Incubation was performed for 1 h at 37°C. Mean values of duplicates are plotted.
Two HPLC runs of 50 acid ethanol extracted pancreatic islets. Panel A: The elution of immunoreactive TRF (hatched columns) and insulin (open columns) is shown. Panel B: Synthetic TRF was added to the extract before the run. The elution of immunoreactive TRF (hatched columns) and glucagon (open columns) is shown.

**HPLC (high pressure liquid chromatography)**

The HPLC apparatus consisted of a Varian Model 8500 with a Varichrom UV-detector, a chart recorder and a loop injector. Column was 5 × 250 mm ODS Hypersil (Shandon Southern, England). The samples were solubilized and eluted at room temperature as follows: i) 2 min buffer A (0.1 mM NaH₂PO₄ · H₂PO₄, PH 2.1), ii) 3 min linear 3% gradient with buffer B (CH₃CN, Rathburn Chemicals), iii) 40 min linear 0.8% gradient with buffer B, and iv) 5 min linear 4% gradient with buffer B. The flow rate was 1 ml/min and the back pressure 80 atm. Absorbance was read at 225 nm. Fractions of 1 ml were collected, evaporated, neutralized with 0.2 mM Na₂HPO₄ (pH 12) and then submitted to the radioimmunoassays.

**Immunoadsorption of the antisera**

For solid phase immunoadsorption of anti-TRF serum, a TRF-bTG-conjugate or bTG (5 mg each) was coupled to CNBr-activated Sepharose 4 B (1 ml gel, Pharmacia, Sweden) according to the manufacturer's instructions.

One ml of each immunoadsorbent was mixed with 200 µl of the diluted antisera at +4°C overnight. After centrifugation at 3000 r.p.m. for 15 min the supernatant was used for immunocytochemical staining. The TRF-binding capacity of the immunoadsorbed antisera was tested (see Fig. 2).

Diluted anti-TRFs, anti-glucagon and anti-insulin sera were incubated overnight with and without 2 µg–3 mg of synthetic TRF, highly purified porcine glucagon and monocomponent human insulin (Novo), respectively. As above, the centrifuged antisera were used for staining. Other controls used for staining were anti-bCG and normal rabbit serum (NRS). Dilutions from 1:50 to 1:300 were done in PBS.

**Immunocytochemistry**

Pancreata from decapitated 25 day old male Sprague-Dawley rats were fixed in 4% neutral formalin for 24 h. We have observed that after formalin fixation these tissues retain about 80% of their alcohol-extractable TRF content. Young rats were used because they have higher pancreatic TRF-concentrations than the adults (Koivusalo & Leppaluoto 1979). After washing with PBS (pH 7.4) for 3 days, the tissue was dehydrated in alcohol and embedded in Paraplast (Lancer, USA). Serial sections of 5 µm were cut and mounted on gelatinized glass slides.
The sections were deparaffinized and brought to PBS through ethanol. In order to block non-specific protein binding, the immunocytochemical procedure was preceded by immersion of the slides in 0.2% bovine serum albumin in PBS at 37°C for 60 min. The following staining schedule, based on the procedure of Sternberger et al. (1970) was used: I) The diluted antiserum (100 μl) was applied to the section and the slides were incubated in a humid chamber at 37°C for 60 min. After this the slides were washed for 15 min in 3 changes of 0.3% Triton X-100 in PBS and one change in PBS. II) The second antibody incubation, with swine anti-rabbit IgG (Dako, Denmark) diluted 1:10, and wash were performed as in the first incubation. III) The third antibody incubation, peroxidase-rabbit antiperoxidase (PAP) complex (Dako), was used at a 1:100 dilutions for 30 min, after which 3 PBS washes followed. IV) The cytochemical peroxidase reaction was developed for 5 min with PBS containing 0.06% 3,3'-diaminobenzidine tetrahydrochloride (Fluka, FRG) to which H₂O₂ was added to give a final concentration of 0.03%. Then followed a wash with H₂O for 5 min. The sections were dehydrated with ethanol, cleared in xylol and mounted with Permount (Fisher, USA). Some sections were also stained with 0.1% toluidene blue. The slides were examined under a Leitz Orthoplan microscope equipped with Leitz Orthomat photomicrographic system.

If Triton X-100 was left out of the wash a faint staining of the islet marginal cells appeared in the NRS controls. Thus Triton X-100 was considered important for the wash. No staining was recognized in slides in which PBS replaced single steps.

Results

Chromatographic studies

As can be seen from Table 2, the total amount of islet IR-TRF was 0.86 ± 0.30 pmol per rat pancreas, which is of the same order as that of the exocrine part. However, when the concentration per mg of protein is calculated, a 10-fold TRF concentration in the islets is observed.

In our reverse phase HPLC studies the biological material was divided into two halves and synthetic TRF added to one. The results of two such runs are seen in Fig. 2, panels A and B. Most of the endogenous IR-TRF was eluted at 22–24 min and some at 15–16 min. The higher peak at 22 min in panel B must be considered as corresponding to synthetic TRF. Hence most of the islet TRF is eluted as synthetic TRF, before glucagon and insulin immunoreactive material, which are eluted at 39–41 and 40–41 min, respectively. The TRF, glucagon and insulin amounts per islets, with a 100% recovery, were 0.053 ± 0.022, 0.061 ± 0.015 and 0.951 ± 0.188 pmol/islet (mean ± SEM, n = 3–6), respectively. There appears to be an almost equimolar content of TRF and glucagon in the islets, but the insulin content is about 15 times higher.

It was found necessary to run synthetic TRF with biological material, because TRF alone was eluted at 15 min. A TRF-analogue, 3-Me-His-TRF, eluted at 17 min in buffer solution.

Immunocytochemical studies

The a-TRF I, when used for immunocytochemical studies, showed positive staining reactions in the

<table>
<thead>
<tr>
<th>TRF</th>
<th>Wash</th>
<th>Islets</th>
<th>Exocrine part</th>
</tr>
</thead>
<tbody>
<tr>
<td>pmol/pancreas</td>
<td>1.85 ± 0.38</td>
<td>0.86 ± 0.17</td>
<td>1.01 ± 0.10</td>
</tr>
<tr>
<td>pmol/mg protein</td>
<td>–</td>
<td>0.81 ± 0.11</td>
<td>0.08 ± 0.01</td>
</tr>
</tbody>
</table>

Fig. 3.

Binding of [¹²⁵I₂]TRF to diluted bTG-Sepharose- (filled circles) and TRF-bTG-Sepharose- (open circles) adsorbed α-TRF I. Incubation was performed overnight at 4°C. Mean values of duplicates are plotted.
Immunocytochemical demonstration of TRF cells in an islet of rat pancreas with the PAP technique. Antiserum a-TRF I, adsorbed with bTG-Sepharose, was used for staining at a dilution of 1:100 (× 250).

Langerhans islets, specially in the marginal cells of the islets (see Fig. 4). Visually, the staining reaction was neither increased or decreased when the antiserum was pre-incubated with synthetic TRF or immunoadsorbed with bTG-Sepharose. However, the TRF-bTG-Sepharose-absorbed antiserum showed a clearly reduced staining activity in the marginal cells, but the faint central staining remained and was hence considered non-specific.

As is seen from Fig. 3 there was a significant binding of immunologically active \([^{125}\text{I}]\text{TRF}\) to bTG-Sepharose-adsorbed antiserum and a binding decrease with dilution of the antiserum. The TRF-bTG-Sepharose-adsorbed antiserum, however, did not show any binding. This shows that the TRF-bTG-Sepharose immunoabsorbent removes the TRF-specific antibodies from the antiserum and evidently this is not due to a non-specific destruc-

Fig. 4.

Fig. 5.
tion of the antibody activity by the immunoadsorbent. The 1/50 dilution of the bTG-Sepharose-adsorbed antiserum gave a higher staining intensity in the histological sections than the 1/200 dilution.

In toluidene blue-stained sections the peroxidase reaction was localized to the cytoplasm of the marginal cells. When compared with Fig. 5, one can see that glucagon also has a marginal localization. The displacement studies, however exclude immunological cross-reactivity between TRF and glucagon (Fig. 1).

The a-insulin stained the central cells of the islet. The insulin- and glucagon-specific staining reactions were abolished when the antisera were pre-incubated with homologous hormones.

The a-TRF II, which was raised against bSA-TRF, stained the whole islet, but when this antiserum was pre-incubated with bSA, the result was the same as that for a-TRF I. The pre-incubation of a-TRF I with bTG did not change the staining activity of a-TRF I.

a-hCG, another hyperimmune antiserum, resulted in no staining.

Discussion

Our finding that pancreatic IR-TRF is at the highest concentration in the islets is in agreement with that of Martino et al. (1978). Our purification of the methanol extracted tissue samples by cation exchange chromatography should exclude most factors interfering with the TRF-RIA. The IR-TRF of the exocrine part and wash is probably due to contamination by small islets or breaking of outer cells during collagenase treatment.

From our HPLC studies it can be concluded that most of the islet immunoreactive TRF is eluted as synthetic TRF. The identity of the TRF immunoreactivity eluted before synthetic TRF is unknown. Methylated His₃-TRF may exist in biological material but 3-Me-His³-TRF was eluted later than synthetic TRF and hence at least this biologically and immunologically active analogue should not exist in our samples. It is also notable that our HPLC-system clearly separates TRF from such larger islet peptides as glucagon and insulin.

Immunocytochemistry with the PAP-technique gave us possibilities of recognizing and comparing low-intensity staining reactions. With this technique we were able to show that peripheral islet cells were stained with our a-TRF I serum; either it was adsorbed or not with bTG immunoadsorbent or neutralized with synthetic TRF. The reaction was clearly reduced when the a-TRF was adsorbed by TRF-bTG immunoadsorbent. It is noteworthy that incubation of the a-TRF with synthetic TRF or TRF-bTG immunoadsorbent inhibited the subsequent binding of [¹²⁵I]TRF, but incubation with bTG immunoadsorbent did not.

Sternberger et al. (1978) have shown that pituitary sections treated with purified LRF-anti-LRF complexes impart a strong staining to gonadotroph granules. Seppälä et al. (1979) have also found that the staining of pancreatic islets with anti-LRF was enhanced when the antiserum was pre-incubated with LRF. Both groups reported that the staining was only prevented when the antiserum was pre-treated with solid phase immunoadsorption and they concluded that the tissue binds LRF-anti-LRF complexes. Our present results concerning TRF could possibly be explained in a similar fashion, i.e. the TRF-anti-TRF complex can bind of the marginal islet cells. However, it is difficult to understand how TRF, after binding to the antibody, could have any site for tissue binding, because our cross-reactivity studies show that both the N- and C-terminals, are required for antibody binding. At the moment a good explanation does not appear to exist for this phenomenon.

The weaker TRF staining compared with that of glucagon could be due to several factors. TRF is a smaller peptide and a weaker immunogen than glucagon and the immunological determinations of small peptides are more easily covered by tissue components. Differences in antigen solubility must also be considered, glucagon being relative insoluble at neutral pH but TRF more soluble. It is also possible that TRF is receptor-bound in pancreas.

Swaab et al. (1977) have noted that the adsorption technique alone is absolutely insufficient to prove specificity for the homologous antigen. We cannot exclude the possibility of cross-reacting substances in the tissue sections. However, our immunochromical studies of chromatographically purified islets indicate the existence of TRF in the rat pancreatic islets and the immunocytochemical studies with the same antiserum suggest the marginal islet cells as the particular localization site.

There is also functional evidence for a local connection for the TRF and glucagon, i.e. it has been shown that TRF enhances the arginine stu-
mulated glucagon release of perfused rat pancreas (Morley et al. 1979). TRF may be one of the 'missing links' in the control of synthesis and release of gut hormones (Grube et al. 1978). A good site for such a regulator in the pancreas, is in the margin of the islet, to mediate information, by a paracrine effect, between the exocrine and the endocrine part.

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References


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