Transthyretin's role in prohormone processing in the islets of Langerhans

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Summary

Transthyretin (TTR) is a transport protein in plasma which transport thyroxine, triiodothyronine, and retinol binding protein. Though found mainly in hepatocytes and the choroid plexus, alpha and beta cells located in the islet of Langerhans produce significant amounts of the protein, however, its function in endocrine pancreatic cells is unknown.

Prohormones are precursors to hormones and can arise into multiple, different hormones at functional maturity. For instance, the same prohormone proglucagon becomes glucagon in the pancreas’ alpha-cells, but becomes glicentin and GLP1 if further processed in the L-cells of the intestine.

While the exact function of TTR in the islets of Langerhans is currently up for debate, its proximity to secretory granules indicates a possible role in prohormone processing. A role in hormone production would ultimately explain the presence of the potentially cytotoxic protein in the pancreas.

The goal of this project is to assess the extent to which transthyretin participates in the production of hormone from prohormone in the islets of Langerhans, specifically alpha cells, by blocking its synthesis using siRNA and comparing levels of prohormones and hormones to those of non-transfected cells.

To accomplish this, endocrine cells were cultured, then transfected with a vector containing TTR siRNA to limit synthesis as well as GFP and Zeocin resistance to aid in selection. Immunohistochemistry was employed to determine expression levels of the various proteins involved in the experiments.

Transfected cell colonies were unfortunately difficult to produce using the particular vector that I did, indicating that something in the vector is cytotoxic to the cell line or that TTR is needed for its survival.
**Introduction**

Electron microscopy conducted by Gunilla Westermark confirmed the presence of TTR in secretory granules of islet cells (Westermark, G.T., Westermark P. 2008). Since previous research indicates that prohormone processing takes place in the secretory granule (Shennan, K.I. *et al.*, 1995), it is a feasible notion that TTR may play a crucial role in the production of biological active hormones from proinsulin, proIAPP, proglucagon, or various other prohormones produced in the islets of Langerhans.

**Islets of Langerhans**

The islets of Langerhans make up the endocrine part of the pancreas and constitute 1-2% of the total pancreas volume. There are five main types of endocrine cells located in the islets, producing hormones integral to neurotransmission and blood homeostasis.

The bulk of islet tissue is beta-cells, representing 65-80% (with relation to cell number in rats) of the islet is insulin producing beta cells. Alpha-cells represent roughly 15-20% of the islet and are chiefly responsible for glucagon production. Delta, PP, and epsilon-cells, producing somatostatin, pancreatic polypeptide and ghrelin, respectively, represent the remaining tissue (Elayat, A.A. *et al.*, 1995).

This study mainly focuses on rodent islets, which differ from human islets in several respects. For instance, the proportion of cell types varies between the two and the arrangement of the cells is quite different. Rodent islets are highly ordered in comparison, with their beta cells concentrated in the interior with the alpha cells mainly located towards the exterior. Human islets, in contrast, show alpha and beta cells arranged along blood vessels in seemingly no particular order (Carbrera, O. *et al.*, 2006). There are many similarities between islets in both species, but it is important to note the differences.

**Alpha-cells**

As stated previously, endocrine alpha-cells excrete glucagon. Glucagon raises blood glucose levels by instigating a series of reactions eventually resulting in the conversion of stored glycogen to glucose in the liver.

**Beta-cells**

Most of the cells in the islets are the insulin producing beta-cells. Insulin regulates glucose uptake in body tissues and therefore blood glucose levels. Diabetes mellitus I and II are associated with the dysfunction of these endocrine cells because they are responsible for insulin production. Aside from insulin, beta-cells produce proteins such as IAPP (Westermark P, *et al.*, 1986) and C-peptide (Y. Ido, *et al.*, 1997) which arises during proinsulin processing.
Prohormone Processing

Several enzymes have been identified that are essential to glucagon’s prohormone processing including proprotein convertase 1/3 (PC1/3), PC2, 7B2, and SAAS.

PC1/3 and PC2 are involved in prohormone cleaving of glucagon precursors. PC2 cleaves products which become mature glucagon (Rouille Y, et al. 1997) while, PC1/3 is most active in enteroendocrine L-cells and gives rise to GLP-1, GLP-2 and oxyntomodulin (Rouille Y, et al. 1997). 7B2 serves as a chaperone for PC2 and is crucial for PC2 reactivity (Benjannet S. et al. 1998). SAAS functions as a PC1 inhibitor. Over-expression has lead to obesity in mice due to elevated blood glucose levels though it is suspected this role is independent of the peptide’s inhibitory function (Wei S, et al. 2004).

The prohormone processing of glucagon takes place in the ER secretory granules of alpha-cells. Hormone producing cells, such as those located in the islets of Langerhans, require secretory granules for regulated exocytosis (Palade G. 1975). The granules gradually increase in acidity due to V-ATPase activity (Finbow ME and Harrison MA. 1997).

Though much is known about the processing of proglucagon, the effect of TTR on either proglucagon itself or the proteins necessary for the maturation of glucagon are currently unclear.

TTR

Curiously, TTR has been detected in the secretory granules, a region of the cell where glucagon is processed. Since TTR serves no known function that would necessitate its presence in alpha cells, there is a need to explain its presence. A plausible explanation is that TTR might participate in prohormone processing.

![Fig 1. TTR tetramer (source: http://en.wikipedia.org/wiki/Image:Uno_side.png). Rigid beta sheets form the backbone of potentially harmful amyloid formations if monomers reassemble incorrectly (Skoulakis, 2003).](image)
Immunohistochemistry

The immunohistochemistry performed in my experiments involved binding of a primary antibody to a certain antigen and its presence was visualized by a secondary antibody containing a fluorophore which could be detected under a specific wavelength of light. This study used Alexa 488, detected under a blue light, and Alexa 546, detected under green.

In order for the antibodies to penetrate the cell membrane and bind to their specific antigens, the membrane must be perforated with saponin, which makes holes in the membrane large enough for the antibodies to enter. Cells are generally fixed in order to preserve the integrity of the cell and prevent decay. Cells containing the bound antibodies are then treated with DAPI (4',6-diamidino-2-phenylindole) which binds to nucleic acids and emits a strong luminescence under UV light, enabling the nuclei to be observed.

Since DAPI was used to stain the nuclei, the proteins’ location relative to the nucleus can be determined.

Aims of the study

The goals of this study were to obtain stable transfectants, optimize dilutions of antibodies to acquire the best possible fluorescent microscopy photos, design and confirm the effectiveness of primers to be used in PCR experiments, and to ultimately compare protein concentrations of transfected and untransfected cells to hopefully find a link between TTR presence and glucagon expression.
Results

Cell culture

Alpha tc-1 and beta tc-6 cells were successfully cultivated in flasks and 12-well plates. The beta cells appeared to have much lower resilience and experienced much higher levels of cell death than the alpha cells. This coupled with the fact that their growth rate seemed much lower than that of the alpha cells and little, if any, TTR is found in beta cells lead me to abandon beta cells in the experiments completely and continue with only alpha-cells.

Transfection optimization

Two methods of transfection were attempted, a PEI method as well as a protocol using the QIAGEN Attractene transfection reagent. Experiments testing transfection using the Attractene with multiple concentrations of both reagent and psiRNA-hH1GFPzeo vector showed little difference, except at very low and very high concentrations where no transfection and cytotoxicity was observed, respectively.

Typical cells transfected with attractene can be seen in figure 2. Because of poor results with both methods, establishing colonies of stable transfectants was impossible.

Two different cell lines, CHO-1 and wild-type COS 677, were transfected using the PEI method as well as the Attractene protocol as a control for the previous transfections to evaluate transfection conditions and vector toxicity. The PEI method again showed poor results, while roughly 25-40% of the cells in the Attractene wells were successfully transfected. The 1x concentrations of both vector and transfection reagent seemed to have the highest percentage of transfected cells. Transfected cells from the COS 677 cell line can be seen in figure 2.
Fig 2. Cells that underwent Attractene transfection. A) A long exposure time under 488 nm and 40x magnification reveals a dim greenish cloud along with several over-exposed flecks. B) Nuclei from the cells of A are visible under UV light, 40x magnification C) Mutant COS 677 transfected by the same means as A and B under normal light, 20x magnification. D) Successfully transfected cells from C are visible under 488 nm, 20x magnification

**Immunohistochemistry**

Paraformaldehyde fixed cells were incubated with primary antibodies, secondary antibodies, and DAPI. Various concentrations of primary and secondary antibodies were tested in order to determine the best possible means to assess the presence of TTR, insulin, and glucagon.

Originally, the goal was to compare transfected cells to normal cells of the same line to get a qualitative picture of the differences between protein concentrations. Since stable transfectants were not obtained, photos of non-transfected cells (figure 3) could not be compared to transfected cells.

All labeling was successful. The optimal dilutions for primary and secondary antibodies were 1:500 and 1:2000 for DAPI.
mRNA detection

RT-PCR was used to produce cDNA from mRNA fragments roughly 200 bp in length coding for mouse CGRP, mouse glucagon, mouse insulin 1, mouse insulin 2, rat TTR, and mouse TTR (table 1). Strong bands appeared on insulin 1, rat TTR, and mouse TTR.

The appearance of a band would indicate the presence of mRNA meant to code for its respective protein. The results of the gels acquired from transfected and non-transfected cells were to be compared. If bands appeared for protein mRNA in this experiment on the transfected cells, yet the immunohistochemistry experiment failed to show expression of the mature protein, this would indicate that TTR somehow had an influence on the protein’s processing.

Since glucagon was very relevant to my work but showed no amplification, a new primer pair was designed and tested. The agarose gel from the experiment is pictured in figure 4. All primers ordered from TAG Copenhagen, Denmark.
Table 1. Corresponding genes, sequences, annealing temperature (Tm) in °C, and length of product in bp of primers designed in the experiment. All sequences are *Mus musculus* except “Rat TTR” from the species *Rattus norvegicus*.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Left Primer</th>
<th>Tm</th>
<th>Right Primer</th>
<th>Tm</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucagon A</td>
<td>CCCTCTGTCTACACCTGTTC</td>
<td>54.65</td>
<td>GCCTTGTTCTTCCATCTC</td>
<td>55.36</td>
<td>206</td>
</tr>
<tr>
<td>CGRP</td>
<td>GAAAGGCTGATGAAAGACAC</td>
<td>54.89</td>
<td>ATCAACAGGATGTGTATG</td>
<td>54.84</td>
<td>202</td>
</tr>
<tr>
<td>Insulin 1</td>
<td>AACCATTGTTAGGTTGATG</td>
<td>54.92</td>
<td>TGCTCTTCTCAGGTGTATAT</td>
<td>54.96</td>
<td>203</td>
</tr>
<tr>
<td>Insulin 2</td>
<td>TCTTCTACACATCCATGTCC</td>
<td>55.86</td>
<td>GTAGTGGGTGGGTCTAGTTGC</td>
<td>54.71</td>
<td>203</td>
</tr>
<tr>
<td>TTR</td>
<td>GTCTCTCTGATGTCCAAAGTC</td>
<td>55.01</td>
<td>GTACGATTTGCTGCTGCAGTT</td>
<td>54.55</td>
<td>206</td>
</tr>
<tr>
<td>Rat TTR</td>
<td>AATCCAAGTGTCCTCTGATG</td>
<td>54.50</td>
<td>CCAGTCTACCTGTCACC</td>
<td>54.97</td>
<td>201</td>
</tr>
<tr>
<td>Glucagon B</td>
<td>ACGCTCAAAACGAGCAAAAA</td>
<td>54.00</td>
<td>TGACGTGGCAATGTTGTT</td>
<td>52.00</td>
<td>300</td>
</tr>
</tbody>
</table>

The primers most-crucial for the study, TTR and glucagon, were confirmed to amplify DNA fragments of expected size.

![Fig 4. Lane 1: ladder, lane 2: glucagon B primer, lane 3: glucagon A, lane 4: TTR primer, lane 5: control. cDNA obtained via RNA extraction from alpha-cells and RT-PCR.](image)

Again, results from non-transfected cells could not be compared to transfected cells as significant numbers of transfected cells could not be obtained.
Discussion

Though alpha tc-1 colonies of stable transfectants could not be produced, the same transfection method which failed during the psiRNA experiments resulted in successfully transfected CHO-1 and wild-type COS 667 cells using the FRET vector. Alpha tc-1 and beta tc-6 cells have successfully been transfected using similar methods in the past (Mee L., et al., 2009). The vectors have also been expressed in other types of cells as well (Paulsson J.F. et al., 2008) (Hernández V.H., Bueno D., 2005). Therefore, TTR is either essential to cell viability, or the vector itself is cytotoxic to the alpha tc-1 cell lines used in the experiment.

It is interesting to note the differences in successful transfections using PEI and the Attractene transfection reagent. Besides the reagent itself, one way in which the methods differed is in that cells in the PEI protocol were “starved” for 5 hours in serum-free medium in order for them to more readily incorporate the foreign DNA via endocytosis. Though this seems to work under other circumstances, in my case leaving the cells in a nutrient-rich medium containing serum may have played a role for the greater number of transfected cells in the more successful Attractene protocol.

Because colonies of stable transfectants could not be produced using the psiRNA-hH1GFPzeo vector, later stages of the study such as quantification of protein number and comparisons to wild-type cells could not be completed as planned. Despite this, several parts of this experiment yielded useful results.

First, optimal dilutions for transfection reagents, antibodies, culture media, DNA, and DAPI for the various experiments were determined. By varying the concentrations of solution elements throughout the experiments, I could compare results and discover which of them worked the best.

Secondly, primers were designed that effectively bound to mouse insulin, mouse glucagon, mouse TTR, and rat TTR coding genes. These sequences can be used for future experiments on those particular proteins.

Exposure time and settings for microscopy photos were optimized for different wavelengths and GFP intensity was tested using GFP antibodies (Sigma). It was confirmed that the intensity was, in fact, greater using the anti-bodies, but they were not necessary if the cells were actually transfected. This knowledge would be very useful in future experiments and reduce the time and resources needed to complete them.

The methods for the experiment, aside from the transfections, were proven to be effective. For example, cell cultures were established, cells were fixed, proteins were successfully labeled, and amplified cDNA was synthesized.

A possible source of error is contamination. Viral, bacterial, or fungal growth in the culture medium would have adverse effects on the cells. Steps were taken to avoid this, such as changing medium in the tissue culture room, but the risk remains. Another possible source of error is denaturation of enzymes or antibodies used in the experiment. If not stored properly, proteins can change to conformations different from the active state.
Future research on this subject should attempt to transfect cells using a different vector or blocking TTR synthesis in a different manner. Another possible method could be introducing TTR-blocking siRNA using “virus-mimetic polymeric micelles” (Xiong X.B., 2010). In this experiment, the RNA was introduced directly into the cell rather than coded for by incorporating a plasmid into the cell’s DNA. If part of the vector was cytotoxic, using a synthetic molecule to deliver the siRNA without forcing the cell to produce it would solve the problem.

If colonies of cells with a lower amount of TTR could be produced, the further portions of the experiments such as protein quantification would then be possible to perform.
Materials and Methods

Cell Culture

Alpha-cells used in these experiments were acquired from the continuous Alpha tc-1 cell line (ATCC, American tissue culture collection). They must adhere to a surface and cannot be grown in solution. This quality makes them easier to use in microscopy studies because they will adhere to microscope slides placed on the bottom of the well in the culture plates.

Beta-cells in these experiments were acquired from the Beta tc-6 cell line (ATCC, American tissue culture collection) and were treated identically to their alpha-cell counterparts with respect to culture, transfection, and immunohistochemistry.

Rat insulinoma cells were used during the RNA extraction and RT-PCR portions of the experiment as a control to test the specificity of the primers. There are several differences in sequence between mouse and rat TTR and if the mouse primer bound to both it would indicate that a more specific primer was needed.

In order to successfully reproduce, the culture medium must contain several things. Fetal calf serum contains the nutrients the cells require. Antibiotics are used to kill bacteria and prevent infection. Beta-mercaptoethanol is used to reduce disulfide bridges of cysteine in the serum so it can be incorporated into cell proteins. Transfected cell medium contained Zeocin, which is toxic to cells without resistance incorporated into their DNA. This raises the ratio of transfected to non-transfected cells.

Roughly 1mL of Sigma Trypsin-EDTA x10 solution was added to culture flasks containing roughly 10mL of tc-6 liquid cell culture RPMI-1640 with 10% FCS, 1% PST, 1% sodium pyruvate, and 0.1M beta-mercaptoethanol and incubated 10 minutes at 37ºC. The culture was then mixed by pipetting and 7mL was transferred to a 15mL falcon tube and centrifuged in a Heraeus Labofuge 400 at 900g for 10 minutes. The supernatant was discarded and the pellet re-dissolved in 14mL medium, then distributed between wells in either a 24 or 12 well plate containing either 13mm or 22mm glass slides, respectively and incubated at 37ºC.

Transfection

The introduction of nucleic acids into cells is otherwise known as transfection. Though several methods of accomplishing this exist, I relied on chemical means to introduce the vectors into the cell via endocytosis. After the vector is successfully incorporated into the cell’s DNA, the cell will produce the macromolecules encoded in the vector.

A popular chemical means of transfection, discovered in 1995, is the polyethylenimine or PEI method (Boussif, O. et al., 1995). PEI forms a positively charged complex with the DNA to be introduced into the cell and binds with negatively charged cell surface residues. After the cell brings the complex into the cytoplasm through endocytosis, osmotic pressure breaks the vesicle, the complex dissociates, and the vector DNA is incorporated cell’s DNA (Akinc, A. et al., 2004).
The other method used was the QIAGEN Attractene transfection reagent, which “is a nonliposomal lipid that enables transfection of all adherent cells” (QIAGEN).

In the first method, cells were cultured in 12-well plates and incubated in serum-free media for 1.5 hours. A solution of 17.5 µL of 20% glucose, 2.5 µg DNA vector, and sterile water up to 70 µL was prepared. 8.4 µL was then added and the solution was incubated at room temperature for 30 minutes. 10 µL of solution was added to each well and the plates were placed at 37°C for 4.5 hours. 150 µL FCS was then added to each well and the plates were stored at 37°C.

For the second method, cells were cultivated on well plates and transfected according to the Attractene Transfection Reagent Traditional Protocol (QIAGEN).

**psiRNA-hH1GFPzeo**

The cells were transfected with the psiRNA-hH1GFPzeo vector purchased complete with the TTR siRNA insert (InvivoGen) (figure 4). Because green fluorescent protein (GFP) will be produced in a successfully transfected cell using my particular vector, viewing cells under a wavelength of 488 nm will reveal whether or not the cell is transfected. Transfected cells can then be separated from non-transfected cells, allowing further stages of the experiment to take place.

![psiRNA-hH1GFPzeo vector](image)

**Fig 5.** psiRNA-hH1GFPzeo vector used in transfection. (Invivogen). GFP::zeo aids in gauging transfection efficiency. Various promoters ensure gene expression.
**Immunolabeling**

Cells were fixed by replacing the culture medium with 4% paraformaldehyde in phosphate buffered saline (PBS) for 30 minutes. PBS was composed of 137mM NaCl, 2.7mM KCl, 10mM Na$_2$HPO$_4$, 1.76mM KH$_2$PO$_4$. Cells were rinsed three times in PBS for 15 minutes, BSS two times for 15 minutes, and perforated with 0.1% saponin in BSS for 15 minutes. BSS was composed of 137mM NaCl, 5.4mM KCl, 1.4mM Na$_2$HPO$_4$, 0.6mM KH$_2$PO$_4$, 0.8mM MgSO$_4$, and 0.78mM CaCl$_2$. Cells were incubated at room temperature over-night on a rocking table in an antibody solution composed of a 1:500 dilution of antibodies to 0.1% saponin.

Cells were then rinsed three times in 0.1% saponin in BSS and wells were filled with 1:500 dilutions of secondary antibodies in 0.1% saponin in BSS. Plates were wrapped in foil and placed on a shaker for two hours then rinsed three times in 0.1% saponin in BSS for 20 minutes and twice in PBS for 20 minutes.

Slides were mounted using 1:1 glycerol in PBS with 1:2000 DAPI.

**RNA Extraction**

RNA extraction was performed using the manufacturer’s protocol for the QIAzol lysis reagent (QIAGEN). Purification was achieved using the RNeasy MinElute Cleanup kit protocol (QIAGEN). Mouse liver was used a positive control for TTR presence.

**RT PCR**

Reverse transcriptase polymerase chain reaction is a means to produce a large number of copies of DNA from an mRNA template.

First, the RNA is reverse transcribed using the enzyme reverse transcriptase, producing a complimentary strand of cDNA. The cDNA is then amplified using standard PCR techniques.

The method is an effective means to confirm the presence of mRNA, which is often difficult to detect given its short half-life.

The Primer3 design tool at www.justbio.com was used to create primers for 200 bp fragments of DNA. Sequences were selected if they had an annealing temperature close to 55ºC, were roughly 20 bp long, and spanned across introns.

Primer sequences were obtained for the following proteins: mouse CGRP, mouse glucagon, mouse insulin 1, mouse insulin 2, rat TTR, and mouse TTR.

cDNA was produced using the QuaniTect reverse transcription kit according the manufacturer’s protocol.

Subsequent amplification achieved using 2.5µL 10x PCR buffer, 1µL dNTP (10mM), 0.5µL forward primer (100pmol/ul), 0.5µL reverse primer (100pmol/ul, 0.2µL Taq polymerase (Saween), and 15.5µL of water per sample. Samples were then incubated at 94ºC for 5 minutes and underwent 40 cycles of 94ºC for 5 minutes, 46ºC for 45 seconds, and 72ºC for 45 seconds. Finally samples were incubated at 72ºC for 7 minutes and held at 4ºC.
Each primer pair was tested using RNA extracted from alpha tc-1 cells, beta tc-6 cells, RIN cells and mouse liver, known to contain TTR.
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References


