The Thyroid Hormone Transport Protein Expression Cloning Strategies

Het Schildklierhormoon Transport Eiwit
Expressie Clonerings Strategieën

PROEFSCHRIFT

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The studies reported in this thesis were carried out under the direction of Prof. Dr. G. Hennemann in the laboratory of the Thyroid Hormone Research Unit of the Department of Internal Medicine III and Clinical Endocrinology (head Prof. Dr. J.C. Birkenhäger), Erasmus University Medical School, Rotterdam, The Netherlands.

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Ter nagedachtenis aan mijn Oma,
die nooit heeft kunnen begrijpen wat ik nu eigenlijk deed.

CONTENTS

Voorwoord

Één van de belangrijkste onderdelen van het doen van wetenschappelijk onderzoek is samenwerking. Niet alleen in het laboratorium, maar ook aan het bureau. Slechts aan de hand van reeds bekende methoden en resultaten kan een goede onderzoekslijn voortgezet worden. En juist hierin is het laboratorium waar ik mijn promotie onderzoek heb mogen doen zo sterk. Iedereen is altijd bereid met raad en daad klaar te staan. Daarvoor wil ik alle medewerkers van ons lab van harte bedanken. Daarnaast zou ik een aantal collegae nog wat nadrukkelijker willen bedanken.

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List of abbreviations

5'N	5'-nucleotidase
ATP	adenosine triphosphate
$BrAcT_3$	N-bromoacetyl-T_3
$BrAcT_4$	N-bromoacetyl-T_4
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CHAPS	3-[(3-chloroamidopropyl)dimethyl-ammonio]-1-propanesulfonate
CMPF	3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid
cRNA	complementary ribonucleic acid, *in vitro* transcribed RNA
DIT	diiodotyrosine
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EBNA-1	Epstein Barr virus nuclear antigen 1
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
FCS	fetal calf serum
FSH	follicle stimulating hormone
G418	geneticin, neomycin analogue
G6P	glucose-6-phosphatase
hCG	human chorionic gonadotropin
HepG2	human hepatoblastoma cell line
hGH	human growth hormone
IgG	immunoglobulin G
IU	international units
kD	kilodalton
Km	Michaelis constant
LDL	low density lipoprotein
LH	luteinising hormone
MIT	monoiodotyrosine
mRNA	messenger ribonucleic acid
NB	nuclear binding
ND	not detectable
NEFA	non esterified fatty acid
NS	not significant
NTI	non thyroidal illness
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEPCK	phosphoenol pyruvate carboxykinase

PTU	propylthiouracil
RAI	radioactive iodine treatment
rT_3	3,3',5'-triiodothyronine, reverse T_3
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SHBG	sex hormone binding globulin
T_3	3,3',5-triiodothyronine
T_3G	T_3 glucuronide
T_3S	T_3 sulphate
T_4	3,3',5,5'-tetraiodothyronine, thyroxine
TBG	thyroxine binding globulin
TBS	tris buffered saline
TCB	total cellular binding
TED	tris EDTA DTT buffer
TPO	thyroid peroxidase
TRE	thyroid hormone responsive element
TRH	thyrotropin-releasing hormone
TRIAC	triiodothyroacetic acid
Tris	tris(hydroxymethyl)aminomethane
TSH	thyroid-stimulating hormone, thyrotropin
TTBS	tween tris buffered saline
TTR	transthyretin, thyroxine binding pre-albumin (TBPA)
Vmax	maximum velocity

LEGENDS

**	:	$p<0.001$
#	:	$p<0.005$
*	:	$p<0.01$
+	:	$p<0.025$

Chapter 1

Introduction

Introduction

1. Physiology of thyroid hormone bioactivity

1.1 Thyroid hormones

Thyroid hormones are unique in that they contain large amounts of the trace element iodine (see Fig. 1). The thyroid gland, one of the largest organs specialised in endocrine function, actively accumulates iodide upto concentrations which are 30-40 times higher than in plasma. Intrathyroidal iodide is oxidised by H_2O_2, catalysed by thyroid peroxidase (TPO), and incorporated into tyrosyl residues of thyroglobulin. Dependent on the level of iodination, monoiodotyrosine (MIT, one iodine bound) or diiodotyrosine (DIT, two iodines bound) can be formed.

Within the thyroglobulin molecule, two DIT residues can be coupled to form 3,3',5,5'-tetraiodothyronine (thyroxine, T_4). By the same mechanism, though less efficient, one MIT residue can be coupled to a DIT residue to form 3,3',5-triiodothyronine (T_3). Both reactions are catalysed by thyroid peroxidase (TPO). The produced hormone-residues are released via endocytosis of colloid droplets and subsequent intrathyroidal lysosomal degradation of the thyroglobulin molecule. T_3 and T_4 are released into the circulation, whereas MIT and DIT are deiodinated intrathyroidally in order to conserve iodide [1].

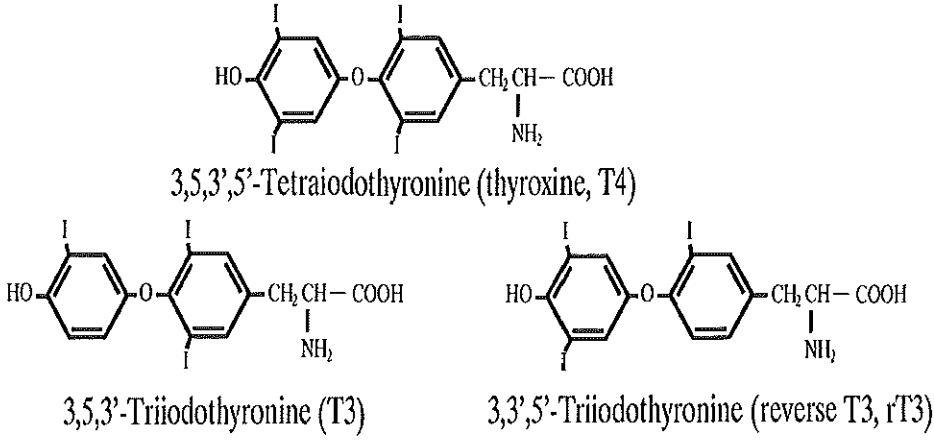

Figure 1 *Structures of thyroid hormones. Outer-ring deiodination of thyroxine results in the production of biologically active T_3, whereas inner-ring deiodination produces the biologically inert metabolite reverse-T_3. (from : Greenspan, 1994 [1])*

Thyroxine is the main secretory product of the thyroid gland. It has only little, if any, biological activity [2] and is considered to be a prohormone. The biological effects of thyroid hormone are attributed to 3,3',5-triiodothyronine (T_3). Normally, approximately 80% of total triiodothyronine production is accounted for by extrathyroidal deiodination of circulating thyroxine [3, 4]. In rats, however, the contribution of the thyroid and peripheral tissues to the pool of T_3 remains unclear. Intrathyroidal conversion of thyroxine to T_3 may play a major role in rats [5]. Alternatively, circulating thyroxine can be deiodinated peripherally to biologically inert 3,3',5'-triiodothyronine (rT_3) [6]. The thyroid secretes virtually no rT_3.

1.2. The hypothalamo-pituitary-thyroid axis

Thyrotropin-releasing hormone (TRH), a pyroglutamyl-histidyl-prolinamide tripeptide, is synthesised by neurons in the supra-optic and supra-ventricular nuclei of the hypothalamus. After storage in the median eminence it is secreted into the pituitary portal system (Fig. 2). TRH controls synthesis and release of thyroid-stimulating hormone (TSH) by thyrotropes in the pituitary gland through a G protein-coupled system.

Thyroid-stimulating hormone (TSH or thyrotropin) is an approximately 28 kD heterodimeric glycoprotein, consisting of one α- and one ß-subunit. The α-subunit is common to FSH, LH, and placental hCG. Pituitary TSH secretion is regulated by TRH, controlling glycosylation and release, and intrathyrotrope T_3 levels, controlling TSH transcription and release.

TSH has several effects on the thyroid cell, most of which are mediated through the G protein-coupled adenylate cyclase-cAMP system. The phosphatidyl inositol pathway is also stimulated in some TSH actions, but less strongly. TSH stimulation results in thyroid cell morphology changes like increased size and increased thyroid vascularity. Iodide uptake and organification increase, and thyroid hormone secretion increases. Thyroglobulin- and thyroid peroxidase transcription are stimulated, resulting in increased H_2O_2 generation and increased iodide oxidation with subsequent incorporation into MIT, DIT, T_3, and T_4, leading to increased thyroid hormone synthesis. Also the thyroid hormone secretion pathway is stimulated [1].

The hypothalamo-pituitary-thyroid axis shows several levels of regulation, as illustrated in Figure 2. Increased serum T_3 or T_4 levels suppress TRH and TSH release, whereas decreased levels increase TRH and TSH release. These effects result from regulation at both the transcriptional level as well as at the post-translational level [1,7]. There is still some debate concerning the question whether thyroxine can exert direct effects on TRH or TSH release, or whether its effects are due to intracellular T_3 produced

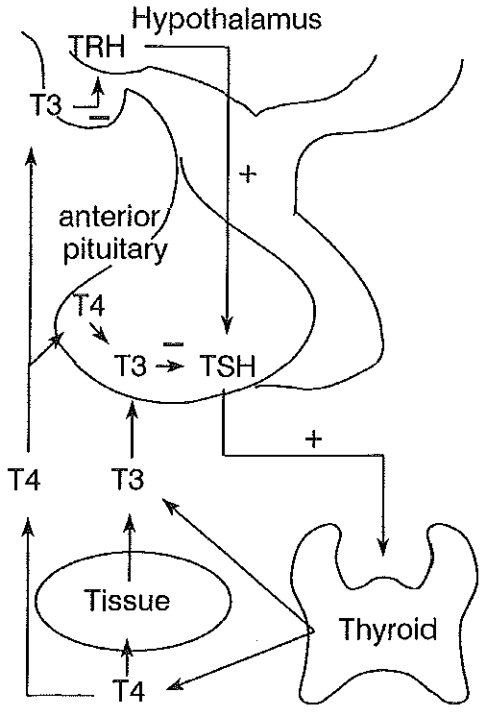

Figure 2 *Schematic overview of the hypothalamo-pituitary-thyroid axis. TSH synthesis and release is stimulated by hypothalamic TRH. TSH is secreted by anterior pituitary thyrotropes, and stimulates the thyroid gland to produce and secrete thyroid hormones. Serum T_3 is mainly derived from peripheral T_4 deiodination. Intra-pituitary T_3 is derived from serum T_3 and local deiodination of serum T_4. Both TRH and TSH synthesis and release are inhibited primarily by T_3. (from : Greenspan, 1994 [1])*

by local deiodination of T_4. Also direct effects of hyperthyroidism on membrane lipid composition, influencing membrane fluidity have been postulated [8, 9, 10], although this seems much less pronounced in brain [11].

1.3 The classical concept of transcriptional regulation

The thyroid hormone receptor is a member of a large family of intracellular hormone receptors. The product of *c-erbA*, a cellular homologue of the viral oncogene *v-erbA*, binds T_3 with high affinity and specificity. The structures of these intracellular hormone receptors show many similarities. All have an evolutionary very well conserved DNA binding domain, and a much less conserved carboxy-terminal hormone binding domain. At the N-terminal side of the DNA binding domain a third domain has been identified, which probably plays a role in modulating receptor-DNA interaction [12, 13, 14].

In man, there are at least two different genes coding for the thyroid hormone receptors. The *c-erbA* α-gene, located on chromosome 17, codes for the α1- and α2-form of the receptor. These two different forms result from alternative splicing. A second gene is localised on chromosome 3 and codes for *c-erbA* ß [15]. In rat a second form of *c-erbA* ß has been identified. This ß2-receptor has so far only been identified in rat pituitary [16].

Following cellular entry, T_3 is bound to the intracellular receptor. The hormone receptor complex binds to specific thyroid hormone responsive elements (TRE) located in the regulatory sequences of thyroid hormone responsive genes. Binding of this complex increases or decreases the rate of transcription of these genes, depending on which gene is regulated [13, 17]. Table I shows a number of genes which are regulated by thyroid hormones. Increased or decreased expression of proteins influences the biological activity of these proteins *in vivo*, resulting in the biological effects of thyroid hormone.

1.4 Serum binding proteins

Due to their hydrophobic nature, circulating thyroid hormones are mainly associated with serum binding proteins. Less than 1% is circulating in its free form. The three main proteins responsible for serum binding of thyroid hormones are thyroxine binding globulin (TBG), transthyretin (TTR, previously known as thyroxine binding pre-albumin, TBPA), and albumin. TBG binds approximately 70% of circulating T_4, and is a 54 kDa glycoprotein. Transthyretin binds approximately 10% of circulating T_4, and is a 55 kDa non-glycosylated protein, composed of four identical subunits. Albumin binds approximately 20% of circulating T_4, has a molecular weight of 66 k and contains no carbohydrates [18]. The binding protein ratios may change dramatically under different (patho-) physiological conditions.

In addition to the three main serum binding proteins, thyroid hormones are bound to lipoprotein components [18, 19] and thyroid hormone autoantibodies [18]. Erythrocytes may also serve as a blood-carrier for T_3, as has been suggested for rat erythrocytes [20].

The so-called 'free hormone hypothesis', originally proposed by Robbins and Rall [21] states that only the free hormone in plasma can cross cell-surface membranes and exert biological effects. This implies that any change in serum binding of thyroid hormone which affects the free serum concentration of thyroid hormone results in altered cellular uptake and subsequently altered biological activity of thyroid hormones [22].

1.5 Transmembrane transport of thyroid hormones

It has long been thought that thyroid hormones enter cells by passive diffusion [23, 24]. However, it has now been shown by several independent laboratories, using different types of cells and organisms, that transport of thyroid hormones across the lipid bilayer of

the plasma membrane involves an energy dependent, saturable and stereo-specific transport mechanism [25-45].

As most circulating T_3 in man is derived from intrahepatic deiodination of thyroxine, transport of thyroxine from plasma into the hepatocyte may play an important role in the regulation of thyroid hormone bioactivity. Also, as most long-term biological effects of thyroid hormone are mediated via the intracellular thyroid hormone receptor(s), the importance of trans-plasma membrane transport of these hormones seems obvious. Finally, metabolic clearance of thyroid hormones involves intracellular deiodination, conjugation, deamination or ether-bond cleavage [46], all of which can only take place after cellular entry of the hormones.

Inhibition of transmembrane transport of thyroid hormones by circulating compounds, reduced intracellular energy stores, or changes in expression of the transport protein(s) may have pronounced effects on intracellular thyroid hormone concentration and thus thyroid hormone bioactivity. Examples of these phenomena will be given throughout the rest of this thesis.

1.6 Biological effects of thyroid hormones

Already since the studies of Magnus-Levy in 1895 [47] the action of thyroid hormones has been traditionally linked to an augmentation in respiration. Nowadays it has been recognised that thyroid hormones have profound effects on many enzymes and almost all organ systems, and that they play an important role in cell growth and differentiation [48].

As mentioned previously, most long term biological effects of thyroid hormone are mediated through binding of hormone to the nuclear thyroid hormone receptor with subsequent modulation of gene expression. Table I summarises several genes which are regulated by thyroid hormone.

Gene	Species (organ)	Regulation	Refs
TSH ß-subunit	mouse, rat, human	decrease mRNA	7, 49, 50
Pre-pro-TRH	human	decrease mRNA	7
Growth hormone	rat	increase mRNA	51
α-Myosin HC	rat	increase mRNA	51
Malic enzyme	rat	increase mRNA	51, 52
MyoD1	mouse	increase mRNA	53
PEPCK	rat	increase transcription	54
Glucose transporter	rat (liver cell line ARL15)	increase mRNA	55
Glucokinase	rat	increase mRNA	56
mitochondrial α-glycerophosphate dehydrogenase	rat (liver)	increase mRNA	57
Mitochondrial cyt-c oxidase	rat (hepatoma cell line, HTC)	increase mRNA (no increase nuclear encoded oxidase !)	58
Apolipoprotein A1	rat	increase mRNA	59
SHBG	human liver (HepG2)	increase mRNA	60
EGF	mouse	increase mRNA	61
α1-Adrenoreceptor	rat (vascular bed)	decrease receptor density	62
ß-Adrenoreceptor	rat (vascular bed)	increase receptor density	62
Na/K-ATPase	rat, human	increase mRNA sub-units	63, 64
Interferon responsiveness of lymphoid cells	mouse (spleen)	increase by T_3 and T_4 (fully expressed after 30 minutes)	65
Type I deiodinase	rat (liver)	increase mRNA	66
Type II deiodinase	rat (brain)	decrease activity (cytoskeleton)	67

Table I *Examples of thyroid hormone responsive genes with their response to thyroid hormones. Different genes are influenced in opposite directions and through different mechanisms.*

Some of the physiological implications of these complex changes in gene expression can be summarised as follows.

Lack of fetal thyroid hormone secretion results in impaired brain development and skeletal maturation (cretinism). T_3 increases oxygen consumption and heat production, except in brain, spleen and testis. T_3 stimulates cardiac muscle contractility and increases heart rate, like in hyperthyroidism. Sensitivity to catecholamines is increased in hyperthyroidism. In severe hypothyroidism, hypoventilation may occur, occasionally requiring assisted ventilation. Thyroid hormones increase 2,3,-diphosphoglycerate content of erythrocytes, allowing increased oxygen dissociation from haemoglobin and thus increasing oxygen availability to tissues. Thyroid hormones stimulate gut motility, increase bone turn-over and increase bone resorption. Hyperthyroidism increases hepatic gluconeogenesis and glycogenolysis as well as intestinal glucose absorption. Low density lipoprotein (LDL) receptors increase, resulting in decreased cholesterol levels. Metabolic turnover of many hormones and pharmacological compounds is increased [1, 68-70].

In general, hyperthyroidism results in increased protein turnover and increased energy expenditure. Therefore, thyroid hormone is considered to be an energy demanding hormone. Reduced thyroid hormone levels will result in reduced energy expenditure and increased protein conservation.

2. Thyroid hormone bioactivity during non-thyroidal illness and fasting

2.1 Changes in serum thyroid hormone parameters

In man, acute or chronic illness not related to the thyroid gland (non-thyroidal illness, NTI) often result in decreased serum T_3 levels. This so-called 'low-T_3 syndrome' or 'sick euthyroid syndrome' is found for instance in patients with liver disease, after stress or surgery, in patients with renal failure , in the elderly sick, and also during caloric deprivation. In addition, ingestion of a number of drugs, including amiodarone, corticosteroids, d-propanolol, propylthiouracil, and X-ray contrast agents, are known to induce a 'low-T_3 syndrome'. Despite the decreased serum T_3 concentration, TSH concentrations are usually normal. Serum free T_4 is slightly elevated in mild NTI, but falls in more severely ill patients. Serum reverse T_3 concentrations are often elevated [71].

2.2 Generation of low-T_3 syndrome

The low-T_3 syndrome in NTI patients is considered to be an adaptation mechanism to the situation of metabolic stress. The lowered serum T_3 results in reduced energy expenditure and increased protein conservation. This will protect organ function and support the recovery of the patients [71, 72].

The changes in thyroid hormone concentrations observed in NTI result from changes in serum binding, cellular entry, and intracellular deiodination of thyroid hormones. The reduced total T_4 with increased free T_4 results from decreased serum T_4 binding capacity, but also from the presence of circulating inhibitors of T_4 binding to serum binding proteins [73].

In vitro studies using primary cultures of rat hepatocytes have shown inhibition of T_4 transport by serum of NTI patients. These studies indicate that 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) and indoxyl sulfate, in concentrations present in sera of uraemic patients, inhibit cellular transport and subsequent deiodination of thyroxine without direct effects on intracellular type I deiodinase [74]. In sera of non-uraemic patients, bilirubin and non-esterified fatty acids (NEFA), in combination with the reduced serum albumin concentration, were shown to inhibit cellular transport of thyroxine in the same system [75]. It has been suggested that increased NEFA concentrations, in addition to a decrease of cellular ATP, is responsible for the attenuated cellular transport of thyroxine during caloric restriction in obese subjects [76].

Deiodinative activation of T_4 to T_3 can be reduced by direct effects on intrahepatic type I deiodinase, as for example by d-propanolol [77] or propylthiouracil [46]. This will result in decreased T_3 production and decreased rT_3 clearance. Also, a decrease in expression of type I deiodinase activity, as reported for starvation in rats [78], will result in decreased intrahepatic T_3 production. This is most likely due to the hypothyroidism induced by fasting [79]. Therefore, the changes in thyroid hormone parameters found in NTI are often attributed to alterations in type I deiodinase activity [80, 81, 82].

2.3 Serum TSH concentration

Despite the decreased serum T_3 concentration in the low-T_3 syndrome, there is no increase in serum TSH. In addition to central mechanisms influencing TRH secretion [83], intra-pituitary T_3 production is increased by an increase in expression of type II deiodinase [67, 84, 85]. It has recently been shown that CMPF and indoxyl sulfate do not inhibit the uptake of thyroxine in cultured rat anterior pituitary cells [86], in contrast to hepatic uptake of this hormone. As the serum T_4 concentration is often normal or elevated in NTI, the uptake of T_4 into the pituitary is not inhibited, and type II deiodinase activity is increased, the intra-pituitary T_3 concentration might be in the normal range.

Using the same system of cultured rat anterior pituitary cells it has been shown that, based on the free hormone concentration, triiodothyroacetic acid (Triac) is more potent than T_4 or T_3 in suppressing TSH secretion [87]. Therefore, the increased Triac concentrations found in fasting and NTI [88, 89] may be responsible for the suppressed TSH in the low-T_3 syndrome found during calorie restriction.

TSH-suppressive effects of triiodothyronine sulfate (T_3S), which is increased during NTI [90] were not found in the cultured rat anterior pituitary cell system. In addition to the relatively low uptake of this compound into the cells, it has little if any effect on the TRH-induced TSH response in this system [91].

3. Potential benefits of cloning the thyroid hormone transport protein

3.1 A new group of steroid hormone transport proteins

As mentioned previously, it has long been thought that thyroid hormones cross the lipid bilayer of the plasma membrane by simple diffusion [23, 24]. It has now generally been accepted that this is not the case for thyroid hormones [25]. Also, it has been suggested that Na/K-ATPase is involved in the uptake of corticosterone by isolated rat liver cells [92]. Therefore, it is not unlikely that there are specific transport proteins which are responsible for the uptake of most lipophilic hormones, including thyroid hormones and steroid hormones such as glucocorticoids and testosterone.

As transport of thyroid hormone is the best characterised transport system of this group, it is the best candidate for cloning. Sequencing of this protein might lead to the identification of a family of transport proteins.

3.2 Homology screening

Based on an amino acid sequence it would be possible to select probes for homology screening. This would allow isolation of thyroid hormone transport proteins from different tissues or species. One of the hypotheses in our laboratory is the presence of different transport proteins in liver and pituitary gland. This hypothesis is based on physiological data, and should be confirmed by biochemical and molecular biological data. Sequence analysis of thyroid hormone transport proteins of different species might reveal evolutionary well conserved domains, which would allow selection of probes for identification of other members of this group of transport proteins analogues to G protein-coupled receptors [93, 94].

3.3 Structure function relationship

Elucidation of the genetic code of the thyroid hormone transport protein would also

offer the possibility to study structure-function relationships. Using site-directed mutagenesis, the transport characteristics of altered thyroid hormone transport proteins can be studied. Based on these data, important domains within the protein can be identified. This will not only increase our understanding about the transport process itself, but also is a first step towards computer modelling of the thyroid hormone binding site. Knowledge of the three-dimensional structure of this binding site allows the design of highly specific thyroid hormone uptake antagonists [94]. This will be discussed in paragraph 3.6 of this chapter. Also, expression of high amounts of thyroid hormone transport protein is essential to study three-dimensional structures. Due to the hydrophobic character of the protein, purification from plasma membranes is difficult. Addition of an immuno-tag before expression in an *in vitro* system may allow large scale purification of the transport protein.

3.4 Regulation of gene expression

Based on an identified sequence, expression of mRNA in different tissues could be studied. Both in animal models as well as in healthy volunteers and patients, the expression of the transport protein can be studied. Regulation of expression of thyroid hormone transport protein is important with respect to the general adaptation in situations of metabolic stress, i.e. the low-T_3 syndrome. Studies could be conducted to analyse its role in the regulation of bioactivity in health and disease.

3.5 Generation of epitope-specific antibodies

Based on sequence information, it would be possible to synthesise peptide fragments. These fragments could be used to raise highly specific antibodies. These antibodies could be used to study expression of the transport protein in different tissues, and could be used for *in vitro* and *in vivo* uptake studies.

3.6 Thyroid hormone uptake antagonists

Based on the three-dimensional structure of the thyroid hormone binding site, it might be possible to synthesise highly specific thyroid hormone uptake antagonists. These antagonists could be used for acute treatment of hyperthyroidism, and the often life-threatening situation called 'thyroid storm' which can occur following radioactive iodine treatment (RAI) or in undiagnosed hyperthyroidism. The major advantages of such antagonists are that they would act extracellularly, possibly reducing harmful side-effects, and that they have an acute effect. The cellular uptake of thyroid hormone is inhibited, as opposed to the current antithyroid medication which inhibits thyroid hormone synthesis. These medications exert a sufficient effect only after weeks or sometimes even months.

4. Cloning strategies

4.1 From gene to protein to biological activity

In order to isolate a gene that codes for a particular protein, the biological characteristics of this protein have to be known. Only on the basis of these properties, unknown genetic sequences can be related to the expression of a particular protein. Also, biological function can be used to identify genetic sequences. Unknown genetic sequences are introduced into a biological system, expressed as functional proteins, and analysed for their biological properties.

4.2 Biological characteristics of the thyroid hormone transport protein

For the transmembrane thyroid hormone transport protein, many physiological characteristics are known. This plasma membrane protein is expressed in most cells. It is best characterised in hepatocytes, as the liver is the most important organ for extra-thyroidal conversion of thyroxine to triiodothyronine [25]. The protein has high affinity for thyroid hormones, with Km values of 61 nmol/l for T_3 and 1.4 nmol/l for T_4 (37 ^{0}C, rat hepatocytes) [25]. Preincubation of rat hepatocytes with the covalent affinity label N-bromo-acetyl-T_3 results in a decreased transmembrane transport of T_3 [95]. Transmembrane transport is sodium- and temperature dependent, and saturable. It is rate limiting in total cellular uptake and metabolism in rat hepatocytes [28], and inhibited by a monoclonal antibody raised against rat hepatocytes [26]. Uptake of T_3 by human liver derived HepG2 cells is inhibited by benzodiazepine compounds, which are proposed to be competitive inhibitors without being a substrate for transport [96].

Many other characteristics have been described for different cell types. For rat erythrocytes, involvement of the tryptophan transport system T has been proposed [37, 39]. In rat pituitary GH4C1 cells involvement of the neutral amino acid transport system L has been suggested [97]. Both systems describe Km values in the micromolar range, which suggest that these are not the same systems as the high affinity transport systems described for hepatocytes (nanomolar range).

4.3 Proposed cloning strategies

Based on the biological characteristics, several strategies can be suggested in order to identify the genetic sequence coding for the thyroid hormone transport protein.

The functional characteristic of transport of thyroid hormone from plasma into the cell can be used as a marker for expression cloning. In cells which contain functional type I deiodinase, the cellular production of iodide after intracellular deiodination can be used to

monitor cellular entry. Iodide production can be rapidly determined using LH20 chromatography of cell culture media [98]. If the introduced foreign genetic material codes for the thyroid hormone transport protein, there will be more transport protein expressed in the plasma membranes of these cells. This will increase the cellular entry of thyroid hormone, and subsequently increase the amount of iodide produced by these cells. Thyroid hormone uptake and metabolism of cells that will be used for overexpression of the transport protein have to be characterised prior to transfection with foreign genetic material. If these cells are suitable for overexpression of the transport protein, a eukaryotic expression system has to be established. We have chosen for the recently developed pDR2 system. This vector is replicated in the cytoplasm of permissive cells, which not only increases the copy number but also allows rescue of plasmids from positive clones.

The presence of the protein in the plasma membrane, combined with its affinity for N-bromo-acetyl T_3, offers the possibility to covalently label the transport protein in intact cells, isolate the plasma membranes, and analyse the labelled proteins. The purified protein(s) can be used for protein microsequencing and design of specific oligonucleotide probes, or for raising antibodies to screen (bacterial) expression libraries [94].

In addition, the affinity of the thyroid hormone transport protein for benzodiazepines has been used to construct an affinity chromatography column. Rat liver plasma membrane proteins were applied to this column and eluted with high concentrations of T_3. The purified proteins were used to raise antibodies in rabbits. These antisera were tested for T_3 uptake inhibition in human liver derived HepG2 cells [99]. We also produced mouse monoclonal antibodies which were raised against rat hepatocytes [26]. These antibodies were screened for hepatocyte binding and T_3 uptake inhibition. The antisera and antibodies can possibly be used to screen bacterial expression libraries.

In cells that do not contain deiodinating enzymes, intracellular binding of thyroid hormone can be used to monitor cellular entry. An increase in thyroid hormone transport protein will increase cellular entry, and consequently increase total cellular binding. For this strategy we have used *Xenopus laevis* oocytes. These huge cells have been used for the expression of many plasma membrane transport proteins [100]. The oocytes are injected manually with a total pool of mRNA of the tissue of interest. As the thyroid hormone transport protein is highly expressed in rat liver, we have compared water-injected and rat liver mRNA-injected oocytes with respect to their T_3 uptake. The main advantage of this system is that it starts with a total pool of mRNA expressed in single cells. These cells are analysed individually for expression of the protein of interest. An indication of potential results is obtained before the actual cloning procedures have started.

5. Scope of the thesis

In this thesis the development of a cloning strategy for the transmembrane thyroid hormone transport protein is described. Based on the extensive physiological

characterisation of this transport system, several strategies are proposed and initiated.

Overexpression of the transport system in its hepatic environment using the eukaryotic expression vector pDR2 is proposed in Chapter 2. In order to allow correct interpretation of thyroid hormone uptake and metabolism data by transfected cells, wild-type HepG2 cells had to be characterised with respect to their thyroid hormone handling. These data are described in Chapters 2.1 and 2.2. Optimisation of DNA transfection into these cells is described in Chapter 2.3.

Partial purification of the transport protein from human liver derived HepG2 cells has been proposed in order to allow protein microsequencing or raising of specific antibodies. For this purpose a highly selective technique based on polycationic beads has been used. Chapter 3 describes the N-bromo-acetyl T_3 affinity labelling of plasma membrane proteins isolated using this technique.

Chapter 4 describes the characterisation of several available antibody preparations. Antibodies were tested for transport inhibition in rat hepatocytes or human liver derived HepG2 cells, and for their ability to recognise antigens on denaturing Western blots.

Expression of the transport protein by injection of rat liver mRNA into *Xenopus laevis* oocytes is described in Chapter 5. The uptake of T_3 by water-injected oocytes is compared to mRNA-injected oocytes.

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Chapter 2.1

Reduced T_3 deiodination by the human hepatoblastoma cell line HepG2 caused by deficient T_3 sulfation.

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Reduced T_3 deiodination by the human hepatoblastoma cell line HepG2 caused by deficient T_3 sulfation.

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Summary

Type I deiodination of T_3 sulfate occurs at a Vmax that is 30-fold higher as compared to T_3, both in rat and in human liver homogenates. We now present data showing lack of T_3 deiodination by a human liver derived hepatoblastoma cell line, HepG2, caused by deficient T_3 sulfation. Cellular entry of T_3 was assessed by its nuclear binding after whole cell incubation. In spite of the presence of type I deiodinase, as confirmed by T_4 and rT_3 deiodination in homogenates, no deiodination of T_3 could be detected. Since HepG2 cell homogenates also deiodinated chemically synthesized T_3 sulfate (T_3S) and inhibition of type I deiodination by propylthiouracil (PTU) did not cause T_3S accumulation in whole cell incubations, we conclude that (i) HepG2 cells show reduced T_3 deiodination caused by deficient T_3 sulfation, and (ii) sulfation of T_3 is an obligatory step prior to hepatic deiodination.

Introduction

Carrier mediated transport of thyroid hormones across the plasma membrane has been shown in a large number of tissues and cells of many species [1-4]. Transport of thyroid hormone from the extracellular compartment to the intracellular sites is a prerequisite for (i) the conversion of thyroxine (T_4), the main secretory product of the thyroid gland, to biologically active 3,3',5-triiodothyronine (T_3) by the intracellular deiodinases [5,6], and (ii) the binding of plasma derived T_3 to nuclear receptors to exert its biological effects [7].

Although T_3 uptake and metabolism have been studied extensively in rat and human brain derived cell lines [4,8], rat erythrocytes [9] and hepatocytes [10-13], and in human fibroblasts [14], there are, to our knowledge, no reports concerning deiodination of T_3 in intact human hepatocytes. In the rat, T_3 transported into the liver is upon entry into the intracellular compartment conjugated to T_3 sulfate (T_3S)[12]. Studies using rat hepatic microsomal fractions comparing T_3 and T_3S as substrates for type I deiodinase showed that sulfation of T_3 increases the Vmax of deiodination 30-fold [15]. In human liver microsomes prior sulfation of T_3 also resulted in a 30-fold increase of its inner-ring deiodination by type I deiodinase [16]. Inhibition of type I deiodinase by incubation in the presence of propylthiouracil (PTU) is known to prevent deiodination of T_3-sulfate, thereby leading to the accumulation of this compound [13].

Since it is difficult to obtain viable human hepatocytes, we decided to use the human hepatoblastoma cell line HepG2 to study T_3 metabolism in human liver derived cells. These cells have been shown to retain many of the specialized functions which are usually lost upon culturing. They still possess many metabolizing enzymes, and are therefore thought to be a suitable model for human hepatocytes [17].

Part of this work has been presented at the 19th annual meeting of the European

Thyroid Association in Hannover, Germany [18].

Materials and Methods

Cell Culture

HepG2 cells were obtained from Dr. Knowles, the Wistar Institute, Philadelphia, USA. Cells were grown in a 5 % CO_2, 37 °C, humidified incubator in Dulbecco's Modified Eagle Medium/Nutrient Mixture Ham's F12 (1:1 vol/vol) supplemented with 10 % fetal calf serum (FCS), penicillin (100 IU/ml), streptomycin (100 µg/ml), and glutamine (2.4 mmol/l). Cells were subcultured at confluence using trypsinization (0.05 % trypsin, 0.002 % collagenase, 0.02 % EDTA). All tissue culture chemicals and plastics were obtained from GIBCO/BRL (European division, Breda, The Netherlands).

Whole Cell incubations

Subconfluent cultures of HepG2 cells were harvested using trypsinization, and transferred to six-wells plates.

Iodide production : After reaching confluence, medium was replaced with 2 ml incubation medium per well (culture medium without FCS, with 0.5 % BSA, with or without 0.5 mmol/l ouabain or 100 µmol/l PTU) for 30 min. Incubation was started by replacing medium with 1 ml fresh incubation medium containing 90,000 cpm/ml 3'-L-[^{125}I]T_3 (24 pmol/l, Amersham International, U.K., 3070 µCi/µg). Cells were incubated for 90 min (ouabain experiments) or 17 hours (PTU experiments) in a humidified 5 % CO_2 incubator. Parallel incubations without cells were performed to correct for spontaneous deiodination. After incubation, medium was removed for further analysis. Cells were washed with 2 ml of ice-cold phosphate buffered saline (PBS, pH 7.4), and lysed in 0.1 N NaOH. Total cellular binding (TCB) was determined by measuring cell lysate radioactivity. The media were analyzed for iodide, conjugates, and remaining iodothyronines using Sephadex LH-20 chromatography (Pharmacia Fine Chemicals, Uppsala, Sweden), as described previously [13]. Iodide production is expressed as percentage of total radioactivity added to the column. As TCB was below 5%, the radioactivity applied to the column approximated total radioactivity added to the medium. All tracers used were always LH-20 purified on the day of the experiment.

Studies in primary cultures of rat hepatocytes were performed as described previously [10] for comparison with results in HepG2 cells.

Nuclear Binding : Nuclear binding experiments were performed essentially as iodide production experiments, with the following modifications: after reaching confluence cells were washed with incubation medium, followed by direct incubation with 1 ml incubation medium containing 120,000 cpm/ml 3'-L-[^{125}I]T_3 (32 pmol/l, Amersham, U.K., 3070 µCi/µg) for 120 min. Non-specific cellular uptake was determined by addition of 10 µmol/l unlabelled T_3 (Henning GmbH, Berlin, Germany). After incubation, the medium was discarded and the cells were washed twice with 2 ml ice-cold PBS. Cells were then scraped from the wells with a rubber police-man, and resuspended in 2 ml ice-cold PBS. All following procedures were performed on ice. Radioactivity of cells was measured to determine TCB.

The cells were pelleted by centrifugation (300 g, 4 °C, 7 min), and subsequently solubilized in 1 ml PBS, 0.5 % Triton X-100, as described previously [2]. After 2 min of continuous vortexing, nuclei were spun down (900 g, 4 °C, 5 min), and washed twice in PBS, 0.5 % Triton X-100. After the final wash, radioactivity of the nuclear pellet was measured to determine nuclear binding (NB).

Type I deiodinase assays

In order to study deiodination of iodothyronines in cell homogenates, HepG2 cells were harvested and washed twice with 10 ml ice-cold TED buffer (10 mmol/l Tris, 3 mmol/l EDTA, 5 mmol/l dithiotreitol, pH 7.5). The cells were lysed by sonication. Human liver homogenates were prepared by mincing pieces of normal human liver (Dept. of Surgery, Academic Hospital Dijkzigt) in TED buffer, followed by sonication. Protein concentration was determined using a commercially available kit based on the method of Bradford (Bio-Rad Laboratories B.V., The Netherlands). Homogenates were stored at -80 ^{0}C until further use.

Type I deiodination assays were performed by incubation of [^{125}I]-T_3 (22 nmol/l, 3070 µCi/µg), [^{125}I]-T_3S (22 nmol/l from [^{125}I]-T_3), [^{125}I]-T_4 (35 nmol/l, 1500 µCi/µg) or [^{125}I]-rT_3 (55 nmol/l, 1200 µCi/µg) in the presence of 167 nmol/l unlabelled iodothyronine (Henning GmbH, Berlin, Germany) for 60 min at 37 ^{0}C with 100 µg protein in 200 µl TED. The reaction was stopped by addition of an equal volume of 12 % trichloro-acetic acid (TCA). Precipitated protein was spun down, and the supernatants were analyzed using LH-20 chromatography, as described previously [13].

Miscellaneous

Ouabain and nuclear binding experiments were standardized for DNA-contents, determined with Hoechst dye, as reported previously [19]. PTU experiments were standardized for protein contents, as described above. To monitor quality of cells after incubation, ATP was determined in perchloric acid (PCA) cell extracts using the luciferine/ luciferase system (Lumac Adenylate Energy Charge Kit). [^{125}I]-T_3 sulfate was synthesized by reaction of [^{125}I]-T_3 with chlorosulfonic acid, as described previously [13].

Results

Comparison of T_3 deiodination in primary cultures of rat hepatocytes and HepG2 cell-cultures clearly shows the absence of iodide production in HepG2 cells in spite of comparable total cellular binding (TCB), as can be seen from Figure 1. Ouabain inhibited both iodide production (20.43 ± 0.27 to 17.93 ± 0.74 % Iodide/80 µg DNA, $p < 0.025$) and TCB (6.22 ± 0.19 to 5.76 ± 0.03 fmol [^{125}I]-T_3/80 µg DNA, $p < 0.01$) in rat hepatocytes, and TCB in HepG2 cells (4.38 ± 0.07 to 3.89 ± 0.04 fmol [^{125}I]-T_3/80 µg DNA, $p < 0.001$), in agreement with previous reports showing dependence of the uptake process on a sodium gradient over the plasma membrane [5]. ATP concentrations did not change significantly during preincubation or incubation with tracer T_3 (data not shown). The lower TCB in HepG2 cells is due to higher DNA concentrations in HepG2 cells caused by chromosome amplifications [20]. When expressed per confluent well, TCB of rat hepatocytes approximately equals HepG2 TCB.

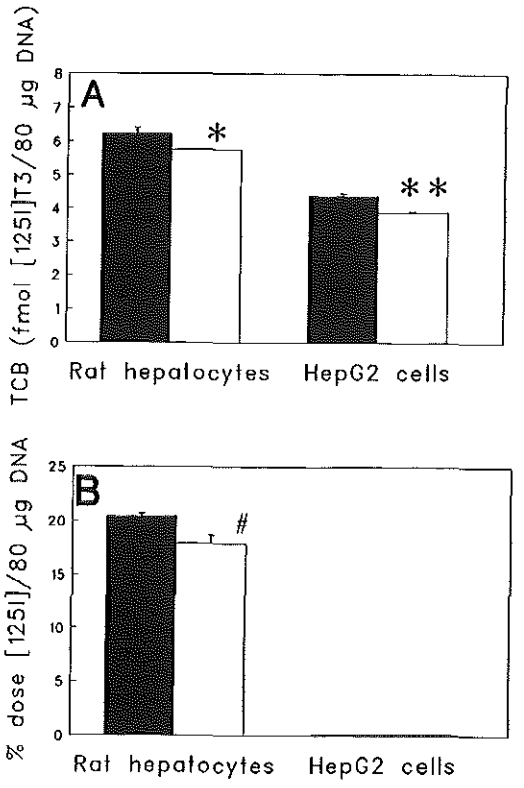

Figure 1 *Cultures of HepG2 cells were incubated for 90 min with [^{125}I]-T_3 and analyzed for total cellular binding (TCB) and iodide production. Results were compared with primary cultures of rat hepatocytes. Even though HepG2 cells show comparable TCB (panel A), there is no iodide production by HepG2 cells (panel B). TCB was slightly decreased by ouabain (0.5 mmol/l) in rat hepatocytes and HepG2 cells, as well as iodide production by rat hepatocytes.*
*■ : control, □ : ouabain, #: p<0.025, *: p<0.010, **: p<0.001*

To confirm cellular entry of T_3 in HepG2 cells, T_3 nuclear binding was determined. Figure 2 shows nuclear binding of T_3 following incubation of intact HepG2 cells with radiolabelled T_3. Incubation of the cells in the presence of 10 µmol/l unlabelled T_3 reduced nuclear binding by nearly 100 %.

Since T_3 does enter the cells, the lack of deiodination has to be explained either by lack of deiodinase activity or by absent sulfation of T_3, as this process has been shown to occur prior to its deiodination in rat hepatocytes [21]. Therefore, HepG2 homogenates were tested for type I deiodinase activity. Table I shows the deiodinase activities of HepG2 cell homogenates. There is hardly any deiodination of T_3 (0.07 ± 0.03% 125I/ 100 µg protein.hr), but good deiodination of T_4, rT_3 and T_3S (7.90 ± 1.20, 40.7 ± 6.90 and 6.06 ± 0.17 % 125I/ 100 µg protein.hr, respectively), confirming the presence of type I deiodinase.

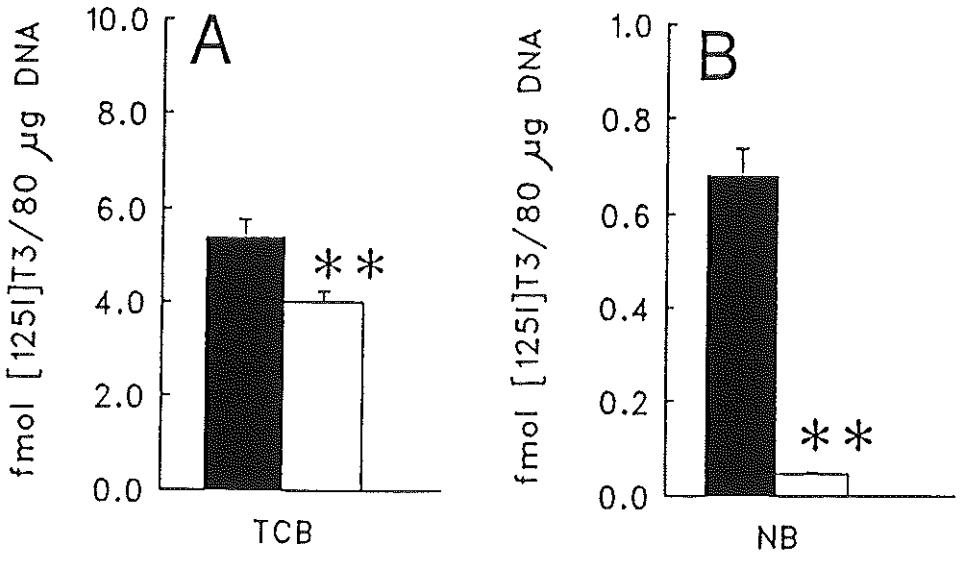

Figure 2 *To asses cellular entry of T_3, whole cell incubations of HepG2 were performed. Cells were incubated for 120 minutes with [^{125}I]-T_3 in the presence or absence of 10 µM unlabelled T_3. Total cellular binding of [^{125}I]-T_3 (panel A) was markedly lower in the presence of 10 µmol/l unlabelled T_3, whereas nuclear binding (panel B) was reduced by nearly 100%.*
*■ : control, □ : 10 µmol/l unlabelled T_3, ** : p<0.001*

Thus, a deficiency in T_3-sulfation has to be presumed. In order to allow measurement of T_3S production in whole cell incubations, type I deiodinase activity was inhibited by addition of 100 µmol/l PTU. Since T_3S is deiodinated by type I deiodinase, PTU causes accumulation of this compound [13]. Figure 3 compares the effect of PTU on iodide production and T_3S production by rat hepatocytes and HepG2 cells. Iodide production by rat hepatocytes is reduced from 61.3 ± 1.17 % to 20.6 ± 1.47 % dose [125-I]/mg protein ($p<0.001$), whereas T_3S increases from 3.5 ± 0.59 to 19.8 ± 0.81 % dose [125-I]-T_3S /mg

Substrate	HepG2 % ^{125}I/100 µg protein.hr	Human Liver % ^{125}I/100 µg protein.hr
T_3	0.07 ± 0.03*	1.20 ± 0.03
T_4	7.90 ± 1.20	12.1 ± 1.10
rT_3	40.7 ± 6.90*	44.5 ± 1.80
T_3S	6.06 ± 0.17	17.3 ± 0.14

Table I *Type I deiodinase activity in HepG2 and human liver homogenates. Values represent mean ± SEM (n=3 or * n=6).*

protein ($p<0.001$). HepG2 cells do not show any significant accumulation of T_3S in the presence of PTU. Iodide production slightly decreases from 0.7 ± 0.04 to 0.18 ± 0.13 % dose [125-I]/mg protein ($p<0.05$), whereas T_3S decreases from 0.6 ± 0.06 to 0.5 ± 0.05 % dose [125-I]-T_3S /mg protein (not significant).

Discussion

The most important finding of the present study is the lack of iodide production from T_3 by intact HepG2 cells, in spite of cellular entry of the hormone. Cellular entry of T_3 was confirmed by the presence of the hormone at nuclear sites after whole-cell incubations, as also reported by Robbins and coworkers [2]. This nuclear binding was nearly absent in the presence of 10 µmol/l unlabelled T_3 (93 % reduction of NB), which can be explained by an almost complete saturation of the cellular uptake system and displacement of radiolabelled T_3 at nuclear sites by unlabelled T_3. The observation that only 28 % of TCB is displaced by the same concentration of unlabelled hormone may be explained by the high non-specific capacity of the outer cellular matrix [5].

Since type I deiodinase activity is present in HepG2 cell-homogenates, as assessed by the production of iodide from T_4, rT_3, and T_3S, we suggest that there is a deficient T_3 sulfation in the HepG2 cell line. Homogenates of normal human liver, on the other hand, do deiodinate T_3 (see Table I), demonstrating that sulfation of T_3 is intact in normal human hepatocytes. The lower T_3S deiodination activity that we found in HepG2 homogenates as compared to human liver homogenates (see Table I) was accompanied by a lower T_4 and rT_3 deiodination activity. Thus, an overall lower type I deiodinase activity in HepG2 cells has to be presumed. This somewhat lower activity, however, should still be sufficient to produce measurable iodide production from T_3 in whole cell-incubations.

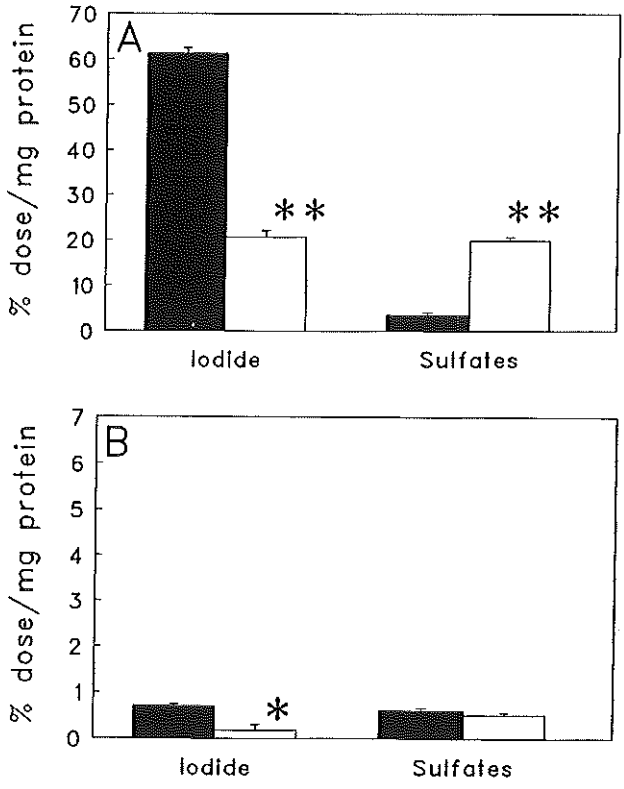

Figure 3 *Cultures of rat hepatocytes (panel A) or HepG2 cells (panel B) were incubated for 17 hours with [125-I]-T_3 in the presence or absence of 100 µmol/l PTU. Media were analyzed for iodide and T_3-sulfate production using LH-20 chromatography. Iodide production by rat hepatocytes is strongly reduced in the presence of PTU, with a concomitant increase in T_3-sulfate ($p<0.001$). HepG2 cells do show little reduction in iodide production ($p<0.05$), but there is no change or even a decrease in T_3-sulfate.*
■ *: control,* □ *: 100 µmol/l PTU, * : $p<0.05$, ** : $p<0.001$*

Whole cell experiments in the presence of PTU, in order to inhibit type I deiodination of intracellularly formed T_3S, also showed absence of T_3S accumulation in HepG2 cells as compared to rat hepatocytes. The very little amount of iodide formed by HepG2 cells during the 17 hr incubation was inhibitable by PTU, suggesting type I deiodination of T_3 without prior sulfation. If there would have been prior sulfation, the T_3S should have increased.

We conclude that (i) HepG2 cells transport T_3 across the plasma membrane, and (ii) HepG2 cells do not deiodinate T_3 because of deficient T_3 sulfation, since T_3S is readily deiodinated by HepG2 cell-homogenates, and incubation in the presence of PTU does not increase T_3S accumulation. This deficiency appears to be specific for HepG2, in contrast to normal human liver homogenate. In spite of the fact that HepG2 cells have been reported

to retain many liver-specific metabolizing enzymes [17], we have to conclude that this is not the case for T_3-sulfation. Nevertheless, this absence of T_3-sulfation allows us to conclude that even though HepG2 has type I deiodinase activity, prior T_3-sulfation is obligatory for T_3 deiodination by this enzyme. This mechanism has originally shown to be operative in rat hepatocytes by using sulfate free incubation medium [22].

Thus, these experiments show for the first time that, without artificially depleting sulfate availability to the cells, sulfation of T_3 is an obligatory step prior to deiodination. Since HepG2 is a human liver derived cell line, these data suggest that also in human, as shown for rat [15], sulfation of T_3 indeed has profound effects on the deiodination of this hormone. Also, many drugs are metabolized via hepatic conjugation. Sulfation of 7-hydroxycoumarin by HepG2 has been described [17], which, in combination with the present findings, points to substrate selectivity of sulfotransferase activity in hepatocytes. Studies using different substrates for sulfotransferase should allow further characterization of the T_3 sulfation deficiency in HepG2.

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Chapter 2.2

Uptake and metabolism of 3,5,3'-triiodothyronine (T_3) and 3,3',5'-triiodothyronine (rT_3) by human liver derived cells ; HepG2 cells as a model for thyroid hormone handling by human liver.

J. Clin. Endocrinol. Metab., submitted

Uptake and metabolism of 3,5,3'-triiodothyronine (T_3) and 3,3',5'-triiodothyronine (rT_3) by human liver derived cells ; HepG2 cells as a model for thyroid hormone handling by human liver.

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Short running title : Thyroid hormone handling by human HepG2 cells.

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Abstract

The uptake of 3,5,3'-triiodothyronine (T_3) and 3,3',5'-triiodothyronine (rT_3) was studied in human liver derived HepG2 cells. The results showed a saturable, time-dependent and ouabain-sensitive increase in nuclear bound T_3. The effects of ouabain (0.5 mmol/l) and unlabelled T_3 (10 nmol/l and 10 µmol/l) were much more pronounced at the nuclear level, suggesting the presence of a non-specific component in total cellular binding. Comparison of rT_3 metabolism in HepG2 cells and primary cultures of rat hepatocytes showed an approximately 10-fold lower iodide production in HepG2 cells. Iodide production was decreased in the presence of ouabain, and almost absent in the presence of propylthiouracil (PTU, 100 µmol/l). Initial uptake data confirmed the presence of a carrier mediated uptake system for both T_3 and rT_3. Metabolism data indicated functional type I deiodinase activity in HepG2 cells, the presence of glucuronidating enzymes, and the absence of thyroid hormone sulfotransferase activity. Based on these data we propose that HepG2 cells provide an appropriate model for thyroid hormone handling by human liver. In addition, we suggest that in human liver sulfation of thyroid hormone, and therefore deiodination of T_3, is only of minor importance.

Introduction

Both in man and rat the conversion of thyroxine (T_4), the main secretory product of the thyroid gland, to the biologically active 3, 5, 3'-triiodothyronine (T_3), takes place in the liver. After entrance of T_4 into the cellular compartment, it can either be activated by deiodination to T_3, or inactivated by deiodination to 3,3',5'-triiodothyronine (reverse triiodothyronine, rT_3) (1-3). In the liver, both reactions are mediated via type I-deiodinase, which has recently been cloned (4). Alternatively, the iodothyronines can be conjugated by glucuronyltransferases, with subsequent excretion into the bile, or sulfotransferases (1).

Many studies on the hepatic entry of thyroid hormone have been performed using primary cultures of rat hepatocytes (5-9), rat liver derived cell-lines (10,11), and rat liver *in vivo* (12). It has been shown that the uptake process is carrier-mediated, being saturable, stereo-specific, energy- and temperature dependent. This process has also been shown to be operative in other types of cells of several species (13-20), and is not only essential for the intracellular activation of T_4, but also for the biological effects of circulating T_3, which is mediated via specific nuclear receptors.

Recently we reported data on thyroid hormone transport in primary cultures of human hepatocytes (21). Furthermore, transport into human fibroblasts (22), neuroblasts (23), and glioma cells (24) has been described. Because of difficulties in routinely obtaining viable human hepatocytes we decided to study the human liver derived cell-line HepG2, in which only uptake and nuclear binding of T_3 has been studied (23,25).

HepG2 cells have been shown to retain many of the liver-specific metabolic

enzymes, and are thought to be a suitable model for human liver cells (26). In this report we studied T_3 and rT_3 uptake and deiodination in human HepG2 cells and discussed the results with those obtained in the previously used rat and human hepatocyte-model. As we have shown that there is no deiodination of T_3 in HepG2 cells due to deficient T_3 sulfation (27), we have used nuclear binding of T_3 to study internalization of this hormone.

Materials and Methods

Materials

L-[3'-125I]T_3, and I-[3'-5'-125I]rT_3 (both specific activity >1200 µCi/µg) were obtained from Amersham International (Aylesbury, Buckinghamshire, UK). Unlabelled T_3 and rT_3 were obtained from Hennig (Berlin, Germany). Sephadex LH-20 was obtained from Pharmacia (Uppsala, Sweden). All tissue culture chemicals and plastics were obtained from GIBCO/BRL (European Div, Breda, The Netherlands). All other chemicals were purchased from Sigma Chemical Co (St Louis, MO).

Methods

Cell Culture

HepG2 cells were obtained from Dr. Knowles, the Wistar Institute, Philadelphia. Cells were grown in a 5 % CO_2, 37 °C, humidified incubator in Dulbecco's Modified Eagle Medium/Nutrient Mixture Ham's F12 (1:1 vol/vol) supplemented with 10 % fetal calf serum (FCS), penicillin (100 IU/ml), streptomycin (100 µg/ml), and glutamine (2.4 mmol/l). Cells were subcultured at confluence using trypsinization (0.05 % trypsin, 0.002 % collagenase, 0.02 % EDTA).

Whole Cell incubations

Subconfluent cultures of HepG2 cells were harvested using trypsinization, and transferred to six-wells plates. After reaching confluence, medium was replaced with 2 ml incubation medium per well (culture medium without FCS, with 0.5 % BSA, with or without 0.5 mmol/l ouabain, or 100 µmol/l PTU) for 30 min. Incubation was started by replacing medium with 1 ml fresh incubation medium containing the same additions and 90,000 cpm/ml of 125I-labelled iodothyronine (total T_3 concentration 24 pmol/l, total rT_3 concentration 60 pmol/l). Cells were incubated for indicated periods of time in a dark humidified 5 % CO_2 incubator at 37 °C. Parallel incubations without cells were performed to correct for spontaneous deiodination. After incubation, medium was removed for further analysis. Cells were washed with 2 ml of ice-cold phosphate buffered saline (PBS, 137 mmol/l NaCl, 2.7 mmol/l KCl, 8.1 mmol/l Na_2HPO_4, 1.5 mmol/l KH_2PO_4, pH 7.4), and lysed in 0.1 N NaOH. Total cellular binding (TCB) was determined by measuring cell lysate radioactivity. The media were analyzed for iodide, conjugates, and remaining iodothyronines using Sephadex LH-20 chromatography, as described previously (28). All tracers used were always LH-20 purified on the day of the experiment.

For comparison, studies with primary cultures of rat hepatocytes were performed as described earlier (8).

Nuclear binding

Experiments were performed essentially the same as whole cell incubations with the following modifications, as described previously (25). After reaching confluence, cells were preincubated as described above in the presence and absence of 0.5 mmol/l ouabain, 10 nmol/l or 10 µmol/l unlabelled T_3, followed by incubation with 1 ml incubation medium

containing the same additions and 150,000 cpm/ml 3'-L-[^{125}I]T_3 (total T_3 concentration 40 pmol/l) for indicated periods of time. After incubation, the medium was discarded and the cells were washed twice with 2 ml ice-cold PBS. Cells were scraped from the wells using a rubber police-man, and resuspended in 2 ml ice-cold PBS. All following procedures were performed on ice. Radioactivity of cells was measured to determine TCB. The cells were pelleted by centrifugation (300 g, 4 ^{0}C, 7 min), and subsequently solubilized in 1 ml PBS, 0.5 % Triton X-100. After 2 min of continuous vortexing, nuclei were spun down (900 g, 4 ^{0}C, 5 min), and washed twice in PBS, 0.5 % Triton X-100. After the final wash, radioactivity of the nuclear pellet was measured to determine nuclear binding (NB).

General

All metabolism experiments were standardized for protein contents, determined in the individual cell lysates using a commercially available kit based on the method of Bradford (Biorad, The Netherlands). Nuclear binding experiments were standardized for cell density. To monitor quality of cells after incubation, ATP was determined in perchloric acid (PCA) cell extracts using the luciferin/luciferase system (Lumac Adenylate Energy Charge Kit). For several experiments the protein/DNA ratio was determined in order to verify whether protein contents indeed reflects cell number. DNA concentration was determined using Hoechst dye, as described previously (29). Data are expressed as mean ± SD for incubations assayed in triplicate, with each experiment performed at least three times. Statistical analysis of data was performed using Student's t-test for unpaired observations.

Results

All metabolism incubations were standardized for protein contents, as these values can routinely be obtained for each individual sample without the need for parallel incubations. For control incubations, protein/DNA ratios of 1.44 ± 0.24 mg protein per µg DNA are found, without significant changes by any of the incubation conditions. Therefore, we feel that this is a very useful standardization technique. Nuclear binding experiments were standardized for cell density, as in these experiments the cells are used for the isolation of nuclei, excluding the possibility of lysing cells in NaOH for protein determination.

Figure 1 shows the time-dependent increase in total cellular binding of T_3. The presence of 10 µmol/l unlabelled T_3 significantly reduced T_3 uptake during the entire time course ($p<0.001$). Incubations in the presence of 0.5 mmol/l ouabain start to differ significantly from controls after 120 minutes or longer ($p<0.001$), similar to incubations in the presence of 10 nmol/l unlabelled T_3 ($p<0.01$ at 2 hr and $p<0.001$ at 17 hr).

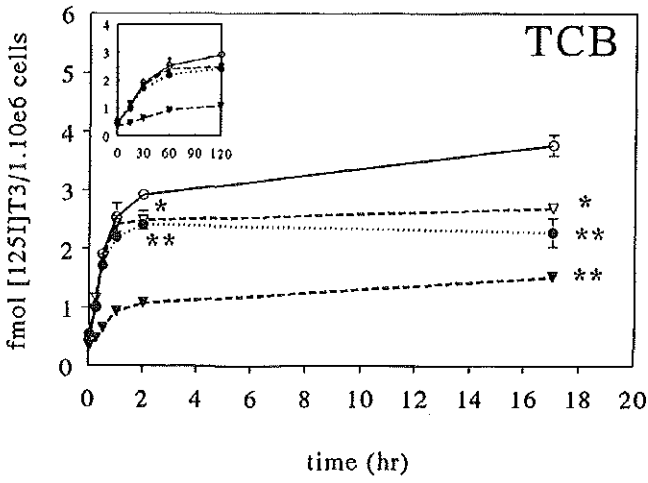

Figure 1 *Total cellular binding (TCB) of [^{125}I]-T_3 (total T_3 concentration 40 pmol/l in culture medium containing 0.5 % BSA) by HepG2 cells at 37 ^{0}C. The inset shows the initial uptake for 0 to 120 minutes. Total cellular binding is decreased in the presence of 0.5 mmol/l ouabain (●) and in the presence of 10 nmol/l (▵) and 10 µmol/l (▲) unlabelled T_3. Ouabain and 10 nmol/l unlabelled T_3 incubations start to differ significantly from control incubations (○) after 120 minutes ($p<0.001$, and $p<0.01$ respectively), whereas incubations in the presence of 10 µmol/l unlabelled T_3 are significantly lower during the entire time course ($p<0.001$).*
*** : $p<0.001$, * : $p<0.01$*

After 17 hr the total cellular binding is decreased by 39.7 ± 4.6 % in the presence of 0.5 mmol/l ouabain, and by 28.5 ± 1.8 % in the presence of 10 nmol/l unlabelled T_3.

Figure 2 shows the time dependent increase in nuclear bound T_3. Uptake of T_3 into HepG2 cells was studied using nuclear binding of radiolabelled T_3 as proof that T_3 has entered the cells. Nuclear binding increases as a percentage of the total cellular binding from 4.1 ± 1.0 % at 15 minutes to 19.6 ± 0.71 % at 17 hr. In the presence of 10 µmol/l unlabelled T_3, nuclear binding does not increase above background during the entire time course. Incubations in the presence of 10 nmol/l unlabelled T_3 differ significantly from control incubation after 15 minutes or longer ($p<0.01$ and $p<0.001$ respectively). Incubations in the presence of 0.5 mmol/l ouabain differ significantly from control incubations after 120 minutes or longer ($p<0.005$ and $p<0.001$ respectively). After 17 hr the nuclear binding is decreased by 71.5 ± 4.5 % in the presence of ouabain, and by 92.6 ± 0.6 % in the presence of 10 nmol/l unlabelled T_3.

Metabolism of rT_3 by HepG2 cells was compared with incubations of primary cultures of rat hepatocytes. Figure 3 shows the iodide and the conjugates production from rT_3 during a 17 hr incubation with HepG2 cells.

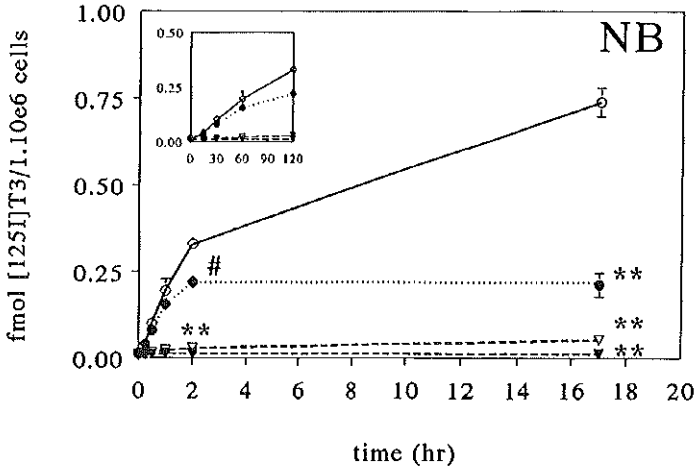

Figure 2 *Nuclear binding of [^{125}I]-T_3 (total T_3 concentration 40 pmol/l in culture medium containing 0.5 % BSA) by HepG2 cells at 37 °C. After incubation, nuclei were isolated by repeated washing of cells with PBS/0.5 % Triton X-100. The inset shows the initial uptake for 0 to 120 minutes. There is a time dependent increase in nuclear bound T_3 in control incubations (○), which is decreased in the presence of 0.5 mmol/l ouabain (●) ($p<0.005$ for 120 min or longer). The decrease in the presence of 10 nmol/l (△) unlabelled T_3 is significant from 15 minutes ($p<0.01$) or longer ($p<0.001$), whereas in the presence of 10 µmol/l (▲) unlabelled T_3 all data are significantly lower than control incubations ($p<0.001$).*

*** : $p<0.001$, # : $p<0.005$*

Iodide production amounts to 15.90 ± 0.80 fmol per mg protein which decreases to 13.56 ± 0.27 fmol/mg protein in the presence of 0.5 mmol/l ouabain ($p<0.01$) and to 2.75 ± 0.34 fmol/mg protein in the presence of 100 µmol/l PTU ($p<0.001$). Conjugates production in these incubations decreases from 2.43 ± 0.08 fmol/mg protein to 1.48 ± 0.07 fmol/mg protein in the presence of ouabain ($p<0.001$), without changes in the presence of PTU (2.09 ± 0.39 fmol/mg protein, not significant).

Iodide and conjugates production from rT_3 during a 90 minutes incubation with rat hepatocytes is shown in Figure 4. Iodide production decreases from 17.63 ± 1.14 fmol/mg protein to 1.99 ± 0.04 fmol/mg protein in the presence of PTU ($p<0.001$), without significant changes in the presence of ouabain (17.60 ± 1.47 fmol/mg protein). Conjugates production increases from 0.37 ± 0.03 fmol/mg protein to 3.65 ± 0.09 fmol/mg protein in the presence of PTU ($p<0.001$), but shows no significant changes in the presence of ouabain.

Discussion

In this study the uptake and metabolism of T_3 and rT_3 in human liver derived HepG2 cells was investigated. We show, by measuring nuclear bound hormone, that T_3 enters the

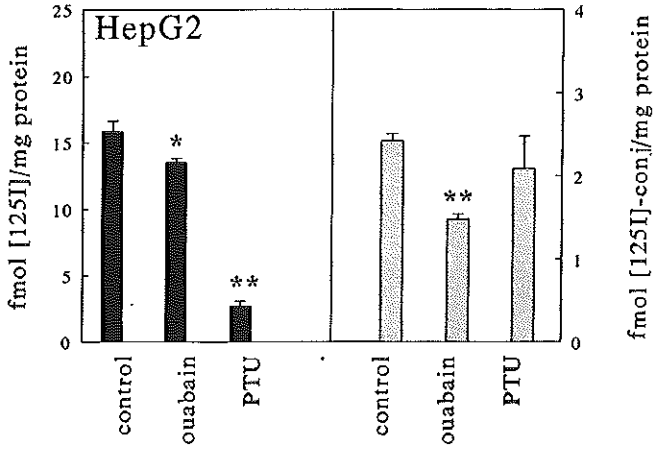

Figure 3 *Metabolism of [^{125}I]-rT_3 (total rT_3 concentration 60 pmol/l in culture medium containing 0.5% BSA) by HepG2 cells during a 17 hour incubation at 37 °C. Iodide production (left axis) decreases in the presence of 0.5 mmol/l ouabain ($p<0.01$) and 100 µmol/l PTU ($p<0.001$). Conjugates production (right axis) decreases in the presence of 0.5 mmol/l ouabain ($p<0.001$), but is not significantly different from control incubations in the presence of PTU.*
*** : $p<0.001$, * : $p<0.01$*

cellular compartment. There is a time dependent increase in total cellular binding of T_3. Initial uptake is linear up to 30 minutes and falls off subsequently. Suppression of linear initial uptake by the high unlabelled T_3 concentration represents saturation of the plasma membrane transport process. As ouabain does not interfere with intracellular binding of thyroid hormone (30), its suppressive effect on T_3 uptake indicates sodium dependency of the plasma membrane transport process. In the same experiments, nuclear bound radioactivity was determined. There is a clear time dependent increase in the percentage of hormone bound to nuclear sites, suggesting movement of T_3 from extra-nuclear sites (plasma membrane and cytoplasma) to the nucleus. The effects of the low concentration of unlabelled hormone (10 nmol/l T_3) and ouabain are much more pronounced at the nuclear level, suggesting partial non-specific and ouabain insensitive binding in the total cellular binding data. This is explained by the fact that there is a substantial amount of T_3 aspecifically bound to the plasma membrane, which does not enter the cells (5). The observation that nuclear bound T_3 is decreased by almost 75% in the presence of ouabain suggests that at least 75% of nuclear T_3 must have entered the cell by ouabain sensitive transport systems, as this concentration of ouabain (0.5 mmol/l) has no influence on cell viability, as monitored by ATP and DNA measurements (data not shown). Our data show that although plasma membrane transport inhibitors have low to moderate effects on total cellular binding, the effects on the actual transport process and thus availability of intracellular thyroid hormone may be substantial.

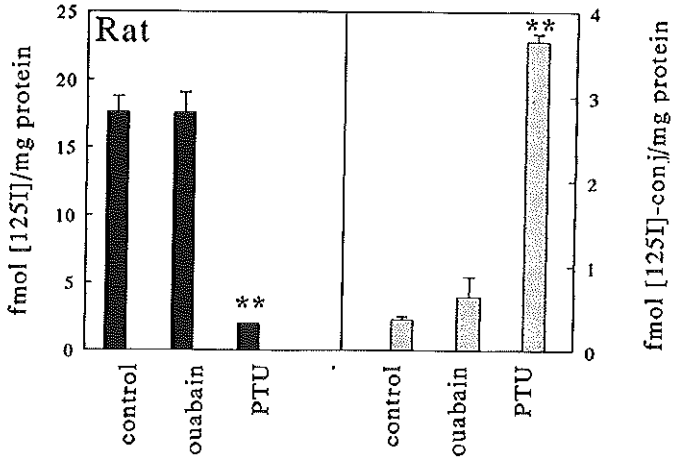

Figure 4 *Metabolism of [^{125}I]-rT_3 (total rT_3 concentration 60 pmol/l in culture medium containing 0.5% BSA) by primary cultures of rat hepatocytes during a 90 minutes incubation at 37 °C. Iodide production (left axis) decreases in the presence of 100 µmol/l PTU ($p<0.001$), but shows no significant changes in the presence of 0.5 mmol/l ouabain. Conjugates production (right axis) shows no significant changes in the presence of 0.5 mmol/l ouabain, and an almost 10-fold increase in the presence of PTU ($p<0.001$).*
*** : $p<0.001$*

At the nuclear level this has its consequences for occupancy of hormone receptors and consequently for initiation of biological effects.

As there is only very little nuclear binding of rT_3, which is also reflected by its very low total cellular binding (data not shown), we have used the intracellular deiodination and conjugation of this hormone to study cellular entry in human liver derived HepG2 cells. Analogous to previous studies on T_3 metabolism in HepG2 cells (27), we have performed parallel incubations with primary cultures of rat hepatocytes for comparison.

Taking the different incubation times into consideration, there is an approximately 10 fold higher rate of deiodination and conjugation in rat hepatocytes versus HepG2 cells. This is not only observed for rT_3, but also for T_4 metabolism. For this reason we were not able to study T_4 metabolism, which would require too long incubation times such that cell growth would be compromised.

In this study we present data of 90 minutes rat hepatocytes incubations, compared to 17 hours human HepG2 incubations. There is substantial rT_3 deiodination in HepG2 cells, comparable to rat hepatocytes, in contrast to the previously reported absence of T_3 deiodination (27). This confirms the presence of functional type I deiodinase activity in HepG2 cells, and is in concert with our previous conclusion that the lack of T_3 deiodination is due to deficient T_3 sulfation. There is a small but significant decrease of iodide production

in the presence of ouabain ($p<0.01$) in HepG2 cells, which is not found in rat hepatocytes. This could be explained by the fact that in the present studies rat hepatocytes were cultured in HepG2 medium. This medium differs slightly from the normal rat hepatocyte medium. Under optimal culture conditions we always find ouabain inhibition of thyroid hormone transport in rat hepatocytes (5-8). In the presence of PTU, however, iodide production by both cell types is reduced dramatically by inactivation of type I deiodinase.

Comparison of conjugates production in HepG2 cells and rat hepatocytes shows a relatively high conjugates production in HepG2 cells in control incubations (2.43 ± 0.08 vs 0.37 ± 0.03 fmol/mg protein, HepG2 vs rat). Conjugates of HepG2 cells contained only glucuronides, whereas conjugates produced by rat hepatocytes contained glucuronides and little sulfates. There is a decrease of conjugates production in HepG2 cells in the presence of ouabain, explained by decreased cellular entry of the hormone, which is not found in rat hepatocyte incubations due to suboptimal incubation conditions (see above). The increase in rT_3 conjugates by PTU in rat hepatocytes is explained by the decreased deiodination of rT_3 by type I deiodinase and consequently increased availability of rT_3 for conjugation. There is no increase in conjugates production by HepG2 cells in the presence of PTU. One theoretical explanation for this phenomenon could be that in control incubations the glucuronyl-transferases in HepG2 cells are already saturated using this rT_3 concentration. This is supported by the observation that in HepG2 control incubations there is a relatively high glucuronidation of rT_3 as compared to rat hepatocytes. A more likely explanation is a shift from rT_2 glucuronides in control incubations to rT_3 glucuronides in PTU incubations. Both compounds accumulate in the same fractions following LH-20 chromatography.

In conclusion, we have shown that there is a time-dependent ouabain-sensitive increase in nuclear binding of T_3 in human liver derived HepG2 cells. These cells do not deiodinate T_3, due to deficient sulfation (27), but do deiodinate rT_3, which enters the cells through an ouabain-sensitive transport system. There is substantial glucuronidation, but no detectable sulfation of rT_3, and no increase in glucuronidation in the presence of PTU.

Combining all data that we have obtained in our laboratory using the human liver derived cell line HepG2 and our previous data concerning the uptake and metabolism of iodothyronines in primary cultures of human hepatocytes (21), we propose that HepG2 cells are a suitable model for the human liver. Both human hepatocytes as well as HepG2 cells show an approximately 10-fold lower rate of metabolism as rat hepatocytes. In addition, both human hepatocytes and HepG2 cells show, in contrast to rat hepatocytes, no iodide production from T_3 and no increase in T_3 conjugates in the presence of PTU. In HepG2 cells we have shown that this is caused by deficient sulfation of T_3 (27), despite sulfation of other compounds in these cells (26). Based on these data we propose that in the human liver deiodination of T_3 is only of minor importance, due to deficient sulfation of this hormone. These observations caution against uncritical use of the rat liver as a model for hepatic thyroid hormone metabolism in man.

Acknowledgements

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Chapter 2.3

Optimisation of calcium phosphate transfection of HepG2 cells: a method to obtain permissive cells for the eukaryotic expression system pDR2

Optimisation of calcium phosphate transfection of HepG2 cells: a method to obtain permissive cells for the eukaryotic expression system pDR2

Introduction

The recently developed expression vector pDR2 is replicated in any cell type that expresses the Epstein Barr Virus Nuclear Antigen-1 (EBNA-1). This trans-acting factor is involved in the intracellular replication of the pDR2-vector based on the Epstein Barr virus origin of replication which is present in this vector. Therefore, any cell expressing EBNA-1 which is transfected with the pDR2 vector will subsequently start replicating this vector without the need for genomic integration [1, 2]. This phenomenon is called episomal replication, which not only increases the level of gene expression by the increased copy number, but also allows rescue of plasmids from positive clones (Fig. 1). Cells expressing EBNA-1 are called permissive cells, and the establishment of these cells is a first requirement before using this system for expression cloning purposes.

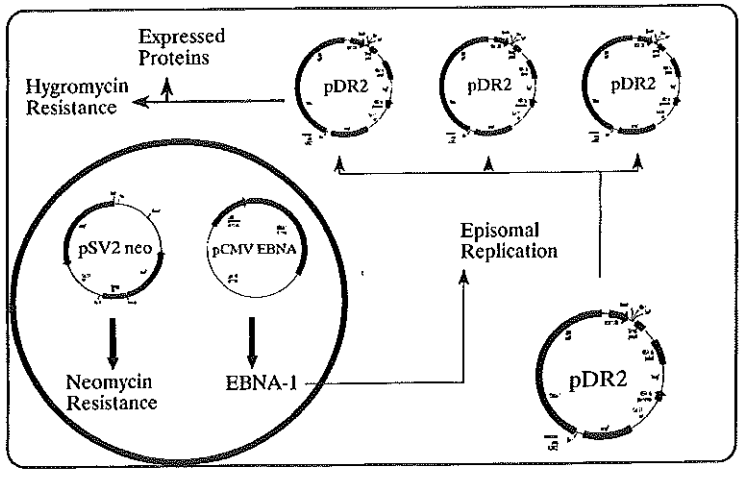

Figure 1 *Schematic overview of a permissive cell. Chromosomal integration of pSV2 neo and pCMV EBNA will result in neomycin resistant cells that express Epstein Barr virus Nuclear antigen (EBNA-1). This protein allows episomal replication (outside the nucleus) of the eukaryotic expression vector pDR2, which not only increases the copy number of this vector, but also allows rescue of plasmids from positive clones. Cells expressing the pDR2 vector can be selected using hygromycin.*

In order to select HepG2 cells which stably express EBNA-1, cotransfection with a vector containing an antibiotic resistance gene has to be performed. In this system a cotransfection of pCMV EBNA and pSV2 neo is used. The expression of EBNA-1 is controlled by the cytomegalovirus (CMV) promoter which is present on the pCMV EBNA vector, whereas the neomycin resistance is obtained from the cotransfected pSV2 neo (Fig. 1). Both plasmids are linearised before transfection, in order to increase chromosomal integration. Plasmids are transfected into the cells using the calcium phosphate precipitation technique, as DNA fragments transfected with this method are integrated into the genome in so-called tandem-repeats [3]. This not only increases transfection efficiency, but also increases the level of expression of integrated DNA fragments.

In this chapter the optimisation of calcium phosphate precipitation in HepG2 cells is described. Growth hormone expressed from the vector pTKGH is used to monitor transfection efficiency. This vector codes for human growth hormone, directed by the constitutive thymidine kinase promoter. Also, optimal neomycin (stable pCMV EBNA transfections, [4]) and hygromycin B (episomal pDR2 transfections, [5]) concentrations are determined for selection of transfected HepG2 cells.

Materials and methods

Materials

All tissue culture plastics were obtained from GIBCO/BRL (European division, Breda, The Netherlands). Plasmids for the pDR2 expression system were obtained from Clontech (Westburg bv, Leusden, The Netherlands). The human growth hormone plasmid pTKGH was obtained from Allegro (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA). The neomycin analogue G418 (geneticin) was obtained from GIBCO/BRL (European division, Breda, The Netherlands). All other chemicals were obtained from Sigma Chemical Co (St, Louis, MO, USA). All plastics and media used are either provided sterile or autoclaved before use. Alternatively, 0.22 µm filters were used for sterilisation. Restriction enzymes were obtained from Boehringer (Mannheim, Germany).

Media and plates for molecular biology

LB medium (10 g/l bactotryptone, 5 g/l bactoyeast, and 8 g/l NaCl in demineralised water) was used for cultures of E. coli, and for preparation of LB plates. SOC medium (2% bactotryptone, 0.5% bactoyeast, 10 mmol/l NaCl, 2.5 mmol/l KCl, 10 mmol/l $MgCl_2$, 10 mmol/l $MgSO_4$, and 20 mmol/l glucose, added as a sterile solution after autoclaving) was used for electroporation of E. coli. TE-buffer (10 mmol/l tris, 1 mmol/l EDTA, pH 8.0) was used for DNA and RNA preparations.

Cell culture

HepG2 cells were obtained from Dr. B. Knowles, the Wistar Institute, Philadelphia, USA. Cells were grown in a 5 % CO_2, 37 ^{0}C, humidified incubator in Dulbecco's Modified Eagle Medium/Nutrient Mixture Ham's F12 (1:1 vol/vol) supplemented with 10 % fetal calf serum (FCS), penicillin (100 IU/ml), streptomycin (100 µg/ml), and glutamine (2.4 mmol/l). Cells were subcultured at confluence using trypsinization (0.05 % trypsin, 0.002 % collagenase, 0.02 % EDTA). Cells were stored in liquid nitrogen using cell culture freezing medium containing DMSO (GIBCO, Breda, The Netherlands).

Transformations and electroporations

Heat shock transformation was performed by growing *E.coli* PC2495 cells on a LB-Mg^{2+} plate (10 mmol/l $MgCl_2$) overnight. Single colonies were transferred to an eppendorf tube containing ice-cold $CaCl_2$ (100 mmol/l, 5 colonies per 200 µl), and placed on ice for 20 minutes. Varying amounts of ligation mixture were added, and tubes were kept on ice for an additional 20 minutes. Following a heat shock of 2 minutes at 42 °C, addition of 800 µl LB medium, and incubation at 37 °C for 45 minutes, the contents was plated onto appropriate plates. Plates were incubated overnight at 37 °C, and colonies of interest were amplified in minipreps.

Electroporation of *E.coli* DH5α was performed by thawing 100 µl of electrocompetent cells (prepared by sequential glycerol washing, according to the manufacturers protocol, Biorad, Breda, The Netherlands) and adding 1 to 2 µl of ligation mixture. After incubation on ice for 1 minute, cells were transferred to an electroporation cuvette (0.1 cm, Biorad, Breda, The Netherlands), and electroporated with 1 pulse of 1.8 kV. Immediately, 1 ml SOC medium was added, and cells were transferred to an eppendorf tube. After incubation for 45 minutes at 37 °C, cells were plated onto appropriate plates. Plates were incubated overnight at 37 °C, and colonies of interest were amplified in minipreps.

Miniprep isolation of plasmid DNA

For small scale preparation of plasmid DNA, minipreps were used. Briefly, transformed bacteria were grown overnight at 37 °C in 2 ml LB/Amp. Cells were harvested by spinning down 1 ml of the bacterial culture in an eppendorf tube (8000 rpm, 1 min). After resuspending the pellet in 200 µl GET buffer (50 mmol/l glucose, 10 mmol/l EDTA, 25 mmol/l tris, pH 8.0), cells were lysed by addition of 400 µl freshly prepared alkaline solution (0.2 mol/l NaOH, 1% SDS) and incubation on ice for 5 min. Subsequently, proteins, high molecular weight RNA and chromosomal DNA were precipitated by addition of 300 µl 7.5 mol/l ammonium acetate and incubation on ice for 10 min. Tubes were spinned for 3 min at 10,000 rpm, and supernatants were precipitated using 0.6 volumes of isopropanol. After incubation at room temperature for 10 min, plasmid DNA was spinned down for 10 min (rmax). The pellet was washed once with 70% ethanol, and dissolved in TE buffer. The remaining 1 ml of bacterial culture was used for glycerol stocks, by adding 1 volume of sterile glycerol and storage at -80 °C.

Plasmids amplifications and preparation

Circularised plasmids were amplified using the Quiagen Maxiprep system (Quiagen, Westburg bv, Leusden, The Netherlands). Appropriate strains of *E.coli* were transformed with the plasmid of interest using electroporation, plated onto LB-Ampicillin (Amp, 50 µg/ml), grown in minipreps (2 ml LB-Amp per vial), analysed by restriction enzyme digestion, and subsequently grown overnight in 500 ml of LB-Amp. Plasmid DNA was isolated according to the manufacturers instructions, and stored at -80C after determination of the DNA concentration by measuring the OD260. Glycerol stocks of bacterial cultures were prepared as described above.

As the pCMV EBNA plasmid was provided as a HindIII-linearised fragment, it was ligated in order to allow amplification in *E.coli*. According to the map provided by the manufacturer CMV EBNA was cloned into the multiple cloning site (MCS) of pUC19, which contains a functional lacZ gene. Linearised pCMV EBNA was ligated by adding 1 µl 10x ligation buffer (Boehringer, Mannheim, Germany), 0.5 µl 10 mmol/l ATP, 1 µl T_4 ligase (1 u/µl, GIBCO/BRL, Breda, The Netherlands), 4 µg of HindIII digested pCMV EBNA and demineralised water to an end-volume of 10 µl. This mixture was incubated overnight at 15 °C, and transformed into competent *E.coli* PC2495 cells by heat shock. Cells were plated

onto LB/Amp IPTG XGal plates, and all white colonies were analysed by restriction enzyme digestion.

For stable transfection experiments, pCMV EBNA and pSV2neo were linearised by digestion with HindIII and EcoRI respectively. Restriction enzyme digestions were performed using the standard enzyme and buffer systems of Boehringer (Mannheim, Germany) followed by phenol / chloroform / iso-amylalcohol (IAA) extraction and ethanol precipitation. Briefly, the digestion mixture was made up to 100 µl with TE buffer and washed twice with 100 µl phenol / chloroform / IAA (Life Technologies, Breda, The Netherlands) and once with 100 µl chloroform. Plasmid DNA was precipitated by addition of 0.1 volume of 3 mol/l NaAcetate and 2 volumes of ethanol absolute, with subsequent incubation at -80C for 2 hr or longer. DNA was pelleted by spinning in an eppendorf centrifuge for 30 minutes at 4C (rmax). The DNA pellet was washed with 70% ethanol (-20C), and dissolved in the appropriate buffer or water. Transfections were performed using 20 µg of linearised pCMV EBNA and 1 µg of linearised pSV2neo. DNA concentrations were made up to 25 µg per 500 µl precipitate using the aspecific vector pCDM8 (Invitrogen, ITK Diagnostics bv, Uithoorn, The Netherlands).

Calcium phosphate precipitation

Calcium phosphate precipitation was performed according to Sambrook etal [3]. The desired amounts of DNA were ethanol-precipitated as described above, and dissolved in 220 µl 0.1 x TE buffer. Calcium phosphate-DNA crystals were formed by adding 250 µl 2 x Hepes buffered saline (2 x HBS, 280 mmol/l NaCl, 10 mmol/l KCl, 1.5 mmol/l $Na_2HPO_4.2H_2O$, 12 mmol/l D(+)-glucose and 50 mmol/l HEPES, pH 7.05) and subsequent slow addition of 31 µl 2 mol/l $CaCl_2$. After incubation at room temperature for 20 minutes, the precipitate was resuspended carefully and transferred to tissue culture flasks. HepG2 cells were grown to 50% confluence, and medium was changed 1 hour before addition of the calcium phosphate precipitate. Routinely, 500 µl of crystals was added to 5 ml medium per 25 cm^2 flask. In some experiments, the effect of the amount of crystals per flask was studied. In these experiments varying volumes were used.

After overnight incubation, cells were submitted to a glycerol shock. Medium of the cells was removed, and 0.5 volume of 15% glycerol in HBSP (0.75 mmol/l Na_2HPO_4, 5 mmol/l KCl, 140 mmol/l NaCl, 6 mmol/l glucose, 25 mmol/l HEPES, pH 7.0) was added. After 1 minute, 0.5 volume of phosphate buffered saline (PBS, 137 mmol/l NaCl, 2.7 mmol/l KCl, 8.1 mmol/l Na_2HPO_4, 1.5 mmol/l KH_2PO_4, pH 7.4) was added. Medium was aspirated, and cells were washed three times with PBS, followed by addition of fresh culture medium. Transfection was monitored by sequential sampling of tissue culture supernatants for hGH determination (pTKGH transfections), or by addition of selection pressure (pSV2 neo/ pCMV EBNA transfections) after different periods of time.

Neomycin and hygromycin concentration curves

In order to determine the appropriate concentrations of neomycin or hygromycin B, HepG2 cells were grown in 24 wells tissue culture plates, and incubated in culture medium containing varying concentrations of geneticin (G418, a neomycin analogue) or hygromycin B. All concentrations were tested in duplicate on two different plates. G418 and hygromycin B were added to individual wells from stocks of 100 µg/µl and 455 units/µl respectively. Medium was changed every two days for one week. After one week, each well was washed with 2 times 1 ml of PBS and remaining cells were lysed in 400 µl 0.1 mol/l NaOH. Protein concentrations were determined in duplicate using the microassay protocol, as described below.

Microassay for protein concentration determination

In order to screen many samples simultaneously, protein determination in 96 wells microtiter-plates was used. Protein samples (cells lysed in 0.1 N NaOH) were diluted to appropriate concentrations in 0.1 mol/l NaOH. Protein concentration was determined according to a commercially available kit based on the method of Bradford (Biorad, The Netherlands). Equilibration curves, always in triplicate, and samples were measured by pipetting 5 µl sample into each well of the 96 wells microtiter-plate. Subsequently, 200 µl of protein determination reagent (1:5 diluted in water, 0.22 µm filtered) was added. Plates were vortexed for 10 minutes, and the OD595 was determined using a Biorad microtiter-plate reader. Calibration curves were calculated using non-linear regression, and protein concentrations of samples were calculated according to the obtained parameters.

Human growth hormone assay

Concentrations of human growth hormone (hGH) were determined using a commercially available kit (Eurodiagnostica bv, Apeldoorn, The Netherlands).

Results and discussion

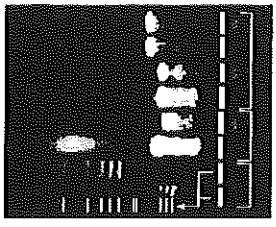

Figure 2 *UV illuminated 0.8% agarose gel of pSV2 neo and pCMV EBNA. Lanes 1 and 2 : markers, lane 3 and 4 : pSV2 neo, non-digested and EcoRI, lanes 5 through 8 : pCMV EBNA, non-digested, EcoRI, HindIII, and EcoRI / HindIII double digested. Molecular weights markers summarised (arrow) : 9.4, 6.6, 4.4, 2.3, 2.0, 1.35, 1.08, 0.87, 0.60, 0.31 kb.*

Ligation and amplification pCMV EBNA

Following ligation and transformation of HindIII linearised pCMV EBNA (provided as such by the manufacturer) 4 individual white colonies were picked for amplification in minipreps. Isolated plasmid DNA was digested using EcoRI, HindIII, or both, and analysed on agarose gel electrophoresis (0.8% agarose, 100 V constant voltage). Figure 2 shows the UV illuminated gel of these digestions. The non-digested pCMV EBNA shows several supercoiled configurations, whereas the EcoRI digested plasmid shows a linear fragment in the 5.5 kb range. Both the HindIII and the HindIII/EcoRI digested plasmids show one linearised fragment in the 2.7 kb range, which most likely represents two different fragments of similar size, and no band in the 5.5 kb range. These data confirm the limited restriction map provided by the manufacturer, as pCMV EBNA consists of the 2.7 kb pUC19 vector, with a 2.75 kb CMV EBNA insert in the HindIII position of the multiple cloning site.

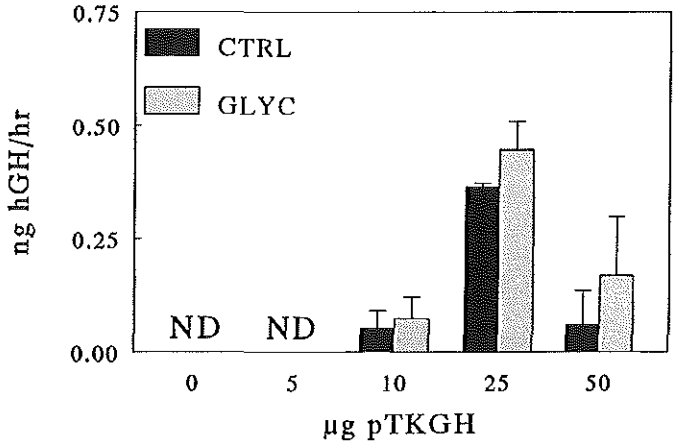

Figure 3 *Effects of varying DNA concentrations and a glycerol shock on transfection efficiency. Calcium phosphate-DNA precipitates were prepared using varying amounts of human growth hormone (hGH) coding vector pTKGH and transfected into human liver derived HepG2 cells. After overnight incubation at 37 °C, cells were subjected to a glycerol shock. Cells were incubated in normal HepG2 medium, and supernatants were analysed for growth hormone production. The highest hGH production was found using 25 µg pTKGH per 500 µl precipitate. In all incubations a glycerol shock increases transfection efficiency. ND = not detectable.*

Digestion with EcoRI linearises the vector, whereas both the HindIII and the double digestion separate the vector and the CMV EBNA insert.

Optimisation of CaPO4 precipitation

Figure 3 shows the effect of varying DNA concentrations for the preparation of precipitates and the effect of a glycerol shock. When transfection efficiency is expressed as pg hGH produced per hour per µg pTKGH transfected, it becomes obvious that transfection using 25 µg of DNA is the most efficient protocol. The CaPO4/DNA precipitate formed with the highest DNA concentration is less efficient in transfecting HepG2 cells. It can also be seen that in all transfections a glycerol shock increases the amount of hGH produced per µg pTKGH.

The effects of varying amounts of CaPO4 precipitate on the transfection efficiency, expressed as ng hGH produced per µg pTKGH, are shown in Figure 4. One large preparation of pTKGH/CaPO4 precipitate was made, and different volumes were added to HepG2 cell cultures.

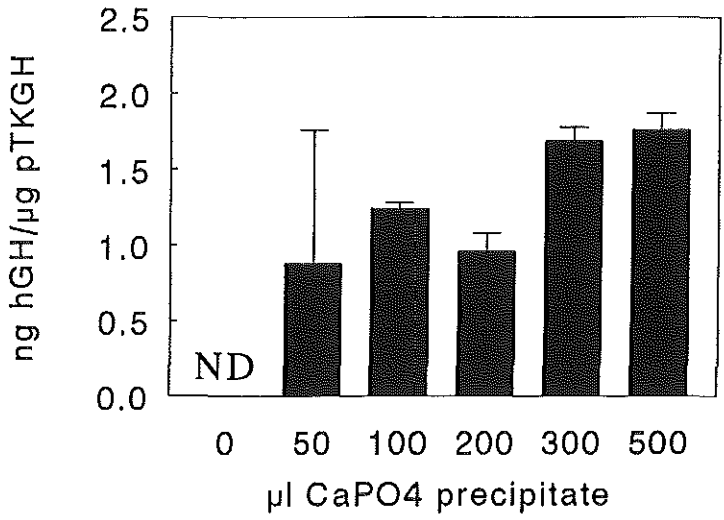

Figure 4 *Effects of varying amounts of calcium phosphate-DNA precipitate on transfection efficiency. Calcium phosphate-DNA precipitates were prepared using 25 µg pTKGH per 500 µl precipitate. Different volumes were transfected into human liver derived HepG2 cells. Cells were incubated overnight, subjected to a glycerol shock, and analysed for hGH production by sampling cell culture supernatants. Transfection efficiency is calculated as ng hGH per µg pTKGH transfected. All volumes seem comparable with respect to transfection efficiency. Only the 50 µl volume shows a high variation, probably due to the small pipetting volume. ND = not detectable.*

Although there are no extreme differences in transfection efficiency (varying from 0.96 ±0.12 to 1.76 ± 0.11 ng hGH per µg pTKGH), the 50 µl transfection showed the largest variations. As both the 100 and 200 µl transfections showed lower efficiencies than the higher volumes, a 500 µl volume was used for all future transfections. This volume combines a relatively high transfection efficiency with easy to use pipetting volumes.

Figure 5 shows a time curve of hGH production in the supernatant of HepG2 cultures. Cells were transfected with 500 µl of CaPO4 precipitate, containing indicated amounts of pTKGH adjusted to an end-concentration of 25 µg DNA per 500 µl using the aspecific vector pCDM8. Control incubations contained 25 µg pCDM8 vector. Samples were taken from the tissue culture medium and analysed for hGH. There is no hGH production in control incubations, and a time dependent increase in the accumulation of hGH in the medium of pTKGH transfected cells. This increase in hGH concentration is almost linear, suggesting a continuous production of hGH from the pTKGH vector. There are hardly any differences in hGH production between the 5 µg and 25 µg pTKGH transfections, which suggests that 5 µg of pTKGH already is enough to cause maximal hGH production in HepG2 cells. This indicates a relatively efficient transfection using the described protocol.

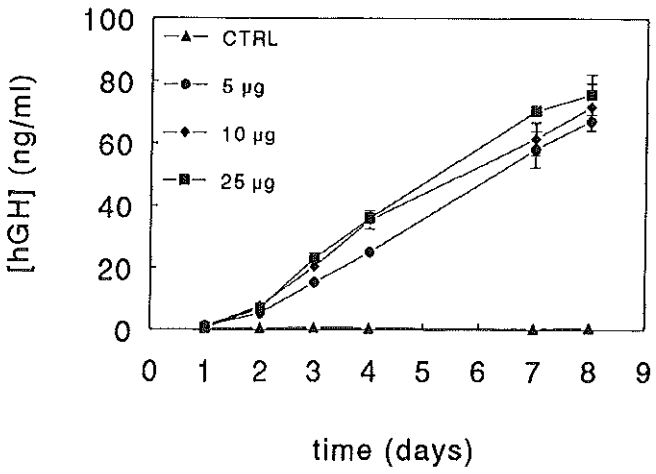

Figure 5 *Time curve of growth hormone production. Calcium phosphate-DNA precipitates were prepared using varying amounts of pTKGH. DNA concentrations were adjusted to 25 µg DNA per 500 µl precipitate using the aspecific vector pCDM8. HepG2 cells were transfected, incubated overnight, subjected to a glycerol shock, and analysed for hGH production by sequential sampling of cell culture supernatants. Control incubations were performed using 25 µg pCDM8 per 500 µl precipitate. In all pTKGH transfected cells a time dependent increase in hGH is found. The small difference between 5 and 25 µg pTKGH transfections suggest an already maximal hGH production using the 5 µg pTKGH.*

In summary, the optimal transfection conditions for CaPO4 precipitation of human liver derived HepG2 cells are transfection of 25 µg of DNA per 500 µl CaPO4 precipitate per 25 cm^2 tissue culture flask, incubation overnight, followed by a glycerol shock for 1 min. Shorter incubation times and longer glycerol shocks were also tested, but none improved the current protocol (data not shown).

Neomycin and hygromycin concentration curves

HepG2 cells have an extremely high tolerance for G418, as shown in Figure 6. To allow selection within one week, a concentration of 2500 to 3000 µg per ml should be used. Previous reports using other cell types indicate concentrations of 200 to 400 µg per ml [2]. This high tolerance of HepG2 cells for G418 could be explained by the fact that they are liver derived cells, and therefore able to intoxicate compounds.

Based on these data, three different concentrations of G418, i.e. 750 µg/ml, 1000 µg/ml and 1500 µg/ml were used for prolonged periods of culture. All three concentrations were able to prevent growth of non-transfected cells, as shown in control transfection experiments. These concentration do not need pH correction of the culture medium.

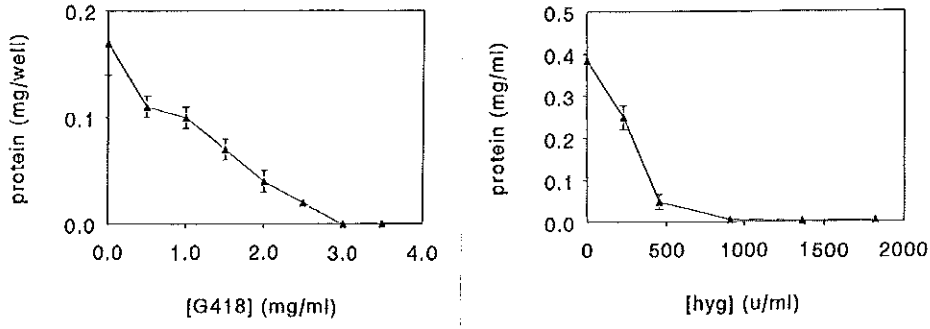

Figure 6 *Neomycin and hygromycin tolerance curves for HepG2 cells. Human liver derived HepG2 cells were grown in 24 wells plates and incubated with different concentration of the neomycin analogue G418 or hygromycin. Medium was changed every two days. After 1 week, cells were washed with phosphate buffered saline, and lysed in NaOH. Cell lysates were used for protein determination. HepG2 cells can tolerate very high G418 concentrations for prolonged periods of time, whereas a hygromycin concentration of 455 units per ml is sufficient to kill most cells within 1 week.*

Hygromycin B can be used at concentrations of 455 u/ml or higher, as also shown in Figure 6. At this concentration no pH adjustments are needed, and non-transfected cells die within one to two weeks.

Stable pCMV EBNA / pSV2 neo transfections

Table I shows the number of colonies obtained with the different selection conditions. Cells were transfected in 25 cm^2 flasks, submitted to a glycerol shock (day 1), and G418 was added on day 4 or day 7. For control incubations (pCDM8 transfected HepG2 cells) selection pressure was applied after 4 days. On day 7, cells were transferred to 75 cm^2 flasks. After 4 weeks, none of the control incubations contained any cells. Groups of cells in pCMV EBNA transfected cells were scored as colonies, and transferred individually to 24 wells plates. Individual clones were grown to confluence in 750 µg/ml G418, and transferred to 75 cm^2 flasks. Only the clones which were growing to a reasonable extend were stored in liquid nitrogen (clones A to M, 12 clones).

[G418] µg/ml	# of colonies control	4 days	7 days
750	0	21	108
1000	0	23	16
1500	0	3	25

Table I : *Number of colonies obtained after transfection with pCMV EBNA and pSV2 neo. Transfected cells were submitted to different concentrations of the neomycin analogue G418 after 4 or 7 days in order to select neomycin resistant clones. Control transfections were performed with the aspecific vector pCDM8, and G418 was added on day 4.*

Conclusions

Using the human growth hormone coding plasmid pTKGH we have determined the optimal calcium phosphate precipitation conditions for HepG2 cells. The optimal DNA concentration for preparation of crystals is 25 µg per 500 µl endvolume. Cells should be incubated with the calcium phosphate-DNA precipitate for at least 12 hours, and should be treated for 1 minute with a glycerol shock. Neomycin concentrations for selection pressure should be very high, ranging from 750 to even 3000 µg G418 per ml.

Due to more promising results using the *Xenopus laevis* strategy (Chapter 5), the stored neomycin resistant clones were not yet tested for episomal replication of pDR2. Whenever the *Xenopus* strategy leads to the identification of a single clone, this particular clone can be tested using the obtained neomycin resistant, and therefore potentially pDR2 permissive HepG2 cells.

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Chapter 3

Rapid isolation of plasma membranes using poly-cationic beads : preliminary data on thyroid hormone affinity labelling of human liver derived HepG2 plasma membranes

Clin. Chim. Acta., submitted

Rapid isolation of plasma membranes using polycationic beads : preliminary data on thyroid hormone affinity labelling of human liver derived HepG2 plasma membranes

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Keywords plasma membrane, polycationic beads, Affigel 731, HepG2, thyroid hormone, liver

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1. Introduction

Transport of thyroid hormones across the plasma membrane is an essential first step in intracellular events such as the deiodination of thyroxine (3,3',5,5'-tetraiodo-L-thyronine, T_4) to bioactive 3,3',5 triiodo-L-thyronine (T_3), the binding of T_3 to its nuclear receptors, and the metabolic clearance of all circulating thyroid hormones. Several independent studies in different tissues have shown that this transmembrane transport of thyroid hormones is a carrier-mediated process [1].

In the present study we show that plasma membranes from the human liver derived cell-line HepG2 can be isolated rapidly using poly-cationic beads. This method is based on chemical interaction between the plasma membrane and poly-cationic residues on the beads, and has been used for several different cell types [2-7]. We suggest that this method is a very rapid and selective alternative for the commonly used centrifugation techniques for plasma membrane isolation. Especially the absence of endoplasmic reticulum contamination is useful for the study of hepatic plasma membrane proteins.

2. Materials and methods

2.1 Plasma membrane isolation

Cell isolation

HepG2 cells were grown to confluency (10-14 75 cm^2) in DMEM/Ham's F12 (1:1 vol/vol) supplemented with 10 % FCS, penicillin (100 IU/ml), streptomycin (100 µg/ml), and glutamine (2.4 mmol/l). Cells were washed with 5 ml ice cold attachment buffer (310 mmol/l sucrose, 20 mmol/l MES buffer, 7:3 vol/vol, adjusted to pH 5.2 with 1 mol/l tris) and scraped from the flasks in 5 ml ice cold attachment buffer using a rubber policeman. Cells were collected by centrifugation in a sterile 50 ml tube (300 x g, 7 min, 4 C), washed three times using 30 ml of attachment buffer, and resuspended thoroughly using a siliconised Pasteurs pipette (Sigmacote, Sigma St Louis, MO, USA). All following procedures were performed on ice.

Attachment of cells to beads

Polyethyleneimine-coated beads (Affigel 731, Biorad, The Netherlands) were allowed to swell overnight and washed three times with attachment buffer. Washed HepG2 cells were made up to a 20 % (v/v) suspension in attachment buffer in a siliconised 12 ml glass tube. Washed beads were made up to a 50 % (v/v) suspension in attachment buffer. Beads were added dropwise to the cell suspension, and incubated for 20 minutes upto overnight at 4 ^{0}C under continuous rotation. Subsequently, unoccupied spaces on the beads were blocked by adding fresh attachment buffer containing 40 U/ml heparin (Leo Pharmaceutical Products bv, Weesp, The Netherlands) for 20 min. The beads were washed twice with attachment buffer to remove unattached cells. Beads were made up to a 50% (v/v) suspension in 10 mmol/l tris pH 7.4.

Membrane isolation

The beads were sonicated on ice for 2 x 10 sec, 10 µm, transferred to a sterile 50 ml tube, and washed using 40 ml ice cold 10 mmol/l tris pH 7.4 until no cellular debris is left.

The final plasma membrane coated beads were resuspended in tris (50 mmol/l, pH 7.5) and stored at -20 °C or used immediately for solubilisation.

Solubilisation of plasma membrane proteins

Plasma membrane coated beads were resuspended in 10 ml detachment buffer (50 mmol/l tris, 1 mmol/l CHAPS, pH 7.5) and incubated on ice for 1 hour with occasional swirling. The beads were vortexed for 2 min, and spinned down (900 x g, 5 min, 4 C). The supernatant was removed, beads were washed once more, and both supernatants were concentrated using centriprep-10 (Amicon Division, Beverly, MA, USA). Concentrated plasma membrane protein solutions were stored at -20 °C or -80 °C.

2.2 Bromo-acetyl affinity labelling and SDS-PAGE of plasma membranes

N-Bromoacetyl-3'-[^{125}I]-T_3 ($BrAcT_3$) and N-Bromoacetyl-3'-5'-[^{125}I]-T_4 ($BrAcT_4$) were synthesised as described previously [8]. For affinity labelling, 500,000 cpm of $BrAcT_3$ or $BrAcT_4$ per incubation was dried down under nitrogen in an eppendorf tube. To start incubation, 100 µl of protein solution was added and tubes were incubated for 10 min at 37 °C. To stop affinity labelling, 50 µl of 3 x SDS-PAGE sample buffer was added, and tubes were incubated at 80 °C for 10 min. Sodium dodecyl sulphate polyacrylamide gelelectrophoresis (SDS-PAGE) was performed according to Laemmli [9]. After staining and destaining, gels were dried and autoradiographed for different periods of time at -80C.

2.3 Marker enzyme studies

Both marker enzyme determinations were modifications of previously reported methods [10,11], adjusted to eppendorf volumes. Glucose-6-phosphatase (G6P) was used as an endoplasmic reticulum marker, and 5'-Nucleotidase (5'N) as a plasma membrane marker. Both assays were monitored by inorganic phosphate release, as described below. As a second marker for endoplasmic reticulum contamination, we used bromo-acetyl affinity labelling of type I deiodinase, as described above.

Preparation of cell homogenates

HepG2 cell homogenates, for marker enzyme control incubations, were prepared by sonication of cells in tris buffered saline (20 mmol/l tris, 0.5 mol/l NaCl, pH 7.5).

Inorganic phosphate determination

Inorganic phosphate reagent was prepared by adding 3.125 ml 36.6 N H_2SO_4 to 96.9 ml of demineralised water, then addition of 1 g ammonium molybdate, and subsequently 8 g Fe(II)SO_4. Any other order will result in precipitates. The reagent was filtered through a 0.45 µm filter, and stored in the dark at 4 °C. To determine the concentration of inorganic phosphate in a sample, 1250 µl of reagent was mixed with 50 µl of sample. After 30 min at room temperature, the OD660 was measured.

3. Results and discussion

3.1 Plasma membrane isolation

Figure 1 shows an example of HepG2 cells attached to Affigel 731 beads after blocking with heparin. There are only few unattached cells remaining, which will be completely removed after sonication and subsequent washing of the beads.

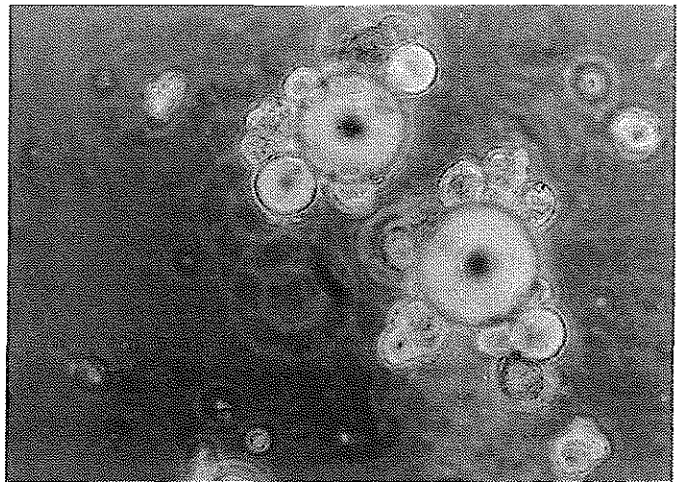

Figure 1 *Attachment of HepG2 cells to Affigel 731 beads prior to lysis of the cells. Unoccupied spaces on the beads are blocked before endoplasmic reticulum is released from the cells.*

It should be noted that only a small proportion of the plasma membrane is actually attached to the bead-surface. Because only this part of the plasma membrane will be isolated, the specificity will be very high, but the recovery very low.

Table I shows the specific activities of G6P and 5'N in HepG2 cell homogenates and four different plasma membrane isolations. Although there is considerable variation among the different isolations, there is a reproducible decrease in endoplasmic reticulum marker (G6P), and increase in plasma membrane marker (5'N). In order to determine the specific activity of 5'N in HepG2 cell homogenates, a relatively high protein concentration had to be used. For the plasma membrane fractions, much lower protein concentrations already showed comparable phosphate production in the 5'N assays.

		glucose-6-phosphatase		5'-nucleotidase	
	protein added (µg)	[Pi] mmol/l	spec.act. µmol/min.mg	[Pi] mmol/l	spec.act. µmol/min.mg
HepG2 homogenate	250	5.17	6.9	0.73	0.9
HepG2 PM #1	14	0.04	0.9	0.09	2.1
HepG2 PM #2	6	0.01	0.4	1.07	58.4
HepG2 PM #3	8	0.02	0.8	0.69	30.2
HepG2 PM #4	9	0.04	1.5	0.70	25.1
Affigel beads	0	ND	0	ND	0

Table I *Specific activities of marker enzymes in HepG2 plasma membranes. Values represent means of duplicates. ND : not detectable.*

3.2 Preliminary data on thyroid hormone transport protein

Figure 2 shows linear density plots of $BrAcT_3$ and $BrAcT_4$ labelled plasma membranes. Both autoradiographs show a labelled protein band of 40 kD, whereas $BrAcT_3$ labelled plasma membranes show an additional 65 kD protein band. None of the plasma membrane fractions shows a major protein band in the 27 kD range, confirming the absence of type I deiodinase.

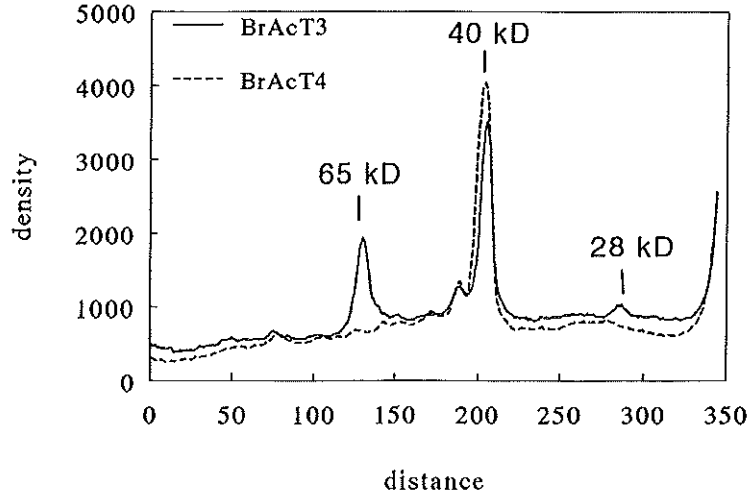

Figure 2 *Linear density plot of bromoacetyl-T_3 and -T_4 affinity labelled HepG2 plasma membranes after 72 hours exposure. A 40 kDa protein band is labelled by both affinity labels, whereas a 65 kDa protein band is specifically labelled by $BrAcT_3$. Both distance and density are expressed as arbitrary units based on the pixels of the scanned autoradiography.*

This gives additional evidence for the absence of endoplasmic reticulum contamination [8].

There is one previous report, describing the presence of a 65 kD $BrAcT_3$ labelled protein band in human placenta plasma membranes [12]. Recently, affinity purification based on structural similarities between thyroid hormones and benzodiazepines revealed the presence of an approximately 60 kD doublet in rat liver plasma membranes [13]. Antibodies raised against these proteins inhibit the uptake of T_3 in HepG2 cells. Recent data on Western blots suggest that the approximately 60 kD doublet recognised by the antibodies corresponds to the 65 kD protein we have labelled with $BrAcT_3$, although labelling does not interfere with antibody recognition (data not shown).

Combining these independent observations, we suggest that the 65 kD protein might represent a potential candidate for the plasma membrane protein that transports T_3

from plasma into the cell. The 40 kD protein labelled by both $BrAcT_3$ and $BrAcT_4$ might represent a specific binding site not related to transport, as in liver T_4 and T_3 are transported via different mechanisms [1].

Although this very rapid and specific technique of plasma membrane isolation offers the possibility to routinely obtain highly purified plasma membrane fractions for analytical purposes, we feel that is not suitable for preparative isolation of plasma membranes. As only that part of the plasma membrane is isolated which is physically in contact with the surface of the bead, it is a relatively inefficient method, unsuitable for large scale purification purposes. For Western blot analysis and other analytical techniques this method of plasma membrane isolation using poly-cationic beads offers a very fast and useful tool.

4. Acknowledgements

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Chapter 4

Characterisation of putative thyroid hormone transport protein antibodies.

Characterisation of putative thyroid hormone transport protein antibodies.

Introduction

In our laboratory several antibodies have been raised by injection of cultured rat hepatocytes in BALB/c mice. The obtained hybridoma cells were screened for antigen recognition by testing hybridoma culture supernatants for rat hepatocyte binding. Positive clones were subsequently screened for inhibition of triiodothyronine (T_3) uptake by measuring total cellular binding of [^{125}I]-T_3 in cultured rat hepatocytes. One antibody, ER22, immunoprecipitated a 52 kD protein band and inhibited transport of T_3 and thyroxine (T_4). A second antibody, ER15 (Dept Internal Medicine I, Erasmus University Medical School, Rotterdam, The Netherlands), recognised two protein bands of 115 and 32 kD, but did not inhibit transport of iodothyronines into rat hepatocytes. Both antibodies showed binding to intact rat hepatocytes, suggesting recognition of plasma membrane antigens [1]. More recently two additional thyroid hormone uptake inhibiting antibodies, 81-1A1-10 and 10C2-1 were raised using the exact same protocol [2].

A fifth antibody, raised against affinity purified rat liver plasma membranes, was kindly provided by Dr. Kragie (ADARC, Harvard Medical School, Boston, USA) who had previously shown that transport of thyroid hormones into human liver derived HepG2 cells is competitively inhibited by benzodiazepines [3]. Based on these data, a benzodiazepine-affinity purification column was constructed and used for the purification of rat liver plasma membrane proteins. Antisera were raised in rabbits against T_3-eluted protein fractions, and screened for inhibition of [^{125}I]-T_3 uptake by measuring total cellular binding in HepG2 cells. The antiserum which was selected, recognised an approximately 60 kD doublet on denaturing Western blots, and inhibited transport of T_3 into HepG2 cells. It was named after the rabbit and the tissue against which it was raised, Lillith for liver [4].

In this chapter, the purification and characterisation of the different antibody preparations is described. Antibody preparations are tested for IgG contents, thyroid hormone uptake inhibition, and antigen recognition on denaturing Western blots. These antibodies might be useful for λgt-11 screening. This system is based on the expression of individual proteins by bacteria, after which they are transferred to nitrocellulose filters and identified by specific antibodies.

Materials and methods

Materials

All tissue culture plastics and media were obtained from GIBCO/BRL (Breda, The Netherlands). Protein A Sepharose CL-4B was obtained from Pharmacia LKB (Uppsala, Sweden). Immunopure® Goat Anti-Rabbit IgG, peroxidase conjugated, and Goat Anti-

Mouse IgG, peroxidase conjugated or alkaline phosphatase conjugated were obtained from Pierce (Rockford, Illinois, USA). All other chemicals were obtained from Sigma Chemical Co (St. Louis, MO, USA). High and low molecular weight markers, both normal and prestained, were obtained from BRL (Breda, The Netherlands). Nitrocellulose sheets (0.45 µm) were obtained from Schleicher & Schnell, Dassel, Germany).

Antibody production

All hybridoma cultures were removed from liquid nitrogen stores and grown in suspension culture at 37 ^{0}C in a humidified 5% CO_2 incubator. In order to improve the isolation of IgG from culture supernatants, protein-free hybridoma medium (PFHMII, GIBCO, Breda, The Netherlands) was used. Supernatants were used for ammonium sulphate precipitation and protein A Sepharose IgG purification, as described below, or stored at -20 ^{0}C.

Due to relatively low and sometimes absent IgG production in hybridoma culture supernatants, permission was asked and obtained (Ethical committee on animal experiments, Medical Faculty, Erasmus University Rotterdam) for the production of ascites in young adult BALB/c mice. One week before immunisation, mice were injected intraperitoneally with 0.1 ml incomplete Freunds adjuvants (Pierce, Rockford Illinois, USA). On day 0, mice were injected intraperitoneally with 1 x 10e6 hybridoma cells in 0.5 ml phosphate buffered saline (PBS, 137 mmol/l NaCl, 2.7 mmol/l KCl, 8.1 mmol/l Na_2HPO_4, 1.5 mmol/l KH_2PO_4, pH 7.4) at room temperature. Whenever ascites production became obvious, mostly after 2 weeks or longer, ascites was removed and mice were sacrificed. Ascites fluids were stored at -80 ^{0}C, or submitted to ammonium sulphate precipitation and protein A Sepharose IgG purification, as described below.

Ammonium sulphate precipitation

In order to increase the IgG concentration in hybridoma culture supernatants or to remove proteins other than immunoglobulins from mouse ascites, ammonium sulphate precipitation was used [5]. Briefly, solutions were centrifuged at 3000 g for 30 min to remove solid materials and remaining cells. Supernatants were transferred to an appropriate glass beaker and placed on a magnetic stirrer in a cold room (4 C). Slowly, 1 volume of saturated ammonium sulphate (pH 7.0, 4 C) was added, and the contents was stirred overnight. Proteins were precipitated by centrifugation at 3000 g for 30 min, 4C, and clear supernatants were transferred to a new glass beaker. IgG was precipitated by a second slow addition of 1 volume of saturated ammonium sulphate (pH 7.0, 4 C), overnight stirring, and centrifugation at 3000 g for 30 min at 4 ^{0}C. The IgG pellet was resuspended in PBS, and dialysed overnight versus two changes of PBS at 4 ^{0}C in order to remove remaining ammonium sulphate. IgG concentrated fractions were used for protein A Sepharose, or stored at -80 ^{0}C.

Protein A Sepharose IgG purification

In order to isolate IgG from tissue culture supernatants or ammonium sulphate precipitated fractions, protein A Sepharose chromatography was used. For different isolations different columns were used, ranging from 5 ml bed volume Pharmacia columns (Uppsala, Sweden) to self-made 0.5 ml bed volume Pasteurs-pipette columns. In order to prevent cross-contamination, none of the columns was used for more than one particular antibody preparation. All purifications were performed in a cold room (4 C) according to the high salt protocol for antibody purification on protein A columns [6]. Briefly, the NaCl concentration of the sample was adjusted to 3.3 mol/l by adding NaCl, and 0.1 volume of 1 mol/l sodium borate pH 8.9 was added. The pH of the sample was adjusted to 8.9 using a concentrated NaOH solution, and applied to a prewashed protein A Sepharose column (3 x

4 column volumes 3 mol/l NaCl, 50 mmol/l sodium borate, pH 8.9). The first flow-through was applied again, before washing the column with 10 column volumes of 3 mol/l NaCl, 50 mmol/l sodium borate pH 8.9 and 10 column volumes of 3 mol/l NaCl, 10 mmol/l sodium borate pH 8.9. Purified IgG was eluted by repeated addition of 0.5 column volume of 100 mmol/l glycine pH 3.0. Samples were collected in eppendorf tubes containing 0.1 elution volume of 1 mol/l tris pH 8.0 in order to adjust the pH to neutral. Fractions were analysed for IgG contents using OD280 measurement and dot-blot analysis.

Dot blot analysis

In order to determine whether a sample contains IgG, or whether a specific antigen is recognised by a particular IgG, dot blot analysis was used. Nitrocellulose and Whatmann paper were wetted in tris buffered saline (TBS, 20 mmol/l tris, 0.5 mol/l NaCl, pH 7.5) and fitted into the dot-blot apparatus according to the manufacturers instructions (Schleicher & Schnell, Dassel, Germany). The apparatus was attached to a vacuum pump, and individual wells of the dot-blot apparatus were filled with 1 ml of TBS. After the TBS was pulled through the nitrocellulose filter, samples were added. Different volumes and concentrations of antibody preparations were routinely tested using this system. Also, varying concentrations of liver homogenate (prepared by sonication in TBS containing 0.05% v/v Tween-20 (TTBS) or 10 mmol/l tris, 3 mmol/l EDTA, 5 mmol/l dithiotreitol, pH 7.5 (TED)) were transferred to nitrocellulose using this system to test antibody preparations for their ability to recognise rat liver proteins. Individual wells were washed with 1 ml of TBS before removing the nitrocellulose from the apparatus. Filters were blocked and analysed analogues to immunoblots, as described below.

Immunoblotting

All antibodies were tested for their ability to recognise proteins on denaturing polyacrylamide gel electrophoresis blots (SDS-PAGE blots). Both the Protean II as well as the miniprotean system (Biorad, The Netherlands) were used. SDS-PAGE was performed according to Laemmli [7], and proteins were transferred electrophoretically to nitrocellulose using the Hoefer Transphor system (Hoefer Scientific Instruments, San Francisco, USA) for large Protean II gels or the mini-transblot system (Biorad, The Netherlands) for small miniprotean gels. After removing a strip for protein staining, the nitrocellulose filters were marked and blocked by incubation for 20 min at room temperature in 2% w/v gelatin in TBS. Blocked filters were washed twice in TBS containing 0.05% v/v Tween-20 (TTBS) and incubated for 45 min at room temperature in different dilutions of antibodies (1:10 to 1:1000 in TTBS/ 1% w/v gelatin). Blots were washed two times 5 min in TTBS, and incubated for 30 min at room temperature with different dilutions of the appropriate secondary antibody (1:1000 to 1:5000 in TTBS/ 1% w/v gelatin). Subsequently, blots were washed two times 5 min with TTBS and once for 5 min in TBS before staining with the appropriate reagent. For alkaline phosphatase conjugated antibodies the reagent was prepared by adding 3.3 mg Nitro Blue Tetrazolium and 1.6 mg 5-bromo-4-chloro-3-indolyl phosphate to 10 ml 0.2 mol/l tris, 10 mmol/l $MgCl_2.6H_2O$, pH 9.1. For horseradish peroxidase conjugated antibodies a commercially available substrate kit containing 3,3'-diaminobenzidine tetrahydrochloride (DAB) was used (Pierce, Rockford, USA).

Protein staining immunoblots

Nitrocellulose strips were stained for protein using Amidoblack 10B (Merck, Darmstadt, Germany). Strips were fixed in 6% trichloroacetic acid (TCA) for 5 min, stained in 0.1% amidoblack in 45% methanol and 10% acetic acid for 5 to 10 min, and destained in 45% methanol and 10% acetic acid until sufficient contrast was obtained. Destained strips were washed in water, and air-dried.

Bromo-acetyl affinity labelling and SDS PAGE analysis

N-Bromoacetyl-3'-[^{125}I]-T_3 ($BrAcT_3$) was synthesised as described previously [8]. For affinity labelling, 500,000 cpm of $BrAcT_3$ per incubation was dried down under nitrogen in an eppendorf tube. To start incubation, 100 µl of rat liver microsomes (prepared according to Visser etal [9]) was added and tubes were incubated for 10 min at 37 ^{0}C. To stop affinity labelling, 50 µl of 3 x SDS-PAGE sample buffer was added, and tubes were incubated at 80 ^{0}C for 10 min. SDS-PAGE and autoradiography were performed as described above.

Thyroid hormone uptake assays

The effects of preincubation and incubation in the presence of antibodies was studied using primary cultures of rat hepatocytes and cultures of human liver derived HepG2 cells. Rat hepatocytes were obtained from male Wistar rats by collagenase perfusion of the liver, and thyroid hormone uptake was studied as described previously [10]. Briefly, cells were incubated in 6 wells tissue culture plates in HamsF10 containing 0.5% bovine serum albumin (BSA) in the presence of [^{125}I]-T_4 or [^{125}I]-T_3. After incubation at 37 ^{0}C for different periods of time, media were analysed for iodide, conjugates, and remaining iodothyronines. HepG2 cells were incubated in 6 wells tissue culture plates in Ham's F12/DMEM (1:1 v/v) containing 0.5% BSA in the presence of [^{125}I]-T_3. After 120 min at 37 ^{0}C, cells were washed using ice-cold PBS and removed from the wells using a rubber policeman. Cells were counted in a γ-counter to determine total cellular binding, before isolation of nuclei by sequential washing in PBS / 0.5% Triton X-100 [11]. Nuclear bound radioactivity was measured to determine nuclear binding.

Results and discussion

Dot blot analysis of hybridoma culture supernatants

In order to test whether the new hybridoma cultures were producing detectable amounts of IgG, and to test whether these antibodies were able to detect human liver antigens on native immunoblots, dot blot analysis was performed. As there were no hybridoma cells available for ER22, this antibody was not tested. Human liver TED homogenate (1 mg/ml in TED), human liver TTBS homogenate (1 mg/ml in TTBS) and HepG2 TTBS homogenate (1 mg/ml in TTBS) were blotted onto nitrocellulose filter, blocked as described above, and analysed using the different primary antibodies. As positive controls the individual primary antibodies were blotted, blocked, and stained using the appropriate secondary antibody. A second positive control was performed by using goat anti-human liver lipase (GαLLP), which was kindly provided by Dr. M. Breel (Dept of Internal Medicine III, EUR, The Netherlands). Negative controls were performed by testing liver homogenates without primary antibodies. Data are shown in Table I.

spot	1st Ab	2nd Ab	signal	spot	1st Ab	2nd Ab	signal
HL TED	81-1A1	GαM PO	-	HL TED	ER15	GαM PO	-
HL TTBS	81-1A1	GαM PO	-	HL TBS	ER15	GαM PO	-
HepG2	81-1A1	GαM PO	-	HepG2	ER15	GαM PO	-
HL TED	10C2-1	GαM PO	-	HL TED	none	GαM PO	-
HL TTBS	10C2-1	GαM PO	-	HL TBS	none	GαM PO	-
HepG2	10C2-1	GαM PO	-	HepG2	none	GαM PO	-
HL TED	GαLLP	SαG PO	+	81-1A1	none	GαM PO	-
HL TTBS	GαLLP	SαG PO	+	10C2-1	none	GαM PO	+
HepG2	GαLLP	SαG PO	+	ER15	none	GαM PO	-

Table I *Dot blot analysis of hybridoma cell tissue culture supernatants. Only the positive control goat anti liver lipase (GαLLP) recognises human liver antigens. Staining of individual IgG fractions revealed the absence of mouse IgG in 81-1A1 and ER15 supernatants.*

Table I shows the absence of detectable IgG in tissue culture supernatants (TCSN) of 81-1A1-10 and ER15, whereas the IgG which is present in 10C2-1 TCSN is unable to detect antigen in any of the human liver homogenates. The positive control GαLLP is able to detect antigen in all three human liver homogenates. These data were confirmed by ammonium sulphate precipitation and protein A Sepharose purification, followed by SDS-PAGE analysis. Only the 10C2-1 supernatants contained heavy and light chain IgG (data not shown).

As 81-1A1-10 is one of the antibodies which should be able to inhibit transport of thyroid hormone into rat hepatocytes, and as the hybridoma cultures did not produce any IgG, we started the production of ascites in young adult BALB/c mice using new cultures of 81-1A1-10.

Dot blot analysis of new 81-1A1 cultures and immunoprecipitation HepG2 homogenate

Dot-blot analysis of new cultures of 81-1A1-10 hybridoma cells revealed that passages 2, 3 and 4 show the presence of IgG in their TCSN, whereas no IgG is detectable in protein-free hybridoma medium (PFHMII, negative control). These 81-1A1-10 cells were grown in suspension and used for injection into BALB/c mice. Ascites was produced and purified using ammonium sulphate precipitation and protein A purification. Purified ascites IgG was tested for thyroid hormone uptake inhibition, using the rat hepatocyte system.

Thyroid hormone uptake inhibition experiments

In order to test the available antibodies for inhibition of thyroid hormone uptake, different IgG preparations were tested for their effects on iodide and conjugates production from [^{125}I]-T_4 and [^{125}I]-T_3 by primary cultures of rat hepatocytes. Table 2 shows iodide production for T_4 and T_3, with their corresponding p-values when compared to control incubations. Comparison to mouse IgG incubations results in the same conclusions.

Antibody	T_4	p	T_3	p
Control	12.36 ± 0.60		29.18 ± 2.57	
Ouabain	10.07 ± 0.27	0.001	16.51 ± 1.42	0.001
ER22 Ascites	10.47 ± 0.53	0.005	33.06 ± 3.27	NS
ER15 Ascites 83	12.02 ± 0.15	NS	29.49 ± 4.30	NS
ER15 Ascites 88	10.54 ± 0.80	0.005	30.64 ± 3.34	NS
81-1A1-10 TCSN	11.90 ± 0.91	NS	30.15 ± 2.21	NS
81-1A1-10 pur. ascites	12.60 ± 0.29	NS	31.88 ± 2.62	NS
mouse IgG	12.37 ± 0.64	NS	31.49 ± 1.67	NS

Table 2 : Iodide production from [^{125}I]-T_4 and [^{125}I]-T_3 by primary cultures of rat hepatocytes. Ouabain shows a significant decrease in iodide production for both hormones. ER22 ascites and ER15 ascites 88 show significant inhibition of iodide production from [^{125}I]-T_4. No significant changes in conjugates were found (data not shown).

Iodide production from [^{125}I]-T_4 is significantly reduced in the presence of ER22- or ER15 ascites 88 (both $p<0.005$). Iodide production from T_4 and T_3 is also significantly reduced in the presence of ouabain (both $p<0.001$, positive control of the test system). Mouse IgG (1 mg/ml, 1:100 dilution) shows no significant changes when compared to control incubations.

None of the other antibody preparations, including the new 81-1A1-10 TCSN or the new 81-1A1-10 purified ascites from these cells, show any significant decrease in iodide production. Also, no significant changes were found in conjugates production, excluding effect on intracellular type I deiodinase (data not shown).

Western blots of rat liver microsomes using Lillith antiserum

In order to test whether the proteins which are recognised by Lillith antiserum are also labelled by the covalent affinity label $BrAcT_3$, and in order to see whether this affinity labelling interferes with antigen recognition, we performed affinity labelling of rat liver microsomes using $BrAcT_3$ with subsequent Western blotting and autoradiography using

Lillith antiserum. For negative controls 1:100 dilutions of normal rabbit serum were used. Figure 1 shows the Western blots of increasing amounts of rat liver microsomes, in the presence and absence of $BrAcT_3$ labelling. The inset shows the corresponding autoradiograph of the highest protein concentration.

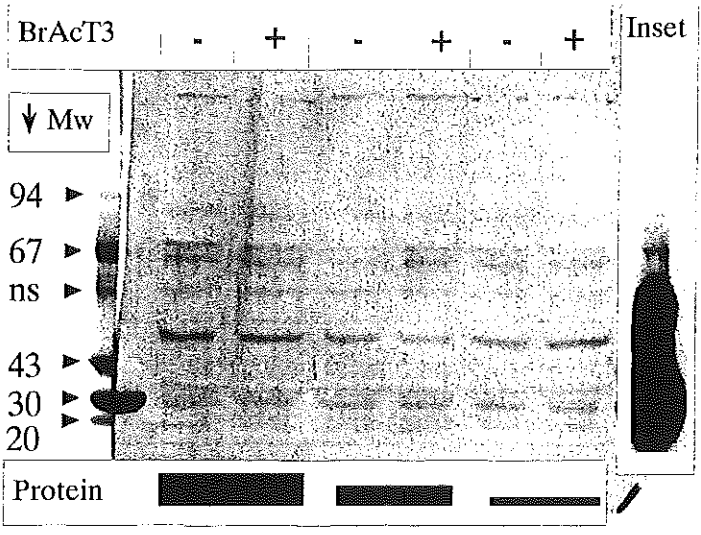

Figure 1 *Immunoblot of rat liver microsomes using Lillith antiserum. Rat liver microsomes (62.5, 75, and 87.5 µg protein/lane) were labelled using BrAc-[^{125}I]-T_3 and separated using SDS-PAGE. Proteins were blotted onto nitrocellulose, blocked using gelatin, and incubated with 1:100 dilution of Lillith antiserum. After staining with goat-anti-rabbit second antibody, the Western blot was used for autoradiography. In addition to the reported doublet which we find to be in the 67 kD range, we also identified three additional bands of 85, 58 and 50 kD respectively. The 50 kD band is very pronounced, even at lower protein concentrations. The band in the 25 kD range is also recognised by normal rabbit serum. Labelling with $BrAcT_3$ did not interfere with antigen recognition. Autoradiography revealed the presence of a protein of similar molecular weight as the 67 kD doublet recognised by Lillith antiserum (inset).*

In addition to the reported approximately 60 kD doublet, which we find to be just below the 67 kD marker, we also identified three additional bands. One band, at approximately 50 kD, is already present at the lowest protein concentrations. Two other bands, of approximately 58 kD and 85 kD, are much weaker but present. As Dr. Kragie tested Lillith antiserum on rat liver plasma membranes, the strong 50 kD protein might represent a protein called protein disulphide isomerase (PDI), a microsomal protein previously mistaken for type I deiodinase due to its high affinity for $BrAcT_3$ [12]. Possibly this protein is also recognised by antibodies raised against affinity purified plasma membranes. An approximately 25 kD band which is recognised by Lillith antiserum is also present in normal rabbit serum incubated Western blots.

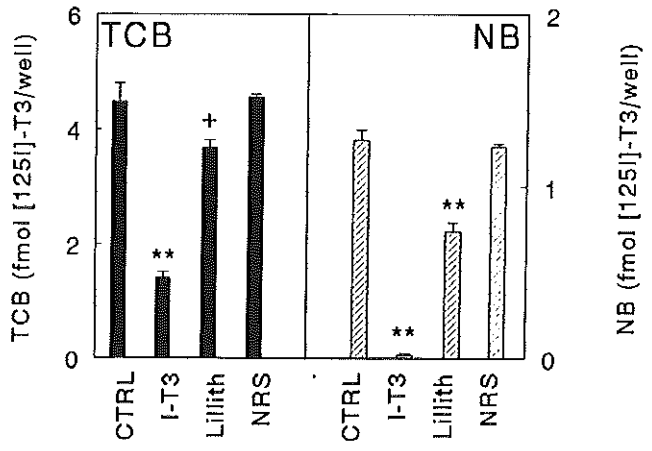

Figure 2 *Thyroid hormone uptake inhibition by Lillith antiserum. Human liver derived HepG2 cells were incubated in the presence and absence of 1:100 dilutions of Lillith antiserum. The effects on total cellular binding and nuclear binding of [^{125}I]-T_3 was studied. Total cellular binding was significantly lower than control incubations or incubations in the presence of 1:100 dilutions of normal rabbit serum ($p<0.025$). This effect was even more pronounced at the nuclear level ($p<0.001$).*
*** : $p<0.001$, + : $p<0.025$*

The recognition of the 60 kD doublet is not influenced by $BrAcT_3$ affinity labelling. Apparently, covalent labelling does not interfere with epitope recognition.

In order to verify whether Lillith antiserum inhibits transport of thyroid hormone, total cellular binding and nuclear binding of [^{125}I]-T_3 by human liver derived HepG2 cells was studied.

Thyroid hormone uptake inhibition by Lillith antiserum

Figure 2 shows total cellular binding and nuclear binding of [^{125}I]-labelled T_3 in the presence and absence of unlabelled T_3 or 1:100 Lillith antiserum dilution. As a negative control 1:100 dilutions of normal rabbit serum were used. There is a significant decrease of total cellular binding ($p<0.025$) in the presence of Lillith antiserum, which is even more pronounced at the nuclear level ($p<0.001$). This suggest an effect on transmembrane transport, as we have previously shown that there is an aspecific component in the total cellular binding data due to aspecific extracellular binding of T_3 to the plasma membrane (Chapter 2.2 of this thesis) which is not present at the nuclear level. There are no significant changes in the presence of 1:100 diluted normal rabbit serum.

Conclusions

Combining that hybridoma cells are no longer available for ER22, that 81-1A1-10 cells no longer produce inhibiting IgGs, that ER15 IgG shows little inhibition of T_4 transport in spite of the fact that it is supposed to be a non-inhibiting antibody, and that none of the antibodies inhibits transport of T_3, we conclude that these antibodies are not suitable for a λgt-11 expression cloning strategy.

As Lillith antiserum has been shown to specifically recognise proteins on denaturing Western blots, and as this antiserum also inhibits transmembrane transport of T_3, we conclude that this antiserum would be useful for the screening of a λgt11 expression library. In addition, the comparable molecular weights of the protein recognised on Western blots, the protein labelled in rat liver microsomes by $BrAcT_3$, and the protein labelled by $BrAcT_3$ in HepG2 plasma membranes (Chapter 3 of this thesis) suggest that this protein might very well represent a common protein involved in transport of thyroid hormone.

Even though the antiserum recognises several proteins of different molecular weight, including an aspecific band which is also recognised by normal rabbit serum, it should still be possible to identify clones of interest using this antibody preparation. Isolated clones can always be analysed using alternative screening methods, such as expression in the *Xenopus* system (Chapter 5 of this thesis).

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Chapter 5

Expression of the triiodothyronine transport protein in *Xenopus laevis* oocytes.

Expression of the triiodothyronine transport protein in *Xenopus laevis* oocytes.

Introduction

A very elegant, and increasingly popular expression cloning system is based on the injection of RNA into oocytes of the South African clawed frog *Xenopus laevis* [1]. Due to the relatively large size of the oocytes, cells can be injected manually with foreign genetic material and screened individually. *Xenopus laevis* oocytes have been shown to be excellent tools for the expression and functional analysis of proteins of many different origins, ranging from human to avian sarcoma virus, *Escherichia coli* and even plant material [2]. The oocytes are able to efficiently translate injected messenger RNAs (mRNA) and perform a wide variety of post-translational modifications including N-acetylation, N-glycosylation, hydroxylation, poly-protein cleavage, covalent- and non-covalent subunit assembly, removal of signal sequence, haem addition and phosphorylation [2].

Many trans-plasma membrane transport proteins have been cloned or are being cloned using the *Xenopus* system [3-11]. Also, the protein responsible for the intrahepatic deiodination of thyroxine to 3,5,3'-triiodothyronine (T_3), the so-called type I deiodinase, has recently been cloned using this system. Even though this protein is a seleno-cysteine protein, containing a UGA stop-codon which also codes for seleno-cysteine, the *Xenopus* system is still able to translate this into functional type I deiodinase protein [12].

An additional important advantage of the *Xenopus* system compared to other expression cloning systems is the possibility to start with injection of a total pool of mRNAs. If injection of the entire pool of mRNAs results in functional expression of the protein of interest, enrichment of mRNA by size fractionation will even increase the relative biological activity, provided that the protein does not consist of two or more subunits encoded by different mRNAs of different size. Following the construction of a directional cDNA library of size fractionated mRNA, subdivided pools of the library are tested for expression of the protein of interest using *in vitro* cRNA transcription and injection into oocytes. By continuously subdividing positive pools a single clone can be obtained.

In this chapter, the expression of the triiodothyronine transport protein in *Xenopus laevis* oocytes is described. Most uptake experiments were performed in the laboratory of Prof. Heini Murer at the University of Zurich, Department of Physiology, Switzerland, and were guided by Dr. Daniel Markovich. Several different mRNA isolations were tested. Positive controls were performed by injection of *rBAT* cRNA. This clone codes for system $b^{0,+}$-like amino acid transport, and expresses sodium independent transport of L-arginine, L-cysteine, and L-leucine [11]. Uptake of [^{3}H]-l-Arg was used to assay for *rBAT* cRNA expression. Also, the uptake of [^{125}I]-T_3 by *mtr* cRNA-injected oocytes was tested. The *mtr* clone was isolated from the ascomycete *Neurospora crassa* and codes for neutral aliphatic

and aromatic amino acids transport, including transport of phenylalanine and tryptophan. The cloned sequence is named *mtr*, as mutations in this locus result in methyl-tryptophan resistance [13, 14]. As transport of thyroid hormones has been associated with transport of aromatic amino acids by several independent laboratories [15-19], we were interested to see whether *mtr* expression would induce [^{125}I]-T_3 uptake in the *Xenopus* system.

Materials and methods

Animals

Two to three years old adult *Xenopus laevis* females were obtained from H. Kähler (Institut für Entwicklungsbiologie, Hamburg, Germany) or from J. Narroway (Hubrecht Laboratory, Utrecht, The Netherlands). Frogs were maintained in a water-filled tank with three dark sides at a temperature of 15 to 16 °C. A 12 hour day-night schedule was maintained in order to reduce seasonal variations in oocyte quality. Frogs were fed twice a week. The water was changed immediately after feeding, with water from a second tank which was filled one day before in order to dechlorinate.

Total RNA isolation

Total RNA, required for isolation of poly-A+ RNA, was isolated from male Wistar rat liver according to two different protocols. For both protocols, rats were sacrificed by decapitation. The liver was perfused with approximately 25 ml of ice-cold phosphate buffered saline (PBS, 137 mmol/l NaCl, 2.7 mmol/l KCl, 8.1 mmol/l Na_2HPO_4, 1.5 mmol/l KH_2PO_4, pH 7.4) before removal and was always immediately transferred into liquid nitrogen. Livers were stored at -80 °C.

Using the first protocol, as described by Chomczynski [20], liver fragments were grinded in liquid nitrogen, and transferred into a sterile tube. While the tissue was thawing, appropriate amounts of solution D was added (4 mol/l guanidinium thiocyanaat (GTC), 25 mmol/l sodium citrate pH 7.0, 0.5% sarcosyl, 0.1 mol/l ß-mercaptoethanol). DNA was sheared using a 18 gauge needle, and 50 µl NaAc pH 4.0 was added. Subsequently, 500 µl phenol and 100 µl chloroform was added, and tubes were kept on ice for 15 min. After centrifugation for 15 min (eppendorf centrifuge, rmax, 4 C) the water phase was transferred to a new tube. RNA was pelleted using 1 volume of isopropanol and incubation at 4 °C overnight. Pellets were washed once more with solution D, precipitated with isopropanol, and washed with 75% ethanol. Final RNA pellets were dissolved in water or TE buffer (10 mmol/l tris, 1mmol/l EDTA, pH 8.0), and concentrations were determined by measuring the OD260. RNA was stored at -80 °C as an ethanol precipitate by addition of 0.1 volume of 3 mol/l sodium acetate and 2 volumes of ice-cold ethanol absolute (-20C), or used immediately for mRNA isolation.

The second protocol was a commercially available protocol, based on cesium-trifluoroacetate (Cs-TFA) gradient centrifugation (RNA extraction kit, Pharmacia P-L Biochemicals, Uppsala, Sweden). Besides the RNase inhibiting properties of guanidinium thiocyanaat, which was also present in the Chomczynski protocol, the Cs-TFA prevents RNase activity during the final steps of the RNA isolation [21]. Briefly, liver fragments were treated as described above, and lysed in the provided extraction buffer (1 g of liver per 18 ml of buffer). DNA was sheared using a 16 gauge needle, and samples were loaded onto Cs-TFA containing RNase free centrifugation tubes (autoclaved or incubated with 3% H_2O_2 overnight). After centrifugation for 16 hr at 125,000 x g (15 C) using a SW-41 Ti rotor (Beckman, Mijdrecht, The Netherlands), the supernatants were removed carefully and RNA pellets were dissolved in TE buffer. RNA concentrations were determined by measuring the

OD260. RNA was stored at -80 °C as an ethanol precipitate or used immediately for mRNA isolation.

mRNA isolation

For the isolation of poly-A+ RNA from total rat liver RNA preparations, several different protocols were used. All were based on hybridisation of the 3'- poly-A+ tail of mRNAs to an oligo-dT stretch attached to solid carriers. One method was based on oligo-dT-cellulose (Boehringer, Mannheim, Germany), according to Sambrook etal [22]. All other methods were based on commercially available kits for mRNA isolation. The mRNA separator kit (Clontech Laboratories, Palo Alto, USA) was based on prepacked oligo-dT columns and the SCIGEN mRNA isolation kit (Scigen, Kent, UK) was based on oligo-dT(18) attached to paramagnetic cellulose particles. Isolations were performed according to the manufacturers instructions. All use the ionic strength protocol, with hybridisation under high salt conditions, and elution of mRNA using a low ionic strength buffer.

In vitro transcription

In order to prepare cRNA for positive controls, *rBAT* was amplified in *E.coli* and plasmid DNA was isolated using the Quiagen Maxiprep system (Quiagen, Westburg bv, Leusden, The Netherlands). Isolated plasmid DNA was linearised by XhoI digestion (Boehringer, Mannheim, Germany) using the standard buffers supplied for this enzyme. After restriction enzyme digestion, the volume of the incubation mixture was made up to 100 µl with TE buffer, and 100 µl of phenol/ chloroform/ isoamyl alcohol (IAA) was added (Life technologies, Breda, The Netherlands). Contents was mixed well, tubes were centrifuged for 2 min at rmax (eppendorf centrifuge), and the water phase was extracted once more with 100 µl phenol/chloroform/IAA and once with 100 µl chloroform. The final water phase was used for ethanol precipitation, as described above. Linearised *rBAT* was checked using 0.8% agarose gel electrophoresis, and used for cRNA synthesis.

In vitro transcription was performed using the Ampliscribe^{tm} T7, T3 and SP6 transcription kit (Epicentre technologies, Madison, USA), according to the T3 protocol for synthesis of capped RNA. For capping, the m7G[5']ppp[5']G cap analog was used (Epicentre technologies, Madison, USA). After transcription, the DNA template was digested using RNase-free DNase I, and incubation mixture was phenol extracted as described above. RNA was precipitated by addition of an equal volume of 5 mol/l ammonium acetate, incubation on ice for 30 min, and centrifugation at 4 °C for 10 min rmax (eppendorf centrifuge). This method selectively precipitates RNA while leaving DNA, protein, and unincorporated NTPs in the supernatant. RNA pellets were dissolved in TE buffer and stored at -80 °C. If RNA was to be stored for longer than 1 month, it was stored as an ethanol precipitate at -80 °C. Transcript integrity was verified using 1% agarose/TBE gel electrophoresis.

Oocyte isolation and RNA-injections

Ovarian fragments were removed from *Xenopus* females under MS22 anaesthesia (1 g/l 3-aminobenzoic acid ethyl ester, Sigma, St.Louis, USA in tap water) and hypothermia. Small lumps containing 20-50 oocytes were washed in calcium-free ORII (82.5 mmol/l NaCl, 2 mmol/l KCl, 1 mmol/l $MgCl_2$, 10 mmol/l Hepes adjusted with tris to pH 7.5), and incubated for two times 90 minutes in collagenase A or B (Boehringer, Mannheim, Germany, 2 mg/ml in ORII) on a rotator in order to remove follicular layers. The oocytes were washed thoroughly five times with ORII followed by five times with modified Barth's solution (MBS, 88 mmol/l NaCl, 1 mmol/l KCl, 0.82 mmol/l $MgSO_4$, 0.4 mmol/l $CaCl_2$, 0.33 mmol/l $Ca(NO_3)2$, 2.4 mmol/l $NaHCO_3$, 10 mmol/l Hepes/Tris pH 7.4, containing 100 IU/ml penicillin and 100 µg/ml streptomycin). The oocytes were sorted manually on morphological

criteria, such as size, polarisation, and pigmentation. Healthy looking stage V-VI oocytes were transferred to 6 well tissue culture plates and incubated in MBS at 18 °C in the dark.

The next day, healthy looking oocytes stage V-VI oocytes were injected semi-automatically with 50 nl water (control) or 50 nl mRNA (50 ng mRNA / oocyte for rat liver mRNA or 1 ng cRNA / oocyte for *rBAT* positive control). Also, a commercially available rat liver mRNA preparation (50 ng mRNA / oocyte male Sprague-Dawley outbred rat liver, Clontech, Palo Alto, USA) was tested. In Zürich, the Inject+Matic system (J.A. Gabay, Geneva, Switzerland) was used, whereas in Rotterdam the Nanoject system (Drummond Scientific Co., Broomall, USA) was used. Injected oocytes were maintained at 18 °C for 3 to 5 days, with changes of MBS solution every 1-2 days.

Transport assays

Oocytes were divided into groups of 10 oocytes each. After washing of the oocytes for 1 min in sodium free solution A (100 mmol/l Choline chloride, 2 mmol/l KCl, 1 mmol/l $CaCl_2$, 1 mmol/l $MgCl_2$, 10 mmol/l Hepes/tris pH 7.5), the cells were transferred to a 4 ml tube and incubated for 1 hour at 25 °C in 100 µl solution A containing different concentrations of substrate at the desired specific activity of the isotope (50 µmol/l at 10 µCi/ml for [^{3}H]-l-Arg and 5 or 10 nmol/l at 10 or 20 µCi/ml respectively for [^{125}I]-T_3). Incubations were performed in the presence (100 mmol/l NaCl) or absence (100 mmol/l choline chloride) of sodium. For thyroid hormone uptake,incubations in the presence and absence of protein (0.1% bovine serum albumin, BSA) were also performed.

After the incubation the uptake solution was removed, and oocytes were washed three times with 3 ml of ice-cold stop-solution (solution A) containing 0.1 % BSA. Oocytes were transferred to new tubes and counted as a group or individually for [^{125}I]-T_3 uptakes, or were transferred individually to scintillation vials containing 200 µl of 10% sodium dodecyl sulphate (SDS) and allowed to shake vigorously for 45 min at room temperature for [^{3}H]-l-Arg uptakes. Dissolved oocytes were supplemented with 3 ml of scintillation fluid (Pico-Fluor™ 15, Packard Instrument bv., Groningen, The Netherlands) before measurement of radioactivity.

Statistics

Experiments were performed with groups of 5 or 10 oocytes. Data are expressed as fmol per 10 oocytes, or as fmol per oocyte ± standard deviation. Statistical significance was evaluated by Student's t test for unpaired observations.

Results

Figure 1 shows the uptake of [^{3}H]-l-Arg by water- and *rBAT* cRNA-injected oocytes. The experiments in Zürich were performed with oocytes from frogs that were different from those in Rotterdam. After injection of cRNA, the oocytes were incubated for 3 days before assaying for l-Arg uptake. Uptake solutions contained 50 µmol/l l-Arg at 10 µCi/ml, in the absence of sodium and protein. There was a significant increase in [^{3}H]-l-Arginine uptake by *rBAT* cRNA-injected oocytes, both for oocytes obtained from frogs in Zürich ($p<0.001$, $n=6$) as well as in Rotterdam ($p<0.001$, $n=10$).

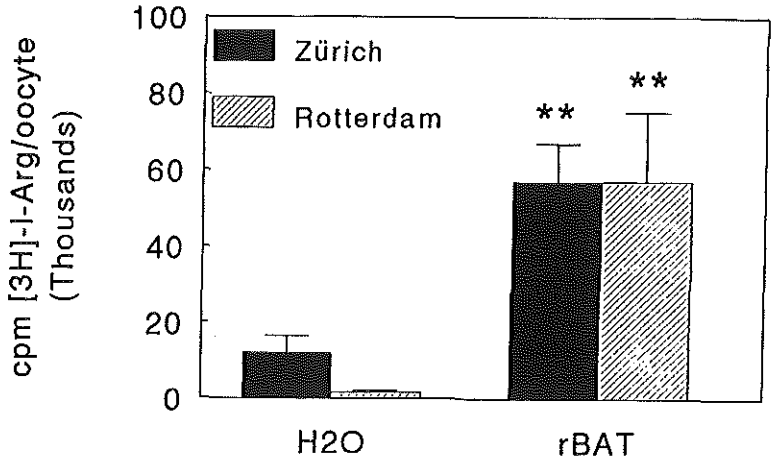

Figure 1 *[^{3}H]-l-Arginine uptake by rBAT injected oocytes. As a positive control for the expression of plasma membrane protein form injected mRNA, the arginine transport protein coding rBAT was used. Oocytes were injected with 1 ng cRNA per oocyte, incubated for 3 days, and analysed for [^{3}H]-l-Arginine uptake. Both the oocytes obtained from frogs in the Zürich laboratory as well as the oocytes obtained from the frogs in our own laboratory showed a significant increase in [^{3}H]-l-Arg uptake as compared to water-injected oocytes.*
*** = p<0.001*

The increase in [^{125}I]-T_3 uptake by mRNA-injected oocytes is shown in Table I. Experiments were performed in Zürich, three days after injection, in the presence and absence of 0.1% BSA. All uptake solutions contained sodium. Four different mRNA preparations were compared to water-injected oocytes. The mRNA preparation O (CS-TFA RNA isolation and Clontech mRNA kit) showed an increase in [^{125}I]-T_3 uptake, both in the presence and absence of albumin in the uptake solution. Data are expressed as fmol [^{125}I]-T_3 per 10 oocytes, as in these experiments the oocytes were not counted individually. In the absence of protein, the amount of [^{125}I]-T_3 bound to the oocytes is much higher than in the presence of albumin.

RNA prep	fmol [^{125}I]-T_3 per 10 oocytes	
	+BSA	- BSA
H_2O	8.89	42.02
Dan	7.80	36.37
CT	9.50	39.63
N	9.58	38.78
O	11.41	59.54

Table I : *[^{125}I]-T_3 uptake by rat liver mRNA-injected oocytes. Oocytes were incubated for 3 days before performing the uptake experiments. Uptake solution contained 10 µCi [^{125}I]-T_3 per ml (total T_3 concentration 5 nmol/l), in the presence or absence of 0.1% BSA. All experiments were performed in the presence of sodium. Cells were incubated as described in materials and methods.*

Table II shows [^{125}I]-T_3 uptake by oocytes in sodium containing protein-free uptake solution after 5 days of incubation. Oocytes were incubated with 10 µCi or 20 µCi [^{125}I]-T_3, resulting in total T_3 concentrations of 5 and 10 nmol/l respectively. Three mRNA preparations showed an increase in [^{125}I]-T_3 uptake. In addition to mRNA O, mRNA preparations CT (Clontech commercially available rat liver mRNA) and N (a second Cs-TFA, Clontech mRNA kit preparation) also showed an increase as compared to water-injected oocytes. Increase of the substrate concentration from 5 to 10 nmol/l total T_3 (i.e. free T_3, since there is no protein present in the uptake solution) resulted in an increase in [^{125}I]-T_3 uptake by all groups of oocytes, including water-injected.

RNA prep	fmol [^{125}I]-T_3 per 10 oocytes	
	10 µCi	20 µCi
H_2O	22.36	79.08
Dan	22.55	71.86
CT	39.04	152.28
N	28.40	119.60
O	55.72	139.78

Table II : *[^{125}I]-T_3 uptake by rat liver mRNA-injected oocytes. Oocytes were incubated for 5 days before performing the uptake experiments. Uptake solution contained 10 µCi or 20 µCi [^{125}I]-T_3 per ml (total T_3 concentration 5 and 10 nmol/l respectively), in the absence of protein. All experiments were performed in the presence of sodium. Cells were incubated as described in materials and methods.*

The effects of the presence or absence of sodium (sodium replaced by choline) in the uptake solution is shown in Figure 2. Oocytes were injected with 50 ng mRNA O per oocyte, and incubated for five days. Data are expressed as fmol [^{125}I]-T_3 per oocyte. Uptake solutions contained 10 or 20 µCi [^{125}I]-T_3 (5 or 10 nmol/l respectively) and no protein. At both substrate concentrations, mRNA-injected oocytes showed a significant increase in T_3 uptake. In the presence of sodium, T_3 uptake increased from 2.05 ± 0.62 to 4.51 ± 1.18 fmol T_3/oocyte ($p<0.001$, 10 µCi [^{125}I]-T_3, n=10) and from 6.29 ± 2.17 to 11.80 ± 1.62 fmol T_3/oocyte ($p<0.005$, 20 µCi [^{125}I]-T_3, n=5). In the absence of sodium, T_3 uptake increased from 1.74 ± 0.60 to 2.78 ± 0.52 fmol T_3/oocyte ($p<0.001$, 10 µCi, n=10) and from 4.33 ± 1.24 to 10.23 ± 2.51 fmol T_3/oocyte ($p<0.005$, 20 µCi, n=5). Only at the 10 µCi dose of [^{125}I]-T_3 in mRNA-injected oocytes a significant difference between sodium and choline containing incubations is seen (4.51 ± 1.18 in the presence of sodium versus 2.78 ± 0.52 in the presence of choline, $p<0.001$, n=10).

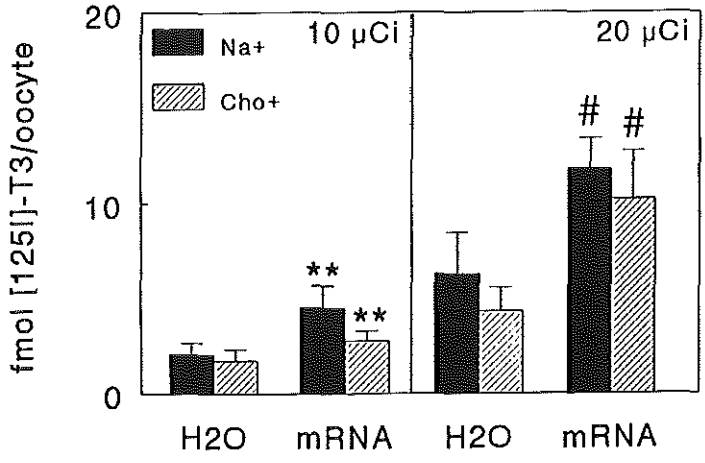

Figure 2 *Effects of sodium and substrate concentration on [^{125}I]-T_3 uptake. Oocytes were injected with 50 ng rat liver mRNA per oocyte, incubated for 5 days, and analysed for [^{125}I]-T_3 uptake. Incubations were performed in the presence and absence of sodium, using 10 µCi or 20 µCi [^{125}I]-T_3 per ml (5 and 10 nmol/l total T_3 respectively, in the absence of protein). Injection of rat liver mRNA resulted in a significant increase of [^{125}I]-T_3 uptake as compared to water-injected oocytes. In the absence of sodium this increase was lower, but still significantly higher than water-injected oocytes. Increased substrate concentration results in increased T_3 uptake, both in water-injected and mRNA-injected oocytes. In the presence of sodium, the mRNA induced T_3 uptake increases from 2.46 ± .041 to 5.51 ± 1.21 by increasing the substrate concentration two-fold.*
*** = $p<0.001$, # = $p< 0.005$*

Figure 3 shows the increase in [^{125}I]-T_3 uptake by mRNA-injection into oocytes obtained from frogs in the Rotterdam laboratory. Oocytes were injected with rat liver mRNA (preparation O, 50 ng/oocyte), *rBAT* cRNA (1 ng/oocyte), or *mtr* cRNA (1 ng/oocyte), and incubated for 4 days at 18 ^{0}C. Oocytes were analysed for [^{3}H]-l-Arg uptake, as shown in Figure 1, or for [^{125}I]-T_3 uptake, as shown in Figure 3. Uptake solutions contained 10 µCi [^{125}I]-T_3 (5 nmol/l total T_3) and no protein, both in the presence and absence of sodium. Uptake of [^{125}I]-T_3 in the presence of sodium increased from 2.23 ± 0.27 to 4.07 ± 0.83 fmol T_3/oocyte in mRNA-injected oocytes and 3.91 ± 0.66 fmol T_3/oocyte in *mtr* injected oocytes (both $p<0.001$, n=10). In the presence of choline, T_3 uptake increased from 1.91 ± 0.45 to 3.22 ± 0.43 fmol T_3/oocyte in mRNA-injected oocytes ($p<0.001$, n=10), and showed no significant increase in *mtr* injected oocytes (2.52 ± 0.70 fmol T_3/oocyte). Both mRNA-injected oocytes as well as *mtr* injected oocytes showed a significant decrease in T_3 uptake in the absence of sodium (4.07 ± 0.83 to 3.22 ± 0.43 fmol T_3/oocyte for mRNA-injected oocytes, $p<0.01$, n=10, and 3.91 ± 1.66 to 2.52 ± 0.70 fmol T_3/oocyte for *mtr* injected oocytes, $p<0.001$, n=10).

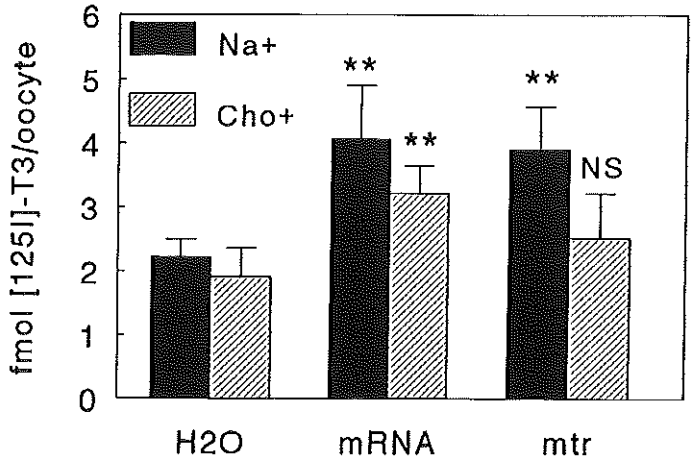

Figure 3 *[^{125}I]-T_3 uptake by rat liver mRNA and mtr cRNA-injected oocytes. Cells were injected with 50 ng per oocyte rat liver mRNA or 1 ng per oocyte mtr cRNA, incubated for 4 days, and analysed for T_3 uptake in the presence and absence of sodium using 10 µCi [^{125}I]-T_3 per ml. Rat liver mRNA-injected oocytes showed a significant increase in T_3 uptake as compared to water-injected oocytes, both in the presence and absence of sodium. Mtr cRNA-injected oocytes showed a significant increase in the presence of sodium. In the absence of sodium no significant increase of T_3 uptake was found.*
*** = $p<0.001$, NS = not significant*

Conclusions

In all independent experiments in both laboratories, injection of rat liver mRNA resulted in an increase in [^{125}I]-T_3 uptake as compared to water-injected oocytes. The best results were obtained using the Cs-TFA RNA isolation kit (RNA extraction kit, Pharmacia P-L Biochemicals, Uppsala, Sweden) followed by poly A+ selection using the mRNA separator kit (Clontech Laboratories, Palo Alto, USA).

Comparison of different experiments using the same mRNA preparation shows an uptake of approximately 4 to 6 fmol T_3 per oocyte per hour at a substrate concentration of 5 nmol/l [^{125}I]-T_3 in the absence of protein (59.54 fmol T_3/10 oocytes, Table I; 55.72 fmol T_3/10 oocytes, Table II; 4.51 ± 1.18 fmol T_3/oocyte, Fig. 2; and 4.07 ± 0.83 fmol T_3/oocyte, Fig. 3). In most experiments this is twice as high as the uptake in water-injected oocytes.

Increased substrate concentration results in an increase in [^{125}I]-T_3 uptake. This is confirmed by the experiments performed in the presence and absence of protein. In the absence of albumin the free T_3 concentration increases, resulting in an increased [^{125}I]-T_3 uptake. Increasing the substrate concentration in protein-free uptake solution from 5 nmol/l to 10 nmol/l not only increases the [^{125}I]-T_3 uptake by mRNA-injected oocytes, but also of water-injected oocytes (Fig.2). This is most likely due to an increase in aspecific extracellular binding resulting from the increased substrate concentration, and to an increase in [^{125}I]-T_3 uptake by already present transport mechanisms. Nevertheless, the mRNA induced [^{125}I]-T_3 uptake increases from 2.46 ± 0.41 to 5.51 ± 1.21 (mean ± SEM, n=10 and n=5 respectively) by increasing the substrate concentration from 5 to 10 nmol/l total T_3 (protein-free).

The effect of the presence or the absence of sodium in the uptake solution remains unclear. Although all incubations show a decrease in [^{125}I]-T_3 uptake in the absence of sodium, including water-injected oocytes, most of these decreases are not significant. In addition, all mRNA-injected oocytes differ significantly from water-injected oocytes, even in the absence of sodium (Figs. 2 and 3). This suggest the presence of different transport mechanisms with different sodium dependence. In addition, these different transport mechanisms are also present in water-injected oocytes.

Finally, we have one experiment suggesting an increase in [^{125}I]-T_3 uptake by *mtr* cRNA. Although these results have to be reproduced in order to be conclusive, the data seem promising. Possibly the tryptophan transporter is one of the sodium dependent transport mechanisms responsible for the cellular entry of T_3 in this system.

As we always find very high binding of [^{125}I]-T_3 to damaged oocytes (data not shown), we propose that total cellular binding of T_3 to *Xenopus laevis* oocytes is not limited by intracellular binding capacity. Therefore we feel confident that the increase in [^{125}I]-T_3 uptake is due to an increase in cellular uptake, as opposed to an increase in intracellular binding. Future experiments will be conducted in order to confirm cellular entry of the hormone, as the presented data can also be explained by an increase in extracellular

binding, e.g. expression of an extracellular receptor. Of course, identification of such an extracellular receptor would also be of great interest.

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Chapter 6

General Discussion

General Discussion

This general discussion consists of two parts divided into three separate paragraphs. The first two paragraphs discuss the results described in this thesis, whereas the third and last paragraph is an extension of the introduction focusing on the role of the thyroid hormone transport protein in thyroid hormone bioactivity.

6.1 Summary of the initiated cloning strategies

The studies described in this thesis were performed in order to provide a basis for cloning of the protein that transports thyroid hormones from plasma into the cell. Based on the genetic code of this protein, tissue specific expression and regulation of gene expression can be studied. It also offers the possibility to study structure function relationships, by altering the genetic code of the protein and relating the changes to the biological characteristics of the expressed protein. Ultimately, specific thyroid hormone uptake antagonists could be designed based on the three-dimensional structure of the thyroid hormone transport protein.

Cloning of the thyroid hormone transport protein would also offer a new and powerful tool to increase our understanding of thyroid hormone bioactivity in general, and its regulation in health and disease in particular. It would give new insights in the complex relationships between hypothalamo-pituitary-thyroid control of serum thyroid hormone concentrations, extra-thyroidal deiodination and conjugation of thyroid hormones, and the role of trans-plasma membrane transport in all of these processes.

Based on the known biological characteristics of the thyroid hormone transport protein, four independent cloning strategies were proposed (Chapter 1. Introduction). The first strategy was based on overexpression of the transport system in its 'normal environment', i.e. the hepatic transport protein in the hepatocyte system. We decided to use human liver derived HepG2 cells, as previous experiments in our laboratory suggested low transport of iodothyronines in these cells. In Chapters 2.1 and 2.2 the characterisation of these cells is described, whereas Chapter 2.3 describes the establishment of so-called permissive HepG2 cells.

Chapter 2.1 describes the absence of iodide production from T_3 by HepG2 cells. In our laboratory, the secretion of iodide into medium is often used to monitor transmembrane transport of thyroid hormones [1-3]. After cellular entry, thyroid hormones are deiodinated by deiodinases, or conjugated by glucuronyl-transferases and sulfotransferases. In fact, these processes are closely interrelated, as for example for T_3. The deiodination of this hormone is preceded by sulfation, which increases the Vmax for deiodination by type I deiodinase almost 30-fold. Inhibition of type I deiodinase results in accumulation of T_3 sulfate, which can be analysed in the medium of the cells. Glucuronidation of T_3 results in

the stable metabolite T_3G, which is also secreted into the medium [4].

Using human liver derived HepG2 cells, only very little amounts of iodide were produced using T_3 as a substrate. Cellular entry of T_3 was confirmed by nuclear binding of this hormone. Incubations of HepG2 cell homogenates using rT_3 as a substrate confirmed the presence of functional type I deiodinase. Using T_3 as a substrate, there was no accumulation of conjugates in the presence of propylthiouracil (PTU), an inhibitor of type I deiodinase. This lead us to conclude that there is a deficient sulfation of T_3 in HepG2 cells, in spite of previous reports showing sulfation of other compounds in these cells. The lack of sulfation decreases the rate of deiodination of T_3, and the iodide production found in HepG2 cells most likely results from direct deiodination of T_3 by type I deiodinase.

Additional experiments using T_3 and reverse-T_3 (rT_3) as substrates by HepG2 cells are described in Chapter 2.2. Time curves of total cellular binding and nuclear binding of T_3 in the presence and absence of ouabain, a compound that interferes with the sodium gradient and which is known to inhibit thyroid hormone transport into rat hepatocytes [5], showed a much more pronounced effect at the nuclear level. This suggest an aspecific component in the total cellular binding data.

Incubations of HepG2 cells using rT_3 as a substrate showed considerable iodide production. This hormone is directly deiodinated by type I deiodinase, without prior sulfation. Comparison with primary cultures of rat hepatocytes revealed an approximately 10-fold lower rate of iodide production in HepG2 cells, which might reflect species differences in metabolic rate in general. Therefore we compared 90 min incubations of rat hepatocytes to 17 hour incubations of HepG2 cells. Iodide production was decreased in the presence of ouabain and in the presence of PTU. Again, there was no increase in conjugates in the presence of PTU, in contrast to rat hepatocytes. There was, however, a relatively high rT_3 glucuronides production in control incubations, indicating the presence of glucuronyl transferase activity in HepG2 cells.

Summarising all HepG2 uptake and metabolism data, these cells closely resemble human hepatocytes, which were previously studied in our laboratory [3]. There is normal cellular entry of T_3, but hardly any deiodination due to lack of sulfation. Cellular entry of rT_3 is also present, resulting in intracellular deiodination and iodide secretion into the medium. The uptake and metabolism of thyroxine (T_4) was also studied, but required too long incubation times to enable measurement of T_4 metabolism. In view of the observed 10-fold difference in metabolic rate as compared to rat hepatocytes, HepG2 cells would require a 10 day incubation with [^{125}I]-T_4, as the standard incubation time for rat hepatocytes using T_4 as a substrate is 20 hours.

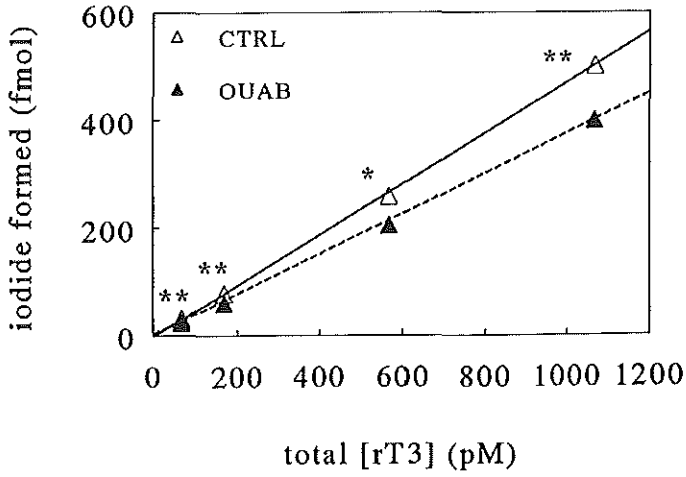

Figure 1 *Increase in iodide production with increased extracellular rT_3 concentration. Human liver derived HepG2 cells were incubated with varying concentrations of rT_3 in the presence and absence of ouabain. Iodide production was measured after 17 hr incubation by LH20 chromatography of cell culture media. Incubations in the presence of ouabain showed significantly lower iodide production for all rT_3 concentrations. Data are expressed as mean ± SD. ** = p<0.001, * = p<0.01*

Although the presence of thyroid hormone transport proteins in HepG2 cells was not favourable with respect to using these cells for expression of the exact same protein, we did test whether an increase in cellular entry of thyroid hormone would increase iodide production. For this purpose rT_3 had to be used as a substrate, as T_3 is not deiodinated. Cellular entry was increased by increasing extracellular rT_3 concentrations in the presence and absence of ouabain. Figure 1 shows the linear increase in the absolute amount of iodide produced with increasing extracellular rT_3 concentrations. This implies that, with these concentrations, intracellular type I deiodinase is not limiting in the amount of iodide produced. Also, the lower iodide production in the presence of ouabain confirms the involvement of a sodium dependent transport system. Based on these data we suggest that if overexpression of the thyroid hormone transport protein results in increased intracellular rT_3 concentrations, this in turn would result in increased iodide production, which can routinely be observed in the medium of these cells.

Therefore, HepG2 cells can be used for the expression cloning strategy as described in Chapter 2.3. For this strategy, permissive HepG2 cells had to be prepared [6]. In order to transfect DNA into HepG2 cells, we have used the calcium phosphate precipitation technique. This technique was optimised using the pTKGH vector, which codes for human growth hormone. The production of growth hormone by transfected cells is used to quantify transfection efficiency. Using the optimised calcium phosphate precipitation protocol, 12 neomycin resistant and therefore potentially EBNA-1 expressing clones were isolated. It

should be realised, however, that neomycin resistance and EBNA-1 expression are encoded by two independent vectors which were co-transfected. All neomycin resistant clones have to be screened for episomal replication of the eukaryotic pDR2 vector by transfecting each single clone with this vector and selecting for hygromycin resistance. EBNA-1 expressing cells should show higher efficiencies in producing stable hygromycin resistant clones, as EBNA-1 allows episomal replication of the pDR2 vector [6]. However, due to more promising results using the oocyte strategy (Chapter 5), these experiments were postponed.

The second strategy was based on purification of the thyroid hormone transport protein, in order to allow protein microsequencing. We decided to use a new technique of plasma membrane isolation, as this technique is not only very rapid, but also reduces the contamination with endoplasmic reticulum as compared to the standard techniques using gradient centrifugation. The technique is based on the chemical interaction between the negatively charged phospholipid heads of the plasma membrane lipid bilayer and the positively charged residues of special beads [7]. These beads are attached to intact cells, before any endoplasmic reticulum can be exposed to the beads surface. Only after blocking of the unoccupied beads surface by heparin the cells are lysed and cellular contents, including endoplasmic reticulum, is washed away. The beads remain with small fragments of highly purified plasma membrane.

Chapter 3 describes the plasma membrane isolation technique, as applied to human liver derived HepG2 cells. In Chapters 2.1 and 2.3, the presence of T_3 transport has been shown, indicating the presence of this protein in HepG2 plasma membranes. Purified plasma membranes showed no glucose-6-phosphatase activity, confirming the absence of endoplasmic reticulum, and an increase in 5'-nucleotidase, suggesting enrichment of plasma membranes [8, 9]. Solubilised plasma membranes were covalently labelled using N-bromo-acetyl T_3 and T_4 ($BrAcT_3$ and $BrAcT_4$), in order to identify the transport protein. SDS-PAGE and autoradiography revealed the presence of a 40 kD protein band which was labelled by both affinity labels, and a 65 kD band labelled only by $BrAcT_3$. No major band could be identified in the 27 kD range, giving additional evidence for the absence of type I deiodinase and therefore endoplasmic reticulum contamination.

Combining these data with the experiments described in Chapter 4, the 65 kD protein might be a good candidate for the T_3 transport protein. Chapter 4 describes the characterisation of several antibody preparations. Although most of these antibodies are no longer available in the laboratory or do not recognise antigens on denaturing Western blots, the Lillith antiserum seems very useful. It inhibits total cellular binding and nuclear binding of T_3 in HepG2 cell incubations, and recognises a protein of similar molecular weight as the protein which was labelled by $BrAcT_3$. In addition, it recognises the protein in its denatured form, and even $BrAcT_3$ labelling does not interfere with epitope recognition. Therefore it is not unlikely that it is able to recognise the transport protein if it is expressed in a bacterial system, such as for example a λgt-11 expression library. Even though it recognises several

other proteins, it offers good possibilities to isolate the thyroid hormone transport protein.

The reason for preliminary interruption of all other strategies is described in Chapter 5. Using injection of rat liver mRNA we were able to increase the total cellular binding of T_3. Previous experiments in our laboratory showed very high T_3 binding to damaged oocytes, suggesting a high intracellular binding capacity. The rat liver mRNA-induced increase of total cellular binding therefore has to be accounted for by an increase in cellular entry of the hormone, and thus to either an increased expression of a transport protein, or expression of an extracellular binding protein or receptor.

In water-injected oocytes, a thyroid hormone uptake system seems to be present. Part of this uptake system is sodium dependent, as substitution of sodium by choline always results in decreased total cellular binding of T_3. Injection of rat liver mRNA increases both the sodium dependent as well as the sodium independent system(s). In spite of the fact that water-injected oocytes also express thyroid hormone transport protein(s), the almost two-fold increase by rat liver mRNA should be sufficient to allow the construction of a cDNA library from size-fractionated mRNA. Screening of pools of this library should finally result in individual clones, some of which might express sodium dependent or sodium independent transport of thyroid hormone. Screening using T_3 or T_4 as a substrate might also differentiate between the thyroid hormone transport inducing clones.

Summarising all proposed strategies, the *Xenopus laevis* strategy seems to be the most promising. It not only gives direct biochemical and molecular biological evidence for the presence of a thyroid hormone transport protein coding mRNA in rat liver, but also offers the possibility to identify clones of a cDNA library based on the functional expression of this protein.

6.2 Disadvantages of the proposed strategies

The pDR2 strategy has as major disadvantages that the HepG2 cells already show intrinsic transport of thyroid hormones. Therefore it will be difficult to detect minor increases in iodide production resulting from increased cellular entry of the substrate. Although the *Xenopus laevis* oocytes also show intrinsic transport of thyroid hormone, this system already gives an indication of the increase in thyroid hormone uptake in the first phase of the strategy. The pDR2 system will only give results after plating of the entire cDNA library and transfection of HepG2 cells with pools of this library. In addition, it is difficult to estimate the dilution of cDNA clones for each transfection experiment. In contrast to the *Xenopus* system, it is not possible to start with transfecting the entire pool of cDNAs. Therefore, a compromise has to be made between the number of bacterial plates one can use per experiment, and the number of colonies which can be transfected into one population of HepG2 cells before losing the signal. Also, if the transport protein is composed of different subunits, the screening of subdivided pools might abolish the expression of functional thyroid hormone transport protein. Of course this is also true for the *Xenopus* system, but

this can be circumvented. By injecting the total pool of mRNA, the presence of functional protein can be tested. If size fractionation results in loss of the signal, the presence of different subunits of different sizes can be postulated. Combining the different size fractionated fractions should result in reconstitution of the functional thyroid hormone transport activity. Although laborious, this procedure can be maintained throughout the entire strategy.

The major disadvantages of the plasma membrane isolation technique are the very low recovery of plasma membrane proteins and the difficulties associated with designing degenerate oligonucleotide probes. Fist of all, sufficient highly purified protein has to be provided in order to determine part of the amino acid sequence. Even though it is very well possible that the 65 kD band labelled by $BrAcT_3$ represents a single protein, there could still be a very low amount of protein present in this band. The autoradiograph only shows a signal after 72 hours of incubation, and no proteins can be seen on the corresponding SDS-PAGE gel. As the current methods of protein microsequencing need fmole amounts of protein blotted onto protein binding filters, it might be very difficult to achieve these concentrations of pure protein.

Even if this would be possible, the protein microsequencing will result in a partial amino acid sequence. Based on these data oligonucleotide probes can be synthesised. It should be realised however, that many amino acids have different codons. The amino acid leucine for example is coded for by UUA UUG CUU CUC CUA CUG. Therefore, one amino acid sequence might result in many different oligonucleotide probes. Screening of a library using these probes can subsequently result in many completely unrelated clones, and clones which code for only part of a protein. Therefore, all of the identified clones have to be tested in an expression system, to confirm their ability to code for the thyroid hormone transport protein, based on functional criteria.

Raising antibodies against the 65 kD plasma membrane protein seems irrelevant at the moment, as the Lillith antiserum is already available for this approach. This antibody is characterised on functional criteria, and can be used to screen a bacterial expression library. Nevertheless, some unrelated proteins might be recognised, resulting in false positive clones. Therefore, all identified clones should be tested in an expression system to confirm their ability to express functional thyroid hormone transport protein. This will also exclude clones which code for only (part of) the antigenic epitope of the protein. The main advantage of this strategy is that it is the least laborious and therefore the most inexpensive approach.

The *Xenopus laevis* strategy has as major disadvantage the risk of separating different subunits if the functional thyroid hormone transport protein would be a hetero-polypeptide complex. An additional problem is the apparently inevitable seasonal variation in oocyte quality. This latter problem has appeared during the introduction of the system in our laboratory, and was circumvented by performing the experiments in Dr. Murers laboratory, Zurich, Switzerland. In addition, the frogs are sensitive to environmental

changes, such as new housing. An acclimatisation time of several months has to be taken into account. Also the distinction between expression of an extracellular binding plasma membrane protein and a trans-plasma membrane transport protein has to be made. However, both this distinction and the potential subunit problem are common to all proposed cloning strategies. The presence of intrinsic transport of thyroid hormones is unfortunate, but not in conflict with this strategy. The mRNA-induced increase in T_3 uptake should be sufficient to allow construction and screening of a cDNA library.

6.3 The role of the thyroid hormone transport protein in thyroid hormone bioactivity

In the introduction of this thesis, the role of transmembrane transport of thyroid hormones in thyroid hormone bioactivity has been discussed in order to illustrate the need for elucidation of the genetic code of this protein. It is now generally accepted that thyroid hormones cross lipid bilayers through an energy dependent and stereospecific transport system [10]. Although there is accumulating evidence for extranuclear effects of T_3 and possibly T_4 [11-14], most of the biological effects of thyroid hormones are mediated through binding of T_3 to its nuclear thyroid hormone receptor [15-17].

Figure 2 schematically illustrates the cellular events involved in regulation of thyroid hormone bioactivity. In addition to regulation of thyroidal secretion of thyroid hormones and the effects of circulating compounds on binding of thyroid hormones to serum proteins, these intracellular events are extremely important with respect to thyroid hormone bioactivity.

In the introduction of this thesis, most of these regulatory processes have been described, except for the cytosolic binding proteins. There are only few reports describing the presence of intracellular transport of thyroid hormone [18-23], in spite of its potential importance for thyroid hormone bioactivity. In fact, its importance seems to be as much underrated as transmembrane transport of thyroid hormones once was.

In order to understand the mechanisms involved in the generation of the so-called low T_3 syndrome, which often occurs in man during acute or chronic illnesses not related to the thyroid gland, all of the individual steps involved in thyroid hormone bioactivity have to be considered. Even though one particular step may not be rate limiting for thyroid hormone bioactivity in one particular physiological condition, this may very well be so in other circumstances. In serum of non thyroidal illness (NTI) patients, for example, several compounds have been identified which inhibit the cellular entry of thyroxine. Even though these compounds do not affect the deiodinase enzyme, deiodination of thyroxine to bioactive T_3 is reduced as a result of decreased substrate availability for the deiodinase enzyme [24-26]. In addition, the experiments described in Chapters 2.2 and 4 of this thesis suggest that only minor effects on total cellular uptake of thyroid hormone may have pronounced effects on intracellular thyroid hormone concentrations, and therefore on biological activity.

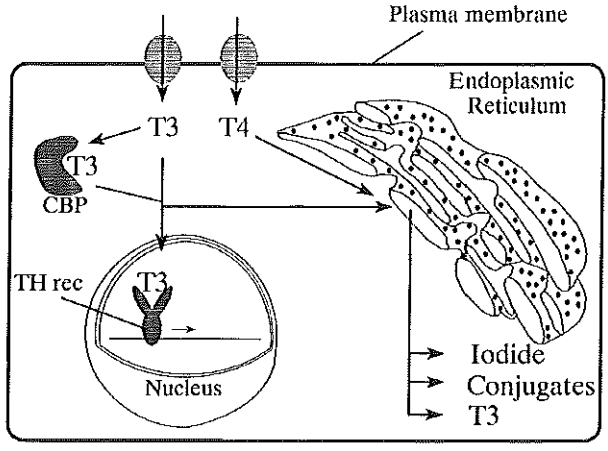

Figure 2 *Schematic overview of the cellular events involved in regulation of thyroid hormone bioactivity. Thyroid hormone enters the cells by an energy dependent transport system. In the cytoplasm it is bound by cytoplasmic binding proteins (CBP), and transported to the nucleus or the endoplasmic reticulum. In the nucleus, T_3 binds with its nuclear receptor and alters the transcription of thyroid hormone responsive genes. In the endoplasmic reticulum, thyroid hormones are deiodinated or conjugated, resulting in the production of iodide, conjugates, and 'lower' iodothyronines. Deiodination of T_4 results in the production of bioactive T_3. In brain this is the major source of intracellular T_3, whereas in man the hepatic deiodination of T_4 is responsible for upto 80% of total serum T_3 production.*

The often unchanged TSH concentrations during NTI can also be explained according to the schematic overview shown in Figure 2. In our laboratory it has recently been shown that the compounds that inhibit cellular entry of thyroxine in rat hepatocytes have no effect on cellular entry of this hormone in primary cultures of rat pituitary cells [27]. In addition, pituitary cells contain the type II deiodinase enzyme, which is responsible for intra-pituitary T_3 production. In contrast to the hepatic type I deiodinase enzyme, which shows down-regulation of enzyme activity in NTI, the type II deiodinase activity increases under these conditions. Therefore, the combination of normal serum T_4 levels with unaffected cellular entry of T_4 and increased intracellular deiodination could, despite the low serum T_3, result in normal intra-thyrotrope T_3 levels, and therefore unaffected TSH secretion [14, 28, 29]. It should be noted however, that the increased intrapituitary type II deiodinase activity in hypothyroidism is mainly attributed to increases in somatotrope- and lactotrope type II deiodinase activity, and not to thyrotrope type II deiodinase activity [30].

The sequence of events which result in the generation of the low T_3 syndrome may be different in rat and human. Also, the initiation of the low T_3 syndrome during fasting and NTI may be different.

In rat, the decreased serum T_3 concentration found in fasting is primarily due to diminished thyroidal secretion of thyroxine [31]. This results in hypothyroidism, which in its turn results in decreased hepatic type I deiodinase activity and increased pituitary type II deiodinase [14, 32].

In man, however, no changes are found in serum T_4 concentration during fasting [33], whereas serum T_3 is decreased and serum rT_3 is increased. Serum free fatty acids, preferably called non-esterified fatty acids or NEFA, are increased. In this case, the hypothyroidism is initiated by a decrease in hepatic type I deiodinase activity. This is caused by a decreased availability of intrahepatic reduction equivalents due to increased fatty acid metabolism. Reduction equivalents are essential for the regeneration of the type I deiodinase enzyme following the oxidative deiodination of thyroid hormone. Therefore, reduced availability of reduction equivalents will result in reduced type I deiodinase activity. In addition, it has been shown that NEFA inhibits transport of thyroxine from plasma into hepatocytes, resulting in decreased substrate availability for the intracellular deiodinase enzyme [25, 26]. Both mechanisms result in decreased serum T_3 and increased serum rT_3. These effects are amplified by the subsequent decrease in hepatic type I deiodinase activity and increase in pituitary type II deiodinase activity resulting from hypothyroidism in general.

The low T_3 syndrome during NTI in man may be initiated by the accumulation of thyroid hormone transport-inhibiting compounds in serum. In uraemic patients, for example, serum concentrations of CMPF and indoxyl sulfate are sufficiently high to inhibit transport of thyroxine from plasma into the hepatocyte [24]. This will decrease peripheral T_3 production, reducing serum T_3 concentration, and subsequently resulting in decreased hepatic type I deiodinase and increased pituitary type II deiodinase activities. In non-uraemic patients, a similar sequence of events is initiated by the accumulation of bilirubin and NEFA [25]. In rats there is, as to now, no proper model for NTI. Nevertheless, the previously mentioned thyroid hormone transport inhibition by CMPF, indoxyl sulfate, bilirubin, and NEFA have been shown using primary cultures of rat hepatocytes [24-26].

In order to resolve the very complex interactions between the different tissues involved in thyroid hormone bioactivity, a better understanding of the mechanisms involved in transmembrane transport of thyroid hormone is essential. Therefore, cloning of the thyroid hormone transport protein would offer a very useful tool to study the role of thyroid hormone transport in the regulation of thyroid hormone bioactivity. It offers the possibility to study the expression of this protein during different (patho-) physiological conditions, and will give more insight in possible tissue or species differences. Ultimately, thyroid hormone uptake antagonists can be designed in order to mimic the beneficial effects of the low T_3 syndrome during any form of non-thyroidal disease.

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Summary

Summary

The work presented in this thesis provides a basis for the cloning of the protein that transports thyroid hormones from the exterior into the cell. As thyroxine (T_4), the main secretory product of the thyroid gland, has only little intrinsic biological activity, most of thyroid hormone bioactivity depends on extra-thyroidal deiodination of T_4 to biologically active 3,5,3'-triiodothyronine (T_3). The liver is the major source of peripheral T_3 production in man. In addition to some direct effects of thyroid hormone, most biological effects are mediated through binding of T_3 to its nuclear receptor followed by changes in expression of thyroid hormone responsive genes.

As both the deiodinase enzymes and the thyroid hormone receptors are localised intracellularly, thyroid hormones have to cross the lipid bilayer of the plasma membrane in order to be deiodinated or to exert their biological effects. Therefore, transport of thyroid hormone across the plasma membrane may play an important role in the regulation of thyroid hormone bioactivity.

Cloning of the thyroid hormone transport protein would offer a very useful tool to study the role of thyroid hormone transport in this regulation of thyroid hormone bioactivity. It offers the possibility to study the expression of this protein during different (patho-) physiological conditions, and will give more insight into possible tissue or species differences. Ultimately, thyroid hormone uptake antagonists could be designed, which have potential use for the acute treatment of hyperthyroidism.

Based on the known biological characteristics of the transport protein, four independent cloning strategies were proposed.

Chapter 2 describes the pDR2 eukaryotic expression vector strategy, in which the hepatic thyroid hormone transport protein is (over-) expressed in a hepatocyte system, i.e. human liver derived HepG2 cells. An increase in transmembrane transport would increase intracellular thyroid hormone concentrations, and thereby increase substrate availability for intracellular deiodination. This can be monitored routinely using LH-20 chromatography of cell culture supernatants. Chapters 2.1 and 2.2 describe the characterisation of 'wild-type' HepG2 cells with respect to thyroid hormone uptake and metabolism, whereas Chapter 2.3 described the establishment of permissive HepG2 cells for the pDR2 expression system.

Chapter 3 describes the purification of the putative thyroid hormone transport protein by isolation of HepG2 plasma membranes. Using a new technique based on polycationic beads, a 65 kD protein was identified using the covalent affinity label N-bromoacetyl T_3. Amino acid sequencing of this protein offers the possibility to deduce part of the nucleotide sequence, and synthesise oligonucleotide probes accordingly. Alternatively, antibodies can be raised against this protein.

Chapter 4 describes the characterisation of several antibodies which are available in our laboratory. One antiserum, named Lillith, recognised a protein of approximately 65 kD,

and inhibited transport of T_3 in human liver derived HepG2 cells. This antiserum can be used to screen a bacterial expression library, e.g. a λgt11 library.

Chapter 5 describes the expression of T_3 transport in *Xenopus laevis* oocytes by injection of rat liver mRNA. These huge cells can be injected manually with the entire pool of mRNAs, and screened individually. Injection of rat liver mRNA resulted in an almost twofold increase in [^{125}I]-T_3 uptake, which was partly sodium dependent. Using size fractionation, an enriched cDNA library can be constructed. This cDNA library can than be screened by injection of cRNA into *Xenopus laevis* oocytes, or can be used for any of the other strategies.

As the *Xenopus* strategy has as major advantage that the functional criterium of thyroid hormone transport is used for screening, and that this signal is already obtained in the first phase of the expression cloning procedures, it seems to be the most promising strategy at the moment. Although there are some serious disadvantages, such as the possibility of separating essential subunits during the size fractionation or the cDNA library construction, these disadvantages are common to all proposed strategies. Therefore, all efforts to clone the thyroid hormone transport protein are directed towards the *Xenopus* expression system.

Samenvatting

Samenvatting

Middels het in dit proefschrift beschreven werk is een basis gelegd voor de clonering van het eiwit dat schildklierhormoon van buiten de cel naar binnen transporteert. Omdat thyroxine (T_4), het voornaamste produkt van de schildklier, zelf slechts weinig biologische activiteit heeft, berust het merendeel van de schildklierhormoon bioactiviteit op extra-thyreoidale dejodering van T_4 naar het biologisch actieve 3,5,3'-triiodothyronine (T_3). De lever is de belangrijkste plaats van perifere T_3 produktie in de mens. Naast enkele directe effecten van schildklierhormoon, berusten de meeste biologische effecten op binding van T_3 aan een nucleaire receptor, gevolgd door veranderingen in genexpressie van schildklierhormoon gevoelige genen.

Omdat zowel de dejoderende enzymen als de schildklierhormoon receptoren binnen in de cel liggen, moet schildklierhormoon de plasma membraan passeren alvorens gedejodeerd te kunnen worden of zijn biologisch effect uit te kunnen oefenen. Daarom kan transport van schildklierhormoon over de plasma membraan een belangrijke rol spelen in de regulatie van schildklierhormoon bioactiviteit.

Het cloneren van het schildklierhormoon transport eiwit levert een belangrijk hulpmiddel voor de bestudering van de rol van dit eiwit in de regulatie van schildklierhormoon bioactiviteit. Het biedt de mogelijkheid om de expressie van dit eiwit te bestuderen tijdens verschillende (patho-) fysiologische condities, en geeft meer inzicht in mogelijke weefsel- en species verschillen. Op de langere termijn is het wellicht mogelijk om specifieke antagonisten van schildklierhormoon opname te maken, welke gebruikt zouden kunnen worden voor de acute behandeling van hyperthyreoidie.

Op basis van reeds bekende biologische karakteristieken van het transport eiwit zijn vier onafhankelijke clonerings strategieën voorgesteld.

Hoofdstuk 2 beschrijft de pDR2 eukaryote expressie vector strategie, waarbij de schildklierhormoon transporter uit de lever tot (over-) expressie wordt gebracht in een levercel systeem, nl. de humane lever cellijn HepG2. Een toename in transmembraan transport zal de intracellulaire schildklierhormoon concentratie doen toenemen, en daardoor de beschikbaarheid van substraat voor intracellulaire dejodering verhogen. Dit kan routinematig gemeten worden door LH20 chromatografie van celcultuur supernatanten. De Hoofdstukken 2.1 en 2.2 beschrijven de karakterisatie van 'normale' HepG2 cellen met betrekking tot schildklierhormoon opname en metabolisme, terwijl Hoofdstuk 2.3 het verkrijgen van 'permissive' ofwel 'ontvankelijke' HepG2 cellen voor de pDR2 expressie vector beschrijft.

Hoofdstuk 3 beschrijft de zuivering van een eiwit dat mogelijk het schildklierhormoon transport eiwit is, door isolatie van HepG2 plasma membranen. Op basis van een nieuwe techniek welke gebruik maakt van polycatione beads, en door middel van covalente labelling met het affiniteit label N-bromoacetyl T_3 werd een 65 kD eiwit geïdentificeerd.

Bepaling van de aminozuur volgorde van dit eiwit biedt de mogelijkheid een gedeelte van de nucleotide volgorde te herleiden, om daar vervolgens oligonucleotide probes tegen te maken. Het is ook mogelijk antilichamen op te wekken tegen dit eiwit.

Hoofdstuk 4 beschrijft de karakterisatie van verschillende antilichaam preparaten welke in ons laboratorium beschikbaar zijn. Een antiserum, welke de naam Lillith heeft gekregen, herkent een ongeveer 65 kD eiwit en remt het transport van schildklierhormoon in de humane lever cellijn HepG2. Dit antilichaam kan gebruikt worden om een bacteriële expressie bank te screenen, bijvoorbeeld een λgt11 bank.

Hoofdstuk 5 beschrijft de expressie van T_3 transport in *Xenopus* leavis oocyten door injectie van rat lever messenger RNA. Deze zeer grote cellen kunnen handmatig geïnjecteerd worden met de totale populatie van mRNAs, om vervolgens individueel gescreend te worden. Injectie van rat lever mRNA resulteerde in een bijna tweevoudige stijging in [^{125}I]-T_3 opname, welke gedeeltelijk natrium gradiënt afhankelijk was. Door gebruik te maken van grootte selectie van mRNAs kan een verrijkte cDNA bank gemaakt worden. Deze bank kan dan vervolgens gescreend worden door injectie van cRNA in *Xenopus* leavis oocyten, of voor elk van de andere strategieën.

Omdat de *Xenopus* leavis strategie als belangrijkste voordeel heeft dat screening gebaseerd is op het functionele criterium van schildklierhormoon transport, en omdat er al een signaal gevonden wordt in de eerste fase van de expressie clonerings procedure, lijkt het op dit moment de meest veelbelovende strategie. Ondanks een aantal belangrijke nadelen, zoals de mogelijkheid dat essentiële subunits tijdens de grootte selectie of cDNA bank constructie gescheiden worden, zijn deze nadelen ook van toepassing op elk van de andere voorgestelde clonerings strategieën. Daarom word momenteel alle energie voor het cloneren van het schildklierhormoon transport eiwit op de *Xenopus* leavis strategie gericht.

Curriculum Vitae

Paulus Gerardus Johannes van Stralen werd op 20 September 1967 geboren te Nijmegen. Het diploma Voorbereidend Wetenschappelijk Onderwijs werd in 1985 behaald aan het Dukenburg College te Nijmegen. Aansluitend hierop begon hij aan de studie Biologie aan de Katholieke Universiteit Nijmegen waar op 30 Januari 1990 het doctoraal Toegepaste Richting behaald werd. De doctoraalfase omvatte de hoofdrichting Microbiologie (Prof. Dr. Ir. Vogels, Dr. Keltjens) en de nevenrichtingen Toegepast (Dr. Broekkamp, Dr. Jenk, Organon International bv, Dept CNS, Oss), Biochemie (Prof. Dr. van de Ven, Dr. Ir. van den Ouweland), en Human Cytogenetics (Dr. Sheer, Imperial Cancer Research Fund, London, UK).

Per 1 februari 1990 werd hij aangesteld als onderzoeker in opleiding (OIO) door de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO), gebied Medische Wetenschappen. In dit dienstverband is tot juni 1994 onderzoek gedaan op de Afdeling Inwendige Geneeskunde III en Klinische Endocrinologie van het Academisch Ziekenhuis Dijkzigt te Rotterdam. De resultaten van dit onderzoek zijn in dit proefschrift verwerkt.