A mutation which disrupts the hydrophobic core of the signal peptide of bilirubin UDP-glucuronosyltransferase, an endoplasmic reticulum membrane protein, causes Crigler-Najjar type II

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Abstract Crigler-Najjar (CN) disease is caused by a deficiency of the hepatic enzyme, bilirubin UDP-glucuronosyltransferase (B-UGT). We have found two CN type II patients, who were homozygous for a leucine to arginine transition at position 15 of B-UGT1. This mutation is expected to disrupt the hydrophobic core of the signal peptide of B-UGT1. Wild type and mutant B-UGT cDNAs were transfected in COS cells. Mutant and wild type mRNA were formed in equal amounts. The mutant protein was expressed with 0.5% efficiency, as compared to wild type. Mutant and wild type mRNAs were translated in vitro. Wild type transferase is processed by microsomes, no processing of the mutant protein was observed.

Key words: Bilirubin; UDP-glucuronosyltransferase; Signal peptide; Endoplasmic reticulum; Crigler-Najjar

1. Introduction

Bilirubin is the toxic breakdown product of the protoporphyrin part of the heme group of proteins such as hemoglobin and cytochromes. Bilirubin is conjugated with glucuronic acid in the liver, and the resulting water soluble bilirubin glucuronides are excreted into bile, and subsequently the feces. Glucuronidation of bilirubin is catalyzed by the hepatic enzyme bilirubin UDP-glucuronosyltransferase (B-UGT) (EC 2.4.1.17) [1].

Crigler-Najjar disease (CN) is an autosomal recessive inherited disease, caused by the complete or partial absence of B-UGT. CN disease is characterized by high serum levels of unconjugated bilirubin, which, when not treated, ultimately leads to brain damage and death [2]. Two subtypes of CN disease exist; patients with type I CN have no detectable B-UGT activity. Type II CN is less severe, patients have residual B-UGT activity and serum bilirubin levels can usually be lowered by treatment with phenobarbital [2,3].

UDP-glucuronosyltransferases (UGT) are a family of proteins involved in the detoxification of many endo- and exogenous substances. UGTs are anchored to the endoplasmic

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reticulum (ER) membrane by a single hydrophobic helix located near the carboxyl terminus of the protein. A short carboxyl terminal stretch of amino acids is thought to be exposed to the cytoplasm. The bulk of the protein, including the active site, is thought to be oriented towards the lumen of the ER [4,5]. B-UGT is encoded by a complex gene, UGT1, from which at least six different isoforms, UGT1A to UGT1F, are generated by a mechanism of alternative splicing [1,6,5].

Signal peptides fulfil a critical role in the targeting of proteins to the ER and translocation of proteins across the ER membrane. One of the common features of signal peptides is a hydrophobic core of at least six amino acids. The signal recognition particle (SRP) interacts with the hydrophobic region of the signal peptide of the growing nascent polypeptide. The translation stops and the complex of ribosome, SRP, and nascent chain is targeted to the ER. At the ER membrane, SRP interacts with a receptor, and protein synthesis continues with simultaneous translocation of the protein across the ER membrane [7,8].

Rat liver UGTs are processed by microsomes; they are glycosylated, and possess a cleavable signal peptide [9,10]. When a human phenol UGT, the UGT1F gene product, was expressed in *Escherichia coli*, cleavage of a signal peptide by bacterial signal peptidase occurred [11]. Human B-UGT1, the UGT1A gene product, is glycosylated [12], and a signal peptide of 27 amino acids is predicted.

Signal peptide mutations that cause human disease have thus far only been identified in the secretory proteins: preprovasopressin [13], preproparathyroid hormone [14,15], and coagulation factor X [16]. In vasopressin and factor X, mutations were found respectively in the −1 and −3 positions of the carboxyl terminus of the signal peptide. These mutations block the cleavage of signal peptide by signal peptidase but not targeting and translocation across the ER membrane. In parathyroid hormone a mutation has been found which disrupts the hydrophobic core of the signal peptide, and which has been shown to block targeting and translocation across the ER membrane.

We found two CN type II patients who were homozygous for the same signal peptide mutation. The mutation is expected to give rise to a leucine to arginine substitution, at position 15 in the middle of the hydrophobic core of the predicted B-UGT1, UGT1A, signal peptide. CN disease caused by the 15-Arg/B-UGT mutation is inherited in an autosomal recessive manner, and causes a relatively mild form of hyperbilirubinemia. The mutation in the signal peptide of B-UGT inhibits translocation of the nascent chain across the ER membrane.

2. Materials and methods

2.1. Chemicals

Bilirubin and cell dissociation solution was from Sigma, St. Louis, MO, USA. nitroblue tetrazolium, 5-bromo,4-chloro,3-indolyl phosphate, goat anti-mouse immunoglobulins conjugated with alkaline phosphatase were from Biorad, Veenendaal, The Netherlands. Nitrocellulose membranes for Western blotting, 0.45 μm pore size, were from Schleicher and Schuell, Dassel, Germany.

pSVK3 vector was from Pharmacia, Woerden, The Netherlands. Restriction enzymes, T7 capscribe kit, rabbit reticulocyte lysate in vitro translation kit, and dog pancreatic microsomes were from Boehringer, Mannheim, Germany. Tritiated leucine, specific activity 4.4–7.0 TBq/mmol, [^{32}P]ATP, specific activity 110 TBq/mmol, Amplify, and Hybond-N+ membranes for Northern blotting were from Amersham, Amersham, UK. Random primer DNA labelling system was from Gibco BRL, Breda, The Netherlands.

All other chemicals were of analytical grade and were obtained from Merck, Darmstadt, Germany.

2.2. Detection of mutations

Chromosomal DNA from the parents of the CN patients was isolated from blood, and a 1220 bp fragment comprising part of exon 1 of B-UGT1 (UGT1A), and the promoter region, was amplified using PCR. Sequence of the primers:

1 CGTTTCATGAAGGTCTTGGAG

2 GGTCTATATACGACTCGTTC

The signal peptide mutation was initially detected by sequencing of both strands, as described [17]. The presence of the mutation was confirmed using the *MspI* restriction site polymorphism introduced by the mutation. When a *MspI* site is introduced by the mutation, restriction of the PCR fragment with *MspI* yields fragments of 300 and 920 bp.

2.3. In vitro transcription and translation

For the synthesis of mRNA, the cDNA encoding B-UGT1, UGT1A, was cloned in the pSVK3 vector. This vector has a SV40 promoter for eukaryotic expression and a T7 promoter for the generation of mRNAs. Site directed mutagenesis was performed as described previously [17,18].

B-UGT/pSVK3 was linearized with *Hin*dIII, which cuts in the cDNA 400 bp downstream of the termination codon, and capped mRNA was generated using the T7 capscribe kit from Boehringer, Mannheim. In vitro transcribed mRNAs were translated with reticulocyte membranes in the presence of tritiated leucine, according to the instructions supplied by the manufacturer.

SDS PAGE was performed according to standard techniques, as described [3]. The gels were fixed and soaked in Amplify for 20 min, to increase sensitivity. After drying, fluorograms were made by exposing Kodak Biomax MR film (Eastman Kodak, Rochester, NY, USA) for up to 2 weeks to the dried gels.

2.4. Transient expression in COS-7 cells

For the expression studies in COS-7 cells, the B-UGT cDNA was subcloned in the pCMV4 vector [19]. In this vector the expression is driven by the CMV promoter, in addition a translational enhancer is present. Expression levels with pCMV4 were consistently higher than with pSVK3 (results not shown). COS-7 cells were transfected using DEAE dextran as described previously [3]. COS-7 cell homogenates were subjected to SDS PAGE and Western blotting with a monoclonal antibody directed against human UGT1, as described [3]. B-UGT activity was measured using HPLC, as described [3]. Microsomes were prepared from COS-7 cells as described previously [3,12]. β-Galactosidase activity was determined in cell homogenates according to [20]. In all cases mock transfected or untransfected COS-7 cells were used as a control.

RNA was extracted from the COS-7 cells transfected with B-UGT by the method of Chomczinsky et al. [21]. RNAs were stored at −70°C. The RNAs, 20 μg per lane, were separated on a 1.2% agarose gel containing 7% formaldehyde, and stained with ethidium bromide to determine intactness of the ribosomal RNA. The separated RNA was transferred to Hybond-N+ according to [20]. The blots were

hybridized for 16 h with a [^{32}P]ATP labelled 2100 bp B-UGT cDNA fragment, at 65°C in: 0.5 M sodium phosphate buffer, pH 7.5, 7% (w/v) SDS and 1 mM EDTA. Labelling of the cDNA probe with [^{32}P]ATP was performed with a random primer DNA labelling kit from Gibco BRL. After the hybridization, the blots were washed with 0.2×SSC, 0.1% SDS at 65°C. Autoradiographs were made by exposing the blot to Kodak Biomax MR film for 4 h.

3. Results

3.1. Patients

Two unrelated CN type II patients, who were homozygous for the same mutation, were found. A clinical description of both patients was given in [22], where the patients were designated 8 (patient A), and 9 (patient B). This study was approved by the medical ethical committee of the hospital. In the first month of life, patients A and B had serum bilirubin concentrations of 286±44 and 286±16 respectively. Serum bilirubin of the patients currently is 195±2 μM, patient A, and 197±3 μM, patient B. Both patients are treated with phenobarbital, and patient A also receives phototherapy. Treatment with phenobarbital reduced serum bilirubin by 49% and 71% respectively.

Bile of both patients was analyzed with HPLC to determine the presence of bilirubin conjugates [22]. Bile of patient A contained 49% bilirubin monoglucuronide and 10% bilirubin diglucuronide, bile of patient B contained 27% monoglucuronide and 14% diglucuronide. The percentage of unconjugated bilirubin was 41% in patient A, and 59% in patient B. In healthy subjects all bilirubin in bile is conjugated, and more than 70% of bilirubin is present as diglucuronides. The bile analysis of the patients indicates that low B-UGT activity is present in the liver of both patients.

Both patients had a T to G transition at nucleotide 44 of the B-UGT1 gene; this mutation causes a leucine to arginine transition at amino acid 15. In Fig. 1 the nucleotide and amino acid sequences of the postulated signal peptides of wild type B-UGT and 15-Arg/B-UGT are shown. The amino acids comprising the hydrophobic core are underlined. The arginine introduces a positive charge in the hydrophobic core of the mutant signal peptide.

A 1220 bp fragment of the 5' region of the B-UGT1 coding sequence was amplified with PCR, from genomic DNA of the patients' parents. Because the mutation introduces an *MspI* site at position 300 of the PCR fragment, the presence of the mutation could easily be confirmed. Both pairs of parents were heterozygous for the mutation. Since the parents did not have elevated serum bilirubin concentrations, and bile of the parents contained normal amounts of bilirubin glucuronides [22], we concluded that the CN disease caused by the 15-Arg/B-UGT mutation is inherited in a recessive manner.

3.2. Expression of wild type and 15-Arg/B-UGT in COS-7 cells

Expression of 15-Arg/B-UGT in COS-7 cells resulted in low but significant levels of B-UGT activity. Western blotting was performed with the monoclonal antibody WP1 [23], which recognizes human B-UGT. In homogenates of COS-7 cells transfected with wild type B-UGT (lane marked wt), a 51 kDa protein is detected, in homogenates of COS-7 cells transfected with 15-Arg/B-UGT (lane marked mut), no B-UGT could be detected (Fig. 2A). B-UGT assays of homogenates of COS-7 cells transfected with 15-Arg/B-UGT consistently showed a low amount of enzyme activity. To establish

1, Start pre B-UGT

↓

27, end signal peptide

↓

Wild type B-UGT

Met Ala Val Glu Ser Gln Gly Gly Arg Pro <u>Leu Val Leu Gly Leu Leu Leu Cys Val Leu Gly Pro Val Val</u> Ser His Ala

t

atg gct gtg gag tcc cag ggc gga cgc cca ctt gtc ctg ggc c g ctg ctg tgt gtg ctg ggc cca gtg gtg tcc cat gct

g

Met Ala Val Glu Ser Gln Gly Gly Arg Pro <u>Leu Val Leu Gly Arg Leu Leu Cys Val Leu Gly Pro Val Val</u> Ser His Ala
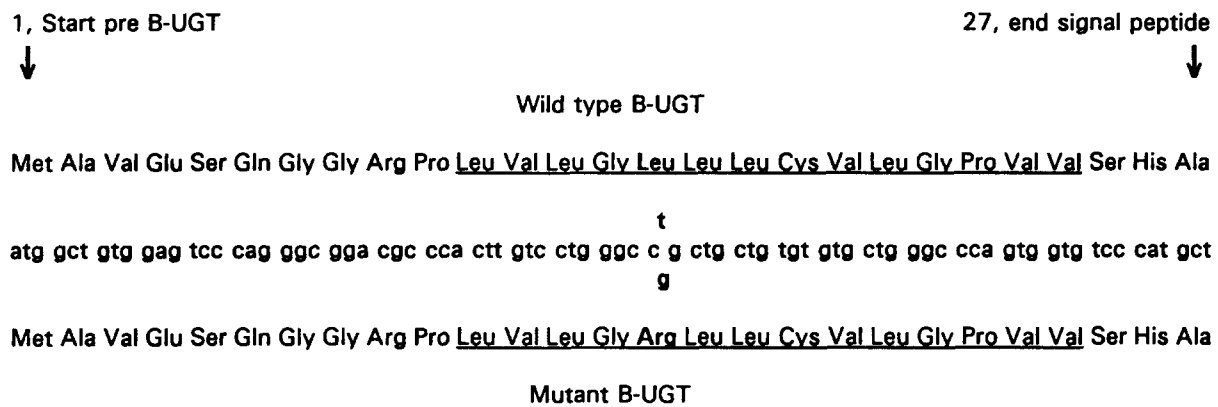
Mutant B-UGT

Fig. 1. Nucleotide and amino acid sequence of the predicted signal peptide regions of wild type and 15-Arg/B-UGT. The residues comprising the hydrophobic core of the signal peptide are underlined.

whether the mutant enzyme was membrane bound, we prepared microsomes from the transduced COS-7 cells. The B-UGT activity was present in the microsomal fraction of the COS-7 cells transfected with wild type and 15-Arg/B-UGT (results not shown). This indicates that the mutant enzyme is present in its normal subcellular localization. In enzyme assays with homogenates from COS-7 cells transfected with wild type B-UGT 15±1% monoglucuronide and 1.6±0.7% diglucuronide were formed. In incubations with 15-Arg/B-UGT, only bilirubin monoglucuronides were formed. To determine the precise amount of residual expression of the 15-Arg/B-UGT protein we performed co-expression studies. Normal or mutant B-UGT cDNAs were expressed in COS-7 cells together with a plasmid containing the *Escherichia coli* β-galactosidase gene. β-Galactosidase and B-UGT activity were measured in COS-7 cell homogenates, and the expression level of B-UGT was corrected for the expression of β-galactosidase. Values were obtained from two independent experiments. Activity of the 15-Arg/B-UGT was 0.5%, with a range of 0.4–0.6%, of the activity of wild type enzyme.

To determine whether the reduced expression of 15-Arg/B-UGT was due to a defect at the transcriptional or translational level, we performed Northern blot analysis of RNA isolated from COS-7 cells transfected with normal or mutant B-UGT (Fig. 2B). B-UGT mRNA was detected using a 2.1 kb probe complementary to B-UGT cDNA. Comparable amounts of B-UGT messenger RNAs were detected in cells transfected with wild type B-UGT, and 15-Arg/B-UGT (lanes marked wt and mut respectively). The defect in the patients with the 15-Arg/B-UGT mutation is therefore not caused by a pretranslational mechanism affecting mRNA levels.

3.3. In vitro transcription and translation of wild type and 15-Arg/B-UGT

Wild type and mutant mRNA were generated by in vitro transcription of pSVK3 vectors which contained the mutant or wild type B-UGT cDNAs. The integrity of the in vitro transcribed mRNAs was confirmed by agarose gel electrophoresis. The mRNAs were translated into the corresponding polypeptides by in vitro translation with rabbit reticulocyte lysates and tritiated leucine. In the absence of microsomes, wild type and mutant B-UGT were translated with the same efficiency, and yielded proteins with identical molecular weights (lanes marked wt and mut respectively). When dog pancreatic microsomes were added to the in vitro translation

mixture, the wild type protein was processed and multiple protein bands were detected (lane marked wt mut). One band of lower molecular weight was observed which could reflect the precursor from which the signal peptide has been cleaved off. The bands of higher molecular weight probably represent intermediate glycosylation products. In vitro translations with the mutant protein showed no processing (lane marked mut mic), and a reduced incorporation of label was consistently seen (Fig. 3). However, if 0.5% of the mutant protein was processed by microsomes, this would probably be below the detection limit of our autoradiograms. The translation products were dependent on the addition of RNA (in the lane marked −), no RNA was added to the translation mixture. When control β-globin mRNA was added to the translation mixture (lane marked +), a protein with the expected molecular mass of 16.5 kDa was generated.

4. Discussion

We have shown that a leucine to arginine transition at amino acid 15, in the middle of the hydrophobic core of the signal peptide of B-UGT, results in a strongly reduced expression of the mutant protein. By expression of wild type and mutant B-UGT cDNA in COS-7 cells, we were able to show that the mutant protein is expressed at a level of 0.5%, compared to that of wild type B-UGT. While transcription of both cDNAs in COS-7 cells was equal, in vitro translation of wild type and mutant mRNAs revealed that the mutant mRNA is not processed by microsomes. The reduced expression of the 15-Arg/B-UGT in the patient is therefore caused by a reduced translation of the mutant mRNA, or by impaired targeting of the mutant protein to the ER, followed by degradation of the mutant protein. In vitro translation of the mutant mRNA, in the absence of microsomes, was normal, but strongly reduced in the presence of microsomes. This suggests that the decreased expression of the mutant protein is caused by an impaired interaction of the mutant protein with the ER translocation machinery. Indirect evidence suggests that the mutant protein is present in the ER; B-UGT activity in COS-7 cells transfected with mutant and wild type B-UGT was recovered in the microsomal fraction. These data correlate with the observations made in the patients. The CN patients homozygous for the 15-Arg/B-UGT mutation had the relatively benign CN type II phenotype. Since bile from the patients contained bilirubin glucuronides, some hepatic B-
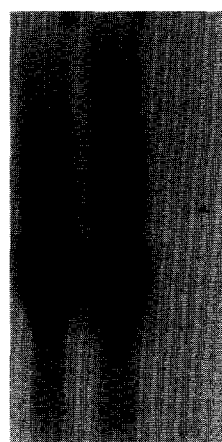
A Western blot

mut   wt   con

51 kDa →
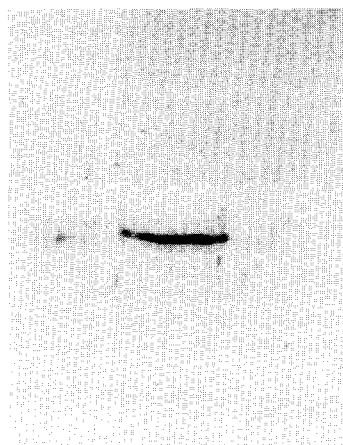
B Northern blot

mut wt con

← 28 S

2.1 kb →

← 18 S

Fig. 2. Western and Northern blots of COS-7 cells transfected with wild type and 15-arg/B-UGT. COS-7 cells were transfected with wild type and 15-Arg/B-UGT, and processed for Western and Northern blotting as described in Section 2. A monoclonal antibody against human UGT was used to detect B-UGT on the Western blot. For detection on the Northern blot a probe comprising the complete B-UGT cDNA, labelled with [^{32}P]ATP, was used. COS-7 cell protein and RNA were isolated from parallel transfections. In the Western blot, 50 μg protein was applied to each lane. In the Northern blot, 20 μg total RNA was applied to each lane: mut, COS-7 transfected with 15-Arg/B-UGT; wt, COS-7 transfected with wild type B-UGT; con, COS-7 mock transfected. Only COS-7 cells transfected with wild type B-UGT show immunoreactivity. Wild type and 15-Arg/B-UGT DNA are transcribed with equal efficiency in COS-7 cells.

UGT expression was present. The observation that the patients can form bilirubin glucuronides is another indication that the residual B-UGT expressed in these patients is present in its normal subcellular localization.

We have previously described a CN type II patient with 4.4% residual activity, who had serum bilirubin levels between 85 μM and 194 μM in the first year of his life. [3]. The patients from this study both had serum bilirubin concentrations of 286 μM in the first year of their lives. In the patients

described in this study, B-UGT expression is therefore probably less than 4.4%, which agrees with the 0.5% residual activity we measured in COS-7 cells.

Three naturally occurring mammalian signal peptide mutations have been described to date. These mutations were found in the secretory proteins, vasopressin, factor X, and parathyroid hormone [13–16]. The signal peptide mutation described in preparathyroid hormone was similar to the 15-Arg/B-UGT mutation, a disruption of the hydrophobic core of the signal peptide. Both in vitro translation and expression of the mutant parathyroid hormone in COS-7 cells showed that the introduction of a single arginine in the hydrophobic core of the signal peptide did not completely inhibit processing. Only when an additional arginine was introduced in the hydrophobic core, processing was abolished completely [15]. In B-UGT, the introduction of a single arginine in the hydrophobic core of the signal peptide already strongly inhibits processing of the mutant protein. This discrepancy could be due to positional effects of the introduction of a positive charge, or to the different nature of the proteins. Besides the fact that parathyroid hormone is a secretory protein and B-UGT a membrane protein, the other obvious difference between parathyroid hormone and B-UGT is the size. Parathyroid hormone has 109 amino acids, and B-UGT is comprised of 533 amino acids. It has been shown that small proteins can be imported cotranslationally into the ER via an SRP/ribosome independent pathway [24]. The relatively high import of the mutant parathyroid hormone as compared to the import of 15-Arg/B-UGT could be due to the fact that the mutant parathyroid hormone can take the SRP/ribosome independent pathway whereas 15-Arg/B-UGT, because of its large size, cannot take this pathway.

[Mw]   –   +   wt   wt   mut   mut
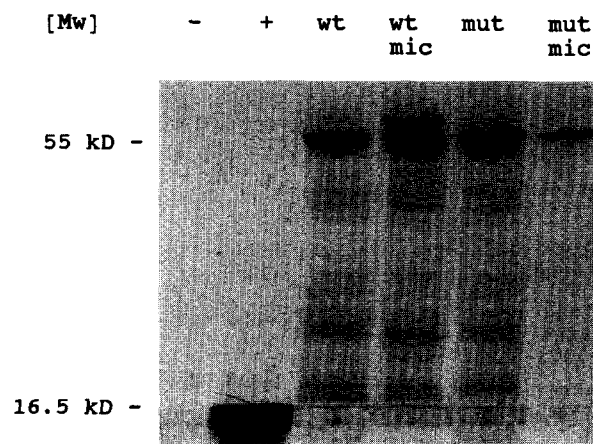   mic   mic

55 kD –

16.5 kD –

Fig. 3. In vitro translation of wild type and 15-Arg/B-UGT mRNA, in the presence and absence of microsomes. Wild type and mutant capped B-UGT mRNAs were translated and analyzed with SDS PAGE and autoradiography, as described in Section 2. –, no RNA; +, control β-globin mRNA; wt, wild type B-UGT mRNA; mut, 15-Arg/B-UGT mRNA; wt mic, wild type B-UGT mRNA, dog pancreatic microsomes; mut mic, 15-Arg/B-UGT mRNA, dog pancreatic microsomes. Translation of wild type and mutant B-UGT mRNA, in the absence of microsomes, yields proteins with identical molecular weights. Only wild type B-UGT is processed by dog pancreatic microsomes.

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