Journal of Steroid Biochemistry & Molecular Biology 123 (2011) 109–114

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Functional characterisation of the H365Y mutation of the 21-hydroxylase gene in congenital adrenal hyperplasia

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ARTICLE INFO

Article history:
Received 4 August 2010
Received in revised form
25 November 2010
Accepted 26 November 2010

Keywords:
Congenital adrenal hyperplasia
Steroid 21-hydroxylase deficiency
CYP21A2
H365Y
R356W
Salt-wasting

ABSTRACT

The study subject was a 13 day-old boy admitted to hospital, with weight loss since birth. He presented with the vomiting and hypotension that are classical features of congenital adrenal hyperplasia (CAH). The most common type of CAH is an autosomal recessive disorder caused by mutations in the 21-hydroxylase (CYP21A2) gene. To examine the CYP21A2 gene, gene-specific PCR was carried out, followed by sequencing. The baby was shown to be a compound heterozygote H365Y/R356W for two CYP21A2 gene mutations each inherited from a different parent. One of the mutations has not previously been functionally characterised. The mutations were reconstructed in an expression plasmid and characterised *in vitro* after transient transfection into human embryonic kidney (HEK293T) and hepatoblastoma (C3A) cell lines followed by measurement of enzyme activity. The CYP21A2 H365Y mutant exhibited minimal 21-hydroxylase activity to convert 17-hydroxyprogesterone to 11-deoxycortisol or progesterone to 11-deoxycorticosterone. Western immunoblotting indicated that the H365Y enzyme was produced in more variable amounts than wild type; in particular, the H365Y mutant protein may be unstable and/or subject to a more rapid degradation by the human proteosome as well as catalytically inefficient. The double mutant genotype with a severe mutation on each allele is compatible with the clinical presentation.

1. Introduction

Congenital adrenal hyperplasia (CAH) is an autosomal recessive disorder caused by mutations in genes encoding enzymes that are responsible for steroid biosynthesis in the adrenal cortex. Clinical manifestations of the condition vary from a severe salt-wasting, potentially fatal disease to a mild, late-onset disease. The most common form of CAH occurring in more than 90% of cases is steroid 21-hydroxylase deficiency (Integrated relational Enzyme database number EC 1.14.99.10). The disease has been assigned Online Mendelian Inheritance in Man number 201910. The gene encoding 21-hydroxylase cytochrome P450 is known as CYP21A2, Human Genome Nomenclature Committee assigned number HGNC:2600. Two-thirds of 21-hydroxylase-deficient CAH individuals are compound heterozygotes for mutations in the CYP21A2 gene [1]. The major enzyme activity of the protein is the conversion of 17-hydroxyprogesterone to 11-deoxycortisol while its progesterone to 11-deoxycorticosterone activity is also important.

The CYP21A2 gene is located on the short arm of chromosome 6 (6p21.3). The genetics are complicated by the presence, 30 kilobases upstream on the same chromosome, of a non-functional pseudogene (CYP21P) sharing 98% nucleic acid sequence homology with CYP21A2. Mutations arise by a number of mechanisms including deletions, duplications and gene conversion [2]. There can also be spontaneous new mutations, indeed 5% of disease-causing mutations are new [3] and many of these are unique to the family where they were initially discovered.

Genotype phenotype correlation has been studied. Transient cell transfection technology is the most common method to measure *in vitro* activity of mutant proteins [4,5] and there is usually (but not always) correlation between severity of symptoms and residual 21-hydroxylase activity from the corresponding mutated plasmid [6–11]. The CYP21A2 p.Arg356Trp (R356W) mutation is one of the known pseudogene-derived mutations and may have serious effects on the phenotype because the resulting protein has been shown to have no *in vitro* activity [12].

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[1] The authors are deeply saddened by the death of A. Michael Wallace in August 2010. He was a major contributor to this study and is sorely missed both scientifically and personally.

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doi:10.1016/j.jsbmb.2010.11.013

We determined the causative mutations for a salt-wasting proband, then used site-directed mutagenesis on the wild type CYP21 plasmid vector to create two plasmids, each separately reproducing one of the errors. The H365Y *in vitro* phenotype was determined using transient transfection in two different human cell lines. The CYP21A2 H365Y mutation had previously been detected using peptide mass signature, and noted as a "new" putatively deleterious mutation [13].

2. Materials and methods

2.1. Proband and family

A 13 day-old boy was admitted to a Scottish paediatric hospital. He had 17% weight loss since birth and presented with features of classical salt-wasting congenital adrenal hyperplasia including a grossly elevated serum 17-hydroxyprogesterone level of 542 nmol/l (the reference range for stressed or premature infants is <30 nmol/l and for proven CAH is >50 nmol/l). DNA was obtained from the baby and his parents. His parents were unrelated to each other. Informed consent for genetic diagnosis and studies was obtained.

2.2. Sequencing of proband and family members

Allele specific PCR was carried out prior to sequencing. Four PCR products were prepared, exploiting the major changes between gene and pseudogene in exons 3 and exons 6. The gene specific primers and PCR conditions were as described [14]. PCR products were purified by electrophoresis on a 1% agarose gel, followed by excision and purification after dissolving the gel slice using QIAquick PCR purification kit (Qiagen, Crawley, UK). Purified DNA was sent to MWG Biotech Germany (now known as Eurofins) along with the appropriate sequencing primers which were the same primers used for the PCR and an internal primer in exon 8, CAHe8seqf, sequence 5′-aggaggagctagaccacg-3′. PCR products were sequenced in both orientations.

2.3. Construction of the Cyp21A2 mutant plasmids

The parental wild type plasmid pcDCYP21A2 was a generous gift from Dr. Bon-chu Chung [12] and is referred to as phc21 in the original paper. This was used as a positive control for the *in vitro* assays (where it is called WT CYP21A2) as well as the vector from which the mutations were made. All primers for mutagenesis and sequencing of plasmid were obtained from Eurofins MWG Operon, (Ebersberg, Germany). Site-directed mutagenesis was performed with the primers listed in Table 1 using the QuikChange Mutagenesis kit (Stratagene, Amsterdam, Netherlands) according to the manufacturer's instructions. The positions of the introduced mutations were verified by DNA sequencing of the CYP21A2 H365Y and CYP21A2 R356W plasmids. Additional sequencing, using all nine primers [12] was carried out to confirm that no unexpected mutations had been introduced into the coding sequence of the plasmids. Sequencing analysis of the CYP21A2 plasmids was performed by DNASHEF (Edinburgh, UK).

Table 1
Primers used for site directed mutagenesis.

Name	Primer	Sequence
R356W	Forward	5′-ggtgctgcgcctg**t**ggcccgttgtgcc-3′
R356W	Reverse	5′-ggcacaacgggcc**a**caggcgcagcacc-3′
H365Y	Forward	5′-cttagccttgcccc**t**accgcaccacacggcc-3′
H365Y	Reverse	5′-ggccgtgtggtgcgg**t**agggcaaggctaag-3′

The base pair to be changed is indicated in bold, and the codon affected is underlined.

2.4. Plasmid DNA purification, cell culture and transfections

The DNA plasmid was extracted from lysed competent cells and was purified for transfection using the endofree plasmid maxi kit (Qiagen, Crawley, UK). The HEK293T human embryonic kidney cell line was purchased from the ECACC and all cell culture media and supplements from Lonza Ltd., Wokingham, UK. The human C3A hepatoblastoma cells, derived from HEPG2 cells, were obtained from ATCC, Rockville, MD, USA. HEK293T cells were cultured in DMEM supplemented with 4.5 g/l glucose, 2 mM glutamine, 25 mM HEPES 100 U penicillin/50 μg streptomycin/ml and 10% fetal bovine serum (FBS), at 5% CO_2 in air at 37 °C. C3A cells were cultured in DMEM F-12 (DMEM: Ham's F-12 1:1 mix), 10% FBS, 100U penicillin/ml and 50 μg streptomycin/ml, 1% sodium pyruvate. Cells were transiently transfected with the CYP21A2 plasmid constructs or a pCMV vector encoding β-galactosidase using FuGENE HD reagent according to the recommended manufacturer's protocol (Roche Diagnostics Ltd., Burgess Hill, UK). Transfection efficiency of each cell line was confirmed by measuring LAC-Z expression in the β-galactosidase vector-transfected cells 48 h after transfection. Plasmid pcDCYP21 containing cloned wild type normal CYP21A2 cDNA was used as a positive control and untransfected cells were used as a negative control.

2.5. Assay of 21-hydroxylase activity

21-Hydroxylase enzyme activity in the intact transfected cells was determined by adding 0.5 μmol/l progesterone containing 0.05 μCi [^{3}H] progesterone tracer or 0.5 μmol/l of 17-hydroxyprogesterone containing 0.05 μCi [^{14}C] 17-

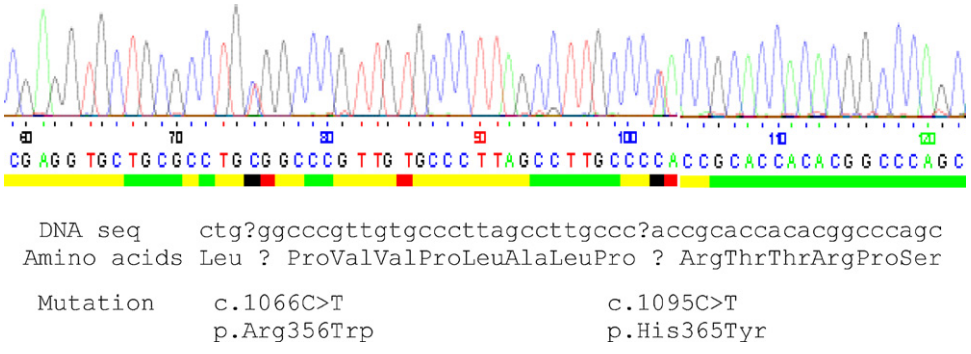

Fig. 1. Sequence of Exon 8 from proband. Sequence of PCR product 4 for proband in forward orientation using primer CAHe8seqF. Both changes are heterozygote, with a cytosine (C) to thymine (T) change resulting in p.Arg356Trp (R356W) on one chromosome and another C–T change resulting in p.His365Tyr (H365Y) on the other.

hydroxyprogesterone as tracer to the cell culture medium 24, 48, 72 or 96 h post-transfection as indicated. Tritium- and [^{14}C]-labelled steroids were obtained from Dupont-NEN (Stevenage, UK). The reaction was terminated after 6 or 24 h by the removal of the cell media and its addition to dichloromethane (1:6, v/v). After extraction, steroids were separated by thin layer chromatography (Alugram SIL GLUV254, precoated sheets 20 × 20 cm Cat. NO. A006109/0276, Camlab Ltd., Cambridge, UK; solvent system chloroform/ethyl acetate, 4:1, v/v) and quantified on a BioScan Radio-Imager (LabLogic Systems Ltd., Sheffield, UK).

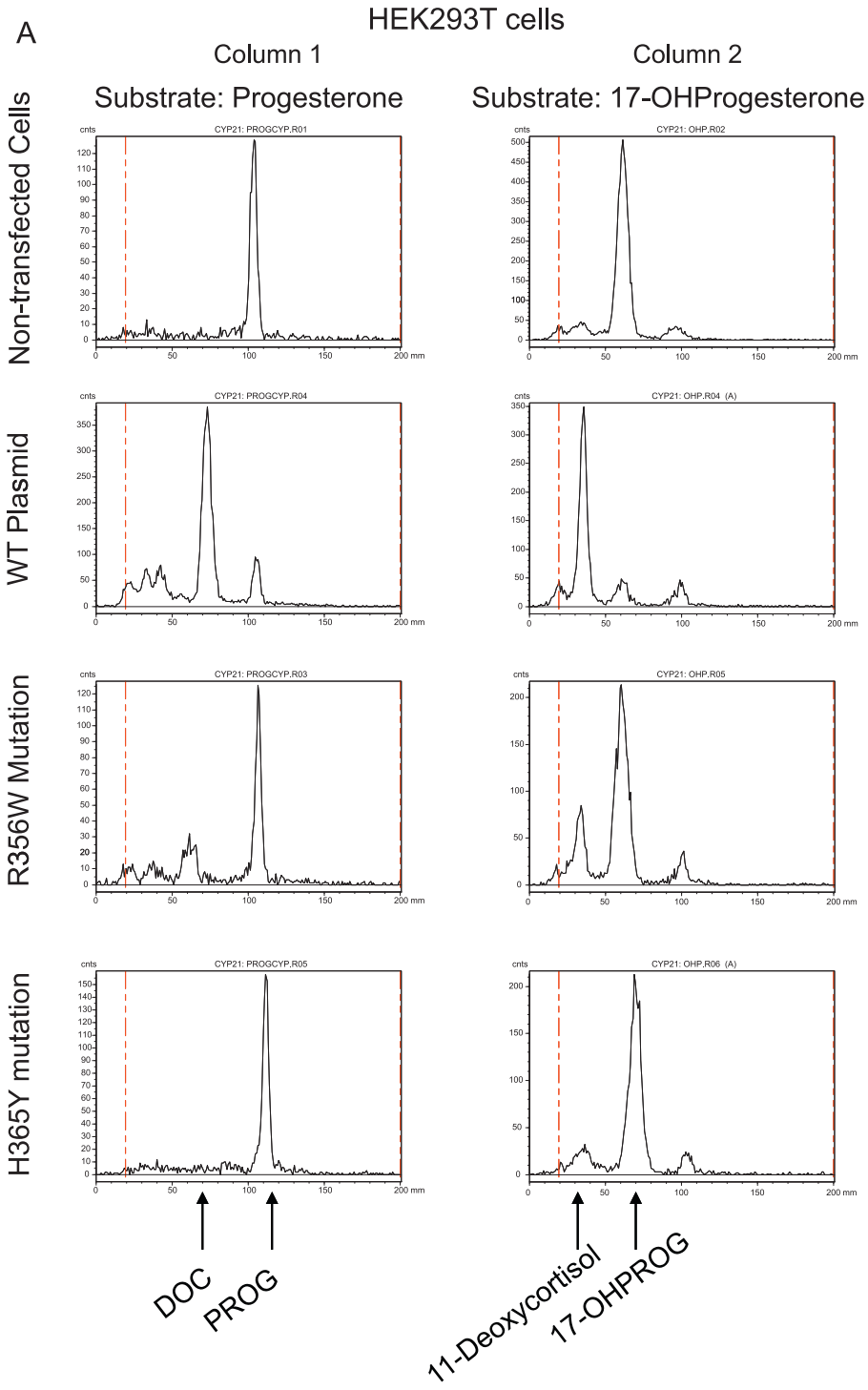

Fig. 2. Comparison of 21-hydroxylase activities illustrated on thin-layer radiochromatograms after transfection of (A) HEK293T and (B) C3A cells with wild type (WT) and mutant CYP21A2 plasmids. A. Column 1: radiolabelled progesterone (PROG) conversion to 11-deoxycorticosterone (DOC) in 24 h. Column 2: radiolabelled 17-hydroxyprogesterone (17-OHPROG) conversion to 11-deoxycortisol in 24 h, as detailed in Section 3.2, in HEK293T cells transfected with WT, R356W and H365Y CYP21A2 plasmids. B. Comparison of 21-hydroxylase activities of C3A cells illustrated on thin-layer radiochromatograms 24 and 48 h post-transfection with WT and H365Y mutant CYP21A2 plasmids. Radiolabelled progesterone (PROG) conversions to 11-deoxycorticosterone (DOC) in a 6 h assay period commencing 24 or 48 h post-transfection, as detailed in Section 3.2, are illustrated.

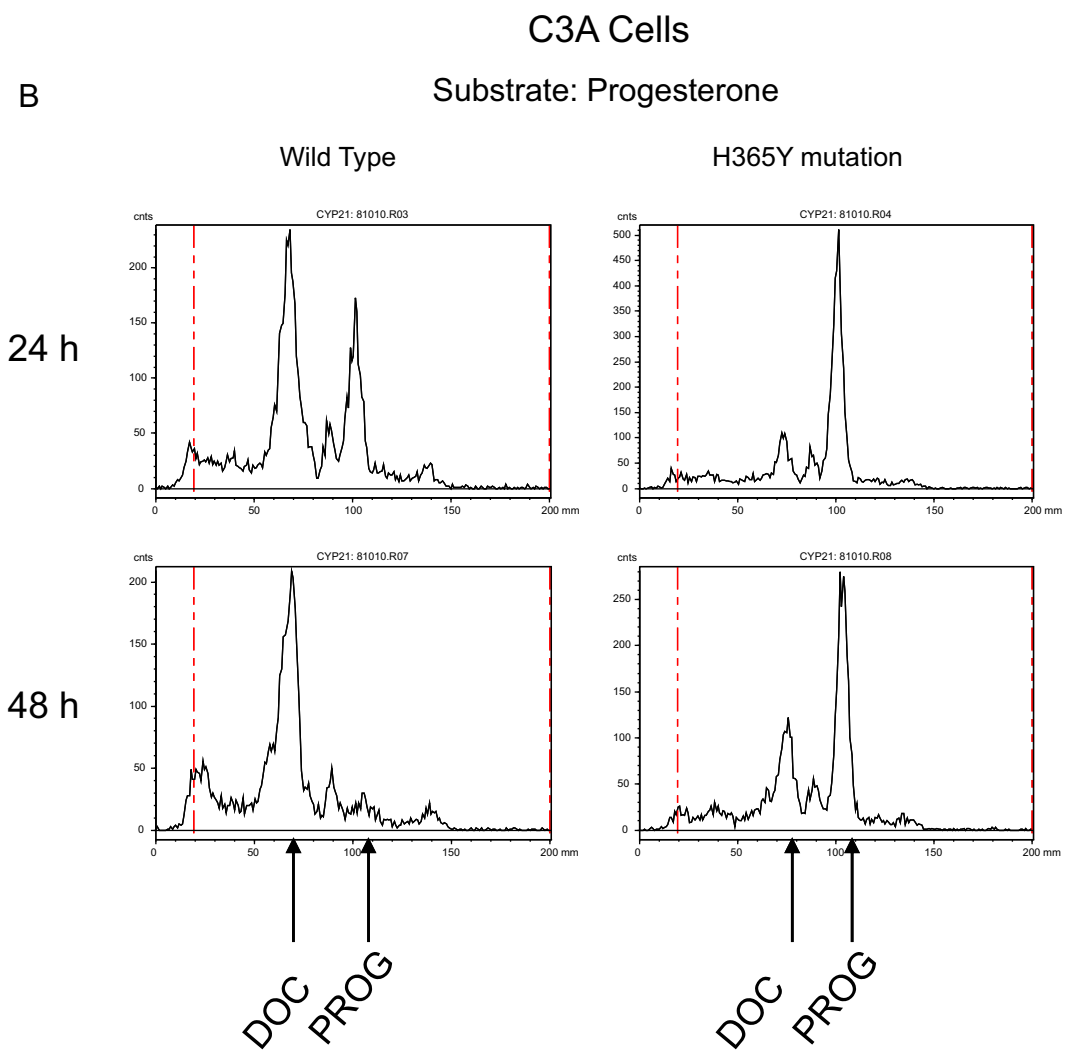

Fig. 2. (*Continued*).

2.6. Western immunoblot analysis

After the 24 h incubation to assess activity the transfected and control cells were washed twice with ice-cold phosphate-buffered saline and homogenised in lysis buffer (50 mM Tris, 0.1% SDS, 1% sodium deoxycholate containing Complete Protease Inhibitor (Roche Diagnostics Ltd., Burgess Hill, UK). The protein concentration was determined by the dye-binding assay of Bradford [15]. Cell homogenates (15 μg) were resolved on a 12% acrylamide gel and electro-blotted onto an Immobilon-P membrane (Sigma-Aldrich, Poole, UK). Western analysis was carried out after blocking the membrane for 1 h in PBS containing 5% (w/v) dried non-fat milk and 0.05% Tween 20. Overnight incubation at 4 °C in the primary goat anti-human CYP21A2 antibody Cat No sc-48466 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; at a dilution of 1:2000 in blocking buffer was followed by a 1 h incubation in horseradish peroxidase conjugated anti-sheep IgG at 1:25,000 in blocking buffer. The signal was visualised using an Immobilon Western Detection Kit (Millipore, Watford, UK).

3. Results and discussion

3.1. Proband DNA sequencing

Genomic sequence results from PCR product 4 of the proband's DNA showed 2 mutations both in exon 8 (Fig. 1). R356W has previously been detected, and the H365Y mutation had been proposed earlier as a "new" mutation [13]. Our discovery of this latter mutation was in a completely separate family.

No other potentially causative mutations were detected. The proband was homozygote for the Leu insert in exon 1, c.27-28CTG as described by [16,17], is homozygous for the A allele of the g.655A/C>G of the intron 2 polymorphism [18], and is homozygous rare for the known p.Lys102Arg (K102R) or c.305G>A non-pathogenic variant [16]. The proband is also heterozygote at two previously described loci in exon 1, but both of these mutations are silent c.115T>C (p.=) and c.135A>C (p.=). As well as this, the proband was heterozygous at two positions unrelated to splicing function in each of introns 2 and 3. From sequence data alone it is therefore likely that the H365Y mutation could be a causative mutation associated with expression of CAH.

3.2. Transfection, 21-hydroxylase radiometric activity assay and Western immunoblot analysis

HEK293T and C3A cell lines were used to assess 21-hydroxylase activities from the transfected CYP21 plasmids. Results are shown in Fig. 2. In HEK293T cells after 24 h incubation with progesterone, progesterone was largely converted to 11-deoxycorticosterone by the WT CYP21A2 gene product, whereas only a very small amount of conversion for CYP21A2 R356W was seen and after transfection with CYP21A2 H365Y negligible conversion was

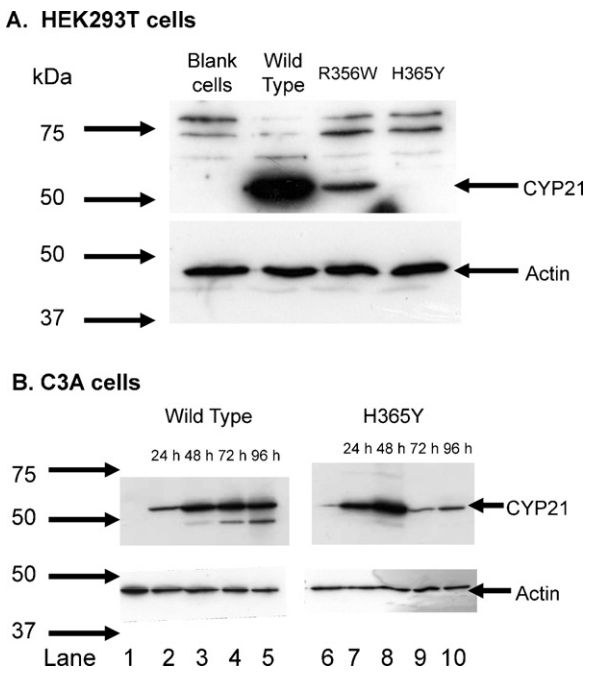

Fig. 3. Western immunoblots of transfected cell lysates. Membranes were first probed with anti-hCyp21A2 antibody followed by probing using an anti-β-actin antibody to ensure equivalent protein loading and transfer of gel/membranes lanes. Migration of molecular weight (kDa) standards are indicated at the arrows. A. Transfected HEK 293T cells. Lane 1: untransfected cell lysate. 2: WT CYP21A2 transfected cells. 3: mutant R356W CYP21A2 transfected cells. 4: mutant H365Y CYP21A2 transfected cells. B. Transfected C3A cells harvested at 24, 48, 72 and 96 h post-transfection with either WT CYP21A2 or mutant H365Y CYP21A2 plasmid.

observed (column 1). Transfected HEK293T cells incubated with 17-hydroxyprogesterone revealed a low amount of activity associated with the CYP21A2 R356W mutant and negligible activity from the CYP21A2 H365Y mutant (Fig. 2A, column 2). When C3A cells were transfected with CYP21A2 H365Y mutant and wild type plasmid, similar results were seen in these cells except that a somewhat greater (albeit still small) amount of substrate conversion was seen with the CYP21A2 H365Y mutant.

These relative activities were reflected in the quantities of CYP21A2 protein seen in the Western blot of transfected HEK293 cells (Fig. 3A) which suggests that the H365Y mutant protein was unstable. After transfection of C3A cells with the H365Y mutant plasmid and evaluation of protein levels at various times post-transfection, revealed that the mutant protein accumulated at a similar rate to wild type enzyme during the early, rapid transient phase of plasmid expression; however, at 72 and 96 h post-transfection the levels of H365Y protein was markedly reduced compared to wild type enzyme levels (Fig. 3B). Taking together the outcomes of the H365Y mutant activity and protein levels, it appears that the mutant enzyme is not only inactive but also less stable than the wild type enzyme.

3.3. The H365Y mutation: origins

Sequencing of DNA from the proband showed that he was a H365Y/R356W compound heterozygote. Both his parents were shown by sequencing to be carriers, his father carried the CYP21A2 R356W mutation and mother, the H365Y mutation. Most mutations causing CAH come from the pseudogene but this one appears not to derive from the pseudogene. Ethnicity of the USA-based carrier of this mutation was not reported [13]. As our Scottish discovery of this mutation was completely independent from this, it would be useful to know its frequency in different populations. There are examples in the literature of "rare" CYP21A2 mutations appearing in apparently unrelated individuals and some of these apparently rare mutations may in fact be sufficiently common to warrant inclusion in a screening programme [19]. Sequencing the whole gene and DNA from the parents is useful because it helps to avoid misinterpretation which could be caused by two mutations on the same allele [20].

3.4. Phenotype and genotype correlation

The proband presented with the features of classical salt-wasting CAH including an elevated serum 17-hydroxyprogesterone level. The R356W CYP21A2 mutation had previously been shown to result in 21-hydroxylase deficiency *in vitro* [12] and in several studies, summarised in [21], is generally associated with the salt-wasting form of the disease. Therefore the phenotype of the proband is likely to be dependent on the level of function of the protein encoded by the H365Y mutant. Our *in vitro* transfection and expression results showed the CYP21A2 H365Y mutant plasmid resulted in even less enzyme activity than the CYP21A2 R356W plasmid in all situations indicating that the additional presence of this mutation is likely to be associated with the salt-wasting aspect of the disease. The different enzyme activities measured after transfection of the CYP21A2 R356W plasmid in the two separate cell lines is an interesting observation that may relate to the *in vitro* translational efficiency of expression and/or the stability of the protein in these cell lines. The study of the CYP21A2 H365Y mutant is also suggestive that not only is the mutant functionally inactive but also appears unstable in these mammalian cell lines. This suggestion cannot be directly addressed *in vivo* without access to an affected patient's adrenals. Transient cell transfection technology, while a valuable tool, can only provide one level of understanding of the complex controlled gene expression occurring in the human body.

3.5. The H365Y mutation: importance

Congenital adrenal hyperplasia is an autosomal recessive disease; therefore the severity of the disease is most likely to reflect the residual function of the milder mutation in any patient. In this case, R356W was known to be a severe mutation and the therefore the salt wasting presentation of the proband, as well as the *in vitro* results suggest that H365Y has a severe effect on the protein function. Had this mutation been detected in compound heterozygote with a mild allele, it could not have been classified using clinical evaluation [22].

Acknowledgements

We are very grateful to Dr. Bon-chu Chung (Taiwan) for the expression vector pcDCYP21and to Ms. Anne White of the Molecular Diagnostic Lab., Royal Infirmary of Edinburgh for sequencing of the site directed mutagenesis constructs of the CYP21A2 plasmid.

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