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Cytochrome P450 CYP3A in marsupials: Cloning and characterisation of the second identified CYP3A subfamily member, isoform 3A78 from koala (*Phascolarctos cinereus*)

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ABSTRACT

Cytochromes P450 (CYPs) are critically important in the oxidative metabolism of a diverse array of xenobiotics and endogenous substrates. Previously, we cloned and characterised the CYP2C, CYP4A, and CYP4B gene subfamilies from marsupials and demonstrated important species-differences in both activity and tissue expression of these CYP enzymes. Recently, we isolated the Eastern grey kangaroo CYP3A70. Here we have cloned and characterised the second identified member of marsupial CYP3A gene subfamily, CYP3A78 from the koala (*Phascolarctos cinereus*). In addition, we have examined the gender-differences in microsomal erythromycin N-demethylation activity (a CYP3A marker) and CYP3A protein expression across test marsupial species. Significant differences in hepatic erythromycin N-demethylation activity were observed between male and female koalas, with the activity detected in female koalas being 2.5-fold higher compared to that in male koalas ($p<0.01$). No gender-differences were observed in tammar wallaby or Eastern grey kangaroo. Immunoblot analysis utilising anti-human CYP3A4 antibody detected immunoreactive proteins in liver microsomes from all test male and female marsupials including the koala, tammar wallaby, and Eastern grey kangaroo, with no gender-differences detected across test marsupials. A 1610 bp koala hepatic CYP3A complete cDNA, designated CYP3A78, was cloned by reverse transcription-polymerase chain reaction approaches. It displays 64% nucleotide and 57% amino acid sequence identity to the Eastern grey kangaroo CYP3A70. The CYP3A78 cDNA encodes a protein of 515 amino acids, shares approximately 68% nucleotide and 56% amino acid sequence identity to human CYP3A4, and displays high sequence similarity to other published mammalian CYP3As from human, monkey, cow, pig, dog, rat, rabbit, mouse, hamster, and guinea pig. Collectively, this study provides primary molecular data regarding koala hepatic CYP3A78 gene and enables further functional analyses of CYP3A enzymes in marsupials. Given the significant role that CYP3A enzymes play in the metabolism of both endogenous and exogenous compounds, the clone provides an important step in elucidating the metabolic capacity of marsupials.

1. Introduction

Cytochromes P450 (CYPs) comprise a superfamily of membrane-bound haemoproteins that catalyse the oxidative metabolism of a diverse array of xenobiotic compounds and endogenous substrates. CYPs are regulated in a tissue-specific and temporal manner and are critically important in detoxifying and eliminating drugs, chemicals and environmental pollutants that may be encountered during an organism's lifetime. In mammals, various CYPs also participate in the biosynthesis and metabolism of steroids. According to the latest published CYP update, 7232 named CYPs and 896 pseudogenes have been identified from at least 85 eukaryote (including vertebrates, invertebrates, protists, fungi and plants) and 20 prokaryote species. Due to the pace of CYP research, a database of newly identified CYPs is maintained at http://drnelson.utmem.edu/CytochromeP450.html (Nelson, 2010). According to this database, 18 mammalian CYP families comprising 43 subfamilies have now been identified. CYP3A gene subfamily is of considerable interest as it displays an exceptional functional diversity, is inducible by many chemicals, and is highly

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expressed in the liver and gastrointestinal tract of most species studied (Nebert and McKinnon, 1994; Gibson et al., 2002; Wolbold et al., 2003; Burk and Wojnowski, 2004). In addition, CYP3A participates in the bio-activation of several structurally diverse xenobiotics and endogenous compounds such as the sex steroids, oestradiol, progesterone and testosterone (Anakk et al., 2003).

The CYP3 gene family is comprised of CYP3A, CYP3B, CYP3C, and CYP3D subfamilies. However, mammalian CYP3 members are only found in CYP3A (Nelson, 2010). While CYP3A subfamily exhibits broad substrate specificity, these enzymes show few dramatic substrate specificity differences. This can provide useful leads for the identification of possible residues for site-directed mutagenesis.

In humans, CYP3A is commonly considered the most important of all drug-metabolising enzymes as at least 60% commonly used drugs are metabolised by CYP3As. Example of drugs that are CYP3A substrates include macrolide antibiotics erythromycin and triacetyloleandomycin, calcium channel blockers nifedipine and diltiazem, steroidal agents, immunosuppressive cyclosporine, and carcinogens benzo(a)pyrene and aflatoxin B1 (Wrighton et al., 1985; Guengerich et al., 1986; Kronbach et al., 1988; Waxman et al., 1988; Shimada et al., 1989; Gallagher et al., 1994). CYP3A activity is often assessed using midazolam administration and the erythromycin breath test, with at least two probe substrates suggested for investigating potential drug–drug interactions in vitro (Kenworthy et al., 1999; McCrea et al., 1999; Wang et al., 2000; Atkins et al., 2001; Rivory and Watkins, 2001; Tucker et al., 2001; Fayer et al., 2002).

Generally, mammalian CYP3A has very few specific probes that allow us to determine its activity (Kashiwada et al., 2007). To date, 6β-testosterone hydroxylase is a well known bioindicator widely used to determine the CYP3A activity in mammals, whereas erythromycin N-demethylase is a marker for various CYPs, but also a strong marker for mammalian CYP3A (Christen et al., 2009; Li et al., 2008; James et al., 2005; Vaccaro et al., 2003, 2005; Fayer et al., 2002). CYP3A cDNA was first isolated from human liver (Komori et al., 1988) and subsequently cloned from rabbit (Potenza et al., 1989), dog (Ciaccio et al., 1991), rat (Miyata et al., 1993), guinea pig (Mori et al., 1993), mouse (Itoh et al., 1994), hamster (Alabouch et al., 1996), pig (Nissen et al., 1998), cow (Natsuhori et al., 1997), monkey (Carr et al., 2004; Booth-Genthe et al., 2005; Matsunaga et al., 2005; Carr et al., 2006), and recently Australian marsupial (El-Merhibi et al., 2010a). Multiple isoforms of CYP3A have been found in humans (Wrighton and Vandenbranden, 1989; Westlind et al., 2001; Strausberg et al., 2002), rats (Wang et al., 1996; Matsubara et al., 2004), mice (Sakuma, 2000; Sakuma et al., 2000), and canine (Fraser et al., 1997).

Australia's native marsupial fauna comprises a vast array of carnivorous and herbivorous species exhibiting unique physiological, reproductive and dietary characteristics. A number of marsupial species possess the ability to ingest and metabolise a range of dietary compounds which would be toxic to other mammalian species. As a result, it has been hypothesised that some marsupial species may have evolved novel mechanisms and pathways for the detoxification of ingested dietary compounds (McLean and Foley, 1997; El-Merhibi et al., 2008). Relatively few studies have investigated the mechanisms governing the metabolism of both xenobiotic compounds and endogenous substrates by Australian marsupial species at the molecular level. Consequently, very few complete CYP450 sequences have been characterised for any of these unique mammals.

Previously, we cloned and characterised the CYP2C, CYP4A, and CYP4B gene subfamilies from a number of marsupial species. We found several important species-differences in both activity and tissue expression of these CYP enzymes, and recently have isolated the kangaroo CYP3A70 (Ngo et al., 2000, 2006, 2010; Jones et al., 2008; El-Merhibi et al., 2010a; Milic et al., 2011). We have now cloned and characterised the second identified CYP3A member from marsupials, the koala (*Phascolarctos cinereus*), a 1579 bp complete CYP3A cDNA, designated CYP3A78. In addition, we have investigated the gender-differences in the expression of CYP3A proteins and CYP3A activity in a number of marsupials, using Western blot analysis and erythromycin N-demethylation assay, respectively. We have also examined the inhibition of erythromycin N-demethylation activity in marsupial microsomes by troleandomycin, a CYP3A inhibitor.

2. Materials and methods

2.1. Materials

Polyclonal goat anti-human CYP3A4 IgG was kindly provided by Professor Mick McManus (University of Queensland). HRP (horseradish peroxidase) strepavidin conjugated anti-goat secondary IgG was

Table 1
Details of oligonucleotide primers.

Name	Sequences (5′-3′)	Direction	Origin[a]	Accession number
a. Primers used for RACE reactions.				
UPM[c]	CTAATACGACYCACTATAGGGCAA GCAGTGGTAACAACGCAGAGT (0.4 μM)	Sense	–	–
	CTAATACGACTCACTATAGGGC (2 μM)	Sense	–	–
k3Af	TGA AAG AA/T/CA/G TG/CT TTG GG/A/TG CCT ACA GC	Sense	–	–
k3Ar	AC/TC AC/TC ACC A/GC/TA/G/C/T GA/T/CC CCT TTG GGA ATG	Antisense	–	–
k3A783′f	GATTCCCAGGCCACAAATGACCCTG	Sense	(+)1139–(+)1166	HQ595724[b]
k3A785′r	GGAGGCCTTGTTGGGTAGATTACTGTC	Antisense	(+)1296–(+)1330	HQ595724[b]
b. Primers used for isolation of koala CYP3A78 complete cDNA. The start and stop codons are shown in bold and restriction sites are underlined.				
kCYP3A78f	ACGCGCTAGCTAATCAGAGGC**ATG**GG	Sense	(−)21–(+)5	HQ595724[b]
kCYP3A78r	GATGAGAAGGGCGGCCGC**AGT**GAAGAGT	Antisense	(+)1536–(+)1508	HQ595724[b]
c. Sequencing primers				
M13	GTAAAACGACGGCCAGT	Sense	–	–
M13r	CACACAGGAAACAGCTATGACCATG	Antisense	–	–
T_3	AATTAACCCTCACTAAAGGG	Sense	–	–
T_7	GTAATACGACTCACTATAGGGC	Antisense	–	–

[a] Corresponding to the position of the nucleotides relative to the start codon (designated +1).
[b] GenBank accession number of the koala complete CYP3A78 mRNA (El-Merhibi1 et al., 2010b, direct submission). The primers were derived from the koala partial CYP3A78 nucleotide sequence obtained previously utilising degenerate primers designed based on conserved regions of aligned sequences of human (NM_017460; BC033862; NM_000765; AF337813), monkey (AB124894; AB206123; DQ022197; AY334551; AY635466), dog (NM_001003340; NM_001003338), pig (NM_214423), cow (Y10214), rat (L24207; U46118; AB084894), mouse (D26137; AB033414; AB039380), hamster (D86951), rabbit (J05034), and guinea pig (D16363) CYP3A cDNAs.
[c] UPM: Universal Primer Mix, for more details refer to SMART® RACE cDNA Amplification Kit User Manual (Clontech, Palo Alto, CA, USA).