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CHARACTERIZATION OF THE NOVEL DEFECTIVE *CYP2C9*24* ALLELE

Author's names:

Darja Herman, Vita Dolzan, Magnus Ingelman-Sundberg

Institutions:

Institute of Biochemistry, Faculty of Medicine, Ljubljana, Slovenia (D.H., V.D.)

Section of Pharmacogenetics, Department of Physiology and Pharmacology, Karolinska Institute, Stockholm, Sweden (D.H., M.I.-S.)

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Corresponding author:

Vita Dolzan, Institute of Biochemistry, Faculty of Medicine, Vrazov trg 2, SI-1000 Ljubljana, Slovenia; phone: +386 1 543 76 69; fax: + 386 1 543 76 41;

e-mail: vita.dolzan@mf.uni-lj.si

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- *Results and Discussion*: 755

Abbreviations: CYP – cytochrome P450, HEK293 – human embryonic kidney cell line

Abstract

CYP2C9 is one of the major drug-metabolizing enzymes and it is involved in the oxidative metabolism of approximately 10% of clinically important drugs, among which some such as anticoagulant warfarin, have a narrow therapeutic index. The human *CYP2C9* gene is highly polymorphic. We found a new sequence variation in exon 7 of *CYP2C9* gene (*1060G>A*) resulting in a substitution of acidic amino acid Glutamate to basic Lysine (E354K) when translated. The allele, designated *CYP2C9*24*, was present in heterozygous state in one warfarin treated patient. To characterize the *CYP2C9*24* allele, we expressed the wild-type and CYP2C9.24 protein in a recombinant yeast expression system and in HEK293 cell system. CO difference spectra were recorded on dithionite-reduced microsomes and protein was determined by Western-blotting. Transfection with CYP2C9.1 cDNA resulted in detectable CYP2C9 protein in yeast or HEK293 cells, whereas only small amounts of the protein were detected in yeast transfected with CYP2C9.24 cDNA. A strong differential absorption peak at 450 nm was observed with microsomes of yeast transfected with CYP2C9.1 cDNA, while no peak was detected with microsomes of yeast transfected with CYP2C9.24 cDNA or empty pYeDP60 plasmid. These results suggest that CYP2C9.24 may be improperly folded, both in yeast and mammalian cells, resulting in improper heme incorporation and rapid intracellular degradation. The data obtained in the expression systems are cosistent with our findings *in vivo*. In conclusion, we have identified a novel defective CYP2C9 variant allele of potential importance for drug metabolism *in vivo*.

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Cytochrome P450 (CYP) 2C9 is involved in the metabolism of more than 100 currently used drugs, corresponding to about 10% to 20% of commonly prescribed drugs (Kirchheiner and Brockmoller, 2005). Some of them, e.g. anticoagulant warfarin, have a narrow therapeutic index (Miners and Birkett, 1998). Clinically available warfarin is a racemic mixture and CYP2C9 is responsible for the metabolism of more potent *S*-enantiomer (Kaminsky and Zhang, 1997). The human *CYP2C9* gene is highly polymorphic (http://www.imm.ki.se/CYPalleles/). The two most common variant alleles in Caucasian populations are *CYP2C9*2*, with a point mutation in exon 3 (430C>T) and *CYP2C9*3*, with a point mutation in exon 7 (1075A>C). Both are associated with a decrease in the catabolic activity of the enzyme (Rettie et al., 1994; Sullivan-Klose et al., 1996). It was shown in many studies that the patients with polymorphic alleles require significantly lower doses of warfarin and are more susceptible to bleeding complications than the carriers of two wild-type alleles (Kirchheiner and Brockmoller, 2005).

We have previously reported a novel *1060G>A* sequence variant in exon 7 of *CYP2C9* gene, leading to substitution of acidic amino acid Glutamate to basic Lysine (E354K). The new allele, designated *CYP2C9*24*, was present in heterozygous state in one warfarin treated patient who was also a heterozygous carrier of the *CYP2C9*2* allele. As exon 3 and 7 are separated by a long distance (>39 kbp) we could not assess whether these two alleles were located on the same or on separate allele.

The carrier of the *CYP2C9*24* allele was 77 years old and was treated with warfarin due to atherosclerotic cardiomyopathy with atrial fibrillation. The index patient had the lowest daily warfarin dose requirement (1.5 mg/day) and the lowest (*S*)-warfarin clearance (0.809 ml/min) when compared to a group of warfarin patients matched with the index patient for age, indication, drug co-treatment and *CYP2C9*1/*2* heterozygous genotype (mean (SD) warfarin

dose 3.04 (1.16) mg/day and (*S*)-warfarin clearance 2.400 (0.864) ml/min) (Herman et al., 2006).

To asses the functional importance of the *CYP2C9*24* allele, we expressed the wild-type and CYP2C9.24 protein in a recombinant yeast expression system and in mammalian HEK293 cell system.

Methods

Preparation of CYP2C9 expression constructs

The yeast expression vector pYeDP60 which contained the wild-type *CYP2C9* cDNA (AstraZeneca, R&D, Umeå, Sweden) was used as a template to generate mutated *CYP2C9*24* cDNA. QuickChange® Site-Directed Mutagenesis Kit (Stratagen, La Jolla, California, USA) was used to introduce *1060G>A* mutation with mutagenic oligonucleotides mut2C9_F (5'-CAGATGCTGTGGTGCACAAGGTCCAGAGATACATTG-3') and mut2C9_R (5'-CAATGTATCTCTGGACCTTGTGCACCACAGCATCTG-3'). The *CYP2C9*1* and *CYP2C9*24* alleles were amplified from pYeDP60 vector using primers that introduced *KpnI* restriction site before the start codon (5'-ATAGGTACCATGGATTCTCTTGTGGTCC-3') and *EcoRI* site after the stop codon (5'- ATAGAATTCTCAGACAGGAATGAAGCAC-3'). Fragments were purified by QIAquick Gel Extraction Kit (Qiagen, Valencia, California, USA) and subcloned into pcDNA3.1 (Invitrogen Life Technologies, Rockville, MD, USA). All the constructs were verified by sequencing.

Expression in yeast

pYeDP60 containing *CYP2C9*1* or *CYP2C9*24* cDNA were transfected by lithium acetate procedure in *Saccharomyces cerevisiae* strain W(R) that overexpresses yeast reductase (Pompon et al., 1996). Yeast cells transfected with empty pYeDP60 were used as a negative control. After 2-4 days of incubation at 28°C the colonies were transferred to a new SGI plate (l g/l Casamino acids, 7 g/l yeast nitrogen base, 20 g/liter glucose, 20 mg/liter tryptophan) making 2-3 cm long lines and incubated for additional 2-4 day at 28°C until the yeast had grown thick. Then the yeasts were transferred into 20 ml SGI medium and grown over night at 28°C until $OD_{600\ nm}$ was around 10. The culture was poured into 230 ml YPGE medium (10 g/l yeast extract, 10 g/l BactoPeptone, 5 g/l glucose, 2% ethanol) and grown at 28°C to

$OD_{600\ nm}$ around 10-13. Induction was started by the addition of 28 ml of 20% galactose and the growth was continued until $OD_{600\ nm}$ reached 30 (Pompon et al., 1996).

The yeast cells were recovered by centrifugation, washed once with distilled water and resuspended in TEK buffer (50 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.1 M KCl). Cells were spun down again and resuspended in 2.5 ml TES buffer (50 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.6 M sorbitol), with added Complete Protease Inhibitor Cocktail (Roche Applied Science, Pentzberg, Germany). The yeast cells were mechanically disrupted using glass beads. To the crude extract 5 ml of cold TES buffer was added three times and the supernatant was withdrawn and centrifuged at 15 000g for 10 min at 4°C. The supernatant was then transferred to a new tube and centrifuged at 34 000g for 1 h at 4°C. The pellet, consisting of microsomes, was dissolved in 2.5 ml of cold TEG (50 mM Tris-HCl pH 7.4, 1 mM EDTA, 20% glycerol) (Pompon et al., 1996). The protein concentration was determined according to Lowry *et al.* (Lowry et al., 1951). The carbon monoxide-binding spectra analysis was recorded on dithionite-reduced microsomes (Omura and Sato, 1964). Yeast transfection and all the subsequent analysis were repeated three times.

Expression in HEK293 cells

Cells were grown at 37°C in atmosphere of 5% CO_2 in Minimum Essential Medium supplemented with FBS, sodium pyruvate, non-essential amino acids and antibiotics (Invitrogen Life Technologies, Rockville, MD, USA). When HEK293 cells reached 80-90% confluency they were transiently transfected with pcDNA3.1 constructs using Lipofectamine™ 2000 Transfection Reagent (Invitrogen Life Technologies, Rockville, MD, USA). Empty pcDNA3.1 was used as a negative control. After 60 h, the cells were harvested, homogenized, and fractionated by centrifugation (Karlgren et al., 2004). The protein concentration of microsomal fraction was determined according to Lowry *et al.* (Lowry et al.,

1951) and the carbon monoxide-binding spectra analysis was recorded on dithionite-reduced microsomes (Omura and Sato, 1964). Transfection of HEK293 cells and all the subsequent analysis were repeated three times.

Western blot analysis

Microsomes from transfected yeast or HEK293 cells, corresponding to 30 μg of protein, were separated on 10% SDS-polyacrilamide gels and transferred to a Hybond-C extra membrane (Amersham Biosciences, Uppsala, Sweden). The membrane was first incubated with a CYP2C9 antibody diluted 1:5000 (BD Bioscience, San Jose, California, USA) and then with the secondary antibody conjugated with horseradish peroxidase diluted 1:2000 (BD Bioscience). SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) was used for detection. Molecular weight marker was The BenchMark Prestained Protein Ladder (Invitrogen Life Technologies, Rockville, MD, USA). Membranes were scanned using luminescent image analyzer LAS-3000 (Fujifilm Life Science, Stamford, CT, USA).

Results and Discussion

Most of the genes encoding enzymes involved in drug metabolism are genetically diverse. *CYP* genes, in particular, have higher density of polymorphic sites and higher genetic diversity than any other human genes examined. Among subfamilies involved in phase I drug metabolism CYP2 displays the highest level of genetic diversity. It is particularly important to predict the function of non-synonymous sequence variation in *CYP* genes and to correlate that function to phenotypic observation because of their role in drug metabolism and adverse drug reactions (Solus et al., 2004).

In the present study we have characterized a rare variant *CYP2C9*24* allele that was previously found in one warfarin treated patient (Herman et al., 2006). We found it interesting since the *1060G>A* mutation resulted in substitution of Glutamate to Lysine (E354K) within the region where many genetic polymorphisms were reported to affect enzyme activity (*CYP2C9*3*, *CYP2C9*4* and *CYP2C9*5*). Indeed, the patient, who was a heterozygous carrier of *CYP2C9*24* allele, had slower metabolism of *S*-warfarin and consequently required lower warfarin dose than other warfarin treated patients, matched for age, indication, drug co-treatment and *CYP2C9*1/*2* heterozygous genotype (Herman et al., 2006), indicating that *1060G>A* mutation may be of functional importance.

To study a potential functional role of *CYP2C9*24* allele, *CYP2C9* cDNA with and without *1060G>A* mutation were first expressed in yeast *S. cerevisiae* strain W(R) since it was previously shown that CYP2C9 protein can be efficiently expressed in this system (Yasar et al., 2001). Carbon monoxide difference spectra analysis of microsomal fractions revealed a strong peak at 450 nm for yeast transfected with pYeDP60 constructs containing wild-type *CYP2C9* cDNA (Fig. 1A), indicating the presence of correctly folded P450 enzyme (about 400 pmol/mg microsomal protein). On the other hand, no peak at 450 nm was observed for yeast transfected with *CYP2C9*24* cDNA or with empty pYeDP60 plasmid. By western

blotting we showed that transfected yeast produced highly detectable level of CYP2C9.1 protein, whereas only a small amount of CYP2C9.24 protein was seen in all independent replications of transfection (Fig. 1B). These results suggested that CYP2C9.24 protein may be improperly folded in yeast cells, presumably due to improper heme incorporation and therefore might be subjected to a rapid intracellular degradation. Although it could also be possible that protein synthesis was decreased or immunoreactivity of the variant protein changed, decreased protein levels and faster proteolytic degradation rate was recently reported in variant CYP1B1 allele with N453S substitution (Bandiera et al., 2005).

To further test this hypothesis, we heterologously expressed wild-type and mutated CYP2C9 proteins in mammalian cells HEK293. Carbon monoxide difference spectral analysis of the microsomal fraction obtained from transfected HEK293 cells showed that only wild-type CYP2C9.1 protein was correctly folded (60 pmol/mg microsomal protein) (Fig. 2A), whereas no P450 CO-spectrum was observed for the cells transfected with *CYP2C9*24* cDNA or with empty pcDNA3.1 plasmid. Western blot analysis revealed that only cells transfected with wild-type construct produced detectable levels of CYP2C9.1 in all independent replications of transfection (Fig. 2B). These results were confirmative of those obtained from the yeast expression system. The only difference was that in HEK293 expression system not even a small amount of incorrectly folded protein could be detected. Similar discrepancies have been reported between yeast and mammalian system (Oscarson et al., 1999), suggesting that yeast may be a more permissive expression system for poorly stable proteins.

E354 is positioned in the carboxy-terminal portion of the helix K and is a part of the absolutely conserved EXXR sequence which may be involved in stabilizing the core structure. This sequence is buried in the interior of the protein and participates in a hydrogen bond network, together with the amino acids residues from the meander region (Fig. 3). Meander region is also highly conserved in structure (Graham and Peterson, 2002). The substitution of

Glutamate to Lysine would disrupt the salt bridges between E354, R357 and M406 from the meander region. Bigger size of the Lysine is also inconsistent with tight packing interactions with the amino acid residues that surround E354. Therefore, the substitution would probably result in disruption of the interaction of helix K with the meander region and would lead to protein misfolding or instability, which was also suggested by our experimental data.

In conclusion, our data suggest that the substitution of acidic amino acid Glutamate to basic Lysine may lead to incorrectly folded CYP2C9.24 protein that is rapidly degraded in both, yeast and mammalian cells. Furthermore, our data support our observations of lower warfarin dose requirements and lower (S)-warfarin clearance in a patient with *CYP2C9*24* allele (Herman et al., 2006).

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Footnotes

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Reprint requests:
Vita Dolzan, Institute of Biochemistry, Faculty of Medicine, Vrazov trg 2, SI-1000 Ljubljana, Slovenia;
phone: +386 1 543 76 69; fax: + 386 1 543 76 41; e-mail: vita.dolzan@mf.uni-lj.si

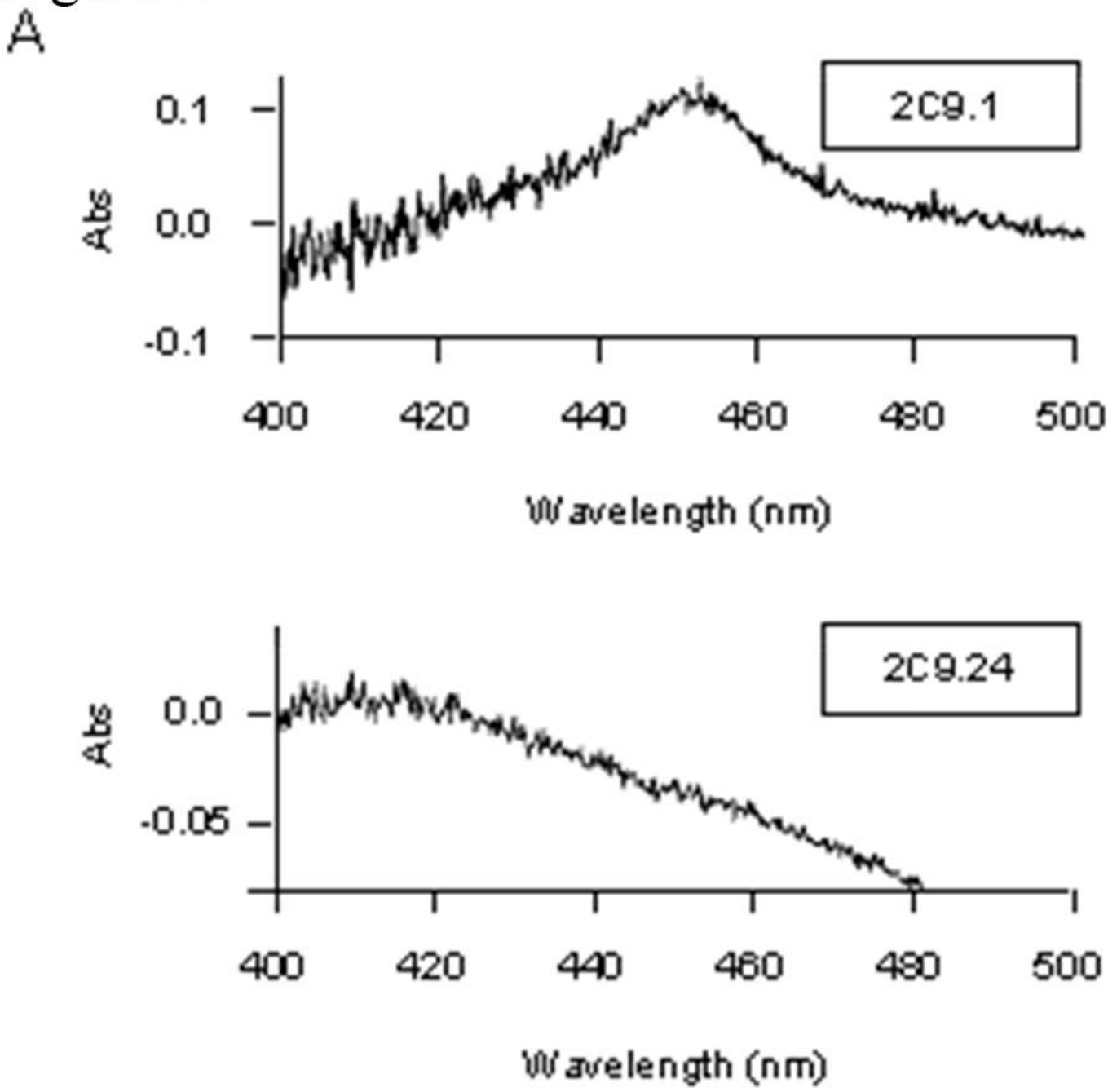
Legends to Figures

Fig. 1 Analysis of CYP2C9 expressed in yeast. (A) Carbon monoxide-binding spectra analysis of yeast cells transformed with wild-type *CYP2C9*1* and *CYP2C9*24* cDNA cloned in pYeDP60 (V60) expression vector. (B) Western blot analysis of CYP2C9 using CYP2C9 antibody. Microsomes were prepared from yeast cells transformed with *CYP2C9*1* and *CYP2C9*24* cDNA or empty pYeDP60. 30μg of protein was loaded per lane.

Fig. 2 Analysis of CYP2C9 expressed in mammalian HEK293 cells. (A) Carbon monoxide-binding spectra analysis of HEK293 cells transformed with wild-type *CYP2C9*1* and *CYP2C9*24* cDNA cloned in pcDNA3.1 expression vector. (B) Western blot analysis of CYP2C9 using CYP2C9 antibody. Microsomes were prepared from HEK293 cells transformed with *CYP2C9*1* and *CYP2C9*24* cDNA or empty pcDNA3.1. 30μg of protein was loaded per lane.

Fig. 3 View of glutamate residue at position 354 present in the helix K. E354 forms hydrogen bonds (indicated by dashed lines) to R357 and together they form hydrogen bonds to the backbone of the meander region. This figure was produced from CYP2C9 crystal structure image (http://www.rcsb.org/pdb/explore.do?structureId=1OG5) using DeepView (http://www.expasy.org/spdbv/).

Figure 1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400
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Wavelength (nm)
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Abs
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420
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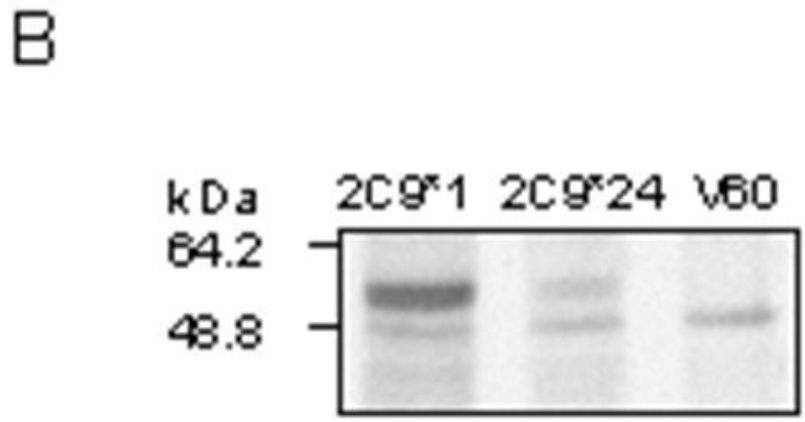
480
500
Wavelength (nm)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2C9*24
V60
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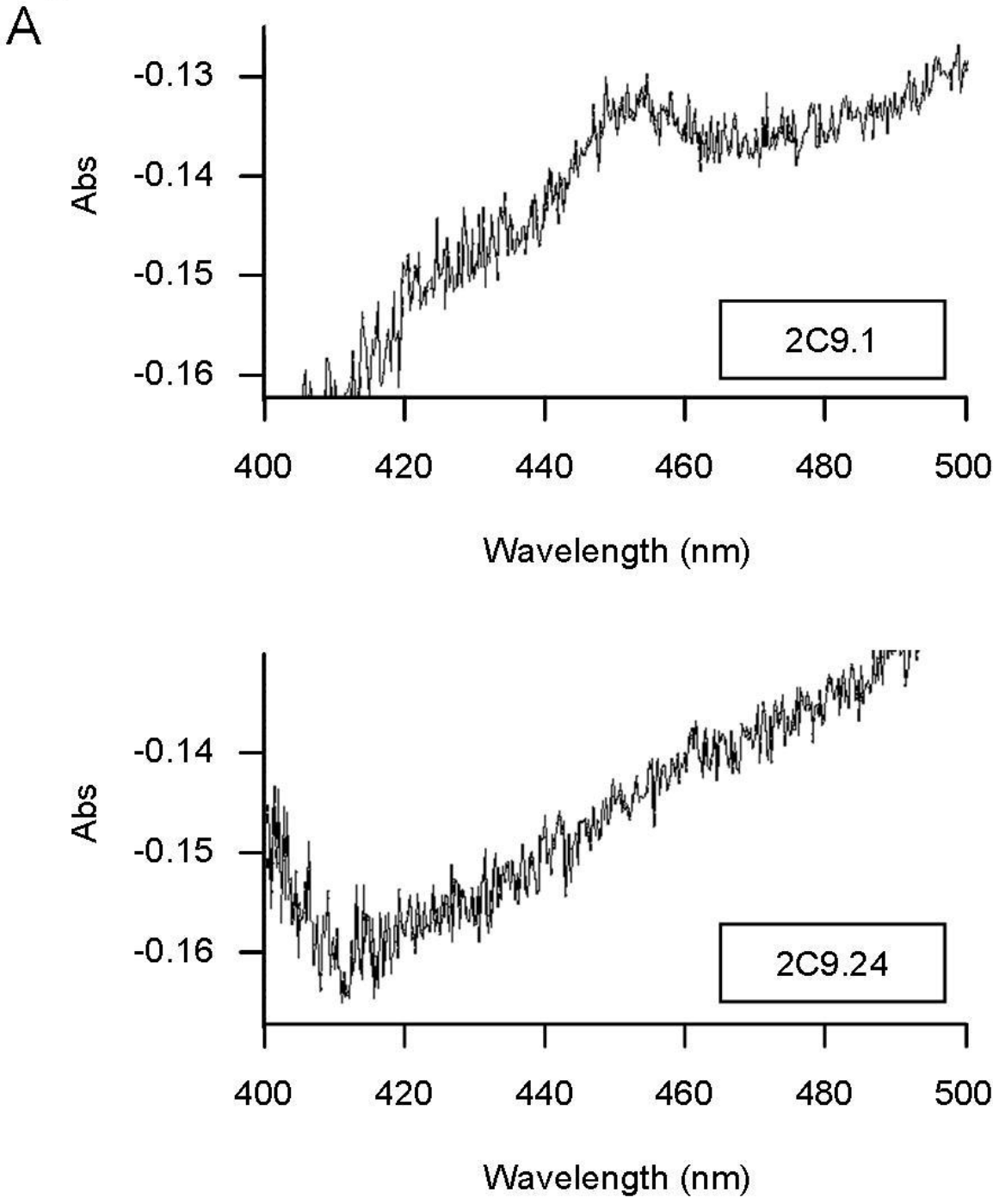
48.8

Figure 2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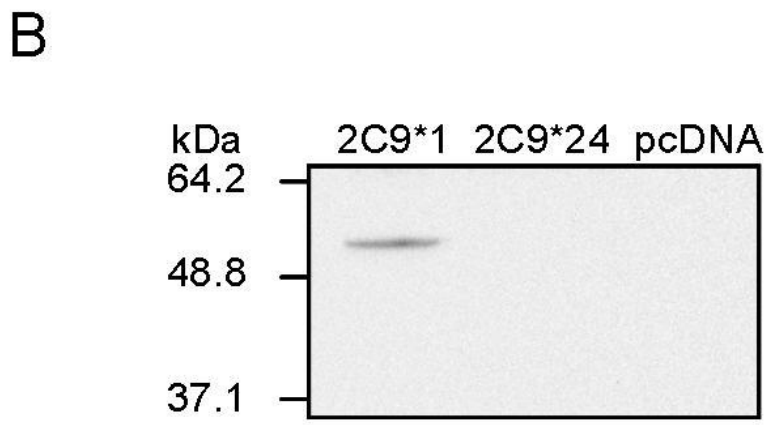
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pcDNA
64.2
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Figure 3