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ORIGINAL ARTICLE

Extensive protein interactions involving cytochrome P450 3A4: a possible contributor to the formation of a metabolosome

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CYP3A4, cytochrome P450, metabolosome, nano-LC-MS/MS, protein–protein interactions

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Abstract

Cytochrome P450 (CYP) 3A4 is a membrane protein that catalyzes hydroxylation of endogenous and exogenous substrates. Protein–protein interaction is a crucial factor that regulates the function of enzymes. However, protein–protein interactions involving human CYPs have not been fully understood. In this study, extensive protein–protein interactions involving CYP3A4 were determined by a shotgun analysis of immunoprecipitate utilizing anti-CYP3A4 antibody. Our shotgun analysis revealed that 149 proteins were immunoprecipitated with anti-CYP3A4 antibody in human liver microsomes. We further determined that 51 proteins of 149 proteins were specifically immunoprecipitated with the anti-CYP3A4 antibody. Our analysis demonstrated that other CYP isoforms are interacting with CYP3A4, which is in agreement with previous findings. Based on our current and previous findings, we propose that drug-metabolizing enzymes such as CYP3A4 and UDP-glucuronosyltransferase 2B7 form a metabolosome, which is a functional unit of metabolism consisting of multiple metabolism-related proteins.

Abbreviation

CPR, NADPH-cytochrome P450 reductase; CYP, cytochrome P450; ER, endoplasmic reticulum; LC-MS/MS, liquid chromatography–mass spectrometry; UGTs, UDP-Glucuronosyltransferases.

Introduction

Cytochrome P450s (CYPs; EC 1.14.x.x) are important membrane-bound enzymes that metabolize a number of endogenous and exogenous substrates. Human CYP is a superfamily of enzymes that are divided into 18 families with a total of 57 isoforms (Lewis 2004). The *CYP3A4* gene is located on chromosome 7q22.1 and is the main CYP isoform responsible for the metabolism of more than 50% of clinically used drugs (Williams et al. 2004). As CYPs are called as phase I drug-metabolizing enzymes, there is also a group of phase II drug-metabolizing enzymes that catalyze the conjugation of their substrates. UDP-Glucuronosyltransferases (UGTs; EC 2.4.1.17), which are the major phase II drug-metabolizing enzymes, metabolize drugs by transferring the glucuronic acid moiety of UDP-glucuronic acid to the substrates (Dutton 1980; Mackenzie et al. 2005).

While the active site of CYPs locates in the cytoplasmic side of endoplasmic reticulum (ER), the active site of UGT proteins locates in the luminal side of ER (Shepherd et al. 1989). Whereas it has been reported that CYPs and UGTs functionally interact with each other, as CYPs catalyze hydroxylation of substrates so that UGT can transfer glucuronic acid to the hydroxyl group of the substrates (Nakajima and Yokoi 2005; Zheng et al. 2007). Physical interactions between CYPs and UGTs have been demonstrated by immunoprecipitation assays utilizing anti-CYP3A4 and anti-UGT2B7 antibodies (Fremont et al. 2005; Ishii et al. 2007; Takeda et al. 2009), supporting that CYPs and UGTs are interacting with each other to cooperatively metabolize the substrates. Not only UGTs but also other drug-metabolizing enzymes, such as epoxide hydrolase 1, have been reported to interact with CYPs (Taura et al. 2000).

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However, the physiological role of these protein interactions is still largely unknown.

Metabolosome, which is a functional unit of metabolism, is the multimolecular assembly composed of metabolizing enzymes, transport-related proteins (transporters, channels or pumps), regulatory proteins, scaffold proteins, and other functional cellular components, which are assembled by means of multiple protein–protein interactions and/or protein–lipid interactions (Mori et al. 2011). The fact that human CYPs have been reported to interact with other proteins such as UGTs and epoxide hydrolase led us to investigate whether CYPs form a metabolosome assembled by extensive protein–protein interactions. In this study, large-scale analysis of protein–protein interaction involving CYP3A4 was carried out by shotgun liquid chromatography–mass spectrometry (LC-MS/MS) proteomic analysis of immunoprecipitated proteins to identify proteins interacting with human CYP3A4 in human liver microsomes.

Materials and Methods

Chemicals and reagents

Human liver microsomes were obtained from BD Gentest (Woburn, MA). Rabbit anti-human CYP3A4 antibody (H00001576-D01) was purchased from Abnova (Taipei, Taiwan). Control antibodies (IgG from rabbit serum) were purchased from Sigma–Aldrich (St Louis, MO) and Abcam (Cambridge, MA). Immunoprecipitation Kit Dynabeads ProteinG was purchased from Invitrogen (Carlsbad, CA). All other chemicals and solvents were of analytical grade or the highest grade commercially available.

Immunoprecipitation and Nano-LC-MS/MS analysis of the immunoprecipitate

Immunoprecipitation assay and nano-LC-MS/MS analysis were conducted as described before (Fujiwara and Itoh 2014).

Results

Identification of proteins immunoprecipitated with human CYP3A4 antibody

CYP3A4 is one of the major CYP isoforms involved in metabolism of a wide variety of drugs in the human liver. To identify proteins that interact with CYP3A4, human liver microsomes were subjected to an immunoprecipitation assay with rabbit anti-human CYP3A4 antibody, followed by a shotgun analysis of the immunoprecipitate by an LC-MC/MC analysis. Our shotgun analysis revealed that 149 proteins including human CYP3A4 were coimmunoprecipitated with anti-human CYP3A4 antibody (Fig. 1). Human CYP3A4 has been reported to interact with human UGTs and epoxide hydrolases. In this study, peptide sequences of these proteins were observed in the LC-MC/MC analysis of the immunoprecipitate, showing the reproducibility of the previous findings. Several peptide sequences of other proteins such as protein transport protein Sec61 were also obtained in the shotgun analysis. These data indicate that CYP3A4 might interact with multiple proteins to form a metabolosome.

To exclude the possibility that the anti-CYP3A4 antibody used in the immunoprecipitation assay nonspecifically reacted to proteins in human liver microsomes, we incorporated our previous data into the current study. We have previously carried out immunoprecipitation assays with two different control rabbit IgG, followed by shotgun analysis (Fujiwara and Itoh 2014). It was revealed that more than 100 proteins were nonspecifically interacted with IgG in the human liver microsomes. Table S1 shows the list of proteins specifically coimmunoprecipitated with the anti-human CYP3A4 antibody.

While the list of proteins coimmunoprecipitated with the anti-CYP3A4 antibody contains several mitochondrial, outer membrane, and extracellular proteins, it also contains a number of proteins that are present in the ER such as CYP3A5, CYP2A6, CYP4F2, and bile acyl-CoA synthetase (Table 1).

Discussion and Conclusions

CYPs are the predominant proteins that are involved in metabolism of a wide variety of drugs. Due to a greater interest especially in the biomedical field, the insight into the molecular mechanism of CYPs-catalyzed metabolism (i.e., hydroxylation) of the substrate has been thoroughly investigated. Protein–protein interaction is known as a crucial factor that regulates the function of the proteins. CYPs have been reported to interact with a microsomal protein, NADPH-cytochrome P450 reductase (CPR). Indeed, these interactions are required for CYP catalysis (Bernhardt 2006). The physical and functional interactions between CYP and CPR have been widely demonstrated. Recent studies also revealed that CYPs interact with other drug-metabolizing enzymes expressed in ER such as UGTs and epoxide hydrolase (Taura et al. 2000; Ishii et al. 2007). It was further demonstrated that the protein–protein interaction between CYPs and UGTs affected their enzymatic activities (Ishii et al. 2014). Not only with other proteins, but CYPs also interact with CYP enzymes themselves to form homo- and heterodimers (Subramanian et al. 2009, 2010; Davydov 2011; Reed and Backes 2012). The presence of these complexi-

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(A)

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   1MALIPDLAME TWLLLAVSLV LLYLYGTHSH GLFKKLGIPG PTPLPFLGNI
  51LSYHKGFCMF DMECHKKYGK VWGFYDGQQP VLAITDPDMI KTVLVKECYS
 101VFTNRRPFGP VGFMKSAISI AEDEEWKRLR SLLSPTFTSG KLKEMVPIIA
 151QYGDVLVRNL RREAETGKPV TLKDVFGAYS MDVITSTSFG VNIDSLNNPQ
 201DPFVENTKKL LRFDFLDPFF LSITVFPFLI PILEVLNICV FPREVTNFLR
 251KSVKRMKESR LEDTQKHRVD FLQLMIDSQN SKETESHKAL SDLELVAQSI
 301IFIFAGYETT SSVLSFIMYE LATHPDVQQK LQEEIDAVLP NKAPPTYDTV
 351LQMEYLDMVV NETLRLFPIA MRLERVCKKD VEINGMFIPK GVVVMIPSYA
 401LHRDPKYWTE PEKFLPERFS KKNKDNIDPY IYTPFGSGPR NCIGMRFALM
 451NMKLALIRVL QNFSFKPCKE TQIPLKLSLG GLLQPEKPVV LKVESRDGTV
 501SGA
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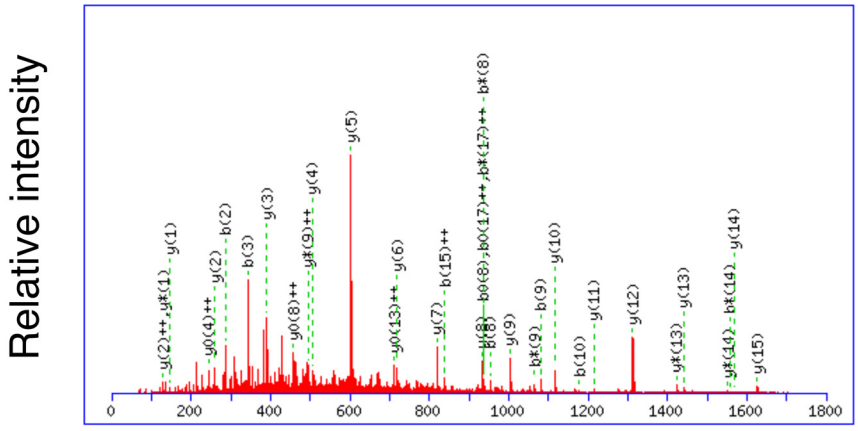
(B)



(C)

#	b	b++	b*	b*++	b0	b0++	Seq.	y	y++	y*	y*++	y0	y0++	#
1	100.0757	50.5415					V							21
2	286.155	143.5811					W	2294.1162	1147.5617	2277.0896	1139.0485	2276.1056	1138.5564	20
3	343.1765	172.0919					G	2108.0369	1054.5221	2091.0103	1046.0088	2090.0263	1045.5168	19
4	490.2449	245.6261					F	2051.0154	1026.0113	2033.9889	1017.4981	2033.0048	1017.0061	18
5	653.3082	327.1577					Y	1903.947	952.4771	1886.9204	943.9639	1885.9364	943.4719	17
6	768.3352	384.6712			750.3246	375.6659	D	1740.8837	870.9455	1723.8571	862.4322	1722.8731	861.9402	16
7	825.3566	413.1819			807.3461	404.1767	G	1625.8567	813.432	1608.8302	804.9187	1607.8462	804.4267	15
8	953.4152	477.2112	936.3886	468.698	935.4046	468.206	Q	1568.8353	784.9213	1551.8087	776.408	1550.8247	775.916	14
9	1081.4738	541.2405	1064.4472	532.7273	1063.4632	532.2352	Q	1440.7767	720.892	1423.7501	712.3787	1422.7661	711.8867	13
10	1178.5265	589.7669	1161.5	581.2536	1160.516	580.7616	P	1312.7181	656.8627	1295.6916	648.3494	1294.7075	647.8574	12
11	1277.595	639.3011	1260.5684	630.7878	1259.5844	630.2958	V	1215.6653	608.3363	1198.6388	599.823	1197.6548	599.331	11
12	1390.679	695.8431	1373.6525	687.3299	1372.6685	686.8379	L	1116.5969	558.8021	1099.5704	550.2888	1098.5864	549.7968	10
13	1461.7161	731.3617	1444.6896	722.8484	1443.7056	722.3564	A	1003.5129	502.2601	986.4863	493.7468	985.5023	493.2548	9
14	1574.8002	787.9037	1557.7736	779.3905	1556.7896	778.8985	I	932.4757	466.7415	915.4492	458.2282	914.4652	457.7362	8
15	1675.8479	838.4276	1658.8213	829.9143	1657.8373	829.4223	T	819.3917	410.1995	802.3651	401.6862	801.3811	401.1942	7
16	1790.8748	895.941	1773.8483	887.4278	1772.8643	886.9358	D	718.344	359.6756	701.3175	351.1624	700.3334	350.6704	6
17	1887.9276	944.4674	1870.901	935.9542	1869.917	935.4621	P	603.3171	302.1622	586.2905	293.6489	585.3065	293.1569	5
18	2002.9545	1001.9809	1985.928	993.4676	1984.944	992.9756	D	506.2643	253.6358	489.2377	245.1225	488.2537	244.6305	4
19	2133.995	1067.5011	2116.9685	1058.9879	2115.9844	1058.4959	M	391.2374	196.1223	374.2108	187.609			3
20	2247.0791	1124.0432	2230.0525	1115.5299	2229.0685	1115.0379	I	260.1969	130.6021	243.1703	122.0888			2
21							K	147.1128	74.06	130.0863	65.5468			1

Figure 1. Peptide sequence analysis of human CYP3A4. Six peptide sequences of human CYP3A4 were obtained with the shotgun analysis of the immunoprecipitate with anti-CYP3A4 antibody (A). The middle panel indicates the MS/MS spectra of the peptide (B), whereas the bottom panel indicates ion tables (C).

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Table 1. ER Proteins that were coimmunoprecipitated with anti-CYP3A4 antibody.

No.	Accession	Score	Mass	Number of peptide sequences	emPAI	Protein name
2	CP3A4_HUMAN	140	57705	6	0.21	Cytochrome P450 3A4
7	S61A1_HUMAN	87	52687	1	0.07	Protein transport protein Sec61 subunit alpha isoform 1
9	SSRG_HUMAN	73	21067	1	0.18	Translocon-associated protein subunit gamma
10	NDUB4_HUMAN	70	15256	1	0.26	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 4
12	ERP29_HUMAN	64	29032	1	0.13	Endoplasmic reticulum resident protein 29
14	S27A5_HUMAN	61	76420	2	0.1	Bile acyl-CoA synthetase
15	CO4A_HUMAN	61	194247	1	0.02	Complement C4-A
16	CISD2_HUMAN	59	15497	2	0.56	CDGSH iron-sulfur domain-containing protein 2
18	AT2A3_HUMAN	52	115444	1	0.03	Sarcoplasmic/endoplasmic reticulum calcium ATPase 3
21	SSRD_HUMAN	50	19158	1	0.2	Translocon-associated protein subunit delta
22	NDUAC_HUMAN	49	17104	1	0.23	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 12
23	FAS_HUMAN	48	275877	1	0.01	Fatty acid synthase
24	AT1A1_HUMAN	48	114135	1	0.03	Sodium/potassium-transporting ATPase subunit alpha-1
27	GLU2B_HUMAN	43	60357	1	0.06	Glucosidase 2 subunit beta
28	GANAB_HUMAN	43	107263	1	0.03	Neutral alpha-glucosidase AB
36	CP3A5_HUMAN	26	57357	1	0.06	Cytochrome P450 3A5
38	TM109_HUMAN	25	26194	1	0.15	Transmembrane protein 109
39	CP4F2_HUMAN	23	60442	1	0.06	Leukotriene-B(4) omega-hydroxylase 1
41	CP2A6_HUMAN	22	56636	1	0.07	Cytochrome P450 2A6
47	STRUM_HUMAN	18	135113	1	0.03	WASH complex subunit strumpellin

ties of the protein–protein interaction in ER suggests that CYP3A4 might be involved in the formation of a metabolosome, a functional unit of metabolism consisting of multiple metabolism-related proteins.

Most of the metabolites of endogenous and exogenous compounds produced by CYPs are subsequently metabolized by phase II drug-metabolizing enzymes such as UGTs to further increase in their hydrophilicity. The fact that the majority of CYPs are localized on the cytoplasmic and UGTs on the luminal side of the ER membrane leads us to believe that their substrates and metabolites need to be efficiently translocated across the ER membrane. However, CYPs and UGTs solely possess a transmembrane helix, which make it impossible to form a pore, suggesting that other proteins might be involved in the transport of the hydrophilic substrates/metabolites across the ER membrane. In this study, it was revealed that a number of microsomal proteins were interacting with CYP3A4 in the microsomal fraction (Table 1). While the functional relationship between CYP3A4 and the interacting proteins is largely unknown, the formation of a metabolosome might contribute to the accelerated translocation of the hydrophilic substrates/metabolites across the ER membrane. This hypothesis is supported by the finding that several translocators such as Sec61 and translocon-associated proteins were coimmunoprecipitated with CYP3A4 in this study (Table 1). While the primary role of the translocators is to translocate polypeptides across the ER membrane, it has been reported that Sec61 was also involved in efflux of a small molecule, calcium, from the ER (Lang et al. 2011). The functional relationship between CYP3A4 and proteins listed in Table 1 needs to be further investigated in the future.

To exclude the proteins nonspecifically interacting with the antibody used in this study, we incorporated our previous data (Fujiwara and Itoh 2014), which showed that more than 100 proteins were nonspecifically interacted with IgG in the human liver microsomes. However, many mitochondrial, outer membrane, and extracellular proteins such as ATP synthase and transhydrogenase were still included in the list of proteins that were found to interact with CYP3A4 (Table S1). While it is possible that those proteins are transiently present in the ER to interact with CYP3A4, it could be a result of indirect/nonspecific interactions.

In conclusion, we performed a shotgun analysis of immunoprecipitate obtained with anti-CYP3A4 antibody by utilizing nano-LC-MS/MS analysis. Twenty ER proteins were newly identified as proteins interacting with CYP3A4 in human liver microsomes. These proteins might be associated with a formation of a metabolosome involving CTP3A4. Functional roles of newly identified proteins interacting with CYP3A4 in metabolism and translocation of substrates or metabolites need to be examined in future studies.

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Disclosures

None declared.

References

Bernhardt R (2006). Cytochromes P450 as versatile biocatalysts. J Biotechnol 124: 128–145.

Davydov DR (2011). Microsomal monooxygenase as a multienzyme system: the role of P450-P450 interactions. Expert Opin Drug Metab Toxicol 7: 543–558.

Dutton GJ (1980). Acceptor substrates of UDP glucuronosyltransferase and their assay. Pp. 69–78*in* G. J. Dutton, ed. Glucuronidation of drugs and other compounds. CRC Press, Boca Raton, FL.

Fremont JJ, Wang RW, King CD (2005). Coimmunoprecipitation of UDP-glucuronosyltransferase isoforms and cytochrome P450 3A4. Mol Pharmacol 67: 260–262.

Fujiwara R, Itoh T (2014). Extensive protein-protein interactions involving UDP-glucuronosyltransferase (UGT) 2B7 in human liver microsomes. Drug Metab Pharmacokinet 29: 259–265.

Ishii Y, Iwanaga M, Nishimura Y, Takeda S, Ikushiro S, Nagata K, et al. (2007). Protein-protein interactions between rat hepatic cytochromes P450 (P450s) and UDP-glucuronosyltransferases (UGTs): evidence for the functionally active UGT in P450-UGT complex. Drug Metab Pharmacokinet 22: 367–376.

Ishii Y, Koba H, Kinoshita K, Oizaki T, Iwamoto Y, Takeda S, et al. (2014). Alteration of the function of the UDP-glucuronosyltransferase 1A subfamily by cytochrome P450 3A4: different susceptibility for UGT isoforms and UGT1A1/7 variants. Drug Metab Dispos 42: 229–238.

Lang S, Erdmann F, Jung M, Wagner R, Cavalie A, Zimmermann R (2011). Sec61 complexes form ubiquitous ER Ca2+ leak channels. Channels (Austin) 5: 228–235.

Lewis DF (2004). 57 varieties: the human cytochromes P450. Pharmacogenomics 5: 305–318.

Mackenzie PI, Bock KW, Burchell B, Guillemette C, Ikushiro S, Iyanagi T, et al. (2005). Nomenclature update for the mammalian UDP glycosyltransferase (*UGT*) gene superfamily. Pharmacogenet Genomics 15: 677–685.

Mori Y, Kiyonaka S, Kanai Y (2011). Transportsomes and channelsomes: are they functional units for physiological responses? Channels (Austin) 5: 387–390.

Nakajima M, Yokoi T (2005). Interindividual variability in nicotine metabolism: C-oxidation and glucuronidation. Drug Metab Pharmacokinet 20: 227–235.

Reed JR, Backes WL (2012). Formation of P450 · P450 complexes and their effect on P450 function. Pharmacol Ther 133: 299–310.

Shepherd SR, Baird SJ, Hallinan T, Burchell B (1989). An investigation of the transverse topology of bilirubin UDP-glucuronosyltransferase in rat hepatic endoplasmic reticulum. Biochem J 259: 617–620.

Subramanian M, Low M, Locuson CW, Tracy TS (2009). CYP2D6-CYP2C9 protein-protein interactions and isoform-selective effects on substrate binding and catalysis. Drug Metab Dispos 37: 1682–1689.

Subramanian M, Tam H, Zheng H, Tracy TS (2010). CYP2C9-CYP3A4 protein-protein interactions: role of the hydrophobic N terminus. Drug Metab Dispos 38: 1003–1009.

Takeda S, Ishii Y, Iwanaga M, Nurrochmad A, Ito Y, Mackenzie PI, et al. (2009). Interaction of cytochrome P450 3A4 and UDP-glucuronosyltransferase 2B7: evidence for protein-protein association and possible involvement of CYP3A4 J-helix in the interaction. Mol Pharmacol 75: 956–964.

Taura KI, Yamada H, Hagino Y, Ishii Y, Mori MA, Oguri K (2000). Interaction between cytochrome P450 and other drug-metabolizing enzymes: evidence for an association of CYP1A1 with microsomal epoxide hydrolase and UDP-glucuronosyltransferase. Biochem Biophys Res Commun 273: 1048–1052.

Williams JA, Hyland R, Jones BC, Smith DA, Hurst S, Goosen TC, et al. (2004). Drug-drug interactions for UDP-glucuronosyltransferase substrates: a pharmacokinetic explanation for typically observed low exposure (AUCi/AUC) ratios. Drug Metab Dispos 32: 1201–1208.

Zheng Y, Sun D, Sharma AK, Chen G, Amin S, Lazarus P (2007). Elimination of antiestrogenic effects of active tamoxifen metabolites by glucuronidation. Drug Metab Dispos 35: 1942–1948.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Proteins that were coimmunoprecipitated with anti-CYP3A4 antibody. Proteins specifically immunoprecipitated with the anti-CYP3A4 antibody are shown. Proteins expressed in the ER are highlighted with a red color.

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