Original Article

Functional characterization and substrate specificity of a novel gene encoding zinc finger-like protein, ZfLp, in *Xenopus laevis* oocytes

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ABSTRACT — In the present study, we isolated and determined the pharmacological characteristics of a novel gene encoding the zinc finger-like protein (ZfLp). The isolated cDNA consisted of 1,581 base pairs that encoded a 526-amino acid protein. The amino acid sequence of ZfLp is 96% identical to that of zinc finger protein 415 isoform 5 (ZNF415-5). Reverse-transcription (RT)-polymerase chain reaction (PCR) analysis revealed that the ZfLp mRNA is expressed in the breast, lung, stomach, small intestine colon and ovary, but not in the liver. When expressed in *Xenopus laevis* oocytes, ZfLp mediated the high affinity transport of [³H]paclitaxel (taxol) in a sodium-independent manner ($K_m = 336.7 \pm 190.0$ nM). The uptake of [³H]paclitaxel (taxol) by ZfLp was *trans*-stimulated by glutarate and glutathione (GSH). A *cis*-inhibition experiment revealed that ZfLp-mediated transport of [³H]paclitaxel (taxol) is inhibited by several organic solutes specifically clotrimazole. Using several clotrimazole derivatives, we found that N-benzylimidazole would be a minimum unit for producing the inhibition of ZfLp-mediated drug uptake. Our results may provide insights into the novel role of soluble protein, such as ZNF, in the human body. Our results, therefore, would be expected to facilitate research on the novel role of ZNFs and on the discovery of novel drugs for targeting ZNF-related proteins such as ZfLp.

Key words: Zinc finger-like protein, Drug transporter, Organic solutes, Toxicity

INTRODUCTION

The zinc finger proteins (ZNFs) are a single class of transcription factors in the human genome. ZNFs constitute the largest individual family of nucleic acid-binding proteins (Looman *et al.*, 2002). A zinc finger has a conserved motif of 28 amino acids, which is often repeated within a protein and may be involved in protein-protein or DNA-protein interactions (Klug and Rhodes, 1987; Wu, 2002; Lupo *et al.*, 2011). ZNFs can coordinate one or more zinc ions to stabilize their folds. In addition, these proteins are structurally diverse and exist among proteins that perform a broad range of functions in various cellular processes such as repair, replication, translation, transcription, metabolism, signaling, cell proliferation and apoptosis (Mitchell and Tjian, 1989). Thus, ZNFs play central

roles in regulating the expressions of a variety of genes.

Drug concentration in the blood and the target organs regulate several sequential steps such as transport, intracellular processing, elimination, excretion, and metabolism. Although these processes have been studied in the liver, kidney, brain, small intestine and placenta, the uptake step is an important process because many different kinds of drug carrier proteins are known to regulate the drug concentrations in these organs (Saier, 2000; Van Aubel *et al.*, 2000; Leonard *et al.*, 2003; Leonessa and Clarke, 2003; Rizwan and Burckhardt, 2007). Several different kinds of xenobiotic transporters have been isolated and well characterized. To date, 51 solute carrier (SLC) families have been classified (<http://www.bioparadigms.org/slc/intro.htm>). Among them, the *SLC21A* and *22A* families are considered to be most important molecules

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for drug clearance from the body. For example, LST-1/OATP1B1[*SLCO1B1*] transports statins (Simonson *et al.*, 2004; Kameyama *et al.*, 2005), ezetimibe glucuronide (Nozawa *et al.*, 2005; Oswald *et al.*, 2008), SN-38 (Nozawa *et al.*, 2005), and valsartan (Maeda *et al.*, 2006). Human organic anion transporter 2 (hOAT2[*SLC22A7*]), 5 (hOAT5[*SLC22A10*]) and 7 (hOAT7[*SLC22A9*]) are also considered to be a key molecule in hepatic handling of organic anions since this protein mediates the transport of methotrexate, theophylline, erythromycin, prostaglandin E₂ (PGE₂), cAMP, α -ketoglutarate, azidodeoxythymidine (AZT), tetracycline and *p*-aminohippurate (PAH) (Sun *et al.*, 2001; Babu *et al.*, 2002; Takeda *et al.*, 2002; Kobayashi *et al.*, 2005a; Shin *et al.*, 2007).

With regard to the secondary structure of these transporters, many investigators have predicted there are 10-12 transmembrane domains (TMDs) (Sekine *et al.*, 1997; Sweet *et al.*, 1997; Cha *et al.*, 2000, 2001; Hagenbuch and Meier, 2004; Koepsell and Endou, 2004). Likewise, the ATP-binding cassette (ABC) transporter family, such as breast cancer resistance protein (BCRP[*ABCG2*]), is predicted to have 6 TMDs (Leslie *et al.*, 2005). In addition, we have proposed that human organic solute carrier partner 1 (hOSCP1, formally named organic solute carrier protein 1) is predicted to have 3 TMDs (Kobayashi *et al.*, 2005b). The secondary structure model of 4F2hc is predicted to have a single transmembrane domain (Wells *et al.*, 1992; Kanai *et al.*, 1998). Similarly, related to b⁰+ amino acid transporter (rBAT), the lambda light chain of human immunoglobulin surface antigen-related gene (IgLC-rG), and ribosomal protein L3 (RPL3) are predicted to have one and two TMDs, respectively (Bertran *et al.*, 1992a, 1992b; Wells *et al.*, 1992; Lee *et al.*, 1993; Kanai *et al.*, 1998; Kobayashi *et al.*, 2005b, 2010a and 2010b). Thus, the TMD signature seems to be an important structural signature(s) for activating drug transport.

In the present study, we examined the transport activity of a novel gene encoding zinc finger-like protein (ZfLp) using a *X. oocyte* expression system. Although ZNFs plays an important role in regulating the expression of many kinds of genes, our results indicate that isolated ZfLp may function as a drug carrier protein. Our findings would provide new insights into a novel function of ZNF, especially a ZNF-related gene such as ZfLp.

MATERIALS AND METHODS

Materials

[³H]Paclitaxel (taxol) (20 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). [³H]5-Fluorouracil (5-FU) (13 Ci/mmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA, USA). Deoxycytidine [5'- α -³²P]triphosphate (dCTP) (111 TBq/mmol) was obtained from Muromachi Yakuhin Kaisha, LTD (Tokyo, Japan). N-Triphenylimidazole, N-diphenylmethylimidazole, and N-phenylpropylimidazole were synthesized as previously described (Kobayashi *et al.*, 1993). N-Benzylimidazole and clotrimazole were purchased from Sigma-Aldrich, Co. (St. Louis, MO, USA). All other chemicals not listed here were of the highest grade commercially available.

Construction of cDNA library and isolation of ZfLp

A nondirectional cDNA library was prepared from human breast poly (A)⁺ RNA using the Superscript Choice System (Life Technologies, Gaithersburg, MD, USA) and was ligated into a phage vector λ ZipLox *Eco*RI arms (Life Technologies). Human breast poly (A)⁺ RNA was purchased from BD Biosciences Clontech (Palo Alto, CA, USA).

An Expressed Sequence Tag (EST) data base search for "Query SLCO2A1" (GenBank accession number NM_005630) and an EST clone (BU944345) were identified. After PCR amplification of this EST clone (left primer, 5'-ATACGGACAGACTGGGATGC-3'; right primer, 5'-GAGGTGGCTTCCAGTACAGC-3'), the PCR product was labeled with [5'- α -³²P]dCTP by random priming (¹⁷Quick Prime Kit, Amersham Pharmacia Biotech), and the library was screened with an EST clone as a probe under low stringency conditions. Replicate filters of a phage library were hybridized overnight in a hybridization solution (50% formamide; 5x standard saline citrate (SSC); 3x Denhardt's solution; 0.2% sodium dodecyl sulfate (SDS); 10% dextran sulfate; 0.3 μ g/ml denatured salmon sperm DNA; 2.5 mM sodium pyrophosphate; 25 mM 4-morpholineethane sulfonic acid (MES); 0.03% Antifoam A; pH 6.5) at 37°C overnight. The filters were washed in 3x SSC and 0.5% SDS at 37°C. cDNA inserts in positive λ ZipLox phages were recovered in a plasmid pZL1 vector by *in vitro* excision.

cDNA sequence

Double-stranded cDNA of isolated clones were sequenced in both directions. Deleted clones, obtained by a KiloSequence deletion kit (Takara, Tokyo, Japan),

and specifically synthesized oligonucleotide primers were used for sequencing ZfLp cDNA, which was sequenced by the dye terminator method using a dye primer cycle sequencing kit (ver. 3.1., Applied Biosystems, Foster City, CA, USA) and automated Applied Biosystems 310 DNA sequencer. The sequence and the phylogenetic tree were analyzed using DNASIS®-Pro, Ver. 2.02 (HITACHI Software Engineering, Yokohama, Japan).

cRNA synthesis and functional characterization of ZfLp in *Xenopus laevis* oocytes

Selection and isolation of stage V and VI defolliculated *X. laevis* oocytes was performed as previously described (Kobayashi *et al.*, 2010a, 2010b). Collagenase A (Boehringer Mannheim, Mannheim, Germany) was used to remove the follicular layer at a final concentration of 2.0 mg/ml in a oocyte Ringer 2 (OR2) solution (83 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), pH 7.5). The ZfLp cDNA was linearized with KpnI, and capped cRNA was transcribed *in vitro* by Sp6 RNA polymerase. Defolliculated oocytes were microinjected with 50 ng of *in vitro* transcribed cRNA and incubated for two days in a modified Barth's solution containing gentamicin (50 µg/ml) at 18–20°C. Uptake experiments of radiolabeled substrates, as indicated in each experiment, were performed in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, pH 7.4) at room temperature. Oocytes were incubated in 150 µl of the same solution containing radiolabeled substrates. For Na⁺-dependent transport experiments, we used ND96 buffer containing lithium chloride, choline chloride, and mannitol with an equivalent amount of sodium chloride. Concentration-dependent uptake experiments of [³H]paclitaxel (taxol) in oocytes expressing ZfLp were performed at final concentrations of 1, 10, 20, 50, 100, 200 and 400 nM. Time-dependent transport of [³H]paclitaxel (taxol) via ZfLp were performed at 15, 30, 60, 120 and 150 min. The compound was incubated with expressing ZfLp oocytes for 1 hr at room temperature, stopped with ice-cold ND96 solution and washed 5 times. Three oocytes were transferred to one scintillation vial and dissolved in 200 µl 10% SDS. A scintillation cocktail was added, and radioactivity was counted. Counts in cRNA minus uninjected oocytes were subtracted from the counts in ZfLp cRNA-injected oocytes. Data are presented as mean ± S.E., except for kinetic constants for which the error represents the error of the fit. K_m indicates the Michaelis-Menten constant (nM). The experiments were repeated with oocytes from at least five to six frogs.

RT-PCR analysis

ZfLp cDNA was amplified by RT-PCR using the sense primer 5'-GACCAACGCGATAGAAGAGG-3' and antisense primer 5'-ATTCAAGGCTTTGTCGCACT-3' to yield a 312 fragment. RT-PCR was performed under the following conditions: 1 cycle at 60°C for 30 min, 1 cycle at 94°C for 2 min, 35 cycles at 94°C for 1 min, annealing at 53°C for 1.5 min followed by a final extension at 49°C for 10 min according to the manufacturer's instructions (BD Biosciences Clontech, Palo Alto, CA, USA).

Quantification of ZfLp mRNA expression

Total RNAs from 7 human tissues (breast, lung, liver, stomach, small intestine, colon, and ovary) were purchased from BioChain Institute, Inc. (San Leandro, CA, USA). RNAs were reverse-transcribed and real-time PCR was operated on a realplex² system (ependorf Japan, Tokyo, Japan) using the TaqMan Gene Expression Assay for ZNF415 (Applied Biosystems, Rotkreuz, Switzerland). Constitutively expressed human glyceraldehyde-3-phosphoate dehydrogenase (G-3-PDH) was measured as an internal control for sample normalization (Applied Biosystems).

cis-Inhibition study

For inhibition experiments, oocytes expressing ZfLp were incubated for 1 hr in ND96 solution containing 100 nM [³H]paclitaxel (taxol) in the presence or absence of inhibitors at a final concentration of 10 µM. Cyclosporine A, miconazole, clotrimazole, troleandomycin, azithromycin, erythromycin, N-triphenylimidazole, N-diphenylmethylimidazole, N-benzylimidazole, and N-phenylpropylimidazole were directly dissolved in ND96 solution from the stock solution. These inhibitors were dissolved in dimethyl sulfoxide (DMSO) and diluted to a final concentration as described above. The final concentration of DMSO in the assay medium did not exceed < 1%.

Statistical analysis

Kinetic data from experiments measuring the uptake of radiolabeled substrates were fit to the Michaelis-Menten equation by nonlinear least-squares regression analysis with standard errors derived from these curves (GraphPad Prism Version 4.01). Comparisons of data measuring initial rates of uptake of radiolabeled substrates in the presence and absence of inhibitors were performed by one-way ANOVA (GraphPad Prism Version 4.01), or unpaired Student's *t* test.

RESULTS

Because the *SLC2A1/SLCO2A1/OATP2A1* (NM_005630) gene is expressed ubiquitously in the human body (<http://www.bioparadigms.org/slc/menu.asp>), we assumed that this gene is a good tool for isolating a novel gene. After multiple rounds of screening using [³²P]dCTP-labeled EST clone (BU944345) under low stringency condition, we isolated several clones. Two clones had overlapping identical sequences, identical to ZfLp; the other clones had approximately 80% similarity with Ras and Rab interactor 2 (CAI19340), ras inhibitor (AAA36553), hypothetical protein (CAB66858), Ras and Rab interactor 2, isoform CRA_b (EAX10217), unnamed protein product (BAG52947) at the amino acid level. The deduced amino acid sequence of ZfLp exhibited < 10% identical to that of the *SLC2A1/SLCO2A1/OATP2A1*. The nucleotide and deduced amino acid sequences of ZfLp are shown in Fig. 1A. ZfLp cDNA consist of 1,581 nucleotides and codes for 526 amino acids with a calculated molecular mass of 60.4 kDa. There are four consensus sequences for *N*-glycosylation sites (Asn⁸⁸, Asn¹⁰³, Asn⁴²⁵⁸, Asn⁴⁸¹), fourteen PKC sites (Thr⁹⁰, Ser¹⁰⁵, Thr¹⁵⁵, Thr⁹⁰, Ser¹⁶¹, Thr²⁰³, Ser²²⁵, Thr³⁰⁰, Ser³⁰⁹, Ser³²², Ser⁴⁰⁰, Ser⁴⁷⁷, Thr⁴⁹⁰, Ser⁵⁰⁵), nine zinc finger C₂H₂ sites (Cys²¹⁷, Cys²⁴⁵, Cys²⁷³, Cys³²⁹, Cys³⁵⁷, Cys³⁸⁵, Cys⁴⁴¹, Cys⁴⁶⁹, Cys⁴⁹⁷). Rose hydropathy analysis (Window 11) of ZfLp protein revealed that this protein has several TMDs (data not shown). However, Kyte and Doolittle (Window 11), TMPred (http://www.ch.embnet.org/software/TMPRED_form.html), Janin (Window 11) and SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/sosui_submit.html) hydropathy analyses predicted that ZfLp protein has no hydrophobic stretches long enough to span the membrane (data not shown).

When we submitted the ZfLp cDNA sequence to Genbank, a similar sequence from humans had been deposited to GenBank (GenBankTM/EMBL/DDBJ: BAA92059, unnamed protein product, Homo sapiens). The amino acid sequence of ZfLp is 96% identical to that of ZNP415-5. We also identified several similar human gene products such as EAW72118, ABI51311, EAW72117, EAW72115, NP_060825, NP_001129510, ABI51312, ABI51310, EAW72116, AAH63880, BAG53073, XP_541449, NP_001166145, AAK37403, NP_115973, BAF82683, BAG53339, and BAG37173 that exhibited approximately 87% and 96%, respectively, predicted amino acid identity. Therefore, we constructed the phylogenetic tree using some of these similar sequences in blast program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and found that ZfLp is positioned within the human zinc

finger protein superfamily (Fig. 1B). These findings lead us to conclude that isolated cDNA is a novel gene that belongs to the human zinc finger protein.

Based on the Rose hydropathy analysis, we hypothesized that ZfLp might have a transport function. ZfLp cRNA-injected *Xenopus* oocytes were used for the transport characterization. Although ZfLp cRNA-injected oocytes exhibited no transport of several organic solutes such as [³H]allopurinol, [³H]probenecid, [³H]valproate, [¹⁴C]*p*-aminohippuric acid, [¹⁴C]tetraethylammonium, [¹⁴C]theophylline, [¹⁴C]nicotinamide, [¹⁴C]6-mercaptopurine, [¹⁴C]salicylate (data not shown); however, we found that oocyte expressing ZfLp exhibited the transport of [³H]5-FU and [³H]paclitaxel (taxol) to about 2.1 and 4.3 fold that of the control oocytes, respectively (Table 1). Our results indicate that [³H]paclitaxel (taxol) and [³H]5-FU would be good substrates for determining the pharmacological characterization of ZfLp.

We subsequently examined the transport characterization of ZfLp using [³H]paclitaxel (taxol). Firstly, we tested the effect of pH on the uptake of [³H]paclitaxel (taxol) by ZfLp-expressing oocytes. As shown in Fig. 2A, there was a different in [³H]paclitaxel (taxol) transport with a pH at 7.5; the activity was decreased by pH 5.5. Thus, the uptake of [³H]paclitaxel (taxol) mediated by ZfLp is sensitive to pH. Based on this finding, we next examined the concentration-dependent transport of [³H]paclitaxel (taxol) via ZfLp (Fig. 2B). The ZfLp-mediated uptake of [³H]paclitaxel (taxol) showed saturable kinetics and could be modeled by the Michaelis-Menten equation. Nonlinear regression analyses yielded *K_m* values of 336.7 ± 190.0 nM. Time-dependent transport of [³H]paclitaxel (taxol) via oocytes expressing ZfLp was subsequently investigated and revealed that the transport activity increased linearly until 15 min and reached maximum at 60 min of incubation (Fig. 2C). The effect of extracellular cation on the uptake of [³H]paclitaxel (taxol) was studied (Fig. 2D). The uptake of [³H]paclitaxel (taxol) via ZfLp was not affected by the replacement of extracellular sodium with lithium, choline, and mannitol, indicating that transport of [³H]paclitaxel (taxol) via ZfLp exhibited an Na⁺-independent carrier protein. We next examined the *trans*-stimulatory effect of GSH and glutarate to elucidate whether these endogenous compounds *trans*-stimulate the transport of [³H]paclitaxel (taxol) via ZfLp. As shown in Fig. 2E, transport of [³H]paclitaxel (taxol) by ZfLp exhibited the *trans*-stimulatory effect in the presence of both GSH and glutarate.

In order to elucidate the distribution and the localization of ZfLp mRNA in the human body, we chose some human tissues and performed RT-PCR analysis. A band

Isolation and pharmacological characterization of a novel gene, ZfLp

Fig. 1. cDNA and amino acid sequences and phylogenetic tree analysis of the zinc finger-like protein (ZfLp). A, cDNA and deduced amino acid sequences of the ZfLp. B, Phylogenetic relationship between zinc finger-like protein and several ZNF-related proteins. The phylogenetic tree was constructed using DNAsis-Pro. Version 2.02 (HITACHI Software Engineering). Branch length is drawn to scale. ZfLp, zinc finger-like protein.

with 312 bp was detected in the breast, lung, stomach, small intestine, colon, and ovary (Fig. 3A). No apparent PCR band was detected in human liver. To elucidate further detailed expression of the *ZfLp* gene, we subsequent-

ly performed real-time PCR analysis. The mRNA expression of this clone exhibited a rank order of the ovary (Ov) > lung (Lu) > breast (Br) > stomach (Sto) > colon (Col) > small intestine (SI) = liver (Li) (Fig. 3B). Thus, we found

Fig. 2. Functional characterization of ZfLp in *Xenopus laevis* oocytes. A, Effect of pH on [³H]paclitaxel (taxol) transports in ZfLp-expressing oocytes. One-hour uptake of 100 nM [³H]paclitaxel (taxol) was measured in Na⁺ buffers adjusted to pH values at 5.5 and 7.5. □, un-injected; ■, ZfLp cRNA-injected. B, Concentration-dependence of ZfLp-mediated uptake of [³H]paclitaxel (taxol). The uptake rate of [³H]paclitaxel (taxol) by the control and ZfLp-expressing oocytes for 1 hr were measured at variable concentrations. □, un-injected; ▲, ZfLp cRNA-injected. ●, ZfLp cRNA-injected–un-injected. C, Time-dependent uptake of [³H]paclitaxel (taxol) in ZfLp-expressing oocytes. The uptake of 100 nM [³H]paclitaxel (taxol) in oocytes expressing ZfLp was measured during 2.5 hr of incubation. □, un-injected; ▲, ZfLp cRNA-injected. ●, ZfLp cRNA-injected–un-injected. D, Effect of extracellular cation on [³H]paclitaxel (taxol) uptake by ZfLp. The uptake rates of [³H]paclitaxel (taxol) (100 nM) by control oocytes or ZfLp-expressing oocytes for 1 hr were measured in the presence or absence of extracellular Na⁺. Extracellular Na⁺ was replaced with an equimolar concentration of choline, lithium, and mannitol. E, *trans*-Stimulatory effect of glutamate and glutathione (GSH) on the transport of [³H]paclitaxel (taxol) via ZfLp. 100 nM [³H]paclitaxel (taxol) was used for the uptake experiments. Oocytes expressing ZfLp were preloaded with 10 μM glutamate or 10 μM GSH for 1 hr before starting the uptake experiment. *trans*-Stimulatory effect was observed in the presence of glutamate and GSH. □, non-injected; ■, ZfLp cRNA-injected. Data are expressed as fmol/oocyte/hr. Values are mean ± S.E. of 12–18-oocyte determinations. The significance between control and ZfLp cRNA-injected oocytes was determined by the unpaired *t* test (**p* < 0.01). Other experimental conditions and details are described under “Materials and Methods”. GSH, glutathione.

Table 1. Uptake of [³H]5-FU and [³H]paclitaxel (taxol) by ZfLp-expressing oocytes

Tracer	Concentration (nM)	Non-injected (fmol/oocyte/hr)	ZfLp (fmol/oocyte/hr)	Fold increase
[³ H]5-FU	100	36.01 ± 4.56	75.35 ± 3.39*	2.1
[³ H]Paclitaxel (taxol)	100	14.33 ± 1.75	61.77 ± 2.16*	4.3

After two days incubation, uptake experiments were performed in an ND96 solution for 1 hr. Values are mean ± S.E. of 12-15 oocyte determinations. The significance between control (non-injected) and ZfLp-cRNA-injected oocytes was determined by the unpaired *t* test (**p* < 0.05).

that there was a marked difference of the expression of ZfLp mRNAs among human tissues.

To find the substrate specificity of ZfLp, the *cis*-inhibitory effect of various compounds by ZfLp-mediated uptake of [³H]paclitaxel (taxol) was subsequently investigated. As shown in Fig. 4, the inhibition of the ZfLp-mediated transport of [³H]paclitaxel (taxol) exhibited a rank order of troleandomycin > erythromycin > azithromycin > clotrimazole.

Because clotrimazole exhibited the highest inhibitor for ZfLp-mediated uptake, we next studied the *cis*-inhibitory effect of various clotrimazole derivatives by ZfLp-mediated uptake of [³H]paclitaxel (taxol). As shown in Fig. 5, inhibitory effects were observed when adding N-triphenylmethylimidazole, N-diphenylmethylimidazole and N-benzylimidazole whereas N-phenylpropylimidazole did not inhibit the transport of [³H]paclitaxel (taxol) via ZfLp, suggesting that, N-benzylimidazole would be a structural determinant for the inhibition of ZfLp-mediated drug uptake.

DISCUSSION

The ZNFs are the single largest class of transcription factors in the human genome. Previous studies have revealed that ZNFs are involved in transcriptional activation and regulation of apoptosis (Klug and Rhodes, 1987; Looman *et al.*, 2002; Wu, 2002; Lupo *et al.*, 2011). Alternative splicing emerges as a major mechanism of generating protein diversity. The present study describes the isolation and transport characterization of a novel gene encoding the zinc finger-like protein, ZfLp. Transport experiments using a *X.* oocyte expression system revealed that ZfLp transports [³H]paclitaxel (taxol) and [³H]5-FU in pH-, concentration-, time-dependent and sodium-independent manners. The uptake of [³H]paclitaxel (taxol) via ZfLp was *trans*-stimulated by GSH and glutarate. *cis*-Inhibition experiment revealed that N-benzylimidazole exhibits a minimum structure for the inhibition of ZfLp-mediated

drug transport by clotrimazole. The present paper is the first evidence concerning the transport of organic solutes via the ZNFs-related gene in the human body.

Phylogenetic tree analysis revealed that the *ZfLp* gene belongs to the ZNF superfamily, and to ZNF415 in particular. Cheng *et al.* (2006) have reported that five different ZNF415 isoforms (ZNF415-1, -2, -3, -4, and -5) are expressed in adult tissues but exhibit different levels of expression by RT-PCR analysis. Their report indicates that ZNF415-5 mRNA is expressed in the liver. We examined, based on their findings, the tissue distribution and the expression of the *ZfLp* gene. RT-PCR analysis revealed that the ZfLp mRNA was expressed in the breast, lung, stomach, small intestine, colon and ovary in human adult tissues. However, we observed that no PCR band is detected in the liver, indicating that the expression of ZfLp is tissue-specific. Further, real-time PCR analysis revealed that the expression level of ZfLp mRNA in the small intestine and colon are low. In this respect, further study is needed to determine the reason the expression levels of ZfLp mRNA are exhibited in a differential pattern in human tissues.

With respect to the transmembrane domains (TMDs), we have reported that OSCP1/Oscp1, the lambda light chain of human immunoglobulin surface antigen-related gene (IgLC-rG), ribosomal protein L3 (RPL3) and human nucleoside transporter 1 (hNT1) are predicted to have at least one TMD (Kobayashi *et al.*, 2005a, 2005b, 2010a and 2010b; Umemoto *et al.*, 2009). These transporters are predicted to be clarified as soluble proteins (<http://bp.nuap.nagoya-u.ac.jp/sosui/>). On the other hand, human organic solute transporters such as SLCO21A/Slco21a, SLCO22A/Slco22a and SLC3A1/2 have several membrane-spanning domains. Similarly, ABC transporters are also predicted to have 6 to 12 membrane spanning domains (Solbach *et al.*, 2006). One possible structural feature for activating the drug transport seems to require the transmembrane signature. We examined, therefore, the secondary model of ZfLp protein using Rose

Fig. 3. Localization, distribution and expression of ZfLp mRNA in various human tissues. A, Localization and Distribution of ZfLp mRNA by RT-PCR. The first strand cDNA prepared from human breast, lung, liver, stomach, small intestine, colon and ovary total RNA (Biochain) was used as a template for PCR amplification. The PCR products were electrophoresed on 1.0% agarose gel and visualized with ethidium bromide. The ZfLp-specific PCR product was obtained from human breast, lung, stomach, small intestine, colon and ovary. No PCR product was found in the liver. B, Expression of ZfLp mRNA by real-time PCR. The expression of the ZfLp in human various normal tissues exhibited a rank order of ovary (Ov) > lung (Lu) > breast (Br) = stomach (Sto) > colon (Col) > small intestine (SI) = liver (Li).

(Window 11), Janin (Window 11), Kyte and Doolittle (Window 11), SOSUI (<http://bp.nuap.nagoya-u.ac.jp/sosui/>) and TMpred analyses (http://www.ch.embnet.org/software/TMPRED_form.html). Rose hydropathyl analy-

Fig. 4. *cis*-Inhibitory effect of ZfLp-mediated [³H]paclitaxel (taxol) uptake by various compounds. The concentration of [³H]paclitaxel (taxol) was 100 nM and those of inhibitors in the assay medium were 10 μ M. The values are expressed as a percentage of ZfLp-mediated [³H]paclitaxel (taxol) uptake in the absence of inhibitors. Clotrimazole exhibited a strong inhibitor for ZfLp-mediated [³H]paclitaxel (taxol) uptake. Data are expressed as the mean $\pm$ S.E. for 18-21 oocytes.

sis predicted that there are several transmembranes; however, no membrane-spanning domains were predicted by other analyses, suggesting that, at least partly, ZfLp may have transmembrane domain(s).

Based on previous papers (Kanai *et al.*, 1998; Ballatori *et al.*, 2005; Kobayashi *et al.*, 2005a, 2005b, 2010a and 2010b; Solbach *et al.*, 2006), we conducted uptake experiments using ZfLp-cRNA-injected oocytes. Surprisingly, we found that ZfLp mediates the transport of [³H]paclitaxel (taxol) and [³H]5-FU. In addition, we observed that transport properties of [³H]paclitaxel (taxol) via ZfLp are exhibited in pH-, concentration-, time-dependent manners, and Na⁺-independent manner. Further, we observed that oocytes expressing ZfLp do not transport PAH and TEA (data not shown), suggesting that either anionic or cationic moieties would not be necessary for the transport recognition of ZfLp. Several possibilities regarding the ZfLp-mediated drug transport have been raised as follows; first, ZfLp could be a new transporter with an atypical structure; secondary, ZfLp could be a subunit or associated protein of a transporter which is endogenously expressed in the oocytes; thirdly, ZfLp could be an intracellular protein which influences the expression of an endogenous transporter; fourthly, over-expression of ZfLp increases or stimulates uptake of an endogenous transporter in a non-specific manner; there-

Fig. 5. *cis*-Inhibitory effect of ZfLp-mediated [³H]paclitaxel (taxol) uptake by clotrimazole and its structurally related derivatives. The concentration of [³H]paclitaxel (taxol) was 100 nM and those of inhibitors in the assay medium were 10 μ M. The values are expressed as a percentage of ZfLp-mediated [³H]paclitaxel (taxol) uptake in the absence of inhibitors. N-Benzylimidazole exhibited a minimum structure for producing the inhibition of ZfLp-mediated [³H]paclitaxel (taxol) uptake by clotrimazole. No inhibitory effect was observed when adding N-phenylpropylimidazole. Data are expressed as the mean $\pm$ S.E. for 18-21 oocytes.

fore, further study is needed to resolve on these subject. It would be interesting to elucidate whether ZfLp is coupled to other unidentified proteins or whether ZfLp stimulates unidentified transport proteins. To distinguish whether ZfLp is a transporter/transporter or subunit/transporter associated protein, immunohistochemical analysis would be helpful to determine the subcellular localization of ZfLp.

Several endogenous compounds have been identified as the driving force of drug transporters. Sekine *et al.* (1997) and Sweet *et al.* (1997) have reported that dicarboxylates is an exchanger for stimulating rat Oat1 (rOat1[*Slc22a6*])-mediated drug uptake. Pajor *et al.* (1995) have revealed that dicarboxylate also acts as the driving force of sodium-dicarboxylate cotransporter-1 (NaDC-1[*Slc13a2*]). Li *et al.* (2000) have reported that intracellular GSH is an exchange substrate and GSH stimulates the transport of organic anions via Oatp2 (Oatp1a4[*Slc10a4/Slc21a5*]). On

the other hand, Mahagita *et al.* (2007) have reported that GSH has no effect on the uptake or efflux via OATP1B1/OATP-C/LST-1/OATP2[*SLC01B1/SLC21A6*]. Recently, we have reported that cyclosporine A and glutathione (GSH) act as the driving force of IgLC-rG (Kobayashi *et al.*, 2010a). Furthermore, Bahn *et al.* (2008) have reported that hOAT10[*SLC22A13*]-mediated uptake of [¹⁴C]urate is *trans*-stimulated by GSH. Thus, some organic solutes act as a driving force. To elucidate the driving force of ZfLp-mediated uptake, we subsequently examined GSH and dicarboxylate (glutarate) as a driving force of ZfLp. We found that a *trans*-stimulatory effect was observed when adding GSH and glutarate. Our results indicate that both endogenous substrates function as the driving force of ZfLp-mediated uptake and may act as an exchanger in the human body.

We subsequently studied the *cis*-inhibitory effect of ZfLp-mediated uptake by several organic solutes to clarify the substrate specificity. The transport of [³H]paclitaxel (taxol) via ZfLp was significantly inhibited by the addition of troleandomycin, erythromycin, azithromycin and clotrimazole whereas cyclosporine A and miconazole did not inhibit ZfLp-mediated uptake of [³H]paclitaxel (taxol). Because clotrimazole exhibited the strongest inhibitor for ZfLp-mediated [³H]paclitaxel (taxol) transport, we subsequently conducted the same experiment using clotrimazole and its structurally related compounds (N-triphenylmethylimidazole, N-diphenylmethylimidazole, N-benzylimidazole, and N-phenylpropylimidazole) in order to elucidate further precise substrate specificity. We found that a similar inhibitory effect was observed when adding N-triphenylmethyl-, N-diphenylmethyl-, and N-benzyl-imidazoles, whereas the transport of [³H]paclitaxel (taxol) mediated by ZfLp did not interact with N-phenylpropylimidazole, suggesting that N-benzylimidazole would be a minimum structural unit for the inhibition of ZfLp-mediated drug uptake by clotrimazole.

There are five ZNF415 isoforms (ZNF415-1-5) known to date (Cheng *et al.*, 2006). Phylogenetic tree analysis revealed that the amino acid sequence deduced from ZfLp cDNA is positioned within a member of the ZNFs. Therefore, we assumed that ZfLp may be a novel isoform of ZNF415, presumably ZNF415-6. Cheng *et al.* (2006) have reported that ZNF415-1 and -5 have the highest expression in adults, while ZNF415-3 has the lowest expression level (2006). On the other hand, ZNF415-2 and -4 both have a moderate level of expression. The ZNF415-1 protein is localized in the nucleus whereas the other isoforms are localized in both the nucleus and cytoplasm. Cheng *et al.* (2006) suggests these ZNF415 isoforms may have different roles (2006). Moreover, Eferl

and Wagner have reported that all isoforms of ZNF415, except ZNF415-1, can inhibit AP-1 and p53 activity (Eferl and Wagner, 2003). AP-1 is a dimeric transcription factor which is mainly composed of either a Jun-Jun homodimer or a Jun-Fos heterodimer. In addition, AP-1 regulates the expression of multiple genes essential for apoptosis, differentiation, cell proliferation, cell cycle control, and tumorigenesis (Eferl and Wagner, 2003). The p53 pathway is composed of many kinds of genes and products that respond to a wide variety of stress signals. These responses to stress include apoptosis, cellular senescence or cell cycle arrest (Levine *et al.*, 2006). Taking these facts into consideration, ZfLp may also regulate the activity of these transcription factors and may play an important role in cell cycle control or apoptosis.

In conclusion, we describe the isolation, functional characterization, and substrate selectivity of a novel human zinc finger-like protein, ZfLp. Phylogenetic tree analysis suggests that isolated ZfLp is a new isoform of ZNFs, presumably ZNF415-6. Using a *X. oocyte* expression system, we discovered that ZfLp functions as a drug carrier protein. Our results may provide insights into the novel role of soluble protein such as ZNF and may contribute to the elucidation of molecular mechanism(s) of the [³H]paclitaxel (taxol) toxicity in the human body. Our results, therefore, would be expected to facilitate research on the novel role of ZNFs and on the discovery of novel drugs for targeting ZNF-related proteins such as ZfLp.

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