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ABC Protein Transport of MRI Contrast Agents in Canalicular Rat Liver Plasma Vesicles and Yeast Vacuoles

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The mechanism of excretion into bile of hepatospecific magnetic resonance imaging (MRI) contrast media employed labeled Gd-reagents EOB.DTPA, BOPTA, B 20790 (iopanoate-linked), and B 21690 (glycocholate-linked) for measurement in rat liver canalicular plasma membrane vesicles and yeast vacuoles. The presence of ATP gave threefold greater transport of B 20790 and B 21690 than of EOB.DTPA and BOPTA. In yeast vacuoles the ATP stimulatory effect was eightfold with B 20790 and fivefold greater for B 21690, whereas in YCF1- or YLLO115w-deleted yeast cells the transport was significantly reduced and absent from double mutants, YCF1 and YLLO15w. The transport was similar in wild-type and deletant cells for B 21690; taurocholate gave 85% inhibition. These data suggest that bilary secretion of structurally related MRI agents depend on molecular structure. The findings are suggestive as of possible value for clinical diagnosis of inherited hyperbilirubinemias and other liver disorders. © 2001 Academic Press

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Abbreviations used: DTPA, diethylenetriaminepentacetic acid; BOPTA, 4-carboxy-5,8,11-tris(carboxymethyl)-1-phenyl-2-oxa-5,8,11-triazatridecan13-oic acid; EOB-DTPA, (*S*)-*N*-[2-[bis(carboxymethyl)amino]-3-(4-ethoxyphenyl)propyl]-*N*-[2-[bis(carboxymethyl) amino]ethyl]glycine; B 20790, 18-[[3-(2-carboxybutyl)-2,4,6-triiodophenyl]amino]-3,6,9-tris(carboxymethyl)-11,18-dioxo-3,6,9,12-tetraazaoctadecanoic acid; B21690, *N*-[(3β,5β,7α,12α)-3-[[13-carboxy-6,9,12-tris(carboxymethyl)1,4-dioxo-3,6,9,12-tetraazatridecyl]amino]-7,12-dihydroxy-24-oxocholan-24-yl]glycine.

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Recently data indicate that "traffic ATPases" in species ranging from *Escherichia coli* to humans are all members of a unique transporter superfamily ATP binding cassette (ABC) proteins (1, 2). This class of proteins plays a significant role in both human health and disease. Thus, Dubin-Johnson syndrome and cystic fibrosis are examples of genetic diseases caused by mutations in ABC genes (MRP2 and CFTR, respectively) (3, 4). For most of the ABC proteins, however, the physiological substrates are not yet known, so that their clinical significance and role in cellular physiology are not clear.

Several ABC proteins mediate biliary excretion, most notably MRP2 which is responsible for the canalicular secretion into bile of conjugated bilirubin (5), exogenous organic anions and glutathione conjugates. Other MRP proteins are involved in export of compounds across the basolateral membrane of the hepatocyte. These are upregulated during cholestasis (6), ostensibly to protect the hepatocyte from accumulating toxic cholephiles whose biliary secretion is impaired.

In *Saccharomyces cerevisiae*, the MRP/CFTR subfamily is the second largest group of ABC proteins and comprises six members (7). Each member of this subfamily exhibits homology to one of four mammalian proteins: CFTR (cystic fibrosis transmembrane conductance regulator, a chloride channel) (4), MRP1 (human multidrug resistance-associated protein) (8), SUR (sulfonylurea receptor implicated in insulin secretion) (9), and MRP2/cMOAT (canalicular multispecific organic anion transporter) (5), indicating structural and functional similarity (1, 7). Among the six members, YCF1 is best characterized; it has been demonstrated to act as an ATP dependent pump for glutathione S-conjugates in yeast vacuoles (10, 11). YCF1 appears to be a good model for the human multidrug resistance-associated protein MRP1. The *YLL015* gene has a high

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degree of identity with *YCF1*, suggesting similar function and pattern of expression for the coded protein (12).

Indirect evidence indicates that some MRI contrast agents (Gd-EOB.DTPA and Gd-BOPTA) are substrates for MRP2/cMOAT (13, 14). In the present paper, we have investigated the transport by ABC proteins of four MRI agents with different molecular structure, using two experimental models as rat liver canalicular plasma membrane vesicles and *S. cerevisiae* vacuoles, and found that the transport of the four compounds tested was mediated by distinct ABC proteins belonging to the MRP family.

MATERIALS AND METHODS

Chemicals. BOPTA (15), B 20790 (16), and B 21690 (17) were prepared as previously described, whereas EOB.DTPA was prepared with minor modifications of the reported procedure (18). Gadolinium complexes of the 4 ligands were prepared according to routine complexation procedures. $^{147}PmCl_3$ in HCl was purchased from ICN (Irvine, CA), and used to prepare stock solutions of ^{147}Pm-BOPTA, ^{147}Pm-EOB.DTPA, ^{147}Pm-B 20790 and ^{147}Pm-B 21690 with a specific activity of 89.6 mCi/mmol. Labeled solutions of the complexes (hereafter named Gd-EOB.DTPA, Gd-BOPTA, Gd-B 20790 and Gd-B 21690) were obtained by adding the appropriate amount of the solution of ^{147}Pm complex to the solution of the corresponding Gd complex. ^{147}Pm and Gd-B 21690 were prepared as sodium salts. All other ^{147}Pm and Gd complexes were prepared as salts with 1-deoxy-1-(methylamino)-D-glucitol (MGH). The purity of each compound was greater than 99%, as assessed by HPLC. Zymolyase 100T was a product of ICN Biochemicals (Milan, Italy). Yeast extract and Bactopeptone were obtained from Difco (Milan, Italy). ATP Magnesium salt, and all other chemicals (analytical grade) were purchased from Sigma (Milan, Italy).

Isolation of canalicular plasma membrane vesicles from rat hepatocytes. Female Wistar rats (60–90 days old, 200–250 g) were allowed free access to a standard laboratory chow (Nissan, Milan, Italy) and tap water, and were housed in an environment of constant temperature and humidity with alternating 12 h cycles of light and darkness. Liver plasma membrane vesicles highly enriched in the canalicular domain (cLPMV) were prepared according to the method of Meier *et al.* (19). Enrichment in the canalicular domain was assessed by measuring canalicular marker enzymes (5′-nucleotidase and Mg-ATPase), as reported previously (20). The enzyme activities were increased more than 60-fold over those of the starting homogenate. In agreement with previous reports (21), contamination by basolateral plasma membranes, assessed by the activity of Na^+/K^+-ATPase, was negligible. cLPMV were stored in liquid nitrogen until use (within 4 weeks), thawed at 37°C and revesiculated by passage 20 times through a 25-gauge needle (22). Protein concentration was measured by using the bicinchoninic acid protein assay kit (Sigma, Milan, Italy) with bovine serum albumin as standard.

Isolation of yeast vacuoles. The haploid wild-type *S. cerevisiae* strain, S288C, grown in YPD medium (1% yeast extract, 2% bactopeptone and 2% glucose) was used (23). Yeast vacuoles were prepared by the Ficoll flotation procedure (24). Using a glass Potter homogenizer, the intact vacuoles were vesiculated in 20 mM 2-(*N*-morpholino) ethanesulfonic acid–Tris buffer (MES-Tris), pH 6.9, containing 10 mM $MgCl_2$ and 50 mM KCl. An equal volume of 10 mM MES-Tris pH 6.9, containing 5 mM $MgCl_2$ and 25 mM KCl, was added to this suspension, and vacuolar membrane vesicles were collected by centrifugation at 37,000*g* for 30 min at 4°C. The pellet, containing vacuolar vesicles, was resuspended in the above buffer containing 5% glycerol (v/v) and stored in liquid nitrogen until use. The purity of each preparation was assessed by measuring marker enzyme activities for cytoplasm and cell organelles (24). The activity of the vacuolar membrane marker enzyme, α-mannosidase, was enriched consistently more than 45-fold in the vacuolar fraction compared to total homogenate. Contamination from cytosol was not detected, and the contamination with other cell components ranged from 1 to 5%. Protein content in the samples was determined as above.

Effect of ATP on uptake of MRI contrast agents. Uptake of labelled Gd-complexes was measured by a standard rapid filtration technique (24;25;25). Transport by cLPMV was measured in 100 μl of 10 mM Tris-HCl buffer, pH 7.4, which contained 250 mM sucrose and 50 to 300 μM MRI contrast agents, in the presence of 5 mM ATP (except for the "No ATP"). When *S. cerevisiae* vacuoles were used, sucrose was substituted with 600 mM sorbitol. The reaction was started by addition of vesicles or vacuoles (20–30 μg of membrane protein) to the transport solution. After 15–600 s, uptake was stopped by the addition of 1 ml of ice cold Tris-sorbitol buffer, and the sample was vacuum filtered in less than 5 s through pre-wetted nitrocellulose filters (type HA, 0.45 μm, Millipore, Bedford, MA). The filters were then washed twice with 2.5 ml of ice-cold stop buffer, air-dried, and 10 ml of scintillation solution added (Filtercount, Packard, Groningen, The Netherlands). Radioactivity was measured in a liquid scintillation spectrophotometer (Beta V, Kontron, Milan, Italy), with automatic quench correction using external reference standards.

Uptake was also measured at 4°C and found to be identical either with or without ATP. Specific uptake was calculated as total uptake at 37°C minus uptake at 4°C. Specific ATP-dependent uptake was calculated as the difference between the values found in the presence and the absence of 5 mM ATP.

Strains and media. *S. cerevisiae* strains used were: S288C (α, *SUC2, mal, mel, gal2, CUP1*) obtained from the Yeast Genetic Stock Center (Berkeley, CA, USA), JBG1 (α, Δ*ycf1::G, SUC2, mal, mel, gal2, CUP1*), JBG2 (α, Δ*yll015w::XkanMX4X, SUC2, mal, mel, gal2, CUP1*) and JBG12 *ycf1::G*, (α, Δ*yll015w::XkanMX4X, SUC2, mal, mel, gal2, CUP1*). Yeast cultures were grown on YPD. Geneticin (G 418) resistant cells were grown on YPD plates containing 200 mg/liter of G418 (Gibco BRL, Gaithersburg, MD) (26).

PCR synthesis of disruption cassette. The deletion cassettes were constructed according to Baudin *et al.* (27) and the EUROFAN knock-out standard guidelines, with the dominant resistance marker gene, *kanMX4,* included in vector YCpGKG and YCpXKX which allow marker recycling for multiple gene disruption (28). The amplification of cassettes for the two different gene disruption experiments was synthesised with the following primers: YCF1-for and YCF1-rev for disruption of *YCF1*, YLL015w-for and YLL015w-rev for that of *YLL015w*, described in Table I.

Each primer consisted of 40 nucleotides homologous to the appropriate flanking region of the genomic target locus, and of 20 nucleotides homologous to the ends of the *kanMX4* cassette. Templates for amplification of disruption cassettes used in the haploid strain were: YCpGKG for disruption of *YCF1* and YCpXKX for that of *YLL015w*. Amplification parameters were 1 min 30 s at 94°C, 30 s at 94°C, 30 s at 55°C, and 2 min at 72°C for 27 cycles, and finally 7 min at 72°C. The PCR product was directly used to transform yeast strains, after digestion of the circular plasmid DNA template to avoid false positive.

Transformation of yeast. Growth conditions and lithium acetate transformation were according to Gietz and Woods (29) using about 500 ng of PCR product. Transformed cells were grown at 30°C on YPD for 3–4 h and plated on YPD plates containing 200 mg/liter G 418 (26). Each large colony was streaked out on YPD-G 418 plates after 2 days of incubation at 30°C to identify the correct transformants.

TABLE I

Oligonucleotides Used for PCR Analysis

Oligonucleotide	Sequence
YLL015w-for	5′-GACAATGTCTTCACTAGAAGTGGTAGATGG-GTGCCCCTATAAAAATAGGCGTATCACGAG-3′
YLL015w-rev	5′-CGAGGGTGAATCGAATTCCCTCACACTACC-CTGGTCAAGATCGATGATAAGCTGTCAAAC-3′
YLL015w.F1	5′-CTGCTATCCCACAATGTTGA-3′
YLL015w.R1	5′-CTGGTTAGGATCTTTGATTT-3′
YLL015w.F2	5′-CCACCTGAAGCACAGTCCAT-3′
YLL015w.R2	5′-GAGCTCATTTCTATCTGTCT-3′
YCF1-for	5′-GGGGTATCGTACTACCGTAAAGAACAAGAA-AATGGCTGGTAAAAATAGGCGTATCACGAG-3′
YCF1-rev	5′-GACCAAACCAGCCTCCATGCACAGTGAATA-GAACAATGATTCGATGATAAGCTGTCAAAC-3′
YCF1.F1	5′-GATCTACTGGTCAGGAATCT-3′
YCF1.R1	5′-GTTCTGCATATAGCCCATGA-3′
YCF1.F2	5′-GAGCGAGGCACCTTTAATAG-3′
YCF1.R2	5′-GGCATGTGGATTATCCTTGC-3′
K1	5′-TACAATCGATAGATTGTCGCAC-3′
K2	5′-AGTCGTCACTCATGGTGATT-3′

Verification of correct ORF replacement by PCR. Correct ORF replacement was verified by colony PCRs according to Huxley (30). Oligonucleotides YCF1.F1, YCF1.R2 and YLL015w.F1, YLL015w.R2 were designed to bind outside the two target loci, while oligonucleotides YCF1.R1, YCF1.F2 and YLL015w.R1, YLL015w.F2 within the target loci and oligonucleotides K1 and K2 within the marker *kanMX4* (Table I). PCR analysis of the transformants showed one band characteristic for the mutated allele, obtained with the primers YCF1.F1-K1 or K2-YCF1.R2 and YLL015w.F1-K1 or K2-YLL015w.R2. Amplification parameters were the same as described above.

Construction of the double mutant yeast. Strain S288C was transformed with the linear cassette for disruption of *YCF1*, containing the FRTs carrying the mutation G and the *kanMX4* gene. The disrupted strain (JBG), checked by PCR, was grown for 10 generations in YPD liquid medium and kept shaking in stationary phase for two days (24). Cells were then plated on YPD and replicas plated on YPD-G418. The pop-out of the *kanMX4* marker gene was demonstrated by colony PCR, leaving behind a single FRT with the mutation G. The derived strain was called JBG1. The disruption of the second gene (*YLL015w*) was carried out with the appropriate linear cassette containing the marker *kanMX4* and the FRTs with the mutation X. Transformants were selected for G 418 resistance and the correct replacement was verified by PCR analysis. The new strain obtained was called JBG12.

Statistical analysis of the data. All studies were performed at least in triplicate with two or more vacuole/vesicle preparations and the results are expressed as mean ± S.D. of the six or more values obtained.

RESULTS

ATP Dependence of MRI Contrast Agent Uptake in cLPMV

The chemical structures of the compounds tested are shown in Fig. 1. The structurally similar compounds Gd-EOB.DTPA and Gd-BOPTA have been studied extensively, and the former is in clinical use. Gd-B 20790 and Gd-B 21690 differ by an elongated side-chain, and the fifth COO^- group on a second side-chain is appended to the ring system (Fig. 1). In Gd-B 20790, an iopanoic acid residue has been covalently linked, while in Gd-B 21690 a bile acid (glycocholate) was linked.

Figure 2 shows the time course of uptake by cLPMV of Gd-B 20790 (panel A) and Gd-B 21690 (panel B) at a final substrate concentration of 133 μM in the presence or absence of ATP. In the absence of ATP, total uptake increased only slightly over 10 min, and was almost three times higher for Gd-B 20790 than for Gd-B 21690. Moreover, intercept with the *y* axis was also almost three times greater for Gd-B 20790 than for Gd-B 21690 (268 vs. 95 pmol/mg protein), indicating a higher binding of Gd-B 20790 to the membranes. For each compound, the addition of 5 mM ATP stimulated transport significantly.

When each of the four MRI agents was tested at a same concentration (133 μM), the specific ATP-dependent uptake after 3 min was comparable for Gd-EOB.DTPA and Gd-BOPTA but over three times

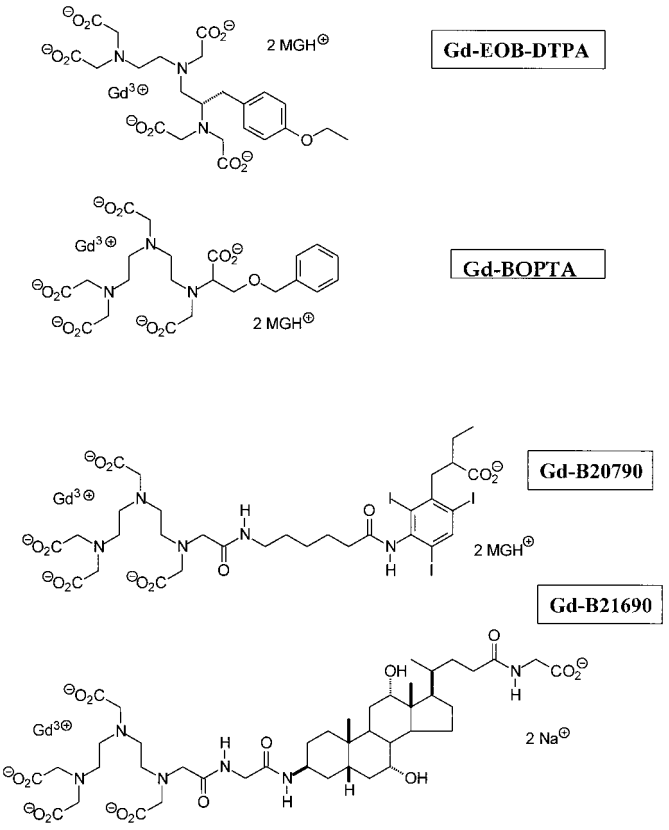

FIG. 1. Chemical structure of magnetic resonance imaging contrast agents. Each compound has five anionic groups, three of which are neutralized by binding to Gd^{3+}, leaving each compound with a net of two negative charges to form salts with 1-deoxy-1-(methylamino)-D-glucitol (MGH^+) (Gd-BOPTA, Gd-EOB.DTPA, and Gd-B 20790) or Na^+ Gd-B 21690. The iopanoate sidechain in Gd-B 20790 and the glycocholate side chain in Gd-B 21690 are highlighted by shaded rectangles.

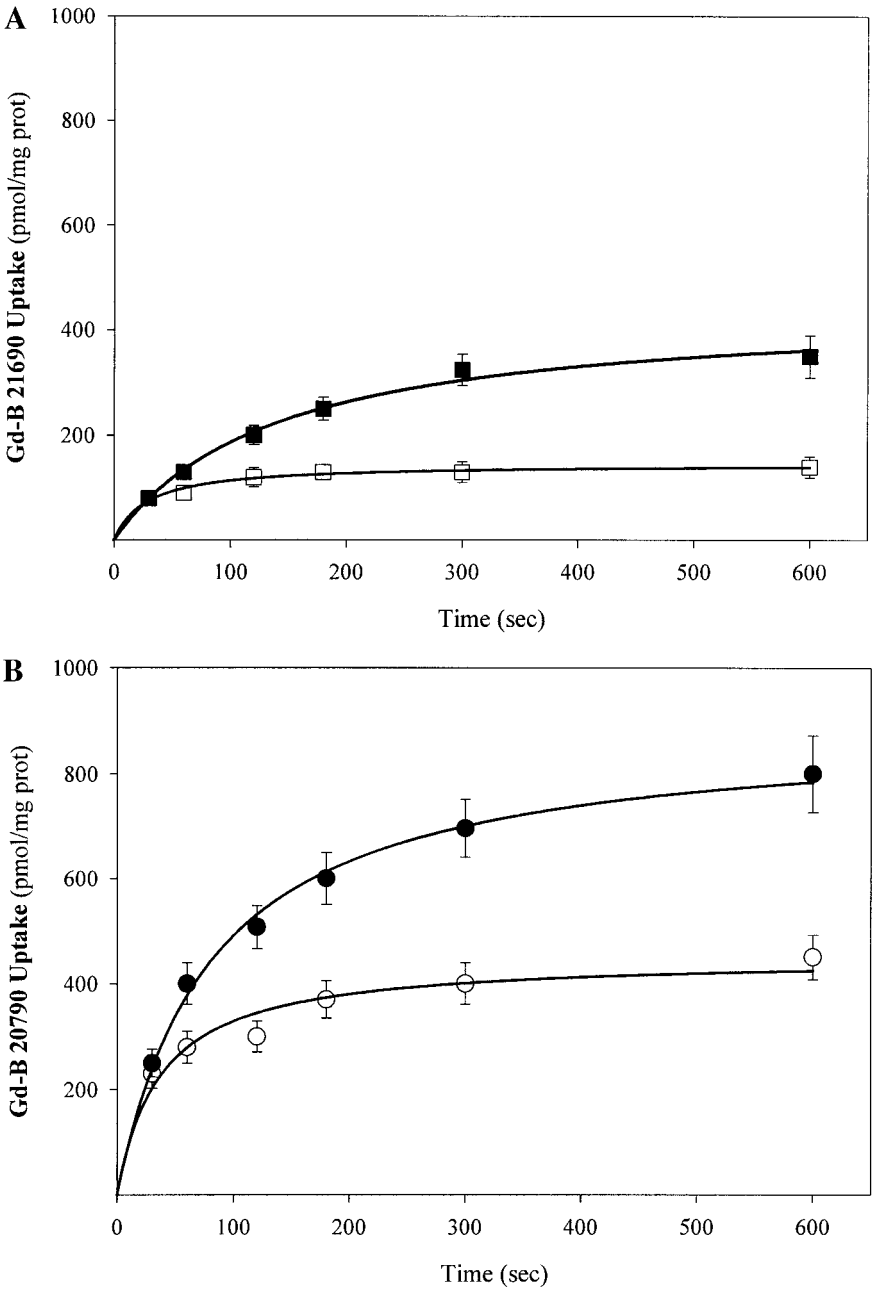

FIG. 2. ATP-dependence of Gd-B 21690 (A) and Gd-B 20790 (B) rat liver canalicular plasma membrane vesicles, each at a final substrate 133 μM. Filled symbols show the specific uptake measured in the presence of 5 mM ATP; open symbols indicate the specific uptake obtained in the absence of ATP. Each point represents mean ± S.D. of 3 determinations, in at least two vesicle preparations.

higher for the compounds with iopanoate Gd-B 20790 and glycocholate Gd-B 21690 sidechains (Table II).

ATP-Dependent Uptake of MRI Contrast Agents into Yeast Vacuoles

Table III reports the ATP-dependent transport of MRI contrast agents in yeast vacuoles. Transport of the four compounds was measured at a constant concentration of 150 μM. In line with what was observed in cLPMV (Table II), the specific ATP-dependent uptake of Gd-B 20790 and Gd-B 21690 was significantly greater ($P < 0.001$) than for Gd-EOB.DTPA and Gd-BOPTA. Unlike what was found in cLPMV, the ATP-dependent uptake was greater ($P < 0.01$) for Gd-B 20790 than for Gd-B 21690. As also shown in Table III, the addition of 50 μM taurocholate did not produce any inhibition in the uptake of compounds Gd-EOB.DTPA, Gd-BOPTA and Gd-B 20790, but inhibited by 85% the

TABLE II

ATP-Dependent Uptake of MRI Agents by Rat Canalicular Liver Plasma Membrane Vesicles

MRI contrast agent	Specific ATP-dependent uptake (pmol/mg protein)
(I) Gd-EOB.DTPA	30 ± 4
(II) Gd-BOPTA	27 ± 3
(III) Gd-B 20790	110 ± 12*
(IV) Gd-B 21690	98 ± 11*

Note. Total specific uptake over 3 min was measured as the difference in specific uptake obtained in the presence and in the absence of 5 mM ATP. All substrates were measured at a concentration of 133 μM. Data are expressed as mean ± SD of at least three experiments, each performed with at least two different plasma membrane preparations.

* $P < 0.001$ vs Gd-EOB.DTPA and Gd-BOPTA.

Gd-EOB.DTPA vs Gd-BOPTA, and Gd-B 20790 vs Gd-B 21690 not significant.

transport of the glycocholate-linked compound Gd-B 21690.

Transport in Yeast Mutants

Table IV reports data obtained in vacuoles derived from wild type and mutant yeast strains, either singly deleted for the gene *YCF1* (strain JBG1) or *YLL015* (strain JBG2), or doubly-deleted for both these genes (strain JBG12). To confirm comparability between the different strains, taurocholate uptake was measured in the presence of ATP. At a concentration of 50 μM, the ATP dependent uptake of ^{3}H-taurocholate was found to be 765 ± 99 pmol/mg prot/min in all strains.

As reported in Table IV, the ATP-dependent transport of Gd-BOPTA and Gd-B 20790 into vacuoles of the *YCF1* deletant strain (JBG1), was significantly decreased (62–64%) when compared with the wild type; transport of Gd-EOB.DTPA was also decreased significantly (37%), although to a lesser extent. Similar results were observed when the *YLL015w* deletant strain (JBG2) was used. When both *YCF1* and *YLL015w* genes were deleted (strain JBG12), the uptake of the three compounds (I–III) was almost fully abolished (91–98%). On the contrary, none of the gene deletions affected significantly the ATP-dependent transport of IV.

DISCUSSION

The data presented indicate that the biliary secretion of all four MRI contrast agents studies is an ATP-dependent process, mediated by ABC transporters. Previous data showed impaired biliary secretion of compounds Gd-EOB.DTPA and Gd-BOPTA in TR$^-$ rats (13, 14), which genetically lack the mrp2/cMoat canalicular transporter (31). These findings were regarded as evidence that compounds Gd-EOB.DTPA and Gd-BOPTA were secreted into bile *via* this ABC protein. Our data expand those observations with the demonstration that, in rat liver plasma membrane vesicles enriched in the canalicular fraction, ATP stimulates to a comparable extent the transport of Gd-EOB.DTPA and Gd-BOPTA. In addition, data obtained in single or double deleted yeast vacuoles indicate that an ABC protein similar to mrp2 mediates the ATP-dependent transport of these two MRI contrast agents in yeast.

The similar chemical structure of the two new MRI contrast agents we tested (compounds Gd-B 20790 and Gd-B 21690), led us to anticipate a similar, if not identical, molecular mechanism for their biliary excretion. Compared to Gd-EOB.DTPA and Gd-BOPTA, Gd-B 20790 and Gd-B 21690 compounds show higher lipophilicity and protein binding. Accordingly, they should be transported by the liver better than the less hydrophobic compounds, Gd-EOB.DTPA and Gd-BOPTA. We reported previously that Gd-B 20790 and Gd-B 21690, but not Gd-EOB.DTPA and Gd-BOPTA, are substrates for the human Organic Anion Transporter Polypeptide (OATP) present at the basolateral plasma membrane of the hepatocyte (32), confirming the greater liver-specificity of the two new MRI contrast agents, Gd-B 20790 and Gd-B 21690. Our new data indicate that the insertion of an iopanoate Gd-B 20790 or a glycocholate Gd-B 21690 residue in the side chain of the MRI agent also leads to a 3-fold increase in the ATP-dependent uptake of the agent by cLPMV, as compared to Gd-EOB.DTPA and Gd-BOPTA. Since all four agents are bivalent organic anions, it seemed likely that MRP proteins would be involved in their biliary secretion. Compound Gd-B 21690, however, contains a glycocholate side-chain, so that its possible

TABLE III

Taurocholate Inhibition and Specific ATP-Dependent MRI Uptake by Yeast Vacuoles

MRI contrast agent	Specific ATP-dependent uptake (pmol/mg protein)		Inhibition by taurocholate (percent of control)
	No taurocholate	+50 μM taurocholate	
(I) Gd-EOB.DTPA	110 ± 15	116 ± 20	None
(II) Gd-BOPTA	101 ± 12	107 ± 14	None
(III) Gd-B 20790	880 ± 90*	870 ± 95	None
(IV) Gd-B 21690	562 ± 53*,¶	116 ± 14#	85 ± 10#

Note. Total specific uptake over 3 min was measured as the difference in specific uptake obtained in the presence and in the absence of 5 mM ATP. All substrates were measured at a concentration of 150 μM. Inhibition was tested by adding 50 μM taurocholate to the uptake buffer. Data are expressed as mean ± SD of at least three experiments, each performed with at least two different plasma membrane preparations.

* $P < 0.001$ vs Gd-EOB.DTPA and Gd-BOPTA.

¶ $P < 0.01$ vs Gd-B 20790.

# $P < 0.001$ vs uptake in the absence of taurocholate.

TABLE IV

ATP-Dependent Uptake of MRI Agents by Wild-Type and Mutated Yeast Strains

	Deletion (strain)			
MRI agent	Wild type	ΔYCF1 (JBG1)	ΔYLL015w (JBG2)	ΔYCF1 & LL015w (JBG12)
(I) Gd-EOB.DTPA	228 ± 19	144 ± 17* (63 ± 6%)	56 ± 14* (24 ± 6%)	21 ± 14* (9 ± 6%)
(II) Gd-BOPTA	236 ± 24	86 ± 13* (36 ± 9%)	132 ± 11* (56 ± 5%)	4 ± 7* (2 ± 3%)
(III) Gd-B 20790	1717 ± 213	648 ± 92* (38 ± 5%)	692 ± 58* (40 ± 3%)	90 ± 60* (5 ± 3%)
(IV) Gd-B 21690	1400 ± 176	1250 ± 168 (89 ± 12%)	1388 ± 161 (99 ± 12%)	1303 ± 80 (93 ± 6%)

Note. Total specific uptake over 5 min was measured as the difference in specific uptake obtained in the presence and absence of 5 mM ATP. All substrates were measured at a concentration of 250 μM. The data in parentheses are the residual uptake in the mutated strain as a percentage of uptake in the wild-type strain. Data are expressed as means ± SD of at least three experiments, each performed with at least two different plasma membrane preparations.

* $P < 0.01$ vs wild type.

transport by canalicular bile salt transport systems (31) should be considered.

To better define the involvement of ABC proteins in the biliary transport of MRI contrast agents, we also tested transport of the four MRI agents by *S. cerevisiae* strains with knockouts of specific MRP-like proteins. This eukariotic cellular model has the advantages of a fully sequenced genome (7), which can easily be manipulated genetically. Yeast vacuoles have been shown to display ATP-dependent transport systems for other organic anions such as glutathione S-conjugates (12), bile acids (33), and (more recently) unconjugated bilirubin (24). In wild-type yeast vacuoles, all the four MRI contrast agents tested were taken up *via* an ATP stimulated mechanism. As in cLPMV, the specific ATP-dependent, transport of Gd-B 20790 and Gd-B 21690 was significantly greater than that of Gd-EOB.DTPA and Gd-BOPTA. The transport of Gd-B 20790 was, by contrast, about 40% higher than that of Gd-B 21690. Most notably, transport of Gd-B 21690, but not of the other three MRI agents, was inhibited (~85%) by taurocholate. Collectively, these findings support the conclusion that the transport of Gd-B 21690 involves a mechanism different than the transport of the other three MRI agents, which lack bile acid moieties in their side-chains.

Since Gd-EOB.DTPA and Gd-BOPTA are substrates for rat mrp2 (13, 14), the role of several related members of the yeast MRP family was investigated. Among the six MRP genes expressed in yeast, *YCF1* and *YLL015w* are those showing the highest homology to the mammalian MRP1 and MRP2 (1). The involvement of an MRP protein in the transport of Gd-EOB.DTPA, Gd-BOPTA and Gd-B 20790 was confirmed by the experiments in yeast, in which the deletion of *YCF1* or *YLL015w*, resulted in significant decreases in the ATP-dependent transport of these three agents. Most convincingly, the double knockout strain, which lacked both *YCF1* and *YLL015w* gene products, showed no residual transport activity of Gd-EOB.DTPA, Gd-BOPTA and Gd-B 20790. These data indicate that the proteins encoded by both *YCF1* and *YLL015w* genes account additively for the ATP-dependent transport of Gd-EOB.DTPA, Gd-BOPTA and Gd-B 20790 by yeast vacuoles. Since yeast *YCF1* and *YLL015w* show 40% homology with the mammalian transporters, MRP1 and MRP2 (1, 12), it may be concluded that Gd-B 20790, as well as Gd-EOB.DTPA and Gd-BOPTA (13, 14), is secreted into bile *via* canalicular MRP protein(s). In addition, these data confirm that *YLL015w* gene product is involved also in the vacuolar transport of MRI contrast agents, in addition to that of bile pigments (24).

In contrast to compounds Gd-EOB.DTPA, Gd-BOPTA and Gd-B 20790, the ATP-dependent transport of Gd-B 21690 in yeast vesicles was unaffected in either single or double deletion yeast mutants. This observation, together with the inhibition by taurocholate exclusively of uptake of Gd-B 21690 into yeast vacuoles, supports the conclusion that the biliary transport of Gd-B 21690 is mediated by a different carrier than those involved in transport of Gd-EOB.DTPA, Gd-BOPTA and Gd-B 20790. The presence of a glycocholate sidechain only in Gd-B 21690 suggests that ATP-dependent transport of Gd-B 21690 might be mediated by the yeast bile acid transporter (yBAT) (33). Since, unlike in mammalian organs, the yeast bile acid transporter belongs to the MRP family (1), it will be necessary to test directly whether the biliary excretion of Gd-B 21690 in humans is mediated by the homologous ABC protein, SPGP (34).

Compound Gd-BOPTA is currently used for clinical diagnostic procedures. The availability of new MRI contrast agents which can be excreted into bile by

different mechanisms may be useful to select the most effective MRI agent for the study of disorders in which a genetic defect of one or more of the canalicular ABC transporters is present, e.g., the MRP2 deficiency in the Dubin–Johnson syndrome (3). The specificity of different MRI agents for different canalicular transport mechanisms might be useful also for drug targeting in more common disorders.

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