MOLECULAR AND CELLULAR BIOLOGY, Sept. 2004, p. 7612–7621 Vol. 24, No. 17
0270-7306/04/$08.00+0 DOI: 10.1128/MCB.24.17.7612–7621.2004

Mrp4 Confers Resistance to Topotecan and Protects the Brain from Chemotherapy

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Received 6 April 2004/Returned for modification 29 May 2004/Accepted 12 June 2004

The role of the multidrug resistance protein MRP4/ABCC4 in vivo remains undefined. To explore this role, we generated Mrp4-deficient mice. Unexpectedly, these mice showed enhanced accumulation of the anticancer agent topotecan in brain tissue and cerebrospinal fluid (CSF). Further studies demonstrated that topotecan was an Mrp4 substrate and that cells overexpressing Mrp4 were resistant to its cytotoxic effects. We then used new antibodies to discover that Mrp4 is unique among the anionic ATP-dependent transporters in its dual localization at the basolateral membrane of the choroid plexus epithelium and in the apical membrane of the endothelial cells of the brain capillaries. Microdialysis sampling of ventricular CSF demonstrated that localization of Mrp4 at the choroid epithelium is integral to its function in limiting drug penetration into the CSF. The topotecan resistance of cells overexpressing Mrp4 and the polarized expression of Mrp4 in the choroid plexus and brain capillary endothelial cells indicate that Mrp4 has a dual role in protecting the brain from cytotoxins and suggest that the therapeutic efficacy of central nervous system-directed drugs that are Mrp4 substrates may be improved by developing Mrp4 inhibitors.

The brain is protected by two permeability barriers, the blood-brain barrier and the blood-cerebrospinal fluid (CSF) barrier (14, 32). The blood-CSF barrier is anatomically defined by the choroid plexus, located in the lateral, third, and fourth ventricles. Anionic and cationic transporters expressed by the choroid plexus epithelial cells are thought to prevent entry by certain compounds. The mechanism of this function differs depending on the selective expression of the transporters on the apical versus the basolateral surface of the cells. The ATP-binding cassette (ABC) transporter Mrp1 localizes in the basolateral membrane of choroid plexus epithelial cells (30, 46) but is not expressed in endothelial cells in the brain capillaries.

The endothelial cells of the brain's capillaries are tightly joined to form a hydrophobic permeability barrier (32) termed the blood-brain barrier. Pgp expression in these cells limits the movement of hydrophobic cationic drugs from the blood into the brain (36, 42, 43). However, in vitro, these capillary endothelial cells also transport organic anions unidirectionally toward the capillary lumen in an energy-dependent fashion (5, 25, 41). Therefore, the capillary endothelial cells appear to express an unidentified anionic ABC transporter. Currently, it is unknown whether an anionic ABC transporter is expressed at functional levels in vivo in the endothelium of brain capillaries.

The ABC transporter Mrp4, originally described as a nucleotide transporter (37), is known to transport a diverse array of compounds (2, 7, 34) and is capable of transporting organic anions as well as antiviral and antiretroviral compounds that do not easily penetrate the central nervous system (CNS) (2, 3, 9, 27, 37). Mrp4 expression was previously demonstrated on the basolateral membrane of the prostate gland and the apical membrane of the kidney (21, 44). Studies in cultured epithelial cells have demonstrated basolateral localization of Mrp4 (22). Transporters typically route to one surface in polarized cells. For instance, the Mrp (ABCC) subfamily members localize to either the basolateral or apical membrane, but not to both. MRP1 is restricted to the basolateral membrane of the choroid plexus and intestine, whereas MRP2 is found on the apical membrane in the intestine and liver (26, 29). Mrp4 might be unique among the Mrp transporters in having cell- or tissue-dependent polarized expression, but the biological importance of this unique ability to localize either apically or basolaterally remains unknown.

We have developed *Mrp4* knockout mice, and here we report their first use to show that Mrp4 is expressed in the lumen of brain capillaries and in the basolateral membrane in the choroid plexus epithelium. In vivo, Mrp4 restricts topotecan movement from the blood into the CSF and from the capillaries into the brain tissue by virtue of its unique ability to traffic to either the apical or basolateral membrane. We further show that Mrp4 overexpression confers resistance to the camptothecin topotecan. These studies have specific therapeutic implications for targeting the CNS that might harbor tumors but have more general implications in CNS therapy because of the expanding array of important drugs known to be transported by Mrp4.

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MATERIALS AND METHODS

Choroid tissues. Human choroid plexus tissue was obtained from the tissue bank of St. Jude Children's Research Hospital and from commercially available

human tissue arrays (ResGen/Invitrogen). Mouse tissues were dissected from the lateral and fourth ventricles with a stereo microscope.

Immunohistochemistry reagents. 3′-Diaminobenzidine tetrahydrochloride (DAB), avidin-biotin blocking reagents, hematoxylin, and streptavidin-horseradish peroxidase were obtained from DakoCytomation. Goat serum, rabbit serum, biotin-labeled goat anti-rabbit and rabbit anti-rat immunoglobulin antibodies, and rabbit immunoglobulin G were from Vectorlabs. Hydrogen peroxide (30%), an aminoethyl carbazole kit, and rat immunoglobulin G were from Sigma. Horseradish peroxidase-labeled goat anti-rat immunoglobulin antiserum was obtained from Santa Cruz Biotech. Citrate buffer came from Zymed Laboratories.

Construction and genotyping of the targeting vector. A 3′ fragment from the mouse *Mrp4* cDNA was used to screen a bacterial artificial chromosome genomic library; one clone (18832) was selected for development of the targeting vector. The exon and intron junctions of the mouse *Mrp4* gene (23) were used in developing the targeting vector. The 5′ arm consisted of a 2.7-kb BamHI/XhoI fragment 5′ of exon 27 that was ligated into the pKONTKV1901 vector (Stratagene). The 3′ arm was generated by PCR with the bacterial artificial chromosome clone as the template. One primer contained an artificial XhoI site and stop codons engineered into exon 27. The other primer contained a SalI site in exon 28. These two primers were used to amplify a 7-kb fragment that was then subcloned into the SalI site of the pKONTV1901 vector. The fragment was verified by DNA sequence analysis. The targeting vector was linearized by NotI and then electroporated into 129/SVJ-derived embryonic stem (ES) cells. Genomic DNA from 487 ES clones that survived 2 weeks of G418 selection was screened first by PCR analysis and subsequently by Southern blot analysis after digestion with BamHI. Twenty-four clones had undergone the correct homologous recombination. Chimeric mice were generated by injecting independently isolated ES clones into C57BL/6 blastocysts. All experiments were approved by the St. Jude Children's Research Hospital animal care and use committee.

Antibody generation, immunoblotting, and immunohistochemistry. The polyclonal anti-mouse Mrp4 antiserum (Mrp4-mp) was created by immunizing rabbits with a keyhole limpet hemocyanin-conjugated 17-residue peptide (NTS NGQ PSA LTI FET AL) derived from the C terminus of the murine Mrp4/Abcc4 sequence (23) and purified by peptide affinity column. We also used an anti-human MRP4 antibody (Mrp4-hp) that was cross-reactive with a homologous region of mouse Mrp4 (2, 37).

Antibodies were generated by constructing a fusion gene encoding the *Escherichia coli* maltose-binding protein and a fragment of human MRP4 (amino acids 372 to 431 [FP M_4I]) in the pMAL-c vector as described previously (11, 35). The immunization and fusion protocols have been described previously (11, 35). Briefly, a 12-week-old female Wistar rat was injected with approximately 100 μg of the fusion protein. Three booster injections were given. Cells obtained from draining popliteal lymph nodes and the spleen of the rat were fused with Sp_20 mouse myeloma cells. Supernatants were screened for hybridoma cells containing monoclonal antibodies on enzyme-linked immunosorbent assay plates coated with specific fusion protein and, as a control, on plates coated with an irrelevant fusion protein. Several hybridomas produced antibodies that reacted with the fusion protein under Western blot conditions.

Two antibodies, M_4I-10 and M_4I-80, were selected for further analysis. Their isotype was determined by using isotype-specific second-step reagents (Nordic, Tilburg, The Netherlands). The reactivity of these monoclonal antibodies to the approximately 180,000-kDa full-length Mrp4 was shown by Western blot analysis with fractions of Mrp4-overexpressing HEK 293 cells (49). The monoclonal antibodies also reacted with the mouse orthologue, Mrp4, in Western blot analysis of kidney protein fractions from wild-type and *Mrp*$^{-/-}$ mice. Antibody binding was detected by using horseradish peroxidase-labeled rabbit anti-rat immunoglobulin antiserum (Dako, Copenhagen, Denmark) and 5-amino-2-hydroxybenzoic acid (Merck, Darmstadt, Germany) with 0.02% H_2O_2 as a chromogen.

Lysates and/or crude membrane fractions of cell lines and tissues were prepared and analyzed as previously described (2, 37). Protein concentration was determined with the Bio-Rad protein assay (8) obtained from Bio-Rad Laboratories (Hercules, Calif.). Proteins were size fractionated on a 7% polyacrylamide gel and transferred to a nitrocellulose membrane by electroblotting. The membrane was blocked and incubated with the primary antibody at the appropriate dilution and then with the secondary horseradish peroxidase-labeled anti-rat immunoglobulin antiserum (Dako). Enhanced chemiluminescence (Amersham) was used to detect immunoreactive protein. Ponceau S staining was used to ensure equivalent protein loading.

Mrp4 expression was analyzed immunohistochemically in 4-μm sections of formalin-fixed, paraffin-embedded tissue. The tissue sections were cleared of paraffin, rehydrated with xylene and ethanol, and then boiled in a pressure cooker in 0.1 M citrate buffer (pH 6.0) to reconstitute antigenicity. The samples were treated with peroxidases, avidin, and biotin to block nonspecific reaction. The tissue sections were incubated with the primary antibody diluted in 5% goat serum and then with the secondary antibody in 1% goat serum. The sections were stained with streptavidin-horseradish peroxidase, DAB, and hematoxylin (as a nuclear counterstain).

Frozen tissue sections (4 μm) were air dried overnight and fixed for 7 min in acetone at room temperature. The slides were incubated with primary antibody for 1 h at room temperature and then with horseradish peroxidase-labeled goat anti-rat immunoglobulin antiserum (Santa Cruz). Color development was obtained with 0.4 mg of aminoethyl carbazole per ml and 0.02% H_2O_2 as a chromogen.

Distribution and pharmacokinetics of topotecan. Mice were injected with 2 mg of topotecan (GlaxoSmithKline) per kg via the tail vein. Blood was obtained by either retro-orbital puncture or cardiac puncture. Urine, bile, and selected tissues were collected. Samples were processed as previously described (18, 39, 47). The pharmacokinetic parameters of topotecan were estimated by using the software NONMEM (6). Data from *Mrp4*$^{+/+}$ and *Mrp4*$^{-/-}$ mice were modeled simultaneously with a two-compartment model (18). Nonlinear mixed-effects modeling was used to determine whether the absence of Mrp4 was a significant covariate of the topotecan pharmacokinetic parameters (16).

Intracellular accumulation of topotecan. Saos-2 cells containing either pcDNA3 or pcDNA3-MRP4 were plated (10^5 cells per well) in a six-well plate. At 70% confluence, the cells were washed with phosphate-buffered saline, and the medium was replaced with fresh medium containing topotecan but no antibiotics or phenol red. At the time points designated in the Results, the medium was removed, the cells were washed twice with ice-cold phosphate-buffered saline, and ice-cold water was added to lyse the cells. Topotecan was extracted from the lysate with methanol and assayed by high-pressure liquid chromatography (P. J. Houghton, G. S. Germain, F. C. Harwood, J. D. Schuetz, C. F. Stewart, E. Buchdunger, and P. Traxler, submitted for publication). Values were normalized to total protein content by using bovine serum albumin as a standard (8).

Cytotoxicity. Cells expressing human MRP4 (Saos 2-MRP4) or the vector only (Saos 2-pcDNA3.1) (2) were cultured in 96-well plates in medium without phenol red or antibiotics. An aqueous stock solution of topotecan was diluted with medium, added to the wells, and mixed. The cells were incubated for 6 h, the medium was removed, and the cells were washed and grown to 90% confluence. Within each cell line, the viability of treated and untreated cells was compared by a [4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) (Sigma) or Alamar Blue (BioSource) assay (4, 10).

Vesicular transport assays. Vesicle-containing fractions prepared from cells engineered to express Mrp4 (2) were incubated with 15 μM ^{3}H-labeled estradiol-17β-glucuronide (PerkinElmer) and various amounts of topotecan for 5 min at 37°C, essentially as described previously (49).

Microdialysis. The surgical procedures, implantation of the cannula and microdialysis probe, and method of assaying topotecan concentration in the brain have been described elsewhere (M. Leggas, Y. Zhuang, J. Welden, Z. Self, C. M. Waters, and C. F. Stewart, submitted for publication).

Statistical analysis. Data were analyzed by two-way analysis of variance with Bonferroni adjustment for multiple pairwise comparisons with the SigmaStat package (SPSS Science).

RESULTS

Generation of Mrp4-deficient mice. Both the human and mouse *MRP4* genes contain 31 exons (23). We targeted exon 27, which encodes a region between the conserved Walker A and B motifs that appears to be essential for communication between these motifs (12) and that facilitates ATP hydrolysis. The *Mrp4* gene was disrupted in murine ES cells by replacing exon 27 with a neomycin resistance cassette by homologous recombination (Fig. 1a). To ensure disruption of *Mrp4* expression, we used an oligonucleotide primer that had three in-frame stop codons engineered into exon 27. Quantitative reverse transcription-PCR analysis revealed 100-fold less Mrp4 mRNA in the *Mrp4*$^{-/-}$ mouse kidney than in the *Mrp4*$^{+/+}$ mouse kidney. Disruption of *Mrp4* in ES cells was confirmed by Southern blot analysis (not shown), and disruption of the *Mrp4* allele in mice was shown by PCR analysis of tail DNA (Fig. 1a).

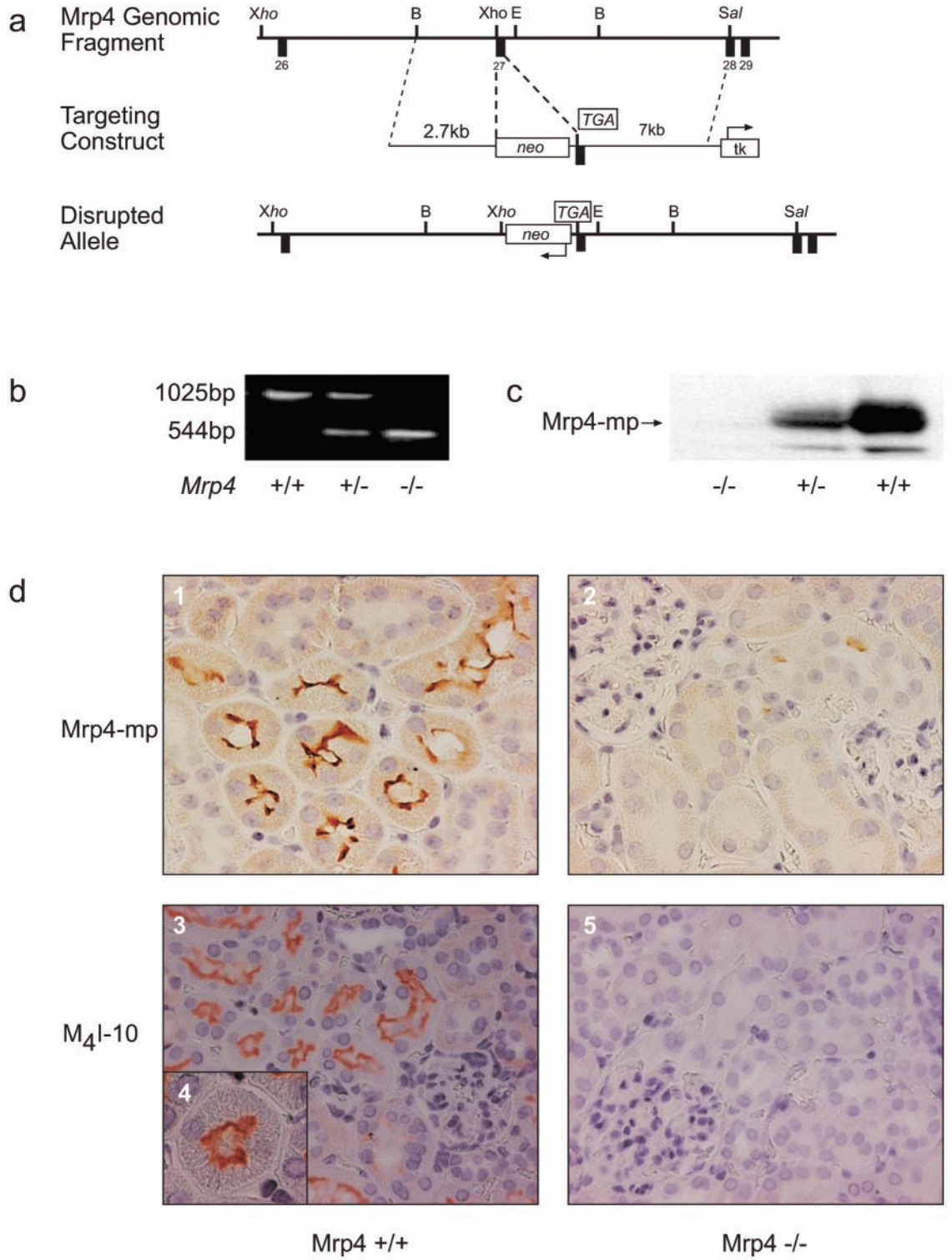

FIG. 1. Targeted disruption of the *Mrp4* locus causes loss of renal Mrp4 expression. (a) Targeting construct design and locus. Xho, XhoI cleavage site; B, BamHI cleavage site; E, EcoRI cleavage site; Sal, SalI cleavage site; neo, position of neomycin phosphotransferase gene; TGA, stop codons engineered into exon 27 (see text). Exon numbers are shown. (b) Representative *Mrp4* genotype analysis of *Mrp4* wild-type, heterozygous, and nullizygous mice. (c) Immunoblot with anti-mouse Mrp4 shows Mrp4 expression in kidney membranes from $Mrp4^{+/+}$ and $Mrp4^{+/-}$ but not $Mrp4^{-/-}$ mice. (d) Immunohistochemical analysis of Mrp4 expression in paraffin-embedded kidneys from $Mrp4^{+/+}$ and $Mrp4^{-/-}$ mice. The murine anti-Mrp4 polyclonal antiserum Mrp4-mp was used in panels 1 and 2. The monoclonal anti-Mrp4 antibody M_4I-10 was used in panels 3 and 5. The inset (panel 4) shows the proximal tubules at greater magnification.

We tested the effect of *Mrp4* gene disruption on renal expression of Mrp4 protein. In immunoblot analyses of crude membrane fractions, loss of one allele resulted in a 50% reduction in immunoreactive Mrp4, and deletion of both alleles resulted in complete loss of expression (Fig. 1c). No additional intact or truncated Mrp4 protein was detected on the *Mrp4*$^{-/-}$ immunoblot with either the polyclonal antibody Mrp4-mp or the monoclonal antibody M4I-10. Immunohistochemical analysis with these two new anti-Mrp4 antibodies detected Mrp4 in the apical membranes of the proximal renal tubules (Fig. 1d, panels 1, 3, and 4), in agreement with a previous report that human MRP4 is localized to the proximal renal tubules (44).

The offspring of crossbred *Mrp4*$^{+/-}$ mice had a normal Mendelian ratio, indicating that Mrp4 is not required for normal development. *Mrp4*$^{-/-}$ mice bred normally, produced normal-size litters with the expected male-female ratio, and gained weight normally for as long as 15 months. Microscopic histology of brain, liver, lung, and kidney tissues revealed no abnormalities in *Mrp4*$^{-/-}$ mice. In standard clinical assays, the mice had normal hemoglobin concentrations and packed-cell volumes, and liver function values (serum transaminases, alkaline phosphatase, and total bilirubin) were within normal limits. Notably, the absence of Mrp4 resulted in no apparent defects in renal function: serum concentrations of urea, nitrogen, creatinine, total protein, and electrolytes were within normal limits.

Topotecan excretion in the absence of Mrp4. The abundant renal expression of Mrp4 and the renal elimination of topotecan (18, 44, 48) prompted us to evaluate the elimination of intravenously administered topotecan in six mice of each genotype (*Mrp4*$^{+/+}$ and *Mrp4*$^{-/-}$). The mice were given an injection of 2 mg of topotecan per kg via the tail vein, and their plasma topotecan concentrations were measured at intervals. Topotecan elimination followed the expected biphasic pattern (48) in *Mrp4*$^{-/-}$ and *Mrp4*$^{+/+}$ animals. At 6 h, the plasma concentration of both forms of topotecan (hydroxy acid and lactone) was 50% higher in the *Mrp4*$^{-/-}$ than in the *Mrp4*$^{+/+}$ animals. We developed a pharmacokinetic model based on the topotecan elimination kinetics to predict differences between the *Mrp4*$^{+/+}$ and *Mrp4*$^{-/-}$ animals in the tissue penetration of topotecan. From this model, we estimated tissue penetration rate constants of 0.35 in the *Mrp4*$^{+/+}$ and 0.65 in the *Mrp4*$^{-/-}$ animals. These rate constants predict more rapid drug penetration into the tissues of the *Mrp4*$^{-/-}$ animals, consistent with the higher topotecan concentration observed in some tissues of the *Mrp4*$^{-/-}$ animals (see below). A similar concentration of topotecan was present in the bile of the *Mrp4*$^{-/-}$ and *Mrp4*$^{+/+}$ mice 6 h postinjection. In contrast, the urine topotecan concentration was 36% lower in the *Mrp4*$^{-/-}$ animals than in the *Mrp4*$^{+/+}$ animals at 6 h.

Mrp4 overexpression decreases topotecan accumulation and toxicity. Although topotecan exists as an anion, it is not a known substrate of Mrp4. Because of the decreased urine concentration of topotecan in the *Mrp4*$^{-/-}$ animals, we next examined the ability of topotecan to inhibit Mrp4-mediated transport of a known substrate. Membrane vesicles prepared from cells engineered to express human MRP4 were incubated with the radiolabeled substrate 2-estradiol-17β-glucuronide, and ATP-dependent transport was measured (48). Topotecan inhibited MRP4-mediated transport of the substrate in a dose-dependent manner (Fig. 2a). Accumulation of topotecan was then compared in cells programmed to express human MRP4 or the empty vector (2). Cells overexpressing human MRP4 accumulated much less topotecan (Fig. 2b) and were much less sensitive to topotecan (estimated 50% inhibitory concentration, 1 versus 5 μM; $P < 0.05$). Therefore, Mrp4 can confer resistance to topotecan, as also shown by cell viability assays (Fig. 2c). The decreased intracellular concentration of topotecan in MRP4-overexpressing cells is consistent with the known expression of MRP4 in the plasma membrane (Fig. 2d). These results indicate that topotecan is an Mrp4 substrate and that its overexpression contributes to topotecan resistance.

Mrp4 expression in the choroid plexus and cerebral vasculature. After systemic administration of 2 mg of topotecan/kg, the whole-brain concentration of topotecan was almost six times as high in the *Mrp4*$^{-/-}$ mice as in the *Mrp4*$^{+/+}$ mice (Fig. 3a). Immunoblot analysis demonstrated the presence and absence of Mrp4 in whole-brain protein extracts from *Mrp4*$^{+/+}$ and *Mrp4*$^{-/-}$ mice, respectively (Fig. 3b). Because of recent reports that some Mrp4 substrates are removed from the brain by an organic anion transporter, we assessed whether Mrp4 is expressed in the choroid plexus. We compared the level of Mrp4 expression in choroid plexus lysate with that in kidney crude membrane fractions by immunoblot with an antibody to human Mrp4 (Mrp4-hp) (1, 2) that cross-reacts with mouse Mrp4 and a newer antibody to the extreme C terminus of mouse Mrp4 (Mrp4-mp). Both antibodies detected Mrp4 in the kidney and choroid plexus of *Mrp4*$^{+/+}$ mice but not *Mrp4*$^{-/-}$ animals (Fig. 4a).

To estimate the relative expression of Mrp4 in the choroid plexus compared to the kidney, we loaded different amounts of choroid plexus lysate on the gel and compared the intensity of the resulting bands with that of a band representing a known amount of kidney membrane. The choroid plexus appeared to contain at least four to five times as much Mrp4 as the kidney (Fig. 4b) in this rough estimate. In the choroid plexuses of *Mrp4*$^{+/+}$ and *Mrp4*$^{-/-}$ animals, we found at both low (Fig. 4c, panels 1 and 3, respectively) and high (Fig. 4c, panel 2) magnification that Mrp4 was localized to the basolateral surface of the choroid plexus. We then used the anti-Mrp4 monoclonal antibody to identify Mrp4 expression in the choroid plexus from a different strain of mouse (FVb), from rats, and from humans (Fig. 4d, panels 1 to 4). In all three species, Mrp4 was localized to the basolateral membrane of the choroid plexus, a location that would allow it to restrict brain penetration of topotecan.

The comparatively high topotecan levels in the brain of *Mrp4*$^{-/-}$ mice could also reflect the absence of Mrp4 expression in brain capillaries. Cultured brain capillary endothelium shows anion transport activity (5, 25, 41). We used the monoclonal anti-Mrp4 antibody to evaluate Mrp4 expression in brain capillaries in frozen and in paraffin-embedded tissue sections. Mrp4 was expressed only in the capillaries of *Mrp4*$^{+/+}$ animals (Fig. 5a). To localize Mrp4, we examined the cross-section of a brain capillary from a mouse (Fig. 5b, panel 1) and a rat (Fig. 5b, panel 2) at high magnification. In both, the anti-Mrp4 antibody stained the apical membrane of the endothelial cells of brain capillaries). Therefore, Mrp4 is expressed in the basolateral membrane of the choroid plexus epithelial

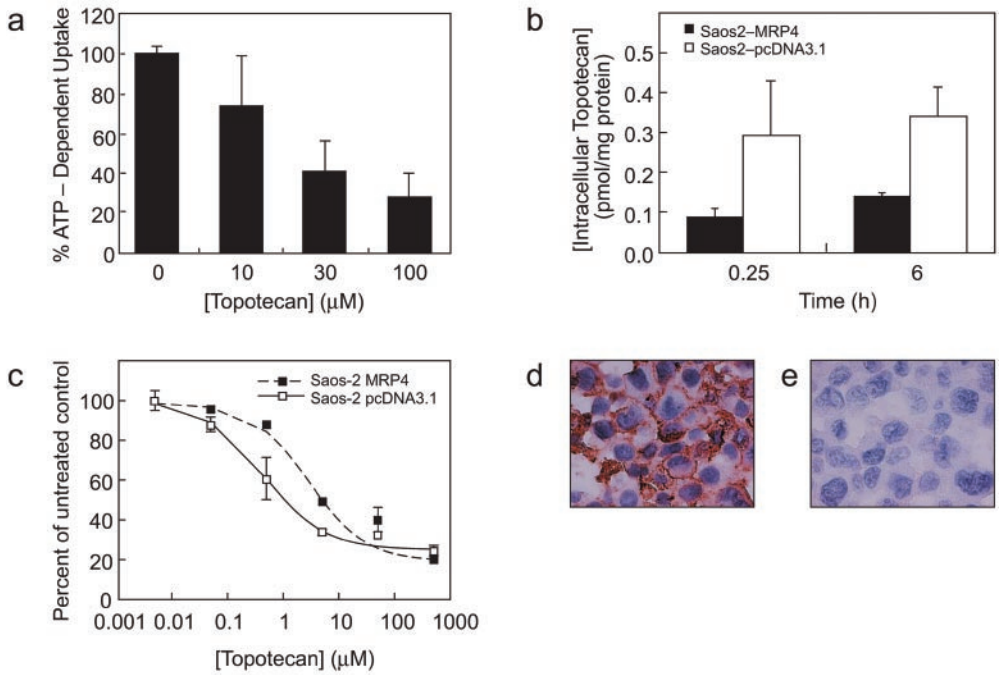

FIG. 2. Topotecan is an Mrp4 substrate. (a) Transport of the Mrp4 substrate estradiol-17β-glucuronide into membrane vesicles prepared from Saos-2 cells engineered to express Mrp4 (13) in the presence of various concentrations of topotecan. (b) The intracellular accumulation of topotecan was assayed in Saos-2 cells transfected with empty vector or stably expressing human MRP4 at the indicated times after treatment. (c) Topotecan sensitivity of the cells shown in panel d, shown as percent cell viability. Localization of Mrp4 in Saos-2 cells stably expressing Mrp4 (d) or transfected with empty vector (e). Bars represent 1 standard deviation.

cells and in the luminal side of brain capillaries, consistent with Mrp4's restricting the brain penetration of its substrates.

Restriction of CSF accumulation of topotecan by Mrp4. If Mrp4 in the basolateral epithelium of the choroid plexus and in the brain capillaries restricts the brain penetration of systemically administered topotecan, we would predict a greater CSF concentration of topotecan in *Mrp4*$^{-/-}$ animals after administration of topotecan. We stereotactically inserted cannulas into the lateral ventricles of mice and obtained CSF samples by microdialysis at 15-min intervals over a 3-h period after injection of 2 mg of topotecan/kg via the tail vein. The CSF topotecan concentration of *Mrp4*$^{-/-}$ mice was almost 10-fold that of *Mrp4*$^{+/+}$ mice at each time point (Fig. 6a). These results directly demonstrate that Mrp4 in the choroid plexus and brain capillaries limits the passage of topotecan from the blood into the CSF. We propose models depicting how Mrp4 restricts brain penetration by topotecan in the choroid plexus (Fig. 6b) and in the brain capillaries (Fig. 6c).

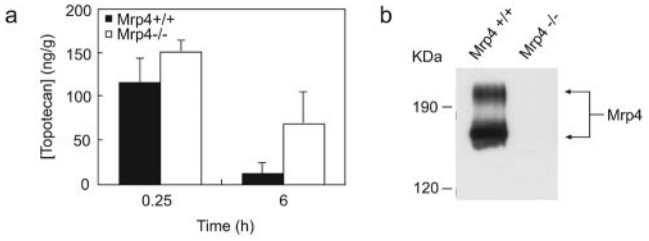

FIG. 3. Topotecan concentration in the brain is higher in the absence of Mrp4. (a) Mean (± standard deviation) concentration of topotecan in the brains of six *Mrp4*$^{-/-}$ (open bars) and six *Mrp4*$^{+/+}$ (solid bars) mice 15 min and 6 h after intravenous injection of 2 mg of topotecan/kg. (b) Equal samples of total brain protein (10 μg) from Mrp4$^{+/+}$ and Mrp4$^{-/-}$ animals were fractionated by polyacrylamide gel electrophoresis and treated with the monoclonal anti-Mrp4 antibody M4I-10.

DISCUSSION

Our development of an *Mrp4*-nullizygous mouse allowed us to demonstrate that Mrp4 limits the brain penetration of substrate drugs by exerting strong anionic transport activity in the blood-brain and blood-CSF barriers. Mrp4 functions at two levels, protecting cells from cytotoxic accumulation of topotecan and restricting topotecan penetration into the brain. Mrp4 expression was demonstrated in the basolateral membrane of the choroid plexus epithelium and in the brain capillary endothelium. As a proof of concept, we showed in vivo that Mrp4 effectively limits the movement of topotecan from the blood into the CSF. These findings suggest that Mrp4 functions to inhibit the movement of other toxic anionic compounds into the brain from the blood. Because Mrp4 transports a variety of important chemotherapeutic substrates (1, 7, 34, 37, 44, 45), inhibition of Mrp4-mediated transport might facilitate the brain penetration of therapeutic organic anions used to treat a number of malignancies and other diseases.

The endothelial cells in the lumen of brain capillaries form tight junctions that restrict the penetration of large, strongly hydrophilic compounds (32). These cells also have efflux transporters for cationic compounds (e.g., Pgp) and organic anions (5, 25, 41). It is noteworthy that many of the endogenous anions reportedly effluxed by cultured brain capillary endothe-

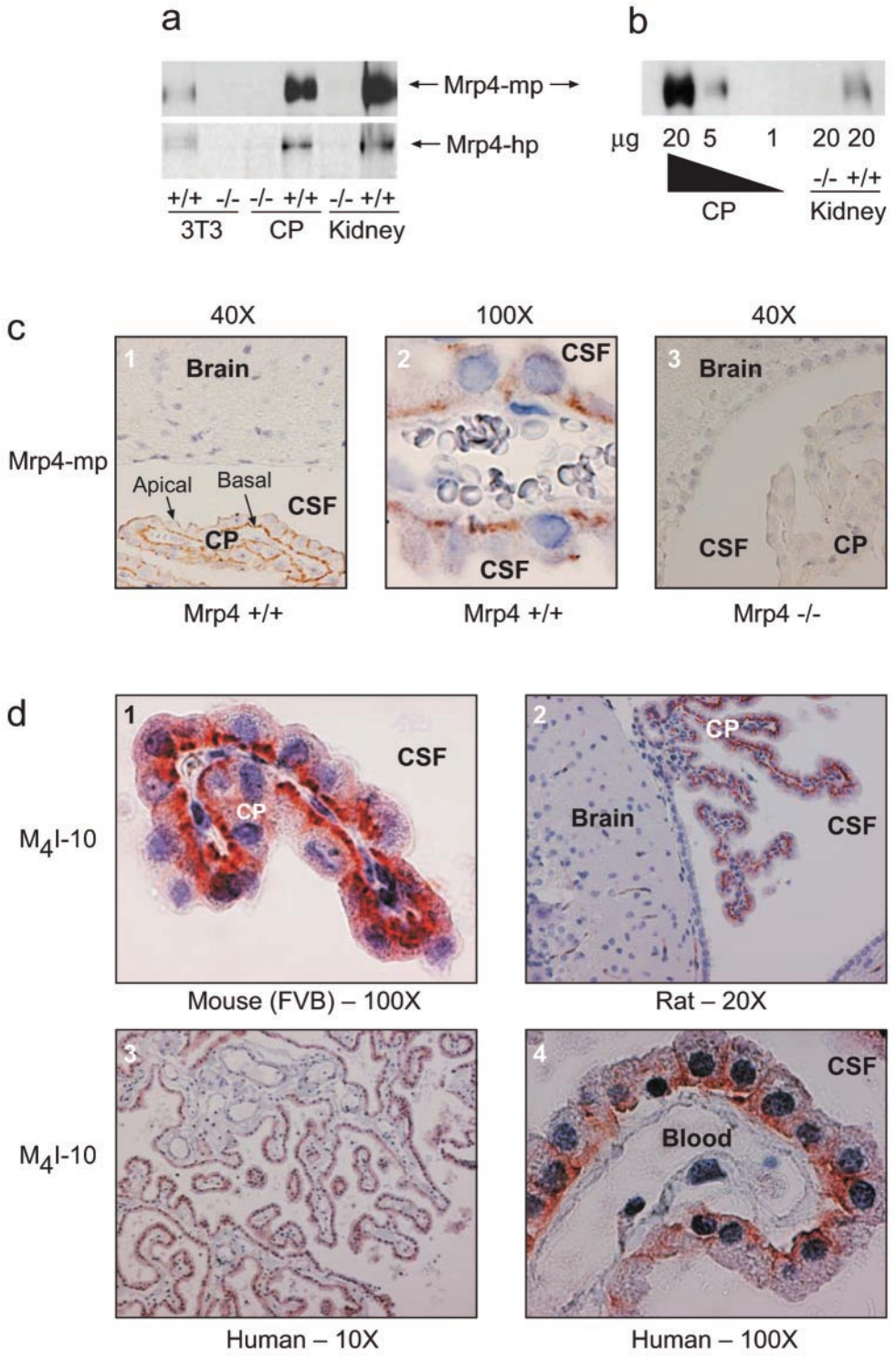

FIG. 4. Mrp4 is highly expressed in the basolateral membrane of the choroid plexus in multiple species. (a) Detection of Mrp4 in the choroid plexus (CP) and kidney of *Mrp4*$^{+/+}$ mice by immunoblot analysis with two different anti-Mrp4 antibodies. 3T3, NIH 3T3 cells transfected with the Mrp4 expression vector or empty vector served as positive and negative controls, respectively. (b) Mrp4 was more abundant in the choroid plexus than in the kidney of *Mrp4*$^{+/+}$ mice, as shown by immunoblot analysis with polyclonal anti-mouse Mrp4 (Mrp4-mp). (c) Immunohistochemical localization of Mrp4 in choroid plexus epithelial cells from *Mrp4*$^{+/+}$, Mrp4$^{+/-}$, and *Mrp4*$^{-/-}$ animals with the murine polyclonal Mrp4 antiserum (Mrp4-mp). The magnification is indicated above each panel. (d) Mrp4 expression detected in the choroid plexus from an FVB mouse, rat, and human (panels 1 to 4, respectively) with the anti-Mrp4 monoclonal antibody (M_4I-10).

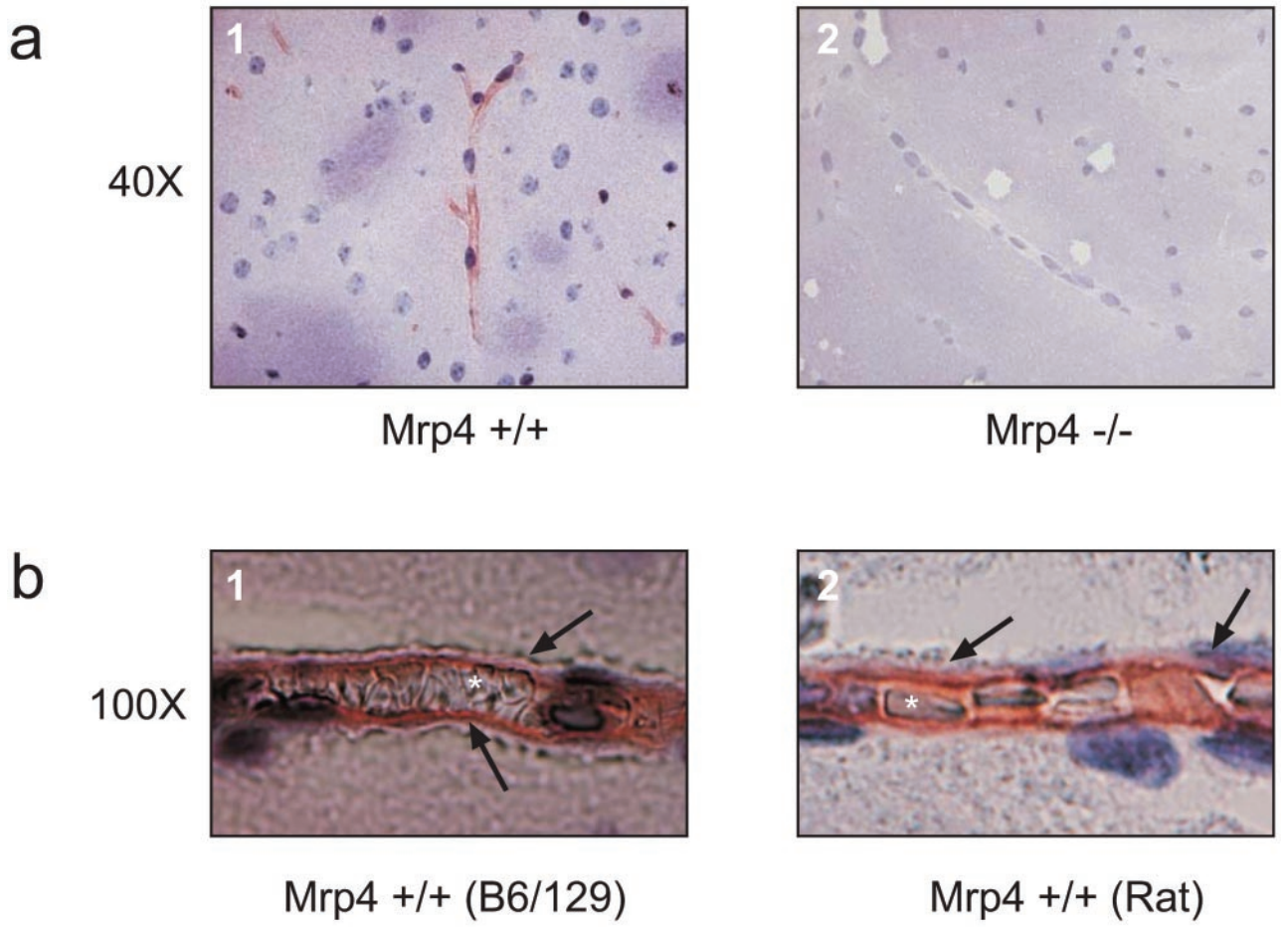

FIG. 5. Mrp4 is expressed in brain capillaries. (a) Frozen tissue sections prepared from the brains of *Mrp4*$^{+/+}$ and *Mrp4*$^{-/-}$ FVB mice were treated with anti-Mrp4 monoclonal antibody. (b) Panel 1 shows a cross-section of wild-type mouse capillary, revealing red blood cells (asterisk), surrounding capillary wall, and luminal Mrp4 staining (red); panel 2 shows a rat capillary, revealing interior red blood cells (asterisk). Arrows indicate the capillary wall and the luminal endothelial cell Mrp4 staining (red). Magnifications are indicated to the left of the panels.

lial cells are Mrp4 substrates (taurocholate, dehydroepiandrosterone 3-sulfate, 2-estradiol-17β-glucuronide, and estrone sulfate) and that the anions are transported into the capillary lumen (17, 19, 20). The expression of Mrp4 in the lumen of brain capillaries is compatible with the premise that Mrp4 protects the brain by restricting the movement of cytotoxic organic anions out of the capillaries.

The choroid plexuses within the lateral, third, and fourth ventricles form a barrier between the blood and the ventricular CSF and are morphologically indistinguishable in *Mrp4*$^{-/-}$ and *Mrp4*$^{+/+}$ mice. Organic anion transporters have been identified in the apical and basolateral membranes of the choroid plexus epithelial cells (24). Basolateral localization places Mrp4 in proximity to the fenestrated capillaries of the choroid plexus, where it can prevent uptake of anionic substances from the blood. Mrp4 may also efflux toxic metabolites from the brain. For example, 1-naphthol is a cytotoxic and genotoxic compound whose glucuronide is effluxed from the choroid plexus epithelium by an ABC-like transporter (40). The fact that naphthol-glucuronide inhibits Mrp4 transport (44) suggests that it is an Mrp4 substrate. Methotrexate is another organic anion and Mrp4 substrate (19). Importantly, the transport of intraventricularly injected methotrexate from the choroid plexus into the blood is inhibited by probenecid, a reported Mrp4 inhibitor (38, 44). Therefore, Mrp4 appears to play a significant but heretofore unrecognized role in the CNS penetration of some cytotoxins and therapeutic agents. Moreover, Bcrp (another efflux transporter of topotecan and methotrexate) is undetectable in murine blood-brain barrier cells (unpublished observation) and is thus unlikely to limit the uptake of these substrates into the brain.

Mrp4's ability to be expressed on either the basolateral or apical membrane was anticipated, based upon previous immunohistochemical analysis showing basolateral localization in the prostate and apical localization in the kidney (21, 44), but the biological importance of Mrp4's unique ability to localize apically or basolaterally was unknown. There are examples of transporters that route to different surfaces in polarized cells; however, among the Mrp (ABCC) subfamily of transporters localization is to either the basolateral or apical membrane, but not both. For instance, MRP1 is restricted to the basolateral membrane in the choroid plexus and intestine, whereas MRP2

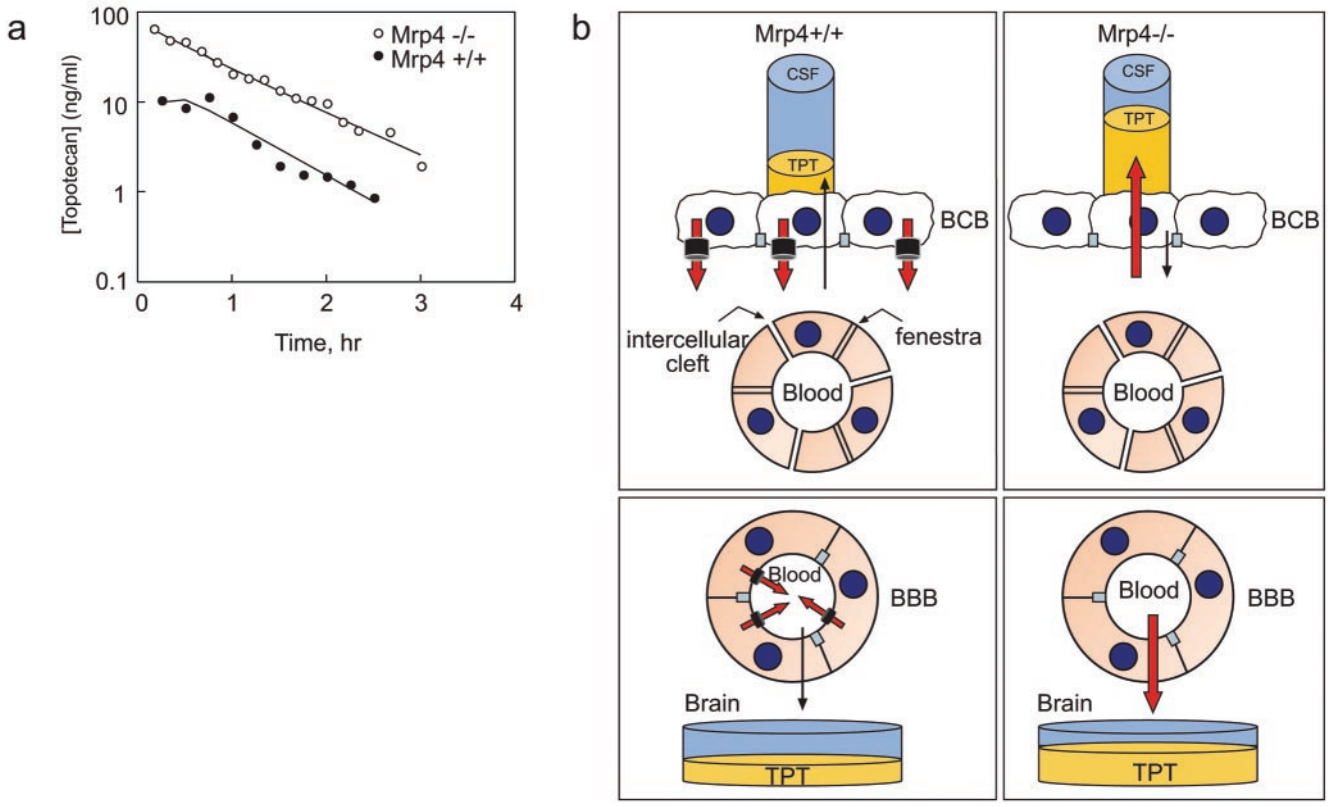

FIG. 6. Expression of Mrp4 in choroid plexus epithelium restricts the CSF uptake of systemically administered topotecan. (a) CSF concentration of topotecan in *Mrp4*$^{+/+}$ (solid circles) and *Mrp4*$^{-/-}$ (open circles) mice at the indicated times after intravenous injection of 2 mg of topotecan/kg. (b, left) Model showing the role of Mrp4 in the basolateral choroid plexus in restricting topotecan (TPT) penetration into the CSF. BCB, blood-CSF barrier. (b, right) Model depicting the role of Mrp4 in capillary endothelial cells in restricting penetration of topotecan into the brain. BBB, blood-brain barrier.

is found on the apical membrane in the intestine and liver (26, 29).

Mrp4 is unique among the Mrp4 subfamily of ABC transporters because its polarized expression is cell and tissue dependent. In the kidney, proximal tubules, and brain capillary endothelial cells, Mrp4 is routed apically, which orients it to vectorially transport its substrates into the lumen. In contrast, Mrp4's basolateral location in the choroid plexus epithelium allows it to exclude the entry of substrates into the CSF from the blood. These differences have led us to speculate that the asymmetrical localization of Mrp4 in polarized cells plays a role in the transport of an important anionic substrate(s) unique to these tissues. An example from the organic anion-transporting polypeptides illustrates this possibility. Oatp2 is expressed at the basolateral membrane in the liver but on the apical membrane in the retina (13). Expression of Oatp2 in the retina is postulated to involve the transport of unusual cationic, amphiphillic retionoids (i.e., *N*-retinyl and *N*-retinylidene ethanolamine) into and out of retinal pigment epithelial cells. Mrp4 localization and orientation in the choroid plexus and capillaries suggest a role in the flux of important physiologic anions from the brain or CSF into the blood. One candidate substrate is prostaglandins. Prostaglandins have recently been demonstrated to be Mrp4 substrates (31). Prostaglandins are synthesized in the choroid plexus (15), undergo circadian variation (28), and are important in mediating the pain response (33). An Mrp4-mediated efflux from the brain would be compatible with controlling prostaglandin levels.

In conclusion, we have shown that Mrp4 restricts the penetration of the brain by the important CNS-active anticancer agent topotecan. This function is linked to Mrp4's dual roles in the blood-CSF and blood-brain barriers. The ability of Mrp4 to block the CNS entry of topotecan is closely linked to its specific localization to either the apical or basolateral membrane. This dual localization of Mrp4 may help to explain the difficulty in identifying the anionic transporters in the CNS in vivo. Our findings provide a new "molecular handle" by which we can better understand not only the efflux of topotecan but also that of other anionic therapeutic compounds whose limited CNS penetration and decreased therapeutic effect may now be explained by the expression of Mrp4. Furthermore, our findings illustrate that Mrp4 overexpression in tumor cells can limit drug penetration and confer topotecan resistance. In total, these findings have important therapeutic implications for the treatment of CNS tumors, which express Mrp4, as well as for therapies targeted toward the brain.

ACKNOWLEDGMENTS

We thank Piet Borst, William Evans, and Mary Relling for constructive comments. In addition, we gratefully acknowledge Sharon Naron for editorial comments and suggestions. We thank A. Schoonderwoerd and M. de Haas for excellent technical assistance with the immuno-

histochemical studies. We thank Jacqueline Mital for help in preparation of the manuscript.

This work was supported by NIH research grants GM60904, ES058571, and CA23099; by Cancer Center support grant P30 CA21745; by Dutch Cancer Society grants NKI 2001-2473 and NKI 1998-1764 (to Piet Borst); and by the American Lebanese-Syrian Associated Charities.

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