Disruption of the Murine Major Vault Protein (MVP/LRP) Gene Does Not Induce Hypersensitivity to Cytostatics[1]

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ABSTRACT

Vaults are ribonucleoprotein particles with a distinct structure and a high degree of conservation between species. Although no function has been assigned to the complex yet, there is some evidence for a role of vaults in multidrug resistance. To confirm a direct relation between vaults and multidrug resistance, and to investigate other possible functions of vaults, we have generated a major vault protein (MVP/lung resistance-related protein) knockout mouse model. The *MVP*$^{-/-}$ mice are viable, healthy, and show no obvious abnormalities. We investigated the sensitivity of *MVP*$^{-/-}$ embryonic stem cells and bone marrow cells derived from the MVP-deficient mice to various cytostatic agents with different mechanisms of action. Neither the *MVP*$^{-/-}$ embryonic stem cells nor the *MVP*$^{-/-}$ bone marrow cells showed an increased sensitivity to any of the drugs examined, as compared with wild-type cells. Furthermore, the activities of the ABC-transporters P-glycoprotein, multidrug resistance-associated protein and breast cancer resistance protein were unaltered on MVP deletion in these cells. In addition, MVP wild-type and deficient mice were treated with the anthracycline doxorubicin. Both groups of mice responded similarly to the doxorubicin treatment. Our results suggest that MVP/vaults are not directly involved in the resistance to cytostatic agents.

INTRODUCTION

The 13 megadalton mammalian vault complex is the largest ribonucleoprotein particle described to date (1). Vaults are predominantly found in the cytoplasm; however, a small percentage of vaults seems to be associated with the nucleus, in particular the nuclear membrane and nuclear pore complex (2–4). Some groups have shown a partial association of vaults with cytoskeletal elements (4, 5). Although the function of vaults remains to be elucidated, the subcellular localization of the complex as well as the distinctive shape, a conserved hollow barrel-like structure with an invaginated waist and two protruding caps (6), has led to the hypothesis that vaults function in cellular transport (7) and/or nucleo-cytoplasmic transport (2–4).

Many reports point to a role for vaults in cellular defense against toxic compounds and consequently in MDR.[3] Relatively high vault levels have been found in tissues that are frequently exposed to xenobiotic elements, such as epithelia of the gastrointestinal tract and lung. Furthermore, the expression of the MVP, also referred to as LRP, was associated with a non-P-gp-mediated multidrug resistant phenotype in human cancer cell lines (8–11). In addition, MVP expression closely reflected known chemoresistance characteristics in broad panels of unselected tumor cell lines and untreated clinical cancers of different histogenetic origins (8, 12). Several clinical studies indicate that expression of MVP is associated with a poor response to chemotherapy, but other studies have failed to show a correlation between MVP expression and clinical drug resistance (reviewed in Ref. 13). Direct evidence for the involvement of vaults in drug resistance was presented by Kitazono *et al.* (14, 15). They showed that increased levels of MVP in the colon carcinoma SW620 cells paralleled increased resistance to doxorubicin, etoposide, and Taxol. This resistance could be partly overcome by expression of *MVP*-specific ribozymes. However, expression of MVP, stably transfected in the ovarian carcinoma cell line A2780, leads to increased numbers of vault particles, but does not confer a drug resistance phenotype (16, 17).

Vaults consist of three proteins, a M_r 100,000 MVP, and two minor vault proteins of M_r 193,000 (VPARP) and M_r 240,000 (TEP1), as well as many copies of a small untranslated RNA (vRNA) of 88–141 bases. Interestingly, the TEP1 subunit is also associated with the telomerase complex (18, 19). Within this complex, its function is unknown, but it was shown that TEP1 binds to telomerase RNA. However, the recently generated *TEP1* knockout mice do not show abnormalities in telomere length nor telomerase activity (20). In these mice, vault-like particles were observed that appear structurally intact but have a decreased density in the caps. It was found that TEP1 is capable of binding vRNA, and is responsible for the stabilization of vRNA and its binding to vaults (21). VPARP contains a functional PARP domain. Several proteins with such a domain have been identified; PARP1, the best-studied protein of this group, has been shown to play a role in base excision repair. Also, other proteins with PARP activity might be involved in DNA damage repair and genomic stability (reviewed in Ref. 22). Other than its association with the vault complex, VPARP is also located in the nucleus, which might point to an involvement in DNA damage repair as well. VPARP has been shown to ADP-ribosylate itself and MVP (23). The identification of other substrates of VPARP might give insight in the function of VPARP and/or the vault complex. However, the bulk of the vault complex is made up by the MVP. Expression of MVP in insect cells that do not express VPARP and TEP1 gives rise to vault-like particles (24), a finding that illustrates the importance of the MVP in the formation of the vault complex. Therefore, absence of MVP will most likely result in a disruption of the whole vault particle and, hence, eliminate or severely impair its function.

Here we describe the generation of a MVP-deficient mouse model. We revealed previously the genomic organization of the murine *MVP* gene (25), which enabled us to construct a targeting vector that was used to disrupt one allele of the *MVP* gene. Subsequently, we generated MVP-deficient mice that were studied extensively for any physical abnormalities. To investigate the potential role of vaults in the cellular resistance to chemotherapy, several *in vitro* drug sensitivity assays were performed using cells and cell lines derived from the *MVP* knockout model. In addition, the *in vivo* toxicity of doxorubicin

Received 7/15/02; accepted 10/10/02.

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[1] Supported by the Dutch Cancer Society, Grant #EUR 98-1754.

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[3] The abbreviations used are: MVP, major vault protein; PARP, poly(ADP-ribose) polymerase; TEP1, telomerase-associated protein; ES, embryonic stem; VPARP, vault poly (ADP-ribose) polymerase; vRNA, vault RNA; LRP, lung resistance-related protein; P-gp, P-glycoprotein; MRP, multidrug resistance-associated protein; BCRP, breast cancer resistance protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DCFDA, carboxyfluorescein diacetate.

was tested in wild-type and knockout mice to examine the effect of MVP absence on the systemic protection against chemotherapy.

MATERIALS AND METHODS

Generation of the Targeting Construct and MVP-deficient ES Cells

The *MVP* gene was isolated from genomic E14 DNA and mapped as described (25). By Southern blot analysis and DNA sequencing the intron-exon structure was determined. The targeting construct consisted of two genomic fragments, a 2.6-kb *Bal*I-*Tth*III1 fragment containing intron 3 and part of exon 4, and a 5-kb *Eco*RI-*Sal*I fragment containing exons 8, 9, and 10. These fragments were cloned into pBS-KSII (Stratagene, La Jolla, CA). Between the two homologous arms we placed a phosphoglycerate kinase-driven neomycin cassette, in antisense direction with respect to the *MVP* transcription. To facilitate the screen for ES cells that underwent homologous recombination, the construct was flanked by the negative selection marker herpes simplex virus-thymidine kinase (see Fig. 1*A*). Before electroporation of the ES cells, the vector was linearized by digestion with *Not*I. The culturing and manipulation of ES cells were performed essentially as previously described (26). In short, a total of 12×10^6 E14 ES cells, derived from a 129/Ola background, were mixed with 25 μg of linearized target vector and electroporated with a pulse of 117 volt and 1200 μF for 10 ms. After a 10-min incubation at room temperature, the cells were plated in 10-cm^2 dishes and incubated at 37°C, 5% CO_2. The following day, the cells were divided over eight dishes, and incubated in G418 and ganciclovir containing medium (300 μg/ml and 100 μg/ml, respectively). The clones surviving this selection were isolated, expanded in 24-well plates, and genotyped by Southern blot analysis of *Bam*HI-digested DNA, using a 300-bp probe located just upstream of exon 3 (Fig. 1*A*).

For experimental purposes, the remaining intact *MVP* allele was also disrupted using the same targeting construct but with the neomycin cassette replaced by a hygromycin cassette. Double-targeted ES cells were initially selected on a medium containing hygromycin only (100 μg/ml) and later examined for disruption of both *MVP* alleles using the 300-bp probe mentioned above.

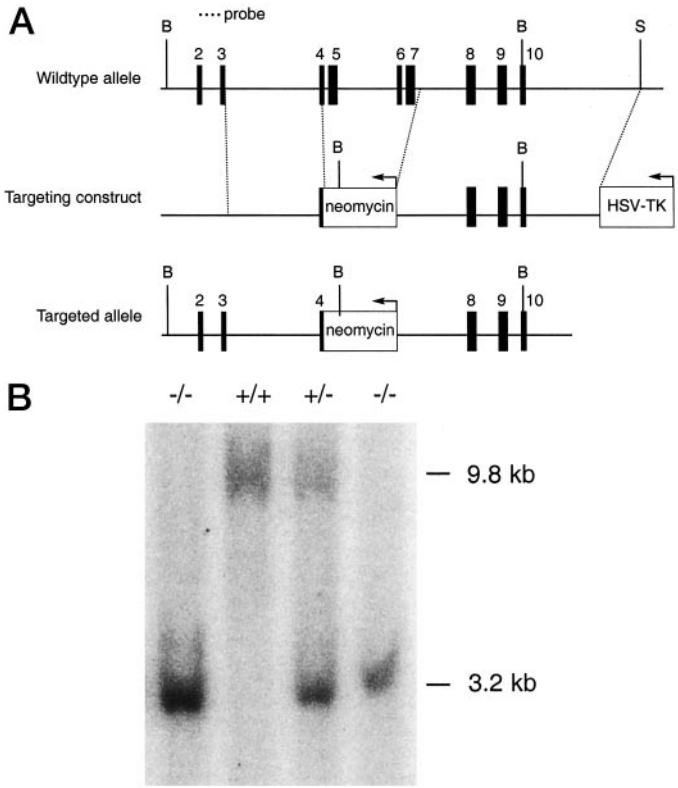

Fig. 1. Strategy of *MVP* gene disruption. *A*, schematic presentation of the gene targeting strategy, showing wild-type allele, targeting construct with the position of the neomycin resistance and the thymidine kinase cassettes, and the recombinant allele after gene disruption. The *dotted line on top* indicates the position of the probe used for genotyping ES cell clones and mice. B, *Bam*HI; S, *Sal*I restriction enzyme sites. *B*, Southern blot analysis of wild-type, targeted, and double-targeted ES cells. *Bam*HI digested genomic DNA was hybridized with the probe depicted in *A*. Hybridizing fragments from wild-type (+/+) and *MVP* knockout (−/−) alleles are 9.8 kb and 3.2 kb, respectively.

Generation and Genotyping of *MVP* Knockout Mice

Chimeric mice were generated by injecting the manipulated 129/Ola-derived, $MVP^{+/-}$ ES cells into C57BL/6 blastocysts as described (26). Heterozygous offspring of the chimeric mice was used to generate MVP-deficient ($MVP^{-/-}$), heterozygous ($MVP^{+/-}$), and wild-type ($MVP^{+/+}$) animals. Two sets of primers were used to genotype the mice by PCR on DNA isolated from tails. One primer set specific for the wild-type allele (forward 5′-GGCTGGTCCGATCCGTCGGTCCTTATCTCCC and reverse 5′-CAGTAGTGTCGAGGTCCCAGGGTGGTGATG) results in a 520-bp fragment spanning exons 6 and 7. The second primer set amplifies a 200-bp fragment of the neomycin cassette (forward 5′-TACTCGGATGGAAGCCGGTC and reverse 5′-AGTCGATGAATCCAGAAAAG). PCR conditions were: 10 min 94°C, followed by 30 cycles of 1 min 94°C, 1 min 65°C, and 1 min 72°C, followed by 10 min at 72°C. The PCR strategy was validated by comparing the results from a Southern blot analysis with the PCR-based data (data not shown). All of the animals used in this study were of a mixed genetic background of 129/Ola and C57BL/6 strains, backcrossed for one to four generations to C57BL/6. Littermate wild-type animals were used as controls, except in the *in vivo* doxorubicin experiment, where the large numbers of animals included restricted the use of controls exclusively consisting of littermates. In this experiment the groups were composed of animals from several litters matched for age and sex, and all derived from fourth-generation backcrosses with C57BL/6.

Western and Northern Blot Analysis

Tissues from $MVP^{+/+}$, $MVP^{+/-}$, and $MVP^{-/-}$ littermate animals were homogenized in a 10 mM sodium phosphate buffer (pH 7.4) containing 0.25% (v/v) Triton X-100 and proteinase inhibitors, using an Ultra-Turrax T25 (IKA-Labortechnik). The tissue lysates were spun briefly to pellet insoluble material. Protein concentrations were determined by the bicinchoninic acid method (Pierce, Rockford, IL). MVP was detected using a polyclonal antibody raised against human MVP (9) and the enhanced chemiluminescence detection system (Amersham-Pharmacia, Uppsala, Sweden).

Total RNA was purified from $MVP^{+/+}$, $MVP^{+/-}$, and $MVP^{-/-}$ littermate animals using RNAzol (Campro Scientific, Berlin, Germany) following the recommendations of the manufacturer. Ten μg of total RNA was subjected to formaldehyde-agarose gel electrophoresis as described (27). Subsequently the size-fractionated RNA was blotted onto Hybond-N$^+$ filters (Amersham, Little Chalfont, United Kingdom). ULTRAhyb (Ambion, Austin, TX) was used as blocking and hybridization mix according to the recommendations of the manufacturer. The blot was probed overnight with an *in vitro* transcribed antisense [^{32}P]-labeled MVP RNA probe, at 68°C, and subsequently washed for 2×5 min in $2 \times$ SSC, 0.1% SDS and 2×15 min in $0.1 \times$ SSC, 0.1% SDS at 68°C. As a loading control, afterward the same blot was probed with a radiolabeled 18 S rRNA oligonucleotide probe (5′-CTTTCGCTCTGGTCCGTCTTGCGCCGGTCC).

Vault Purification and Analysis

Vaults were purified from mouse livers as described previously (6), using a protocol adapted to the limited quantities and size of the mouse livers (21). Electron microscopy of uranyl acetate-stained vaults was carried out as described (28).

Isolation of Immature Bone Marrow Cells

Femurs and tibia were isolated from $MVP^{+/+}$ and $MVP^{-/-}$ littermates. The bones were cleaned and crushed in a sterile mortar to extract the cells using HBSS with 5% FCS, penicillin (100 units/ml), and streptomycin (100 mg/ml). The cells were filtered through a 100-mm cell strainer (Becton Dickinson, Franklin Lakes, NJ) and spun down at $500 \times g$ for 10 min. The cell pellet was resuspended in 50 ml of HBSS, and incubated at 37°C and 5% CO_2 for 60 min. To separate the immature proliferative cells from the more mature bone

marrow cells, the cells were harvested and resuspended in 300 ml of Ca^{2+}/Mg^{2+}-free PBS, and mixed with 2.7 ml of a 55% Percoll (Amersham-Pharmacia) solution. This mixture was loaded on a Percoll gradient, consisting of 3 ml of 85%, 2 ml of 62%, and 2 ml of 55% Percoll in Ca^{2+}/Mg^{2+}-free HBSS. The gradient was centrifuged for 30 min at 1650 $\times$ *g* using slow acceleration and no brakes. The upper fraction, containing the most immature cells, was collected. To induce proliferation, the cells were prestimulated in Cellgro medium (Boehringer, Heidelberg, Germany) containing 10 ng/ml thrombopoietin (Genentech, South San Francisco, CA), 10 ng/ml granulocyte macrophage colony-stimulating factor (R&D, Minneapolis, MN), 10 ng/ml interleukin 3 (R&D), 100 ng/ml stem cell factor (Amgen, Thousand Oaks, CA), and 50 ng/ml FLT3 (Immunex, Seattle, WA). After 2 days the cells were harvested and used in proliferation assays. During the proliferation assays the cells were cultured in medium containing the growth factors mentioned above.

Functional Tests for P-gp, MRP1, and BCRP

The activities of P-gp, MRP1, and BCRP were monitored by fluorescence-activated cell sorter analysis. Three $\times$ 10^5 wild-type and knockout ES cells or immature bone marrow cells were incubated in DMEM with or without 2 mM of the P-gp inhibitor PSC 833 (Novartis Pharma), 100 mM of the MRP1 inhibitor MK571 (Alexis, Lausen, Switzerland), or with 200 nM of the BCRP modulator Ko143 (29). After a 30–60 min incubation at 37°C, 5% CO_2, 200 ng/ml Rhodamine-123 (Sigma-Aldrich), 100 nM DCFDA (Sigma-Aldrich), or 100 nM BODIPY-prazosin (Molecular Probes Inc., Eugene, OR), substrates for P-gp, MRP1, or BCRP, respectively, was added. The cells were incubated with the substrates for an additional 75 min, after which the cells were mixed with 2 ml of ice-cold PBS supplemented with 0.1% sodium azide and spun at 650 $\times$ *g*. Before analyzing the cells by fluorescence-activated cell sorter, 8 μl of 5 nM TOPRO-3 (Molecular Probes) was added. 8226S and the P-gp-overexpressing derivative 8226D6 (30), GLC4 and the drug-resistant derivative expressing MRP1, and vaults GLC4-ADR (31) and MCF-7 and the drug-resistant, BCRP-overexpressing cell line MCF-7 AdVp3000 (32) were included as positive and negative controls for P-gp, MRP1, and BCRP function, respectively.

Analysis of Drug Sensitivity

Cell Survival Assay. The sensitivity to anticancer agents was tested in $MVP^{+/+}$ and $MVP^{-/-}$ ES cells using a MTT microtiter plate assay (33). In brief, on day 1, 3000 cells were seeded in 50 μl medium/well on gelatin-coated, flat-bottomed 96-well plates. After an incubation of 6 h at 37°C, 5% CO_2, drugs were added at various concentrations. After 3 days of incubation, 10 μl of a 5 mg/ml MTT solution in 0.9% NaCl was added in each well, and the incubation continued for an additional 6 h at 37°C, 5% CO_2. The formazan crystals produced by viable cells were resuspended by adding 100 μl/well of acidified isopropanol, after which the absorbance at 562 nm was measured on a plate reader. At least 12 different drug concentrations were used to determine IC_{50} values.

Cell Proliferation Assay. Proliferation of bone marrow cells from $MVP^{+/+}$ and $MVP^{-/-}$ littermate mice was tested in a [^{3}H]thymidine incorporation assay. For this purpose, immature bone marrow cells, isolated by a Percoll gradient and prestimulated for 48 h, were seeded at a density of 20,000 cells/well in a microtiter plate in 100 μl of medium containing growth factors and incubated at 37°C, 5% CO_2. Drugs were added 24 h later in various concentrations, and the cells were incubated for an additional 8 h. Then [^{3}H]thymidine (25 μCi in 25 μl medium) was added to each well, and the cells were incubated overnight. Cells were transferred to a glass fiber filter using a filtermate 196 cell harvester (Packard Bioscience, Groningen, The Netherlands), and the radioactivity on the filters was determined by a topcount microplate scintillation counter (Packard Bioscience).

***In Vivo* Toxicity of Doxorubicin.** Ten or 15 mg doxorubicin/kg bodyweight was administered to *MVP* knockout animals, and age- and sex-matched wild-type animals by i.v. injection in the tail. The animals were examined daily for signs of discomfort and illness. Body weights were determined daily, and blood cell counts were performed on days 3 and 8 after injection. At day 5 after injection, the animals that received the 10 mg/kg doxorubicin dose and PBS injected controls were sacrificed and examined thoroughly for histopathologic abnormalities using standard protocols.

RESULTS

MVP-deficient Mice Are Viable and Appear Normal. ES cell clones surviving the selection medium were genotyped by digestion with *Bam*HI followed by Southern blot analysis. This resulted in a 9.8-kb and a 3.2-kb hybridizing fragment for the wild-type and recombinant allele, respectively (Fig. 1*B*). A probe at the 5′ end of the construct was used to confirm the disruption of the *MVP* allele. The presence of additional integrations was excluded by using a probe based on the neomycin cassette (results not shown). Before the $MVP^{+/-}$ ES cells were injected into blastocysts, the number of chromosomes present in metaphase spreads was counted to verify the karyotype was stable.

Chimeric animals, resulting from blastocyst injection, were backcrossed to C57BL/6 mice to obtain heterozygous animals. These heterozygous mice were intercrossed to produce $MVP^{+/+}$ and $MVP^{-/-}$ littermates. The genotype of the animals was determined by Southern blot analysis and/or PCR on tail DNA (compare Fig. 1*B*). The disruption of *MVP* was confirmed at RNA and protein level. A Northern blot analysis on total RNA isolated from liver from wild-type, heterozygous, and MVP-deficient animals demonstrated that no *MVP* transcripts were detectable in the knockout tissue. Although the loading was not completely equal, judged from the 18S rRNA signal, a decreased signal was found in the heterozygous tissue when compared with the normal tissue (Fig. 2*A*). On Western blot, the knockout tissues contained no detectable MVP, and the heterozygous tissues showed intermediate levels of protein expression (Fig. 2*B*). Similar results were observed with other organs (data not shown).

To verify that the vault particle is indeed disrupted on the loss of MVP, we biochemically fractionated wild-type and *MVP* knockout mouse livers to purify vaults. In the final purification step, the vaults were purified over a single cesium chloride gradient. Vaults were recovered from gradient fractions by dilution and pelleting at 100,000 $\times$ *g*, and analyzed by electron microscopy (Fig. 2*C*). The typical barrel-shaped vault particles were clearly present in the wild-type liver fractions but could not be detected in the corresponding fractions obtained from *MVP* knockout livers. This indicates that MVP cannot be substituted for by another protein, which is in agreement with our previous findings that the murine genome contains a single *MVP* gene (25).

The *MVP* knockout mice appeared viable, were born at Mendelian ratios, bred normally, and did not show clear physical abnormalities up to 16 months of age. Animals of different ages were histologically examined, with special attention to tissues that normally express relatively high levels of MVP/vaults (*i.e.*, lung, liver, and gastrointestinal tract). No microscopical differences were observed between knockout and wild-type littermates (data not shown). Also, no differences were observed in bone marrow and peripheral blood cell counts, and in serum levels of glucose, blood urea, cholesterol, and creatine (results not shown).

Drug Sensitivity in MVP-deficient Cells. A correlation between vault expression and drug resistance has been shown in cell lines as well as for primary human tumors. To examine whether vaults influence the cellular defense against drugs, we tested the sensitivity of ES cells and immature bone marrow cells to various cytostatic agents. Both ES and immature bone marrow cells were shown to express MVP/vaults, whereas no MVP could be detected in $MVP^{-/-}$ ES and bone marrow cells derived from $MVP^{-/-}$ mice (Fig. 3). The survival of $MVP^{-/-}$ ES cells in response to increasing concentrations of cytostatic drugs was compared with the survival of the parental $MVP^{+/+}$ ES cells in a MTT assay. To determine the drug concentration IC_{50} value, 12 different concentrations of each drug were tested. Although a broad range of drugs, exerting toxicity in different man-

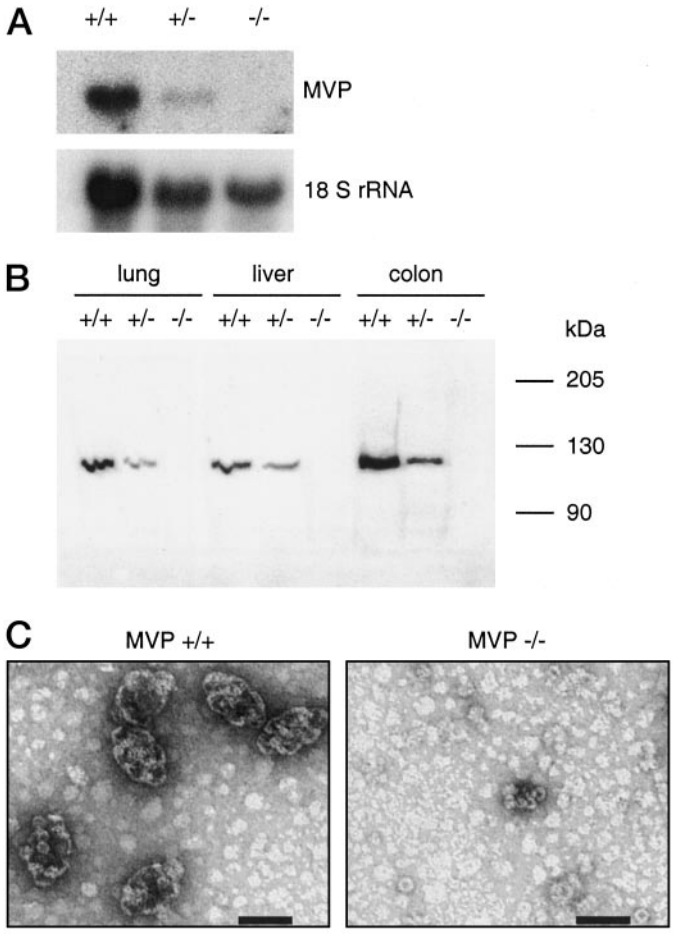

Fig. 2. Absence of *MVP* transcript and protein confirms *MVP* gene disruption and results in absence of vault particles. *A*, Northern blot analysis of total RNA from the liver of wild-type (+/+), heterozygous (+/−), and *MVP* knockout (−/−) littermates hybridized with a MVP antisense RNA probe. The *lower panel* shows the same blot hybridized with a control 18 S rRNA probe. *B*, Western blot analysis of protein lysates from several tissues from wild-type (+/+), heterozygous (+/−), and *MVP* knockout (−/−) littermates for MVP expression. Equal amounts of protein (40 μg) were loaded in each *lane*. *C*, livers from wild-type (+/+) and *MVP* knockout (−/−) mice were fractionated to purify vault particles. Shown are electron micrographs of negatively stained vault preparations that were obtained from these livers. Vault particles with the typical barrel-shaped morphology could easily be detected in the wild-type liver fractions, but were absent in the corresponding fractions of the *MVP* knockout livers. *Scale bars* correspond to 50 nm.

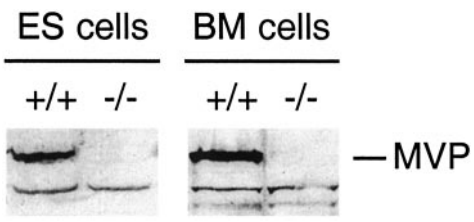

Fig. 3. Expression of MVP in ES cells and bone marrow (*BM*) cells. Western blot analysis of protein lysates from wild-type (+/+) and *MVP* knockout (−/−) ES cells and bone marrow cells. Equal amounts of protein were loaded in each *lane*.

ners, was included, no statistically significant differences in IC_{50} values were observed between wild-type and knockout cells for any of the cytostatics (Table 1). To investigate the effect of drugs on the proliferative capacity of wild-type and knockout cells, we used immature bone marrow cells, induced to proliferate in a [^{3}H]thymidine incorporation assay. The proliferation after a 24-h incubation with increasing concentrations of cytostatic agents was expressed as a percentage of the proliferation measured in untreated cells. The drug concentrations at which the proliferation is decreased by 50% are listed in Table 2. In all of the cases, the inhibition of cell proliferation induced by the cytostatics appears unaltered in MVP-deficient cells.

To test whether the absence of MVP results in altered expression of other multidrug resistance-related proteins such as P-gp, MRP1, and BCRP, the activities of these membrane pumps were examined in wild-type and knockout ES cell lines and bone marrow cells. For this purpose, we measured the intracellular retention of fluorescent substrates in the presence or absence of P-gp, MRP1, and BCRP modulators by flow cytometry. The ratio of substrate retained in the cell in the presence and absence of their modulators is a measure of the activity of these membrane pumps. Table 3 summarizes the activities of the three proteins on ES cells and immature bone marrow cells. Both ES cells and bone marrow cells appear to exhibit a low activity of P-gp and BCRP, whereas addition of the MRP1 modulator MK571 clearly increased the intracellular amount of fluorescent substrate, indicating that MRP1 is functionally present on the surfaces of these cells (Fig. 4). However, the absence of MVP does not lead to an increased activity in any of the MDR-related proteins tested. To investigate whether the inhibition of MRP1 has more severe consequences for the MVP knockout cells than for the MVP wild-type cells, the sensitivity to doxorubicin and etoposide in the presence of the MRP1 modulator MK571 was tested in a MTT assay. Doxorubicin and etoposide were chosen because these agents are known substrates

Table 1 *In vitro cytotoxicity of several cytostatics in $MVP^{+/+}$ and $MVP^{-/-}$ ES cells*

Given are average IC_{50} values ± SD calculated from two to three independent experiments ($n = 4$–6). Note that some assays were carried out in the presence of 50 μM of the MRP1 inhibitor MK571. No statistically significant differences were observed between IC_{50} values in wild-type and knockout cells as determined by the Mann-Whitney test.

Agent	IC_{50} (ng/ml)	
	$MVP^{+/+}$ ES cells	$MVP^{-/-}$ ES cells
Doxorubicin	7.28 ± 1.0	6.15 ± 0.77
Doxorubicin + 50 μM MK571	3.6 ± 1.0	4.3 ± 0.5
Etoposide	2.38 ± 0.08	2.0 ± 0.08
Etoposide + 50 μM MK571	1.0 ± 0.08	0.9 ± 0.2
Vincristine	0.86 ± 0.2	0.83 ± 0.053
Methotrexate	11.4 ± 0.58	12.9 ± 2.3
Ifosfamide	$466 \times 10^3 \pm 87 \times 10^3$	$471 \times 10^3 \pm 35 \times 10^3$
Mitoxantrone	0.53 ± 0.08	0.7 ± 0.2
Gemcitabine	10.05 ± 0.8	10.35 ± 1.1
Taxotere	0.38 ± 0.1	0.30 ± 0.023
Cisplatinum	198 ± 37	228 ± 52
Melphalan	244 ± 71	299 ± 34
Cytosine arabinoside	151 ± 36	244 ± 88
Dexamethason	$25 \times 10^3 \pm 211$	$35 \times 10^3 \pm 1330$

Table 2 *Inhibition of proliferation of $MVP^{+/+}$ and $MVP^{-/-}$ bone marrow cells in response to several cytostatic agents*

Presented are the drug concentrations at which proliferation is reduced to 50% as compared to untreated cells. Given are average values ± SD calculated from two independent experiments ($n = 4$–6). No statistically significant differences were observed between 50% inhibition values in wild-type and knockout cells as determined by the Mann-Whitney test.

Agent	Drug concentration (μg/ml)	
	$MVP^{+/+}$ bone marrow cells	$MVP^{-/-}$ bone marrow cells
Doxorubicin	0.131 ± 0.026	0.119 ± 0.027
Etoposide	2.73 ± 1.47	2.84 ± 1.47
Vincristine	0.047 ± 0.008	0.055 ± 0.004
Methotrexate	0.046 ± 0.006	0.039 ± 0.0015
Ifosfamide	2423 ± 270	1991 ± 514
Mitoxantrone	0.0073 ± 0.0016	0.0081 ± 0.0021
Gemcitabine	0.029 ± 0.011	0.0183 ± 0.001
Taxotere	0.0058 ± 0.0006	0.0054 ± 0.0004
Cisplatinum	7.06 ± 2.27	6.23 ± 2.48
Melphalan	5.06 ± 1.19	3.93 ± 1.53
Cytosine arabinoside	0.11 ± 0.02	0.11 ± 0.003
Dexamethason	322 ± 36	326 ± 44

Table 3 *Functional expression of P-gp, MRP1, and BCRP in $MVP^{+/+}$ and $MVP^{-/-}$ cells*

Depicted are the ratios calculated from the intracellular fluorescent substrates in the absence and presence of the specific modulators PSC 833, MK571, and Ko143. Values given are averages of two independent experiments.

	Bone Marrow		ES cells	
	$MVP^{+/+}$	$MVP^{-/-}$	$MVP^{+/+}$	$MVP^{-/-}$
P-gp	1.45	1.51	1.21	0.95
MRP1	10.41	10.48	7.5	8.8
BCRP	1.34	1.09	1.18	1.15

of MRP1. Although the IC_{50} values of both doxorubicin and etoposide were clearly reduced by adding MRP1 inhibitor (Table 1), this effect was not found to be more pronounced in the MVP-deficient cells.

Toxicity of Doxorubicin *in Vivo.* Although the disruption of the *MVP* gene did not result in an altered sensitivity to drugs in bone marrow and ES cells, it is possible that MVP/vaults play a role in the protection of a specific organ. Such an observation was reported for the P-gp-deficient mice, in which absence of P-gp in the blood-brain barrier led to accumulation of ivermectin in the brain, resulting in a 100-fold increased sensitivity level to this agent (34). We tested the *in vivo* toxicity of doxorubicin in *MVP* knockout mice, because the relation between drug resistance and MVP expression was most obvious for anthracyclines (14, 15). We examined the general appearance, weight loss, and blood cell numbers of 21 wild-type and 23 knockout mice treated with a single i.v. doxorubicin injection of 15 mg/kg bodyweight. The MVP-deficient animals did not show increased symptoms of illness and discomfort when compared with wild-type control animals. The number of WBCs was slightly decreased 3 days after injection in both groups when compared with noninjected animals, but had recovered in both wild-type and knockout mice on day 8 (data not shown). The effects of the doxorubicin treatment on the bodyweights are presented in Fig. 5. Both $MVP^{+/+}$ and $MVP^{-/-}$ animals showed loss of weight during the first days after injection, but the average weight loss was similar in both groups, and all of the animals were able to stabilize and started to recover their weights. Furthermore, the damage to organs after injection of 10 mg doxorubicin/kg bodyweight was investigated extensively, with special attention to organs that normally exhibit a high expression of MVP. Effects of the doxorubicin treatment, such as hydropy in the liver and the disruption of the morphology of the villi in the ileum, were visible in both knockout and wild-type organs (5 wild-type *versus* 5 knockout animals), but the levels of the induced tissue damage were similar (data not shown).

DISCUSSION

We have successfully generated a MVP knockout mouse. The *MVP* mRNA and protein are undetectable in the tissues of the knockout animals and severely reduced in heterozygous animals. Moreover, vault particles could no longer be detected in the *MVP* knockout tissues.

Both the high degree of evolutionary conservation and the complex structure of vault particles, as well as its broad distribution in tissues suggest an important function in cellular processes. Surprisingly, the *MVP* knockout animals do not show any obvious abnormalities, breed normally, and appear healthy. Apparently, vaults do not play an essential role in the development of mice and in cellular processes in a normal healthy environment. However, vaults could be involved in a strategic mechanism needed for survival after induction of cellular

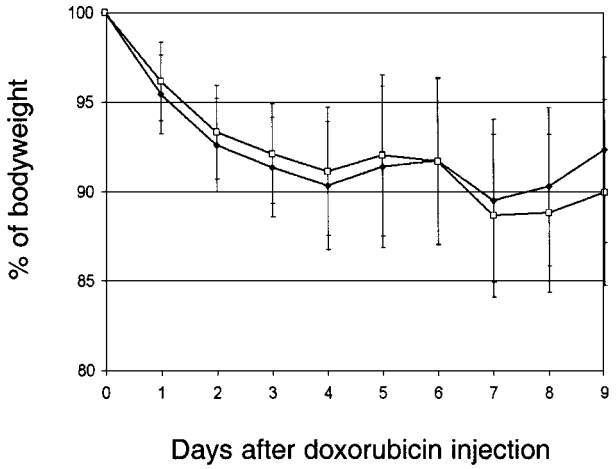

Fig. 5. Effects of doxorubicin treatment on bodyweights of MVP wild-type (*WT*) and deficient (*KO*) mice. Depicted is the average bodyweight of mice i.v. injected with 15 mg/kg doxorubicin, as percentage of the initial bodyweight before treatment; *bars*, ± SD. Body weights were measured daily after injection of doxorubicin. (♦ = WT, □ = KO; n = 21 and 23, respectively).

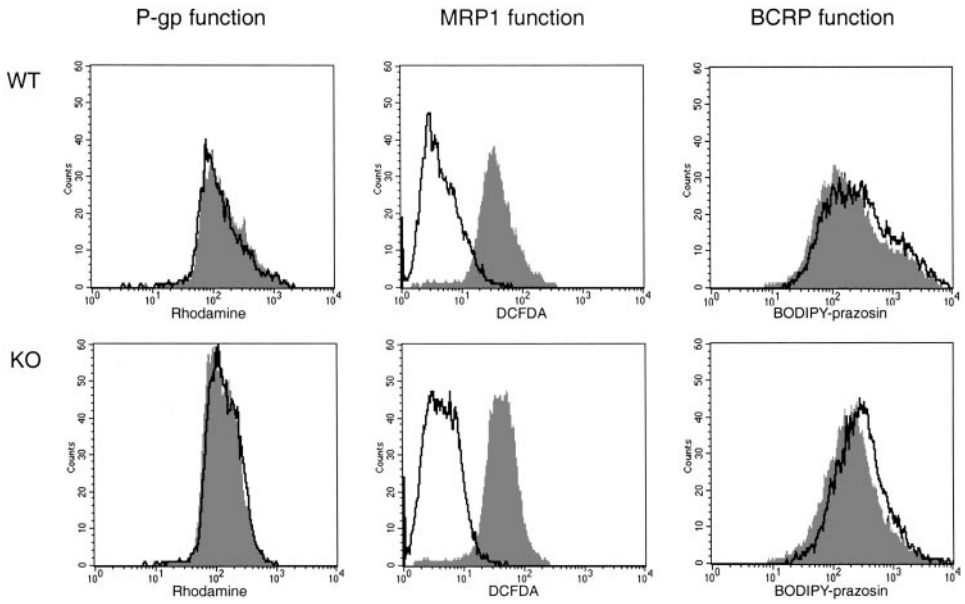

Fig. 4. Functional expression of MDR-related proteins in wild-type and *MVP* knockout cells. The activities of P-gp, MRP1, and BCRP are presented as the intracellular amounts of Rhodamine-123 (P-gp substrate), DCFDA (MRP1 substrate), or BODIPY-prazosin (BCRP substrate) in wild-type (*WT*) and MVP-deficient (*KO*) ES cells in presence (*gray plot*) or absence (*black plot*) of the modulators PSC 833, MK571, and Ko143.

stress or damage. Because the cellular defense against cytotoxic agents may partly rely on vaults, as was suggested in several clinical studies and experiments using MDR cell lines, we have examined a potential function of vaults in multidrug resistance. To this purpose, two different cell types derived from the *MVP* knockout model were tested in two different assays. Firstly, the survival of $MVP^{+/+}$ and $MVP^{-/-}$ ES cells, when exposed to different concentrations of cytostatics, was examined in a MTT assay. Similar experiments have been performed in *MRP1* deficient ES cells by Lorico *et al.* (35), who observed that disruption of *MRP1*, a MDR-related membrane transport protein, leads to hypersensitivity to etoposide and teniposide and, to a lesser extent to doxorubicin. Decreased IC_{50} values of up to 4-fold were reported. In our experiments, no significant difference in IC_{50} values and no difference in the survival curves of MVP-deficient and wild-type ES cells were observed. Secondly, the effects of cytostatic agents on the proliferative capacity of immature bone marrow cells isolated from knockout and wild-type littermate animals were examined in a [^{3}H]thymidine incorporation assay. The inhibition of proliferation in response to all of the administered drugs was similar in wild-type and MVP-deficient cells. Finally, although more extensive research using several cytotoxic agents at different concentrations should be performed to determine the *in vivo* sensitivity of the *MVP* knockout mice, the systemic protection to doxorubicin seems unaltered in MVP-deficient mice as compared with the wild-type control mice. Siva *et al.* (17) suggested that vaults may be necessary, although not sufficient for drug resistance. The data presented in this study confirm these findings, and even suggest that MVP and intact vaults are not necessary for the resistance to a broad range of anticancer drugs.

In contrast, in a number of cell line models, MVP expression has been found to correlate with resistance to several cytostatic agents. A clear relation between MVP expression levels and resistance to doxorubicin, etoposide, Taxol, and vincristine was reported by Kitazono *et al.* (14, 15). Colon cancer SW-620 cells with induced MVP expression were 6-fold more resistant to doxorubicin, 9-fold to etoposide, 8-fold to Taxol, and 2-fold to vincristine, as determined by a MTT assay. When *MVP* transcript levels were decreased by the expression of *MVP*-specific ribozymes, the cells lost the acquired resistance. A mechanism for the action of MVP/vaults proposed by the authors is the efflux of doxorubicin from the nucleus. However, because not all of the drugs investigated in their study have nuclear targets, this only explains part of the acquired resistance of the SW-620 cells. The results of these studies also imply that by losing MVP, cells become more susceptible to doxorubicin, etoposide, and Taxol. However, the MVP-deficient ES and bone marrow cells used in our study were not more susceptible to these cytostatic agents or to any other agent tested.

A possible explanation for the unexpected lack of a drug-sensitive phenotype in the MVP-deficient mice is that vaults only contribute moderately to drug resistance and that the absence of MVP is compensated for by other MDR proteins, like ABC-transporters such as P-gp, MRP family members, or BCRP. However, in the MVP-deficient cell systems used in this study, no increase of activity of P-gp, MRP1, and BCRP was observed. A functional assay showed a considerable activity of MRP1 in both ES cells and bone marrow cells, but this activity did not change after disruption of *MVP*. Addition of a MRP1 inhibitor indeed increased the susceptibility of ES cells for the MRP1 substrates, doxorubicin and etoposide, but the effects were identical in MVP wild-type and deficient cells. Hence, it is not likely that the effects of the disruption of MVP are compensated for by P-gp, MRP1, or BCRP. However, it cannot be excluded that other ABC-transporters or unknown mechanisms of drug resistance become up-regulated on disruption of *MVP*. Furthermore, our experiments did not include long-term exposure of cells to low doses of cytostatic agents. Although a direct involvement of vaults in MDR could not be demonstrated in the *MVP* knockout mouse model, vaults might still be involved in the protection against chronic exposure to drugs. On the other hand, the up-regulation of MVP that has been described in both *in vitro* and clinical studies might be a response to cytostatics but not directly related to the resistance to these agents. Vaults may be involved in other cellular processes that also become activated on exposure to toxic agents, such as apoptosis and/or a general stress response. In addition, some evidence for a role of vaults in cellular differentiation was given by Schroeijers *et al.* (36). The observed up-regulation of vaults during the development of dendritic cells and the impaired function of dendritic cells treated with antibodies against MVP suggest that vaults play a role in the development, maturation, and functioning of dendritic cells. We are currently investigating the development and antigen-presenting capacity of dendritic cells derived from the MVP knockout mice.

Although the major component of the vault particle is absent, and vault particles are no longer detected, the remaining components TEP1, VPARP, and vRNA might still interact and possibly fulfill a functional role. However, the levels of the additional vault components seem to be severely reduced in MVP knockout tissues.[4] Besides the vault-associated fractions of TEP1 and VPARP, also their levels outside the vault complex, *i.e.*, associated with the telomerase complex and the nuclear portion of VPARP, may be affected by the disruption of *MVP*. If this is the case this might indicate a functional link between vaults and the telomerase complex in addition to the simple sharing of the TEP1 subunit. Likewise a possible function of nuclear VPARP in DNA damage repair might also be impaired. Determination of the levels of the minor vault proteins, their subcellular localization and the mechanism responsible for the decrease in their concentrations upon *MVP* disruption could give important clues on the still unrevealed function of the vault complex.

ACKNOWLEDGMENTS

We thank Drs. Mirjam Hermans and Gijsbertus T. van der Horst for excellent advice and helpful discussions, and Manja Muijtjens for technical assistance during the blastocyst injections.

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