

## Correspondence

### Functional analysis of MRP1 cloned from bovine<sup>1</sup>

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Multidrug resistance protein 1 (MRP1), a member of the ATP-binding cassette (ABC) family of membrane transport proteins, functions as an energy-dependent efflux pump that extrudes many kinds of xenobiotics out of cells [1,2]. MRP1 can transport a wide range of natural chemotherapeutic agents including anthracyclines, *Vinca* alkaloids and epipodophyllotoxins, thus conferring multidrug resistance on the cells. The cDNA of MRP1 has been cloned from mice as well as humans, and comparative analyses of the respective proteins have helped to elucidate the domains critical for the activity of this transporter. Although the amino acid identity between human and mouse MRP1 is very high (88%), mouse MRP1 cannot confer resistance to doxorubicin (Dox), and has poor activity to transport 17 $\beta$ -estradiol 17-( $\beta$ -D-glucuronide) (E<sub>2</sub>17 $\beta$ G) when compared with human MRP1 [3]. Mutational analyses of mouse and human MRP1 revealed that two amino acid residues are critical for the activity of MRP1. One is glutamic acid (Glu) at the position 1089 (Glu<sup>1089</sup>) in human MRP1 (corresponding to glutamine (Gln<sup>1086</sup>) in mouse MRP1) [4], the other is threonine (Thr<sup>1242</sup>) (alanine, Ala<sup>1239</sup> in mouse MRP1) [5]. The former residue is mainly related to the ability to confer resistance to Dox. The mutation of Gln<sup>1086</sup> in mouse MRP1 to Glu markedly increased the resistance to Dox, while the mutation of Glu<sup>1089</sup> in human MRP1 to Gln decreased the resistance. On the other hand, the latter residue is mainly related to the transport activity of certain organic anion conjugates, such as E<sub>2</sub>17 $\beta$ G. The cDNA cloning and functional characterization of orthologs of MRP1 would help to clarify the mechanisms for substrate recognition and transport of this transporter.

We cloned bovine MRP1 cDNA from the mammary gland of a lactating cow. The open reading frame of bovine MRP1 contains 4590 nucleotides encoding 1530 amino acid residues (Fig. 1A). The amino acid sequence of bovine MRP1 shows 91% and 87% identity, respectively, with that of the human and mouse orthologs. Alignment of the amino acid sequences of these orthologs revealed that the amino acid residue of bovine MRP1 corresponding to Glu<sup>1089</sup> in human MRP1 is Gln<sup>1088</sup>, while the residue corresponding to Thr<sup>1242</sup> in human MRP1 is Thr<sup>1241</sup>. Northern blotting of poly(A)<sup>+</sup> RNA from various bovine tissues showed that bovine MRP1 is expressed in heart, spleen, lung, kidney, skeletal muscle and mammary gland, while the expression is weak in brain and liver (data not shown). This tissue profile of expression resembles that reported for humans and for mice.

To characterize the functions of bovine MRP1 and compare them with those of the human ortholog, we obtained cells expressing bovine MRP1 (KB/bMRP1) and those expressing human MRP1 (KB/hMRP1), and the drug-resistance profiles

of KB/bMRP1 and KB/hMRP1 were investigated by comparing relative resistance to vincristine (Vcr), actinomycin D (AcD), colchicine (Col), vinblastine (Vbl), Dox and VP16 (Fig. 1B). The expression of MRP1 protein in each cells was confirmed by immunoblot analysis (Fig. 1C). The levels of resistance to Vcr, AcD and Col of KB/bMRP1 were about a half of those of KB/hMRP1, while, judging from the immunoreactivity, the expression levels of bovine MRP1 protein in the KB/bMRP1 cells were also about a half of the levels of human MRP1 protein in the KB/hMRP1 cells (Fig. 1B,C). Both cells showed relatively low resistance to Vbl. These results suggest that bovine and human MRP1 conferred resistance to Vcr, Col, AcD and Vbl with approximately the same efficiency. However, KB/bMRP1 showed no increase in resistance to Dox (1.0  $\pm$  0.4-fold), although KB/hMRP1 exhibited a 5.3  $\pm$  1.2-fold increase, suggesting that bovine MRP1 is much less effective in conferring resistance to Dox than human MRP1. In contrast, KB/bMRP1 showed higher resistance to VP16 (4.9  $\pm$  0.7-fold) than KB/hMRP1 (2.9  $\pm$  0.3-fold), although the expression level of bovine MRP1 in KB/bMRP1 was lower than that of human MRP1 in KB/hMRP1, suggesting that bovine MRP1 is better able to confer resistance to VP16 than human MRP1. The difference between them in the ability to confer resistance to Dox and to VP16 suggests that the substrate specificity of bovine MRP1 is clearly different from that of human MRP1, although the amino acid identity between them is about 90%.

The poor ability to confer resistance to Dox of bovine MRP1 resembles that of mouse MRP1, and is likely due to the fact that the amino acid in bovine MRP1 corresponding to Glu<sup>1089</sup> in human MRP1 is Gln<sup>1088</sup> as in the mouse ortholog. Our results support the notion that this amino acid residue of MRP1 plays an important role in the activity of this transporter, especially in the ability to confer resistance to Dox [4].

It was revealed by mutational analyses of human and mouse MRP1 that the combination of Gln<sup>1086</sup> and Ala<sup>1239</sup> in mouse MRP1 and Glu<sup>1089</sup> and Thr<sup>1242</sup> in human MRP1 is specifically required to confer resistance to Vcr and VP16 [5]. Bovine MRP1 has a unique combination of critical amino acid residues, Gln<sup>1088</sup> like in mouse MRP1 and Thr<sup>1241</sup> as in human MRP1. This combination is the same as that of mutants of human and mouse MRP1 which conferred decreased resistance to both Vcr and VP16 [4,5]. However, the ability of bovine MRP1 to confer resistance to Vcr is similar to that of human MRP1. Moreover, the ability of the bovine MRP1 to confer resistance to VP16 was higher than that of the human ortholog. Our results suggest that there would be other residues that contribute to the drug resistance besides those at positions 1089 and 1242 in human MRP1. Those residues might be one or some of unconserved residues between the bovine and human or bovine and mouse orthologs.

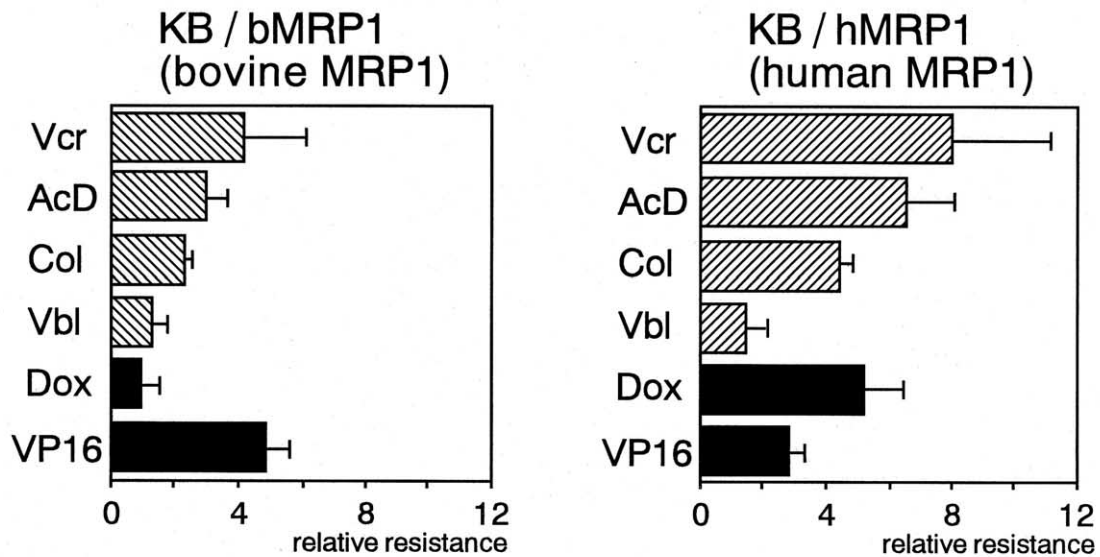
Based on studies using MRP1 knockout mice, it has been suggested that one of the physiological functions of MRP1 is to protect important tissues or organs in the host against xenobiotics, such as the blood–testis barrier [1]. We showed that MRP1 is expressed in the mammary gland of lactating cow, and cloned its cDNA from the tissue. We also found that

**A**

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1 MALRDFCSVDGSDLFEWENVNTWNTSNPDFTKCFQNTVLVWVPCSYLWVCFPFYFLYLSSHDRGYIQMTHLNKAKTALGFLWLWVWADLIFYSFWERSMGK
101 LLAPVFLVSPDLLGITMLLATPLIQIERRRGVQSSGIMLTFWLIALLCALAILRSKIMTALKEDARVDVFRDVTFFIYFSLVLIQLVLSCFSDRSPLFSE
201 TINDPNPCPESSASFLSRITFWWIITGMMVQGYRQPLESTDLWLNKEDTSEQVVPVLVKNWKKCEAKSRKQPVKIVYSSKDPAPKPGSSKVDVNEEEAAL
301 IVKCPQKERDPSLFKVLKTFGPFYFLMSPLFKAVHDLMMFAGPEILKLLINFVNDKKAPEWQGYFYTALLFISACLQTLVLHQYFHCIFVSGMRKTAVI
401 GAVYRKALVITNAARKSSSTVGEIVNLMSVDAQRFMDLATYINMIWSAPLQVILALYLLWLNLGPSVLAGVAVMVLVPLNAVMMAMTKTYQVAHMKSKDN
501 RIKLMNEILNGIKVLKLYAWELAFKDKVLAIRQEELKVLKKSAYLAAVGTFWVCTPFLVALSTFAVYVTVDENNILDAQAFVSLALFNILRFPNLILP
601 MVISSIVQASVSLKRLRVFLSHEDLDPSIQRRPIKDAGATNSITVKNATFTWARNDPPTLHGITFVPEGSVAVVGVQCGCKSSLLSALLAEMDKVEG
701 HVTVKGSAVYVPPQAWIQNISLRENILFGRQLQERYKAVVEACALPDLEILPSGDRTEIGEKGVLSSGGKORVSLARAVYCDSDVYLLDDPLSAVDA
801 HVGKHIFENVIGPKLLKNKTRLLVTHAISYLPQMDVIVMSSGGKISEMGSYQELLARDGAFAEFLRTYASAEQEQQPEDGLAGVGGPGKEVKQMENG
901 LVTDTAGQMQRQLSSSSYSRDVSHHTSTABLRKPGPTEETWKLVEADKAQTGQVKSIVYWDYMKAIGLFISFLSIFLFLCNHVASLVSNYWLSLWTD
1001 DPIVNGTQEHQVRLSVYVQALGISQGITVFGYSMAVSIIGGIFASRRLHLDLHNVLRSPISFFERTPSGNLVNRFSKELDTVDSDMIPQVLIKMFMSLFNV
1101 IGACIIILLATPMAAVIIPPLGLIYFFVQRFYVASSRQLKRLESVSRSPVSHFNETLLGVSVIRAFEEQERFIRQSDLVDENQKAYYPSIVANRWLAV
1201 RLECVCNIVLFPASLFAVIRSHLSAGLVGLSVSYSLQVTTIYNLWLRMSSEMETNIVAVERLKEYSETEKEAPWQIQDMAPPKDWPQVGRVFRDYGLR
1301 YREDLDLVKHNIVTIDGGEKVGIVGRITGAGKSSLTLGLFRIKESAEGETIIDDINIAGIHLHLRFKTIITIPQDPVLFSGSLRMNLDPFSQYSDEEVWT
1401 SLELAHLKGFVSALPDKLNHECAEGGENLSVGRQRLVCLARALLRKTLLVLDLDEATAAVDLETTDDLIQSTIRTFDDCTVLTIAHRLNTIMDYTRVIVLD
1501 KGEIQEWGSPDLLQQRGLFYSMKDSGLV
    
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**B**



**C**

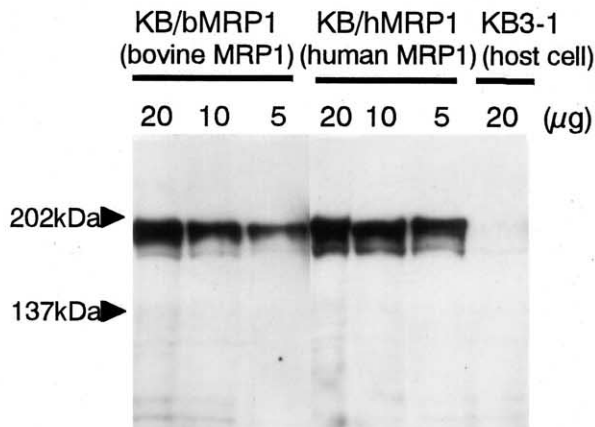


Fig. 1. A: Deduced amino acid sequence of cloned bovine MRP1. Double underlining, Walker A and B motifs and the active transport family signature (C), which are conserved characteristics of the nucleotide-binding domain of ABC transporters. Single underlining, the epitope sequence of the monoclonal antibody MRPM6, which is perfectly conserved in bovine MRP1. The amino acid residues corresponding to Glu<sup>1089</sup> and Thr<sup>1241</sup> in human MRP1 are indicated by arrows. B: The drug-resistance profiles of cells expressing bovine MRP1 and those expressing human MRP1. The expression vector pIRES2-EGFP (Clontech) containing each MRP1 cDNA, which has a neomycin resistance gene for the selection of stable transformants, was introduced into the drug-sensitive human carcinoma line KB3-1. Cells were first selected in the presence of 0.8 mg/ml of geneticin for 10 days, and then with 5 ng/ml of Vcr to obtain cells expressing a detectable amount of bovine or human MRP1. By reverse transcription-polymerase chain reaction analysis, it was confirmed that bovine and human MRP1 mRNAs were certainly expressed in cells transfected with bovine MRP1 cDNA (KB/bMRP1) and those transfected with human MRP1 cDNA (KB/hMRP1), respectively. Relative resistance was calculated by comparing the IC<sub>50</sub> (the drug concentration necessary to inhibit cell growth by 50%) for each of the cells expressing the bovine or human MRP1 to the IC<sub>50</sub> of the host cell KB3-1. Each value is the mean of three to four separate experiments. The IC<sub>50</sub> for KB3-1 was 4.96 ng/ml for Vcr, 1.22 ng/ml for AcD, 4.06 ng/ml for Col, 2.16 ng/ml for Vbl, 33.8 ng/ml for Dox and 542 ng/ml for VP16. C: Expression of bovine and human MRP1 protein in KB/bMRP1 and KB/hMRP1 cells. Membrane proteins (5, 10, 20 µg) prepared from cells expressing bovine MRP1 (KB/bMRP1), cells expressing human MRP1 (KB/hMRP1), and KB3-1 host cells were resolved on a 7% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel and reacted with the monoclonal antibody MRPM6 raised against human MRP1 as a probe for bovine MRP1 as well as human MRP1, because the epitope sequence of MRPM6 is perfectly conserved in the bovine ortholog [2].

there was lower transfer of antimony, one of the substrates of MRP1, from blood to milk in lactating mice (manuscript in preparation). These results suggest that MRP1 protein expressed in the mammary gland would play a role in protecting milk against xenobiotics, and that bovine MRP1 would be one of the genes that influence the quality of daily milk. The exact sites of expression of MRP1 in the mammary gland and the extent to which this molecule contributes to milk quality remain to be elucidated.

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<sup>1</sup> The sequence reported in this paper has been submitted to GenBank with the accession number AB082124.

**Abbreviations:** MRP1, multidrug resistance protein 1; Vcr, vincristine; Vbl, vinblastine; Dox, doxorubicin; AcD, actinomycin D; Col, colchicine; Glu, glutamic acid; Gln, glutamine; Thr, threonine; Ala, alanine

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