Transport of leukotriene C₄ by a cysteine-less multidrug resistance protein 1 (MRP1)

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Overexpression of multidrug resistance protein 1 (MRP1), an ATP-binding cassette protein, causes multidrug resistance. We developed a functional cysteine-less version of MRP1 that provides a framework for detailed biochemical and biophysical studies. The 18 Cys residues of a truncated MRP1 (tMRP1; lacking the first multispinning transmembrane domain) were replaced with Ala to generate Cys-less tMRP1 (CL tMRP1). CL tMRP1 expressed in *Saccharomyces cerevisiae* membranes displayed high-affinity ATP-dependent transport of the MRP1 substrate leukotriene C₄. Compared with full-length MRP1, the

K_m for leukotriene C₄ transport by CL tMRP1 was increased ~3-fold, while V_{max} was not affected. Thus a functional CL tMRP1 can be expressed using a low-cost and rapid-generation yeast expression system. This Cys-less protein can be used for biochemical, spectroscopic and structural studies to elucidate the mechanism of drug transport by MRP1.

Key words: ATP-binding cassette (ABC) protein, Cys-less, cystic fibrosis transmembrane conductance regulator (CFTR), MRP, multidrug resistance, nucleotide-binding domain.

INTRODUCTION

Multidrug resistance of cancer cells is an important clinical problem, characterized by resistance to the cytotoxic effects of a variety of chemically unrelated agents [1]. Overexpression of ATP-binding cassette (ABC) proteins, such as P-glycoprotein (MDR1, ABCB1) and multidrug resistance protein 1 (MRP1; ABCC1) causes multidrug resistance [1,2]. These multidrug resistance proteins are ATPases that pump the chemotherapeutic agents out of the cells, allowing them to survive in the presence of the cytotoxic agents [1,2]. MRP1 is expressed ubiquitously, and may also participate in the regulation of redox potential (via glutathione transport), ion channels and inflammatory responses (via transport of prostaglandins, leukotrienes and cytokines), as well as in the transport of endo- and xeno-biotics, and the distribution of phospholipids in membranes [3–5].

Most mammalian ABC proteins consist of two multispinning transmembrane domains (TMD₁ and TMD₂), with one nucleotide-binding domain following each TMD (see Figure 1A). MRP1, however, belongs to an ABC subfamily (ABCC) in which several members contain one additional TMD (TMD₀), which is joined to TMD₁ by a linker, L₀ [3] (Figure 1). The function of TMD₀ is unknown, but it has been shown that it is not necessary for drug transport [6].

Although significant effort has been devoted to the study of MRP1, basic aspects of the structure and function of the protein are still poorly understood. Recently the crystal structure of two bacterial ABC proteins has been solved [7,8], but the similarities between these bacterial proteins and eukaryotic ABC proteins, such as MRP1, are unclear [9,10]. In any case, detailed biochemical and structural studies will ultimately be needed to elucidate how MRP1 works. A variety of spectroscopic and biochemical techniques have been used to obtain detailed information on the structure and function of membrane proteins, taking advantage of the availability of highly specific thiol reagents and the possibility of placing cysteine residues of

recombinant proteins at specific locations. Unfortunately, the use of these powerful techniques frequently requires the generation of a functional protein devoid of Cys residues. MRP1 has 25 Cys residues, and recent observations indicate that replacement of Cys⁷ produces a protein with little or no function [11], suggesting that the probability of obtaining a functional Cys-less MRP1 is low. With these issues in mind, we took advantage of the observation that an MRP1 lacking TMD₀ retains its drug transport function [6], and have engineered a truncated protein devoid of Cys residues that is well expressed in yeast and is functional. This Cys-less protein can be used in biochemical, spectroscopic and structural studies to elucidate the mechanism of drug transport by MRP1.

EXPERIMENTAL

Plasmid construction and mutagenesis

Recombinant MRP1 proteins were expressed in *Saccharomyces cerevisiae* using the plasmid YEpHis (a gift from Dr Marwan Al-Shawi) [12], under the control of the constitutive yeast plasma membrane H⁺-ATPase (PMA1) promoter. The 23 bp PMA1 promoter sequence preceded the start codon of all constructs [12]. Full-length MRP1 and tMRP1 (MRP1 lacking TMD₀, truncated before the start of L₀ at Asp²⁰⁴), with a FLAG epitope at the N-terminus and a His₁₀ tag at the C-terminus, were engineered by a combination of PCR and ligation to oligonucleotide adaptors, using full-length MRP1 cDNA in the plasmid pRc/RSV-MRP (a gift from Dr P. Borst and Dr M. Kool) [13]. In tMRP1, the starting Met is followed by a FLAG tag and the MRP1 sequence starting at Asp²⁰⁴ (see Figures 1A and 1B). To generate Cys-less tMRP1 (CL tMRP1; Figure 1B), all cysteine residues of tMRP1 were substituted with alanine by site-directed mutagenesis using the Quick Change Multisite Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, U.S.A.). Cys → Ala mutations were preferred to the more conservative

Abbreviations used: ABC, ATP-binding cassette; CL tMRP1, Cys-less tMRP1; L₀, linker region between TMD₀ and TMD₁ of MRP1; LTC₄, leukotriene C₄; MRP1, multidrug resistance protein 1; tMRP1, MRP1 lacking TMD₀ (truncated before the starting of L₀ at Asp²⁰⁴); TMD, multispinning transmembrane domain.

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Figure 1 Schematic representation of the recombinant proteins

(A) Basic topological and domain structure of MRP1. TMD₀, TMD₁, TMD₂, L₀ and the nucleotide binding domains (NBD1 and NBD2) are represented. The arrow points to Asp²⁰⁴, the residue of tMRP1 immediately following the starting Met. (B) Diagrams of MRP1, tMRP1 and CL tMRP1. The tags at the N-terminus (FLAG epitope) and C-terminus (10 His residues) are illustrated. The approximate position of each of the 18 Cys residues that are replaced with Ala in CL tMRP1 is shown.

Cys → Ser substitutions, because the latter resulted in an inactive P-glycoprotein [14]. All sequences were confirmed at the DNA Sequencing Facility of the Protein Chemistry Laboratory of the University of Texas Medical Branch. Details on plasmid engineering are available at <http://www.BiochemJ.org/bj/370/bj3700357add.htm>.

Expression of MRP1 in yeast

Expression of the recombinant proteins was carried out using the protease-deficient yeast strain BJ5457 (*leu⁻*). Yeast were transformed by the lithium acetate method, and transformants were selected on solid minimal medium lacking leucine. For initial screening, randomly picked transformants were grown at 25 °C in 20 ml of SD-Leu selection medium, and MRP1 expression was examined by Western blotting of crude membranes. Expression on a larger scale was performed in baffled flasks at 25 °C in SD-Leu medium containing 15% (v/v) glycerol. Glycerol was used as a chemical chaperone to increase the expression of the recombinant proteins, as described in [12,15]. In pilot experiments, we determined that 15% (v/v) was the optimal glycerol concentration to increase MRP1 expression.

Membrane vesicle preparation

All procedures, starting with harvesting of the cells, were performed at 4 °C, unless noted otherwise. Cells were harvested and diluted 5-fold (w/v) in homogenization buffer containing 50 mM Tris/HCl, pH 7.5, 250 mM sucrose, 1 mM β-mercaptoethanol, 1 mM PMSF, 1 mM EGTA, 5 mM EDTA and one tablet of yeast protease inhibitors (Roche)/50 ml. Cells were lysed with a French Press (Thermo Spectronic, Rochester, NJ, U.S.A.), and the lysate was centrifuged at 3500 *g* for 10 min and then at 14000 *g* for 20 min. The resulting supernatant was

centrifuged at 200000 *g* for 1 h, and the pellet was resuspended and homogenized in 100 ml of buffer containing 10 mM Tris/HCl, pH 7.5, 250 mM sucrose, 1 mM β-mercaptoethanol and 1 mM PMSF. After centrifugation at 200000 *g* for 1 h, the pellet was resuspended in transport buffer (10 mM Tris/HCl, pH 7.5, and 250 mM sucrose) or storage buffer [10 mM Tris/HCl, pH 7.5, 25% (v/v) glycerol, 1 mM β-mercaptoethanol and 1 mM PMSF]. Membrane preparations at a concentration of 10–20 mg/ml were homogenized vigorously with a glass homogenizer, and were divided into aliquots before storage at –80 °C.

Immunodetection of MRP1

Western blots were performed as described previously [16], using the specific anti-MRP1 monoclonal antibody QCRL-1 (Kamiya Biochemical Co., Seattle, WA, U.S.A.).

Leukotriene C₄ (LTC₄) uptake into membrane vesicles

The ATP-dependent uptake of LTC₄ into membrane vesicles was assayed by rapid filtration, as described in [17]. Briefly, membrane vesicles were thawed, diluted in transport buffer and passed 18–20 times through a 27-gauge needle. Transport assays were carried out at room temperature, in a 110 μl volume of transport buffer containing 110 μg of protein, 4 mM ATP or AMP, 10 mM MgCl₂, an ATP-regenerating system (consisting of 10 mM phosphocreatine and 100 μg/ml creatine kinase) and [³H]LTC₄/LTC₄ (14.5 nCi). Reactions were initiated by the addition of membrane vesicles. At the desired times, 20 μl aliquots were removed and mixed with 1 ml of ice-cold transport buffer to stop the reaction. The samples were spotted on to nitrocellulose filters (0.2 μm; Whatman, Ann Arbor, MI, U.S.A.), the filters were rinsed immediately with 2 × 5 ml of ice-cold transport buffer under vacuum, and the radioactivity of the filters was measured.

Figure 2 Expression and function of the recombinant proteins in yeast membranes

(A) Representative Western blots of membranes from yeast transformed with vector alone, MRP1, tMRP1 and CL tMRP1 are shown. Membranes (20 µg per lane) were electrophoresed on a 7% (w/v) Tris/acetate gel and the anti-MRP1 antibody QCRL-1 was employed for the immunoblots. Each panel shows lanes from the same gel. (B) ATP-dependent uptake of LTC₄ into yeast membranes. The uptake of 100 nM LTC₄ was measured in the presence of AMP or ATP at 23 °C, and ATP at 4 °C. Data were obtained in the linear region of uptake. Means ± S.D. of triplicate measurements from three separate experiments are shown. See the Experimental section for additional details.

LTC₄ uptake values given represent the difference between uptake in the presence of ATP and of AMP in parallel experiments, i.e. ATP-dependent LTC₄ uptake. Uptake, expressed in pmol/min per mg, was determined in the linear region. In pilot experiments, we determined that our set-up allowed for accurate and reliable measurement of LTC₄ uptake over periods of ≥ 5 s. We measured uptake over 20 s in most cases. Since near the V_{max} the uptake values for MRP1 and tMRP1 were about twice the values at 100 nM, we used 10 s uptake measurements to assay the kinetics of transport by MRP1 and tMRP1. Preliminary experiments using 10 s uptake measurements showed a reduced affinity of CL tMRP1 for LTC₄, and thus we decided to use 20 s uptake measurements for the experiments with membranes containing CL tMRP1 in order to increase the signal-to-background ratio. Because of the difficulty in obtaining parallel duplicate or triplicate determinations of 10 and 20 s uptake, experiments with the same preparation were started at different times, and the data were averaged. In all cases, data were obtained from at least two different preparations.

RESULTS AND DISCUSSION

Cys-less MRP1 is well expressed in yeast and is functional

Our main goal was to develop a modified MRP1 that is devoid of cysteine residues and has significant drug-transporting ability. MRP1 contains 25 Cys residues, at least one of which, i.e. Cys⁷, is critical for drug transport [11]. Since the function of TMD₀ is unknown, but this domain is not necessary for drug transport,

Figure 3 Comparative transport of LTC₄ by MRP1, tMRP1 and CL tMRP1

(A) Time course of LTC₄ uptake by tMRP1. Averages of triplicate measurements of the ATP-dependent uptake of 50 nM LTC₄ into the membranes are shown. (B) Kinetics of LTC₄ uptake. Uptake of LTC₄ was measured in the linear region, for 10 s (MRP1 and tMRP1) or 20 s (CL tMRP1). Data are averages of duplicate determinations in two different experiments from different preparations. S.D.s are shown only when they are larger than the symbols. Data were fitted to the Michaelis–Menten equation.

we decided to generate a Cys-less MRP1 without TMD₀. Figure 2(A) shows that both tMRP1 and CL tMRP1 were well expressed in yeast membranes.

The immunoblot results indicate that none of the 18 Cys residues in tMRP1 are essential for expression of the protein. However, it is possible that the Cys-less protein is expressed, but is unable to carry out drug transport. To address this question, we screened the membranes containing MRPs for ATP-dependent uptake of the well known MRP1 substrate LTC₄ [6,17,18]. Figure 2(B) shows that, in the presence of MgATP, the membranes containing MRP1, tMRP1 and CL tMRP1 transported LTC₄ at rates significantly higher than those of cells transformed with vector alone, and that AMP and Mg²⁺ could not support efficient LTC₄ uptake into the membranes.

It has been shown that the MRP1-mediated uptake of LTC₄ into membranes is highly temperature sensitive, and the significant decrease observed at 4 °C has been used as an indication of uptake, as opposed to binding [19]. Consistent with MRP1-mediated transport, lowering the temperature from 23 °C to 4 °C produced a large decrease in the ATP-dependent association of LTC₄ with membranes containing MRP1, tMRP1 or CL tMRP1 (Figure 2B). Taken together, these results show that none of the

25 Cys residues of MRP1 are essential for expression of or substrate transport by this protein.

Cys-less MRP1 transports LTC₄ with high affinity

Figure 3(A) shows that the ATP-dependent uptake of 50 nM LTC₄ into membranes containing tMRP1 was linearly related to time for the first 30 s. Essentially the same results were obtained in membranes containing MRP1 and CL tMRP1 (results not shown). Figure 3(B) illustrates the comparative kinetics of LTC₄ uptake into membranes containing MRP1, tMRP1 or CL tMRP1. The kinetics of LTC₄ uptake into membranes containing MRP1 or tMRP1 were similar, with $V_{\max}$ and K_m values of 115 pmol/min per mg and 93 nM respectively for MRP1, and 99 pmol/min per mg and 131 nM respectively for tMRP1. The K_m values are comparable with those measured in a variety of preparations, including mammalian cell membranes [6,17–19]. The calculated $V_{\max}$ for LTC₄ transport by membranes containing CL tMRP1 was 127 pmol/min per mg, a value similar to those for membranes containing MRP1 and tMRP1. However, the K_m was 301 nM, 2–3-fold higher than those for MRP1 and tMRP1. These results show that CL tMRP1 can transport LTC₄ with high affinity. It is remarkable that the $V_{\max}$ for LTC₄ transport was similar for MRP1, tMRP1 and CL tMRP1, and that the only detected effect of the Cys → Ala substitutions was a modest (~ 2–3-fold) increase in the K_m for LTC₄.

Summary

In the present work we have developed a Cys-less version of MRP1 which is functional and is expressed well in yeast. MRP1 expression was performed in the yeast *S. cerevisiae* using a novel MRP1 expression/purification system that is simple, rapid, comparatively inexpensive and suitable for the production of large amounts of purified and functional protein. Based on unpublished observations from our laboratory and the results of the present studies, we estimate that > 3 mg of CL tMRP1 purified to homogeneity can be obtained from a 16 litre fermentor grow. Future biochemical, spectroscopic and structural studies based on CL tMRP1 will help to elucidate the mechanism of drug transport by MRP1.

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