Characterization of binding of Leukotriene C4 by Human Multidrug Resistance Protein 1: Evidence of differential interactions with NH2- and COOH-proximal halves of the protein*

Yue-Ming Qian, Wei Qiu, Mian Gao, Christopher J. Westlake, Susan P.C. Cole¥, and Roger G. Deeley¶

Cancer Research Laboratories, Queen’s University,
Kingston, Ontario, Canada K7L 3N6

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¥ A Senior Scientist of Cancer Care Ontario.

¶ Stauffer Research Professor of Queen’s University.

Send correspondence and reprint requests to: Roger G. Deeley, Ph.D., Cancer Research Laboratories, Botterell Hall, Queen’s University, Kingston, Ontario, Canada K7L 3N6. Tel: 613-533-2981; Fax: 613-533-6830; E-mail: deeleyr@post.queensu.ca
The abbreviations used are: MRP1, multidrug resistance protein 1; P-gp, P-glycoprotein; CFTR, cystic fibrosis transmembrane conductance regulator; ABC, ATP-binding cassette; LTC₄, leukotriene C₄; Sf21, Spodoptera frugiperda; β-gus, β-glucuronidase; GSH, reduced glutathione; ATPγS, adenosine 5’-O-(3-thiotriphosphate); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody; NBD, nucleotide binding domain; MSD, membrane spanning domain; CL, cytoplasmic loop; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PBS, phosphate-buffered saline; IACI, N-(hydrocinchonidin-8’-yl)-4-azido-2-hydroxybenzamide; IAARh123, iodoaryl azidoRhodamine123; AG-A, Agosterol-A

ABSTRACT

Multidrug Resistance Protein 1 (MRP1) is capable of actively transporting a wide range of conjugated and unconjugated organic anions. The protein can also transport additional conjugated and unconjugated compounds in a GSH or S-methyl GSH stimulated manner. How MRP1 binds and transports such structurally diverse substrates is not known. We have used $[^3]$H leukotriene C$_4$ (LTC$_4$), a high-affinity glutathione conjugated physiological substrate, to photolabel intact MRP1, as well as fragments of the protein expressed in insect cells. These studies revealed that: i) LTC$_4$ labels sites in the NH$_2$- and COOH-proximal halves of MRP1, ii) labeling of the NH$_2$-half of MRP1 is localized to a region encompassing membrane spanning domain (MSD)2 and nucleotide binding domain (NBD)1, iii) labeling of this region is dependent on the presence of all or part of the cytoplasmic loop (CL3) linking MSD1 and MSD2, but not on the presence of MSD1, iv) labeling of the NH$_2$-proximal site is preferentially inhibited by S-methyl GSH, v) labeling of the COOH-proximal half of the protein occurs in a region encompassing transmembrane helices 14-17 and appears not to require NBD2 or the cytoplasmic COOH-terminal region of the protein, vi) labeling of intact MRP1 by LTC$_4$ is strongly attenuated in the presence of ATP and vanadate, and this decrease in labeling is attributable to a marked reduction in LTC$_4$ binding to the NH$_2$-proximal site, and vii) the attenuation of LTC$_4$ binding to the NH$_2$-proximal site is a consequence of ATP hydrolysis and trapping of Vi-ADP exclusively at NBD2. These data suggest that MRP1 mediated transport involves a conformational change, driven by ATP hydrolysis at NBD2, that alters the affinity with which LTC$_4$ binds to one of two sites composed, at least in part, of elements in the NH$_2$-proximal half of the protein.
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INTRODUCTION

Development of multidrug resistance is a frequent impediment to the effective treatment of cancer. Although many different mechanisms are involved, multidrug resistance in cultured tumor cells appears most frequently to be associated with increased expression of the ABC transporter proteins, P-glycoprotein (P-gp) and/or Multidrug Resistance Protein (MRP)₁ (1-3). As in cells that overexpress P-gp, drug accumulation in cells with elevated levels of MRP1 is reduced, supporting the notion that the multidrug resistance phenotype caused by both of these proteins involves increased drug extrusion (4,5). However, in contrast to P-gp, demonstration of MRP1-mediated active transport of unmodified chemotherapeutic drugs such as vincristine and daunorubicin in vitro, using inside-out membrane vesicle systems, requires the presence of GSH in addition to ATP (6-9).

More recently, we have shown that stimulation of MRP1 mediated transport by GSH or certain of its analogs is not restricted to unmodified hydrophobic drugs but may also include some organic anion conjugates, such as estrogen sulfates and a glucuronidated tobacco-derived nitrosamine (10,11). However, unlike xenobiotics such as vincristine and verapamil (7,12), these anionic conjugates appear not to stimulate GSH transport (10,11). The mechanisms by which compounds such as vincristine and verapamil stimulate GSH transport remain unclear, although several hypotheses have been proposed (3,10,11,13,14).

The most well characterized substrate of MRP1 is the cysteinyl leukotriene, leukotriene C₄ (LTC₄). Studies with mrp1⁻/⁻ mice have confirmed that LTC₄ is an endogenous substrate for mrp1. These studies have shown that lack of the protein results
in an impaired LTC$_4$ mediated inflammatory response and that mrp1 mediated efflux of LTC$_4$ is involved in regulating dendritic cell migration to lymph nodes (15,16). To date, LTC$_4$ remains the highest affinity MRP1/mrp1 substrate that has been identified ($K_m$ ~100 nM) and many MRP1 structure-function studies have been based on LTC$_4$ transport activity. Examples include reconstitution of LTC$_4$ transport activity by heterologous co-expression of the NH$_2$- and COOH-proximal halves of MRP1 (17) and identification of regions essential or dispensable for function (18-20).

Topology studies of MRP1 have revealed that MRP1 and P-gp share a similar core structure consisting of two membrane-spanning domains (MSD2 and MSD3) and two nucleotide-binding domains (NBD1 and NBD2) (21). The primary distinguishing characteristic of MRP1 and its related proteins, MRPs 2, 3, 6 and 7, is an additional NH$_2$-terminal region forming a membrane-spanning domain (MSD1) with five transmembrane helices (1,21-23). This region is linked to the remainder of the protein by a relatively large cytoplasmic loop (CL), designated CL3, of approximately 130 amino acids (21). We have shown previously that deletion of MSD1 plus approximately 35 or 95 amino acids of the CL3 eliminates LTC$_4$ transport activity (18). However, transport activity could be restored to both NH$_2$-terminally truncated proteins by co-expressing a fragment that contains MSD1 and the first 95 amino acids of CL3, but not by a fragment containing MSD1 and only the first 35 amino acids of CL3(18). Similarly, an internal deletion of 53 amino acids within CL3 also inactivates the protein. These studies strongly suggest that the physical integrity of a certain portion of CL3 is essential for LTC$_4$ transport. A similar conclusion was reached by Bakos et al (19) who demonstrated that a truncated protein lacking MSD1 but retaining essentially all of predicted CL3, MRP1$_{204}$-
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1531, retained considerable LTC₄ transport activity (19). The NH₂-terminus of such a truncated protein is very close to that predicted for a common four-domain ancestor of the MRPs and the more distantly related cystic fibrosis transmembrane conductance regulator (CFTR) suggesting that MRP related proteins with an additional MSD may have evolved by fusion of a gene encoding an already functional transporter with a gene or genes encoding other integral membrane proteins (24).

Although previous studies have defined several regions of MRP1 that are required for LTC₄ transport (18-20), it is presently not known whether these regions are essential for initial binding of substrate, or are required for some subsequent step in the transport process. To define regions of MRP1 involved in LTC₄ binding, we took advantage of single and dual-baculovirus expression vectors, either to produce truncated forms of MRP1 in Sf21 cells, or to co-express two/three fragments of the protein, which were then photolabeled by [³H] LTC₄. Using this approach, we have shown that: i) cooperativity between the NH₂- and COOH-proximal halves of MRP1 is required for high affinity LTC₄ binding, ii) LTC₄ photoaffinity labels sites in both the NH₂- and COOH-proximal halves of the protein, iii) all or part of the region of CL3 between amino acids 204 and 281 is essential for LTC₄ binding although it is not a site of photoaffinity labeling, iv) the affinity of LTC₄ binding to the region containing MSD2 and NBD1 is selectively decreased when Vi-ADP is trapped at NBD2, and v) the decrease in LTC₄ binding observed under vanadate trapping conditions occurs in the absence of ATP hydrolysis by NBD1.
EXPERIMENTAL PROCEDURES

**Materials**-[14,15-^3^H]-LTC^4^-binding by MRP1

Life Sciences and fluorographic reagent Amplify® from Amersham (Oakville, Ontario, Canada). Nucleotides, GSH and S-methyl GSH were purchased from Sigma. GammaBind Plus Sepharose was from Pharmacia Biotech.

**Generation of Constructs** - Recombinant donor plasmids encoding the full-length MRP1 and either of the NH\_2-proximal half-molecule and the COOH-proximal half-molecule of MRP1 have been described (17). To introduce cDNA fragments encoding truncated half-molecules of MRP1 into pFASTBAC Dual vectors, the same strategy as described for generation of a construct capable of expressing both halves of the protein (25) was used. The previously described pFB-\Delta N (MRP932-1531), pFB-MRP900-1143, pFB-MRP1138-1531 and pFB-MRP1061-1531 were individually linearized with SalI, blunted with Klenow fragment, and then digested with KpnI. The recovered SalI*-KpnI fragments were ligated to pFASTBAC Dual which had been digested with SmaI and KpnI to give pFBDual-MRP932-1531, pFBDual-MRP900-1143, pFBDual-MRP1138-1531 and pFBDual-MRP1061-1531. These constructs were further digested with SalI and XbaI and ligated to the SalI-XbaI fragments that were isolated from pFB-MRP1-932, pFB-MRP1-1097 and pFB-MRP1-1138 to generate pFBDual-MRP1-932/MRP900-1143, pFBDual-MRP1-932/MRP1138-1531, pFBDual-MRP1-1097/MRP1061-1531 and pFBDual-MRP1-1138/MRP1138-1531. The dual-expression vector carrying cDNA fragments encoding the amino acids 204-653 and 932-1531 of MRP1 (pFBDual-MRP204-653/MRP932-1531) was constructed by one-step deletion: pFBDual-MRP204-
932/MRP932-1531 was digested with *Eco*I and *Xba*I, made blunt-ended using Klenow fragment, and then ligated after removal of the deleted fragment.

Construct pBSMRP-fc-ATG, which was described previously (17), was linearized with *Bam*HI, made blunt-ended using Klenow fragment and then digested with *Sac*I, generating one fragment encoding amino acids 1-281 of MRP1. The *Sac*-*Bam*HI* fragment was ligated to pFBDual vector that had a blunted end of *Hind*III*-site and a cohesive end of *Sac*-site to produce pFBDual MRP1-281. Translation of the inserted fragment terminated at a stop codon in the vector resulting in the addition of six amino acids, QLVEKY. pFBDual MRP1-281 was linearized with *Sma*I and *Kpn*I and ligated to the *Sall*-*Kpn*I fragment isolated from pFB-MRP281-1531 to produce pFBDual MRP1-281/MRP281-1531.

Constructs of pFB-MRPΔ(228-280) and pFB-MRP281-1531, which were described previously (18), were digested with *Sall* and *Sph*I to release *Sall*-*Sph*I fragments. These fragments were ligated to pFBDual-MRP1-932/MRP932-1531 that had been linearized with the same restriction enzymes to produce pFBDual-MRP1-932 (Δ228-280)/MRP932-1531 and pFBDual-MRP281-932/MRP932-1531.

**Viral Infection and Membrane Vesicle Preparation** – Generation of recombinant bacmids and baculoviruses and conditions used for viral infection were described previously (17). Membrane vesicles were prepared by nitrogen cavitation and sucrose gradient centrifugation, as described (6,10).

**Western blot analysis of MRP1 and LTC₄ transport assay** – Membrane vesicle proteins were electrophoresed on 5-15% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE), transferred to Immobilon-P membranes (Millipore, Bedford, MA) and
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probed with mAbs QCRL-1, MrPr1 or MrPm6, which we have demonstrated recognize the NH₂-proximal part of the protease sensitive region connecting NBD1 to MSD3 (amino acids 918-924), CL3 (amino acids 228-237) and the COOH-terminus of MrP1 (amino acids 1511-1520), respectively (26,27). Immunodetection was performed with the enhanced chemiluminescence Western blotting system from Amersham. LTC₄ transport was assayed in membrane vesicles at 23°C in the presence of 4 mM ATP or AMP using a rapid filtration technique, as described previously (6).

Photoaffinity Labeling of MrP1 with [³H] LTC₄ – Insect cell membrane vesicles (75 µg protein in 35 µl) were incubated with [³H] LTC₄ (0.25 µCi, 200 nM) at room temperature for 10 min, frozen in liquid nitrogen and UV-irradiated, as described (10). Radiolabeled vesicles were analyzed on a 5-15% (or as indicated) gel by SDS-PAGE prior to fluorography. [³H] LTC₄ photolabeling of tryptic fragments of native MrP1 in cell membranes from the multidrug resistant small cell lung cancer cell line, H69AR, was also performed as above after membrane vesicles (120 µg protein) were treated with trypsin at various trypsin/protein ratios (1:800-1:25)(22). For immunoprecipitation experiments, LTC₄-labeling was carried out using 150 µg of membrane proteins and 0.5 µCi [³H] LTC₄. After UV irradiation, membrane proteins were solubilized in PBS containing 1% CHAPS at 4°C for 3 h and insoluble fraction was removed by centrifugation. MAbs QCRL-1 and MrPm6 (1 µg each) were then added to the supernatant of solubilized membrane proteins and incubated at 4°C overnight. Antibody-associated proteins were absorbed with GammaBind Plus Sepharose for 1 h and the beads were washed 4 times with cold PBS. Immunocomplexes were then solubilized and analyzed as above.
RESULTS

Initial characterization of $[^3]H\text{LTC}_4$ labeling of MRP1 expressed in Sf21 cells

Initially, membrane vesicles from Sf21 insect cells expressing intact MRP1 were examined to establish that the protein could be specifically and efficiently photolabeled with $[^3]H\text{LTC}_4$, as we and others have shown previously using MRP1 enriched membrane vesicles from mammalian cells (6,28). A prominently labeled 170-kDa protein, consistent with the size predicted for core glycosylated MRP1 (1,17) was detectable by fluorography following SDS-PAGE of total membrane protein from cells infected with baculovirus encoding full length MRP1, with very little labeling of any other proteins between 70 and 200-kDa. No comparable labeling was observed in membranes prepared from control cells infected with baculovirus encoding β-glucuronidase (β-gus). In addition, unlabeled LTC$_4$ (6 µM) abolished $[^3]H\text{LTC}_4$ labeling of the 170 kDa protein confirming the specificity of the binding (Fig. 2B). Previously, we have demonstrated that LTC$_4$ selectively stimulates ATP binding by NBD1 of MRP1 but the influence of nucleotide on LTC$_4$ binding has not been determined (25). Consequently, photoaffinity cross-linking with $[^3]H\text{LTC}_4$ was performed using previously established ATP binding conditions in the absence and presence of 4 mM ATPγS, a poorly-hydrolyzable ATP analog. No effect of the nucleotide analog on LTC$_4$ binding was observed (Fig. 2C).

Association of the NH$_2$- and COOH-proximal halves of MRP1 is required to form a high affinity LTC$_4$ binding site
MRP1 fragments comprised of 1-932 and 932-1531 have been shown previously to be capable of associating to form a functional transporter (see Fig. 1 for predicted secondary structure of MRP1 and illustration of various MRP1 constructs) (17). To examine LTC_4 binding by these fragments, cells were infected with either a dual expression vector encoding both MRP1_{1-932} and MRP1_{932-1531} or with vectors encoding one or the other fragment. Membrane vesicles were then prepared from the infected cells and immunoblotted with mAbs QCRL-1 and MRPm6 to detect the NH_2- and COOH-halves of the protein, respectively (Fig. 3A). When membrane vesicles prepared from cells infected with the dual expression vector were photolabeled with [³H] LTC_4, strong labeling of a protein corresponding in size to the NH_2-proximal fragment and weaker but readily detectable labeling of a protein with the predicted size of the COOH-proximal fragment was observed (Fig. 3B). Confirmation of the identities of the proteins labeled with LTC_4 in cells co-expressing both halves of the protein was obtained by immunoprecipitation with a combination of two mAbs, QCRL-1 and MRPm6, recognizing the NH_2- and COOH-terminal halves of MRP1, respectively (Fig. 3C).

To investigate whether labeling of both half molecules was the consequence of autonomous binding by independent sites in each half of the protein, we examined membranes from cells expressing comparable levels of one or the other fragment. Although much reduced in intensity, weak photolabeling of the NH_2-terminal half of the protein could be detected in the absence of the COOH-terminal fragment (Fig. 3B). Labeling of the COOH-terminal half was also markedly decreased. However, because of the presence of endogenous proteins with similar electrophoretic mobility to this fragment that were very weakly labeled with [³H] LTC_4, we were unable to definitively
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determine whether or not a very low level of binding to the COOH-terminal half of the protein may also occur. Overall, these data demonstrate that high affinity binding of LTC₄ requires association of both halves of the protein and that the NH₂-proximal half alone may be capable of binding LTC₄ but with much lower affinity than the complete protein.

Co-expression of other combinations of fragments which fail to form a functional transporter, in some cases, displayed labeling on one or two fragments. However, when compared with the results obtained with the full-length protein or by co-expressing fragments MRP1₁-₉₃₂ with MRP1₉₃₂-₁₅₃₁, the intensity of labeling was in general much reduced (summarized in Table 1 or shown in Fig. 3D).

[^3H]-LTC₄ labeling of tryptic fragments of native MRP1

To confirm that sites in the NH₂- and COOH-proximal halves of the intact MRP1 expressed in mammalian cells were also labeled with LTC₄, photolabeling experiments were carried out with membranes prepared from drug resistant H69AR lung cancer cells from which MRP1 was originally cloned (1). We have shown that MRP1 constitutes up to 5% of membrane proteins in these cells (29). When H69AR membranes were subjected to mild trypsinolysis and[^3H] LTC₄ cross-linking followed by SDS-PAGE and fluorography, two fragments of 75-80 kDa and 55-60 kDa were labeled (Fig. 4). Immunoblotting using MRP1 specific antibodies with known epitopes indicated that these two fragments corresponded to regions between approximately amino acids 900-1531 (MSD3 and NBD2) and 250-900 (MSD2 and NBD1), the fragment with the larger apparent Mr resulting from glycosylation at position 1006 in MSD3 ((22) and data not
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shown). These results are consistent with the experiments using recombinant MRP1 expressed in insect cells as shown above. They confirm that [³H] LTC₄ labels sites in both halves of MRP1 and, that the NH₂-proximal fragment corresponding to MSD2 and NBD1 (aa 320-930) and part of the cytoplasmic loop connecting it to MSD1 (CL3) is more strongly labeled than the COOH-proximal 75-80 kDa tryptic fragment corresponding to MSD3 and NBD2.

Involvement of cytoplasmic loop 3 in the interaction of MRP1 with LTC₄

We have previously shown that removal of amino acids 1-229 or 1-281 of MRP1, or the region between them, eliminates LTC₄ transport activity (18). In contrast, co-expression of MRP1₁-₂₈₁ with either NH₂-terminally truncated protein or elimination of only amino acids 1-203 results in a transporter that retains 70-80% of the activity of full length MRP1 (18,19). Overall, these data indicate that the NH₂-proximal portion of CL3 between amino acids 203 and 281 contains a region that is essential for transport activity. To determine whether this region was also required for LTC₄ binding, an array of truncated fragments of MRP1 (see Fig. 1), either expressed alone or co-expressed with complementary fragments, were photocrosslinked with [³H] LTC₄. As shown in Fig. 5B, expression of MRP₁₂₈₁₋₁₅₃₁ alone resulted in only weak photolabeling. The extent of labeling of this fragment increased markedly when it was co-expressed with MRP₁₁-₂₈₁ (Fig. 5A and 5B). MRP₁₁-₂₈₁ was not labeled, either when co-expressed with MRP₁₂₈₁₋₁₅₃₁ or when expressed alone, consistent with data from trypsinolysis experiments suggesting that the NH₂-proximal LTC₄ cross-linking site is in MSD2 or NBD1. This was confirmed
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by the observation that a fragment comprised of amino acids 281-932 was clearly labeled by [³H] LTC₄ when expressed together with MRP₁⁻²⁸¹ plus MRP₁⁻⁹³₂⁻¹⁵₃₁ (Fig. 5C).

Consistent with the retention of transport activity by MRP₁⁻²⁰⁴⁻¹⁵₃₁, this fragment labeled almost as efficiently as full length MRP₁ (Fig. 5D). Topology studies predict that the region 1-192 comprises MSD₁ while the region 204-281 belongs to CL₃. As shown in Fig. 5E, internal deletion of significant portion of CL₃ (region 228-280) in full length MRP₁ reduced but did not completely eliminate LTC₄ labeling of the protein. When a comparable deletion was made in MRP₁⁻⁹³₂ and the fragment was co-expressed with MRP₁⁻¹⁵₃₁, it was apparent that the residual binding was primarily to the COOH-proximal half of the protein (Fig. 5F). A similar result was obtained when MRP₁⁻²⁸¹⁻⁹³₂ was co-expressed with MRP₁⁻¹⁵₃₁ (Fig. 5C). Thus, although the region of CL₃ between amino acids 203-281 is essential for transport activity and is directly or indirectly involved in binding of LTC₄ to the NH₂-proximal half of the protein, it is not an absolute requirement for binding to the COOH-proximal site.

[^³H] LTC₄ labeling site in the COOH-terminal half may be mainly localized within the region 1061-1295

To investigate what region of MRP₁⁻⁹³₂⁻¹⁵₃₁ was required for binding of LTC₄ to the COOH-proximal site, we examined labeling of a fragment, MRP₁⁻¹₂⁹₅, which is predicted to contain only approximately 50 cytoplasmic amino acids following the end of TM17. Although the transport activity of this truncated fragment lacking NBD₂ was completely eliminated, the extent of LTC₄ labeling was similar to that of intact MRP₁ (Fig. 6A), suggesting that NBD₂ and the cytoplasmic tail of MRP₁ is not required for
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LTC₄ binding and crosslinking. In addition, MRP₁⁽¹⁰⁶₁-₁⁵₃₁⁾ could also be labeled when it was co-expressed with MRP₁⁽¹⁻₁⁰⁹⁷⁾. Together, these two observations suggest that the COOH-proximal site is within the region 1061-1295 which includes TMs 14-17 (Fig. 6B).

**Effects of S-methyl GSH on [³H] LTC₄ labeling of dual halves of MRP1**

Because of the ability of GSH to stimulate transport of some MRP1 substrates (6-11), it has been proposed that the protein contains a region capable of specific but relatively low affinity binding of free GSH that may interact allosterically with another region capable of binding the ‘second’ substrate (3,6,10,13). If such a model is correct, the glutathione moiety of LTC₄ might be expected to interact with the GSH binding region of the protein and that it may be possible to compete for this interaction with free GSH or S-methyl GSH. Consequently, we investigated whether S-methyl GSH competed for binding to either or both half molecules. With concentrations of S-methyl GSH between 0.3-1 mM, a modest but detectable increase in LTC₄ labeling of the COOH-terminal half of the protein was observed. However, at 3-10 mM, S-methyl GSH concentration-dependently inhibited labeling, preferentially of the NH₂-terminal half of the protein (Fig. 7A). Densitometry of [³H] LTC₄ labeling of both halves of MRP1 in the absence and presence of S-methyl GSH is shown in Fig. 7B. The results suggest that determinants in the NH₂-terminal half of the protein may be primarily responsible for interaction with GSH and the glutathione moiety of LTC₄.
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**Differential accessibility of the N- and C-halves of MRP1 to [³H] LTC₄ after vanadate trapping**

In human P-gp, it has been demonstrated that ATP hydrolysis in the presence of vanadate, results in trapping of MgADP•Vi in NBD1 or NBD2 effectively locking the ATPase in a catalytic transition state. Under these conditions, the ability to bind substrates, as determined by photolabeling with [³H] azidopine and [¹²⁵I] iodoarylazidoprazosin, is much reduced (30). In the case of [¹²⁵I] iodoarylazidoprazosin, which labels two sites on the protein, the COOH-proximal site was preferentially affected (31). Unlike P-gp, in which Vi trapping of ADP occurs relatively efficiently at both NBDs, previous studies with MRP1 have revealed that Vi induced trapping of ADP occurs preferentially at NBD2 with very little trapping being detectable at NBD1. Consequently, we examined the effect of vanadate induced trapping of ADP on [³H] LTC₄ labeling of intact MRP1 as well as the NH₂- and COOH-half molecules.

Under conditions used for vanadate trapping, labeling of the intact protein, even in the presence of ATP alone, was clearly decreased and in the presence of vanadate and ATP, labeling was further reduced (Fig. 8A). When the dual-expressed halves of the protein were photolabeled by [³H] LTC₄ under the same conditions, a clear difference in the extent of inhibition was observed between the NH₂- and COOH-halves (Fig. 8B). ATP and the combination of ATP and vanadate, markedly reduced photolabeling of the NH₂-half but had very little effect on labeling of the COOH-half.

Consistent with the suggestion that the two NBDs of MRP1 fulfill different functional roles, our previous studies demonstrated that Walker A mutations, K684M and K1333M, in NBD1 and NBD2, respectively, had different effects on the ability of the
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protein to transport LTC₄ (25). Consequently, we used the Walker A mutants to determine whether one or both NBDs had to be capable of ATP hydrolysis to observe the decrease in LTC₄ binding that occurs under vanadate trapping conditions. As shown in Fig. 8C, either the double mutation K684M/K1333M or the single mutation K1333M abolished the ability of vanadate and ATP to inhibit LTC₄ binding, whereas mutation K684M did not. Thus, the data indicate that the reduced labeling of the NH₂-half of MRP1 with LTC₄ results exclusively from the trapping of ADP at NBD2 and is unaffected by nucleotide binding and/or hydrolysis at NBD1.
DISCUSSION

Previous photolabeling studies of MRP1 have utilized two iodinated azido derivatives, a quinoline based drug, N-(hydrocinchonidin-8'-yl)-4-azido-2-hydroxybenzamide (IACI) (32), and iodoaryl azidoRhodamine123 (IAARh123) (33). These high specific activity compounds have the advantage of labeling the protein relatively efficiently which has allowed the identification of small, cross-linked proteolytic fragments presumed to form part of the site or sites to which IACI and IAARh123 bind. Both compounds preferentially label fragments that contain either TM helices 10 and 11, or 16 and 17 (34). Consistent with the labeling of a fragment containing TM17, we have shown recently that mutations of both conserved and non-conserved amino acids in this helix markedly affect drug resistance and the ability to transport the well characterized MRP1 substrate, E₂₁βG (35). However, these mutations have little effect on the transport of LTC₄, indicating that different amino acid residues may be more important for the binding of this compound.

Unlike many MRP1 substrates, IACI and IAARh123 are not conjugated organic anions. The latter is actually cationic at physiological pH and both compounds bind to P-gp (36,37). The affinity with which they bind to MRP1 is also not known and in contrast to some other compounds that have been shown to be transported by MRP1, their interaction with the protein is not stimulated by GSH. However, photolabeling of MRP1 by both IACI and IAARh123 is reduced in the presence of a molar of excess LTC₄, suggesting that the sites with which they interact overlap at least partially with those involved in binding the conjugated leukotriene. Whether the two sites labeled by IACI and IAARh123 are indicative of conformationally distinct forms of a single binding...
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pocket or two functionally linked but physically separate sites is not known. Other regions of the protein required for substrate binding have not been identified. Very recently, somewhat different photolabeling results have been reported using an azido derivative of the polyhydroxylated sterol acetate, Agosterol-A (AG-A) (38). The parent compound has been shown to competitively inhibit LTC₄ transport in a GSH dependent manner and photoaffinity labeling of MRP1 by the azido derivative is also dependent on the presence of GSH (38). However, it is not known if AG-A can be transported by MRP1. In contrast, to the results obtained with IACI and IAARh123, specific labeling by AG-A is restricted to a site between amino acids 932-1531. Whether the site involves TM helices 16 and 17 has not been established.

LTC₄ contains both conjugated and unconjugated double bonds and is intrinsically photolabile (39). It has been used for photolabeling of intact MRP1 but the regions of the protein where cross-linking occurs, or that are required for high affinity binding have not been identified. This is attributable in large part to practical limitations imposed by the low efficiency with which cross-linking occurs and the low specific activity of [³H] LTC₄ (6,10,28,39,40). In this study, we have extended the utility of [³H] LTC₄ as a photolabeling agent by using baculovirus dual-expression vectors that permit co-expression of close to stoichiometric amounts of various MRP1 fragments in Sf21 cells. We demonstrated previously that LTC₄ transport activity can be reconstituted very efficiently by dual-expression of MRP1₁₋₉₃₂ and MRP1₉₃₂₋₁₅₃₁ and that the kinetics of transport are very similar to those of the intact protein (17,25). Using the same two fragments, our data clearly demonstrate that labeling of the protein by LTC₄ occurs at sites in both halves of the molecule. We were also able to confirm that sites in the NH₂-
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and COOH-halves of the intact fully glycosylated protein were labeled by taking advantage of tryptic mapping analyses and the exceptionally high levels of MRP1 in the human lung cancer cell line H69AR. These studies have shown that MRP1 has at least two trypsin hypersensitive sites, one located in CL3 and the other in the region linking NBD1 to MSD3 (22,26). At low concentrations of trypsin, labeling was confined to two fragments, one containing MSD2 and NBD1 and the other MSD3 and NBD2. As observed when both MRP1 half molecules were expressed in insect cells, LTC₄ preferentially labeled the NH₂-proximal half of the native fully glycosylated protein. This pattern of labeling was not found when each half molecule was expressed individually. Labeling of MRP1₁-₉₃₂ was detectable but extremely weak and labeling of MRP1₉₃₂-₁₅₃₁ was impossible to distinguish from very weak labeling of endogenous proteins. Overall, these data indicate that labeling of both halves of the protein appears not to be attributable to the presence of two autonomous high affinity sites, each formed exclusively by elements in one or the other half of the protein. Photolabeling studies carried out with other combinations of MRP1 fragments, as well as various truncated proteins allow us to place some limits on the sites of photolabeling. These studies suggest that the labeling site(s) in the COOH-half of the protein lie(s) between amino acids 1061-1295 which includes TM helices 14-17 and that NBD2 and the cytoplasmic tail of the protein are not required for high affinity binding of LTC₄.

The fact that weak labeling of MRP₁₁-₉₃₂ with LTC₄ could be detected when the fragment was expressed alone suggests that a site capable of autonomous low affinity binding of LTC₄ exists in the NH₂-proximal half of the protein. What part of the LTC₄ molecule is important for the interaction remains to be established. However, we were
recently able to detect a low but significant rate of ATP-dependent, MRP1-mediated GSH transport in the absence of a second substrate, consistent with the existence of a low affinity site capable of binding GSH alone (10,41). In support of this possibility, GSH and S-methyl GSH also decreased [3H] LTC₄-labeling of intact MRP1 in a concentration-dependent manner in the millimolar range, presumably as a result of competition for protein interactions with the GSH moiety of LTC₄. Thus the binding site for LTC₄ may encompass a low affinity site to which GSH can bind independently (10). In the present study, using the dual-expressed halves of MRP1, we found that at all concentrations, S-methyl GSH inhibited LTC₄ labeling of MRP1₁-₉₃₂ but weakly stimulated labeling of the site in MRP1₉₃₂-₁₅₃₁ at low concentrations and inhibited only at higher concentrations. The observation that S-methyl GSH competes preferentially for LTC₄ labeling of the MRP₁-₉₃₂ supports the possibility that high affinity binding to the site in this region involves interaction of the glutathione moiety of the conjugate with a site that is capable of binding GSH and S-methyl GSH with low affinity.

We have shown previously that a truncated protein lacking MSD1 and most of CL3 (MRP1₂₈₁-₁₅₃₁), or a protein in which a region of CL3 between amino acid 227 and 280 had been deleted, was unable to transport LTC₄ (18). In contrast, a protein lacking only MSD1 (MRP1₂₀₄-₁₅₃₁) has been shown to retain significant levels of transport activity (19). Our results confirm that, as expected, MRP1₂₀₄-₁₅₃₁ is competent to bind LTC₄. More importantly, they demonstrate that MRP1₂₈₁-₁₅₃₁ or MRP1 containing an internal deletion of amino acids 204-281 bind LTC₄ poorly. Experiments with dual expressed half molecules indicate that deletion of half of CL3 eliminates binding to the NH₂-proximal site and reduces binding to the COOH-proximal site. In addition, the NH₂-
and COOH-proximal high affinity binding can be restored by dual-expression of MRP1\(_{1-281}\) and MRP1\(_{281-1531}\), or the triple expression of MRP1\(_{1-281}\), MRP1\(_{281-932}\) and MRP1\(_{932-1531}\). Thus all or part of the region of CL3 from amino acid 204-281 is required for high affinity binding of LTC\(_4\), as well as for labeling of the NH\(_2\)-, and to a lesser extent, the COOH-half of the protein. However, MSD1 and CL3 are not directly photolabeled by LTC\(_4\). This limits the NH\(_2\)-proximal site of labeling to MSD2 or NBD1.

During photolabeling studies of MRP1 using AG-A, Ren et al (38) observed that GSH dependent photolabeling of the COOH-proximal half of MRP1 required CL3 and that a double mutation involving tryptophan 261 and lysine 267 within this loop decreased labeling efficiency approximately 3-fold (38). It was concluded that CL3 is a GSH binding site and that the site labeled by AG-A in the COOH-proximal half of MRP1 represents a binding site for the hydrophobic component of conjugated substrates transported by MRP1. Although this remains a possibility, other data indicate that it may be premature to draw this conclusion. In contrast to the results obtained with AG-A, LTC\(_4\) and the unconjugated IACI and IAARh123 all photolabel sites in both halves of the protein. This suggests that the protein either has two sites for binding these compounds, or a single site that can exist in two different conformations and which involves elements in both halves of the protein. Furthermore, although GSH is not required for high affinity binding of IACI and IAARh123, CL3 from amino acids 204 to 281 is required for the photolabeling of both sites by these compounds (34). Thus CL3 appears essential for high affinity binding of substrates that is both GSH dependent and independent, suggesting that it may contain a region that is critical for the correct folding of the protein. Consequently, confirmation that CL3 forms a GSH binding pocket on the protein will
require corroboration by direct labeling of this region of the protein with appropriate GSH analogues.

The stimulation of LTC₄ binding to MRP1₉₃₂₋₁₅₃₁ at low concentrations of S-methyl GSH may be related to the observation we have made previously that low concentrations of the estrogen sulfates stimulate LTC₄ transport and binding by intact MRP1, while they compete for both transport and binding at high concentrations (10). This behavior is consistent with the existence of positive co-operativity between two allosterically linked substrate binding sites, as proposed for both P-gp and the homodimeric bacterial drug transporter LmrA (42-44). In the case of LmrA, it has been suggested that each subunit of the homodimer cycles alternately, between a low and high affinity binding state. With respect to MRP1, our data suggest that the effect of GSH binding is asymmetric and that low concentrations preferentially enhance labeling of site(s) in the COOH-half of the protein, possibly via an allosteric effect, while directly competing with LTC₄ for binding to a site in the NH₂-proximal half. Differential interaction of the NH₂- and COOH-halves of MRP1 with LTC₄ is further evidenced by the results of vanadate trapping experiments. Previous studies with P-gp have demonstrated that vanadate induced trapping of ADP at either of the NBDs of the protein, markedly diminishes the affinity with which several substrates bind (31,45,46). It has been suggested that this is attributable to ‘locking’ the protein in a transition state involved in the translocation and release of substrate. A similar effect of ADP trapping on substrate binding has been observed with LmrA and a model has been proposed in which the alternate trapping of ADP at each NBD of the homodimer eliminates substrate
binding by the subunit where trapping has occurred (43). The results we have obtained with MRP1 suggest a somewhat different model.

Labeling of both dual-expressed halves of MRP1 occurred in the absence of ATP and was unaffected by the presence of the poorly hydrolysable ATP analog, ATPγS. Under the conditions used for these experiments, we have shown previously that NBD1 of MRP1 binds azido-ATP with high affinity while binding of azido-ATP by NBD2 is barely detectable. These results suggest that loading of NBD1 with ATP has no effect on the interaction of LTC₄ with either of the labeled sites. In contrast to the lack of effect of ATPγS on labeling with LTC₄, we found that the labeling of intact MRP1, like P-gp and LmrA, was attenuated in the presence of ATP under conditions that permitted hydrolysis of the nucleotide triphosphate and that the attenuation was enhanced by the presence of vanadate. However, unlike P-gp, experiments with the dual expressed halves of MRP1 revealed that vanadate trapping markedly diminished labeling of the NH₂-half of MRP1 with little effect on labeling of the COOH-half. Under the conditions used for these experiments, we have shown that the trapping of ADP in the presence of vanadate occurs primarily at NBD2 (25), suggesting that occupancy of this NBD by a Mg•ADP•Vi complex decreases interaction of LTC₄ with the site to which cross-linking occurs in the NH₂-half of the protein. This possibility was supported by labeling experiments carried out with dual-expressed mutant half molecules in which we inactivated NBD1 or NBD2 by mutation of conserved lysine residues known to be essential for nucleotide hydrolysis. In P-gp, such mutations in either NBD completely eliminate transport activity (47). In contrast, we have shown that mutation of K684 and K1333 in NBD1 and NBD2 of MRP1, respectively, have different effects on LTC₄ transport (25). Mutation of NBD1
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reduces transport by 60-70% while mutation of NBD2 essentially eliminates all activity. When labeling experiments were carried out under trapping conditions using dual-expressed MRP1K684M₁₋₉₃₂ and wild type MRP1₉₃₂₋₁₅₃₁, LTC₄ labeling was attenuated to the same extent observed with the dual-expressed wild type half molecules. In contrast, no attenuation was observed when similar experiments were carried out with the MRP1K1333M₉₃₂₋₁₅₃₁ half molecule. These experiments confirm that the structural changes in the protein that result in the attenuation of LTC₄ labeling of the NH₂-proximal half of MRP1 are attributable to trapping of an ADP vanadate complex at NBD2 and that hydrolysis of ATP by NBD1 is not required. By extrapolation to the model proposed for LmrA, this would suggest that trapping of ADP in the NBD of one monomer of the homodimer may reduce substrate binding to the other, rather than the same, subunit. Combined with previous LTC₄ transport data showing that MRP1 retains 30-40% of its transport activity when NBD1 is inactivated, our observations support the suggestion that ATP binding and possibly hydrolysis by NBD1 may serve a regulatory function, rather than being directly involved in the transport process (25,48).
LTC₄-binding by MRP1

### Table 1

Summary of $[^3]$H LTC₄ labeling of membrane vesicles co-expressing two or three fragments of MRP1

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* These two fragments were co-expressed with MRP1₁-281.
LTC₄-binding by MRP1

FIGURE LEGEND

Fig. 1. Illustration of various constructs encoding one or two truncated MRP1 fragments. Approximate locations of predicted individual transmembrane helices are indicated (TM 1-17) as is their proposed organization into three membrane spanning domains (MSDs) (22). Linear representations of the various MRP1 fragments expressed in Sf21 cells are aligned below the schematic of topology of MRP1.

Fig. 2. Initial characterization of [³H] LTC₄ photolabeling of insect cell membrane vesicles expressing MRP1. Membrane vesicles were prepared from sf21 cells infected with baculovirus encoding MRP1 or β-glucuronidase (β-gus). A, membrane proteins (75 µg) were incubated with [³H] LTC₄ (200 nM, 0.25 µCi) at 22°C for 10 min and frozen in liquid nitrogen. Samples were alternately irradiated for 30 sec at 312 nm, followed by snap-freezing in liquid nitrogen, for a total of 10 min. Radiolabeled vesicles were solubilized in Laemmli’s buffer and analyzed on 5-15% gradient gel by SDS-PAGE and fluorography. The sizes of protein standards are indicated in kilodaltons. B, [³H] LTC₄ labeling was performed in the presence of 6 µM unlabeled LTC₄ or vehicle (ethanol) alone (control). C, [³H] LTC₄ labeling was performed in the absence (control) and presence of 4 mM ATPγS.

Fig. 3. [³H] LTC₄ photolabeling of membrane vesicles expressing a single half or both halves of MRP1. Membrane vesicles were prepared from sf21 cells infected with baculovirus encoding either the NH₂-proximal half-molecule (MRP1₁₋₉₃₂) or the COOH-proximal half-molecule (MRP1₉₃₂₋₁₅₃₁) of MRP1 or both halves of MRP1 (MRP1₁₋₁₅₃₁).
LTC₄-binding by MRP1

₉₃₂/MRP₁₉₃₂-₁₅₃₁ or β-glucuronidase (β-gus). A, Membrane proteins (4 µg) were subjected to 5-15% gradient SDS-PAGE and transferred to Immobilon-P membrane prior to immunoblotting with mAbs QCRL1 (left panel) and MRPm6 (right panel). B, [³H] LTC₄ photoaffinity labeling of membrane vesicles from cell infected with dual and single expression vectors was carried out as described in Fig. 2 and in "Experimental Procedures". The positions of labeled proteins with sizes corresponding to MRP₁₁₉₃₂ and MRP₁₉₃₂-₁₅₃₁ are indicated in the figure. Weak labeling of an endogenous protein with a mobility slightly lower than MRP₁₉₃₂-₁₅₃₁ was also detected. C, [³H] LTC₄ labeled membrane vesicles were immuno-precipitated with a combination of MRP₁-specific mAbs QCRL-1 and MRPm6 prior to being subjected to SDS-PAGE and fluorography. D, Membrane vesicles expressing MRP₁₁₂₀₄-₆₅₃/MRP₁₉₃₂-₁₅₃₁ were photolabeled with [³H] LTC₄ as described above. Labeling of MRP₁₉₃₂-₁₅₃₁ was detectable but no labeling MRP₁₁₂₀₄-₆₅₃ was observed.

Fig. 4. [³H] LTC₄ photolabeling of tryptic fragments of native MRP1 in H69AR lung tumor cell membranes. Membrane vesicles (120 µg protein) prepared from multidrug resistant H69AR lung tumor cells were treated with trypsin at the indicated trypsin/protein ratios at 37°C for 30 min. Digested membrane proteins were then incubated with [³H] LTC₄ (0.75 µCi) and cross-linked as described in Fig. 2. Radiolabeled proteins were analyzed on a 3-12% gradient gel by SDS-PAGE followed by fluorography. The largest labeled fragment, C1, corresponds to approximately amino acids 900-1531, while the more intensely labeled fragment N# corresponds approximately to amino acids 250-930.
Fig. 5. Requirement of cytoplasmic loop 3 for efficient labeling of MRP1 with \(^3\)H LTC\(_4\). Experimental conditions and procedures for photoaffinity labeling of membrane vesicles with \(^3\)H LTC\(_4\) were as described in the legend to Fig. 2. A, Photolabeled membrane vesicles from cells infected with vectors encoding \(\beta\)-glucuronidase (\(\beta\)-gus) (negative control), MRP1\(_{1-932}\) co-expressed with MRP1\(_{932-1531}\) (positive control), and MRP1\(_{1-281}\) co-expressed with MRP1\(_{281-1531}\). As observed previously, both MRP1\(_{1-932}\) and MRP1\(_{932-1531}\) were photolabeled with \(^3\)H LTC\(_4\) and the intensity of labeling of the NH\(_2\)-proximal fragment was several fold higher than that of the COOH-proximal fragment. Efficient labeling of MRP1\(_{281-1531}\) also occurred but no labeling of MRP1\(_{1-281}\) was detected. B, Comparison of the efficiency of labeling of MRP1\(_{281-1531}\) when expressed alone or together with MRP1\(_{1-281}\). When expressed alone, labeling of MRP1\(_{281-1531}\) was detectable but weak (left lane) and was enhanced several fold by co-expression with MRP1\(_{1-281}\) (right lane). C, Photolabeling of membranes containing MRP1\(_{281-932}\) co-expressed with MRP1\(_{932-1531}\) and membranes containing MRP1\(_{1-281}\) plus MRP1\(_{281-932}\) and MRP1\(_{932-1531}\). MRP1\(_{281-932}\) was strongly photolabeled by \(^3\)H LTC\(_4\) but only when co-expressed with both MRP1\(_{1-281}\) and MRP1\(_{932-1531}\). D, Photolabeling of membranes containing wild-type MRP1 or MRP1\(_{204-1531}\). Both wild type and NH\(_2\)-truncated proteins were equally well labeled by \(^3\)H LTC\(_4\). E, Effect of an internal deletion in CL3 between amino acids 228-280 on photolabeling of MRP1. The CL3 deletion markedly decreased but did not completely abolish photolabeling with \(^3\)H LTC\(_4\). F, Effect of the CL3 deletion of labeling of co-expressed MRP1\(_{1-932}\) and MRP1\(_{932-1531}\). Deletion of amino
LTC₄-binding by MRP1

acids 228 to 280 essentially abolished photolabeling of MRP1₁₋₉₃₂ and decreased but did not eliminate labeling of MRP1₀₉₃₂₋₁₅₃₁.

Fig. 6. [³H] LTC₄ photolabeling of membranes containing MRP1₁₋₁₂₉₅ or MRP1₁₋₁₀₉₇ co-expressed with MRP1₁₀₆₁₋₁₅₃₁. Experimental conditions were as described in the legend to Fig. 2. A, Deletion of the cytoplasmic COOH-terminal region of MRP1 from amino acid 1296 to 1531 which includes NBD2 had little effect on photolabeling of the truncated protein (right lane) compared with full length MRP1 (left lane). B, Photolabeling of membranes containing co-expressed MRP1₁₋₁₀₉₇ and MRP1₁₀₆₁₋₁₅₃₁ resulted in relatively strong labeling of MRP1₁₀₆₁₋₁₅₃₁ with no detectable labeling of MRP1₁₋₁₀₉₇ (right lane). Labeling of co-expressed MRP1₁₋₂₈₁ and MRP₁₂₈₁₋₁₅₃₁ was used as a positive control (left lane). *, photolabeling of an endogenous protein.

Fig. 7. Effect of S-methyl GSH on [³H] LTC₄ photolabeling of co-expressed MRP1₁₋₉₃₂ and MRP1₀₉₃₂₋₁₅₃₁. A, Membrane vesicles containing co-expressed MRP1₁₋₉₃₂ and MRP1₀₉₃₂₋₁₅₃₁ were incubated with [³H] LTC₄ (200 nM, 0.25 µCi) alone or in the presence of various concentrations (0.3-10 mM) of S-methyl GSH at 22°C for 10 min prior to photolabeling, as described in the legend to Fig. 2 and analysed by SDS-PAGE. B, Relative densities of the photaffinity labeled products shown in panel A were determined by densitometry. The extent of [³H] LTC₄ photolabeling in the absence of S-methyl GSH was arbitrarily set to a value of 100.
Fig. 8. Effects of vanadate trapping of ADP on $[^3\text{H}]\text{LTC}_4$ photolabeling of membrane vesicles containing wild type MRP1, co-expressed wild type MRP1$_{1-932}$ and MRP1$_{932-1531}$, and mutant forms of MRP1$_{1-932}$ and MRP1$_{932-1531}$ containing NBDs incapable of hydrolyzing ATP. Membrane proteins were incubated at 22°C for 10 min with ATP (1 mM) and vanadate (400 µM), alone or in combination, prior to addition of $[^3\text{H}]\text{LTC}_4$ (200 nM) prior to photolabeling as described in the legend to Fig. 2. A, $[^3\text{H}]\text{LTC}_4$ labeling of full-length MRP1 in the presence and absence of vanadate and ATP. B, $[^3\text{H}]\text{LTC}_4$ labeling of co-expressed wild type MRP1$_{1-932}$ and MRP1$_{932-1531}$ in the presence and absence of vanadate and ATP. C, Effect of vanadate trapping on $[^3\text{H}]\text{LTC}_4$ labeling of co-expressed expressed mutant forms of MRP1$_{1-932}$ and MRP1$_{932-1531}$ in which NBD1 or NBD2 had been in activated by mutation of essential Walker A mutations (K684M in NBD1 and K1333M in NBD2).
References


LTC₄-binding by MRP1


LTC₄-binding by MRP1


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**Fig. 1**
Fig 2
Fig 3
Fig 4
Fig 5
Fig 6
Fig 7

A

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N-half

C-half

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Relative density

- N-half
- C-half

Fig 7
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MRP1

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N-half
C-half

C

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N-half
C-half

Fig 8
Characterization of binding of Leukotriene C4 by human multidrug resistance protein 1: Evidence of differential interactions with NH2- and COOH-proximal halves of the protein
Yue-Ming Qian, Wei Qiu, Mian Gao, Christopher J. Westlake, Susan P.C. Cole and Roger G. Deeley

J. Biol. Chem. published online August 15, 2001

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