

Subcellular Localization and Activity of Multidrug Resistance Proteins

Asha Rajagopal and Sanford M. Simon*

Laboratory of Cellular Biophysics, The Rockefeller University, New York, New York 10021

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The multidrug resistance (MDR) phenotype is associated with the overexpression of members of the ATP-binding cassette family of proteins. These MDR transporters are expressed at the plasma membrane, where they are thought to reduce the cellular accumulation of toxins over time. Our data demonstrate that members of this family are also expressed in subcellular compartments where they actively sequester drugs away from their cellular targets. The multidrug resistance protein 1 (MRP1), P-glycoprotein, and the breast cancer resistance protein are each present in a perinuclear region positive for lysosomal markers. Fluorescence-activated cell sorting analysis suggests that these three drug transporters do little to reduce the cellular accumulation of the anthracycline doxorubicin. However, whereas doxorubicin enters cells expressing MDR transporters, this drug is sequestered away from the nucleus, its subcellular target, in vesicles expressing each of the three drug resistance proteins. Using a cell-impermeable inhibitor of MRP1 activity, we demonstrate that MRP1 activity on intracellular vesicles is sufficient to confer a drug resistance phenotype, whereas disruption of lysosomal pH is not. Intracellular localization and activity for MRP1 and other members of the MDR transporter family may suggest different strategies for chemotherapeutic regimens in a clinical setting.

INTRODUCTION

Resistance to chemotherapeutic regimens can be the result of diverse cellular mechanisms, ranging from the reduction of intracellular drug accumulation to the reduction of drug sensitivity. One cellular response commonly associated with drug resistance is the overexpression of drug transporters that belong to the ATP-binding cassette family of proteins. P-glycoprotein (Pgp), multidrug resistance protein 1 (MRP1), and breast cancer resistance protein (BCRP), for example, have each been cloned from a drug-resistant cancer line and are thought to mediate drug efflux from the cell at the plasma membrane (Kartner *et al.*, 1983; Cole *et al.*, 1992; Doyle *et al.*, 1998; Maliepaard *et al.*, 2001). Although some reports have suggested subcellular localizations for these proteins (Marquardt *et al.*, 1990; Shapiro *et al.*, 1998; Van Luyn *et al.*, 1998; Meschini *et al.*, 2000), these drug transporters are generally considered to be cell surface localized and to mediate drug resistance by lowering total intracellular drug concentrations (Stride *et al.*, 1999). In some instances, however, the expression of these drug efflux pumps has no effect on the total cellular accumulation of multidrug resistance substrates. The net intracellular accu-

mulation of the anthracycline doxorubicin, for example, is unaltered by the expression of either MRP1 or Pgp, even if the cellular toxicity of the drug is mitigated by the presence of these transport proteins (Rajagopal *et al.*, 2002). Indeed, MRP1 was first cloned from a doxorubicin-resistant lung cancer line that accumulated as much of the drug as its sensitive parental line (Cole *et al.*, 1991; Cole *et al.*, 1992). The method by which these plasma membrane transporters confer resistance to doxorubicin is still not known.

We have previously studied the *in vivo* activity of Pgp, MRP1, and BCRP, by transiently transfecting cells with plasmids encoding enhanced cyan fluorescent protein (ECFP) conjugates of each protein and then quantifying drug transport activities as a function of cyan fluorescence (Chen and Simon, 2000; Rajagopal *et al.*, 2002). Most drug transport studies are done in cell lines that have been selected for stable expression of one of these drug-resistance proteins. The alternative approach of transient transfection offered a number of advantages. We could study the short-term effects of protein expression and avoid the effects of long-term drug selection, a process that is known to up-regulate other proteins involved in cellular detoxification (Chen *et al.*, 1999) and that could thereby lead to the creation of many different cell lines. In addition, transient transfection creates populations of cells that express the protein of interest to varying degrees, from very high levels to nondetectable ones, and we could thereby study drug transport as a function of protein level. Because the transfection procedure leaves a

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* Corresponding author. E-mail address: simon@mail.rockefeller.edu.

cell population that does not express the protein at all, we could easily ascertain the phenotypes conferred by the protein of interest, because nonexpressing cells were present alongside cells expressing the protein and exposed to identical culture conditions.

The ECFP tag on each protein permitted easy identification of those cells that expressed a drug transporter, and we used fluorescence microscopy to monitor the localization patterns of each protein in living cells. Moreover, because some MDR substrates are naturally fluorescent, we could also monitor the cellular accumulation and subcellular distribution of MDR drugs as a function of drug transporter expression. Finally, when we transiently expressed fluorescent conjugates of MRP1 (Rajagopal *et al.*, 2002), Pgp (Chen and Simon, 2000), and BCRP (our unpublished data) in cells, we saw protein-dependent exclusion of many MDR substrates, both in fluorescence-activated cell sorting and under fluorescent microscopy. Moreover, when MRP1-cyan fluorescent protein (CFP) was examined for its subcellular distribution, we found that under confocal microscopy, the protein localized primarily to the plasma membrane and to a juxtannuclear region (Rajagopal *et al.*, 2002), a distribution pattern previously reported for cells expressing MRP1 (Chang *et al.*, 1997). We therefore have reason to believe that ECFP conjugates of drug transporters are functional and have activity profiles analogous to their wild-type counterparts.

Transiently transfected cells were used to examine the patterns of doxorubicin accumulation in cells expressing MDR transporters. Our data suggests that Pgp, MRP1, and BCRP each promote doxorubicin resistance by actively sequestering the drug from the nucleus, its cellular target, into intracellular vesicles. We find that Pgp, MRP1, and BCRP each localize to intracellular membranes that are frequently positive for lysosomal markers such as cathepsin D and synaptotagmin VII. Moreover, our data indicate that these proteins promote doxorubicin accumulation into these lysosomal vesicles. Finally, by inhibiting drug transport selectively at the plasma membrane, we are able to show that this intracellular activity alone is sufficient to confer a multidrug-resistant phenotype. Thus, MRP1, Pgp, and BCRP, all previously thought to be active primarily at the plasma membrane, also act on internal organelles to sequester an MDR substrate from the nucleus.

MATERIALS AND METHODS

Tissue Culture and Transfection

HeLa cells (ATCC CCL-2) were cultured in DMEM with 4.5g/liter glucose and L-glutamine (Cellgro, Herndon, VA) in 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO). Transfections were performed using FuGENE 6 according to the manufacturer's recommendations (Roche Diagnostics, Brussels, Belgium).

DNA Constructs

The plasmid encoding synaptotagminVII-ECFP was a gift from Norma Andrews (Yale University, New Haven, CT) (Martinez *et al.*, 2000). The plasmid encoding ECFP-cystic fibrosis transmembrane conductance regulator was a gift of David Gadsby (Rockefeller University, New York, NY). The pEYFP-endoplasmic reticulum (ER) and the pEYFP-Golgi vectors were purchased from BD Biosciences Clontech (Palo Alto, CA). The construction of both the

pMDR1-ECFP and the pMRP1-ECFP plasmids has been described previously (Chen and Simon, 2000; Rajagopal *et al.*, 2002). The BCRP cDNA was a generous gift of Doug Ross (University of Maryland, Baltimore, MD) (Doyle *et al.*, 1998); it was subcloned into the EGFPc1 plasmid (BD Biosciences Clontech) between the *EcoRI* and *XhoI* sites. The pECFP-BCRP plasmid was made by replacing the EGFP coding sequence with the sequence of ECFP.

Cross-Linking

BM[P_{EO}]₄ stock was prepared at 28 mM in water and used at a hundredfold dilution in Hanks' buffered saline solution with 10 mM HEPES, pH 7.3 (HHBSS). Cells were exposed to the reagent at 37°C for 10 min. The cross-linking reaction was quenched with 50 mM L-cysteine in HHBSS (quenching buffer) for 5 min at room temperature. Cells were subsequently washed with HHBSS and then assayed for MRP1 activity. Cross-linking with bismaleimido-hexane (BMH) was performed with a 90 mM dimethyl sulfoxide stock used at 1000×. Once again, cells were exposed to BMH for 10 min at 37°C and then resuspended in quenching buffer for 5 min at room temperature, washed in HHBSS, and assayed for MRP1 activity.

Activity Assays and Fluorescence Quantification

MRP1, BCRP, and Pgp activity assays were conducted similarly. Twenty-four to 48 h after transfection, cells expressing fluorescent conjugates of the transporter of interest were washed in HHBSS, incubated in either 50 nM tetramethyl rhodamine ester (TMRE) (Molecular Probes, Eugene, OR) or 10 μM doxorubicin (Calbiochem, La Jolla, CA) for 15 min in a 5% pCO₂ incubator, and observed by fluorescent microscopy. Doxorubicin was washed out before observation; TMRE was not.

Fluorescence Microscopy

Fluorescent images were collected on an IX-70 Olympus microscope, with a 1.4 numerical aperture oil-immersion objective and an ORCA 2 or ORCA ER charge-coupled device-cooled camera (Hamamatsu Photonics, Hamamatsu City, Japan), as described previously (Rajagopal *et al.*, 2002). Deconvolution was performed using a DeltaVision deconvolution microscope, by using a 1.4 numerical aperture oil-immersion, 60× objective. Fluorescent images were analyzed using MetaMorph software (Universal Imaging, Downingtown, PA), and correlation coefficients were acquired directly from this software. To quantify fluorescence intensities on a cell-by-cell basis, bright field images were used to acquire the cell boundary, and MetaMorph software was used to calculate the average intensity within the cell boundary for each fluorophore. The mean of these averages was calculated, along with the SE.

Immunocytochemistry

For fixation, cells were washed with chilled phosphate-buffered saline (PBS), permeabilized with ice-cold methanol for 10 min at 20°C, and rinsed twice with cold PBS. To detect MRP1 or cathepsin D, fixed cells were then incubated with the anti-MRP1 antibody MRP1r1 at 1:200 or with the anti-cathepsin D antibody cathepsin D (antibody-2) (Oncogene Research Products, San Diego, CA) at 1:20 for 1 h. Cells were subsequently washed and incubated for 1 h in anti-rat Alexa594 (Molecular Probes) at 1:1000 for MRP1 or anti-rabbit fluorescein for cathepsin D imaging.

Western Blot Analysis

After treatment with cross-linker or PBS, MRP1-ECFP-transfected cells were dissociated with Cell Stripper (Cellgro), solubilized with 1% Triton X-100, spun on a low-speed centrifuge to remove nuclear debris, and then resolved on a 4–20% gradient gel, by using SDS-PAGE. After electrotransfer onto a membrane (Amersham Biosciences, Piscataway, NJ) by using a semidry electro-blotter, pro-

teins were immunoblotted with the MRP1 anti-MRP1 rat monoclonal antibody (Alexis Biochemicals, San Diego, CA) and an alkaline phosphatase-conjugated anti-rat IgG antibody (Sigma-Aldrich).

Fluorescent Labeling of Subcellular Compartments

To label the recycling endosome, cells were incubated with cy3-transferrin, as described previously (Lampson *et al.*, 2001). To label the lysosomes, cells were transfected with synaptotagmin VII-ECFP or were probed with an anti-cathepsin D antibody. Additionally, Texas Red dextrans were chased into the lysosome as follows: cells were incubated in dextrans for 1 h at 37°C, washed in culture medium, washed again 1 h later, and then allowed to remain in culture medium for 8–12 h as described previously (Jaiswal *et al.*, 2002).

RESULTS

Doxorubicin Accumulates in Regions Positive for MRP1

The expression of Pgp, MRP1, or BCRP reduces cellular sensitivity to doxorubicin (Ueda *et al.*, 1987; Cole *et al.*, 1994; Doyle *et al.*, 1998). Cells transiently transfected with each one of these MDR-ECFP fusion proteins were exposed to doxorubicin, and then the fluorescence levels of doxorubicin and ECFP were quantified on a cell-by-cell basis. As reported previously, expression of neither MRP1 nor Pgp had any effect on total doxorubicin accumulation by fluorescence-activated cell sorting analysis (Rajagopal *et al.*, 2002); cells expressing either MDR-ECFP fusion protein accumulated as much of the drug as cells that had not been transfected. Expression of BCRP-ECFP only marginally decreased total doxorubicin accumulation (our unpublished data). However, in all cases, the expression of a multidrug resistance transporter did change the pattern of drug distribution. In the absence of a drug transporter, cells accumulated doxorubicin in the nucleus, but when cells expressed either MRP1, Pgp, or BCRP, they showed substantially decreased nuclear drug accumulation. Expression of MRP1-ECFP (Figure 1A), for example, resulted in the redistribution of doxorubicin from the nucleus into a region that is peripheral to the nucleus (Figure 1B). This perinuclear region was positive for both MRP1-ECFP and doxorubicin, as seen when the fluorescent images of MRP1-ECFP (green) and doxorubicin (red) are merged, and yellow is taken as an indication of colocalization (Figure 1C). A deconvolved image of a transfected cell reveals that these intracellular regions of MRP1 fluorescence are composed of individual MRP1-containing vesicles (Figure 1D) that colocalize with vesicular doxorubicin fluorescence (Figure 1E). In this case, the merge of the two images (Figure 1F) did not always result in yellow where the vesicles colocalized. Because these images represent living cells with rapidly moving vesicles, 1-s time delays between the acquisition of the images sometimes precluded absolute spatial colocalization. Additionally, the relative intensities of the two fluorophores are not matched, and vesicles labeled with more of one reporter than another do not look yellow.

Infrequently, MRP1-ECFP was localized anomalously in HeLa cells; that is, it was found in regions other than the plasma membrane and the perinuclear region. In extremely rare instances, for example, MRP1-ECFP accumulated in

what seems to be large aggregates within the endo-membrane system (Figure 1G). In this same multi-nucleated MRP1-ECFP expressing cell, doxorubicin was also found distributed throughout the endo-membrane system (Figure 1H) in a pattern very similar to the distribution of MRP1-ECFP (Figure 1I). Doxorubicin only assumed this dispersed subcellular distribution when MRP1-ECFP was likewise dispersed, and never in a cell that was not transfected with an MDR protein. Although this anomalous distribution for MRP1-ECFP was seen less than once per transfection, the observation that doxorubicin also assumed analogous subcellular distributions in these anomalous cells is suggestive of MRP1-mediated transport.

In instances when the MRP1-ECFP plasmid was poorly expressed, the protein was found only in intracellular vesicles, and not at the plasma membrane at all (Figure 1J). This result suggests that intracellular localization of the protein is not an artifact of an overexpression system. Although cells like this one weakly express the protein, and exposure times have to be increased to visualize these cells, nevertheless, even in these cells, MRP1-ECFP-expressing vesicles accumulated doxorubicin. Expression of the protein in this case was, however, not sufficient to exclude the drug from the nucleus (Figure 1, K and L).

Therefore, in addition to the plasma membrane (Figures 1A, 2, and 3), MRP1-ECFP localized to intracellular compartments that were peripheral to the nucleus. Within these vesicles, MRP1-ECFP fluorescence was coincident with doxorubicin fluorescence, a finding that would be consistent with MRP1-mediated sequestration of the drug away from the nucleus. In rare instances, when a cell exhibited an altered pattern of intracellular MRP1 distribution, doxorubicin also assumed this altered pattern, arguing strongly that intracellular MRP1 actively transports doxorubicin.

Cross-Linking Distinguishes the Activity of Two MRP1 Pools

Because MRP1 is expressed both at the plasma membrane and in intracellular vesicles, altered patterns of doxorubicin distribution in MRP1-expressing cells could be the result of the activity of either pool of MRP1. To distinguish the activity of the two pools, we compared the effects of two cysteine based cross-linking reagents on MRP1-mediated transport, the cell-impermeable BM[PEO]₄, and the cell-permeable BMH. If MRP1 activity were sensitive to these reagents, then selectively blocking MRP1 function at the cell membrane with the cell impermeable cross-linker would allow us to assess the role of intracellular MRP1 in doxorubicin sequestration. MRP1 sensitivity to these reagents was gauged with the compound TMRE. TMRE is a positively charged, fluorescent MDR substrate that does not accumulate inside MRP1-expressing cells, but instead, is effluxed from the cell in an MRP1-dependent manner. TMRE is also a live stain dye; drug entry is dependent on the maintenance of plasma membrane potential (Farkas *et al.*, 1989). On cell entry, TMRE accumulates in the mitochondria (Farkas *et al.*, 1989).

When cells were exposed to TMRE (Figure 2, A–C), the MRP1-expressing cell (Figure 2A) accumulated little to none of the drug, whereas cells that did not express detectable levels of the MRP1-ECFP took up the drug in the mitochondria (Figure 2, B and C). Treatment with the MRP1 inhibitor

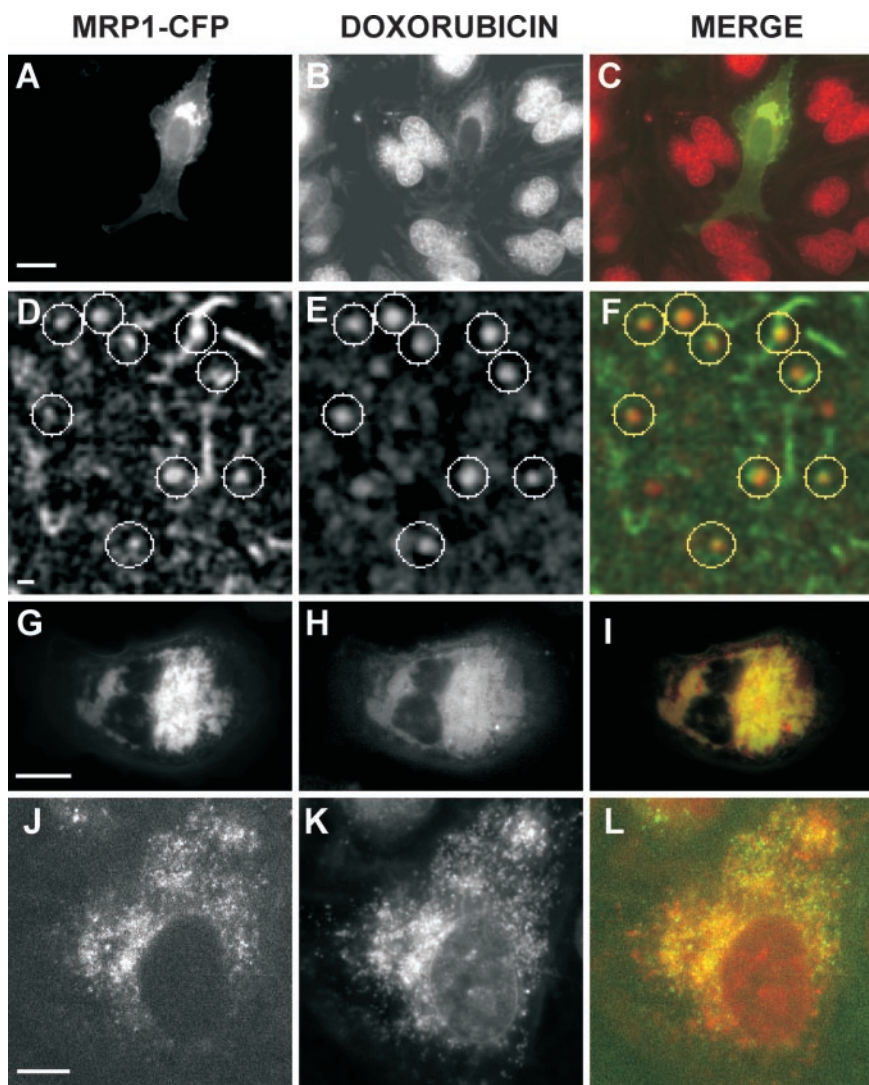


Figure 1. The effect of MRP1-ECFP localization on the distribution of doxorubicin. (A) ECFP fluorescence reveals that a transiently transfected HeLa cell expresses MRP1 both at the plasma membrane and in a juxtannuclear region. Bar, 10 μm . (B) Doxorubicin fluorescence demonstrates that the drug likewise accumulates in a juxtannuclear region in an MRP1-ECFP-expressing cell, whereas the nonexpressing cells surrounding it accumulate the drug in the nucleus. (C) The merge of MRP1-ECFP fluorescence (green) and doxorubicin fluorescence (red) shows the colocalization of doxorubicin and perinuclear localized MRP1-ECFP (yellow). (D–F) An enlarged image of the cell depicted in A–C reveals individual MRP1-ECFP-containing vesicles that also contain doxorubicin. Bar, 1 μm . (G–I) In rare instances when MRP1-ECFP aberrantly collects in the endomembrane system, doxorubicin accumulation is not juxtannuclear but is likewise dispersed. Bar, 10 μm . (J–L) A cell expressing low levels of MRP1-ECFP has little to no plasma membrane ECFP fluorescence and accumulates doxorubicin in a pattern that coincides with intracellular MRP1-ECFP. Bar, 5 μm .

verapamil rendered MRP1-ECFP cells incapable of effluxing TMRE (Figure 2, D and E); all cells accumulated the dye in the mitochondria comparably (Figure 2F). These results (Figure 2, A–E) are consistent with previously published observations that TMRE exclusion from the cell is mediated by MRP1 (Rajagopal *et al.*, 2002).

Cross-linking with the cell-impermeable reagent BM[PEO]₄ affected MRP1-ECFP-expressing cells much as verapamil did; all cells accumulated TMRE to the same extent, regardless of the degree to which they expressed MRP1 (Figure 2, G–I). Likewise, when cells were cross-linked with the cell-permeable reagent BMH, MRP1-dependent efflux of TMRE was also inhibited (Figure 2, J–L). The fact that cells accumulated TMRE after the addition of either BMH or BM[PEO]₄ suggests that cross-linking did not compromise cell viability. The fact that addition of these reagents inhibited MRP1-dependent TMRE transport suggests that cross-linking is sufficient to block MRP1 activity. Moreover, if BM[PEO]₄ is only reacting with MRP1-ECFP at the cell surface, then these results indicate that MRP1 activity at the

plasma membrane is responsible for the absence of TMRE in cells. To determine whether BM[PEO]₄ was inhibiting MRP1 selectively at the plasma membrane, we tested the effect of BM[PEO]₄ addition on subcellular pools of MRP1-ECFP. Because doxorubicin accumulates in MRP1 containing vesicles (Figure 1, D–F), we tested the effect of BM[PEO]₄ treatment on the intracellular distribution of the drug. Used in concert with BMH treatment, this experiment would determine whether BM[PEO]₄ was inhibiting primarily plasma membrane MRP1 and what role, if any, intracellular MRP1 activity played in doxorubicin sequestration. As shown previously, when cells expressing various levels of MRP1-ECFP were exposed to doxorubicin alone, only the MRP1-expressing cell had substantially diminished drug accumulation in the nucleus; the nuclei of all other cells accumulated doxorubicin to the same degree (Figure 1, A–C). However, if we blocked MRP1 function with the MRP1 inhibitor verapamil before doxorubicin administration (Figure 3, A–C), the nuclei of all cells were equally fluorescent with the drug (Figure 3B), regardless of the levels of MRP1-ECFP fluorescence

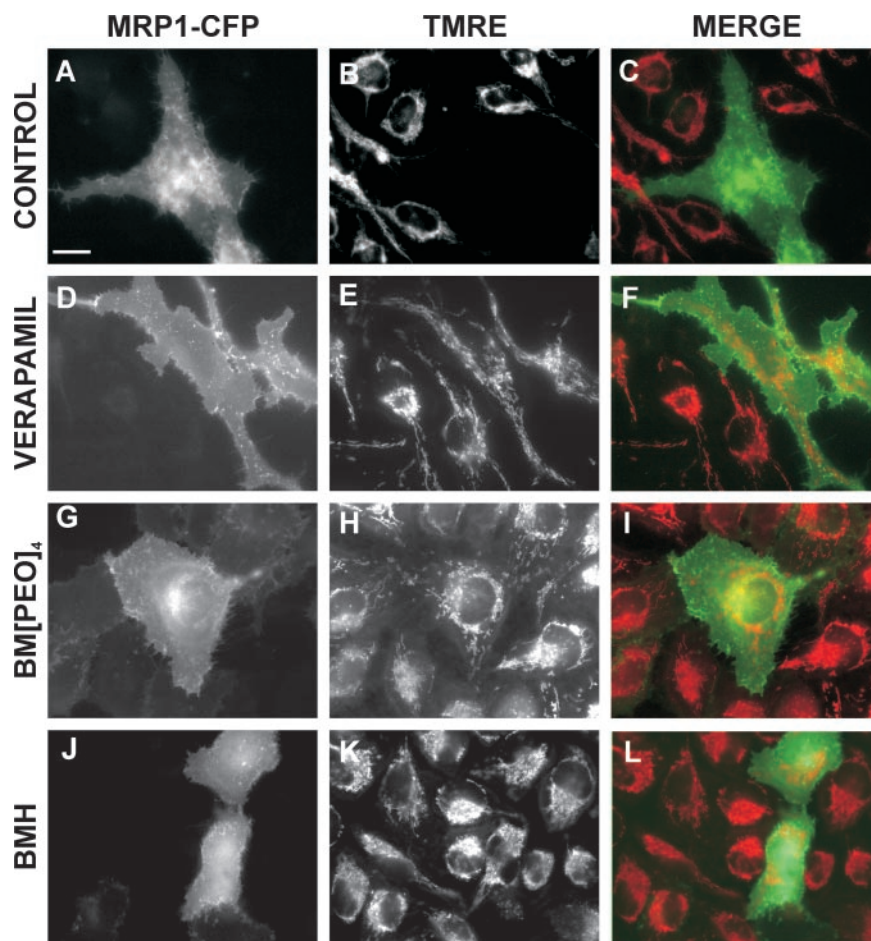


Figure 2. Cross-linking MRP1-ECFP interferes with its ability to transport substrates at the plasma membrane, as assayed by TMRE accumulation. (A–C) MRP1-ECFP expression prevents the intracellular accumulation of TMRE, so that the MRP1-positive cell is not visible under TMRE fluorescence. (D–F) Inhibiting MRP1 with verapamil prevents MRP1-mediated TMRE transport, and the two MRP1-ECFP-expressing cells in this field now accumulate the drug. (G–I) Cross-linking cells with the cell-impermeable reagent BM[PEO]₄ prevents MRP1-mediated TMRE transport so that the MRP1-positive cell accumulates TMRE just like its nonexpressing counterparts. (J–L) Addition of the cell permeable cross-linker BMH also inhibits MRP1 transport of TMRE. Bar, 10 μ m.

(Figure 3, A and C). The fact that doxorubicin sequestration away from the nucleus is sensitive to verapamil suggests MRP1 involvement. When BM[PEO]₄-treated cells were exposed to doxorubicin (Figure 3, D–F), doxorubicin did not accumulate in the nucleus of a cell expressing MRP1-ECFP even though MRP1 activity against TMRE is blocked with this treatment. Indeed, the MRP1-ECFP-expressing cell (Figure 3D) continued to be characterized by perinuclear doxorubicin staining (Figure 3E), which corresponds to the localization of intracellular MRP1 (Figure 3F). In contrast, when cells were exposed to the cell permeable cross-linker BMH, before doxorubicin incubation (Figure 3, G–I), all cells accumulated the drug within the nucleus, much as they did when treated with verapamil. These results reveal that BM[PEO]₄ is affecting the activity of MRP1-ECFP primarily at the plasma membrane, whereas BMH is inhibiting MRP1 throughout the cell. Moreover, these experiments suggest that intracellular MRP1 is responsible for intracellular doxorubicin sequestration, a drug resistance phenotype.

We next tested whether BMH and BM[PEO]₄ are affecting MRP1 activity by directly cross-linking MRP1-ECFP; if so, the electrophoretic mobility of MRP1 should be altered by treatment with either reagent. When cell lysates of MRP1-ECFP-transfected cells were immunoblotted with the anti-MRP1 antibody MRPr1, the antibody recognized a doublet

that migrated near a 250-kDa protein standard. However, when cell lysates of BM[PEO]₄-treated cells were similarly probed, the antibody recognized a much more slowly migrating species of the protein, suggesting that the reagent was cross-linking MRP1 directly. Likewise, when cell lysates of BMH-treated cells were probed with the anti-MRP1 antibody, we saw similar changes in the electrophoretic mobility of the protein (Figure 4A). We have reason, therefore, to believe that cross-linking with either reagent inhibits MRP1 as a result of direct protein modification.

Even if MRP1 is being directly modified by these cross-linking reagents, it is possible that treatment with BM[PEO]₄ or BMH is not inhibiting MRP1, but simply increasing cell permeability to TMRE. To investigate this possibility, we determined the average TMRE accumulation in a population of cells as a function of MRP1 expression, and we determined whether this average was altered by the addition of cross-linker (Figure 4B). When we calculated these averages, we found that neither BM[PEO]₄ nor BMH had any effect on TMRE accumulation in cells that expressed MRP1-ECFP at background levels. We did find, however, that BM[PEO]₄ was able to block MRP1 activity on TMRE almost entirely; all BM[PEO]₄-treated cells accumulated TMRE equivalently, even at high levels of MRP1 expression. On the other hand, BMH inhibition of MRP1 activity was not complete at the

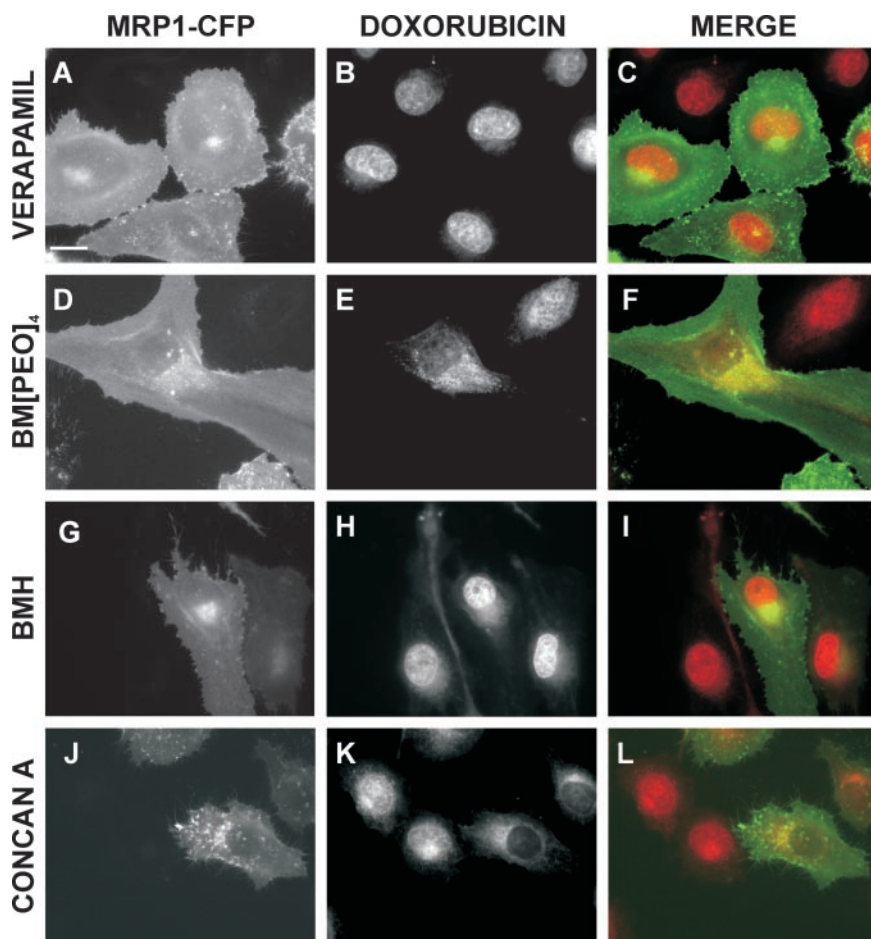


Figure 3. MRP1-ECFP actively sequesters doxorubicin in internal compartments. (A–C) Use of the MRP1 inhibitor verapamil redistributes doxorubicin from the juxtannuclear region to the nucleus of all three cells expressing the protein. (D–F) Addition of the cell-impermeable cross-linker BM[PEO]₄ has no effect on the subcellular distribution of doxorubicin in MRP1-ECFP-expressing cells; the drug is still to be found in juxtannuclear regions that are MRP1-ECFP positive. (G–I) Addition of the cell-permeable cross-linker BMH redistributes doxorubicin to the nucleus of MRP1-ECFP-expressing cells, much as the MRP1 inhibitor verapamil did in A–C. (J–L) Disrupting the organellar pH of cells with concanamycin A does not effect MRP1-ECFP-mediated drug sequestration; doxorubicin is still excluded from the nucleus of MRP1-expressing cells. Bar, 10 μ m.

concentration of BMH used (Figure 4B). However, because BMH enters cells and is free to interact with many intracellular cysteines, it might be more difficult to inhibit MRP1 activity with BMH at a concentration that would not at the same time be lethal to the cells.

When statistical analyses were next performed on BM[PEO]₄-treated cells that were exposed to doxorubicin, we found that the concentration of the cell-impermeable cross-linker that was able to block MRP1 activity on TMRE had a marginal effect on doxorubicin distribution. After treatment with BM[PEO]₄, MRP1-ECFP-expressing cells still showed a statistically significant reduction in nuclear drug accumulation, if not as much as untreated MRP1-ECFP cells (Figure 4C). In contrast, when cells were treated with BMH, the nuclear fluorescence of the drug was not reduced by the expression of MRP1-ECFP, as it was in untreated control cells (Figure 4C). Thus, at a concentration of BMH that was only partially able to block MRP1-mediated TMRE efflux, MRP1 activity against doxorubicin was inhibited. Because BM[PEO]₄ treatment completely inhibited MRP1-ECFP activity at the plasma membrane, as assayed by loss of TMRE efflux, but had little effect on the subcellular localization of doxorubicin, we have reason to believe that the intracellular pool of MRP1-ECFP unaffected by BM[PEO]₄ treatment is responsible for doxorubicin sequestration.

The observation that MRP1 activity can be blocked at the cell surface without disrupting intracellular drug sequestration argues strongly for active, subcellular pools of MRP1. However, many of these intracellular compartments are acidified and acidification of organelles has been implicated as a mechanism for resistance to weak-base chemotherapeutics such as doxorubicin (Wadkins and Roepe, 1997; Altan *et al.*, 1998). For this reason, we also needed to determine whether cellular pH played a role in the vesicular accumulation of doxorubicin. To determine the involvement of cellular pH, we added concanamycin A, an agent that disrupts organellar acidification (Altan *et al.*, 1998). The addition of 100 nM concanamycin A before doxorubicin administration had no effect on drug distribution in MRP1-expressing cells. In a field of cells treated with concanamycin A and doxorubicin (Figure 3, J–L), the three MRP1-expressing cells (Figure 3J) still excluded the anthracycline from the nucleus, whereas nonexpressing cells did not (Figure 3, K and L). These results suggest that MRP1 activity is responsible for doxorubicin sequestration and that changes in cellular pH do not make significant contributions to this phenotype. Furthermore, it indicates that concanamycin A does not affect the activity of MRP1-ECFP.

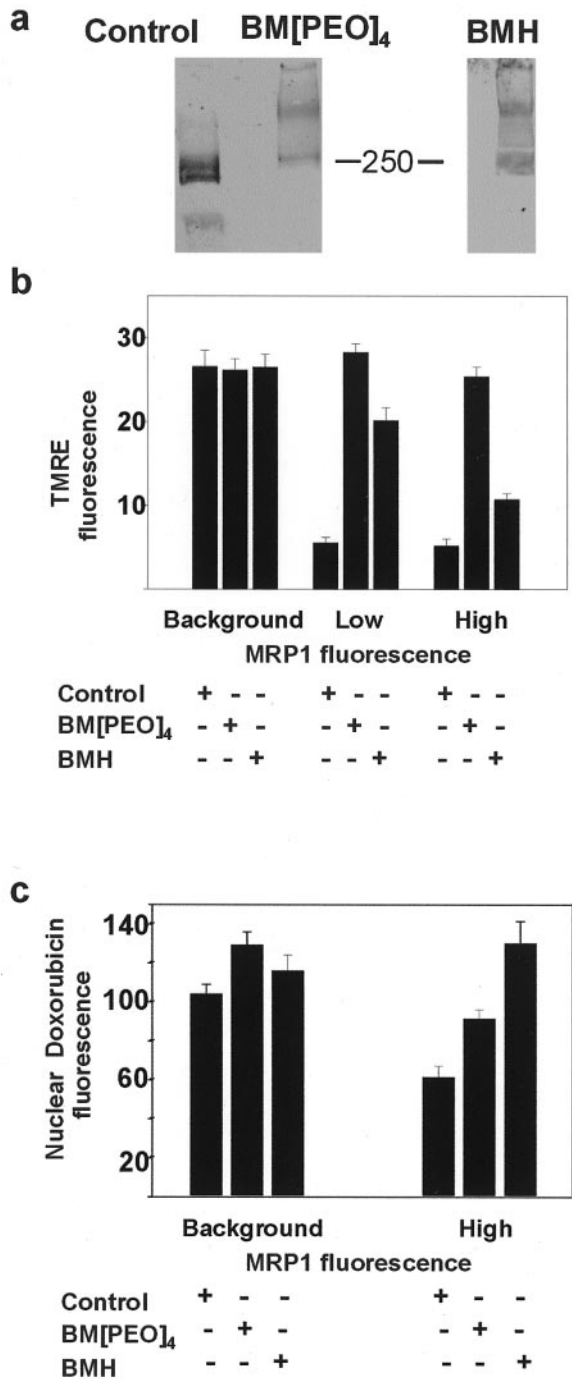


Figure 4. The effect of cross-linking on the electrophoretic mobility and transport activities of MRP1-ECFP. (A) In an immunoblot of MRP1-ECFP-transfected cell lysates, an anti-MRP1 antibody recognizes a doublet whose molecular mass migrates below the 250-kDa protein marker. However, addition of BM[PEO]₄ significantly retards the mobility of MRP1-ECFP. An immunoblot of BMH-treated cells likewise reveals a changed mobility of the protein after cross-linking. (B) MRP1-ECFP activity can be quantified by relating how much TMRE a cell accumulates to how much MRP1-ECFP a cell expresses. Fluorescence functions as a reporter for both MRP1 expression and TMRE accumulation. Neither BM[PEO]₄ nor BMH

Intracellular MRP1 Localizes to Lysosomal Membranes

To characterize the subcellular MRP1-ECFP compartment, we simultaneously transfected cells with fluorescent reporters for various organelles. For example, we coexpressed the lysosomal marker synaptotagmin VII, with an ECFP tag, and MRP1, with an EYFP tag, to determine whether MRP1 resides in the lysosomes. In a deconvolved section of such a doubly transfected cell, we see that MRP1 resides in vesicles (Figure 5A) that also express synaptotagmin VII (Figure 5, B and C). MRP1 might be localizing to the lysosomes not because it is functional there, but because it is subject to protein degradation. However, immunoblots of both MRP1 and MRP1-ECFP-transfected cells are not suggestive of degradation (Rajagopal *et al.*, 2002). It is also possible that the ECFP tag might be directing the fluorescent MRP1 conjugate to lysosomes in a manner that does not represent the trafficking history of the wild-type protein. Immunocytochemistry of cells transfected with wild-type MRP1, however, suggests that even the protein in its unconjugated state localizes to vesicles that are positive for synaptotagmin VII (Figure 5, D–F). As an added assurance, the lysosomal marker cathepsin D also colocalizes with wild-type MRP1 (Figure 5, G–I), an observation that is made especially clear in an enlarged image of a cell probed for both proteins (Figure 5, J–L). Moreover, doxorubicin preferentially accumulates in the lysosomes of cells expressing MRP1; nontransfected cells show markedly decreased doxorubicin accumulation in the lysosomes (our unpublished data).

BCRP and Pgp Localize to Lysosomal Membranes and Doxorubicin-positive Vesicles

We next tested whether other MDR transporters were also found in lysosomes. Cells transfected with BCRP-ECFP, for example, revealed that, like MRP1, this protein was found at the plasma membrane and in intracellular vesicles (Figure 6A). As with MRP1, these BCRP-positive vesicles localized to the lysosomes because they took up fluorescent dextrans that had been chased into the lysosomes (Figure 6, B and C), a method of lysosomal detection that also labels synaptotagmin VII-positive vesicles (our unpublished data). Moreover, as with MRP1, BCRP-containing vesicles were to be found at the periphery of the nucleus (Figure 6D) where they accumulated doxorubicin (Figure 6, E and F). This pattern of protein localization and activity is mirrored by Pgp. Like MRP1, the expression of Pgp does not decrease the net

Figure 4 (cont). increases the permeability of cells to TMRE, because all cells with background MRP1 fluorescence accumulate comparable levels of TMRE. Moreover, cells treated with BM[PEO]₄, regardless of the degree to which they express MRP1, accumulate as much TMRE as untreated, non-MRP1-expressing cells. (C) MRP1-ECFP activity against doxorubicin can be quantified by relating MRP1 expression to the doxorubicin fluorescence inside the nucleus. In BM[PEO]₄-treated cells, MRP1-ECFP still reduces the relative amount of doxorubicin accumulated in the nucleus. For both BM[PEO]₄-treated and untreated cells, MRP1-mediated reduction in nuclear fluorescence is statistically significant ($P < 0.01$ and $p < 0.0001$, respectively). However, all BMH-treated cells have similar amounts of doxorubicin in the nucleus, whether they express MRP1-ECFP or not.

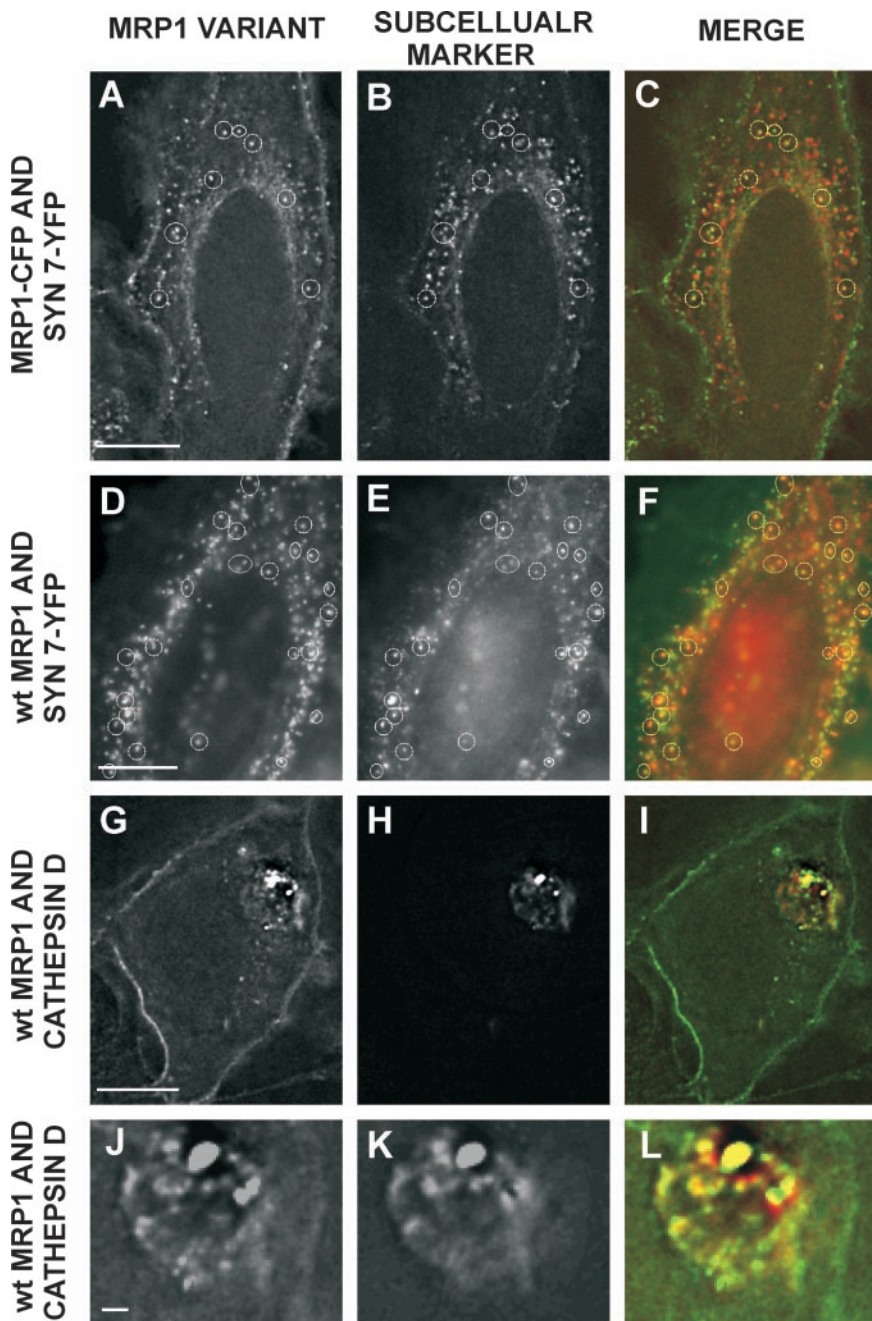


Figure 5. Fluorescently labeled MRP1 and wild-type MRP1 both colocalize with lysosomal markers. (A) An MRP1-YFP-transfected cell expresses MRP1 at the plasma membrane and in intracellular vesicles (white circles). Bar, 10 μm . (B) Cotransfected with synaptotagminVII-YFP, the cell in A also expresses a lysosomal marker in intracellular vesicles (white circles). (C) The merge of the MRP1-YFP fluorescence in A (green) with the synaptotagmin fluorescence in B (red) reveals the degree of colocalization. Images are of living cells with moving vesicles; a delay of at least 1 s separates the images in time. (D–F) The degree of colocalization of wild-type MRP1 (green) and synaptotagminVII (green). Bar, 10 μm . (G–I) Localization patterns of wild-type MRP1 (green) and cathepsin D (red). Bar, 10 μm . (J–L) An enlarged image of the cell depicted in G–I reveals the degree of colocalization of the two proteins. Bar, 1 μm .

cellular accumulation of doxorubicin (Rajagopal *et al.*, 2002). Like MRP1, Pgp-ECFP localized to intracellular vesicles around the periphery of the nucleus (Figure 6G), and these vesicles took up fluorescent dextrans chased into the lysosomes (Figure 6, H and I). Finally, when Pgp-ECFP-expressing cells were exposed to doxorubicin, Pgp-positive vesicles accumulated the drug (Figure 6, J–L). Therefore, we have reason to believe that BCRP and Pgp confer resistance to doxorubicin in a manner similar to MRP1.

The colocalization data presented thus far is primarily based on the visual inspection of two fluorescent signals,

and it is for this reason difficult to discuss the relative degrees of correlation between different experiments. To quantify the information presented in a fluorescent image, we calculated correlation coefficients for regions of the cell in which two fluorescent signals both localize. The r represents the degree to which distinct fluorophores vary their intensities through space in a coordinated manner. Correlation coefficients are expressed on a scale of 0–1, with 0 representing no correlation whatsoever. When correlation coefficients were calculated for the micrographs presented in this study, we plotted them on a line graph and could then see under

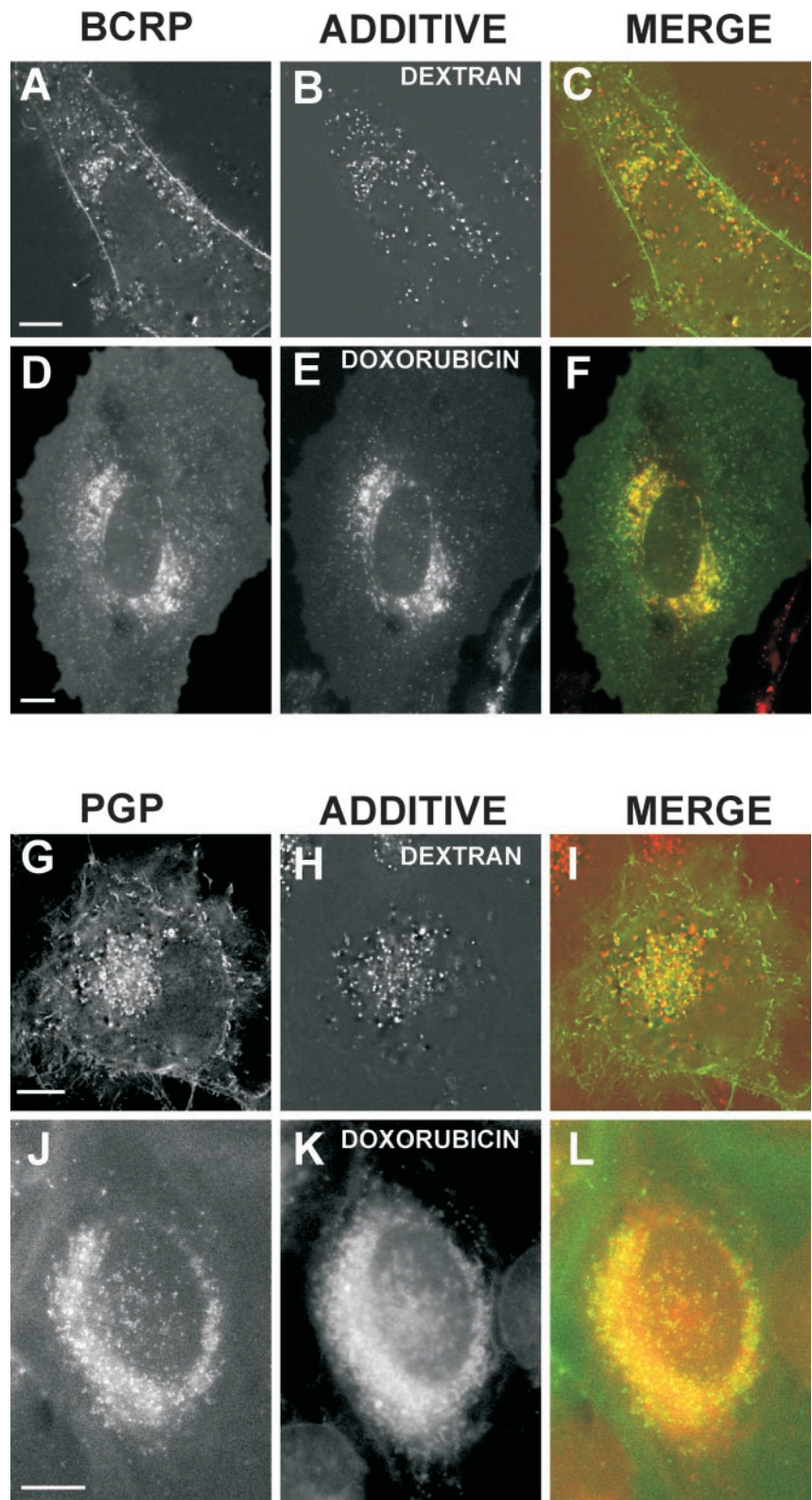


Figure 6. Pgp and BCRP colocalize with a lysosomal marker and accumulate doxorubicin in regions positive for either Pgp or BCRP. Bars, 10 μm . (A) A cell transfected with BCRP-ECFP expresses the protein at the plasma membrane and in intracellular regions. The image is a deconvolved fluorescent section of a cell. (B) Fluorescent dextrans chased into the lysosomes of the cell in A accumulate in intracellular vesicles. (C) The merge of A and B shows the degree to which BCRP-ECFP (green) colocalizes with the lysosomal marker (red). (D–F) The degree of colocalization of BCRP-ECFP in D and doxorubicin in E. (G–I) The degree to which Pgp-ECFP accumulates in intracellular vesicles G that are positive for fluorescent dextrans chased into the lysosomes H. (J–L) Localization patterns of Pgp-ECFP and doxorubicin.

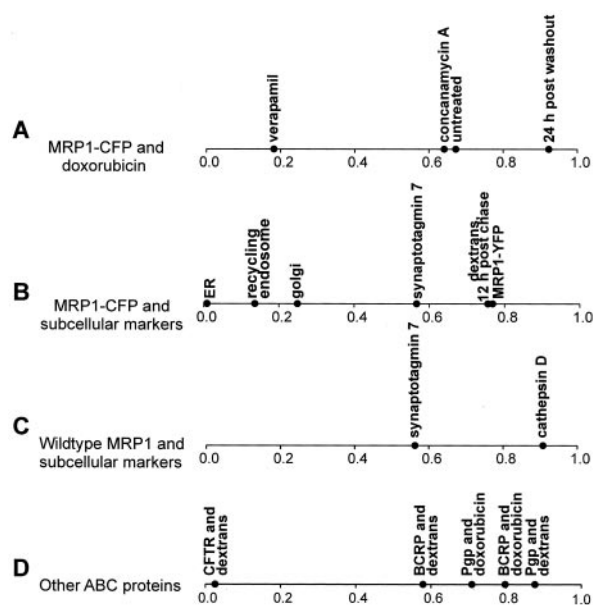


Figure 7. Correlation coefficients quantify degrees of correlation for fluorescent markers. (A) Line graph plotting the correlation coefficients of MRP1-ECFP in differently treated cells. (B and C) Line graphs plotting the correlation coefficients of MRP1-ECFP and wildtype MRP1 with various subcellular markers. (D) Line graph plotting the correlation coefficients of specified ABC proteins and an additive which is either a lysosomal marker or doxorubicin.

what conditions vesicular MRP1 accumulated doxorubicin (Figure 7A), for example, or colocalized with a lysosomal marker (Figure 7, B and C). Using the r of vesicular MRP1-YFP and MRP1-ECFP (0.7677) as an indication of a strong colocalization, we then saw how likely it would be to find MRP1 in vesicles of the Golgi or the ER (Figure 7B). When correlation coefficients for other ATP-binding cassette (ABC) proteins are similarly represented, lysosomal localization and activity were found for only MDR-conferring transporters. The activity of the cystic fibrosis transmembrane conductance regulator, for example, did not show this intracellular localization pattern (Figure 7D).

DISCUSSION

We have demonstrated that the plasma membrane transporter MRP1 has a subcellular localization from which it can promote a drug resistance phenotype. This phenotype is reversible upon inhibition of MRP1 by verapamil but unaffected by alterations in intracellular pH. Using fluorescent markers for the ER, the Golgi, the recycling endosomes, and the lysosomes, we have shown that this intracellular MRP1 activity most likely originates from the lysosomes. Moreover, Pgp and BCRP also localize to lysosomal membranes from which they also transport doxorubicin.

The demonstration of a lysosomal distribution for MRP1, Pgp, and BCRP does not preclude subcellular activity elsewhere. Indeed, this intracellular distribution may be a factor of cell cycle or trafficking history, and these proteins may present themselves in other locations under different condi-

tions. MRP2, for example, has been found in a novel subcellular organelle in nonpolarized hepatic cells (Tuma *et al.*, 2002). However, it would not be unprecedented to propose that MRP1, BCRP, and Pgp all confer doxorubicin resistance primarily from lysosomes. ABC proteins are known to be expressed in the vacuole of nonmammalian systems (Li *et al.*, 1996; Lu *et al.*, 1997), and in mammals, proteins that are not part of the ABC family do confer doxorubicin resistance from lysosomes (Cabrita *et al.*, 1999). Moreover, lysosomes may promote the detoxification of the drug and in this way provide a link between two distinct multidrug-resistance pathways. Whether this subcellular localization pattern of these MDR transporters is a result of overexpression is not known. Insofar as multidrug-resistant cancer cells are known to overexpress ABC transporters such as MRP1, the overexpression system used in this study models itself after a pathophysiological state. However, predominantly intracellular localization patterns for MRP1 have been reported for many normal tissues (Flens *et al.*, 1996; Wioland *et al.*, 2000). Moreover, the finding that cells expressing low levels of MRP1-CFP are characterized by primarily intracellular versions of the protein suggests that vesicular MRP1 is not the result of overexpression (Figure 1, J-L).

Of course, MRP1 activity does not stem entirely from the intracellular organelles; TMRE, for example, is effluxed by MRP1 before it can enter the cell, presumably by plasma membrane localized versions of the transporter. Strangely, the dominant activity of the protein on doxorubicin is on intracellular membranes. Our results suggest that MRP1 may have different activity profiles at different membranes, a difference that could be a function of environment (lipids and cholesterol) or posttranslational modifications (e.g., phosphorylation) that occur at some subcellular compartments. Alternatively, these subcellular compartments may contain other transport mechanisms that act synergistically with MRP1 activity.

Intracellular localization and activity for MRP1 and for other members of the MDR transporter family may suggest different strategies for chemotherapeutic regimens in a clinical setting. To date, inhibitors for these MDR transporters have been selected presumably on the assumption of plasma membrane based efflux mechanisms. MRP1-mediated intracellular drug sequestration may necessitate alternate strategies in the search for MDR inhibitors.

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