Actin disruption inhibits endosomal traffic of P-glycoprotein-EGFP and resistance to daunorubicin accumulation

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One of the major causes of multidrug resistance (MDR) in chemotherapy is overexpression of P-glycoprotein (P-gp), a member of the ATP-binding cassette (ABC) transporter family, a mechanism whereby tumor cells become drug resistant (12, 13). P-gp is encoded by the human mdr1 gene, which is found in many different cancers, including tumors not previously exposed to chemotherapy (11). P-gp, a 170-kDa membrane protein, acts as a drug efflux pump to transport a wide range of structurally and mechanistically different chemotherapeutic drugs out of cancer cells. Consequently, cancer cells survive and continue to proliferate.

P-gp is localized not only on the plasma membrane but also to some extent in intracellular compartments (37). Our previous studies indicated that P-gp is localized in endoplasmic reticulum and Golgi as well as plasma membrane, but intracellular P-gp did not appear to contribute to drug resistance in HeLa cells transfected with P-gp (10). Other studies also showed that intracellular P-gp does not seem to have a major contribution to drug resistance (22). For instance, a human colon carcinoma cell line that expressed P-gp at very high levels intracellularly was sensitive to the P-gp substrates doxorubicin and vincristine (7). Much research has focused on the function of P-gp and ways to overcome P-gp mediated MDR. However, very little is known about the processes of cellular distribution of P-gp and its traffic, and there has been limited exploration of possible ways to alter the subcellular localization of P-gp. Such approaches may provide new insight into alternative ways to overcome MDR in cancer chemotherapy.

Membrane proteins may traffic to the cell plasma membrane via two distinct pathways. In the first, a constitutive/default pathway, membrane protein is incorporated into vesicles and directly moves from Golgi to the plasma membrane along the cytoskeleton. In the second pathway, traffic is through an intracellular endosomal system, with protein vesicles being transported to endosome compartments to form an intracellular pool, followed by further transport to the plasma membrane. The cytoskeleton is involved in trafficking of membrane protein vesicles (36). Among ABC transporters, rat mdr1 and mdr2, which are responsible for biliary secretion of cationic drugs (14) and secretion of phospholipids (22), respectively, were found to be rapidly delivered directly from Golgi to the canalicular membrane (19). Sister of P-gp (SPGP) was shown to traffic to the plasma membrane via an endosomal system (20). Moreover, ABCA1 was associated with an intracellular pool, recycling from this pool to the membrane through the endosomal compartment, indicating it was undergoing endocytosis (33). Kim et al. (17) suggested that there was an intracellular pool of P-gp and that P-gp recycled between the plasma membrane and the intracellular pool, indicating a possible endocytosis pathway. However, the detailed mechanism of P-gp traffic has been little studied and is still unclear.

Cytoskeleton has been shown to be involved in the traffic of a number of membrane proteins (1). Both microtubules and actin provide an intracellular highway for the traffic of membrane proteins. A member of the ABC transporter superfamily, cystic fibrosis transmembrane conductance regulator, appears to be recruited to the apical plasma membrane in a microtubule-dependent manner (35). Microtubules were also shown to play a critical role in the intracellular traffic of human thiamine...
transporter-1 (34) and glucose transporter isof orm 4 (9, 23). On
the other hand, the actin cytoskeleton also has been shown to be
involved in traffic of membrane proteins. Actin filament was
shown to be involved in the traffic from endosome to lysosome in B
cell lymphoma CH27 (5) and in traffic and recycling of H^+–K^+-ATPase (30) and CXC chemokine receptor (38). Re-
cent biochemical interaction studies indicated that P-gp inter-
acts with actin through ezrin/radixin/moesin (ERM) protein, which
 cross-links actin with plasma membrane (24), but the possible
role of actin in the trafficking of P-gp was not addressed. Other
studies showed that P-gp colocalized with raft
markers such as CD44 and CD59 (2). In addition, fluorescence
 resonance energy transfer (FRET) data suggested that CD44,
which binds to actin through ERM protein and resides in close
molecular vicinity to P-gp, may be responsible for the associa-
tion of P-gp and cytoskeleton (2). MR2P, another ABC
transporter, also was reported to interact with actin via
radixin (16, 21). However, previous studies did not investigate the role
of cytoskeleton in the traffic of P-gp, and it is still unclear
whether actin or microtubules facilitate P-gp cellular traffic. It
is therefore critical to examine the consequences of cytoskel-
eton disruption on P-gp cellular localization and function. To
understand the cellular dynamic and traffic of P-gp and to
extend the findings to the development of novel approaches to
overcoming MDR in cancer chemotherapy, we constructed a
P-gp-enhanced green fluorescent protein (EGFP) fusion plas-
mid and established a clonal P-gp-EGFP stable expression
system in human breast cancer MCF-7 cells to enable the
investigation of the pathway of cellular traffic of P-gp in
greater detail. In this study the role of the cytoskeleton in the
P-gp-EGFP intracellular traffic pathway and drug accumula-
tion was examined using confocal fluorescence microscopy and
immunostaining techniques.

MATERIALS AND METHODS

Chemicals and cell culture. Daunorubicin, colchicine, nocodazole,
taxol, cytochalasin D, latrunculin B, cycloheximide (CHX), anti-
human P-gp mouse monoclonal antibody cloneF4, anti β-tubulin
monoclonal antibody, and CY3-conjugated anti-mouse and anti-goat
IgG were obtained from Sigma. Anti-EGFP mouse monoclonal anti-
body and anti-Lamp2 goat polyclonal antibody were purchased from
Santa Cruz Biotechnology. Mouse anti-early endosome antigen 1
(EEA1) monoclonal antibody and mouse anti-Rab11 monoclonal
antibody were purchased from BD Biosciences. Anti-human Golgi
antibody, Golgin-97, was obtained from Molecular Probes. Human
MCF-7 breast cancer cells and the multidrug resistant MCF-7/Adr
cells were obtained from Dr. Mary Bebawy (Faculty of Pharmacy,
University of Sydney). MCF-7/Adr cells stably express wild-type
P-gp, which was initially induced by administration of adriamycin.
Cells were grown in DMEM (GIBCO) with 10% fetal calf serum
(GIBCO) and incubated at 37°C with 5% CO2.

Construction of P-gp-EGFP fusion plasmid and expression of
P-gp-EGFP. Human MDR1 gene was cut from the pAMG6 plasmid
and inserted into pEGFP-N1 vector (Clonetech). Details were de-
scribed in a previous study (10). FuGENE 6 transfection reagent
(Roche Molecular Biomedical) was used for transfection. The stable
transfected MCF-7 cells were first selected in G418 (700 µg/ml)
for 9 wk. The selected cells were then sorted by flow cytometry, and
the cells were seeded in 96-well plates with one cell per well. A single
clonal of the cells was collected and used for the study.

Western blotting. Briefly, MCF-7/P-gp-EGFP, MCF-7/Adr, and
MCF-7 cells were harvested and washed with PBS. Cells were lysed
with lysis buffer (1% Triton X-100, 1 mM PMSF, and 1 mM EDTA
in 50 mM Tris-HCl buffer, pH 7.4) on ice. The cell lysate was
centrifuged at 14,000 rpm at 4°C for 30 min, and the supernatant was
analyzed by SDS-PAGE using 10% polyacrylamide gel. Anti-GFP
monoclonal antibody (1:500) and anti-P-gp monoclonal antibody
cloneF4 (1:200) were used for Western blotting. Anti-mouse alkaline
phosphatase-conjugated IgG (Promega) and Sigma Fast 5-bromo-4-
chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma) was used
for detection.

Cell viability assay. We plated 1 × 104 stable MCF-7/P-gp-EGFP
or MCF-7-sensitive cells into 96-well plates. The cells were mixed
with different concentrations of colchicine or daunorubicin and incu-
bated for 72 h, and then 20 µl of 3-(4,5-dimethyl-2-thiazolyl)-2,5-
diphenyl-2H-tetrazolium bromide (MTT) solution (5 mg/ml) was
added to each well and incubated at 37°C for 4 h. The culture medium
was then removed, and 100 µl of DMSO were added into each well,and
cells were incubated for 20 min. The absorbency was measured
using a Bio-Rad plate reader at 595-nm wavelength. IC50 values were
calculated using the Scientist program (MicroMath Scientific Soft-
ware).

Effects of cytoskeleton inhibitors on the traffic of P-gp-EGFP and
intracellular accumulation of daunorubicin. Stable MCF-7/P-gp-
EGFP cells were grown on coverslips for 18–20 h. The cells were
treated with colchicine (100 µM, 8 h), nocodazole (25 µg/ml, 8 h),
taxol (5 µM, 8 h), cytochalasin D (1 µM, 1 h), or latrunculin B (1 µM,
1 h). The cells were washed in PBS and then fixed in 4% parafor-
maldehyde (PFA) for 10 min. The coverslips were mounted with
Vectashield mounting medium (Vector Laboratories), sealed with nail
polish, and viewed. The localization of P-gp-EGFP was analyzed
using confocal microscopy.

Actin staining with phalloidin-TRITC. Stable MCF-7/P-gp-EGFP
cells were grown on coverslips for 18 h. The cells were then treated
with or without cytochalasin D (1 µM, 1 h) or latrunculin B (1 µM,
1 h). The cells were washed three times in PBS. Cells were fixed
for 5 min in 4% PFA in PBS and then washed three times in PBS. Cells
were permeabilized with 0.1% Triton X-100 in PBS and then washed
again in PBS. Cells were stained with 50 µg/ml TRITC-conjugated
phallloidin for 40 min at room temperature. Cells were then washed
three times in PBS and finally mounted with Vectashield mounting
medium (Vector Laboratories), sealed with nail polish, and viewed
using confocal microscopy.

Dextran staining. Briefly, MCF-7/P-gp-EGFP cells were grown on
coverslips and treated with CHX (10 µg/ml, 24 h). Cells were then
incubated with Alexa Fluor 594-conjugated 10-kDa dextran (Molec-
ular Probes) at 37°C. Cell were washed and fixed in 4% PFA. After
being washed, cells were mounted and sealed. The images were taken
using confocal microscopy.

Immunocytochemistry. MCF-7/P-gp-EGFP or MCF-7/Adr cells
with or without various treatments were washed with PBS, fixed in
4% PFA for 10 min, and then treated with 0.1% Triton X-100 for
20 min. After being washed three times in PBS, cells were incubated with
4% bovine serum albumin for 30 min at room temperature. The
various primary antibodies were added to cells [anti-P-gp cloneF4
(1:200), anti-EEA1 (1:100), anti-Rab11 (1:50), anti-Lamp2 (1:50),
anti-Golgin-97 (1:400), and anti-β-tubulin (1:100)] and incubated for
1 h at room temperature. Cells were washed with PBS and incubated with
secondary antibody conjugated with CY3 (1:100) for 1 h at room
temperature. Finally, the coverslips were rinsed with PBS and then
mounted with Vectashield mounting medium (Vector Laboratories), sealed with nail polish, and viewed.

Intracellular drug accumulation. Cells were cultured on coverslips
for 12 h. Cells were incubated with 1 µM daunorubicin for 30 min,
with or without prior incubation with latrunculin B (1 µM) or
cytochalasin D (1 µM) for 1 h at 37°C. After the culture medium was
removed, the cells were washed in PBS. Intracellular accumulation of
daunorubicin was immediately analyzed using a confocal microscope.

Cell proliferation assay. Stable MCF-7/P-gp-EGFP or MCF-7 cells
were subcultured into six-well plates at a density of 1.8 × 105 cells
per well. Cells were grown overnight. Cells then were incubated with daunorubicin (2 μM, 24 h), latrunculin B (1 μM, 24 h), or cytochalasin D (1 μM, 24 h) alone or with daunorubicin and actin inhibitors (latrunculin B or cytochalasin D) simultaneously. Cells were then harvested and stained with Trypan blue (0.4%). The cell numbers were counted under the microscope. Three individual experiments were conducted, and cell viability relative to control was analyzed. Experimental data were compared using Student’s t-test. Results were considered statistically significant at \( P < 0.05 \).

Confocal microscopy and quantitative analysis. Images were acquired on the Bio-Rad confocal scanning system (Radiance Plus) attached to a Nikon E800 microscope, using a Nikon plan Fluor ×100/1.30 oil objective and the Bio-Rad Laser Sharp 2000 program. Excitation wavelengths (\( \lambda_{\text{ex}} \)) of 488 or 543 nm were used. P-gp-EGFP (\( \lambda_{\text{ex}} = 488 \) nm) was detected at 500–520 nm. Daunorubicin (\( \lambda_{\text{ex}} = 488 \) nm) was detected using a 570-nm long-pass filter. CY3 was excited at 543 nm and then detected using a 570-nm long-pass filter. Dextran was excited at 595 nm and detected using a 615-nm long-pass filter. Control experiments showed each pair of dyes had no overlap in their emission.

For quantitative analysis of the amount of drug uptake into MCF-7/P-gp-EGFP cells, the following analysis was carried out. Control MCF-7 cells (not expressing P-gp-EGFP) in the field of view were used to express the amount of drug uptake as a ratio of fluorescence in expressing cells to that in nonexpressing cells. To allow quantitation, all experiments were carried out under exact conditions of laser intensity and photomultiplier tube gains so that there was no saturation occurring. The data were collected at the same magnification and zoom. Once these conditions were set, all images were collected under the same conditions. It was established that there is no bleed through from the GFP channel to the daunorubicin channel, and hence the data from each channel are representative of only that species. Each layer from the three-dimensional stack was analyzed for the sum of the intensity in the field. For the determination of intracellular fluorescence (EGFP or daunorubicin) intensity, the values for fluorescence in the whole cytoplasmic area were summed and then calculated as fluorescence intensity units per unit surface. The analysis was done using the KS400 image analysis software package (Carl Zeiss Vision).

RESULTS

Stable expression of functional P-gp-EGFP and its characterization. To establish the stable expression of P-gp-EGFP, transfected MCF-7 human breast cancer cells were initially selected in G418 (700 μg/ml) for 9 wk and sorted by flow cytometry. Sorted cells were grown in 96-well plates as one cell per well. A single clone was picked up and used for further studies. The cells were grown in normal DMEM (GIBCO) medium, and stable expression was evaluated by both confocal microscopy (Fig. 1A) and flow cytometry. Flow cytometry results indicated that GFP intensity of stable MCF-7/P-gp-EGFP cells was 20 times higher than the GFP intensity in the control MCF-7 cells (data not shown).

The expression of P-gp-EGFP protein was analyzed using Western blotting. Both anti-P-gp primary antibody (cloneF4) and anti-GFP antibody detected the ~197-kDa P-gp-EGFP fusion protein of MCF-7/P-gp-EGFP cell lysates (Fig. 1B, lane 2, left and right). The wild-type P-gp from cell lysates of MCF-7/ADR cells, detected by anti-P-gp antibody, appeared as an ~170-kDa band (Fig. 1B, lane 1, left), whereas there was no detectable P-gp in the drug-sensitive MCF-7 cells (Fig. 1B, lane 3, left). Furthermore, immunofluorescence assay using anti-P-gp antibody and red fluorescence-conjugated secondary antibody indicated that EGFP completely colocalized with P-gp (data not shown), suggesting that EGFP was a true marker for P-gp in the cells.

To ensure that the stable MCF-7/P-gp-EGFP cells retained their multidrug resistance function, we used the MTT cell viability assay to access the resistance of MCF-7/P-gp-EGFP cells. Compared with sensitive MCF-7 cells, MCF-7/P-gp-EGFP-expressing cells were found to be 14 times more resistant to colchicine and 19 times more resistant to daunorubicin, respectively (Table 1).
These data indicated that stably expressed P-gp-EGFP was a functional drug pump and the EGFP tag did not prevent P-gp from conferring drug resistance. These results are consistent with our previous studies using transient expression of P-gp-EGFP in HeLa cells (10).

Intracellular localization of P-gp-EGFP in stable MCF-7/P-gp-EGFP cells. P-gp-EGFP fluorescence was mainly localized on the plasma membrane of stable MCF-7/P-gp-EGFP cells (Fig. 1A), whereas cells expressing EGFP alone had fluorescence distributed all over the cells, including the plasma membrane, cytosol, and nuclei (data not shown). Furthermore, intracellular P-gp-EGFP also was present in some of the stable MCF-7/P-gp-EGFP cells, as found in multicell analysis in which 31% of the stable cells had intracellular P-gp-EGFP (n = 200). Within these cells, the intracellular P-gp-EGFP fluorescence intensity of the whole visible cytoplasmic region varied between cells (for detail, see MATERIALS AND METHODS), with about 24% of cells having relatively low intracellular fluorescence of P-gp-EGFP (1–4 times higher than the background fluorescence in the field adjacent to the cells), 64% having medium intracellular fluorescence (5–10 times higher), and 12% having high intracellular fluorescence (>10 times higher). With the use of a Golgi marker (Golgin-97), an early endosome marker (EEA1), and a lysosome marker (Lamp2) to examine the intracellular localization, the P-gp-EGFP was found to be mainly colocalized with either Golgi (Fig. 2A), early endosome (Fig. 2B), or lysosome (Fig. 2C) compartments, but not in the recycling endosome (Fig. 2D). Although it was not possible to carry out simultaneous multiple labeling for the intracellular compartments with the equipment, the finding that P-gp-EGFP was not fully colocalized with each of these cellular compartments in the colocalization studies probably means that P-gp-EGFP (unmerged; green) was also present in the remaining compartments at the same time. Of a representative group of multiple cells demonstrating intracellular P-gp-EGFP, ~57% (n = 90) had the intracellular P-gp-EGFP colocalized mainly with EEA1, ~23% (n = 80) mainly with Golgi marker, and
the presence of an intracellular pool of P-gp-EGFP (17).

early endosome is consistent with previous findings suggesting most of the intracellular P-gp-EGFP in the stable cells was largely intracellularly after treatment with cytochalasin D, which appeared as a linear filament (Fig. 3), in contrast to control MCF-7/Adr cells, which had P-gp primarily located on the plasma membrane (Fig. 3H). Notably, overall there was less P-gp on the plasma membrane after treatment with cytochalasin D in the MCF-7/Adr cells (Fig. 3I). However, not all the cells had significantly decreased P-gp on the plasma membrane. The differences may be due to different stages of cell proliferation in the cell population. Thus, in contrast to cells where P-gp-EGFP was just starting to be synthesized, the P-gp would not be rapidly degraded after short treatment with actin inhibitors in cells with large amounts of P-gp already located on the plasma membrane. These results confirm that traffic of P-gp is actin dependent and also indicate that the EGFP tag does not influence the P-gp traffic pathway. In addition, with the use of phalloidin-TRITC staining (red), actin was shown to be completely disrupted after treatment with cytochalasin D or latrunculin B, with hardly any red staining (Fig. 3, K and L), whereas the control cells showed the actin structure to be still intact, with red or yellow (merged with green from P-gp-EGFP) staining in the cells (Fig. 3J).

Accumulation of P-gp-EGFP in early endosome after disruption of actin. In an attempt to establish the nature of the linear filaments observed after treatment with actin inhibitor, immunocytochemistry was performed to obtain information on their localization relative to various cellular compartment markers. The linear filamentous P-gp-EGFP pattern did not colocalize with Golgi and recycling endosome markers (data not shown), whereas the intracellular P-gp-EGFP extensively colocalized with early endosome marker EEA1 (Fig. 4, A–C). This finding demonstrated that P-gp-EGFP remained in the early endosomal compartment when its traffic was disrupted, suggesting that P-gp-EGFP may traffic to the plasma membrane via the early endosomal compartment.

In addition, immunostaining with EEA1 antibody and CY3 secondary antibody demonstrated that early endosome appeared as a linear structure in some untransfected MCF-7 cells (Fig. 4E), suggesting that some of the linear structure also occurred naturally as early endosome. Furthermore, with the

~21% (n = 80) mainly with lysosome marker, indicating that most of the intracellular P-gp-EGFP in the stable cells was localized in early endosome compartments. The localization in early endosome is consistent with previous findings suggesting the presence of an intracellular pool of P-gp-EGFP (17).

Role of cytoskeleton in the traffic of P-gp. To determine whether the cytoskeleton is involved in intracellular traffic of P-gp-EGFP, we used inhibitors of tubulin and actin to examine the role of cytoskeleton in traffic of P-gp-EGFP. Two different tubulin inhibitors (colchicine and nocodazole) and two actin inhibitors (cytochalasin D and latrunculin B) were applied.

Immunocytochemistry with α-tubulin confirmed that the microtubule was disrupted by treatment with tubulin inhibitors, with very little detectable tubulin remaining in the treated cells (red fluorescence in Fig. 3, A–C). P-gp-EGFP remained localized on plasma membrane after MCF-7/P-gp-EGFP cells were incubated with tubulin inhibitors (100 μM colchicine and 25 μg/ml nocodazole for 8 h) (Fig. 3, A–C). Furthermore, P-gp-EGFP remained dominantly on the plasma membrane in the presence of the microtubule polymerization agent taxol (5 μM, 8 h) (Fig. 3D). These results demonstrated that microtubule was not required for P-gp-EGFP to move to the plasma membrane. By contrast, there was extensive intracellular P-gp-EGFP accumulation after MCF-7/P-gp-EGFP cells were treated with the actin inhibitor cytochalasin D (1 μM, 1 h) or latrunculin B (1 μM, 1 h) (Fig. 3, F and G), whereas P-gp-EGFP was mainly on the plasma membrane in control cells (Fig. 3E). After treatment with cytochalasin D or latrunculin B, which completely disrupted the actin filament structure, the intracellular P-gp-EGFP distribution differed from the pattern in control cells. Much of the intracellular P-gp-EGFP presented as linear filamentous patterns after treatment with cytochalasin D or latrunculin B. These results suggest that P-gp-EGFP traffic requires intact actin rather than microtubule. To ensure that the EGFP tag itself did not alter the nature of traffic of P-gp, we studied the localization of wild-type P-gp with or without treatment with cytochalasin D in the multidrug-resistant cell line MCF-7/Adr. Immunocytochemistry with anti-P-gp antibody showed that wild-type P-gp was distributed largely intracellularly after treatment with cytochalasin D, which appeared as a linear filament (Fig. 3I), in contrast to control MCF-7/Adr cells, which had P-gp primarily located on the plasma membrane (Fig. 3H). Notably, overall there was less P-gp on the plasma membrane after treatment with cytochalasin D in the MCF-7/Adr cells (Fig. 3I). However, not all the cells had significantly decreased P-gp on the plasma membrane. The differences may be due to different stages of cell proliferation in the cell population. Thus, in contrast to cells where P-gp-EGFP was just starting to be synthesized, the P-gp would not be rapidly degraded after short treatment with actin inhibitors in cells with large amounts of P-gp already located on the plasma membrane. These results confirm that traffic of P-gp is actin dependent and also indicate that the EGFP tag does not influence the P-gp traffic pathway. In addition, with the use of phalloidin-TRITC staining (red), actin was shown to be completely disrupted after treatment with cytochalasin D or latrunculin B, with hardly any red staining (Fig. 3, K and L), whereas the control cells showed the actin structure to be still intact, with red or yellow (merged with green from P-gp-EGFP) staining in the cells (Fig. 3J).

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In addition, immunostaining with EEA1 antibody and CY3 secondary antibody demonstrated that early endosome appeared as a linear structure in some untransfected MCF-7 cells (Fig. 4E), suggesting that some of the linear structure also occurred naturally as early endosome. Furthermore, with the
use of anti-P-gp antibody and CY3-conjugated secondary antibody, immunostaining was conducted to examine whether similar structures were obtained with the wild-type P-gp in MCF-7/Adr cells. Results showed that linear structures also were observed in some of the wild-type P-gp in ~2.5% of the MCF-7/Adr cells (n = 120 cells) (Fig. 4D), suggesting that the trafficking path of P-gp in stable MCF-7/P-gp-EGFP after treatment with actin inhibitors also is present in wild-type P-gp.

Dynamics of P-gp-EGFP traffic. To confirm that P-gp-EGFP moved to the plasma membrane via early endosome, P-gp-EGFP was transiently expressed in MCF-7 cells, to allow dynamic monitoring of the movement of P-gp-EGFP. Immunofluorescence was used to localize early endosome at 10, 16, 20, 24, and 36 h after transient transfection. Cells with colocalization of P-gp-EGFP and early endosome were counted under a microscope. Previous studies showed that P-gp-EGFP was mostly localized at the endoplasmic reticulum at ~10 h posttransfection, whereas P-gp-EGFP was mainly at the Golgi compartment at 20 h of transfection and predominantly moved to the plasma membrane after 24 h posttransfection (10). The percentage of the cells that colocalized P-gp-EGFP and early endosome was analyzed. Results indicated that at the very early stage of transfection, in which P-gp-EGFP was just being synthesized (~10 h posttransfection), very little intracellular P-gp-EGFP was localized in early endosome, with ~2.5% of cells expressing P-gp-EGFP (Fig. 5A). At 16 h posttransfection, 5% of intracellular P-gp-EGFP was localized with early endosome. At 20, 24, and 36 h posttransfection, the percentage of colocalization of intracellular P-gp-EGFP and early endosome was increased to ~19% (Fig. 5A). This result confirmed that P-gp-EGFP trafficked to the plasma membrane via early endosome after synthesis in endoplasmic reticulum and was modified at Golgi. Furthermore, colocalization of intracellular P-gp-EGFP with lysosome at different times posttransfection was also investigated. Results showed that there was rarely any intracellular P-gp-EGFP colocalized with lysosome at 10, 16, and 20 h posttransfection (1–2%). At 24 and 36 h posttransfection there was 11 and 15% of intracellular P-gp-EGFP colocalized with lysosome, respectively (Fig. 5B). In addition, the percentages of early endosome colocalization of P-gp-EGFP appear to be higher than those for lysosome colocalization (Fig. 5, A and B). These results indicated that P-gp-EGFP moved to the membrane via early endosome and flowed to lysosome later.

Internalization of P-gp-EGFP to early endosome. The pathway for traffic of P-gp-EGFP was also studied with the use of CHX to inhibit new protein synthesis (8, 18, 27, 31). P-gp-EGFP was observed to have intracellular localization after MCF-7/P-gp-EGFP cells were incubated with 10 μg/ml CHX for 24 h (Fig. 6, A and D). Western blots showed that the intact P-gp-EGFP protein level was considerably decreased (~42%) after incubation with CHX (10 μg/ml, 24 h), a concentration used previously under similar conditions (8, 27, 28, 31), indicating that synthesis of P-gp-EGFP was indeed inhibited (data not shown). In addition, the percentage of P-gp-EGFP localized on the plasma membrane was notably decreased after treatment of CHX (Fig. 6, A and D). Previous studies have shown that misfolded P-gp lacked glycosylation and was 20 kDa less than its mature form (25). The misfolded P-gp can be rapidly degraded in ER, with a half-life of only ~2 h (26). After treatment with CHX, Western blotting only detected the 190-kDa mature molecular forms of P-gp-EGFP (data not shown). Moreover, the half-life of P-gp-EGFP was ~18 h in the presence of CHX, indicating that P-gp-EGFP degraded was unlikely to be the misfolded form. Together, the results suggest that CHX caused the degradation of P-gp-EGFP protein. Immunocytochemistry demonstrated that the intracellular P-gp-EGFP was extensively colocalized with early endosome (Fig. 6, A–C) and also with lysosome (Fig. 6, D–F). Among the multiple sample of cells having intracellular P-gp-EGFP, ~40% (n = 60) demonstrated early endosome localization and 55% (n = 60) showed lysosome localization. Increased lysosome localization possibly reflected internalization of much of the P-gp-EGFP for subsequent degradation. Since CHX in these experiments was used to inhibit new P-gp-EGFP synthesis, thereby preventing newly synthesized
P-gp-EGFP from moving into early endosome from Golgi on the way to plasma membrane, the P-gp-EGFP appearing in early endosome could only have come from plasma membrane. This suggests P-gp-EGFP undergoes internalization from plasma membrane to early endosome and subsequently moves to the lysosome for possible degradation.

To further confirm that after treatment with CHX the intracellular P-gp-EGFP came from the plasma membrane via endocytosis, we studied dextran internalization in MCF-7/P-gp-EGFP cells. After treatment with CHX for 24 h, colocalization between intracellular P-gp-EGFP (Fig. 7A) and dextran (Fig. 7B) was observed using confocal microscopy, suggesting that the intracellular P-gp-EGFP was actually endocytosed from the plasma membrane (Fig. 7, A–C).

Disrupting traffic of P-gp-EGFP increases the accumulation of daunorubicin in MCF-7/P-gp-EGFP cells and inhibits the proliferation of MCF-7/P-gp-EGFP. To examine whether actin disruption affects drug accumulation as well as P-gp localization, we incubated MCF-7/P-gp-EGFP cells with either 1 μM latrunculin B or 1 μM cytochalasin D for 1 h, and the accumulation of daunorubicin was analyzed quantitatively using the KS400 image analysis software package (Carl Zeiss Vision). After a short incubation time (30 min), daunorubicin was shown to be located predominantly in the cytosol, and the accumulation of daunorubicin was shown to be significantly increased when MCF-7/P-gp-EGFP cells were treated with cytochalasin D or latrunculin B (Fig. 8, A–C). Quantitation indicated an 8.5- to 7.8-fold greater daunorubicin fluorescence (P < 0.001, n = 3) in cells treated with cytochalasin D or latrunculin B, respectively, than in the nontreated MCF-7/P-gp-EGFP cells (Fig. 8D). After a short incubation time with daunorubicin (30 min), daunorubicin was mainly localized in cytosol. However, after 1 h of incubation, daunorubicin was primarily localized in the nucleus (data not shown). This result is consistent with other studies (3, 6) and indicates that daunorubicin penetrated the P-gp barrier on the plasma membrane after treatment with actin inhibitors, resulting in more cellular accumulation.

To determine whether increased intracellular daunorubicin was available to cause greater cytotoxicity, we examined the proliferation of MCF-7/P-gp-EGFP cells. Results showed that MCF-7/P-gp-EGFP cells proliferated normally (95%) compared with control cells after incubation with daunorubicin alone (2 μM, 24 h), whereas the sensitive cells MCF-7 had only 68% proliferation (P < 0.01, n = 3, Fig. 9), indicating the resistance of MCF-7/P-gp-EGFP cells to daunorubicin. Both MCF-7/P-gp-EGFP and MCF-7 cells had decreased proliferation (~76–83%) after incubation with either cytochalasin D (1 μM) or latrunculin B (1 μM), respectively. However, results showed that the growth of MCF-7/P-gp-EGFP cells was significantly inhibited, with ~60 and ~55% proliferation after incubation simultaneously with actin inhibitors and daunorubinc
bicin, respectively ($P < 0.01$, $n = 3$; Fig. 9), compared with control cells. This was similar to the growth rate in sensitive MCF-7 cells ($56$ and $52\%$, respectively; Fig. 9) after simultaneous treatment with actin inhibitors and daunorubicin. Together, these results suggest that actin inhibitors can disrupt P-gp-EGFP traffic and increase accumulation of the anti-cancer drug daunorubicin, resulting in greater inhibition of proliferation of MCF-7/P-gp-EGFP cells.

**DISCUSSION**

Despite previous biochemical studies demonstrating that P-gp interacts with actin ERM protein and that CD44 may be involved in the interaction (2, 24), there is very little known about how P-gp traffics to the plasma membrane after it is synthesized. In the present investigation, stable expression of P-gp-EGFP was established in MCF-7 human breast cancer cells, and clonal cells were employed to study the traffic and cellular localization of P-gp-EGFP. The P-gp-EGFP fusion protein was functional as a drug pump in a way similar to wild-type P-gp, as demonstrated in experiments showing that the stable cells were resistant to colchicine and daunorubicin, established P-gp substrates. The stable MCF-7/P-gp-EGFP cells were observed to be physically more rounded and clumped than untransfected cells. About one-third of the stable
MCF-7/P-gp-EGFP cells had an intracellular P-gp-EGFP, exhibiting a range from low, medium, and high fluorescence intensity. The immunofluorescent results demonstrated that whereas P-gp-EGFP is localized predominantly in plasma membranes, there is a significant intracellular P-gp-EGFP pool in the stable cells. The intracellular P-gp-EGFP was mainly located at the early endosome compartments and also existed in lysosome and Golgi.

To examine the role of the cytoskeleton in the traffic of P-gp, we applied actin depolymerization agents (cytochalasin D and latrunculin B), microtubule depolymerization agents (colchicine and nocodazole), and a microtubule polymerization agent (taxol) to the stable MCF-7/P-gp-EGFP cells and studied the intracellular localization of P-gp-EGFP. Since cytochalasin D and colchicine are substrates of P-gp, non-P-gp substrate agents such as latrunculin B and nocodazole also were used to avoid possible complications from the export of the inhibitors by the pump. The non-P-gp substrates latrunculin B and nocodazole showed effects similar to those of cytochalasin D and colchicine. The localization of P-gp-EGFP remained mainly at the plasma membrane in the presence of colchicine, nocodazole, and taxol, whereas large amounts of P-gp-EGFP were found to accumulate intracellularly following treatment with cytochalasin D and latrunculin B. These results suggested that the actin filament rather than the microtubule played a role in intracellular traffic of P-gp-EGFP.

We made the interesting observation that in the presence of cytochalasin D or latrunculin B, intracellular P-gp-EGFP staining detected the protein to be largely present as linear filament. Similarly, wild-type P-gp in MDR MCF-7/Adr cells also demonstrated linear filament after treatment with actin inhibitors. (Fig. 3I). Furthermore, wild-type P-gp linear structures were detectable in control MCF-7/Adr cells without any treatment (Fig. 4D), suggesting the linear structures may be involved in normal P-gp trafficking. The intracellular linear filament of P-gp-EGFP did not colocalize with microtubules (data not shown), showing that P-gp-EGFP did not use microtubule as a substitute for its traffic after actin was disrupted. The linear filament of P-gp-EGFP after actin disruption was largely colocalized with EEA1, suggesting that P-gp-EGFP was mainly trapped in the early endosome after traffic was disrupted by the actin depolymerization agents. In addition, under larger magnification, the linear early endosome structure was observed to contain early endosome vesicles that line up as the linear structure (data not shown). Interestingly, immunostaining with the EEA1 antibody also showed a small amount of linear early endosome structures in the untransfected MCF-7 cells (Fig. 4E), again suggesting that linear structures of early endosome also occur naturally.

Dynamic studies showed that the numbers of cells with colocalized intracellular P-gp-EGFP and early endosome or lysosome markers increased with increasing time following transient transfection in MCF-7 cells, indicating P-gp-EGFP moved to the plasma membrane via early endosome after being synthesized and then flowed to lysosome (Fig. 5, A and B). Together, these results demonstrated that P-gp-EGFP traffic to plasma membrane occurred via early endosome and that microtubules did not play a role in the traffic of P-gp-EGFP, with the intracellular traffic of P-gp-EGFP being largely actin dependent. Moreover, disruption of actin resulted in retention of P-gp-EGFP intracellularly. Kim et al. (17) showed that there was an intracellular pool of P-gp and P-gp recycled between the plasma membrane and the intracellular pool, suggesting possible endocytosis of P-gp. However, the precise localization of intracellular P-gp using internal compartment markers was not examined at that time. To investigate whether P-gp-EGFP undergoes endocytosis, the intracellular movement of P-gp-EGFP was examined in stable MCF-7/P-gp-EGFP cells in the presence of CHX, used to inhibit the synthesis of new P-gp-EGFP (4, 15). The observation that P-gp-EGFP was localized intracellularly after treatment with CHX indicated that P-gp-EGFP moved from the plasma membrane into an intracellular compartment. This was supported by our results showing that internalized dextran colocalized with intracellular P-gp-EGFP after treatment with CHX, suggesting that P-gp-EGFP undergoes endocytosis (Fig. 7), with P-gp-EGFP internalizing from plasma membrane into early endosome and lysosome compartments.

The accumulation of daunorubicin significantly increased (8.5- and 7.8-fold, respectively) after MCF-7/P-gp-EGFP cells were treated with cytochalasin D and latrunculin B, most likely as a consequence of disruption of P-gp-EGFP traffic by actin inhibitor. Furthermore, proliferation of MCF-7/P-gp-EGFP cells decreased to 60 and 55% of that of control cells after MCF-7/P-gp-EGFP cells were treated simultaneously with daunorubicin and cytochalasin D or latrunculin B, whereas 97% of MCF-7/P-gp-EGFP cells proliferated after incubation with 2 μM daunorubicin alone for 24 h. These data suggest that interruption of traffic of P-gp-EGFP results in increased accumulation of daunorubicin and concomitantly significantly increased inhibition of cell proliferation. Third-generation P-gp reversal agents, such as LY-335979, are currently in phase III trials. However, some potential limitations with this compound are that phase I results reported some risk of neurotoxicity at high dosage, and the lack of observed pharmacokinetic interaction with doxorubicin may signify a lack of direct effect of the compound on hepatic or renal P-glycoprotein and hence the possibility that it may not affect tumor cell P-glycoprotein (32). Finding new reversal agents to overcome MDR in cancer is always urgent in cancer chemotherapy. Our results suggest that interrupting the intracellular traffic of P-gp-EGFP, and in particular by inhibiting the interaction between P-gp and actin, may provide a potential pathway for overcoming MDR in chemotherapy.

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GRANTS

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REFERENCES

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