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Folate Deprivation Results in the Loss of Breast Cancer Resistance Protein (BCRP/ABCG2) Expression

A ROLE FOR BCRP IN CELLULAR FOLATE HOMEOSTASIS*

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Breast cancer resistance protein (BCRP/ABCG2) is currently the only ABC transporter that exports mono- and polyglutamates of folates and methotrexate (MTX). Here we explored the relationship between cellular folate status and BCRP expression. Toward this end, MCF-7 breast cancer cells, with low BCRP and moderate multidrug resistance protein 1 (MRP1/ABCC1) levels, and their mitoxantrone (MR)-resistant MCF-7/MR subline, with BCRP overexpression and low MRP1 levels, were gradually deprived of folic acid from 2.3 μM to 3 nM resulting in the sublines MCF-7/LF and MCF-7/MR-LF. These cell lines expressed only residual BCRP mRNA and protein levels and retained a poor MRP2 (ABCC2) through MRP5 (ABCC5) expression. Furthermore, MCF-7/MR-LF cells also displayed 5-fold decreased MRP1 levels relative to MCF-7/MR cells. In contrast, BCRP overexpression was largely retained in MCF-7/MR cells grown in MR-free medium containing 2.3 μM folic acid. Loss of BCRP expression in MCF-7/LF and MCF-7/MR-LF cells resulted in the following: (*a*) a prominent decrease in the efflux of Hoechst 33342, a BCRP substrate; (*b*) an ~2-fold increase in MR accumulation as revealed by flow cytometry; this was accompanied by a 2.5- and ~84-fold increased MR sensitivity in these cell lines, respectively. Consistently, Ko143, a specific BCRP inhibitor, rendered MCF-7 and MCF-7/MR cells 2.1- and ~16.4-fold more sensitive to MR, respectively. Loss of BCRP expression also resulted in the following: (*c*) an identical MTX sensitivity in these cell lines thereby losing the ~28-fold MTX resistance of the MCF-7/MR cells; (*d*) an ~2-fold increase in the 4- and 24-h accumulation of [^{3}H]folic acid. Furthermore, MCF-7/MR-LF cells displayed a significant increase in folylpoly-γ-glutamate synthetase activity. Hence, consistent with the mono- and polyglutamate folate exporter function of BCRP, down-regulation of BCRP and increased folylpoly-γ-glutamate synthetase activity appear to be crucial components of cellular adaptation to folate deficiency conditions. This is the first evidence for the possible role of BCRP in the maintenance of cellular folate homeostasis.

Reduced folate cofactors play a key role in one-carbon transfer reactions in the *de novo* biosynthesis of purines and thymidylate (1). As such, normal and neoplastic dividing cells have an absolute requirement for reduced folates in order to properly initiate and complete DNA replication and mitosis (1). Disruption of one-carbon transfer reactions in the folate metabolic pathway by folic acid antagonists (*i.e.* antifolates) is the pharmacological basis for the antitumor activity of methotrexate (MTX)[1] (2) as well as the novel antifolates raltitrexed (3) and pemetrexed (4). Because mammalian cells are devoid of *de novo* biosynthesis of folic acid, they must obtain this folate vitamin from exogenous sources (1). Membrane transport of folates and MTX is mediated by several systems (5, 6). (*a*) The reduced folate carrier (RFC) is the major uptake route that functions as a bi-directional anion exchanger (7, 8) taking up folates through an antiport exchange mechanism with intracellular organic phosphates (9). (*b*) Folate receptors mediate the unidirectional uptake of folate cofactors into mammalian cells via an endocytotic process (5, 10). (*c*) An apparently independent transport system with optimal folate uptake activity at low pH (11–13) is also known. Following uptake, the enzyme folylpoly-γ-glutamate synthetase (FPGS) catalyzes the addition of several equivalents of L-glutamic acid to the γ-carboxyl group in the side chain of folate cofactors and antifolates like MTX (14). This long chain polyglutamate conjugation (up to 10 glutamate residues) is believed to fulfill three cellular functions as follows. (*a*) By rendering folates and antifolates polyanionic, it dramatically increases their cellular retention by preventing efflux (15). (*b*) It increases the binding affinity of folate cofactors and antifolates to some folate-dependent enzymes (4, 16). (*c*) It appears to facilitate, via mitochondrial FPGS activity, the accumulation in mitochondria of folate polyglutamates that are required for glycine biosynthesis (15).

Cellular folate pools are controlled by the above folate influx systems, by FPGS activity, and by ATP-dependent efflux transporters of the ABCC sub-family (17). Recent studies (18–22) have established an increased energy-dependent folate and MTX transport into inverted membrane vesicles isolated from cell lines with MRP1 (ABCC1) through MRP4 (ABCC4) overexpression. This MTX efflux in various tumor cell lines with MRP overexpression resulted in a significant resistance to MTX predominantly upon a short term (≤4 h) antifolate exposure (18–20). The restriction of this antifolate resistance to only a short term drug exposure has been attributed to the ability of these transporters to export only monoglutamate but not polyglutamate conjugates of MTX; indeed, these transport-

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[1] The abbreviations used are: MTX, methotrexate; BCRP, breast cancer resistance protein; MRP, multidrug resistance protein; MR, mitoxantrone; FPGS, folylpoly-γ-glutamate synthetase; LF, low folate; HF, high folate; RFC, reduced folate carrier.

ers failed to transport MTX diglutamates or longer chain polyglutamates (21, 22).

In contrast to the restricted ability of MRP1 through MRP4 to export only monoglutamate forms of folates and MTX, the breast cancer resistance protein (BCRP/ABCG2) has been recently found to transport both mono-, di-, and triglutamate conjugates of folic acid and MTX in membrane vesicles isolated from tumor cell lines with BCRP overexpression (23, 24). This enhanced efflux of MTX in various tumor cell lines with the wild type BCRP (Arg-482) resulted in a marked level of resistance to this drug (25, 26). However, in contrast to the wild type BCRP, two BCRP variants Thr-482 and Gly-482 completely lost their ability to transport folates and MTX (23, 24, 27) and therefore displayed a wild type sensitivity to MTX (24).

Apart from MTX, BCRP also extrudes various anticancer drugs including MR, camptothecin-based and indocarbazole topoisomerase I inhibitors, flavopiridol, and quinazoline ErbB1 inhibitors (28). Furthermore, efflux of anthracyclines is variable and appears to depend on the presence of the above BCRP mutation at position 482.

Here we initiated studies aimed at exploring the possible involvement of BCRP in the maintenance of cellular folate homeostasis. As a first step toward this end, MCF-7 and MCF-7/MR cells with low and high BCRP levels, respectively, were gradually deprived of folic acid. The resulting cell lines growing under low folate (LF) conditions displayed a nearly complete loss of BCRP mRNA and protein expression. Consequently, these cell lines displayed a prominently diminished Hoechst 33342 efflux activity thereby resulting in markedly increased sensitivity to both mitoxantrone (MR) and MTX. Consistent with the ability of BCRP to export folate triglutamates, these LF-adapted cell lines accumulated significantly more [^{3}H]folic acid than their parental counterparts. Furthermore, these cell lines displayed a significantly increased FPGS activity. These results constitute the first evidence that down-regulation of BCRP expression along with increased FPGS activity are essential components of cellular adaptation to folate deficiency conditions. These findings suggest a functional role for BCRP in the maintenance of folate homeostasis.

EXPERIMENTAL PROCEDURES

Chemicals—Folic acid, MTX, and 3,3′-diaminobenzidine tetrahydrochloride were obtained from Sigma. Mitoxantrone hydrochloride was from Cyanamid of Great Britain Ltd. (Gosport, Hampshire, UK). Ko143 was generously provided by Dr. A. H. Schinkel, The Netherlands Cancer Institute, Amsterdam, The Netherlands. Hoechst 33342 was purchased from Molecular Probes (Eugene, OR). [3′,5′,7′,9-^{3}H]Folic acid (69 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA) and purified prior to use as described previously (29).

Tissue Culture and Folic Acid Deprivation—The human breast cancer cell line, MCF-7, and its MR-resistant MCF-7/MR subline (originally termed MCF-7/Mitox, see Ref. 30) with BCRP overexpression (31) were grown as monolayers in RPMI 1640 medium containing 2.3 μM folic acid (Biological Industries, Beth-Haemek, Israel), 10% fetal calf serum (Invitrogen) supplemented with 2 mM glutamine and 100 μg/ml penicillin and streptomycin. The growth medium of MCF-7/MR cells also contained 0.1 μM MR. In order to establish cell lines growing under LF conditions, MCF-7 and MCF-7/MR cells were gradually deprived of folic acid from 2.3 μM (the standard concentration in RPMI 1640 medium) to 3 nM resulting in the sublines MCF-7/LF and MCF-7/MR-LF; this was achieved over a period of 3 and a half months in a folic acid-free RPMI 1640 medium (Biological Industries, Beth-Haemek, Israel) supplemented with 10% dialyzed fetal calf serum (Invitrogen) to which gradually decreasing folic acid concentrations were added. In order to examine the stability of BCRP expression during the omission of MR from the growth medium, MCF-7/MR-HF cells were continuously cultured in MR-free medium containing 2.3 μM folic acid.

The human ovarian carcinoma cell line, 2008, and its sublines stably transduced with MRP1, MRP2, and MRP3 cDNAs were kindly provided by Prof. P. Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands), whereas HEK293/MRP4 and HEK293/MRP5 cells were used as positive controls for MRP4 and MRP5 overexpression, respectively. These cell lines were cultured in RPMI 1640 medium containing 2.3 μM folic acid, 10% fetal calf serum, 2 mM glutamine, and antibiotics.

Growth Inhibition with MR and MTX—For MR growth inhibition, cells (1×10^4–1.75×10^4/well) were seeded in 24-well plates in growth medium containing various concentrations of MR for 3–5 days at 37 °C. For MTX growth inhibition, cells were allowed to attach for 24 h at 37 °C. Attached cells were then exposed to various concentrations of MTX for 4 h at 37 °C, following which the drug-containing medium was aspirated, and three successive 10-min washes in RPMI 1640 containing 10% dialyzed fetal bovine serum at 37 °C were performed. Drug-free medium was added (2 ml/well), and cultures were incubated for 4 days at 37 °C. After incubation with MR or MTX, cells were detached by trypsinization, and the number of viable cells was determined by using trypan blue exclusion.

Western Blot Analysis of MRPs and BCRP Expression—To examine the expression of various MRPs and BCRP in the different cell lines, non-ionic detergent-soluble proteins were extracted from $\sim 2 \times 10^7$ cells in a buffer (250 μl) containing 50 mM Tris, pH 7.5, 50 mM β-mercaptoethanol, 0.5% Triton X-100, and the protease inhibitors aprotinin (60 μg/ml), leupeptin (5 μg/ml), phenylmethylsulfonyl fluoride (10 μg/ml), and EGTA (1 mM). Following 1 h of incubation on ice, the extract was centrifuged at 15,000 $\times$ *g* for 30 min at 4 °C, and the supernatant containing the fraction of Triton X-100-soluble proteins was collected, and protein content was determined using the Bio-Rad protein assay. Proteins (6–60 μg) were resolved by electrophoresis on 7% (for MRPs) or 10% (for BCRP) polyacrylamide gels containing SDS and electroblotted onto a Protran BA83-cellulose nitrate membrane (Schleicher & Schuell). The blots were blocked for 1 h at room temperature in TBS buffer (150 mM NaCl, 0.5% Tween 20, 10 mM Tris, pH 8.0) containing 1% skim milk. The blots were then reacted with the following anti-human MRP monoclonal antibodies (kindly provided by Prof. R. J. Scheper, Vrije Universiteit Medical Center, Amsterdam, The Netherlands): rat anti-MRP1 (MRP-r1; 1:1000), -MRP4 (M_4I-10; 1:500), -MRP5 (M_5I-1; 1:750), and -BCRP (BXP-53; 1:1000) as well as mouse anti-MRP2 (M_2III-5; 1:50) and -MRP3 (M_3II-21; 1:500). Blots were then rinsed in the same buffer for 10 min at room temperature and reacted with horseradish peroxidase-conjugated goat anti-mouse or anti-rat IgG (1:10,000 dilution, Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. Following three 10-min washes in TBS at room temperature, enhanced chemiluminescence detection was performed according to the manufacturer's instructions (Biological Industries, Beth-Haemek, Israel). To normalize for loading differences, the nylon membranes were stripped and reacted with an antibody against β-tubulin (clone 2-28-33 from Sigma; 1:4000).

Immunohistochemistry Studies—Mid-logarithmic monolayers in 24-well plates were washed twice with PBS and fixed with 4% formaldehyde for 10 min. Endogenous peroxidase activity was neutralized by incubation for 20 min in a solution consisting of 4 volumes of methanol and 1 volume of 3% H_2O_2 in double distilled water. The fixed cells were washed twice with PBS, blocked for 1 h at room temperature in PBS containing 1% skim milk, and reacted with an anti-BCRP monoclonal antibody BXP-53 (1:100 dilution). Then a horseradish peroxidase-conjugated goat anti-rat IgG (1:100 dilution) was added. Color development was performed with the chromogen 3,3′-diaminobenzidine (0.6 mg/ml) in a solution containing 50 mM Tris-Cl, pH 7.6, and 0.02% H_2O_2. After counterstaining with hematoxylin, cells were examined with an Olympus BH-2 upright light microscope.

On-line Efflux of Hoechst 33342—Efflux of Hoechst 33342 was measured by using an on-line computerized method. Cells were cultured on glass coverslips that fitted to the wall of a $1 \times 1 \times 4$-cm cuvette (width, depth, and height, respectively). Cells were loaded with 10 μM Hoechst 33342 for 2 h at 37 °C in a phenol red-free RPMI 1640 medium (Invitrogen) until steady state was achieved. Cells were then transferred to ice until the efflux was initiated. To follow Hoechst 33342 efflux, the coverslips were washed twice with ice-cold medium and then placed in a cuvette that contained 3 ml of warm (37 °C) medium. The fluorescence in the extracellular medium originating from the extruded chromophore was monitored on-line with a spectrofluorometer (FluoroMax, SPEX Industries, Edison, NJ). Hoechst 33342 fluorescence was measured every second at an ultraviolet excitation of 318 nm and emission at 460 nm. To correct for fluctuations in the number of cells/slide, cell numbers were determined by adding 0.4 μM of the DNA dye Syto 13 (Molecular Probes, Eugene, OR). The fluorescence of Syto 13 was determined at excitation and emission wavelengths of 485 and 520 nm, respectively. Hoechst 33342 fluorescence was then normalized for the individual Syto 13 signals.

[^{3}H]Folic Acid Accumulation—Adherent cells ($\sim 1 \times 10^6$) in 6-cm

Petri dishes (Nunc) were washed three times in folic acid-free RPMI 1640 containing 10% dialyzed fetal calf serum (Invitrogen). Following an equilibration for 2 h in the same medium at 37 °C, [^{3}H]folic acid (69 Ci/mmol, Moravek Biochemicals, Brea, CA) was added to a final concentration of 1 μM (specific radioactivity 1,200 dpm/pmol) and incubated at 37 °C for 4 and 24 h. Transport was stopped by the addition of 10 ml of ice-cold HBS containing 20 mM Hepes, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, and 5 mM D-glucose, pH 7.4, with NaOH (32). Cells were then washed three times in ice-cold HBS, detached by trypsinization, counted, and centrifuged at 500 × *g* for 5 min at 4 °C, and the radioactivity was determined using an Ultima Gold scintillation fluid and a scintillation spectrometer (Packard Instrument Co.). Controls for accumulation of radiolabeled folic acid contained a 1000-fold excess of unlabeled folic acid (1 mM).

Flow Cytometric Analysis of MR Staining—Exponentially growing MCF-7, MCF-7/MR, and their LF-adapted cell sublines were trypsinized, adjusted to a density of 5×10^5–1×10^6/ml, and incubated in growth medium containing 20 μM MR for 1 h at 37 °C. Cells were then harvested by centrifugation at 4 °C, washed once with ice-cold PBS, and analyzed for mean fluorescence intensity per cell by a FACSCalibur flow cytometer (BD Biosciences). Excitation was at 633 nm and emission at 661 nm. Autofluorescence intensities of unstained cells were recorded and subtracted from those of MR-stained cells.

FPGS Activity Assay—Frozen pellets of 2×10^7 cells grown in the absence of drugs for 3–4 passages were suspended in 0.5 ml of an extraction buffer containing 50 mM Tris-HCl, 20 mM KCl, 10 mM $MgCl_2$, and 5 mM dithiothreitol, pH 7.5. Total cell extracts were obtained by sonication (MSE Soniprep, amplitude 14 μm, three times for 5 s with 10-s intervals at 4 °C) followed by centrifugation at 12,000 × *g* for 15 min at 4 °C. The FPGS activity assay mixture contained 200 μg of protein, 4 mM L-[2,3-^{3}H]glutamic acid (specific activity 6.6 mCi/mmol) and 250 μM MTX in a buffer consisting of 100 mM Tris, pH 8.5, 10 mM ATP, 20 mM $MgCl_2$, 20 mM KCl, and 10 mM dithiothreitol in a final volume of 250 μl (33). Following 2 h of incubation at 37 °C, the reaction was stopped by adding 1 ml of an ice-cold solution containing 5 mM unlabeled L-glutamic acid. Sep-Pak C_{18} cartridges (Millipore, Waters Associates, Etten-Leur, The Netherlands) were used for the separation of free, unreacted L-[^{3}H]glutamate from MTX-[^{3}H]diglutamate. Controls lacking MTX were included in order to correct for polyglutamylation of endogenous folates present in the cell extract.

Semi-quantitative RT-PCR and DNA Sequencing—Following RNA isolation using the TriReagent protocol (Sigma) and cDNA synthesis, a 172-bp human BCRP fragment was PCR-amplified using the upstream primer 5′-TGCCCAAGGACTCAATGCAACA-3′ and the downstream primer 5′-ACAATTTCAGGTAGGCAATTGTG-3′ as described previously (34). The primers for glyceraldehyde-3-phosphate dehydrogenase, and PCR conditions were as described recently (35).

To analyze whether BCRP in parental MCF-7 cells and their MCF-7/MR subline harbored the wild type Arg-482, we performed DNA sequencing of this region using an ABI Prism 310 DNA Sequencer (AME Bioscience). To this end, BCRP primers were designed using the LightCycler Probe Design Software version 1.0 (Idaho Technology Inc.); the upstream and downstream primers are 5′-CAGCGGATACTACAGAG-3′ and 5′-GCCGTAAATCCATATCGTG-3′, respectively. All cell lines were homozygous for the wild type Arg-482.

Statistical Analysis—We used a Student's *t* test to examine the significance of the difference between two populations for a certain variable. A difference between the average of two populations was considered significant if the *p* value obtained was <0.05.

RESULTS

Loss of BCRP Expression in the LF-adapted Cell Lines as Revealed by Western Blot Analysis—The levels of BCRP as well as MRP1 through MRP5 were determined in MCF-7/LF and MCF-7/MR-LF cells relative to their parental counterparts (Fig. 1). Western blot analysis revealed that folate deprivation in MCF-7/LF cells resulted in an 18-fold decrease in BCRP levels, relative to parental MCF-7 cells (Fig. 1*A*); in contrast, no changes were observed in MRP1 levels (Fig. 1*B*). Similarly, whereas MR-resistant MCF-7/MR cells displayed an ~55-fold BCRP overexpression, relative to parental MCF-7 cells, the MCF-7/MR-LF subline expressed only residual BCRP levels (Fig. 1*A*); remarkably, these barely detectable levels of BCRP in MCF-7/MR-LF cells were ~4-fold lower than those present in MCF-7 cells. Furthermore, folate deprivation in MCF-7/MR-LF cells resulted in a simultaneous 5-fold decrease in MRP1 levels, relative to their parental MCF-7/MR cells (Fig. 1*B*). It should be noted that when compared with parental MCF-7 cells, MCF-7/MR cells contained 3-fold less MRP1 levels even before gradual folate deprivation was initiated. Most important, MCF-7/MR-HF cells grown in the absence of MR but in the continuous presence of 2.3 μM folic acid had only a slight decrease in BCRP expression, relative to the near-complete loss of BCRP in MCF-7/MR-LF cells (Fig. 1*A*).

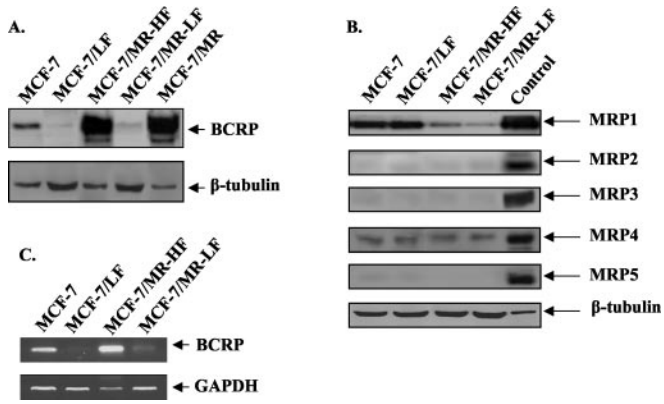

FIG. 1. **Western blot analysis of BCRP as well as MRP1 through MRP5 expression in parental cells and their LF-adapted cell lines.** Aliquots of Triton X-100-soluble membrane proteins (6–60 μg) were separated by electrophoresis on 7.5% polyacrylamide gels containing SDS and electroblotted onto a Protran nylon membrane. Then the membranes were reacted with monoclonal antibodies against BCRP (*A*) or MRP1 through MRP5 (*B*), following which a second peroxidase-conjugated antibody was added, and membranes were developed using a standard ECL procedure. To correct for loading differences, the blots were stripped and reprobed with an antibody against β-tubulin. Note that in order to estimate the barely detectable BCRP expression in the LF-adapted cell lines (*A*), the MCF-7/LF and MCF-7/MR-LF lanes were intentionally loaded with excess protein (60 μg) relative to their parental counterparts (30 μg). The *control lane* in *B* contained membrane protein extracts (6 μg) from cell lines with overexpression of MRP1 through MRP5 as described under "Experimental Procedures." Semi-quantitative RT-PCR analysis was performed in order to estimate BCRP gene expression in the LF-adapted cell lines as compared with their parental cells (*C*). A parallel RT-PCR with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used in order to normalize for the amounts of total cDNA used in each lane (see "Experimental Procedures"). Note that an ~3-fold lower level of MCF-7/MR-HF cDNA was analyzed in order to retain comparability of signal intensity (*C*).

Retention of Poor MRP2 through MRP5 Expression in the LF-adapted Cell Lines—MRP2, MRP3, and MRP4 have the facility to export folates, whereas previously, MRP5 was not shown to export folates and MTX (summarized in Ref. 17); therefore, we determined the levels of these transporters in the LF-adapted cell lines (Fig. 1*B*). MRP2, MRP3, and MRP5 were essentially undetectable in both parental cells and their LF-adapted sublines, whereas MRP4 was expressed at equally low levels in both cells lines (Fig. 1*B*). Reprobing with a β-tubulin monoclonal antibody was used to correct for any differences in the amounts of Triton X-100-soluble proteins that were being analyzed (Fig. 1, *A* and *B*).

Poor BCRP Gene Expression in the LF-adapted Cell Lines as Revealed by RT-PCR—We next examined by semi-quantitative RT-PCR analysis whether the marked loss of BCRP protein levels in the LF-adapted cell lines was because of decreased BCRP gene expression. Parental MCF-7 cells expressed notable levels of BCRP mRNA, whereas MCF-7/MR-HF cells displayed a prominent overexpression of BCRP mRNA (Fig. 1*C*). In contrast, the LF-adapted sublines MCF-7/LF and MCF-7/MR-LF contained only residual levels of BCRP mRNA (Fig. 1*C*, *upper panel*). An RT-PCR of a housekeeping gene (glyceraldehyde-3-phosphate dehydrogenase) confirmed that comparable

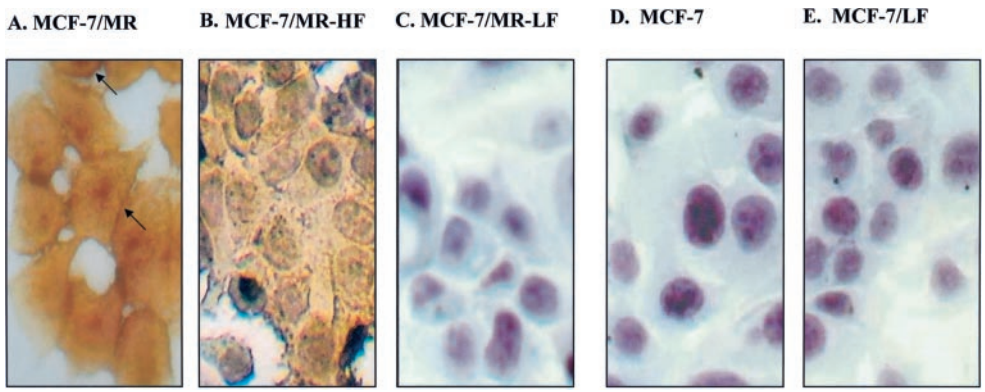

FIG. 2. **Immunohistochemical detection of BCRP expression in parental cells and their LF-adapted cell lines.** Exponentially growing MCF-7/MR (*A*), MCF-7/MR-HF (*B*), MCF-7/MR-LF (*C*), MCF-7 (*D*), and MCF-7/LF (*E*) cells in 24-well culture plates were fixed with 4% formaldehyde and reacted with the anti-BCRP monoclonal antibody BXP-53. Then a horseradish peroxidase-conjugated rabbit anti-mouse IgG was added, and color development was carried out using the chromogen 3,3′-diaminobenzidine. Finally, cells were counterstained with hematoxylin and examined with a light microscope at a ×200 magnification. The *arrows* in *A* denote the plasma membrane localization of BCRP in MCF-7/MR cells growing in the continuous presence of 0.1 μM MR. Note that MCF-7/MR-HF cells (*B*) were grown for 3 and a half months in MR-free medium containing 2.3 μM folic acid.

levels of cDNA were being analyzed in the various cell lines (Fig. 1*C*, *lower panel*).

Loss of BCRP Expression in the LF-adapted Cell Lines as Revealed by Immunohistochemistry—To confirm the loss of BCRP expression in the LF-adapted cell lines, we performed immunohistochemistry with BXP-53, a novel monoclonal antibody to BCRP (Fig. 2). MCF-7/MR cells growing in the continuous presence of 0.1 μM MR displayed an intense cellular staining (Fig. 2*A*) and a clear plasma membrane localization (Fig. 2*A, arrows*). Consistently, MCF-7/MR-HF cells growing in a medium lacking MR but containing 2.3 μM folic acid retained a relatively strong cellular staining (Fig. 2*B*). In contrast, the poor BCRP expression in MCF-7/MR-LF cells (Fig. 2*C*), MCF-7/LF cells (Fig. 2*E*), and the low BCRP levels in parental MCF-7 cells (Fig. 2*D*) were below the level of detection by immunohistochemistry.

Loss of Hoechst 33342 Efflux in the LF-adapted Cell Lines—In order to determine whether the marked loss of BCRP expression in the LF-adapted cell lines was associated with a parallel fall in BCRP export activity, we used an on-line efflux assay (36) of Hoechst 33342. In this functional assay, monolayer cells were loaded with 10 μM of the chromophore Hoechst 33342, an established BCRP efflux substrate (37). After 2 h of loading at 37 °C, the culture medium was replaced by a fluorophore-free medium, and the extent of Hoechst 33342 efflux was continuously monitored by measuring the increase in the fluorescence in the external medium (Fig. 3). MCF-7/MR-HF cells with BCRP overexpression displayed a marked efflux of Hoechst 33342, whereas parental MCF-7 cells with low BCRP expression had a lower efflux. In contrast, the LF-adapted sublines MCF-7/LF and MCF-7/MR-LF cells, which essentially lost BCRP expression, had only a background efflux of Hoechst 33342; curve fitting of these efflux data confirmed the poor efflux of Hoechst 33342 in the LF-adapted cell lines (Fig. 3, *inset*). Thus, loss of BCRP expression was accompanied by a parallel fall in the efflux of Hoechst 33342, an established BCRP substrate.

Accumulation of MR in the LF-adapted Cell Lines as Revealed by Flow Cytometry—We further explored by flow cytometry whether the loss of BCRP expression and efflux function would consistently result in increased MR accumulation in the LF-adapted cell lines (Fig. 4). Indeed, upon a 1-h incubation with 20 μM MR, MCF-7/MR-LF and MCF-7/LF cells displayed statistically significant ($p = 0.003$ and $p = 0.018$, respectively) increases of 1.9- and 2.0-fold in the net accumulation of MR, respectively, relative to their parental cells (Fig. 4).

Sensitivity of the LF-adapted Cell Lines to MR—We therefore examined whether the loss of BCRP expression and MR efflux activity was also accompanied by an increase in MR sensitivity in the LF-adapted cell lines. MCF-7/LF cells that essentially lost BCRP expression exhibited a 2.5-fold increased sensitivity to MR, relative to parental MCF-7 cells (Fig. 5*A*). Consistently, MCF-7/MR-HF cells displayed a 34-fold resistance to MR, relative to parental MCF-7 cells (Fig. 5*B*), whereas MCF-7/MR-LF cells that lost BCRP expression and efflux activity exhibited 84-fold increased sensitivity to MR, relative to their MCF-7/MR-HF counterpart (Fig. 5*B*). Most important, disruption of BCRP efflux activity in MCF-7 and MCF-7/MR-HF cells by the specific and potent BCRP inhibitor Ko143 (38) rendered these cell lines 2.1- and ~16.4-fold more sensitive to MR, respectively (Fig. 5, A and *B*, respectively).

Loss of MTX Resistance in MCF/MR-LF Cells—BCRP was shown to export MTX, thereby conferring resistance to this antifolate, particularly upon short term drug exposure (25, 26). When compared with parental MCF-7 cells, MCF-7/MR cells displayed only 2-fold resistance upon a continuous MTX exposure (Fig. 6*A*) but as high as 28-fold resistance to MTX upon 4 h of antifolate exposure (Fig. 6*B*). In contrast, loss of BCRP expression in MCF-7/MR-LF cells resulted in a complete loss of MTX resistance upon 4 h of drug exposure, thereby resulting in an IC_{50} value (~80 nM) that was identical to that obtained with MCF-7/LF cells (Fig. 6*C*).

Increased Accumulation of [^{3}H]Folic Acid in the LF-adapted Cell Lines—We next examined the ability of the LF-adapted cell lines to accumulate [^{3}H]folic acid as compared with their parental cell lines. After 4 (Fig. 7*A*) and 24 h of incubation (Fig. 7*B*) with 1 μM [^{3}H]folic acid at 37 °C, MCF-7/LF and MCF-7/MR-LF cells displayed an ~2-fold increase in the accumulation of [^{3}H]folic acid relative to their parental counterparts. This increased accumulation of [^{3}H]folic acid after 4 and 24 h of incubation was statistically significant for both MCF-7/LF ($p = 0.015$ and 0.026, respectively) and MCF-7/MR-LF cells ($p = 0.031$ and 0.033, respectively), when compared with their parental cells. In contrast, the trivial elevation in the accumulation of [^{3}H]folic acid after 4 and 24 h in MCF-7/MR-HF cells relative to MCF-7/MR cells was not statistically significant ($p = 0.549$ and 0.249, respectively).

Increased Folylpoly-γ-glutamate Synthetase Activity in the LF-adapted Cell Lines—In a previous study we have shown that gradual folate deprivation achieved by a stepwise increase in the antifolate pressure resulted in a substantial increase in FPGS activity (39) in pyrimethamine-resistant Chinese ham-

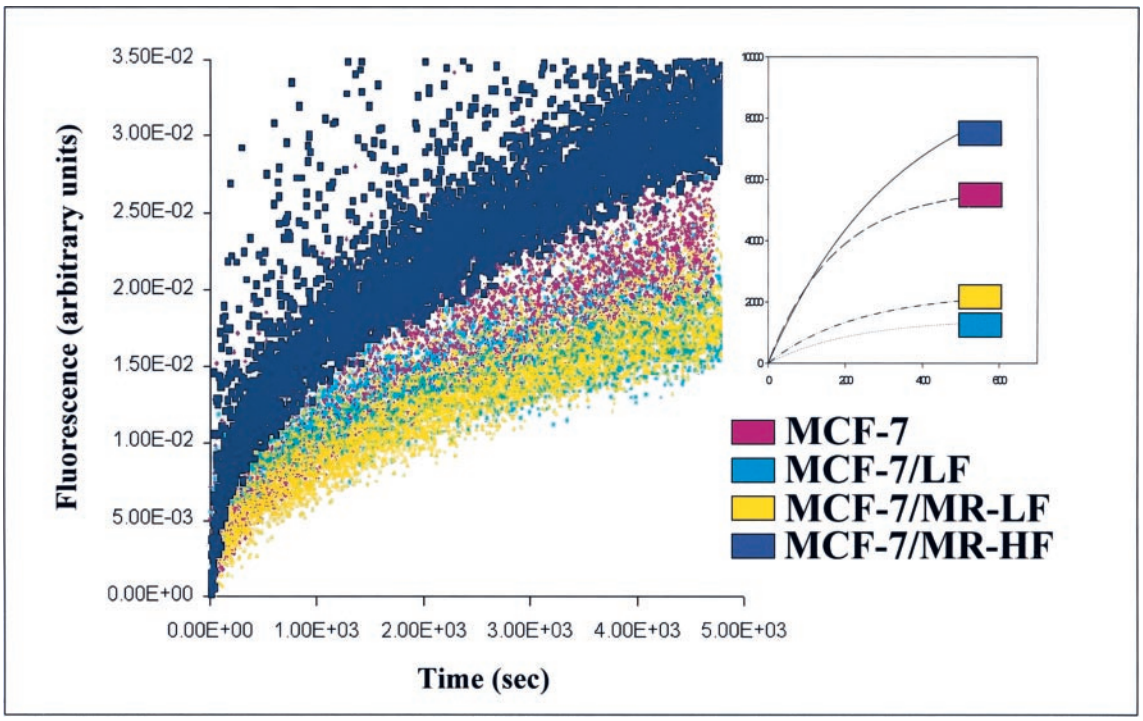

FIG. 3. **On-line efflux of Hoechst 33342 from monolayers of parental and LF-adapted cell lines.** Following loading of MCF-7/MR-HF (*dark blue tracing*), MCF-7/MR-LF (*yellow*), MCF-7 (*purple*), and MCF-7/LF cells (*light blue/green*) with 10 μM Hoechst 33342, cells were washed, and the fluorescence of the extruded chromophore in the extracellular medium was continuously monitored (every second for up to 5000 s) by an on-line computerized spectrofluorometer. The fluorescence depicted was obtained after normalization for differences in the number of cells per well; this was achieved by DNA staining with Syto 13 as described under "Experimental Procedures." *Inset,* curve-fitting of the Hoechst 33342 efflux data was performed as described previously by Wielinga *et al.* (46).

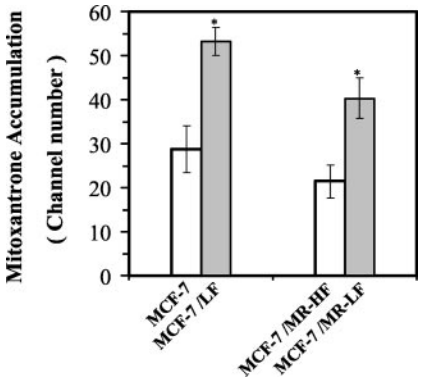

FIG. 4. **Flow cytometric analysis of MR accumulation in parental cells and their LF-adapted cell lines.** Exponentially growing cells were detached by trypsinization and incubated in growth medium containing 20 μM MR for 1 h at 37 °C. Cells were then washed with ice-cold PBS and analyzed for mean linear fluorescence per cell using a flow cytometer. Cellular MR fluorescence was obtained after subtraction of the autofluorescence of unstained cells. Results presented are means ± S.D. of three independent experiments performed in duplicate. The *asterisks* denote statistically significant (Student's *t* test) changes in MCF-7/LF *versus* its parental MCF-7 cells, as well as MCF-7/MR-LF *versus* its MCF-7/MR-HF parental counterpart.

ster ovary cells (40). Here, consistently, folate deprivation resulted in statistically significant increases of 63 ($p = 0.001$) and 20% ($p = 0.008$) in FPGS activity in MCF-7/MR-LF and MCF-7/LF cells, respectively, relative to their parental counterparts (Fig. 8). In summary, the gradual folate deprivation in MCF-7 and MCF-7/MR cells resulted in the loss of BCRP expression and efflux function as well as in a significant increase in the activity of FPGS, the key enzyme responsible for cellular retention of long chain (>3 glutamate residues) folate polyglutamates.

DISCUSSION

Whereas MRP1 through MRP4 export folate and MTX monoglutamates, only BCRP has the facility to extrude both mono-, di-, and triglutamate conjugates of folic acid and MTX (23, 24). We hence undertook the present study in order to explore the possible role of BCRP in folate homeostasis in cells with a ubiquitous expression of MRP1. To this end, we first examined the relationship between folate status and BCRP expression. We therefore performed a gradual folic acid deprivation by using two sets of breast cancer cell lines as follows: parental MCF-7 cells with low BCRP expression and moderate MRP1 levels, as well as their MR-resistant MCF-7/MR subline with high levels of BCRP but low levels of MRP1. Several lines of evidence established that folate deprivation resulted in a dramatic down-regulation of BCRP expression and efflux function. (*a*) Semi-quantitative RT-PCR and Western blot analyses revealed only residual BCRP gene expression and protein levels, respectively, in both LF-adapted cell lines. (*b*) Immunohistochemistry studies demonstrated that MCF-7/MR cells had an intense plasma membrane staining and a marked intracellular staining, whereas their LF-adapted subline MCF-7/MR-LF was devoid of plasma membrane and cytoplasmic staining. (*c*) Although MCF-7/MR-HF and MCF-7 cells displayed a high and low efflux of the BCRP substrate Hoechst 33342, respectively, only residual chromophore efflux was obtained with the LF-adapted sublines. (*d*) Consistently, the LF-adapted cell lines exhibited a significant increase in the net accumulation of MR, relative to their parental cells. (*e*) Folate deprivation resulted in the loss of resistance to the established BCRP substrates MR and MTX in MCF-7/MR-LF cells as well as in a markedly increased sensitivity of MCF-7/LF cells to these drugs. Furthermore, the extent of the increased sensitivity to MR in the LF-adapted cell lines was comparable with that achieved with MCF-7 and MCF-7/MR cells that were treated with Ko143, a potent and specific BCRP inhibitor (38). Based on these cumu-

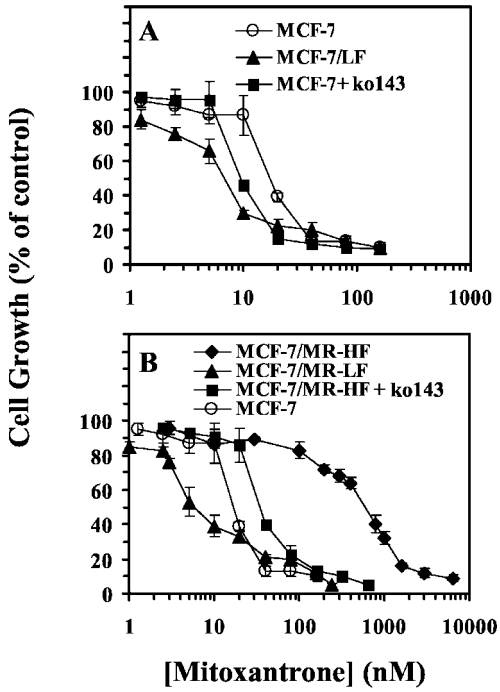

FIG. 5. **Cellular growth inhibition with MR.** Parental MCF-7 and MCF-7-LF (*A*) as well as parental MCF-7, MCF-7/MR-HF, and MCF-7/MR-LF cells (*B*) growing in monolayers in 24-well plates were exposed to various concentrations of MR in the absence or presence of 0.3 μM Ko143, a potent BCRP inhibitor. Following 72 h of incubation at 37 °C, cells were detached by trypsinization, and the number of viable cells was determined using trypan blue exclusion. Results depicted are the means ± S.D. of three independent experiments.

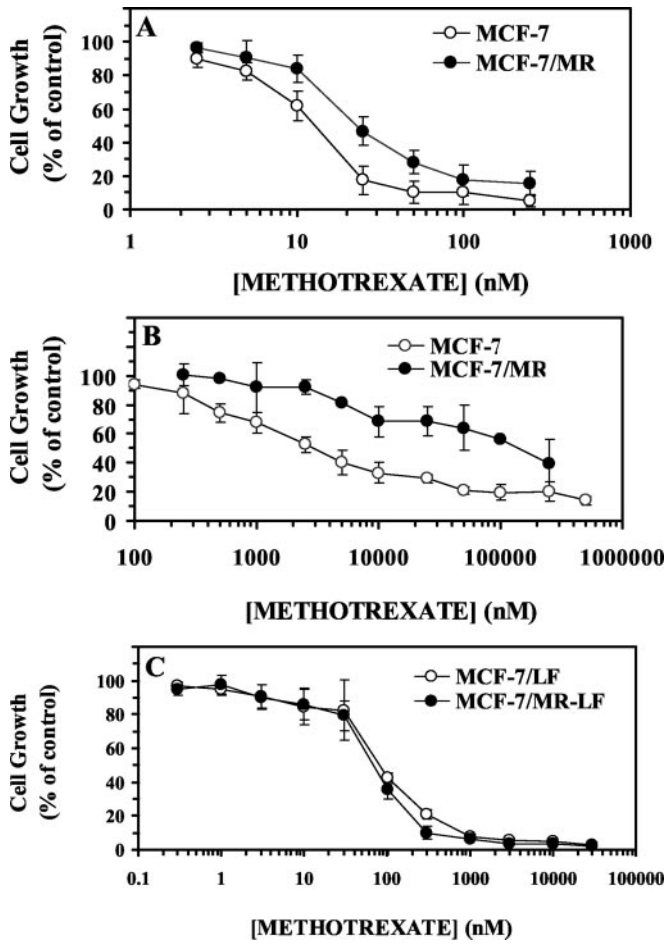

FIG. 6. **Cellular growth inhibition with MTX.** Cells were seeded in 24-well plates and allowed to attach for 24 h at 37 °C. Attached cells were then exposed continuously (*A*) or pulsed for 4 h at 37 °C with various concentrations of MTX (*B* and *C*). Following 4 h of exposure to MTX, the drug-containing medium was removed, and three successive washes each of ~10 min in drug-free medium were performed at 37 °C. Drug-free growth medium was added (2 ml/well), and cultures were incubated for 4 days at 37 °C. Cells were then detached by trypsinization, and the number of viable cells was determined using trypan blue exclusion.

lative data, we conclude that down-regulation of BCRP expression and efflux activity is an essential component of cellular survival under conditions of folate deficiency. These results support the hypothesis that apart from MRP1, BCRP is an important component of folate homeostasis, particularly under conditions of folate deprivation.

We note that loss of BCRP expression and function in MCF-7/MR-LF cells was not the sole adaptive response to folate deprivation. When compared with parental MCF-7 cells, MCF-7/MR-LF cells showed a 14-fold decrease in MRP1 levels. Moreover, the latter cells also displayed a 63% increase in FPGS activity relative to their parental MCF-7/MR-HF counterpart. Thus, MCF-7 cells growing in an excess of folic acid in the growth medium (*i.e.* 2.3 μM) initially had a substantial capacity to actively export folates via MRP1 and BCRP but only poorly via MRP4. In contrast, MCF-7/MR-LF cells growing in an ~770-fold less folic acid in the growth medium essentially lost this ability to export folates via MRP1 and BCRP. It should be emphasized that whereas MRP2, MRP3, and MRP4 have the facility to extrude folates, these transporters were essentially undetectable or poorly expressed in both parental cells and the LF-adapted cell lines. The increased activity of FPGS along with the loss of BCRP and the markedly decreased MRP1 levels under conditions of folate deficiency were presumably crucial adaptations aimed at augmenting cellular folate retention. These findings are in agreement with several *in vivo* and *in vitro* studies that explored the effect of folate deficiency on cellular FPGS activity and the expression of various MRPs. (*a*) Mice fed a low folate diet displayed a 50% increase in liver FPGS activity (41). (*b*) In a recent study (42) we characterized Chinese hamster ovary cells that were subjected to gradually increasing concentrations of the lipid-soluble antifolate, pyrimethamine (40), thereby resulting in a gradually increasing folate deprivation (39). Consequently, these cells displayed a 3–4-fold increase in FPGS activity (39) along with a complete loss of MRP1 expression (42). Furthermore, these cells were also devoid of BCRP and MRP2 through MRP4 even before pyrimethamine selection took place. Hence, in the absence of an ABC transporter that would mediate folate efflux activity, these cells could grow on extremely low concentrations of folates (*e.g.* pM concentrations of leucovorin). (*c*) Recently we have shown (43) that human leukemia cells adapted to grow under extremely low concentrations of leucovorin had a 95% loss of MRP1 expression and folate efflux function along with a 100-fold overexpression of the RFC, the primary folate influx transporter. In conclusion, disruption of folate exporter function via loss of BCRP and/or MRP1 expressions along with a concomitant increase in FPGS activity are apparently essential adaptations to conditions of folate deficiency, thereby resulting in an increased capacity to accumulate and retain cellular folates. We consistently find here that MCF-7/MR-LF and MCF-7/LF cells accumulated significantly higher levels of [^{3}H]folic acid than their parental counterparts.

As mentioned above, we have shown recently (43) that gradual deprivation of leucovorin (5-formyltetrahydrofolate) from the growth medium of human CCRF-CEM leukemia cells resulted in a 95% loss of MRP1 expression. Consistently, replenishment of the latter cells with 5 nM leucovorin resulted in a

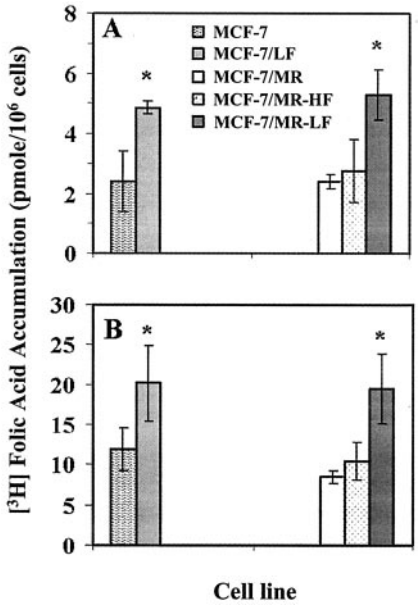

FIG. 7. **[^{3}H]Folic acid accumulation in parental and LF-adapted cell lines.** Monolayer cells were washed with folate-free medium and then incubated for 4 (*A*) and 24 h (*B*) at 37 °C in HBS containing 1 μM [^{3}H]folic acid as described under "Experimental Procedures." Transport was stopped by the addition of 10 ml of ice-cold HBS. Cells were then detached by trypsinization, washed with ice-cold HBS, and the final cell pellet was lysed in 0.2 ml of water, and the radioactivity released was determined using a liquid scintillation spectrometer. The *asterisks* denote statistically significant changes in MCF-7/LF *versus* parental MCF-7 cells, as well as MCF-7/MR-LF *versus* its MCF-7/MR-HF parental counterpart.

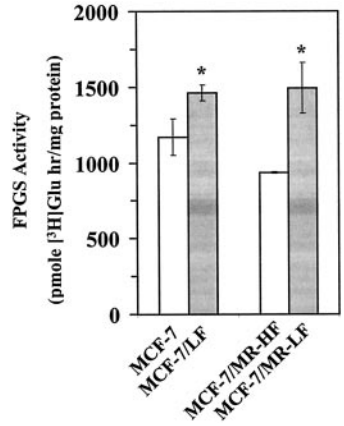

FIG. 8. **Histogram of FPGS activity in parental cells and their LF-adapted cell lines.** The catalytic activity of FPGS in the cytosolic fraction isolated from the various cell lines was determined as described under "Experimental Procedures." Results presented are the means ± S.D. of three independent experiments. The *asterisks* denote statistically significant changes in the LF-adapted sublines when compared with their parental cell lines.

complete restoration of MRP1 expression. In a recent paper (44) we reported that under folate-free conditions, MRP1- and MRP3-overexpressing cell lines were impaired in cellular growth upon a short exposure (4 h) to folic acid or leucovorin, when compared with their parental cells. Furthermore, the folic acid growth stimulation capacity in these cells was dramatically decreased during the pulse exposure to folic acid, when metabolism into rapidly polyglutamatable and hence retainable dihydrofolate and tetrahydrofolate was blocked by trimetrexate, a dihydrofolate reductase inhibitor. In another study (42), as mentioned above, we subjected Chinese hamster ovary cells to gradually increasing concentrations of the lipid-soluble antifolate pyrimethamine (40). This gradually increasing folate deprivation (39) resulted in a complete loss of MRP1 expression (42). Taken together, these findings suggest that down- and up-regulation of the ubiquitously expressed MRP1 can readily influence cellular folate homeostasis, particularly when cellular folate retention by polyglutamylation is attenuated.

Based on the unique substrate specificity of BCRP that is capable of exporting mono-, di-, and triglutamate conjugates of folates, one could predict that expression of substantial BCRP levels would not be compatible with folate deficiency conditions. Indeed, although MCF-7 cells expressed 5-fold more MRP1 than BCRP levels, the LF-adapted subline MCF-7/LF almost completely lost BCRP expression with no change in MRP1 levels. In contrast, following folate deprivation, MCF-7/MR cells with 55-fold BCRP overexpression, relative to parental MCF-7 cells, had a near complete loss of BCRP and MRP1 expression. Hence, elimination of the low expression levels of BCRP in MCF-7/LF cells was apparently sufficient to meet their folate growth requirement. In contrast, the dramatic down-regulation of the initially very high levels of BCRP in MCF-7/MR cells was crucial but apparently not sufficient as MRP1 expression was down-regulated to barely detectable levels. It is possible that this repression in MRP1 expression was achieved in the following manner: when BCRP was decreased to levels that were comparable with those of MRP1, the latter became significant in its contribution to folate efflux, thereby promoting its down-regulation as well. Support for this hypothesis could derive from the fact that replenishment of MCF-7/MR-LF cells with 2.3 μM folic acid for 1 month resulted in restoration of MRP1 expression to levels that exceeded those of parental MCF-7 cells (data not shown). In contrast, no restoration of BCRP expression was observed in these cells. Clearly, resumption of substantial levels of BCRP, an exporter that extrudes the precious triglutamate conjugates of folates, was not consistent with the retention of sufficient cellular folate pools to support cell growth.

Both MRP1 and BCRP are capable of ATP-driven efflux of folate monoglutamates. However, whereas MRP1 is ubiquitously expressed at substantial levels in various tissues, BCRP is relatively poorly expressed, and its pattern of tissue expression is apparently restricted to only a few tissues including placenta, intestine, colon, and the bile canaliculus. Most interesting, all these tissues were reported to express high levels of FPGS mRNA (45) and displayed a relatively high activity of FPGS. Hence, based on the unique folate polyglutamate exporter function of BCRP as well as on the ubiquitous expression of MRP1, it is tempting to speculate that expression of substantial levels of BCRP should be accompanied by adequate levels of FPGS activity in order to ensure sufficient intracellular retention of long chain (>3 glutamate residues) folate polyglutamates.

The present finding of the loss of BCRP expression under conditions of folate deficiency may have potentially important implications for anticancer chemotherapy including MR-containing chemotherapy. First, MR is being used in the treatment of metastatic breast cancer and non-lymphocytic leukemia including acute granulocytic leukemia. Breast cancer patients are initially treated with a chemotherapeutic regimen that contains either MTX or doxorubicin. However, one mechanism of MTX resistance in breast cancer cells may be antifolate drug transport (47, 48). This may be because of the loss of gene expression and function of RFC, the primary transporter for folates and MTX. Consequently, such folate transport-deficient and MTX-resistant cells may suffer from a markedly diminished intracellular folate pool (49). Cell survival on such a shrunken cellular folate pool may be associated with a signifi-

cant down-regulation of MRP1 and/or BCRP (42, 43). Hence, such MTX-resistant breast cancer cells may be most vulnerable to the cytotoxic action of various chemotherapeutic agents including anthracyclines and MR. This consideration may possibly have therapeutic value in overcoming drug resistance of certain tumors that have down-regulated the expression of BCRP under folate deficiency conditions. Second, we have shown here that folate deprivation results in the loss of MRP1 and BCRP expression. As such, one potential strategy to overcome anticancer drug resistance that is based on MRP1 and/or BCRP overexpression could be a transient exposure of the tumors to a folate-deficient diet. This could lead to a marked down-regulation of MRP1 and/or BCRP, thereby resulting in increased sensitivity of the tumors to various chemotherapeutic agents.

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