Breast cancer resistance protein/ABCG2 is differentially regulated downstream of extracellular signal-regulated kinase

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Breast cancer resistance protein (BCRP)/ABCG2 is a drug efflux pump responsible for multidrug resistance in cancer cells. We report that dephosphorylation of extracellular signal-regulated kinase (ERK) by treatment with mitogen-activated protein kinase/ERK kinase (MEK) inhibitors causes two opposing effects, transcriptional upregulation and prompted protein degradation of endogenous BCRP in breast cancer MCF-7 cells. Endogenous BCRP was eventually found to be upregulated. Conversely, treatment with epidermal growth factor was associated with its downregulation in the cells. MEK inhibitors also caused prompted degradation of exogenous BCRP in MCF-7 and gastric cancer NCI-N87 cells that express exogenous BCRP without affecting its transcriptional levels, and potentiated anticancer agents in the cells. A lysosomal inhibitor abolished this prompted degradation of exogenous BCRP, but a proteasome inhibitor did not. Inhibition of p90 ribosomal protein S6 kinase (RSK), one of the downstream effectors of ERK, resulted in transcriptional upregulation of endogenous BCRP but did not affect the protein degradation of exogenous BCRP. The data suggest that BCRP expression is differentially regulated downstream of the MEK-ERK pathway, transcriptionally upregulated through the inhibition of the MEK-ERK-RSK pathway, and post-transcriptionally downregulated through the inhibition of the MEK-ERK-non-RSK pathway. Although the immediate downstream effector of ERK remains to be elucidated, the data provide new insights into regulatory mechanisms of BCRP activity and may assist the development of BCRP-specific expression modulators. (*Cancer Sci* 2009; 100: 1118–1127)

Breast cancer resistance protein (BCRP)/ABCG2 is a half-size ATP-binding cassette transporter[1–3] normally expressed in a wide variety of organs and tissues, such as placenta, intestine, liver, ovary, testis, kidney, and brain, and in hematopoietic stem cells.[1,2,4] The subcellular localization of BCRP occurs in the apical membrane, across which BCRP effluxes its substrates out of cells. BCRP transports certain chlorophyll metabolites and xenobiotics and is presumed to play a protective role against toxic substances and metabolites in the maternal–placental barrier, the digestive tract, and the blood–testis barrier.[5–8] In addition, BCRP mediates the concurrent resistance to chemotherapeutic agents, such as mitoxantrone, SN-38 (an active metabolite of irinotecan), topotecan, and gefitinib, by pumping them out of the cell and lowering their cytotoxic effects.[1–3,9–11] Hence, the BCRP expression status is a significant determinant of sensitivity of cancer cells to its substrate anticancer agents.

To overcome BCRP-mediated multidrug resistance, development of competitive inhibitors of BCRP-mediated drug transport has been proposed.[7,12–14] A second related strategy would be the development of modulators of BCRP expression levels. We previously reported the estrogen-mediated post-transcriptional downregulation of BCRP expression in estrogen receptor α-positive breast cancer cells.[15] However, the mitogenic effects of estrogen on breast cancer might restrict its usefulness as an anticancer agent that targets BCRP.

Mitogen-activated protein kinase (MAPK) cascades, which are activated by a variety of stimuli, including growth factors and cytokines, are key signaling pathways regulating cell proliferation, survival, and differentiation.[16] Among the four major MAPK pathways, the extracellular signal-regulated kinase (ERK) MAPK pathway is critically involved in cancer. Activated ERK phosphorylates and regulates p90 ribosomal protein S6 kinase (RSK) and other numerous substrates.[16] Here, we report that differential BCRP regulation occurs by way of the MEK-ERK pathway. BCRP is transcriptionally upregulated by inhibition of the MEK-ERK-RSK pathway and post-transcriptionally downregulated through inhibition of an as yet undetermined MEK-ERK-non-RSK pathway. We putatively attribute this downregulation to enhanced protein degradation through the endosome–lysosome process. Our data suggest that selective inhibitors of effectors immediately downstream of ERK, other than RSK, in the MEK-ERK pathway might facilitate a BCRP-specific downregulation. Our present findings thus further elucidate the complex regulatory mechanisms of BCRP activities and might provide potentially important information for the future development of BCRP-specific expression modulators.

Materials and Methods

Reagents, cell cultures, and transfection. PD98059, U0126, and bafilomycin A_1 were purchased from Wako (Osaka, Japan). PD98059 and U0126 are reasonably specific, non-ATP-competitive MEK inhibitors widely used for *in vitro* research purposes at doses of 10–20 μM.[16] PD98059 strongly inhibits active MEK1 but not MEK2; U0126 inhibits both MEK1 and MEK2 more effectively than PD98059. Treatment of cells with the MEK inhibitors for 1.5 h caused a remarkable dephosphorylation of ERK1/2 (Fig. S1). As the effects did not continue for 24 h, MEK inhibitors were exchanged with fresh ones every 24 or 48 h as described in the Method sections. Topotecan, MG-132, and SL0101 were purchased from Bosche Scientific (New Brunswick, NJ, USA), Calbiochem (San Diego, CA, USA) and Toronto Research Chemicals (North York, ON, Canada), respectively. SN-38 was generously provided by Yakulto Honsha (Tokyo, Japan).

Human breast cancer MCF-7 cells and human gastric cancer NCI-N87 cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 7% FBS at 37°C in a humidified incubator with 5% CO_2.

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Abbreviations: BCRP, breast cancer resistance protein; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; RSK, ribosomal protein S6 kinase; MEK, MAPK/ERK kinase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MCF-7/BCRP, MCF-7 cells that stably express exogenous BCRP; NCI-N87/BCRP, NCI-N87 cells that stably express exogenous BCRP; EGF, epidermal growth factor; RT-PCR, real-time reverse transcription-PCR.

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The establishment of MCF-7/BCRP cells has been described previously.[15] A *myc*-tagged *BCRP* cDNA fragment was inserted into the pCAGIPuro plasmid to generate the pCAGIPuro-mycBCRP construct. NCI-N87 cells that stably express exogenous BCRP (NCI-N87/BCRP) were established by transfection with pCAGIPuro-mycBCRP using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The transfected cells were selected with 0.3 μg/mL puromycin for 7 days. The mixed populations of resistant cells were used in subsequent experiments. Expression levels of exogenous proteins were found to be unchanged for at least 3 months.

Western blot analysis of ERK1/2. To investigate the effects of FBS and epidermal growth factor (EGF) on ERK1/2 phosphorylation, cells were serum-starved for 24 h and then cultured in DMEM supplemented with 7% FBS or 100 ng/mL EGF for 1.5 h. After harvesting the cells, phosphorylated ERK1/2 was detected using the rabbit polyclonal anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody (Cell Signaling Technology, Danvers, MA, USA) at a 1:200 dilution according to the manufacturer's instructions. The blots were then stripped and reprobed with the rabbit polyclonal anti-p44/42 MAP kinase antibody (Cell Signaling Technology) at a 1:1000 dilution to detect ERK1/2.

Western blot analysis of BCRP. Cells (1×10^6) were incubated in DMEM with or without various concentrations of compounds for 48 h. MEK inhibitors were exchanged with fresh ones 24 h after the start of the treatment. Western blot analysis was carried out as described previously.[15] For detection of endogenous or exogenous BCRP, the blots were incubated with 5 μg/mL of the mouse anti-BCRP monoclonal antibody, BXP-21 (Chemicon, Temecula, CA, USA), or with 2.5 μg/mL of the mouse anti-c-myc monoclonal antibody, 4A6 (Upstate Biotechnology, Lake Placid, NY, USA), respectively. Band intensities were quantified using NIH Image (http://rsb.info.nih.gov/nih-image/) when necessary.

Real-time RT-PCR analysis. Expression levels of endogenous *BCRP* and exogenous *mycBCRP* were examined by RT-PCR. Cells (1×10^6) were incubated in DMEM with various concentrations of compounds for 48 h. MEK inhibitors were replaced with fresh ones 24 h after the start of the treatment. Total RNA extraction, first-strand cDNA synthesis, and RT-PCR of endogenous and exogenous *BCRP* were carried out as described previously with some modifications.[15] Real-time RT-PCR was carried out using SYBR Premix Ex Taq (Takara, Kyoto, Japan) and an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the manufacturers' instructions. *BCRP* mRNA expression levels in the presence of reagents, normalized against those of their corresponding *GAPDH* mRNA levels, were relatively quantified using the $2^{-\Delta\Delta CT}$ method.[17]

siRNA-induced knockdown of RSK. Cells (6.0×10^5) resuspended into 1 mL antibiotic-free DMEM containing 7% FBS were directly transfected with 120 pM siRNA in 6 μL Lipofectamine 2000 dissolved in a total volume of 250 μL of OPTI-MEM (Invitrogen) in accordance with the manufacturer's instructions. ON-TARGETplus SMARTpool Human RPS6KA1, RPS6KA2, and RPS6KA3 (Dharmacon, Chicago, IL, USA) were used for *RSK1*, *2*, and *3* knockdown, respectively, with ON-TARGETplus siCONTROL non-targeting siRNA (Dharmacon) used as the control. The cells were centrifuged at 4 h after transfection, resuspended in culture medium, and cultured for an additional 48 h. Subsequent *RSK1*, *2*, and *3* knockdowns were confirmed by RT-PCR as described above. The primers used were 5′-CCATTGACC-ACGAGAAGAAG-3′ and 5′-AGAGTAGGAGCCCACACCA-3′ for *RSK1* (656-bp fragment), 5′-TCACGGAGGAGGATGTCAA-3′ and 5′-ACTCGGGGTCAAAGTGGAA-3′ for *RSK2* (588-bp fragment), and 5′-AGTGCTGACTGGTGGTCTTT-3′ and 5′-TTCTTCTGTTGGGTCTCTC-3′ for *RSK3* (648-bp fragment). The primers for *RSK1* and *RSK2* were designed in the common nucleotide sequences for transcript variants of the respective genes.

Indirect immunofluorescence analysis of BCRP expression. Cells were treated with 10 μM MEK inhibitors in the absence or presence of 1.0 nM bafilomycin A_1 for 48 h and collected with a cell scraper. The compounds were replaced with fresh ones 24 h after the start of the treatment. The cells were routinely formalin-fixed, paraffin-embedded as a cell block, and processed. Antigen-retrieval was carried out by autoclaving (121°C) for 15 min. The slides were then incubated with 12.5 μg/mL BXP-21 at 4°C overnight followed by incubation with a polyclonal anti-mouse immunoglobulin/FITC secondary antibody (1:20 dilution) (Dako, Glostrup, Denmark) at 4°C overnight. BCRP was detected by fluorescent microscopy as green fluorescence (λ_{ex} 470–490 nm) and cell nuclei counterstained with 0.2 μg/mL 4′,6-diamidino-2-phenylidole (λ_{ex} 330–385 nm) were indicated by purple fluorescence.

Cell growth assay. MCF-7 and MCF-7/BCRP cells were seeded at a density of 3.0×10^4 cells per well in 12-well plates containing 2 mL DMEM. NCI-N87 and NCI-N87/BCRP cells were seeded at a density of 5.0×10^4 cells per well in 24-well plates containing 1 mL DMEM. The medium was supplemented with various concentrations of anticancer drugs in the absence or presence of indicated concentrations of reagents, and cells were cultured for 4 days. The compounds were replaced with fresh ones 2 days from the start of the treatment. The cell numbers in the respective wells were determined using a Coulter Counter (Beckman Coulter, Fullerton, CA, USA).

Intracellular topotecan uptake. Cells were cultured in the absence or presence of 10 μM MEK inhibitors for 48 h. MEK inhibitors were replaced with fresh ones 24 h after the start of the treatment. After trypsinization, cells (5×10^5) were incubated with 40 μM topotecan for 30 min at 37°C, washed in ice-cold PBS, and subjected to fluorescence analysis using FACSCalibur (Becton Dickinson, San Jose, CA, USA). The ratio (%) that represents the fraction of topotecan-treated cells in the M1 area, from which that of control cells was subtracted, was presented. In the M1 area, FL1 is greater than that of the crossing point of the solid and the dotted lines.

Statistical analysis. The two-sided unpaired Student's *t*-test was used to evaluate the statistical significance of differences between two sets of data. Differences were considered significant when the *P*-value was less than 0.05.

Results

Effects of MEK inhibitors on endogenous BCRP expression. Due to low BCRP expression levels and/or low sensitivity of the first antibody used, alteration of endogenous BCRP expression was difficult to visualize using Western blotting under reducing conditions (Fig. S2). Therefore, endogenous BCRP was detected as a dimer of 140 kDa under non-reducing conditions, which generate stronger BCRP signals. PD98059 mildly and U0126 significantly enhanced endogenous BCRP expression in MCF-7 cells in a dose-dependent manner (Fig. 1a). Treatment with 20 μM PD98059 or 10 μM U0126 for 48 h resulted in an approximately 1.5- to 3-fold increase in endogenous BCRP expression.

Quantitative real-time RT-PCR analyses revealed that the treatment with PD98059 for 48 h caused a mild increase and that with U0126 for 48 h resulted in a significant increase in endogenous *BCRP* levels in MCF-7 cells (Fig. 1b).

Effects of EGF on endogenous BCRP expression. We have observed that serum starvation results in marked upregulation of endogenous BCRP, presumably through the post-transcriptional process (Figs 1c, and Fig. S3). The half-lives of endogenous BCRP under serum-starved conditions were calculated to be 76.6 ± 0.90, whereas those in the presence of serum were 62.5 ± 3.51. The half-lives of BCRP in the presence of serum were calculated to be shorter than those determined in our later experiments, and this was possibly due to different experimental conditions. Serum

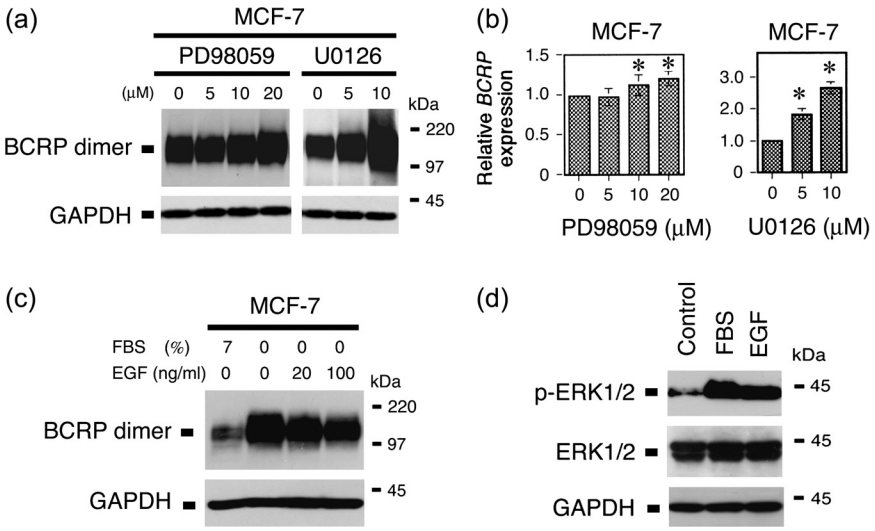

Fig. 1. Effects of MEK inhibitors and epidermal growth factor (EGF) on endogenous breast cancer resistance protein (BCRP) expression in MCF-7 cells. (a) Effects of MEK inhibitors on endogenous BCRP protein expression. Cells were cultured in the absence or presence of various concentrations of reagents for 48 h. Protein sample (30 μg) was loaded in each lane, and Western blot analysis was carried out under non-reducing conditions. BCRP was detected using BXP-21. The data are representative of at least three independent experiments. (b) Real-time RT-PCR analysis of endogenous *BCRP* mRNA in the presence of MEK inhibitors. Cells were cultured in the absence or presence of various concentrations of reagents for 48 h, and total RNA was extracted. First-strand cDNA was synthesized with 50 ng total RNA and was used to amplify *BCRP* cDNA fragments. *BCRP* mRNA levels in the presence of reagents, normalized to those of their corresponding *GAPDH* mRNA levels, were relatively quantified. Each bar represents the mean ± SD of triplicate determinations. Where a vertical bar is not shown, the SD is within the bar graph. The data are representative of three independent experiments. $*P < 0.05$ *versus* untreated cells. (c) Effects of EGF on endogenous BCRP expression. Cells were cultured in the medium with or without FBS in the absence or presence of various concentrations of EGF for 48 h. Protein sample (30 μg) was loaded in each lane, and Western blot analysis was carried out as described above. The data are representative of three independent experiments. (d) Effects of EGF on ERK1/2 phosphorylation. Cells were serum-starved for 24 h, followed by the medium exchange to that supplemented with 7% FBS or 100 ng/mL EGF. Control cells were not subjected to medium exchange. ERK1/2 phosphorylation was examined 1.5 h after the medium change by Western blotting. The data are representative of three independent experiments.

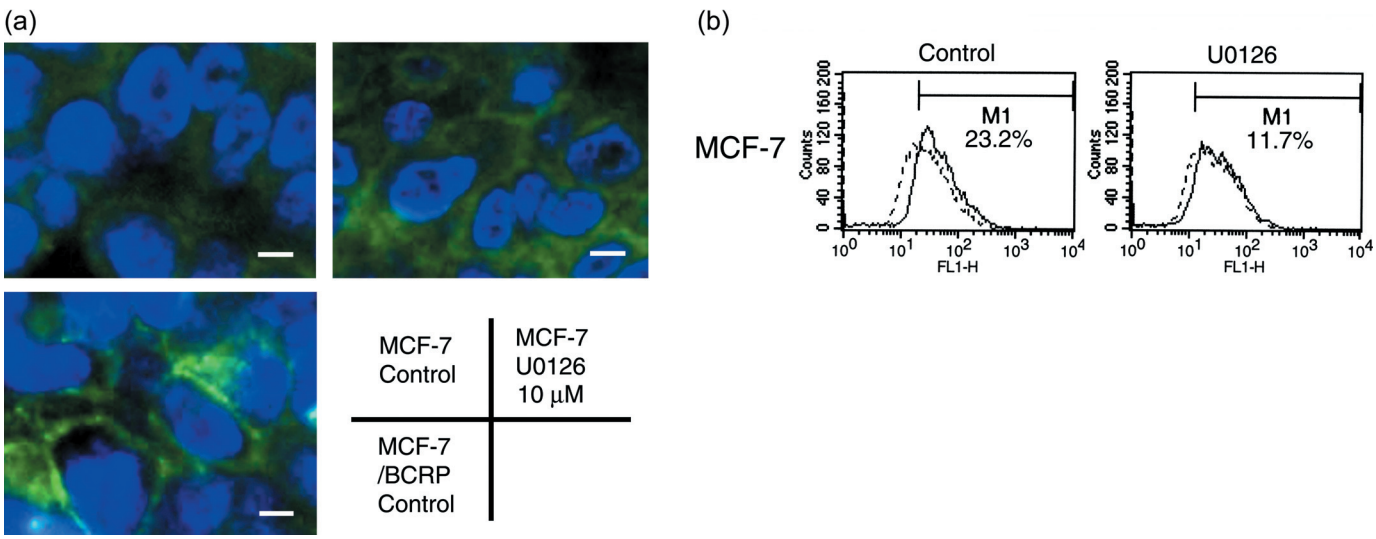

Fig. 2. Effects of U0126 on the subcellular localization of endogenous breast cancer resistance protein (BCRP) and cellular topotecan uptake in MCF-7 cells. Cells were cultured in the absence or presence of 10 μM U0126 for 48 h. (a) MCF-7 and MCF-7/BCRP cells were collected with cell scrapers, formalin-fixed, and paraffin-embedded as a cell block. BCRP was detected by fluorescence microscopy as green fluorescence, while cell nuclei were indicated by purple fluorescence. Scale bar, 10 μm. (b) After trypsinization, MCF-7 cells (5×10^5) were incubated with (solid line) or without (dotted line) 40 μM topotecan for 30 min. After washing, cellular uptake of topotecan was measured by flow cytometry.

starvation of MCF-7 cells resulted in marked upregulation of endogenous BCRP, and EGF counteracted the effects in a dose-dependent manner, suggesting that EGF-mediated ERK1/2 phosphorylation might downregulate endogenous BCRP expression (Fig. 1c). Treatment with 100 ng/mL EGF was nearly as effective as treatment with 7% FBS to phosphorylation of ERK1/2 in MCF-7 cells (Fig. 1d).

Subcellular localization of endogenous BCRP in U0126-treated MCF-7 cells. Upregulated endogenous BCRP in U0126-treated MCF-7 cells was mainly localized at the plasma membrane

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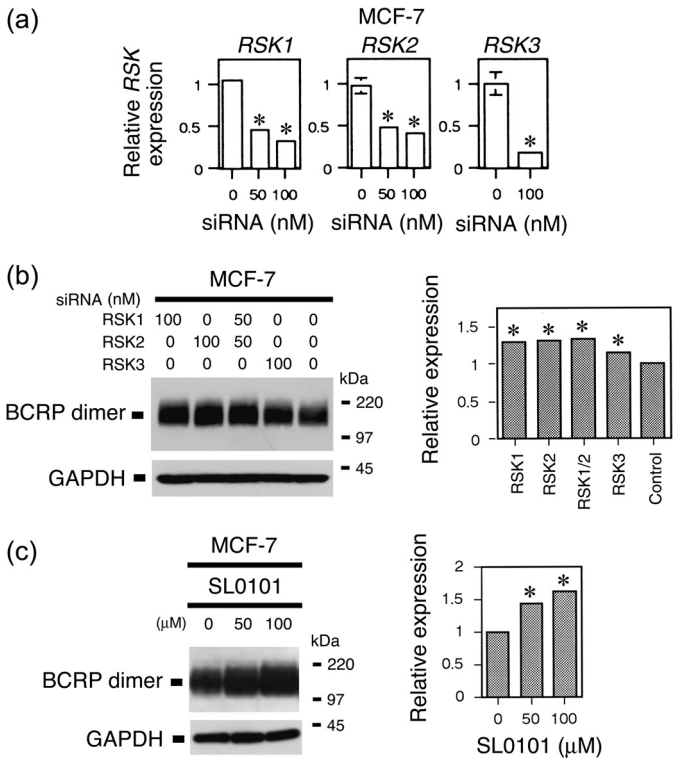

Fig. 3. Effects of p90 ribosomal protein S6 kinase (RSK) inhibitors on endogenous breast cancer resistance protein (BCRP) expression in MCF-7 cells. (a) siRNA-induced knockdown of *RSK*. Cells were transfected with indicated concentrations of siRNA for 4 h and harvested 48 h after transfection. For expression analysis of *RSK* mRNA, total RNA was extracted from harvested cells and RT-PCR of *RSK1, 2, and 3* cDNA fragments was carried out as described in the legend to Fig. 1(b). Each bar represents the mean ± SD of triplicate determinations. Where a vertical bar is not shown, the SD is within the bar graph. The data are representative of three independent experiments. $*P < 0.05$ *versus* control cells. (b) Effects of siRNA-induced *RSK* knockdown on endogenous BCRP protein expression. Protein sample (30 μg) was loaded in each lane, and Western blot analysis was carried out under non-reducing conditions. The band intensities of BCRP were quantified, normalized against those of the corresponding GAPDH, and the relative intensities to those of control cells were determined. Each bar represents the mean ± SD of triplicate determinations. Where a vertical bar is not shown, the SD is within the bar graph. The data are representative of (left) or derived from (right) three independent experiments. $*P < 0.05$ *versus* control cells. (c) Effects of SL0101 on endogenous BCRP expression. Cells were harvested after 48 h of culture in the absence or presence of various concentrations of SL0101, and Western blot analysis was carried out as described above. The data are representative of (left) or derived from (right) three independent experiments. $*P < 0.05$ *versus* control cells.

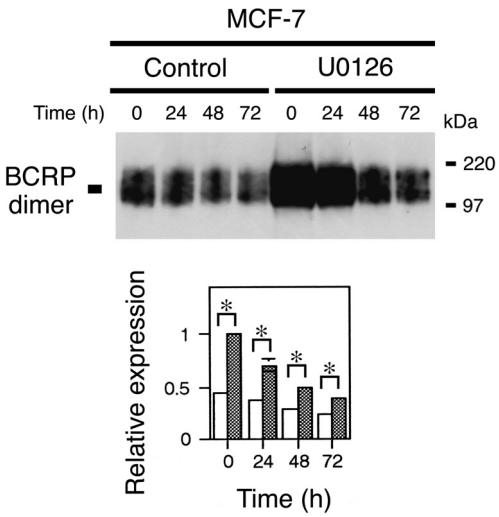

Fig. 4. Effects of U0126 on the degradation of endogenous breast cancer resistance protein (BCRP) in MCF-7 cells. Cells were cultured in DMEM supplemented with 7% FBS in the absence or presence of 10 μM U0126 for 24 h. Then 30 μg/mL cycloheximide was added to the medium, and cell culture was continued for the indicated periods. Cells were harvested at different time points and protein samples were prepared. Western blot analysis was carried out under non-reducing conditions. One-thirtieth parts of the whole-prepared protein was loaded in each lane. The relative intensities to those of U0126-treated cells at the starting point (0 h) were determined. Open bar, control cells. Dotted bar, U0126-treated cells. Each bar represents the mean ± SD of triplicate determinations. Where a vertical bar is not shown, the SD is within the bar graph. The data are representative of (top) or derived from (bottom) three independent experiments. $*P < 0.05$.

(Fig. 2a). Cellular topotecan uptake in U0126-treated cells was decreased compared with that in the untreated cells, suggesting that the upregulated BCRP was functionally expressed at the plasma membrane (Fig. 2b).

Effects of RSK inhibitors on endogenous BCRP expression. RSKs are serine/threonine protein kinases, which positively regulate cell growth. Since phosphorylated ERK1/2 transduces signals to RSKs, effects of RSK inhibitors on endogenous BCRP expression were investigated.[16] Transfection with 100 nM siRNA for *RSK1*, *2*, and *3* reduced mRNA levels by more than 60% for each of these kinases (Fig. 3a), and the knockdown of these *RSKs* all caused mild upregulation of endogenous BCRP (Fig. 3b). These upregulations were caused by a transcriptional process, as revealed by real-time RT-PCR (data not shown).

SL0101 has recently been reported to be a specific inhibitor of RSK1/2.[18] Treatment of MCF-7 cells with 100 μM SL0101 for 48 h resulted in a growth inhibitory effect of less than 30% (data not shown). SL0101 was also found to upregulate endogenous BCRP (Fig. 3c).

Degradation of endogenous BCRP protein in the presence of MEK inhibitor. After treatment with 10 μM U0126 for 24 h, MCF-7 cells were incubated in the presence of 30 μg/mL cycloheximide, a protein synthesis inhibitor, for an additional 0, 24, 48, or 72 h. Endogenous BCRP was found to be more upregulated at the start of the cycloheximide treatment in the presence of U0126 than in the absence of the compound (Fig. 4). Surprisingly, the BCRP degradation rate was found to be greater under the former conditions. The half-lives of endogenous BCRP in MCF-7 cells in the absence or presence of U0126 were calculated to be 82.9 ± 10.5 and 51.3 ± 0.8 h, respectively.

Effects of MEK inhibitors on exogenous BCRP expression. MCF-7/BCRP and NCI-N87/BCRP cells that overexpress exogenous BCRP by the constitutively active LTR or CAG promoters were established (Fig. 5a). LTR and CAG are reportedly stronger promoters than CMV, and LTR might be stronger than CAG. Exogenous BCRP signals were so strong under non-reducing conditions that they were detected as dimers and presumably merged oligomers. Therefore, exogenous BCRP signals were detected as monomers with a molecular weight of 70 kDa under reducing conditions hereafter. Exogenous BCRP expression decreased following treatment with MEK inhibitors for 48 h, in a dose-dependent manner (Fig. 5b). Compared with untreated cells, these cells expressed approximately 5- to 6-fold less exogenous protein after 48 h of treatment with 10 μM PD98059 or U0126.

Real-time RT-PCR analyses revealed that these treatments did not affect the expression levels of exogenous *mycBCRP* mRNA driven by the constitutive promoters in the cells (Fig. S4).

Effects of EGF on exogenous BCRP expression. Similar to the results for endogenous BCRP, serum starvation for 48 h resulted in upregulation of exogenous BCRP in MCF-7/BCRP cells (Fig. 6).

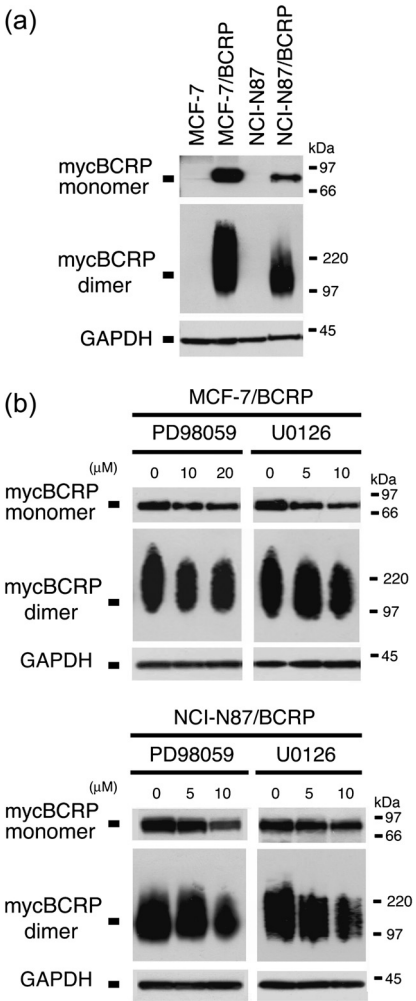

Fig. 5. Effects of MEK inhibitors on exogenous breast cancer resistance protein (BCRP) expression. (a) Establishment of exogenous BCRP-expressing cells. Cells were transduced/transfected with *BCRP* cDNA and selected with appropriate reagents. After harvesting the resistant cells, protein sample (20 μg under reducing conditions and 5 μg under non-reducing conditions) was loaded in each lane and Western blot analysis was carried out. Exogenous BCRP was detected using 4A6. The data are representative of at least three independent experiments. (b) Effects of the MEK inhibitors on exogenous BCRP protein expression. Cells were cultured in the absence or presence of various concentrations of reagents for 48 h. Western blotting was carried out as described above. The data are representative of at least three independent experiments.

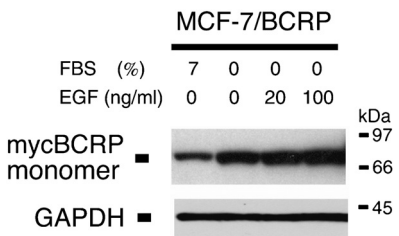

Fig. 6. Effects of EGF on exogenous BCRP expression in MCF-7/BCRP cells. Cells were cultured in DMEM supplemented with or without FBS in the absence or presence of various concentrations of EGF for 48 h. Protein sample (20 μg) was loaded in each lane, and Western blot analysis was performed under reducing conditions. The data are representative of three independent experiments.

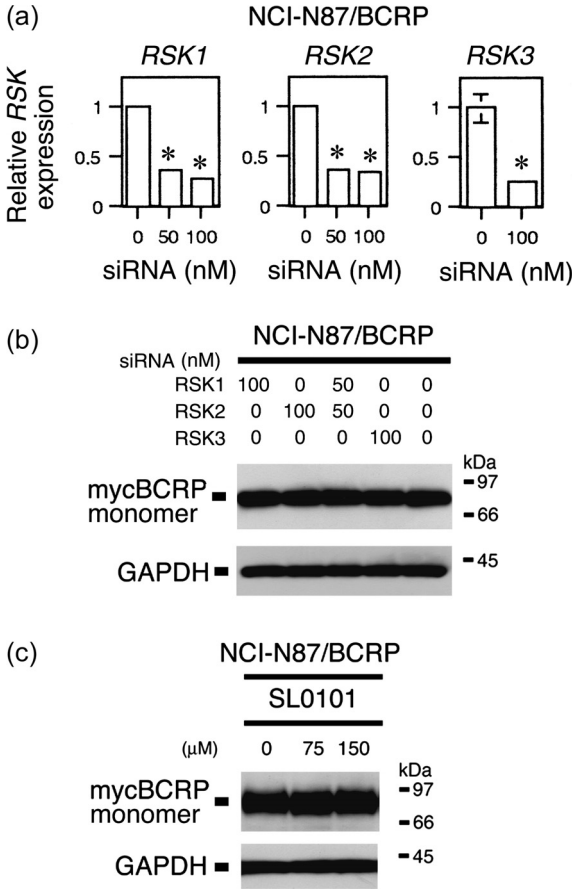

Fig. 7. Effects of p90 ribosomal protein S6 kinase (RSK) inhibition on exogenous breast cancer resistance protein (BCRP) expression in NCI-N87/BCRP cells. siRNA-induced *RSK* knockdown with subsequent RT-PCR and Western blot analysis of exogenous BCRP were carried out as described in the legend to Figs 3(a) and 5(a), respectively. (a) siRNA-induced *RSK* knockdown. Each bar represents the mean ± SD of triplicate determinations. Where a vertical bar is not shown, the SD is within the bar graph. The data are representative of three independent experiments. **P* < 0.05 *versus* control cells. (b) Effects of siRNA-induced *RSK* knockdown on exogenous BCRP protein expression. The data are representative of at least three independent experiments. (c) Effects of SL0101 on exogenous BCRP expression. Cells were harvested after 48 h of culture in the absence or presence of various concentrations of SL0101, and Western blot analysis was carried out under reducing conditions. The data are representative of three independent experiments.

EGF scarcely or only marginally upregulated exogenous BCRP expression levels under serum deprivation over the 48-h period. Effects of EGF on exogenous BCRP turnover might have been masked by serum starvation-mediated BCRP upregulation.

Effects of RSK inhibitors on exogenous BCRP expression. Transfection with 100 nM siRNA for *RSK1*, *2*, and *3* resulted in a greater than 60% reduction in their respective mRNAs in NCI-N87/BCRP cells (Fig. 7a), but did not cause any significant changes in the exogenous BCRP protein levels (Fig. 7b). Treatment of NCI-N87 cells with 150 μM SL0101 for 48 h revealed less than 30% growth suppression (data not shown). In addition, SL0101 did not affect exogenous BCRP levels in NCI-N87/BCRP cells (Fig. 7c).

Degradation of exogenous BCRP protein in the presence of MEK inhibitor. After treatment with 10 μM PD98059 for 24 h, cells were incubated in the presence of 30 μg/mL cycloheximide for an additional 0, 12, 24, 36, and 48 h. The degradation rate of exogenous BCRP protein was approximately 3-fold greater in

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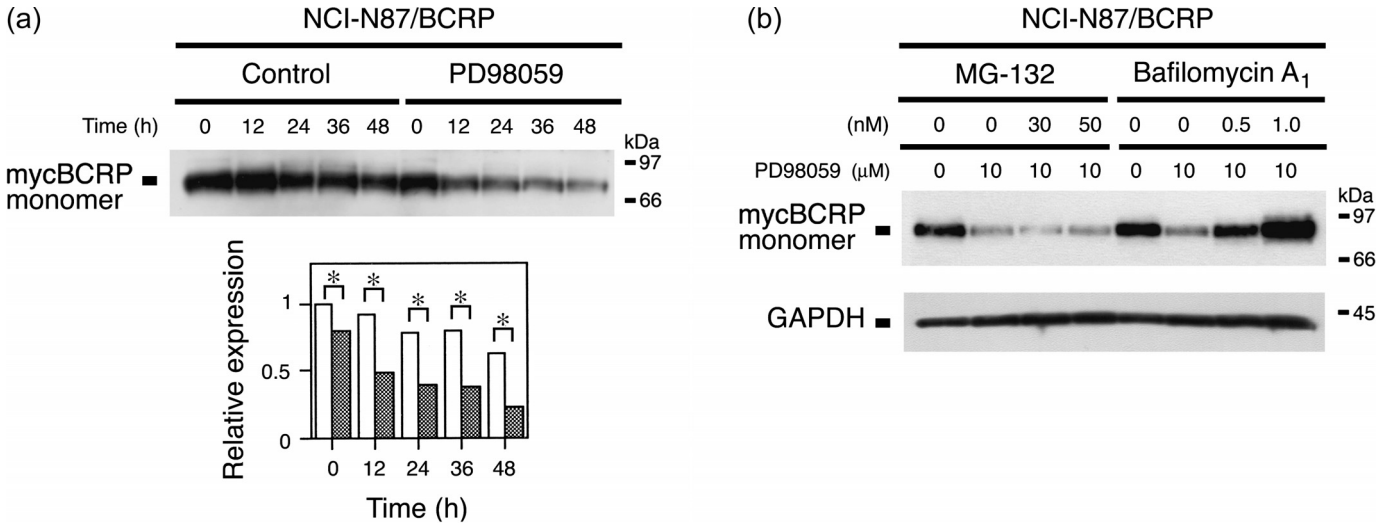

Fig. 8. (a) Effects of PD98059 on the degradation of exogenous breast cancer resistance protein (BCRP). NCI-N87/BCRP cells were cultured in DMEM supplemented with 7% FBS in the absence or presence of 10 μM PD98059 for 24 h. Then 30 μg/mL cycloheximide was added to the medium, and cell culture was continued for the indicated periods. Cells were harvested at different time points and BCRP expression levels were evaluated by Western blot analysis under reducing conditions. One-thirtieth parts of the whole prepared protein was loaded in each lane. The relative band intensities to that of control cells at the starting point (0 h) were determined. Open bar, control cells; dotted bar, PD98059-treated cells. Each bar represents the mean ± SD of triplicate determinations. Where a vertical bar is not shown, the SD is within the bar graph. The data are representative of (top) or derived from (bottom) three independent experiments. *$P < 0.05$. (b) Effects of MG-132 and bafilomycin A_1 on the PD98059-mediated downregulation of exogenous BCRP protein. NCI-N87/BCRP cells were cultured with or without 10 μM PD98059 in the absence or presence of various concentrations of the indicated reagents for 48 h. Cells were harvested, and Western blot analysis was carried out under reducing conditions. Protein sample (20 μg) was loaded in each lane. The data are representative of three independent experiments.

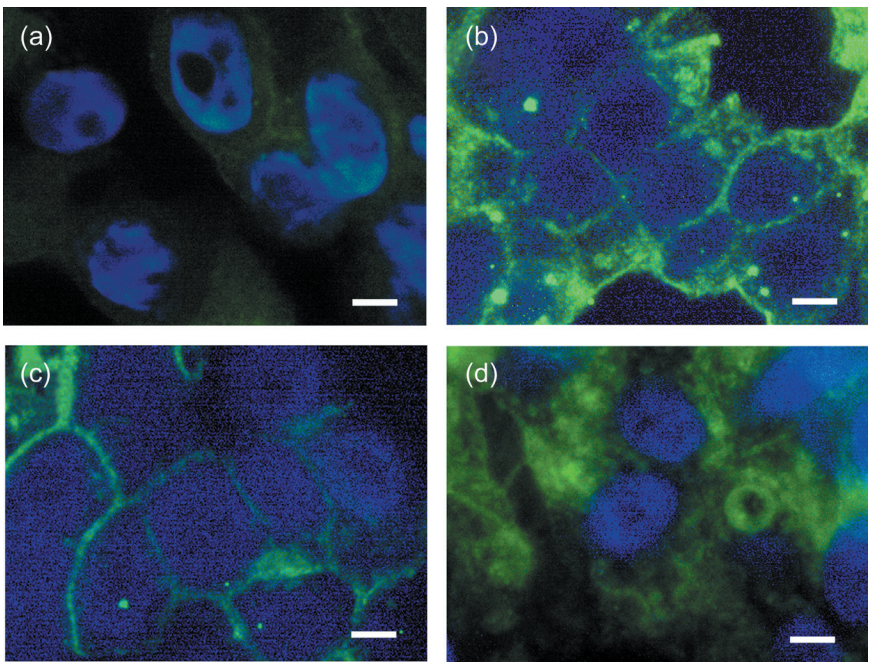

Fig. 9. Effects of PD98059 on the subcellular localization of exogenous breast cancer resistance protein (BCRP) in NCI-N87/BCRP cells. Cells were cultured in the absence or presence of 10 μM PD98059 and with or without 1.0 nM bafilomycin A_1 for 48 h. Immunostaining was carried out as described in the legend to Fig. 2a. BCRP was detected as green fluorescence, and cell nuclei were indicated by purple fluorescence. (a) Untreated NCI-N87 cells; (b) untreated NCI-N87/BCRP cells; (c) NCI-N87/BCRP cells treated with 10 μM PD98059; (d) NCI-N87/BCRP cells treated with 10 μM PD98059 and 1.0 nM bafilomycin A_1. Scale bar, 10 μm. The data are representative of three independent experiments.

the presence of PD98059 than in the absence of this compound (Fig. 8a). The half-lives of exogenous BCRP in the absence or presence of PD98059 were calculated as 78.7 ± 5.8 h and 26.3 ± 2.2 h, respectively.

Next, the effects of the proteasome inhibitor, MG-132, and the lysosome inhibitor, bafilomycin A_1, on exogenous BCRP degradation by MEK inhibitors were investigated. The IC_{50} values of NCI-N87/BCRP cells determined from the 4-day cell growth assay were 76.4 ± 0.04 nM for MG-132 and 1.24 ± 0.06 nM for bafilomycin A_1. Treatment with MG-132 did not significantly affect the MEK inhibitor-mediated BCRP degradation, but bafilomycin A_1 exposure clearly suppressed this degradation in a dose-dependent manner (Fig. 8b).

Subcellular localization of exogenous BCRP in cells treated with MEK inhibitors. Variant BCRP proteins, whose expression levels are significantly lower than those of the wild-type in spite of their similar mRNA levels, have been found to undergo ubiquitin-mediated protein degradation.[19] A putatively misfolded BCRP

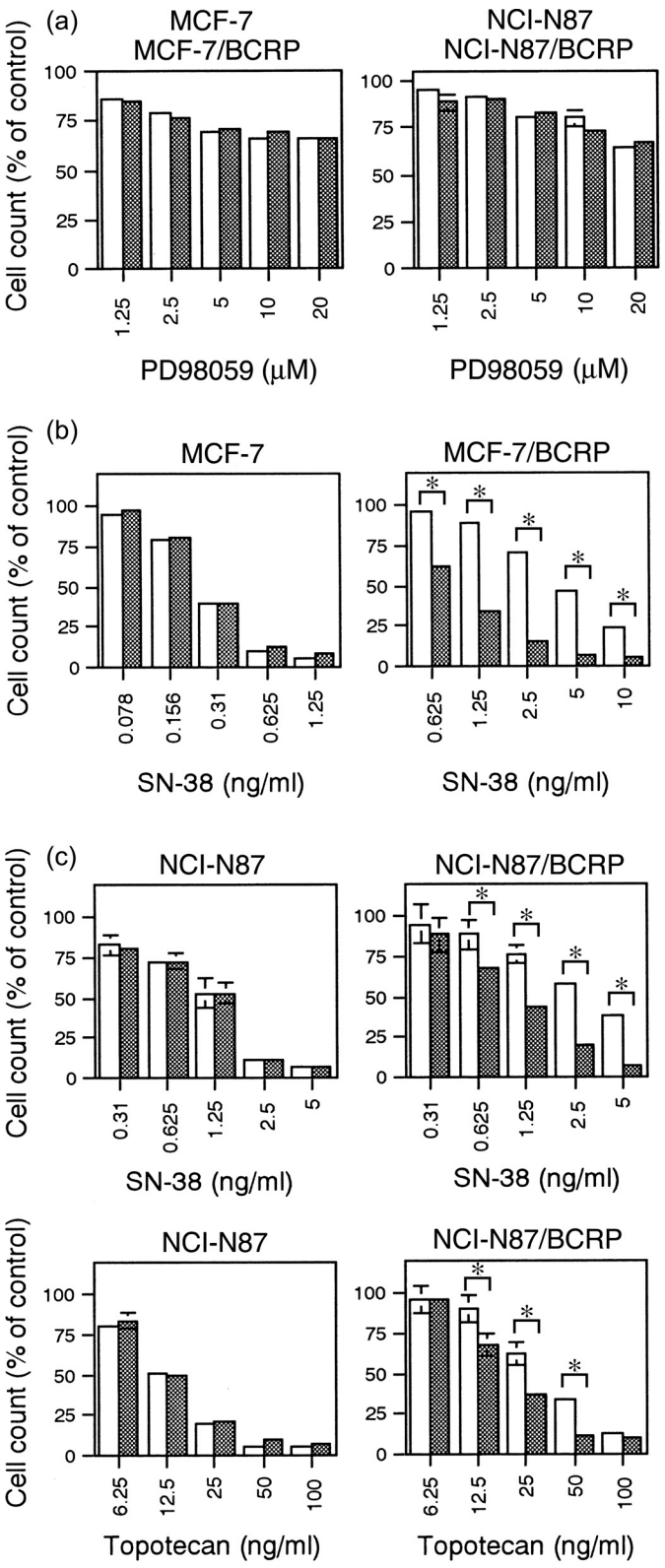

Fig. 10. Effects of PD98059 on anticancer drug resistance. (a) Effects of PD98059 on cell growth. MCF-7 and MCF-7/breast cancer resistance protein (BCRP) cells (3×10^4/well) and NCI-N87 and NCI-N87/BCRP cells (5×10^4/well) were seeded into 12- and 24-well plates, respectively, and cultured in the absence or presence of various concentrations of PD98059 for 4 days. Cell numbers were determined with a Coulter Counter, and presented as percentages relative to those of control cells cultured in the absence of PD98059. Open bars, parental cells; dotted bars, *BCRP*-transduced/transfected cells. The data shown are the means ± SD of triplicate determinations, and are representative of at least three independent experiments. Where a vertical bar is not shown, the SD is within the bar graph. (b,c) Effects of PD98059 on anticancer drug resistance of MCF-7/BCRP and NCI-N87/BCRP cells. Respective cells were seeded as described in the Figure 10(a) legend and cultured with or without 10 μM PD98059 in the absence or presence of increasing doses of specific anticancer agents for 4 days. Cell numbers were determined, and presented as percentages relative to those of control cells cultured in the absence of anticancer agents. Open bars, PD98059-untreated cells; dotted bars, PD98059-treated cells. The data shown are the means ± SD of triplicate determinations, and are representative of at least three independent experiments. Where a vertical bar is not shown, the SD is within the bar graph. *$P < 0.05$.

variant was mainly detectable in intracellular compartments rather than at the plasma membrane, suggesting its impaired intracellular sorting.[19] To gain further insight into the BCRP degradation by MEK inhibitors, its subcellular localization was investigated. Overexpressed BCRP in NCI-N87/BCRP cells was localized mainly at the plasma membrane and partly in the cytoplasm (Fig. 9a,b). Most of the downregulated BCRP signals after PD98059 treatment were still detectable at the plasma membrane (Fig. 9c). Although additional treatment with bafilomycin A_1 restored the expression levels of BCRP protein, these proteins were mainly detected in the intracellular compartment, not at the plasma membrane (Fig. 9d).

Cell growth inhibition studies. Exogenous BCRP expression in MCF-7 and NCI-N87 cells did not significantly affect their sensitivities to cytotoxicities of the MEK inhibitors (Fig. 10a, Fig. S5a). The growth suppressive effects of these agents on *BCRP*-transfected cells and their parental cells were less than 30% at a range of concentrations over the 4-day study periods, except for MCF-7 cells treated with U0126.

Cytotoxicities of BCRP-substrate anticancer agents were found to be only slightly affected by PD98059 in MCF-7 and NCI-N87 cells and by U0126 in NCI-N87 cells, but they were significantly potentiated in their *BCRP*-transduced/transfected cells (Fig. 10b,c, Fig. S5b).

Treatment with PD98059 resulted in little change in the cellular topotecan accumulation in NCI-N87 cells, but a mild increase was evident in NCI-N87/BCRP cells compared with the control cells (Fig. 11a). Treatment of MCF-7/BCRP cells with U0126 also resulted in a mild increase of topotecan uptake (Fig. S6).

Additional lysosome inhibitor treatment did not restore SN-38 resistance in NCI-N87/BCRP cells treated with 10 μM PD98059 (Fig. 11b), consistent with the result shown in Figure 9(d) that suggests a restricted functional recovery of BCRP protein in PD98059-treated cells.

Discussion

We have reported that MEK inhibitors transcriptionally upregulate BCRP but simultaneously enhance its protein degradation. Treatment with these inhibitors caused transcriptional upregulation of BCRP in MCF-7 cells (Figs 1a, 2), but U0126 treatment also enhanced protein degradation of BCRP in the cells (Fig. 4). Despite the two opposing effects, the former probably exceeded the latter and BCRP was eventually found to be upregulated. However, the MEK inhibitors caused marked downregulation of exogenous BCRP, which is driven by the constitutively active LTR or CAG promoters, without affecting its transcription levels in MCF-7/BCRP or NCI-N87/BCRP cells (Fig. 5b and Fig. S4). The downregulation was due to induced protein degradation (Fig. 8a), which could be inhibited by a lysosome inhibitor, bafilomycin A_1 (Fig. 8b). The MEK inhibitors also overcame the multidrug resistance of those cells (Fig. 10b,c, and Fig. S5b).

An siRNA-induced RSK knockdown and SL0101 upregulated endogenous BCRP in the parental cells but did not affect exogenous BCRP levels in the *BCRP*-transduced/transfected

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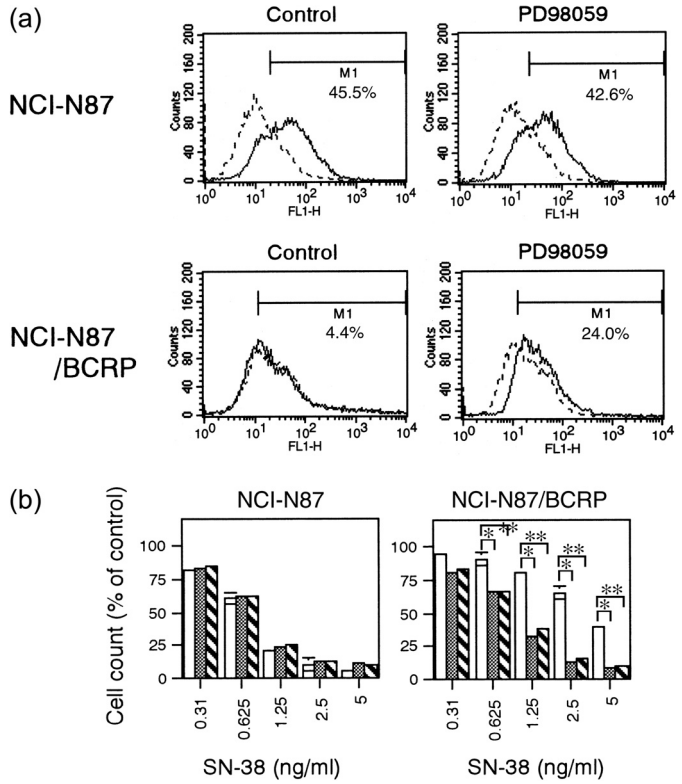

Fig. 11. Effects of PD98059 on cellular topotecan uptake and effects of bafilomycin A_1 on the PD98059-mediated reversal of SN-38 resistance of NCI-N87/breast cancer resistance protein (BCRP) cells. (a) Effects of PD98059 on cellular topotecan uptake. Cells were cultured in the absence or presence of 10 μM PD98059 for 48 h. After trypsinization, cells (5×10^5) were incubated with (solid line) or without (dotted line) 40 μM topotecan for 30 min. After washing, cellular uptake of topotecan was measured by flow cytometry. The data shown are representative of at least three independent experiments. (b) Effects of bafilomycin A_1 on the PD98059-mediated reversal of SN-38 resistance of NCI-N87/BCRP cells. Cells (5×10^4) were seeded into 24-well plates and cultured with or without 10 μM PD98059 and also with or without 1.0 nM bafilomycin A_1 in the absence or presence of increasing doses of SN-38 for 4 days. Cell numbers were determined and presented as percentages relative to those of control cells cultured in the absence of anticancer agents. Open bars, cells cultured without PD98059 or bafilomycin A_1; dotted bars, cells cultured in the presence of 10 μM PD98059; hatched bars, cells cultured in the presence of both 10 μM PD98059 and 1.0 nM bafilomycin A_1. The data are the means ± SD of triplicate determinations, and are representative of three independent experiments. Where a vertical bar is not shown, the SD is within the bar graph. *,**$P < 0.05$.

cells (Figs 3,7). These data suggest that RSK operates as an effector immediately downstream of ERK, involved in transcriptional upregulation of the *BCRP* gene but not the lysosomal degradation of its protein product. The role of RSK1, 2, or 3 in *BCRP* upregulation might not be redundant, as the knockdown of *RSK1*, *2*, or *3* resulted in endogenous *BCRP* upregulation (Fig. 3b).

Several regulatory mechanisms for BCRP expression have been reported. The earlier ones are steroid hormone-mediated mechanisms in specific types of cells harboring the respective receptors, such as breast cancer and trophoblastoid cells.[15,20] In another report, bone marrow cells exposed to hypoxia showed an increase in side population cells, which would express BCRP.[21] BCRP was also induced by hypoxia in mouse Hepalclc7 cells, and human JAR, Saos-2, and OCI-AML3 cells. This regulation may involve the hypoxia-inducible transcription factor complex HIF-1. A nuclear receptor, peroxisome proliferator-activated receptor-γ, has also been found to transcriptionally upregulate BCRP expression in human dendritic cells.[22] Furthermore, it has been reported that the EGF-mediated activation of the MAPK cascade results in a significant increase in BCRP expression in cytotrophoblasts, BeWo cells, and MCF-7 cells at both the mRNA and protein levels.[23] In this report, the BCRP upregulation by EGF was abolished by the PD98059 treatment. However, although not previously reported, we have observed that serum starvation results in upregulation of both endogenous and exogenous BCRP (Figs 1c,6). This upregulation would presumably be due to the post-transcriptional process (Fig. S3). EGF treatment was found to be associated with downregulation of serum deprivation-mediated upregulation of endogenous BCRP. We also found that PD98059 mildly and U0126 markedly upregulated endogenous BCRP in MCF-7 cells (Fig. 1a,b). The reason for this discrepancy is presently unknown. One possible explanation might be differences in the cell lines and the experimental protocols used.

Recently, it has been reported that ubiquitin-mediated protein degradation is involved in removing misfolded BCRP proteins lacking an intramolecular disulfide bond and that the protein level of wild-type BCRP significantly increased when cells were treated with bafilomycin A_1.[19] In addition, the ubiquitin-mediated proteasomal degradation of non-synonymous single nucleotide polymorphism variants of BCRP has been reported.[24] In general, two major proteolytic systems involved in different aspects of protein breakdown exist in mammalian cells, the lysosomal and the non-lysosomal systems.[25] The degradation of plasma membrane proteins as well as of proteins entering the cell by receptor-mediated endocytosis or by pinocytosis takes place in lysosomes.[26] An important non-lysosomal proteolytic pathway is the ubiquitin-proteasome pathway in which soluble nuclear and cytoplasmic proteins are degraded.[25,27] Integral membrane proteins that fail to fold or oligomerize correctly were also found to be degraded by the cytoplasmic proteasome after being exported from the endoplasmic reticulum.[28] In our present study, the MEK inhibitor-mediated BCRP downregulation was abolished by bafilomycin A_1 but not by MG-132 (Fig. 8b). This suggests that BCRP might also be induced to undergo internalization and lysosomal degradation by treatment with the MEK inhibitors. Although the PD98059-mediated BCRP downregulation was abolished by bafilomycin A_1, anticancer drug resistance was not recovered in NCI-N87/BCRP cells (Fig. 11b). In addition, BCRP protein in these cells was found to be internalized and to have accumulated in the cytoplasm after concomitant PD98059 and bafilomycin A_1 treatment (Fig. 9d).

The post-transcriptional downregulation of P-glycoprotein associated with the MEK-ERK-RSK pathway has recently been reported.[29] Endogenous P-glycoprotein levels in human colorectal cancer cells were significantly repressed by MEK inhibitors and/or siRNA-mediated knockdown of RSK1/2/3 in a post-transcriptional manner. Exogenous P-glycoprotein was also remarkably repressed in *MDR1*-transduced human breast cancer cells by the same treatments. Similar to BCRP, this down-regulation was attributable to induced protein degradation. However, the protein degradation event in this case was regulated through the MEK-ERK-RSK pathway, which is not the case for BCRP. This would be a very important consideration for any future development of BCRP-specific expression regulators that do not affect the P-glycoprotein expression levels.

In conclusion, the MEK-ERK signaling pathway is implicated in two opposing regulatory mechanisms for BCRP, transcriptional upregulation and induced protein degradation. As the inhibition of an as yet undetermined MEK-ERK-non-RSK pathway was found to result in enhanced lysosomal BCRP degradation, a search for the immediate downstream effector of ERK, other than RSK, might make it possible to specifically overcome BCRP-mediated drug resistance.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Effects of MEK inhibitors on ERK1/2 phosphorylation. Cells were cultured in DMEM supplemented with 7% FBS overnight, followed by incubation in the absence or presence of 10 μM MEK inhibitors for the indicated periods. Harvested cells were lysed in a buffer containing 2% SDS. Protein sample (10 μg) was loaded in each lane. Phosphorylated ERK1/2 was detected by Western blotting using the anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) antibody, and ERK1/2 was detected using the anti-p44/42 MAP kinase antibody. GAPDH expression was analyzed as a loading control. The data are representative of at least three independent experiments.

Fig. S2. Western blot analysis of breast cancer resistance protein (BCRP) detected by the anti-BCRP antibody. (a) Western blot analysis of endogenous and exogenous BCRP detected by the anti-BCRP antibody, BXP-21. After harvesting cells treated as indicated for 48 h, protein sample (30 μg) was loaded in each lane and Western blot analysis was carried out. Endogenous BCRP with or without exogenous BCRP was detected using BXP-21. The data are representative of at least three independent experiments. (b) Endogenous BCRP expression in MCF-7 and NCI-N87 cells. Western blotting was carried out as described above. NCI-N87 cells express far smaller amounts of endogenous BCRP than MCF-7 cells. The data are representative of three independent experiments.

Fig. S3. Effects of serum starvation on the degradation of endogenous breast cancer resistance protein in MCF-7 cells. After cells were cultured in DMEM supplemented with 7% FBS overnight, medium was exchanged with serum-free medium and the cell culture was continued for an additional 24 h. Then 30 μg/mL cycloheximide was added to the medium, and cell culture was continued for the indicated periods. Protein samples were prepared and Western blot analysis was carried out under non-reducing conditions. One-thirtieth parts of the whole-prepared protein was loaded in each lane. The relative intensities to those of control cells at the starting point (0 h) were determined. Open bar, control cells; dotted bar, serum-starved cells. Each bar represents the mean ± SD of triplicate determinations. Where a vertical bar is not shown, the SD is within the bar graph. The data are representative of (left) or derived from (right) three independent experiments. $*P < 0.05$.

Fig. S4. Effects of MEK inhibitors on exogenous breast cancer resistance protein (*BCRP*) mRNA expression. Treatment of cells with MEK inhibitors, total RNA extraction, and real-time RT-PCR were performed as described in the legend to Fig. 1(b). Each bar represents the mean ± SD of triplicate determinations. Where a vertical bar is not shown, the SD is within the bar graph. The data are representative of three independent experiments.

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Fig. S5. Effects of U0126 on anticancer drug resistance of NCI-N87/breast cancer resistance protein (BCRP) cells. (a) Effects of U0126 on cell growth. Cell culture and treatment with U0126 were performed as in the legend to Fig. 10(a). Cell numbers were determined and presented as percentages relative to those of control cells cultured in the absence of U0126. Open bars, NCI-N87 cells; dotted bars, NCI-N87/BCRP cells. The data shown are the means ± SD of triplicate determinations. Where a vertical bar is not shown, the SD is within the bar graph. (b) Effects of U0126 on anticancer drug resistance. Cells were seeded as described in the legend to Fig. 10(a) and cultured with or without 10 μM U0126 in the absence or presence of increasing doses of specific anticancer agents for 4 days. Cell numbers were determined, and presented as percentages relative to those of control cells cultured in the absence of anticancer agents. Open bars, U0126-untreated cells; dotted bars, U0126-treated cells. The data shown are the means ± SD of triplicate determinations, and are representative of at least three independent experiments. Where a vertical bar is not shown, the SD is within the bar graph. $*P < 0.05$.

Fig. S6. Effects of U0126 on cellular topotecan uptake in MCF-7/breast cancer resistance protein (BCRP) cells. Cells were cultured in the absence or presence of 10 μM U0126 for 48 h. After trypsinization, cells (5×10^5) were incubated with (solid line) or without (dotted line) 40 μM topotecan for 30 min. After washing, cellular uptake of topotecan was measured by flow cytometry. Data of the corresponding parental cells are shown in Figure 2(b).