Regulation of the cell surface expression of human BCRP/ABCG2 by the phosphorylation state of Akt in polarized cells

Tappei Takada, Hiroshi Suzuki, Yukiko Gotoh and Yuichi Sugiyama

Graduate school of Pharmaceutical Sciences, The University of Tokyo, Japan (T.T., H.S., and Y.S)

Department of Pharmacy, The University of Tokyo Hospital, Faculty of Medicine, The University of Tokyo, Japan (T.T., and H.S)

Institute of Molecular and Cellular Biosciences, The University of Tokyo, Japan (Y.G)
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To whom correspondence should be addressed:

Yuichi Sugiyama, Ph.D., Professor and Chair
Department of Molecular Pharmacokinetics, Graduate school of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
Phone +81-3-5841-4770     FAX +81-3-5841-4766
e-mail: yu-one.sugiyama@nifty.com

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Abbreviations:  ABC, ATP-binding cassette;  BCRP, breast cancer resistance protein;  CA, constitutively active;  DN, dominant negative;  EGF, epidermal growth factor;  PI3K, phosphatidylinositol 3-kinase;  PBS, phosphate buffered saline.
Abstract

Human breast cancer resistance protein (BCRP/ABCG2) is believed to act as an efflux pump to protect the body from drugs and toxins. BCRP is known to accept many kinds of endogenous and exogenous compounds as substrates and to be localized on the apical membrane of various tissues. Expression of BCRP is also reported on the side population cells and a recent report suggested involvement of Akt in the modulation of the side population phenotype. In the present study, we have characterized the effect of Akt on the polarized expression of BCRP using LLC-PK1 cells. After treatment with PI3K inhibitors, internalization of stably-transfected BCRP from the apical surface was observed following immunohistochemical staining and the relative expression level of BCRP on the cell surface decreased to 49 ± 14% and 51 ± 8% of the control for LY294002 and wortmannin treatment, respectively. This phenomenon was supported by the observation of internalized BCRP in presence of DN-Akt. When the cells were treated with EGF, the cell surface expression of BCRP was increased to 228 ± 43% of the control accompanied by Akt stimulation. These results suggest that the relative expression of BCRP on the cell surface is regulated by the PI3K-Akt signaling pathway with a positive correlation in polarized cells. Alteration in Akt activities may influence the cellular extrusion of BCRP substrates by modifying epithelial BCRP localization.
Introduction

Breast cancer resistance protein (BCRP/ABCG2) is known to be a half-transporter and is believed to act as a homodimer linked via disulfide bonds under physiological conditions (Allikmets et al., 1998; Doyle et al., 1998; Miyake et al., 1999; Kage et al., 2002). Several studies have shown that BCRP is involved in the transport of antitumor agents, such as topotecan, methotrexate and doxorubicin, so that it contributes to multidrug resistance as P-glycoprotein (P-gp/ABCB1) and multidrug resistance-associated protein 1 (MRP1/ABCC1) (Doyle and Ross, 2003).

In addition, it has been reported that BCRP recognizes other endogenous and exogenous compounds, for example, sulfated conjugates of steroid hormones and drugs (Suzuki et al., 2003), dietary carcinogen PhIP (van Herwaarden et al., 2003) and chlorophyll-derived phototoxins (Jonker et al., 2002). Together with the high expression of BCRP on the apical membrane of the intestine, liver and brain microvessel endothelium, it is now believed that BCRP has a physiological role as an efflux pump to protect the body against a variety of toxic agents (Jonker et al., 2002; Doyle and Ross, 2003; van Herwaarden et al., 2003).

Hoechst 33342, a fluorescent dye useful to sort side population cells which are highly enriched for stem cell activity, is also a substrate of BCRP. Experiments carried out by several investigators have shown high expression of Bcrp1 compared with other ABC transporters in the population (Zhou et al., 2001) and a reduction in the number of
side population cells in Bcrp1-deficient mice (Zhou et al., 2002), identifying BCRP expression as a molecular determinant of the Hoechst 33342 efflux phenotype. Furthermore, a recent report by Mogi et al. (Mogi et al., 2003) indicated that Akt, which functions downstream from phosphatidylinositol 3-kinase (PI3K) as an important mediator of insulin or growth factors, modulates the Hoechst 33342 efflux ability by regulating the expression of Bcrp1.

Under pathophysiological conditions, regulation of transporters has been observed; not only transcriptional regulation of genes, but also differences in the subcellular distribution of proteins. For instance, canalicular ABC transporters such as bile salt export pump (BSEP/ABCB11) and MRP2 (ABCC2) are internalized into subapical domains under cholestatic conditions to protect epithelial cells in the bile duct from the toxic effects of their substrates (Trauner et al., 1997; Paulusma et al., 2000; Stieger et al., 2000). The detailed mechanisms behind these observations remain unclear, although a proper regulation mechanism is crucial for the proper distribution of their substrates. Understanding the mechanism could lead to the development of new treatment strategies.

In the present study, to investigate the effect of Akt activity on the cell surface expression of BCRP in polarized cells, we treated the stable transfectant of human BCRP in LLC-PK1 cells with reagents that modulate Akt activity. Furthermore, we transfected dominant-negative or constitutively-active Akt and observed the subcellular
localization of human BCRP. Western blot analyses and immunohistochemical staining suggest that the apical expression of human BCRP is regulated by the PI3K-Akt signaling pathway.
Materials and Methods

**Materials.** Constitutively active-Akt (CA-Akt) and dominant negative-Akt (DN-Akt) plasmids were kindly provided by Drs. R. Roth and D. Alessi. Epidermal growth factor (EGF) of receptor grade was purchased from Biochemical Technology Inc. (Stoughton, MA). Wortmannin and LY294002 were purchased from Alomone Labs Ltd. (Jerusalem, Israel) and Promega (Madison, WI), respectively. The antibodies used in this study include the monoclonal antibody against BCRP (BXP-21) (Kamiya Biomedical Company, Seattle, WA), anti-Akt (Cell Signaling) and anti-phospho-Akt (Thr308) (Cell Signaling). All other chemicals used were commercially available and of reagent grade.

**Construction of BCRP expressing LLC-PK1 cells.** Wild type BCRP cDNA was purchased from ResGen (#H24176). The complete BCRP cDNA was amplified with the NheI site and Kozak sequence attached at the 5’-end, and with the ApaI site at the 3’-end by PCR, and then inserted into pcDNA3.1(+) vector plasmid (Invitrogen, Carlsbad, CA). BCRP in pcDNA3.1(+) was transfected to LLC-PK1 cells grown on a 6-well plate with FuGene 6 (Roche Diagnostics Corporation, Indianapolis, IN) according to the manufacturer’s instructions. Then, LLC-PK1 cells were selected by culturing them in the presence of 800 µg/ml G418 sulfate (Invitrogen). LLC-PK1 cells stably expressing BCRP were cultured in Medium 199 (GIBCO BRL, Gaithersburg,
MD) with addition of 10% of fetal bovine serum, penicillin (100 units/ml), streptomycin (100 \( \mu \)g/ml), and G418 sulfate (400 \( \mu \)g/ml) at 37°C in an atmosphere containing 5% CO\(_2\) and 95% humidity.

**Immunohistochemical staining.** For immunohistochemical staining, LLC-PK1 cells stably expressing BCRP were plated on a poly-L-coated cover glass (Micro cover glass, 18 x 18 mm and 0.12-0.17 mm thick, Matsunami Glass Ind., Osaka, Japan) at a density of 5 \( \times \) 10\(^5\) cells in 12-well dishes, 72 hr prior to the experiments, after transfection of plasmids containing DN-Akt or CA-Akt when staining for Akt was performed. After fixation with 4% (w/v) paraformaldehyde for 5 min and permeabilization in 1% Triton-X 100 in phosphate-buffered saline (PBS) for 10 min, cells were incubated with BXP-21 diluted 100-fold and polyclonal anti-Akt antibody diluted 200-fold in PBS for 1 hr at room temperature, washed three times with PBS, and then incubated with goat anti-mouse IgG Alexa 488 (Molecular Probes, Inc., Eugene, OR) diluted 250-fold and goat anti-rabbit IgG Alexa 546 (Molecular Probes, Inc., Eugene, OR) diluted 250-fold in PBS with TOPRO-3 iodide (Molecular Probes, Inc., Eugene, OR) for 1 hr at room temperature, and mounted in VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA). The localization of BCRP protein was visualized by confocal laser microscopy (Zeiss LSM-510; Carl Zeiss Inc., Thornwood, NY).
Cell surface biotinylation. LLC-PK1 cells stably expressing BCRP were grown on 24-well plates. Cells were washed with ice-cold PBS containing 0.1 mM CaCl₂ and 1 mM MgCl₂ (PBS-Ca/Mg), then incubated twice with NHS-SS-biotin (1.5 mg/ml; Pierce Biotechnology, Inc., Rockford, IL) at 4°C for 20 min. Then, the cells were washed with PBS Ca/Mg containing glycine (100 mM) and incubated with the same buffer for 20 min at 4°C. After removing the buffer, cells were disrupted with 50 µl lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 1% Triton-X 100, pH7.5) containing 1% SDS and protease inhibitor (0.1 mM PMSF) at 4°C for 30 min. To reduce the SDS concentration, samples were diluted with 450 µl lysis buffer. Then, 50 µl streptavidin-agarose beads (Pierce) was added to the lysate, followed by incubation at 4°C overnight with end-over-end rotation. Following centrifugation, the beads were washed three times with lysis buffer, twice with high salt lysis buffer (50 mM Tris, 500 mM NaCl, 5 mM EDTA, 0.1% Triton-X 100, pH7.5), and once with low salt lysis buffer (50 mM Tris, pH7.5). The biotinylated proteins were released by incubation with 50 µl 3 x SDS loading buffer (BioLabs, Hertfordshire, UK) diluted to 1 x SDS with PBS for 5 min at 60°C. Samples of total cell lysates (15 µl) and biotinylated proteins (20 µl) were subjected to Western blot analysis.

Western blot analysis. The total cell lysates diluted with 3 x SDS loading buffer
or biotinylated proteins were separated on an 8.5% SDS-polyacrylamide gel with a 4.4% stacking gel. The molecular weight was determined using a prestained protein marker (New England BioLabs, Beverly, MA). Proteins were transferred electrophoretically onto a polyvinylidene difluoride membrane (Pall, NY) using a blotter (Bio-Rad Laboratories, Richmond, CA) at 15 V for 1 hr. The membrane was blocked with Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 5% skimmed milk for 1 hr at room temperature. After washing with TBS-T, the membrane was incubated for 1 hr at room temperature in 100-fold diluted BXP-21 or 1000-fold diluted anti-Akt antibody or anti-phospho-Akt antibody. For detection, the membrane was allowed to bind to 5000-fold diluted horseradish peroxidase-labeled anti-mouse IgG antibody or anti-rabbit IgG antibody (Amersham Pharmacia Biotech, Buckinghamshire, UK) in TBS-T for 1 hr at room temperature followed by washing with TBS-T.
Results

**PI3K inhibitors cause internalization of BCRP from the cell surface.** The effect of PI3K inhibitors was investigated using LLC-PK1 cells stably expressing BCRP by immunohistochemical staining and Western blot analysis. Under normal conditions, human BCRP showed apical localization as reported previously (Imai et al., 2003). After treatment with PI3K inhibitors, LY294002 or wortmannin, the cellular localization of human BCRP was altered; some of the protein molecules showed the intracellular localization, although the localization on the apical membrane was also significant (Figure 1). These observations were also confirmed by Western blot analysis. After treatment with LY294002 or wortmannin, the expression level of Akt itself seemed unchanged, whereas hardly any phospho-Akt was observed (Figure 2A). The band density of BCRP in the Western blot analysis of the total cell lysate was almost the same under all conditions. However, the cell surface BCRP, which was detected by the band in the Western blot analysis for biotinylated protein, was reduced after treatment with PI3K inhibitors (Figure 2B). Collectively, as phospho-Akt disappeared, internalization of BCRP from the cell surface was observed.

**EGF treatment increases the cell surface expression of BCRP.** Characterization of the expression of BCRP was also performed after treatment with EGF. A stimulatory effect of EGF on the phosphorylation state of Akt was observed;
the detected band for phospho-Akt appeared stronger after EGF treatment and there seemed to be no difference in the antibody against Akt (Figure 3A). Under Akt-stimulated conditions, the relative ratio of the cell surface expression of BCRP increased compared with the control level (Figure 3B); more BCRP molecules on the cell surface was detected after stimulation by EGF whereas the apparent expression level of BCRP in the total cell lysate appeared to be unchanged.

**Immunolocalization of BCRP in the presence of CA- or DN-Akt.** To investigate the direct effect of Akt on the subcellular distribution of BCRP, the effect of transfection of CA-Akt and DN-Akt was investigated. DN-Akt, which has mutations such as K179A, T308A and S473A in human Akt1, and CA-Akt, the PH domain of which in human Akt1 was substituted by a myristoylation signal sequence (Higuchi et al., 2003), were detected in Western blot analysis with the expected band lengths after transfection of expression vectors (Figure 4) (Masuyama et al., 2001). Subcellular localization of BCRP in the presence of DN-Akt or CA-Akt was characterized by immunohistochemical staining (Figure 5). It was difficult to detect any difference in the cellular localization of BCRP between the control and CA-Akt transfected cells. We could not also observe the alterations in the cellular localization of BCRP in the presence of EGF (data not shown). In contrast, the alterations in the cellular localization of BCRP was observed by transfecting DN-Akt cDNA; green signals for
BCRP appeared not only on the apical surface, but also in the intracellular compartment as was observed after the treatment with PI3K inhibitors (Figure 1).
Discussion

In the present study, we observed the difference in the subcellular localization of human BCRP after treatments which cause alterations in the phosphorylation state of Akt by stimulating or inhibiting the upstream regulator, PI3K; treatment with PI3K inhibitors, LY294002 or wortmannin, induced internalization of BCRP (Figure 1 and 2), and in addition, after incubation with EGF, the relative expression of BCRP on the cell surface increased (Figure 3). Furthermore, after transfection of the dominant-negative Akt cDNA into BCRP-expressing cells, the expression of BCRP seemed to spread to the intracellular domain in addition to the apical localization. Taken together, these observations indicate that the cell surface expression of human BCRP in polarized cells is modulated by the phosphorylation state of Akt.

It is possible that drug-drug interactions could be caused by this regulatory mechanism; if drugs influence the Akt activity, the in vivo transport activity of BCRP may also be modified due to the altered level of cell surface BCRP. Although many compounds are believed to inhibit the function of BCRP, all of them are not reported to inhibit directly the transport function of BCRP; for many of them, a shift in the cytotoxic effect of BCRP substrates (such as topotecan) in BCRP-expressing cells has been shown (Doyle and Ross, 2003). Our hypothesis may be likely to hold true, particularly for EGF receptor inhibitors like CI1033 or Iressa, since they can inhibit the PI3K-Akt signaling pathway (Erlichman et al., 2001; Doyle and Ross, 2003).
Under physiological conditions, in most cases, the subcellular expression of BCRP has been believed to be on the plasma membrane (Rocchi et al., 2000). However, a recent report by Summer et al. (Summer et al., 2003) showed that the Bcrp1 protein in primary bronchial smooth muscle cell cultures and lavaged distal airway cells was confined to the intracellular compartment. Taking this information and our observations into account, BCRP/Bcrp1 localization may be correlated with endogenous Akt activity, although there is no direct evidence to confirm this yet. When Akt activity is modified by factors, such as dietary components or under pathophysiological conditions, the cellular localization of BCRP may also be modified, and consequently, the disposition of the substrate drugs may be altered. For example, green or black tea polyphenols are known to have the ability to down-regulate Akt activity; it is possible that the expression of BCRP in epithelial cells, particularly in small intestinal epithelial cells, could be affected by these compounds, due to high direct exposure (Klein and Fischer, 2002; Pianetti et al., 2002).

Bcrp1-deficient mice are reported to exhibit no biochemical abnormalities under standard housing conditions (Jonker et al., 2002; Zhou et al., 2002). However, they show diet-dependent phototoxicity caused by insufficient protection against chlorophyll-derived dietary phototoxins which are Bcrp1 substrates (Jonker et al., 2002). Our results indicate that the lower activity of Akt causes lower expression of BCRP on the cell surface and, therefore, it is possible that in mice lacking Akt(s) a similar
phenotype may be observed. Although there are some reports on the mice being knocked out of genes encoding Akt1 (Chen et al., 2001; Cho et al., 2001b) or Akt2 (Cho et al., 2001a; Garofalo et al., 2003) or both of Akt1 and Akt2 (Peng et al., 2003), the sensitivity to dietary chlorophyll-breakdown products has not been reported. Analysis of the photosensitive behavior of these mice is an attractive topic for further study.

Besides BCRP, there are a few membrane proteins whose cell surface expression is regulated by Akt: a facilitative glucose transporter 4 (GLUT4) (Foster et al., 2001) and Na⁺/H⁺ exchanger 3 (NHE3) (Lee-Kwon et al., 2001). Their expression on the cell surface increases with stimulation of the PI3K-Akt pathway induced by insulin or growth factors (Janecki et al., 2000; Watson and Pessin, 2001). The effect of stimulation is similar to BCRP, although the cell surface expression under non-stimulated conditions varies depending on the proteins; although GLUT4 is hardly detected on the cell surface in the absence of insulin (Karylowski et al., 2003), approximately 40% of the protein appears on the cell surface under basal conditions in the case of NHE3 (du Cheyron et al., 2003) and BCRP (Figure 3). Despite the observation of increased biotinylated BCRP molecules by Western blot analysis (Figure 3), it is difficult to find any difference in BCRP localization between control and stimulated conditions by immunohistochemical staining performed in the present study. A possible explanation is that, under control conditions, some BCRP may be located in the proximal subapical pool which is difficult to distinguish from the apical membrane. 

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under the immunohistochemical staining conditions used in this study.

Since there is no putative consensus site for phosphorylation by Akt (Brazil and Hemmings, 2001), a direct interaction between BCRP and Akt is unexpected. For consideration of the detailed mechanism involved, information on GLUT4 regulation may be useful. In a recent report, TUG was identified as a putative tether, containing a UBX domain, for the regulation of intracellular sorting of GLUT4 (Bogan et al., 2003). In non-stimulated cells, TUG and GLUT4 form the complex via direct binding, and this complex is largely disassembled by insulin. Because of differences in tissue distribution and the ratio of surface expression, TUG may not be involved in the intracellular regulation of BCRP, however, this example is valuable for indicating future directions for investigation.

In conclusion, we have investigated the effect of Akt stimulation and inhibition on the polarized expression of human BCRP in LLC-PK1 cells. The results obtained by Western blot analyses for biotinylated and total cell lysate specimens suggested that the relative expression of BCRP on the cell surface is correlated with Akt activity. The observations on the inhibition of Akt were also supported by the results of immunohistochemical staining. This is the first report of the regulation of BCRP by the signal transduction cascade in polarized cells and may be useful for the prediction of drug-drug interactions or influence on the distribution of BCRP substrates affected by this regulation.
References


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Legends for figures

Figure 1. **Effect of PI3K inhibitor treatment on the subcellular distribution of BCRP in LLC-PK1 cells.** Subcellular localization of BCRP was examined in LLC-PK1 cells after treatment with PI3K inhibitors. BCRP-expressing LLC-PK1 cells were stained with BXP-21 (green fluorescence) after a 90 min incubation with normal medium (control), medium containing 20 µM LY294002 or 200 nM wortmannin. Nuclei were stained with TOPRO-3 (blue fluorescence). For control and LY294002, the Z-sectioning image is shown and for wortmannin the top part shows the Z-sectioning image with a horizontal line in the en face image and the bottom part shows the en face image.

Figure 2. **Effect of PI3K inhibitors on the cell surface expression of BCRP and phosphorylation state of Akt.** Total cell lysate protein containing both cell surface and intracellular protein (A and B, cell lysate) and biotinylated protein (B, biotinylated) were subjected to SDS-PAGE (8.5%). Treatment conditions for cells are as illustrated in Figure 1. The membrane was incubated with antibody against Akt, phospho-Akt (A) as indicated or with BXP-21 (B). The band density against BCRP was quantified in a luminescent image analyzer and the relative BCRP expression on the cell surface was calculated as the band density in the biotinylated sample divided by that in the cell lysate sample. In panel B, each bar represents the mean ± S.E. of three
Figure 3. **Effect of EGF on the phosphorylation state of Akt and cell surface expression of BCRP.** Total cell lysate protein containing both cell surface and intracellular protein (A and B, cell lysate) and biotinylated protein (B, biotinylated) were subjected to SDS-PAGE (8.5%). BCRP-expressing LLC-PK1 cells were biotinylated after a 30 min incubation with normal medium (control) or medium containing 100 ng/ml EGF. The membrane was incubated with antibody against Akt, phospho-Akt (A) as indicated or with BXP-21 (B). The band density against BCRP was quantified in a luminescent image analyzer and the relative BCRP expression on the cell surface was calculated as the band density in the biotinylated sample divided by that in the cell lysate sample. In panel B, each bar represents the mean ± S.E. of three independent experiments. Statistical significance is indicated as *, which means p <0.05.

Figure 4. **Western blot analysis for Akt in LLC-PK1 cells.** Expression of Akt after transfection of DN-Akt or CA-Akt plasmid was determined by Western blot analysis. Total cell lysate protein from LLC-PK1 cells transfected with DN-Akt plasmid, CA-Akt plasmid or water (mock) was separated by 8.5% SDS-PAGE. The
membrane was incubated with antibody against Akt.

Figure 5. **Immunolocalization of BCRP and Akt in LLC-PK1 cells.**

Subcellular localization of BCRP and Akt were examined in LLC-PK1 cells transfected with water (A), CA-Akt plasmid (B) or DN-Akt plasmid (C). BCRP-expressing LLC-PK1 cells were stained with BXP-21 (green fluorescence) and anti-Akt antibody (red fluorescence). Nuclei were stained with TOPRO-3 (blue fluorescence). In each panel, the top part shows the en face image and the bottom part shows the Z-sectioning image with a horizontal line in the en face image.
Fig. 1

control

LY294002

wortmannin

xz sections

xy plane
Fig. 2

(A) pAkt

(B) Akt

control
LY294002
wortmannin

relative BCRP
(biotinylated/cell lysate)

0
0.5
1

control
LY294002
wortmannin

biotinylated

cell lysate
Fig. 3

(A) pAkt and Akt

(B) Relative BCRP (biotinylated/cell lysate)

Control EGF

Biotinylated cell lysate

Control EGF
Fig. 4

DN-Akt  CA-Akt  mock

(kDa)

175  83  62  48  33
Fig. 5

(A)

xy plane

xz sections

(B)

xy plane

xz sections

(C)

xy plane

xz sections