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Research Paper

Characterization of Polarized Expression of Point- or Deletion-Mutated Human BCRP/ABCG2 in LLC-PK1 Cells

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Purpose. In polarized cells, such as hepatocytes and intestinal epithelial cells, transporters are localized on the apical or basolateral membranes and play important roles in the vectorial transport of their substrates. In the current study, we have aimed to clarify the mechanism for the cellular sorting of human breast cancer resistance protein (BCRP/ABCG2), which is expressed on the apical membrane of many tissues and functions as an efflux transporter.
Methods. After the expression vector, including wild type or mutants of human BCRP cDNA, was transfected into LLC-PK1 cells, immunohistochemical staining and Western blot analyses were performed to characterize the cellular localization and the status of BCRP, respectively.
Results. The transfected cDNA product of wild-type BCRP was expressed on the apical membrane in LLC-PK1 cells. Glycosylation consensus sequences-disrupted mutants showed the apical localization as the wild type, whereas the apical-selective expression disappeared when disulfide bonds could not be formed. Furthermore, examination of the localization of deletion mutants of human BCRP emphasized the importance of some peptide sequences. The region between the N-terminal and ATP-binding cassette and proximal C-terminal region, both of which are well conserved in various animal species, were found to be significant for proper localization.
Conclusions. These results suggest that, although the presence of N-glycan does not affect the localization of BCRP, disulfide bonds and some peptide sequences in both the N- and C-terminals are necessary for the apical expression of BCRP.

KEY WORDS: breast cancer resistance protein; epithelial cells; transporter.

INTRODUCTION

Breast cancer resistance protein (BCRP), also known as ABCG2, is a member of ABC transporter family (1–3). The protein encodes a 655-amino-acid transmembrane protein with a domain organization characteristic of ABC half-transporters, which typically contain six transmembrane segments and one ATP-binding cassette, and requires dimerization with a partner protein to become functional transporters. Many reports indicate that BCRP is expressed as a homodimer combined with disulfide bonds (4,5) and is N-glycosylated in mammalian cells (6,7). From the predicted topology, both the NH_2-terminus and the COOH-terminus are thought to be located in the cytoplasmic side of plasma membrane (7).

BCRP transports various kinds of endogenous and exogenous compounds: drugs such as mitoxantrone or anthracyclines, dyes such as Rhodamine 123 or Hoechst 33342 (8), and several sulfated conjugates (9). In addition to them, recent reports indicate that drastic changes in the bioavailability and tissue distribution of chlorophyll derivatives (10) and carcinogens in food (11) occur in mice lacking Bcrp1. These results suggest that BCRP/Bcrp1, which is expressed on the apical membrane of several tissues, has the physiologic function as an export pump of its substrates.

Genetic disorders caused by mutations in a coding region have been reported for several ABC transporters such as cystic fibrosis caused by mutations in cystic fibrosis transmembrane regulator (CFTR/ABCC7) (12) or Dubin-Johnson syndrome by mutations in multidrug resistance–associated protein 2 (MRP2/ABCC2) (13,14). Mechanism of disorders includes the nonsense mutation and loss of transport activity, and in addition, disrupted intracellular trafficking of transporters, resulting in impaired vectorial transport (15,16). Although involvement of BCRP on genetic disorders has not been reported, protoporphyria or photosensitive dermatitis could be caused by disrupted function of BCRP, which was observed in Bcrp1 –/– mice (10). Accordingly, the regulatory mechanism governing the polarized expression of BCRP would be an important issue, although little knowledge has been reported to date.

In the current study, to characterize the regulatory

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ABBREVIATIONS: ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; PNGase F, peptide N-glycosidase F; GFP, green fluorescent protein; PBS, phosphate buffered saline.

mechanism of polarized expression of human BCRP, we have constructed several kinds of BCRP mutant on putative important factors (N-glycosylation, disulfide bonds, and cytoplasmic domains of the protein). Immunohistochemical staining of the mutants transfected into LLC-PK1 cells suggest that disulfide bonds and some peptide sequences in both the N- and C-terminals are necessary for the apical expression of BCRP, whereas N-glycosylation does not affect BCRP localization.

MATERIALS AND METHODS

Materials

Peptide *N*-glycosidase F (PNGase F) was purchased from New England Biolabs (Beverly, MA, USA), and the digestion procedure was performed as described by the manufacturer. All other chemicals used were commercially available and of reagent grade.

Cell Culture and Transfection

Parental LLC-PK1 cells were cultured in Medium 199 (GIBCO BRL, Gaithersburg, MD, USA) with 10% of fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C with 5% CO_2 and 95% humidity. Vector plasmid containing wild type or mutant BCRP was transfected into LLC-PK1 cells by using FuGene 6 (Roche Diagnostics Corporation, Indianapolis, IN, USA) according to the manufacturer's instructions. For the selection of stable transfectants, cells were cultured in the presence of 800 μg/ml G418 sulfate (Invitrogen, Carlsbad, CA, USA), and for maintenance the culture medium was supplemented with 400 μg/ml G418 sulfate.

Construction of BCRP-Containing Expression Vectors

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 was inserted into pcDNA3.1(+) vector plasmid (Invitrogen). Using the site-directed mutagenesis technique, several mutants of BCRP (N418A, N596A, N418A&N596A BCRP and C592A, C603A&C608A, C592A&C603A&C608A BCRP) were constructed on pcDNA3.1(+) vector. Introduction of mutations was verified by full sequencing. N-terminal and C-terminal deletion constructs were generated by cloning PCR-amplified fragments into the NheI and the ApaI sites of pcDNA3.1(+) in a similar manner with wild-type BCRP. BCRP cDNA, without the termination codon in BCRP-GFP, was inserted into the HindIII and the ApaI sites of a pEGFP-C1 vector plasmid (BD Biosciences CLONTECH, Palo Alto, CA, USA) or into the NheI and the HindIII sites of a pEGFP-N1 vector plasmid (BD Biosciences CLONTECH) for the construction of GFP-BCRP or BCRP-GFP, respectively.

Immunohistochemical Staining

For immunohistochemical staining, LLC-PK1 cells transiently or stably transfected with various BCRP-containing vectors were plated on a poly-L-coated cover glass (Micro cover glass, 18 × 18 mm and 0.12-0.17 mm thick, Matsunami Glass Ind., Osaka, Japan) at a density of 5×10^5 cells in 12-well dishes, 72 h prior to the experiments. 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 the monoclonal antibody against BCRP (BXP-21) (Kamiya Biomedical Company, Seattle, WA, USA) diluted 100-fold in PBS for 1 h at room temperature, washed three times with PBS, and then incubated with goat anti-mouse IgG Alexa 488 (Molecular Probes, Inc., Eugene, OR, USA) diluted 250-fold in PBS for 1 h at room temperature, and mounted in VECTASHIELD Mounting Medium with Propidium Iodide (Vector Laboratories, Burlingame, CA, USA). The localization of BCRP protein was visualized by confocal laser microscopy (Zeiss LSM-510; Carl Zeiss Inc., Thornwood, NY, USA). For detection of GFP-fused BCRP, cells were mounted in VECTASHIELD Mounting Medium with Propidium Iodide after the fixation procedure.

Western Blot Analysis

Membrane fractions were prepared from LLC-PK1 cells stably expressing wild type and mutant BCRP as described previously (17). After indicated treatments, crude membrane fractions were diluted with 3 x SDS loading buffer (BioLabs, Hertfordshire, UK) and 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). Proteins were transferred electrophoretically onto a polyvinylidene difluoride membrane (Pall, East Hills, NY, USA) using a blotter (Bio-Rad Laboratories, Richmond, CA, USA) at 15 V for 1 h. The membrane was blocked with Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 5% skimmed milk for 1 h at room temperature. After washing with TBS-T, the membrane was incubated for 1 h at room temperature in 100-fold diluted BXP-21. For the detection of BCRP, the membrane was allowed to bind to 5000-fold diluted horseradish peroxidase-labeled anti-mouse IgG antibody (Amersham Pharmacia Biotech, Buckinghamshire, UK) in TBS-T for 1 h at room temperature followed by washing with TBS-T.

RESULTS

Expression of Wild-Type BCRP in LLC-PK1 Cells

Expression of wild-type human BCRP was examined by immunohistochemical staining and Western blot analysis. After transfection of expression vector containing wild type BCRP cDNA, BCRP showed apical localization in LLC-PK1 cells (Fig. 1), which reflects the physiologic localization of BCRP in polarized cells (6,10). From the result of the Western blot analysis using crude membrane fractions, the presence of N-glycosylation and disulfide bonds was demonstrated (Fig. 2). Under reducing condition, a band of about 70 kDa was observed as expected and, after treatment with PNGase F, which cleaves the connection between the innermost GlcNAc and asparagine residue, the apparent molecular weight decreased, indicating that BCRP was N-glycosylated in this experimental system. Furthermore, in the absence of reducing agent 2-mercaptoethanol, a band with a higher mo-

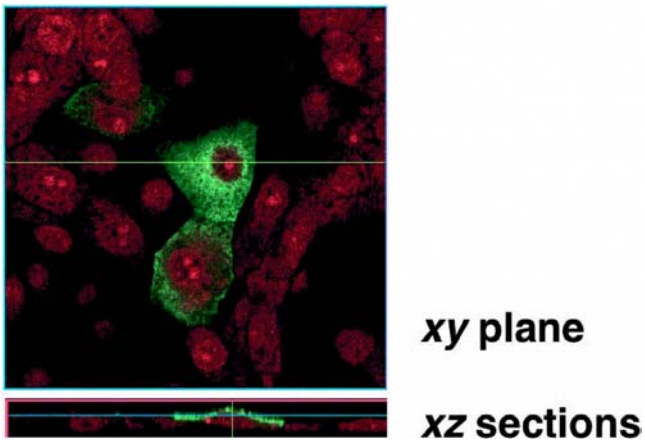

Fig. 1. Immunolocalization of wild-type BCRP in LLC-PK1 cells. LLC-PK1 cells stably transfected with wild-type BCRP were stained with BXP-21 (green fluorescence). Nuclei were stained with propidium iodide (red fluorescence). The top part shows the en face image, and the bottom part shows the Z-sectioning image with a horizontal line in the en face image.

lecular weight appeared, indicating the presence of intermolecular disulfide bonds.

Absence of N-glycosylation Does Not Affect the Localization of BCRP

In human BCRP peptide sequence, there are two putative N-glycosylation sites as illustrated in Fig. 3A. Although the latter asparagine residue is well conserved in all species reported, the former asparagine residue is not present in the mouse orthologue and the consensus motif (NXS/T) is not conserved in rat and porcine BCRP. Western blot analysis revealed that the modification was on the latter asparagine (Fig. 3B); the N418A mutant was N-glycosylated, whereas the N596A and N418A&N596A mutants did not exhibit any alteration in molecular weight following PNGase F treatment. However, the absence of N-glycosylation did not affect the cellular localization of BCRP. All mutants of putative N-glycosylation sites showed apical localization in LLC-PK1 cells (Fig. 3C).

Disulfide Bonds Are Necessary for the Proper Expression of BCRP

Substitution of predicted extracellular cysteine was performed to investigate the effect of disulfide bonds on the cellular localization of BCRP. All of three cysteine residues are conserved in all species reported (Fig. 4A). Western blot analysis demonstrated the disappearance of disulfide bonds in the C592A&C603A&C608A mutant (Fig. 4B). Results of immunohistochemical staining suggested the importance of disulfide bonds on apical localization; a part of C592A mutant product seemed to be located on apical membrane, whereas other mutants appeared intracellularly (Fig. 4C).

Cellular Localization of N-terminal Truncated BCRP

Cellular localization of truncated mutants in the N-terminal region of BCRP was investigated in LLC-PK1 cells. BCRP is predicted to have 60 amino acids in the region from the N-terminal to the ATP-binding cassette and we initially constructed stepwise truncated constructs with 10 amino acids. N10Δ, N20Δ, and N30Δ mutants seemed to be expressed on the apical membrane as the wild type, whereas N40Δ, N50Δ, and N60Δ mutants appeared intracellularly (Fig. 5A). In addition, detailed constructs such as N32Δ and N34Δ exhibited apical expression, and the expression of N36Δ mutant was detected both on the apical membrane and in the intracellular compartment (Fig. 6).

Importance of C-terminal Domains of BCRP for Apical Expression

Truncated constructs of the proximal C-terminal region of human BCRP were also characterized. Removal of only two or three amino acids from the C-terminal was enough to cause impaired trafficking of BCRP (Fig. 7A). Intracellular expression of BCRP-GFP, in contrast to the proper localization of GFP-BCRP as reported (18), supports the importance of the interaction of the proximal C-terminal region with unknown element (s) (Fig. 8).

DISCUSSION

In the current study, we demonstrated the apical localization of human wild-type BCRP and the presence of N-

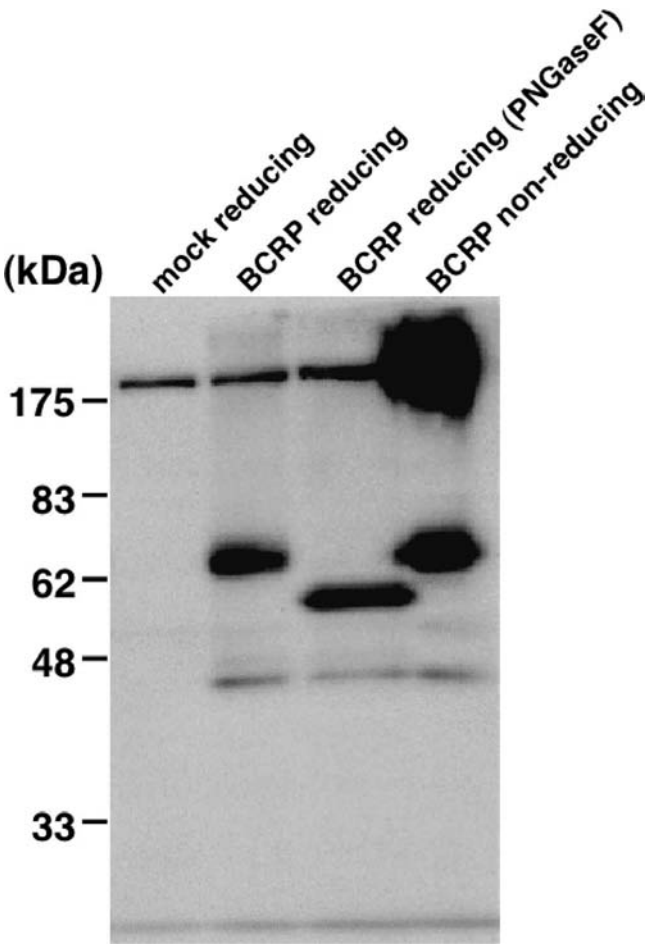

Fig. 2. Western blot analysis of LLC-PK1 cells expressing wild-type BCRP. Crude membrane fractions of LLC-PK1 cells transfected with empty-vector (mock) or wild-type BCRP were loaded after indicated treatments and separated by SDS-PAGE (8.5% separating gel). BCRP was detected by BXP-21. Samples were diluted with the loading buffer in the presence (reducing) or absence (nonreducing) of 2-mercaptoethanol.

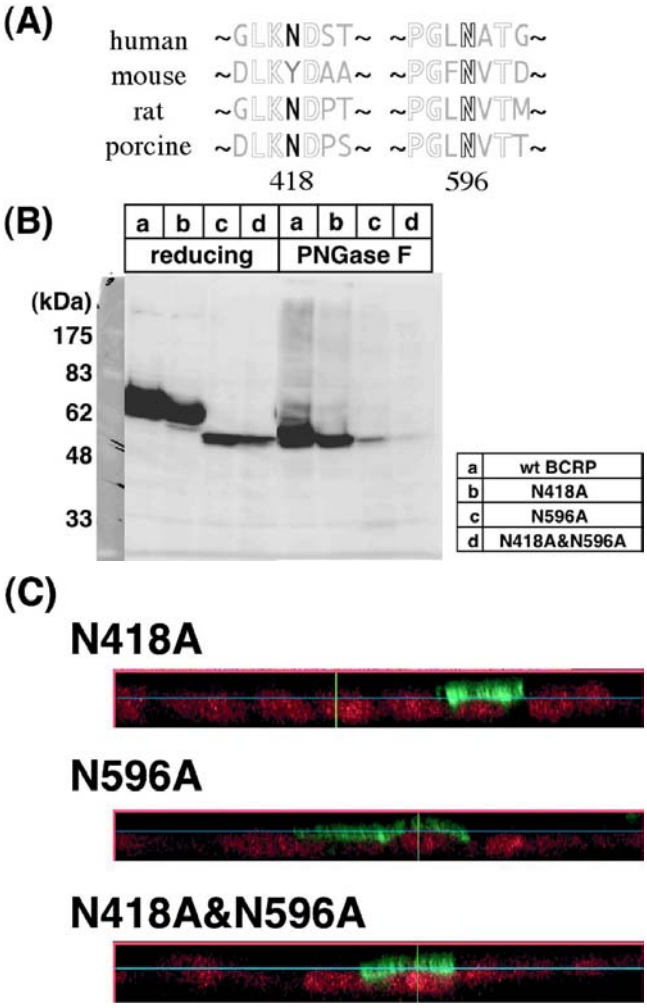

Fig. 3. Effect of the disruption of putative N-glycosylation sites. (A) Sequence alignment of the putative N-glycosylation sites (NXS/T) in BCRP of human, mouse, rat, and pig. Two putative sites exist in the predicted extracellular region of human BCRP. (B) Western blot analysis of the crude membrane fractions of LLC-PK1 cells stably transfected with wild type or point mutants of BCRP. Samples were loaded after indicated treatments and separated by SDS-PAGE (8.5% separating gel). BCRP was detected by BXP-21. (C) Immunohistochemical staining of LLC-PK1 cells stably transfected with point mutants of BCRP. BCRP was stained with BXP-21 (green fluorescence) and nuclei were stained with propidium iodide (red fluorescence). The Z-sectioning image is shown for each mutant.

glycan and disulfide bonds using BCRP-transfected LLC-PK1 cells. Moreover, the importance of several candidates for the regulation of the subcellular localization of BCRP was investigated by immunohistochemical staining of several kinds of point or deletion mutant. Some mutants showed intracellular localization, whereas their cellular localization is expected to be on whole cell membrane if apical targeting or retention signal was lost in these mutants. One possible explanation is the absence of μ1B subunit of AP-1B complex in LLC-PK1 cells, which has been shown to be important for basolateral targeting in polarized epithelial cells (19). Indeed, human BCRP has a putative dileucine signal in the cytoplasm near the transmembrane domain, which is highly conserved in other species and in other ABCG family proteins (20). Because the dileucine domain is known to be one of the basolateral sorting signals (21,22), if similar analyses are performed using a polarized cell system including μ1B, it is possible that the expression of BCRP mutants would be observed on the whole cell surface or the basolateral membrane.

N-glycosylation has been shown to be involved in the apical localization of a number of secretory or membrane proteins (23), whereas exceptions of the involvement have accumulated. For example, in the case of the neuronal glycine transporter GLYT2, N-glycosylation is necessary for the apical expression of proteins (24), while the ecto-nucleotide pyrophosphatase / phosphodiesterase NPP3 does not show the altered subcellular localization in the absence of N-glycan (25). We showed that human BCRP was N-glycosylated in the

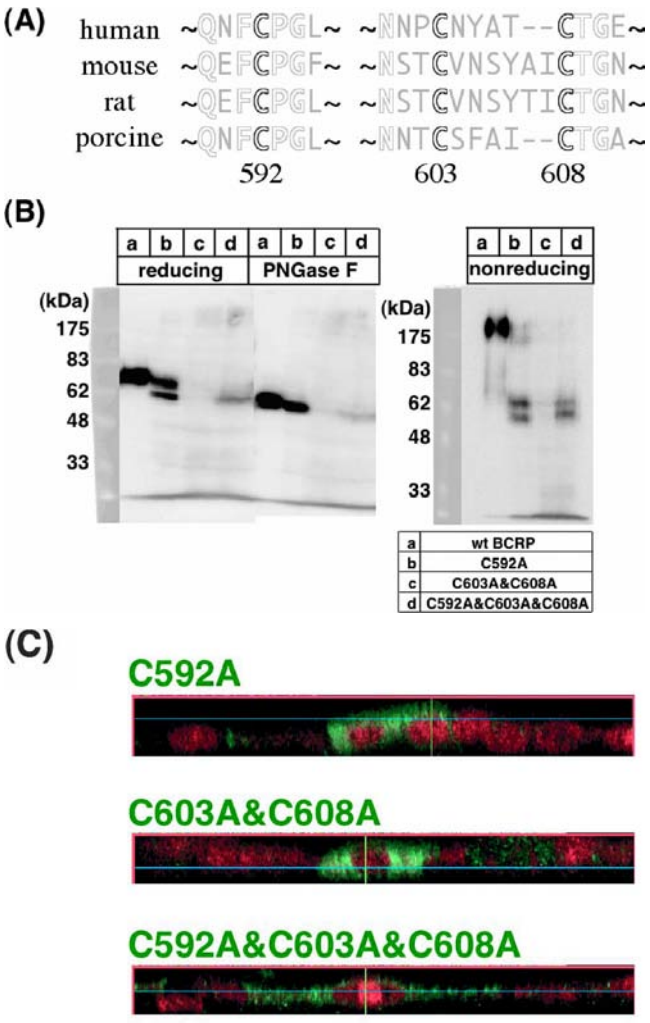

Fig. 4. Effect of disulfide bonds on the expression of BCRP. (A) Sequence alignment of the putative sites of disulfide bond in BCRP of human, mouse, rat, and pig. Three putative sites exist in the predicted extracellular region of BCRP. (B) Western blot analysis of crude membrane fractions of LLC-PK1 cells stably transfected with wild type or point mutants of BCRP. Samples were loaded after indicated treatments and separated by SDS-PAGE (8.5% separating gel). BCRP was detected by BXP-21. (C) Immunohistochemical staining of LLC-PK1 cells stably transfected with point mutants of BCRP. BCRP was stained with BXP-21 (green fluorescence) and nuclei were stained with propidium iodide (red fluorescence). The Z-sectioning image is shown for each mutant.

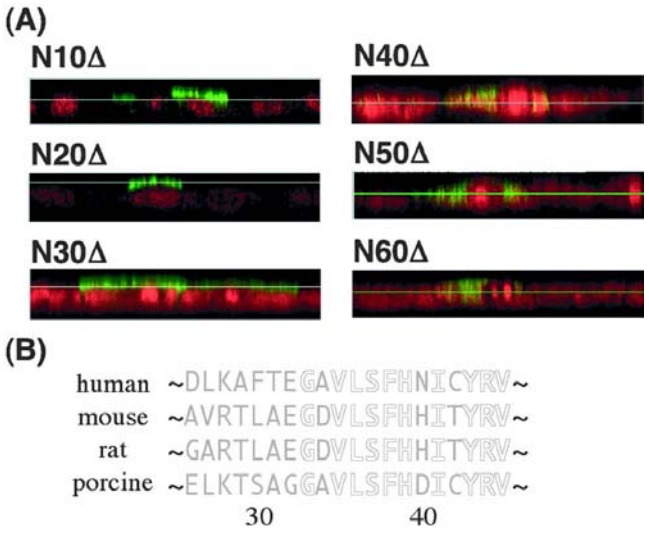

Fig. 5. Cellular localization of N-terminal truncated mutants of BCRP. (A) Immunohistochemical staining of LLC-PK1 cells transiently transfected with N-terminal truncated mutants of BCRP. BCRP were stained with BXP-21 (green fluorescence) and nuclei were stained with propidium iodide (red fluorescence). The Z-sectioning image is shown for each mutant. (B) Sequence alignment of the region in the N-terminus in BCRP of human, mouse, rat, and pig.

596th asparagine, although substitution of the residue for alanine did not lead to any difference on the apical localization of BCRP in LLC-PK1 cells (Fig. 3).

Results of Western blot analyses showed that disulfide bonds are formed in BCRP expressed in LLC-PK1 cells (Fig. 2); a band with a higher molecular weight appeared under non-reducing condition and this band completely disappeared

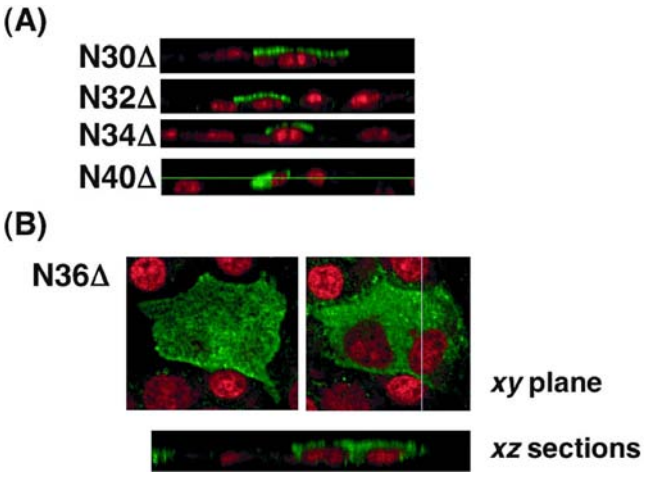

Fig. 6. Immunolocalization of detailed deletion mutants of the N-terminal domain. (A) Immunohistochemical staining of LLC-PK1 cells transiently transfected with N-terminal truncated mutants of BCRP. BCRP were stained with BXP-21 (green fluorescence) and nuclei were stained with propidium iodide (red fluorescence). The Z-sectioning image is shown for each mutant. (B) LLC-PK1 cells transiently transfected with N36Δ BCRP were stained with BXP-21 (green fluorescence). Nuclei were stained with propidium iodide (red fluorescence). 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.

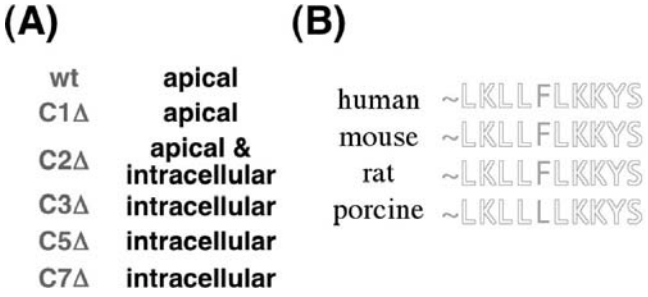

Fig. 7. Immunolocalization of deletion mutants of the C-terminal domain. (A) Summary of the results of immunohistochemical staining of LLC-PK1 cells transiently transfected with C-terminal truncated mutants of BCRP. (B) Sequence alignment of the C-terminal region in BCRP of human, mouse, rat, and pig.

under reducing condition. A report by Kage *et al.* (4) clearly demonstrated that BCRP becomes a homodimer by intermolecular disulfide bonds, in which length of a band under nonreducing condition appeared to be 140 kDa which represents the double of monomer 70 kDa BCRP under reducing condition. However, in our results of Western blot analysis, bands under nonreducing conditions appeared to be more than 175 kDa (Figs. 2 and 4B). A similar observation has been reported (26) using the S1-M1-80 cell line; under nonreducing conditions, several bands with 180 kDa or more were observed. It is speculated that additional protein(s) would be a partner of BCRP in some cell lines or oligomeric BCRP, which could be a tetramer as reported by Xu *et al.* (27), would be detected in our experimental condition.

In the absence of extracellular cysteine disulfide bonds disappeared, while a smaller (~60 kDa) protein was detected in Western blot analyses using crude membrane fractions (Fig. 4B). Because the band length is similar to the length of wild-type BCRP after PNGase F digestion, the smaller band should represent immature BCRP, which might be localized in the endoplasmic reticulum by entrapment of calnexin as Delta F508 CFTR (28). However, because a band with the

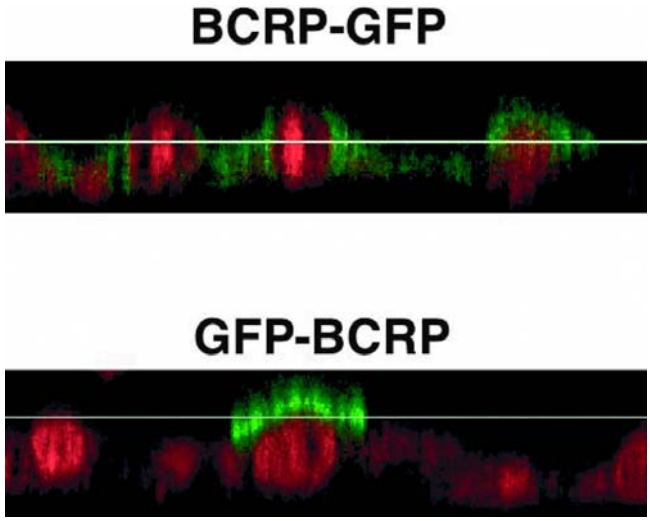

Fig. 8. Cellular localization of BCRP-GFP and GFP-BCRP. LLC-PK1 cells were stably transfected with BCRP-GFP or GFP-BCRP and imaged by confocal microscopy. Nuclei were stained with propidium iodide (red fluorescence). The Z-sectioning image is shown for each construct.

length of matured BCRP (~70kDa) was detected in the C592A&C603A&C608A mutant (Fig. 4B), importance of disulfide bonds for the intracellular localization of BCRP is debatable, although lower stability of protein might have caused weak signals of C603A&C608A and C592A&C603A&C608A mutant (Fig. 4B). As far as the mass balance of band density is concerned, it seems that the strength of bands is much higher under non-reducing condition, whereas the reason remains unknown (Fig. 2). This phenomenon has also been reported in another report (4), and might be explained by a difference in the accessibility of the antibody to the protein.

Some ABC transporters have been reported to have interactions with cytoplasmic proteins which are important for their subcellular localization. Radixin, the dominant ERM protein in liver, has been shown to be necessary for MRP2 expression on the canalicular membrane via direct binding to the carboxy-terminal cytoplasmic domain of MRP2 (29). ERM family proteins are also known to have interactions with P-glycoprotein (P-gp/ABCB1) and have a role as cross-linkers for the association of P-gp with actin filaments (30). Several reports have shown that CFTR or MRP2 interacts with PDZ proteins (31,32) and the lack of its PDZ binding domains results in their mislocalization (33,34). In the current study, we identified putatively important peptide sequences for the proper localization of BCRP. As shown in Figs. 5B and 7B, the regions are highly conserved in all reported orthologues of BCRP, which might indicate the importance of the sequences, while consensus sequences as well-known membrane targeting motifs, such as the PDZ binding domain and the tyrosine motif, are not included in these regions. To clarify a role of these regions on the cellular localization of BCRP is an important subject for further analysis.

In conclusion, we have characterized the polarized expression of human BCRP using point or deletion mutants and results suggest that N-glycan is not significant, whereas disulfide bonds and some peptide sequences in the N- and C-terminals are necessary for the proper localization of BCRP in LLC-PK1 cells. It remains to be disclosed whether these findings could be generalized to other polarized cells. Moreover, in addition to possible association of the putatively important elements with single nucleotide polymorphisms, which have not been reported yet, further analyses of the machinery of the targeting signals would be an important issue.

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