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ORIGINAL ARTICLE

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Absence of *N*-linked glycosylation does not affect plasma membrane localization of breast cancer resistance protein (BCRP/ABCG2)

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Abstract Breast cancer resistance protein (BCRP/ABCG2) is an ATP-binding cassette (ABC) multidrug transporter that confers resistance to various anticancer drugs like topotecan and mitoxantrone. To obtain more insight in its cellular functioning, we investigated phosphorylation and *N*-linked glycosylation of BCRP. In the epithelial Madin-Darby canine kidney (MDCK) cell line, we did not detect phosphorylation of BCRP, in contrast to MRP2, which was phosphorylated. In the ovarian carcinoma cell line T8 also no phosphorylated BCRP was detected. As BCRP in both lines effectively transports drugs, it appears that phosphorylation of BCRP (if it occurs at all) is not needed for drug transport. We further mutated the asparagine residues 418, 557 and 596 in three putative *N*-linked glycosylation motifs of BCRP to alanines. Mutant proteins were expressed in CHO9 and MDCKII cells by transient transfection and characterized by Western blot and immunofluorescence analysis. We found that only BCRP-N596A and a mutant with all three asparagines mutated (triple mutant) were not glycosylated anymore, indicating that only asparagine 596 is normally glycosylated. The mutation of asparagine 596 (or 418) had little effect on the subcellular localization of BCRP, indicating that *N*-linked glycosylation is not essential for routing to the plasma membrane. However, BCRP-N557A and the triple mutant were mainly localized intracellularly, probably in the endoplasmic reticulum, suggesting that this mutation disrupted proper routing of BCRP.

Keywords BCRP · ABCG2 · ABC transporter · *N*-glycosylation · Phosphorylation · Mutations

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Abbreviations ABC: ATP-binding cassette · BCRP: Breast cancer resistance protein · CHO: Chinese hamster ovary · ERM: Ezrin-Radixin-Moesin · MDCK: Madin-Darby canine kidney · MDR: Multidrug resistance · MRP: Multidrug resistance-associated protein · PCR: Polymerase chain reaction · PDZ: PSD-95-Dlh-ZO1 · PKC: Protein kinase C · TAP: Transporter associated with antigen processing

Introduction

The ATP-binding cassette (ABC) transporter family encompasses a large group of membrane proteins able to pump a variety of substrates like lipids, bile salts, peptides and toxic xenobiotics, across lipid membranes in an ATP-dependent manner [9]. Drug-transporting ABC transporters were discovered as proteins able to confer multidrug resistance (MDR). An elevated expression or activity of transporters such as P-glycoprotein, members of the MDR-associated protein (MRP) family and breast cancer resistance protein (BCRP) cause resistance to cells in vitro [6, 17, 29, 34]. Resistance is an important problem in the treatment of tumor cells with chemotherapeutics. The expression of P-glycoprotein in tumors was extensively investigated. Other transporters are also expressed in tumor cells. However, the exact importance of ABC transporters in the resistance of tumors has not been established yet [8, 12, 17]. ABC multidrug transporters lower the effective drug concentration in a cell by pumping the drug out of the cell. By this mechanism, they also influence the systemic bioavailability of certain drugs, and their penetration in various critical organs and tissues [32]. Blocking this process could increase the effect of therapeutics, which are substrates for one or more ABC transporters. More detailed information about structure and cellular function of ABC transporters could therefore improve development of new specific inhibitors.

The molecular mechanisms of P-glycoprotein and members of the MRP family are the most intensively studied examples of ABC drug transporters [4, 5]. More recently, BCRP, also known as ABCG2 or MXR, was found to confer resistance to various anticancer drugs like topotecan and mitoxantrone [1, 2, 29]. While most ABC drug transporters consist of two transmembrane (TM) and two nucleotide-binding (NB) domains, BCRP is a so-called half-transporter, consisting of only one TM and one NB domain. BCRP is mainly localized at the plasma membrane [28, 31] and forms S-S homodimers [22].

Protein phosphorylation by the large family of protein kinases is a common post-transcriptional modification, used in the regulation of cellular processes. Phosphorylation of serine, threonine or tyrosine residues is also found in the ABC transporter family. Phosphorylation of ABC transporters ste6 and ACA1 is important in their activity and stability [24, 25]. Phosphorylation of transporter associated with antigen processing (TAP), an ABC transporter involved in antigen processing, blocks its transport of peptides. After dephosphorylation, the protein is able to transport peptides [25]. P-glycoprotein is phosphorylated on serine residues in its linker region by protein kinase C (PKC). Mutant studies suggest that phosphorylation affects the interaction of certain drugs with P-glycoprotein at lower concentrations, but it does not alter the maximum level of ATPase activity, its dependence on ATP concentration, or the rate of drug transport [15, 16, 33].

A PKC phosphorylation site has also been identified in the C-terminus of MRP2. The site is suggested to play a role in the MRP2 binding to PSD-95-Dlh-ZO1 (PDZ) proteins [18].

Based on sequence analysis using the ProSite database, we detected several putative phosphorylation sites in BCRP [11]. Two PKC sites were positioned in the N-terminus close to the first transmembrane domain (serine 395 and serine 398). We therefore investigated if BCRP was phosphorylated and whether this could play a role in its function.

During synthesis in the endoplasmic reticulum (ER), sugar groups are added to asparagine (*N*-glycosylation) or serine residues (O-glycosylation) of glycoproteins. In the ER and Golgi, this sugar group is modified and extended in several steps. *N*-glycosylation is known to play a role in the stability, targeting and thus activity of several proteins [19]. BCRP is *N*-glycosylated [26, 28] and here we investigated at which residue BCRP is glycosylated and if this glycosylation is important in targeting BCRP to the plasma membrane.

Materials and methods

Plasmid construction

The asparagine of three putative glycosylation sites in BCRP was replaced by an alanine. Site-directed mutagenesis by overlap extension polymerase chain reaction (PCR) [20] was used to generate different amino acid substitutions. BCRP-N418A, BCRP-N557A and BCRP-N596A were made using pcDNA3/BCRP wild type (GenBank accession number AY017168; generously provided by Susan Bates) as template. BCRP-N418A/N557A was made using overlap extension PCR with pGEM-T Easy/BCRP-N418A as template and BCRP-N418A/N557A/N596A was made with pGEM-T Easy/BCRP-N418A/N557A as template again using overlap extension PCR. The oligonucleotides used to introduce mutations are listed below and the nucleotides substituted are underlined.

BCRP-glyc2-for 5′-AA GCT GAT TCT ACT GGA ATC C-3′ (nt 1443-1463)
BCRP-glyc2-rev 5′-CC AGT AGA ATC A GC TTT TAG CCC-3′ (nt 1436-1458)
BCRP-glyc3-for 5′-GTC GCT CTC ACA ACC ATT GC-3′ (nt 1859-1878)
BCRP-glyc3-rev 5′-T GAG A GC GAC CAA CAG ACC-3′ (nt 1850-1868)
BCRP-glyc4-for 5′-GA CTC GCT GCA ACA GGA AAC-3′ (nt 1974-1993)
BCRP-glyc4-rev 5′-TGT TGC A GC GAG TCC TGG-3′ (nt 1970-1987)

All PCR products were inserted into pGEM-T Easy (Promega). Constructs were excised with *Not*I and BCRP fragments were cloned into the *Not*I site of pcDNA3 (Invitrogen). The orientation of the fragments was checked with restriction analysis and sequences were analyzed.

Cell lines

IGROV1 and T8 cells were cultured in RPMI1640 with Hepes (GIBCO), supplemented with 10% fetal bovine serum, 100 units/ml penicillin G and 100 μg/ml streptomycin sulfate. Chinese hamster ovary cells (CHO9) were cultured in Nutrient Mixture F-10 (HAM) with L-glutamine (GIBCO), supplemented with 10% fetal bovine serum, 100 units/ml penicillin G and 100 μg/ml streptomycin sulfate. Madin-Darby canine kidney cells (MDCKII) were cultured in Dulbecco's MEM with glutamax-I (GIBCO), supplemented with 10% fetal bovine serum, 100 units/ml penicillin G and 100 μg/ml streptomycin sulfate. Cells were grown in 5% CO_2 at 37°C. The production of MDCKII transduced with mouse Bcrp1 [21], human BCRP (submitted) or human MRP2 [10] was described elsewhere.

Transfection

CHO9 and MDCKII cells were grown overnight and subsequently transfected with wild type BCRP and mutated constructs using FuGENE6 transfection reagent (Roche) according to the manufacturer. After 4 h, serum-free medium was replaced with complete culture

medium. Transfected cells were incubated overnight at 37°C and analyzed.

Immunoprecipitation and Western blot analysis

Transfected cells were washed twice with cold PBS and lysed in 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1% (w/v) Triton X-100 and protease inhibitors (protease inhibitors cocktail tablet, Roche) for 30 min at 4°C. Lysates were centrifuged and the supernatant was immunoprecipitated at 4°C with monoclonal antibody BXP21, directed against human BCRP [28], coupled to Protein A sepharose CL-4B beads (Amersham Biosciences). Beads were washed twice with RIPA-buffer (100 mM Tris-Cl, 2 mM EDTA, 150 mM NaCl, 0.1% (w/v) SDS, 0.5% (w/v) deoxycholic acid, 0.5% (w/v) Nonidet P-40; pH 8.3) and eluted in 0.1 M 2-mercaptoethanol and 0.1% (w/v) SDS. Samples were boiled for 5 min and one half was incubated overnight at 37°C with 0.2 units *N*-Glycosidase F (Roche) in 0.6 mM Tris-Cl (pH 8.6) and 0.8% (w/v) Triton X-100. The other half was mock-treated. SDS-sample buffer was added and samples were boiled for 5 min. Proteins were separated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes (Schleicher & Schuell). Blots were blocked overnight in 5% Protifar Plus (Nutricia) in PBS and subsequently incubated with monoclonal antibody BXP21 (1:150 in PBTE (1.5 g/l BSA, 0.05% (w/v) Tween 20 and 0.5 mM EDTA in PBS)) and HRP-conjugated goat anti-mouse IgG (1:1,000 in PBTE; Dako). Proteins were visualized using ECL Western blotting detection reagents (Amersham Biosciences).

Immunofluorescence

CHO9 and MDCKII were grown overnight on coverslips and transfected as described above. Transfected cells were washed twice with PBS and fixed in 3% (w/v) paraformaldehyde/PBS for 30 min. After washing with PBS, cells were quenched in 50 mM NH_4Cl/PBS and incubated in blocking buffer [0.2% (w/v) BSA and 0.2% (w/v) saponine in PBS] for 1 h. Cells were then incubated with monoclonal antibody BXP34, directed against human BCRP [31] (1:50 in blocking buffer) and Alexa Fluor 568 goat anti-mouse IgG (1:200 in blocking buffer; Molecular Probes). Coverslips were mounted with Vectashield HardSet Mounting Medium (Vector Laboratories). Cells were analyzed using a Leica Confocal Laser Scanning Microscope.

Metabolic labeling

Cells were washed once with methionine and cysteine free MEM (Sigma). Cells were starved for 30 min at 37°C methionine and cysteine-free MEM, washed and incubated for 2 h in methionine and cysteine-free MEM supplemented with 200 μCi/ml promix (Amersham). After the incubation, cells were treated as described under ''Immunoprecipiation and Western blot analysis'' and analyzed by SDS-PAGE.

Phosphorylation

Cells were washed once with phosphate-free MEM (Sigma). Cells were starved for 30 min at 37°C in phosphate-free MEM, washed and incubated for 2 h in phosphate-free MEM supplemented with 200 μCi/ml [^{32}P] ortho phosphate. Cells were lysed in lysis buffer [1% (w/v) Triton X-100, 10 mM NaF, 25 mM Na-β-glycerophosphate, 1 mM sodium vanadate and protease inhibitors (protease inhibitors cocktail tablet, Roche) in PBS]. Lysates were used in an immunoprecipitation as described under ''Immunoprecipitation and Western blot analysis'' and analyzed by SDS-PAGE.

Results

Phosphorylation of BCRP

To investigate the phosphorylation status of BCRP, we used T8 cells. T8 is a human ovarian carcinoma cell line (IGROV) selected for topotecan resistance that expresses a high level of BCRP [29]. T8 cells were labeled with [^{32}P]ortho-phosphate and a duplicate cell dish was labeled with [^{35}S]methionine/cysteine. Next, BCRP was immunoprecipitated from detergent lysates and analyzed by SDS–PAGE. As a positive control MDCKII cells transduced with MRP2 were used and analyzed in the same way. After labeling cells with [^{35}S]methionine/cysteine, in T8 cells two BCRP bands were detected, one around 72 kDa and one broader band with a slightly higher apparent molecular weight, around 75–80 kD (Fig. 1a). Both bands were PNGase F sensitive ([28] and later in Fig. 3) and represented two glycosylated forms of BCRP, the core-glycosylated and complex-glycosylated form (data not shown). In MRP2 transduced cells also two bands were detected around 220 kDa. After labeling with [^{32}P]ortho-phosphate only the higher MRP2 band was detected, suggesting that only mature MRP2 was phosphorylated. No phosphorylated BCRP band could be detected. To rule out the possibility that the lack of phosphorylation was due to the selection of T8 cells, we repeated the experiment with MDCKII cells transduced with human BCRP. Also, in these cells, no phosphorylated BCRP could be detected (Fig. 1b). In a parallel experiment phosphorylated MRP2 was readily detected. Thus, under conditions where MRP2 was clearly phosphorylated, BCRP was not detectably phosphorylated in either T8 or MDCKII-BCRP cells.

N-linked glycosylation of BCRP

In eukaryotic cells, the *N*-glycosylation motif is characterized by an Asn-X-Thr/Ser sequence, where X can

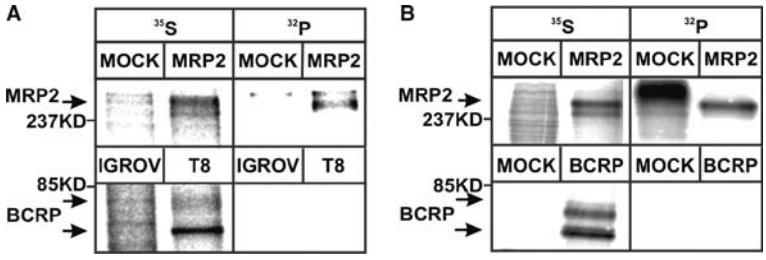

Fig. 1 BCRP is not phophorylated in T8 and MDCKII cells. T8 cells (**A**) or MDCKII cells transduced with *BCRP* (**B**) were labeled with 200 μCi/ml [^{35}S]-methionine/cysteine for 2 h. Duplicate dishes were labeled with 200 μCi/ml [^{32}P]-orthophosphate for 2 h. Cells were lysed and BCRP or MRP2 was immunoprecipitated from the lysates. Immunoprecipitates were analyzed by SDS-PAGE. As control MDCKII cells transduced with *MRP2* were labeled and analyzed in the same way

be any amino acid except proline. Computer analysis of the human BCRP sequence detected four putative *N*-glycosylation motifs. Only consensus sites exposed at some stage to the lumen of the endoplasmic reticulum can be *N*-glycosylated. Using an alignment of the human *BCRP* sequence with mouse *Bcrp1*, we predicted the approximate positions of the TM domains. One putative motif at asparagine 338 was predicted to be positioned intracellularly. For that reason, only asparagine residues 418, 557 and 596 were changed into an alanine by overlapping extension PCR. Computer analysis predicted the positions of these residues in the first (residue 418) and third (residue 557 and 596) extracellular loop, respectively (Fig. 2). Expression vectors containing the mutant DNAs were transiently transfected into CHO9 and MDCKII cells which were subsequently analyzed by Western blot (Fig. 3). In the BCRP-transfected cells, but not in the mock-transfected cells, two bands around 72 kDa and 75 kDa were detected. After PNGase F treatment both bands disappeared and one band (N) was detected around 62 kDa, showing that both bands represented glycosylated BCRP. The higher band represents most likely complex-glycosylated BCRP (G), the lower one the core-glycosylated form (C). Expression of BCRP-N418A resulted in detection of two BCRP bands at the same position as in wild-type BCRP transfectants, indicating that asparagine 418 is not significantly glycosylated.

In BCRP-N557A transfectants, only the lower band was detected. As this band was PNGase F sensitive, BCRP-N557A was still glycosylated. However, only the core-glycosylated form was detected. Expression of BCRPN-596A showed one lower band of 62 kDa which was not PNGase F sensitive. Thus BCRPN-596A was not glycosylated anymore. A protein containing all three mutations [triple (T) mutant] showed the same result as the BCRP-N596A mutant. We conclude that only asparagine 596 of BCRP was glycosylated.

To investigate if *N*-linked glycosylation plays a role in subcellular localization of BCRP we performed immunofluorescence analysis on cells expressing the BCRP mutants. Cells were grown on coverslips, transfected, fixed the next day and incubated with an antibody against human BCRP. To visualize BCRP we used a

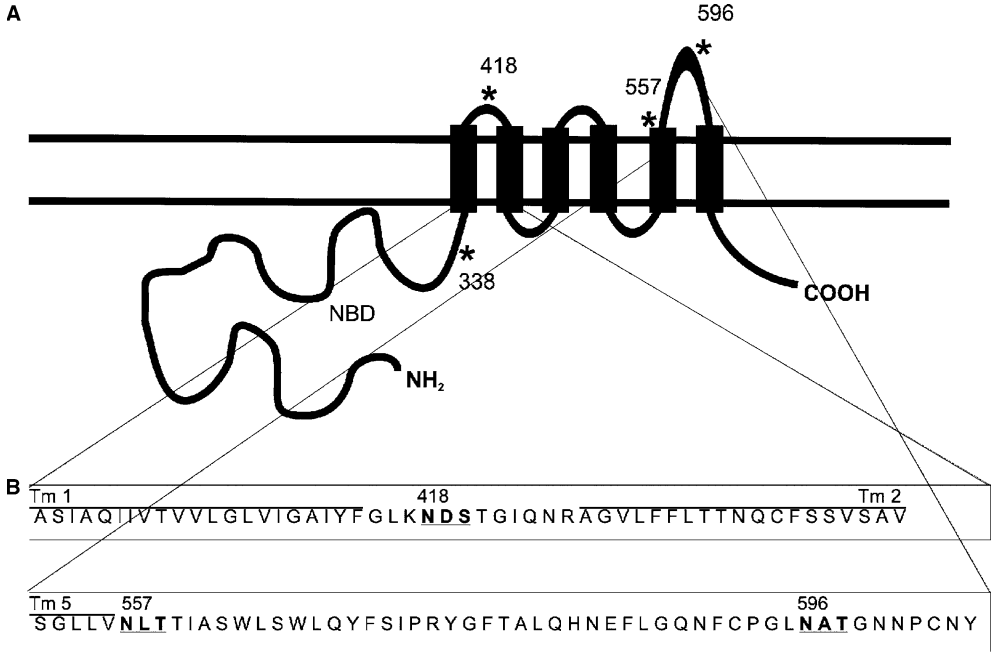

Fig. 2 Four *N*-glycosylation consensus sequences were found in human *BCRP*. (**A**) Two-dimensional model of human BCRP as it is thought to span the plasma membrane. N-terminus and C-terminus and the putative nucleotide-binding domain (*NBD*) are indicated. (**B**) The positions of the four *N*-glycosylation consensus sequences and their surrounding amino acid sequences

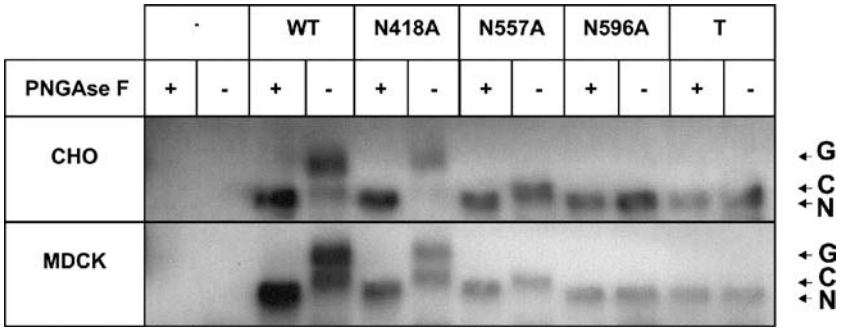

Fig. 3 BCRP-N596A is not glycosylated. Empty vector pcDNA3 or pcDNA3 with BCRP or mutant BCRP, BCRP-N418A, BCRP-N557A and BCRP-N596A, was transiently transfected into CHO9 cells or MDCKII cells using FuGENE6. The next day, cells were lysed, BCRP was immunoprecipitated from the lysates and analyzed by SDS-PAGE and Western blotting. BCRP was detected by using the monoclonal antibody BXP21, raised against human BCRP. *N* non-glycosylated *C* core-glycosylated *G* fully glycosylated

fluorescent second antibody and analyzed the cells by confocal laser scanning microscopy. Both in CHO9 and MDCKII cells, wild-type BCRP was primarily localized at the plasma membrane (Fig. 4). In MDCKII cells also intracellular BCRP was detected. This is in line with the observation that the percentage of core-glycosylated protein (Western blot in Fig. 3) in MDCKII cells was much higher than in CHO9 cells. BCRPN-418A was also mainly found at the plasma membrane in both cell lines. BCRPN-557A, however, showed a different pattern of intracellular staining dispersed throughout the whole cytoplasm. This suggested that BCRPN-557A was primarily localized in the ER. The fact that (Fig. 3) we could only detect the lower, core-glycosylated form of BCRPN557A by Western blotting is in agreement with this finding. Predictably, ER-like staining was also found for the triple mutant. These results suggest that both BCRP-N557A and the triple mutant were not properly targeted to the plasma membrane as a consequence of the N557A mutation. The non-glycosylated BCRP-N596A mutant, however, showed a normal plasma membrane staining in both CHO9 and MDCKII cells, indicating that *N*-glycosylation of BCRP is not important for its plasma membrane targeting.

Discussion

Breast Cancer Resistance Protein is an ABC multidrug transporter that confers resistance to various anticancer drugs [29]. Here we investigated phosphorylation and *N*-linked glycosylation of BCRP.

Phosphorylation of the apical multidrug transporters MRP2 and *P*-glycoprotein was investigated and described [15, 16, 18, 33]. In vitro experiments suggest that phosphorylation of MRP2 by PKC plays a role in its interaction with PDZ proteins [18], proteins involved in scaffolding multiprotein complexes, targeting and regulation of membrane proteins [3, 13]. For example, the Ezrin-Radixin-Moesin (ERM) family of proteins has PDZ domains and crosslinks actin filaments and integral membrane proteins [7]. Experiments in Radixin knockout mice show a role for Radixin in localization of Mrp2 [23]. These results suggest a link between phosphorylation and localization. No exact function for the phosphorylation of P-glycoprotein has been described yet. However, an interaction between P-glycoprotein and ERM has also been described [27].

BCRP is, like MRP2 and P-glycoprotein, an apical multidrug transporter expressed in liver and small intestine [14, 23, 28]. Computer analysis of human BCRP identified several putative phosphorylation sites, including two PKC sites. The similarities between MRP2 and BCRP led us to investigate the phosphorylation status of BCRP. However, under conditions where MRP2 was readily phosphorylated, we could not detect phosphorylated BCRP in either T8 or MDCKII cells. Furthermore, we have also tried to detect any interaction of BCRP with the ERM proteins, but have not been successful so far (data not shown). These results suggest that either BCRP is not phosphorylated at all, or not under the same circumstances as MRP2. Although we studied BCRP phosphorylation in two different cell systems, we cannot fully exclude that phosphorylation of BCRP might occur under different circumstances. More extensive studies in a range of different cell lines and under various growth condition would be needed to address this question.

Our experiments with human BCRP glycosylation mutants showed clearly that only asparagine residue 596 is glycosylated in BCRP and that this modification is not important for targeting of BCRP to the plasma membrane. In mouse Bcrp1 also four potential *N*-linked glycosylation sites are found [1]. Two sites are located intracellularly and two are found in the third extracellular loop (see Fig. 2). When we compared mouse Bcrp1 with human BCRP only asparagine residue 596 in human BCRP, the only glycosylated residue, was conserved at the same position in mouse Bcrp1. Consensus *N*-glycosylation sites around asparagine residues 418 and 557, which are in human BCRP non-glycosylated, are not present at a similar position in mouse Bcrp1. This is in line with our finding that they are not functionally important.

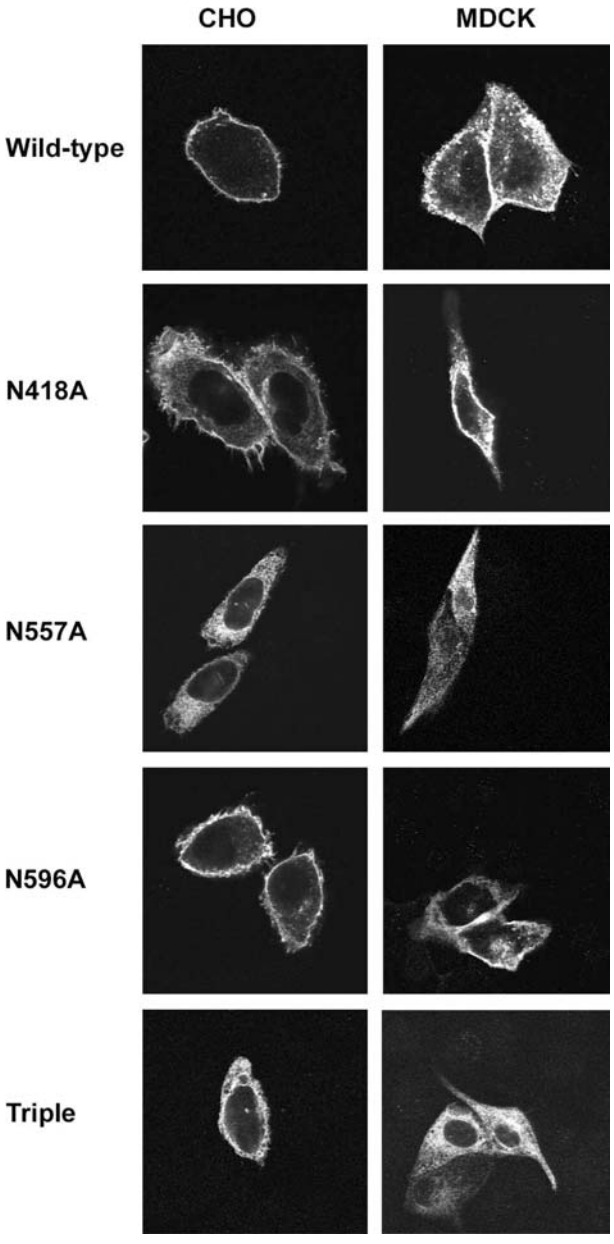

Fig. 4 Wild-type BCRP, BCRP-N418A and BCRP-N596A are localized at the plasma membrane. BCRP-N557A and a triple mutant is localized intracellularly. Empty vector pcDNA3 or pcDNA3 with BCRP or mutant BCRP, BCRP-N418A, BCRP-N557A and BCRP-N596A, was transiently transfected into CHO9 cells or MDCKII cells using FuGENE6. Next day, cells were fixed and incubated with a mouse monoclonal antibody against BCRP. BCRP was visualized by using Alexa Fluor568 goat anti-mouse. The cells were analyzed by confocal laser scanning microscopy

In our experiments, BCRP-N557A was apparently core-glycosylated and localized in the ER. These results suggest that by mutating asparagine 557 the folding and targeting of BCRP was affected. In our computer analysis of TM domains, asparagine residue 557 lies at the border of a transmembrane domain and an extracellular loop. After we made and analyzed our mutants, it was shown by others that mutation of asparagine 557 also influences the resistance of mutant BCRP transfected cells [30] to drugs like mitoxantrone and SN-38. In that study, cells with mutant BCRP expressed at the plasma membrane were selected and the mutation of asparagine 557 into different amino acids resulted in different resistance levels. Asparagine was changed into a histidine (H), aspartic acid (D), glutamic acid (E) and arginine (R). These amino acids are all highly polar amino acids and alanine, as was used in our study, is a hydrophobic residue. However, expression of the mutant BCRP-N557R gave a low expression and the Western blot suggests that only one band was detected instead of the two bands in the other BCRP expressing cells in the same experiment. This suggests that the BCRP-N557R mutant also has a (partial) problem with its folding in the ER. However, an amount of mutant protein still could reach the plasma membrane. We cannot exclude that in our study also a small fraction of BCRP-N557A reaches the plasma membrane. The described difference in resistance of the BCRP-N557H, BCRP-N557D, BCRP-N557E and BCRP-N557R mutants could be partially due to a small difference in folding of BCRP next to a difference in affinity.

Although the absence of *N*-glycosylation did not affect the plasma localization of BCRP in vitro, a role in the stability or folding is still possible. Transgenic overexpression of mutant BCRP in *Bcrp1* knockout mice could for instance be used to study the role of *N*-glycosylation in vivo in more detail.

In conclusion, we could not detect any phosphorylated BCRP in either MDCKII cells or T8 cells. Furthermore, only asparagine residue 596 of human BCRP is *N*-glycosylated. *N*-glycosylation did not affect the plasma membrane localization of BCRP.

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