EXPRESSION, LOCALIZATION, AND FUNCTIONAL CHARACTERISTICS OF BREAST CANCER RESISTANCE PROTEIN IN CACO-2 CELLS

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Received December 21, 2004; accepted February 15, 2005

ABSTRACT:

The function of breast cancer resistance protein (BCRP) and its role in drug absorption, distribution, and elimination has recently been evaluated. The objective of the present study was to examine the expression, localization, and functional characteristics of BCRP in Caco-2 cells, a widely used human intestinal epithelial cell model for investigating intestinal drug absorption. The expression of BCRP in Caco-2 cells was measured by Western blotting using the antibody BXP-21. Localization of BCRP was determined by an immunofluorescence technique using both antibodies BXP-21 and BXP-34. The drug efflux function of BCRP was evaluated via the epithelial transport of methotrexate (MTX) and estrone-3-sulfate (E3S) across Caco-2 cell monolayers in the presence or absence of the BCRP inhibitors Ko143 or GF120918. The drug efflux function of BCRP was measured by the antibody BXP-21. Localization of BCRP was determined by an immunofluorescence technique using both antibodies BXP-21 and BXP-21. The drug efflux function of BCRP was evaluated via the epithelial transport of methotrexate (MTX) and estrone-3-sulfate (E3S) across Caco-2 cell monolayers in the presence or absence of the BCRP inhibitors Ko143 or GF120918.

BCRP in Caco-2 cells was measured by Western blotting using the antibody BXP-21. Localization of BCRP was determined by an immunofluorescence technique using both antibodies BXP-21 and BXP-34. The drug efflux function of BCRP was evaluated via the epithelial transport of methotrexate (MTX) and estrone-3-sulfate (E3S) across Caco-2 cell monolayers in the presence or absence of the BCRP inhibitors Ko143 or GF120918. Both BCRP inhibitors Ko143 and GF120918 increased the apical-to-basolateral (A-to-B) transport of MTX and E3S.

Caco-2 cells may therefore be used as an in vitro model to study the transport characteristics of BCRP.

Breast cancer resistance protein (BCRP) was originally cloned and sequenced from genomic DNA, from highly mitoxantrone-resistant S1-M1-80 human colon carcinoma cells, and from MCF7 AdVp human breast cancer cells selected in doxorubicin (Doyle et al., 1998; Miyake et al., 1999). BCRP is a member of the ATP-binding cassette transporter G family and is also known as ABCG2 or ABCP or MXR (Joritsma et al., 2002; Doyle and Ross, 2003). It is a 655-amino acid polypeptide (72 kDa), containing six putative transmembrane domains and four potential N-glycosylation sites. BCRP is similar to half the duplicated P-glycoprotein (P-gp) or multidrug resistance protein 1 (MRP1) molecule and functions as a homodimer bridged by disulfide bonds (Doyle et al., 1998; Kage et al., 2002).

BCRP is endogenously expressed at a high level in human placenta and to a lesser extent in liver, small intestine and colon, ovary, veins, capillaries, kidney, adrenal, and lung, with little to no expression in brain, heart, stomach, prostate, spleen, and cervix (Doyle et al., 1998; Litman et al., 2001; Maliepaard et al., 2001; Scheffer and Schepfer, 2002). Importantly, BCRP is expressed in the human jejunum at levels considerably higher than those of many other ABC transporters (Taipalensuu et al., 2001). BCRP has been demonstrated to exist on the apical membrane of intestinal epithelium and has limited the oral absorption of topotecan in mice and humans (Jonker et al., 2000; Kruijtzer et al., 2002a). Given the liver and intestinal localization pattern, BCRP, similar to P-gp, may act as a barrier to uptake and absorption and limit the oral bioavailability of drugs as well as mediating hepatobiliary excretion of drugs (Jonker et al., 2000; Joritsma et al., 2002; Kruijtzer et al., 2002b).

Caco-2 cells are derived from human colon cancer cells and exhibit morphological and functional similarities to intestinal enterocytes. It has been widely used as a model of human intestinal epithelium for studies of intestinal drug absorption and metabolism. Many active transport systems such as P-gp (encoded by MDR1 gene, also named ABCB1) and multidrug resistance-associated protein 2 (MRP2, or ABCC2) have been characterized in Caco-2 cells (Makhey et al., 1998; Doppenschmitt et al., 1999; Gutmann et al., 1999). BCRP mRNA has been detected in Caco-2 cells, although its level is 100-fold lower than that in human jejunum (Taipalensuu et al., 2001).

The present study was aimed at characterization of the protein expression, localization, and efflux function of BCRP in Caco-2 cells. We have used BXP-21 monoclonal antibody (mAb) to determine the

ABBREVIATIONS: BCRP, breast cancer resistance protein; ABC, ATP-binding cassette transporter; A-to-B, apical-to-basolateral; B-to-A, basolateral-to-apical; E3S, estrone-3-sulfate; FBS, fetal bovine serum; MDR1, human multidrug resistance gene 1; mAb, monoclonal antibody; MRP, multidrug resistance-associated protein; MTX, methotrexate; Papp, apparent permeability coefficient; P-gp, P-glycoprotein; TEER, transepithelial electrical resistance; GF120918, N-[4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl]-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide; LY335979, zosuquidar trihydrochloride; MK571, 3-[[3-[2-(7-chloroquinolin-2-yl)vinyl]phenyl]-(2-dimethylcarbamoylethylsulfanyl)methylsulfanyl]propionic acid; 2,4-DNP, 2,4-dinitrophenol; FITC, fluorescein isothiocyanate.
BCRP protein expression, and BXP-21 and BXP-34 mAbs to characterize the subcellular distribution of BCRP in Caco-2 cells. In addition, the efflux function of BCRP in Caco-2 cells was determined using estrone-3-sulfate (E3S) and methotrexate (MTX) as substrates. Knowledge of the properties of BCRP in Caco-2 cells is valuable to investigate the absorption mechanism of drug molecules using this in vitro model system.

**Materials and Methods**

Materials. [3H]Estrone-3-Sulfate (specific activity, 45 Ci/mmol) and [3H]methotrexate (specific activity, 33.5 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). GF120918 and LY335979 were synthesized at Millennium Pharmaceuticals, Inc. Ko143 was obtained from the Netherlands Cancer Institute (Amsterdam, Netherlands). MK571 was purchased from Alexis Biochemicals (San Diego, CA). Unlabeled E3S, MTX, prazocin, and 2,4-dinitrophenol (2,4-DNP) were purchased from Sigma-Aldrich (St. Louis, MO). Cell culture media and supplies were obtained from Invitrogen (Carlsbad, CA). BXP-21 and BXP-34 murine monoclonal antibodies were purchased from Signet Laboratories (Dedham, MA). Horseradish peroxidase-conjugated goat anti-mouse IgG was obtained from Abcam Inc. (Cambridge, MA). BXP-21 and BXP-34 mouse polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All supplies for Western blot studies were obtained from Invitrogen.

**Caco-2 Cell Culture.** The Caco-2 cells (passage 18) were obtained from American Type Culture Collection (Manassas, VA). The stock cells were cultured in 150-cm² tissue culture T-flasks for subsequent plating onto 24 Transwell plates (0.33 cm²/well, 0.4-μm pore size; Costar, Cambridge, MA). Briefly, 1 × 10⁵ cells were suspended in 0.2 ml of culture medium [Dulbecco’s modified Eagle’s medium with 0.1 mM nonessential amino acids, 2 mM l-glutamine, 4.5 g/l glucose, and 10% fetal bovine serum (FBS)] and added to the upper chamber of each filter membrane of a Transwell plate. One milliliter of cell-free culture medium was added to the lower chamber. The Transwell plates were then incubated at 37°C in an atmosphere of 5% CO₂ in air and 90% humidity. The cell culture media were changed every other day. Confluent cell monolayers were obtained within 5 to 7 days after plating. The transepithelial electrical resistance (TEER), as measured by an epithelial vol-to-ohm meter (World Precision Instruments, Inc., Sarasota, FL), gradually increased and reached a plateau after day 5, indicating the formation of tight junctions. Monolayers with TEER values greater than 250 ohm · cm² were used. Unless otherwise specified, cell passages between 20 and 40, other than specification (approximately 21–25 days in culture), were used in transport studies to ensure the complete enterocyte-like cell differentiation of the Caco-2 cells.

**Western Blotting.** Cells were scraped and subsequently lysed in hypotonic lysis buffer, consisting of 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1% SDS, 1% Triton X-100 supplemented with protease inhibitors (aprotinin, leupeptin, and phenylmethylsulfonyl fluoride) (Yu and Sinko, 1997). Cell lysates were sonicated and centrifuged at 10,000 rpm for 2 min. The supernatant was aliquoted and stored at −80°C. Protein levels were determined using the BCA protein assay (Pierce Endogen, Rockford, IL). Cell lysates, with and without treatment of reducing agent (7–13% dithiothreitol), were separated on a 4 to 12% gradient polyacrylamide gel and subsequently transferred electrophoretically to a polyvinylidene difluoride membrane. Proteins were hybridized using BXP-21 (1:150 optimal dilution) and horseradish peroxidase-conjugated goat anti-mouse IgG2a (1:1,000), and further visualized by enhanced chemiluminescence (ECL) (Amersham Biosciences Inc., Piscataway, NJ). The protein bands were semiquantified by NIH Image Software.

**Immunohistochemistry.** Cell monolayers cultured on the Transwells were washed with phosphate-buffered saline, pH 7.4; similar phosphate-buffered saline washes were included between each subsequent step. Cell monolayers were fixed in 3.7% (v/v) formaldehyde solution for 10 min at room temperature. Cell membranes were permeabilized by saponin (2%, 2 min at room temperature). Nonspecific binding sites were blocked by incubation in 10% FBS for 45 min. Cells were incubated with anti-human BCRP mAb (BXP-21 or BXP-34 in 1.5% FBS) for 2 h. BCRP staining was revealed by incubation with FITC-conjugated goat anti-mouse antibody for 1 h. The nucleus was stained by propidium iodide for 15 min. After a final wash, cell monolayers were mounted in Vectashield before examination on a Zeiss PASCAL confocal laser scanning system (Carl Zeiss Inc., Thornwood, NY). Control for nonspecific staining was the replacement of BXP-21 or BXP-34 with a nonspecific antibody from the same class, mouse IgG2a.

**Transport Studies in Caco-2 Cells.** Bidirectional transport studies were performed at 37°C in air. Prior to each experiment, the confluent cell monolayers on Transwell inserts were washed and equilibrated for 30 min with transport medium [Hanks’ balanced salt solution containing 10 mM N-2-hydroxyethyl-piperazine-N’-2-ethanesulfonic acid (HEPES) and 10 mM glucose, pH 7.4]. The experiment was initiated by adding a solution containing the test compound to either the apical (for A-to-B transport) or basolateral (for B-to-A transport) compartment. When applicable, inhibitors such as GF120918 (2 μM), prazocin (100 μM), MK571 (50 μM), Ko143 (1 μM), or LY335979 (5 μM) were present in the transport medium of the donor side from the preincubation period throughout the permeability study. At 15, 30, 45, and 60 min, the sample aliquots of receiving solutions were withdrawn from the basolateral side (for A-to-B transport) or from the apical side (for B-to-A transport), and replaced immediately with an equal amount of fresh transport media except at the 60-min time point (the end of the incubation). The samples were mixed with 5 ml of scintillation cocktail and the radioactivity was determined in a liquid scintillation spectrophotometer (Beckman Coulter, Fullerton, CA).

**Data Analysis.** The cumulative amount of drug (Q) on the receiver side was plotted as a function of time. The steady-state flux J was then estimated from the slope (dQ/dt). The apparent permeability coefficient (P_app) of unidirectional flux for the test compound was estimated by normalizing the flux J (mols against the nominal surface area A (0.33 cm²) and the initial drug concentration in the donor chamber C_i (mol/ml), or P_app = J/(A*C_o). The B/A ratio was equal to the P_app value for A-to-B transport (P_app, A-to-B) divided by the P_app value for B-to-A transport (P_app, B-to-A). The kinetic parameters for the E3S transport were estimated by fitting the flux against the donor E3S concentration using a nonlinear regression with a method of least squares fitting (GraphPad Software Inc., San Diego, CA).

Data are expressed as mean ± S.E.M. of three individual monolayers. Tests of significance of differences between mean values were made using a two-tailed unpaired Student’s t test. A probability of less than 0.05 (p < 0.05) was considered to be statistically significant.

**Results**

**Expression and Localization of BCRP.** BCRP protein expression was determined in Caco-2 cell lysate samples via Western blot analysis using BXP-21 as the primary antibody. As shown in Fig. 1A, both the half-transporter BCRP (∼70 kDa) and its dimer (∼140 kDa) were detected unpaired Student’s t test. A probability of less than 0.05 (p < 0.05) was considered to be statistically significant.

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membranes but also in the cytoplasm. On the 5-day-old culture, cells reached confluence and showed increased TEER values (indicative of the tight junction formation); BCRP staining was observed only on the apical membrane, and there was no detectable staining in the cytoplasm, indicating the localization of BCRP on the apical membrane. The same phenomena were also noted during the cell differentiation period (i.e., the 12th and 19th days after cell seeding). When the late passage cells (p59) were grown on the Transwell plates for 12 days, multiple layers were observed as demonstrated by the multiple nuclei staining on the x-z and y-z views (Fig. 2B). BCRP staining, however, was still preferentially shown on the apical side of cell membranes (Fig. 2B). The absence of any green staining in the cell monolayers with mouse IgG2a and FITC-conjugated second antibody served as a negative control.

Efflux Function of BCRP. The function of BCRP as an efflux transporter in Caco-2 cell monolayers was evaluated using a known substrate, E3S (Suzuki et al., 2002, 2003). As shown in Table 1, the P

app value of [\textsuperscript{3}H]E3S (0.02 \mu M) from B-to-A was 46.1 \times 10^{-6} \text{ cm/s} and was 8-fold higher than the P

app value (5.7 \times 10^{-7} \text{ cm/s}) for A-to-B in the 5-day culture (p33). In 12- and 19-day cultures (p33), the P

app values for A-to-B decreased to 4.1 and 4.5 \times 10^{-6} \text{ cm/s}, and the P

app value for B-to-A increased to 63.0 and 58.0 \times 10^{-6} \text{ cm/s}, respectively. In the presence of Ko143 (a BCRP inhibitor), the A-to-B and B-to-A transports of E3S were almost equal, with P

app values around 9 to 11 \times 10^{-6} \text{ cm/s}. Figure 3 showed the comparison of BCRP-mediated [\textsuperscript{3}H]E3S (0.02 \mu M) efflux in both early passage (p36) and late passage (p59) Caco-2 cell monolayers. In p36, the P

app value for A-to-B transport of E3S was 2.3 \times 10^{-6} \text{ cm/s} and was 32-fold less than the P

app value for B-to-A transport. The P

app value increased to 6.1 \times 10^{-5} \text{ cm/s} in the presence of Ko143. However, P

app values of E3S for A-to-B and B-to-A transport in p59 were 16.2 and 37.1 \times 10^{-6} \text{ cm/s}, respectively. The difference in the directional E3S transport was abolished by the BCRP inhibitor Ko143. The TEER value was 4-fold higher in p36 than in p59 in the present cell culture system (Fig. 3).

When [\textsuperscript{3}H]E3S (0.03 \mu M) was coincubated with Ko143 (1 \mu M), GF120918 (2 \mu M) (an inhibitor for BCRP and P-gp), or prazocin (100 \mu M) (an inhibitor for BCRP and P-gp) in Caco-2 cell monolayers, the P

app value of E3S for the A-to-B transport decreased and the P

app value for B-to-A transport increased, resulting in the B/A ratio dropping from 27 to almost unity (Fig. 4). In contrast, the P-gp-specific inhibitor LY335979 (5 \mu M) did not significantly change the directional transport pattern of E3S (p > 0.05) (Fig. 4). The apical or basolateral presence of 2,4-DNP at 200 \mu M (known to inhibit mitochondrial ATP synthesis and deplete cellular ATP; Siekevitz and Potter, 1953) totally inhibited the efflux of E3S with an increase in the A-to-B transport and a decrease in the B-to-A transport (Fig. 4).

Figure 5 showed the unidirectional B-to-A flux (J

B-to-A) of E3S across cultured Caco-2 cells at various concentrations in the absence or presence of a BCRP inhibitor, Ko143 (1 \mu M). The net efflux of E3S across Caco-2 cell monolayers in the B-to-A direction, the difference of B-to-A flux in the presence or absence of BCRP inhibitor (J

net = J

B-to-A - J

A-to-B), was saturable and followed Michaelis-Menten kinetics. The estimated apparent K

m and V

max were 13.1 \mu M and 10.8 pmol/s, respectively.

MTX was chosen to further investigate the BCRP efflux function in the Caco-2 cell monolayers. As shown in Fig. 6, the B-to-A transport of MTX was significantly decreased in the presence of MK571 (a MRP inhibitor; 50 \mu M), Ko143 (1 \mu M), and GF120918 (2 \mu M) (p < 0.05). However, neither the P

app value for A-to-B nor the P

app value for B-to-A of MTX was significantly affected after coincubation with LY335979 (5 \mu M).
The B-to-A efflux of rhodamine 123, which is a substrate for P-gp and mutant BCRP, was significantly decreased in the presence of LY335979 and GF120918 (p < 0.05), but was not changed by coincubating with Ko143 (1 μM) (Fig. 7).

**Discussion**

Western blot analysis of Caco-2 cell lysates demonstrates the presence of both monomer (~70 kDa) and homodimer (~140 kDa) of BCRP in the Caco-2 cells (Fig. 1A). The 140-kDa BCRP complex dissociated to 70-kDa polypeptides in the presence of the reducing agent dithiothreitol, indicating that the BCRP dimer was linked by disulfide bonds. This finding is consistent with the recent discovery by Kage et al. (2002). The same group also demonstrates the necessity of homodimerization for the BCRP function when using a dominant-negative mutation of BCRP with a L554P alteration in the fifth transmembrane domain (Kage et al., 2002).

Immunohistochemical analysis of Caco-2 cells reveals the presence of BCRP on apical and basolateral plasma membranes as well as in the cytoplasm prior to cell confluence (Fig. 2). Upon cell confluence, BCRP was sorted to the “apical membrane” consistent with potential intracellular localization and redistribution of BCRP to the plasma membrane shown in BCRP-overexpressed MCF-7 AdVp3000 and S1-M1-80 drug-resistant cells (Litman et al., 2000). BCRP-transfected MDCK or LLC-PK epithelial cells also exhibited polarized apical localization of BCRP (Jonker et al., 2000; Maliepaard et al., 2001), implying that BCRP may contain a certain endogenous sorting signal, which was recognized by epithelial cells (Jonker et al., 2000; Imai et al., 2003). The polarized apical BCRP distribution in Caco-2 cells continues during the cell differentiation period, which is consistent with the proposed role for BCRP as a secretory detoxifying transporter, contributing to the gastrointestinal epithelial barrier. Results from transport function assays using E3S as the BCRP substrate agreed with immunohistochemical study results. The directional transport of E3S across Caco-2 monolayers was observed immediately after cells reached confluence (5-day culture) and continued throughout the differentiation phase (day 12–19 cell culture) (Table 1). In view of the fact that total BCRP protein expression had not changed over day 5, day 12, and day 19 (Fig. 1B), the higher A-to-B transport and lower B-to-A transport of E3S across Caco-2 cells on day 5, compared with those on day 12 and day 19, indicated that BCRP continuously redistributed to the apical membrane during the differentiation phase.

Similar to P-gp, MRP, and lung-resistant protein (Yu and Sinko, 1997), the expression of BCRP is passage-dependent. The expression...
TABLE 1

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<tr>
<th></th>
<th>( P_{\text{app}} \times 10^6 )</th>
<th>5 days after cell seeding</th>
<th>12 days after cell seeding</th>
<th>19 days after cell seeding</th>
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<tr>
<td></td>
<td>A-to-B</td>
<td>B-to-A</td>
<td>Ratio (B/A)</td>
<td>A-to-B</td>
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<tr>
<td>[^3\text{H}]\text{E3S}</td>
<td>5.7 ± 0.3</td>
<td>46.1 ± 1.0</td>
<td>8.1</td>
<td>9.2 ± 1.4</td>
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<tr>
<td>[^3\text{H}]\text{E3S} + Ko143 (1 \text{μM})</td>
<td>9.2 ± 1.4</td>
<td>9.2 ± 1.4</td>
<td>0.9</td>
<td>11.2 ± 0.7</td>
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<tr>
<td>[^3\text{H}]\text{E3S} + Ko143 (1 \text{μM})</td>
<td>9.2 ± 1.4</td>
<td>9.2 ± 1.4</td>
<td>0.9</td>
<td>11.2 ± 0.7</td>
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Fig. 3. BCRP-mediated efflux of E3S in Caco-2 cells of passages 36 and 59. Ko143 (an inhibitor for BCRP) was used at the concentration of 1 \text{μM}. The transport study was conducted on Caco-2 cell monolayers 12 days after seeding on the Transwell plates. Results obtained from the p36 and p59 Caco-2 cells are labeled as p36 and p59, respectively. (Data expressed as mean ± S.E.M., \( n = 3 \).)

Fig. 4. The effects of efflux pump inhibitors on the transport of E3S. LY335979 (5 \text{μM}), a P-gp-specific inhibitor, did not affect the E3S permeability. However, GF120918 (2 \text{μM}) (an inhibitor for P-gp and BCRP), prazocin (100 \text{μM}) (an inhibitor for P-gp and BCRP), and Ko143 (1 \text{μM}) (a BCRP inhibitor) increased A-to-B transport and decreased B-to-A transport of E3S, indicating that BCRP pumps E3S out of Caco-2 cells. The uncoupling agent 2,4-DNP changed the ratio of B-to-A over to B of E3S from 26.7 to 0.6, suggesting that blocking the mitochondrial ATP synthesis and depleting the cellular energy abolished the BCRP efflux activity. (Data expressed as mean ± S.E.M., \( n = 3 \).)

Fig. 5. B-to-A transport rate of E3S in the presence or absence of Ko143 and net flux of E3S across Caco-2 cells. The efflux of E3S was saturable and the \( K_m \) of E3S for BCRP in Caco-2 cells was 13.2 \text{μM}, which was close to the \( K_m \) (16.6 \text{μM}) determined in BCRP vesicle studies. The \( V_{\text{max}} \) was estimated as 10.8 pmol/s. (Data expressed as mean ± S.E.M., \( n = 3 \).)
the transport of rhodamine 123, indicating that P-gp but not BCRP mediated the MTX efflux across Caco-2 cells. (Data expressed as mean ± S.E.M., n = 3.)

Fig. 7. The effects of efflux pump inhibitors on the transport of the rhodamine 123, LY335979 (5 μM), a P-gp-specific inhibitor, did not affect the MTX permeability. However, GF120918 (2 μM) (an inhibitor for P-gp and BCRP), Ko143 (1 μM) (a BCRP inhibitor), and MK571 (50 μM) (a MRP inhibitor) decreased the B-to-A transport of MTX, indicating that both BCRP and MRP mediated the MTX efflux across Caco-2 cells. (Data expressed as mean ± S.E.M., n = 3.)

In conclusion, both BCRP monomer and dimer are expressed in the Caco-2 cells. BCRP is polarized at the apical side of Caco-2 cells and can efficiently transport its substrate, such as E3S and MTX, out of cells. Therefore, besides BCRP-transfected cell lines, Caco-2 cells can also be used as an in vitro model to study the transport function of BCRP.

Acknowledgments. We thank Dr. Shimoga Prakash for providing us GF120918 and LY335979. Ko143 was kindly provided by Dr. Alfred Schinkel. We also thank Vilmos Csizmadia and Bei-Ching Chuan for technical support.

References


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