Evidence for Different ABC-Transporters in Caco-2 Cells Modulating Drug Uptake

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Purpose. Secretory systems contribute to drug absorption in the gastrointestinal tract. The purpose of this study was the identification of members of the ATP binding cassette superfamily of secretory transport proteins that may potentially modulate drug absorption in Caco-2 cells, which are an important cellular model predicting enteral absorption of drugs.

Methods. Kinetic studies as well as PCR- and Western blot studies with confluent epithelial layers of human Caco-2 cells.

Results. The study demonstrates functional expression of multidrug resistance related protein (MRP) and P-glycoprotein (P-gp) in Caco-2 cells: 1) Efflux studies with the MRP specific substrate glutathionmethylfluorescein (GS-MF) showed functional activity of MRP in Caco-2 cells preloaded with the metabolic precursor of GS-MF, chloromethylfluoresceine-diacetate, CMFDA. Excretion of GS-MF was decreased in presence of the MRP-blocker MK-571. 2) Transport experiments with cyclosporin A demonstrated the functional activity of P-gp. Cellular accumulation was increased in presence of the P-gp blocking agent SDZ-PSC 833. 3) The expression of the 190 kDa protein MRP and the 170 kDa protein P-gp in Caco-2 cells was shown by Western blot analysis with specific monoclonal antibodies. 4) The expression of MRP-mRNA in Caco-2 cells was detected by RT-PCR and compared with the MRP over-expressing cell line H69AR. MRP primers recognize specifically human MRP1 (GenBank accession number L05628), but not all other published sequences of MRP (MRP2-MRP6). P-gp expression on mRNA-level was also confirmed by RT-PCR.

Conclusions. The data demonstrate that besides P-gp, multidrug resistance related protein (MRP) is functionally expressed in Caco-2 cells and contributes to the active excretion of substrates in this cell line.

KEY WORDS: Caco-2 cells; multidrug resistance related protein; MRP; P-glycoprotein; MDR.

INTRODUCTION

According to its barrier function, the intestinal mucosa limits the access of cytotoxic substances to the body via the gastrointestinal tract. Therefore, the intestinal epithelium is equipped with a number of secretory systems such as P-glycoprotein (P-gp) and multidrug resistance related protein (MRP), which modulate the transport of a variety of substances. Both systems belong to the superfamily of ATP-binding cassette (ABC) membrane transport proteins (1–4). Previous studies demonstrate that P-gp is located in the apical membrane of enterocytes and plays an important role in the gastrointestinal tract to prevent entry of xenobiotics from the gut lumen (5–8). The functional expression of P-gp has also been shown in Caco-2 cells, a widely used in vitro absorption model for the screening of novel drugs (9–12). The Caco-2 cell line is a human colon adenocarcinoma cell line that exhibits the functional characteristics of the lower small intestinal tract (13–15). Knowledge about the function of different apically located export pumps with potentially overlapping substrate specificity in this cellular model is of special interest to estimate the extent of absorption in vivo. But little is known about the expression of MRP in Caco-2 cells. MRP is a N-glycosylated, integral membrane phospho-glycoprotein with an apparent molecular weight of 190 kDa. Its function as an ATP-dependent conjugate export pump is intensively investigated and revealed a broad substrate specificity including several amphiphilic anionic endogenous and xenobiotic substances (16–19). The aim of the present study was to investigate, whether MRP is also actively expressed in cultured intestinal epithelial cells, namely Caco-2 cells, and to compare it with the expression of P-gp.

MATERIALS

The MRP-specific substrate MK-571 was from Biomol, Plymouth Meeting, PA, USA. Chloromethylfluorescein-diacetate (CMFDA) was from Molecular Probes, Eugene, OR, USA. All other chemicals were obtained from commercial sources in the highest quality available.

METHODS

Cell Cultures

As a control for MRP expression, the small cell lung cancer cell line H69 and the doxorubicin-selected multidrug-resistant and MRP over-expressing variant of H69, H69AR were used (20). H69 cells were cultured in RPMI 1640 medium (GIBCO) supplemented with 10% heat-inactivated fetal calf serum, 4 mM L-glutamine, and 1mM sodium pyruvate. The H69AR cell line has been maintained in medium containing 0.8 μM adriamycin.

Caco-2 cells (passages 42–48) were cultured with DMEM (Glutamax I) supplemented with 20% (v/v) fetal calf serum, 1% non-essential amino acids, 0.5 mM sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin. After 14–17 days of culture, confluency of the cell monolayers was achieved as determined by microscopy and by measurement of the transepithelial resistance (350–400 Ω cm^2).

Immunodetection of MRP and P-gp

MRP and P-gp were detected by Western blot analysis using the monoclonal antibody (Mab) MRPr1 for MRP (Signet Laboratories, Inc., Dedham, MA, USA) and the Mab C219 (Centocor, Inc., Malvern, PA, USA) for P-gp. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a Mini-Protean II apparatus (Bio-Rad, Zurich, Switzerland). To Caco-2 cell homogenates (1 mg protein ml^{-1}) one fifth volume sample buffer (10% glycerol, 5% SDS, 40 mM DTT, 0.00625%

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bromphenol blue, 62.5 mM Tris/HCl pH 6.8) was added. The samples were loaded onto 10% acrylamide/bisacrylamide gels. After electrophoresis, proteins were transferred electrophoretically (2 hours at a constant amperage of 2 mA × cm^{-2}) to a 0.45 μm pore size nitrocellulose membrane using a Mini Trans-Blot cell (Bio-Rad). The transfer buffer contained 192 mM glycine, 25 mM Tris and 20% methanol. The membrane was blocked overnight at 4°C with 5% powdered skimmed milk in phosphate-buffered saline (PBS) containing 0.3% Tween 20 (PBS-T). Washed membranes were incubated with Mab MRPr1 (1 μg/ml) or Mab C219 (200 ng/ml) in PBS-T, 0.05% Tween 20, 1% bovine serum albumin (BSA) and 1% powdered skimmed milk for 2 hours at 37°C. Washed membranes were incubated for 1 hour at room temperature with horseradish peroxidase-conjugated rabbit anti-rat IgG or rabbit anti-mouse (1:1000) (Dako) in PBS-T containing 0.05% Tween 20, 1% bovine serum albumin (BSA) and 1% milk powder. Membranes were washed in PBS-T and MRP or P-gp was visualized using enhanced chemiluminescence detection (ECL-kit by Amersham, Buckinghamshire, UK).

Polymerase Chain Reaction (PCR) for MRP

Total RNA was isolated from confluent monolayers of Caco-2 cells and from the doxorubicin-selected, multidrug resistant small cell lung cancer cell line H69AR using the RNeasy Mini Kit (Qiagen, Hilden, Germany). After DNaseI digestion the RNA was quantified with a GeneQuant photometer (Pharmacia, Uppsala, Sweden). Its integrity was checked by ethidium bromide agarose gel electrophoresis. The purity of the RNA preparations was high as demonstrated by the 260 nm/280 nm ratio (range 1.8–2.0). One μg of total RNA was reversed transcribed by Superscript II (Gibco Life Sciences, Basel, Switzerland) according to the manufacturer's protocol using random hexamers as a primer.

A total of 50 ng cDNA was used as a template for PCR with a set of primers. Primers were synthesized (Gibco BRL, Basel, Switzerland) for multidrug resistance related protein according to the original sequence of Cole (21): 5′-ATGTCACGTGGAATACCAGC-3′ (forward primer) and 5′-GAAGACTGAACTCCCTTCCT-3′ (reverse), glyceraldehyde phosphate dehydrogenase (GAPDH) primers 5′-ACCACAGTCCATGCCATCAC-3′ (forward) and 5′-TCCCACCACCCTGTTGCTGTA-3′ (reverse) were used as an internal control. The primers recognized specifically human MRP1, but not all other published sequences of MRP (MRP2-MRP6) as indicated by BLAST searches (GenBank accession number L05628 for human MRP1).

For P-gp detection the following primers were used: 5′-GTGCTGGTTGCTGCTTACAT-3′ (forward) and 5′-CCCAGTGAAAAATGTTGCCA-3′ (reverse). PCR was performed with a thermocycler (Biometra, Göttingen, Germany). Each sample was amplified for 35 cycles of denaturation (94°C for 30 s, 60°C for 50 s, 72°C for 50 s). The reaction mixture contained 5 μl of the cDNA template, 1.25 U AmpliTaq gold DNA polymerase (Perkin Elmer, Rotkreuz, Switzerland), 5 μl 10× PCR buffer (Mg^{2+}-free, Perkin Elmer), $MgCl_2$ at a final concentration of 3.5 mM, 4 ml of dNTP reaction mixture (2.5 mM each, Perkin Elmer), 15 pmol of each primer and water to a final volume of 50μl. Each PCR reaction included a 50 μl aliquot of the reaction mixture without cDNA as a negative control. The PCR products were separated by electrophoresis in 1.5 % agarose and visualized by UV in the presence of ethidium bromide.

Uptake Assays

Uptake assays were performed at 20°C using confluent monolayers of Caco-2 cells at day 17. Cells were grown in 24-well cell-culture plates with surface areas of 2 cm^2/well. Cells were washed using Hanks Balanced Salt Solution (HBSS, Gibco, Basel, Switzerland). For the functional assay of P-gp, the cells were preincubated with 1 μM p-glycoprotein inhibitor PSC-833 for 1 hour. After that, cells were incubated 10 or 20 min with 1 μM cyclosporin A, supplemented with tracer amounts of [^{3}H]-cyclosporin A. Then cells were washed with HBSS. The cell monolayers were solubilised in 1% Triton X-100 and the solutions were transferred to scinitillation vials. Radioactivity was determined by liquid scintillation counting (Packard Tri-Carb 2000, Packard, Dreeich, Germany).

Efflux Assays

For measurement of MRP-mediated extrusion from the cells, cells cultured in 24-well plates were loaded with 10 μmol/L CMFDA (Molecular Probes, Eugene, OR, USA) for 60 minutes at 10°C. Thereafter, cells were washed twice with ice-cold HBSS and kept at 10°C in HBSS. CMFDA is metabolized in the cells to glutathione-methylfluorescein (GS-MF). Then, GS-MF efflux from the cells was measured at 37°C or at 10°C in the absence or presence of 2.5 μg/ml MK-571 by adding media of 37°C or 10°C, respectively. At the time intervals indicated in the figure legends, 200μl samples were removed and fluorescence was measured with a fluorescence enzyme-linked immunosorbent assay-plate reader (FluoStar, Tecan, Salzburg, Austria).

Uptake of CMFDA by the cells was assessed by lysing the cells with 1% Triton X-100 in PBS after the 60-minute uptake period and subsequent washes. Fluorescence of the homogenate was determined. The remaining amount of GS-MF in the cells after a time period of 30 min was assessed by lysing the cells with 1% Triton X-100 in PBS. Fluorescence of the homogenate was determined. To quantify the amount of fluorescence, a 25 μol/L GS-MF standard was prepared as follows: 5 U of esterase, 5 U of glutathione S-transferase, 250 μmol/L reduced glutathione, and 25 μmol/L CMFDA were dissolved in 1 ml HBSS medium and incubated at 37°C until no increase in fluorescence was observed. A calibration curve of 0.1–10 μmol/L GS-MF was constructed.

Data Analysis

For data representation and to obtain estimates of kinetic parameters, a non-linear regression program was used (Microcal Origin version 5.0). For statistical comparison, data of groups were compared by analysis of variance (ANOVA). The level of significance was $P = 0.05$. If this analysis revealed significant differences, pairwise comparisons within groups were performed by two-sided unpaired t-tests. P-values were adjusted by Bonferroni's correction for multiple comparisons.

RESULTS

Kinetic assays were performed to investigate the functional expression of MRP and P-gp in the Caco-2 cells. In a first set

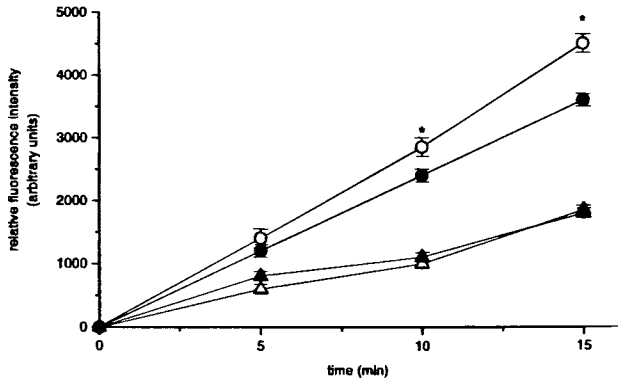

Fig. 1. Time dependent secretion of GS-MF by Caco-2 cell monolayers. ○: secretion by control cells at 37°C; ●: secretion at 37°C by cells preincubated for 1 hour with the specific inhibitor of MRP, MK 571 at 2.5 μg/ml, ▲: secretion by control cells at 10°C; Δ secretion at 10°C by cells preincubated for 1 hour with the specific inhibitor of MRP, MK 571 at 2.5 μg/ml. (* statistical significant different, $p < 0.05$, $n = 8$).

of experiments control cells and cells treated with 2.5 μg/ml MK-571, a specific MRP-blocker, were incubated with CMFDA. CMFDA is metabolized by the cells to yield the glutathione conjugate glutathione-methylfluorescein (GS-MF; 16). GS-MF accumulation in MK-571 treated cells was significantly higher than in control cells. The efflux of GS-MF from was measured at 37°C and 10°C to study MRP-mediated extrusion. Excretion of GS-MF was significantly decreased in the presence of MK-571, indicating blockage of extrusion of GS-MF by MRP (Fig. 1). When the experiment was performed at 10°C, the extrusion of GS-MF was decreased, and no significant difference between control cells and MK-571 treated cells could be observed, suggesting that the secretory function of MRP was not active any more at the low temperature.

When the cells were incubated with cyclosporin A, the cellular accumulation was significantly increased after pretreatment of the cells with PSC-833, a specific p-glycoprotein inhibitor, suggesting that cellular extrusion of cyclosporin A via P-gp was inhibited (Fig. 2).

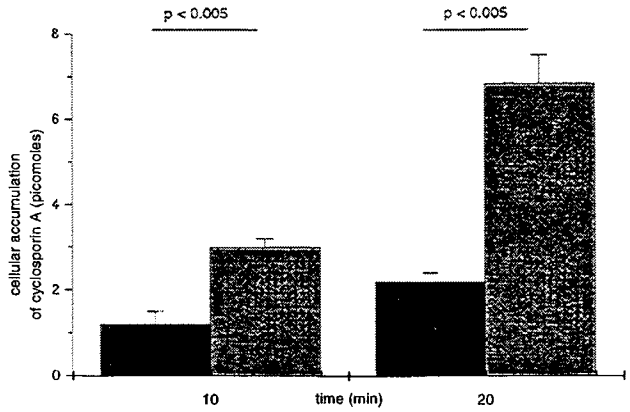

Fig. 2. Time dependent accumulation of cyclosporin A in Caco-2 cells. Black bars: Accumulation in control cells incubated with 1 μM cyclosporin A; gray bars: accumulation in cells which had been preincubated for 1 hour with 10 μM P-gp inhibitor SDZ PSC 833.

The presence of MRP in Caco-2 cell monolayers and reference cell lines was assessed by molecular identification with Western blot analysis by use of a monoclonal anti-MRP antibody (MRPr1). The MRP over-expressing cell line H69AR showed an immunoreaction in the molecular weight range of 190 kDa that was not present in parental H69 cells lacking MRP expression. In Caco-2 cells at the same molecular weight a densely stained band was observed, indicating the presence of MRP in Caco-2 cells (Fig. 3). At 80 kDa the antibody stained another protein that most likely represents a degradation product of MRP (22).

RT-PCR was performed to determine the relative expression of mRNA for the MRP gene in Caco-2 cells. In control experiments the constitutive expression of glyceraldehyde phosphate dehydrogenase (p-GAPDH) was investigated. Figure 4 demonstrates the presence of the MRP1 gene product in the positive control cell line H69/AR as well as in Caco-2 cells. BLAST searches revealed that with the primer pair used human MRP1 (GenBank accession number L05628) is detected selectively and specifically, making it most likely, that this isoform is expressed by Caco-2 cells. Both, the forward and reverse primers recognized the gene product of the isoform MRP1, only.

Western blot analysis with the monoclonal antibody C219 revealed that the P-gp over-expressing cell line MDR-P388 showed an intensive immunoreaction in the molecular weight

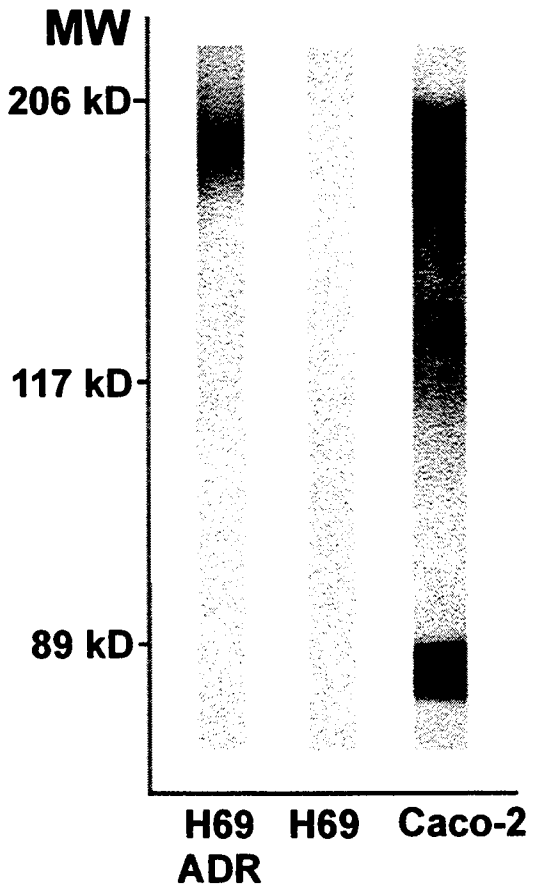

Fig. 3. Western blot detection of MRP in a MRP over-expressing small cell lung cancer cell line (H69ADR) and in Caco-2 cells. MRP cannot be detected in a negative subline of the small cell lung cancer cell line (H69).

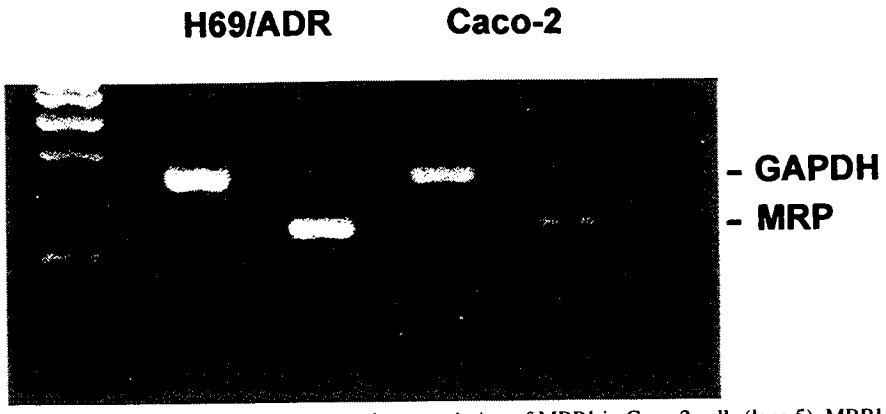

Fig. 4. Polymerase chain reaction reveals transcription of MRP1 in Caco-2 cells (lane 5). MRP1 gene product was compared with the constitutive expression (lane 4) of glyceraldehyde phosphate dehydrogenase. Lane 3 shows expression of MRP1 in the small cell lung cancer cell line H69/ADR compared with the constitutive expression of glyceraldehyde phosphate dehydrogenase (lane 2). Lane 1 represents size markers.

range of 160 kDa that was not present in Par-P388 cells devoid of P-gp expression. When Caco-2 cells were subjected to Western blot analysis a staining in the same molecular weight range was seen, which was indicative for the expression of P-gp in these cells. (Fig 5). PCR analysis (Fig. 6) confirmed the Western blot and indicated that the MDR1-gene product is present in Caco-2 cells. The expression was of a similar magnitude as the constitutive expression of GAPDH.

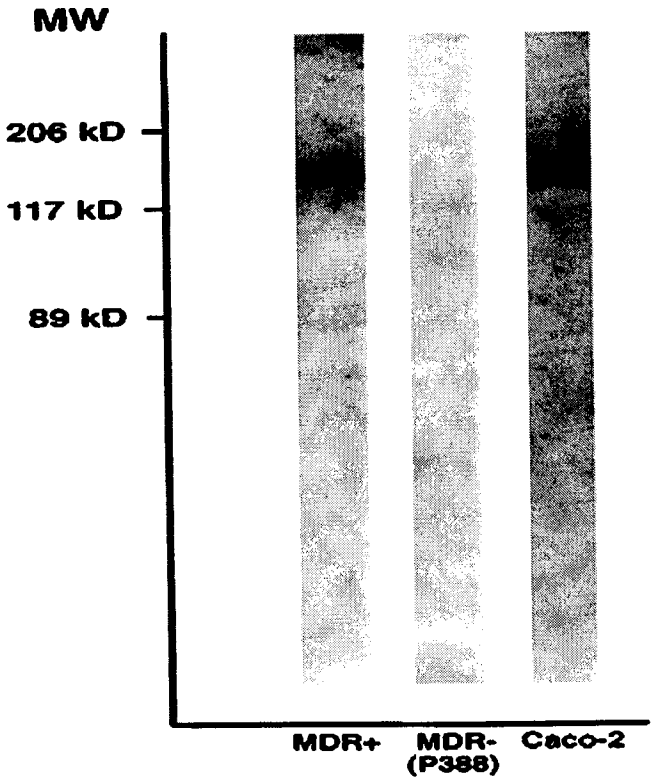

Fig. 5. Western blot detection of P-gp in over-expressing MDR-P388 cells (MDR+), and in Caco-2 cells. Multidrug resistance related protein cannot be detected in a negative subline of murine leukeamia cells (MDR-P388).

DISCUSSION

It is well documented in the literature that the human colon carcinoma cell line Caco-2 functionally expresses the MDR1-gene product P-glycoprotein (6,10,23–26). Caco-2 cells are a widely accepted model to investigate the contribution of p-glycoprotein to overall drug absorption. However, there is an increasing evidence that many substrates of P-glycoprotein are also transported by other active secretory transport proteins, such as the MRP-protein family. At least 6 isoforms of MRP have been identified in animal and man (18) sharing in part the substrate recognition with P-glycoprotein, which becomes of special interest in cytostatic therapy. P-gp has been identified as a transporter of certain cationic and lipophilic compounds and MRP1 as an organic anion transporter that plays also a physiological role in the elimination of glutathione conjugates. Nevertheless, there is considerable overlap in the resistance spectrum of tumor cells, suggesting that both proteins transport important anticancer agents such as doxorubicin, etoposide, and vincristine. Therefore, the question arises, whether MRP is also expressed in Caco-2 cell cultures and whether it participates in drug permeation in that widely used cellular model.

RT-PCR, immunoblot and kinetic analysis were carried out to demonstrate the functional expression of MRP and P-gp. The transport studies were performed with a substrate, which is a MRP but not a P-gp-substrate, namely glutathione-methylfluorescein (16). In the presence of MK-571, which selectively blocks MRP function, the substrate accumulated within the cells. Thus, we assume, that the export of GS-MF has been suppressed by inhibition of MRP-mediated secretion. From this kinetic experiment, no precise distinction is possible between the isoforms of MRP due to their overlapping substrate specificity. But, the PCR and immunoblot studies with a rat antibody against human MRP1 show on a molecular level, that the MRP1-gene product is present in Caco-2 cells.

Cyclosporin has been chosen as a P-gp-substrate because previous data in the literature indicate that it is not a MRP1-substrate: In non P-gp multidrug resistant carcinoma cells, an increase in cellular daunorubicin uptake has been described without simultaneous sensitization to MRP1 substrates (27). In addition, in MRP1 transfected cells only a modestly increased

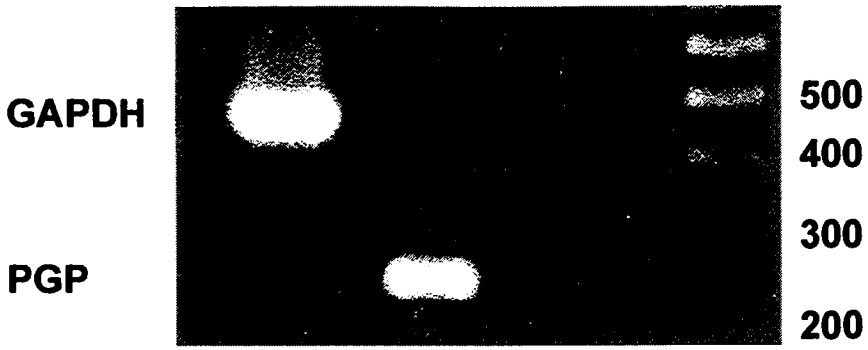

Fig. 6. Polymerase chain reaction reveals transcription of P-gp in Caco-2 cells (lane 2). The MDR1 gene product was compared with the constitutive expression (lane 1) of glyceraldehyde phosphate dehydrogenase. Lane 4 represents size markers.

vincristine accumulation was seen in presence of cyclosporin A (28). Furthermore, photoaffinity labeling studies with bile canalicular plasma membranes from rat liver revealed the incorporation of a photolabile cyclosporin A derivative only into P-gp (29). The kinetic data obtained with cyclosporin A support the molecular evidence for the presence of P-gp on a functional level and confirm previous data about P-gp activity in Caco-2 cells.

In summary, both molecular and functional experiments indicate that P-gp is not the only secretory system being active in Caco-2 cell monolayers. Therefore, our data may help to explain previous findings of a polarized flux of non P-gp-substrates such a calcein across the intestinal barrier and support studies localizing MRP-like proteins in intestinal tissue (30). However, the clinical relevance of MRP1 for the intestinal protection of the body against xenobiotics has to be verified in *in vivo* studies.

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