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Modulation of p-glycoprotein function by caveolin-1 phosphorylation

Stéphane Barakat,*,† Michel Demeule,* Anthony Pilorget,* Anthony Régina,* Denis Gingras,* Loris G. Baggetto† and Richard Béliveau*

*Laboratoire de médecine moléculaire, Hôpital Sainte-Justine, Université du Québec à Montréal, Montréal, Québec, Canada
†Laboratoire Thérapie transcriptionnelle des cellules cancéreuses, Institut de Biologie et Chimie des Protéines, Lyon, France

Abstract

p-glycoprotein (p-gp) is an ATP-binding cassette transporter and its overexpression is responsible for the acquisition of the multidrug resistance phenotype in human tumors. p-gp is localized at the blood–brain barrier and is involved in brain cytoprotection. Our previous work used immunoprecipitation to show that caveolin-1 can interact with p-gp. In this study, we provide evidence that caveolin-1 regulates p-gp transport activity in a rat brain endothelial cell line (RBE4). Down-regulation of caveolin-1 by siRNA reduced the interaction between p-gp and caveolin-1, followed by a decrease in [^{3}H]-Taxol and [^{3}H]-Vinblastine accumulation in RBE4 cells. The latter result showed that down-regulation of caveolin-1 enhanced p-gp transport activity. RBE4 cells were also transfected with Sarcoma in order to modulate caveolin-1 phosphorylation. Overexpression of Sarcoma, a protein tyrosine kinase, stimulated caveolin-1 phosphorylation and increased both [^{3}H]-Taxol and [^{3}H]-Vinblastine accumulation as well as Hoechst 33342 accumulation. Transfection of caveolin-1 inhibits p-gp transport activity. Conversely, transfection of the mutant cavY14F decreased the p-gp/caveolin-1 interaction and reduced accumulation of the two p-gp substrates. Thus, our data show that caveolin-1 regulates p-gp function through the phosphorylation state of caveolin-1 in endothelial cells from the blood–brain barrier.

Keywords: blood–brain barrier, caveolin-1, endothelial cells, p-glycoprotein, phosphorylation, Sarcoma.
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p-glycoprotein (p-gp) belongs to the ATP-binding cassette transporter family of proteins and participates in the expulsion of diverse hydrophobic molecules (Schinkel *et al.* 1996). This transporter is highly expressed at the blood–brain barrier, enabling brain cytoprotection (Demeule *et al.* 2000). p-gp overexpression has been associated with the multidrug resistance (MDR) phenotype acquisition because it reduces the intracellular concentrations of a wide variety of anti-cancer agents (Gottesman *et al.* 2002).

Part of the research on p-gp has focused on its localization in caveolae and, more generally, in detergent-resistant membranes (DRM) (Demeule *et al.* 2000; Ronaldson *et al.* 2004). Caveolae are flask-shaped, invaginated membranes enriched in cholesterol and sphingomyelin, which confer particular physico-chemical properties including insolubility in anionic detergents and low-buoyant density in sucrose gradients (Liu and Anderson 1995; Smart *et al.* 1995; Ahmed *et al.* 1997). Caveolae are known to recruit many signaling proteins (Krajewska and Maslowska 2004). Caveolin-1 (Cav-1), the principal component of caveolae, interacts with and regulates the function of a wide variety of proteins which are known to be involved in cholesterol homeostasis, cell proliferation, and adhesion (Fielding and Fielding 1995; Arnal *et al.* 1999; Cohen *et al.* 2004). When interacting with those proteins, Cav-1 negatively regulates their functions through its caveolin scaffolding domain, corresponding to amino acid 82–101 (Yamamoto *et al.* 1999).

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Address correspondence and reprint requests to Dr Richard Béliveau, Département de Chimie-Biochimie, Université du Québec a Montréal, CP 8888, succursale centre-ville, Montréal, Québec, H3C 3P8, Canada. E-mail: oncomol@nobel.si.uqam.ca

Abbreviations used: BBB, blood–brain barrier; Cav-1, caveolin-1; DRM, detergent-resistant membranes; ECs, endothelial cells; FACS, fluorescent-activated cell sorting; FBS, fetal bovine serum; IP, immunoprecipitation; MDR, multidrug resistance; PBS, phosphate buffered-saline; pcav-1, phosphocaveolin-1; p-gp, p-glycoprotein; RBE4, rat brain endothelial cell line; Src, Sarcoma; VBL, Vinblastine; WT, wild-type.

p-gp has been co-localized with Cav-1 in caveolae using a carbonate-based fractionation method with bovine brain capillary endothelial cells (ECs) using either wild-type (WT) or MDR1 stably transfected Madin-Darby canine kidney cells as well as in CH^RC5 chemoresistant cancer cells (Jodoin *et al.* 2003). p-gp was also co-localized with Cav-1 in Triton-insoluble membrane domains in A549 lung adenocarcinoma cells (Belanger *et al.* 2004). However, the role of the tyrosine-14-phosphorylated Cav-1 remains unknown with regard to p-gp function. p-gp is known to interact with Cav-1, and inhibition of p-gp/Cav-1 interaction by mutagenesis positively modulated p-gp transport activity in COS7 cells (Jodoin *et al.* 2003). Several immunoprecipitation (IP) studies indicated that p-gp could interact with Cav-1 and that this interaction could modulate p-gp transport activity (Cai and Chen 2004; Ronaldson *et al.* 2004). In contrast, others have found that p-gp is localized in raft fractions independent of caveolae by use of different detergents for DRM extractions such as Brij-96 or lubrol (Hinrichs *et al.* 2004; Radeva *et al.* 2005).

In the present work, we demonstrate for the first time that siRNA-mediated down-regulation of Cav-1 stimulates p-gp transport activity in brain capillary-derived ECs. We also show that Cav-1 overexpression inhibits this same transport activity. Furthermore, we provide evidence that tyrosine-14 phosphorylation participates in the regulation of p-gp/Cav-1 interactions and, consequently, has a direct effect on p-gp function.

Materials and methods

Materials

α-Minimal essential medium and F-12 medium were purchased from Gibco-BRL Life Technologies, Inc. (Burlington, ON, Canada). Hoechst 33342 was obtained from Sigma Chemical Co. (St Louis, MO, USA). Fetal bovine serum (FBS) was obtained from HyClone (Logan, UT, USA). Transpass D1 was purchased from New England Biolabs (Ipswich, MA, USA). mAbs directed against Cav-1 and phosphocaveolin-1 (pcav-1) were purchased from BD Transduction Laboratories (Lexington, KY, USA). mAb against p-gp (namely mAb C219) was from ID Labs (London, ON, Canada). mAb against Sarcoma (Src) was from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). An anti-mouse secondary antibody, linked to horseradish peroxidase, was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA) and enhanced-chemiluminescence reagent plus reagents were from NEN™ Life Science Products (Boston, MA, USA). Protein G-Sepharose beads were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). HiPerFect reagents and HP-validated human Cav-1 siRNA design and synthesis (1027400) were performed by Qiagen (Mississauga, ON, Canada). Other biochemical reagents were from Sigma (Oakville, ON, Canada).

Cell culture

Rat brain endothelial cell line (RBE4) -immortalized rat brain microvessel ECs were a gift from Françoise Roux (INSERM, Paris, France) (Regina *et al.* 1999). Cells were plated on type I collagen-coated plates (200 μg/mL) and grown in α-minimal essential medium/Ham's F-12 medium (1 : 1), supplemented with 2 mmol/L glutamine, 300 mg/mL geneticin (G418), 1 ng/mL basic fibroblast growth factor, and 10% heat-inactivated FBS. Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2.

Caveolae purification

Low-density caveolae-enriched domains were isolated by a carbonate-based fractionation method as described previously (25). Briefly, confluent CHRc5 or AuxB1 cells cultured in 100 mm dishes (containing about 10^7 cells) were scraped into 2 mL of 0.5 mol/L sodium carbonate (pH 11) and homogenized extensively using a Dounce homogenizer (10 strokes), a Polytron tissue grinder (three 10 s bursts at medium speed), and a sonicator (three 20 s bursts at 50% maximal power). The resulting homogenate was brought to 45% sucrose by the addition of 2 mL of 90% sucrose in Mes-buffered saline (25 mmol/L Mes, pH 6.5, and 150 mmol/L NaCl) and overlaid with two layers of 35% and 5% sucrose in Mes-buffered saline containing 0.25 mol/L carbonate (4 mL each). The gradient was then centrifuged at 200 000 U*g* for 18 h using a Beckman SW41Ti rotor. For the analysis of the resulting gradient, 1 mL fractions were collected from the top to the bottom of the gradient. Ten or 20 μL from each fraction was subjected to sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis for the detection of p-gp or caveolin, respectively.

siRNA and plasmid transfection

RBE4 cells were seeded in six-well plates or in 60-mm culture plates and cultured at 37°C and 5% CO_2 for 24 h. At 50–60% of confluency, the cells were transiently transfected with serum-free medium containing HiPerFect transfection reagent, using 50 nmol/L siRNA against Cav-1 in 6-well plates or in 60-mm culture plates. 24 h post-transfection, the culture medium was replaced with fresh complete medium and cells were used 48 h after transfection. Using the same procedure, cells were transiently transfected, by Transpass D1 reagent, using 3 to 10 μg of plasmids expressing cav-1, cavY14F, or Src. Three hours post-transfection, the culture medium was replaced with fresh complete medium and cells were used 48 h post-transfection.

FACS analysis

Cav-1 down-regulation in RBE4 cells was examined by fluorescent-activated cell sorting (FACS) analysis. Cells were transfected with siRNA-directed against Cav-1, as previously described. Cells were trypsinized, rinsed once in chilled binding buffer, and fixed with 3.7% formaldehyde for 15 min. Fixed cells were rinsed again with cold binding buffer and permeabilized for 30 min with a 0.1% Triton X-100 solution. After washing, cells were blocked with 1% FBS in binding buffer for 30 min. Cells were washed and resuspended in 200 μL of binding buffer at a final concentration of 10^6 cells/mL. Cells were incubated with control IgG or anti-Cav-1 antibody for 30 min at 4°C, rinsed and incubated with CY-conjugated anti-mouse antibody for 30 min at 4°C. Cells were resuspended in a final volume of 1 mL and analysed by FACS, using a FACScalibur station (BD Biosciences, Mississauga, ON, Canada). Analysis was performed using the Cell Quest Pro software (BD Biosciences).

Hoechst accumulation

Transfected cells in six-well plates were incubated at 37°C with 20 μmol/L Hoechst 33342 (bisbenzimide) in serum-free medium. The fluorescence of the intracellular dye was monitored continuously using a spectrofluorimeter (SpectraMax Gemini; Molecular Devices, Sunnyval, CA, USA) at wavelengths of 355 nm for excitation and 460 nm for emission.

Vinblastine or Taxol accumulation in RBE4 cells

Transfected cells were washed twice with Hank's Balanced Salt Solution (1.3 mmol/L $CaCl_2$, 5.4 mmol/L KCl, 0.44 mmol/L KH_2PO_4, 0.5 mmol/L $MgCl_2$, 0.83 mmol/L $MgSO_4$, 137 mmol/L NaCl, 4.2 mmol/L $NaHCO_3$, 0.34 mmol/L Na_2HPO_4, and 25 mmol/L D-glucose, pH 6.5) and then incubated for 2 h at 37°C in Hank's Balanced Salt Solution containing 50 nmol/L [^{3}H]-Vinblastine (VBL) or [^{3}H]-Taxol. [^{3}H]-VBL or [^{3}H]-Taxol accumulation was stopped by washing the cells five times with ice-cold phosphate buffered-saline (PBS). Cells were lyzed with 0.1% Triton X-100 at 25°C overnight or with 0.1 N NaOH for 1 h. Each sample of about 200 μL was mixed with 10 mL scintillation liquid and the radioactivity in the cell lysate was measured.

Lysate preparation, IP, and western blot analysis

Cells were washed twice with PBS and lysed in buffer containing 50 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 0.1% SDS, 1% NP-40, 0.5% deoxycholate, and protease inhibitors for 30 min at 4°C. Unbroken cells were pelleted by centrifugation at 500 *g* for 10 min. Proteins in cell lysates were quantified by the micro bicinchoninic acid method. For IP, aliquots of lysates (200 μg of protein) were pre-cleared by incubation for 1 h at 4°C with 20 μL of protein G-Sepharose beads (50% in PBS). After centrifugation at 1000 *g* for 3 min at 4°C, supernatants were immunoprecipitated by overnight incubation with 1 μg anti-p-gp at 4°C with agitation. Twenty microlitre of protein G-Sepharose beads were added to the immune complexes for 2 h at 4°C with agitation. Immunoprecipitated p-gp was pelleted by centrifugation at 1000 *g* for 3 min at 4°C. Following three washes of the beads with buffer, aliquots from immunoprecipitated proteins and from cell lysates were solubilized with Laemmli buffer and heated (Cav-1) or not (p-gp) for 4 min at 95°C. Proteins were separated by SDS–polyacrylamide gel electrophoresis followed by semi-dry transfer to polyvinylidene difluoride membranes using standard procedures. Immunodetection of Cav-1 and p-gp was performed with specific antibodies. Horseradish peroxidase-conjugated anti-IgG antibodies were used as secondary antibodies and antigens were detected using chemiluminescence. Blots were exposed to Fuji films and the autoradiograms were scanned with a ChemiImager (Alpha Innotech Corporation, San Leandro, CA, USA).

Results

Down-regulation of Cav-1 inhibits p-gp transport activity

At first, we study the localization of p-gp that was localized in the membranes. Membranes microdomains also called as DRMs were purified by using a sucrose density gradient. Then, p-gp and Cav-1 were detected by immunoblot (Fig. 1a). As expected, p-gp was co-localized with Cav-1 in low-density fraction corresponding to DRMs-enriched fraction.

The regulation of p-gp transport activity was investigated by using siRNA to reduce Cav-1 expression. Cav-1 siRNA were transfected for 24 h into RBE4 cells. Forty-eight hour post-transfection, Cav-1 and p-gp in RBE4 cells were immunodetected with monoclonal antibodies. As shown in Fig. 1a, Cav-1 siRNA induced a strong down-regulation of Cav-1 expression, whereas p-gp expression was unchanged from the control sample. Cav-1 was corresponding to a double band, which is known to be the Cav-1 α and β as indicated in the Fig. 1a. Cav-1 expression was quantified by densitometry analysis (Fig. 1b). Cav-1 expression was decreased by 90% after siRNA transfection. In order to confirm Cav-1 down-regulation mediated by Cav-1 siRNA, Cav-1 expression was determined by FACS analysis. As shown in Fig. 1c, Cav-1 expression decreased by 88% compared with the control. In order to evaluate the level of p-gp/Cav-1 interaction, p-gp was immunoprecipitated and Cav-1 was detected in siRNA transfected RBE4 cells (Fig. 1d). Down-regulation of Cav-1 decreased the binding between Cav-1 and p-gp (Fig. 1d). As 10 μg of total proteins cell lysate were loaded as a positive control and that the IP was realized on 200 μg of total proteins, we could estimate that around 10% of the total p-gp is associated with Cav-1 in RBE4. To evaluate p-gp activity, siRNA-transfected ECs were treated with 50 nmol/L [^{3}H]-VBL or [^{3}H]-Taxol, two p-gp substrates, for 2 h. Drug accumulation within the cells was determined by measuring the intracellular radioactivity of each compound (Fig. 2). Accumulation of [^{3}H]-Taxol and [^{3}H]-VBL were reduced by 35% and 40%, respectively, in Cav-1 siRNA transfected cells as compared with the control cells. These results show that Cav-1 down-regulation by siRNA treatment considerably enhances p-gp transport activity. Thus, Cav-1 down-regulation increased p-gp transport activity by a reduction in p-gp/Cav-1 interaction.

Cav-1 phosphorylation by Src modulates p-gp transport activity

We next investigated the role of Cav-1 phosphorylation on the modulation of p-gp transport activity. Previous studies have shown that Src, a tyrosine kinase implicated in the regulation of signaling pathways, directly participates, when activated, in Cav-1 tyrosine-14 phosphorylation. In particular, it was reported that the activation of Src induces tyrosine-14 phosphorylation of Cav-1 (Labrecque *et al.* 2004). RBE4 cells were transfected with a Src-encoding plasmid. At 48 h post-transfection Src, p-gp, Cav-1, and pcav-1 were detected by immunoblot (Fig. 3a). As determined by densitometry analysis, Src was overexpressed by 1.8-fold in transfected cells as compared with control cells (Fig. 3b). p-gp and Cav-1 were also detected and their expressions were similar after Src transfection (Fig. 3b). In

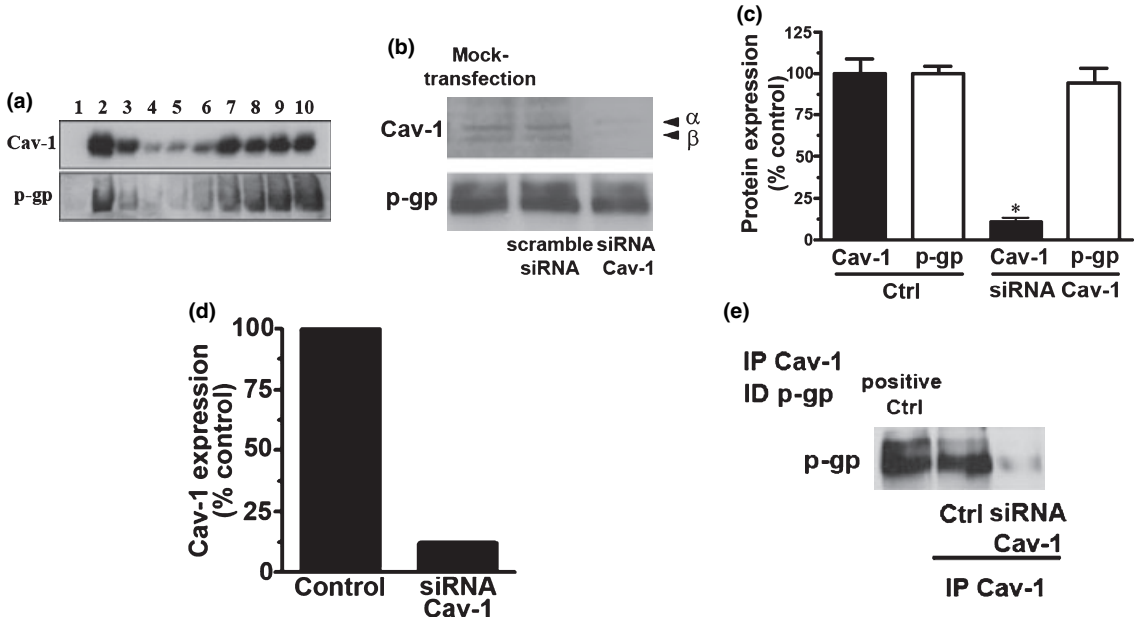

Fig. 1 Localization of p-glycoprotein (p-gp) and caveolin-1 and down-regulation of caveolin-1 using siRNA in brain capillary endothelial cells. (a) Cholesterol-enriched membranes (such as caveolae and raft) purification was realized by using a sucrose density gradient. Twelve fractions were recovered. Twenty microlitre of each fraction were deposited in a sodium dodecyl sulfate–polyacrylamide gel electrophoresis. p-gp and caveolin-1 were immunodetected after an electro-transfer. (b) Rat brain endothelial cell line cells were transfected with siRNA against caveolin-1 using Hiperfect transfection reagent. 10 μg protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis 8% and 15% respectively, for the immunodetection of caveolin-1 and p-gp. mAb C219 and mAb anti-caveolin-1 were used for the immunodetection of p-gp and caveolin-1, respectively. (c) Caveolin-1 and p-gp expression were quantified by densitometry analysis. Data represent the means ± SD of results obtained from three different experiments performed in triplicate. Asterisk denotes statistical significance (p value < 0.05, Student's t-test). (d) Caveolin-1 down-regulation mediated by siRNA was determined by FACS analysis. Caveolin-1 expression was measured using anti-caveolin-1 mAb after cells were treated with 0.1% Triton X-100. Then, cells were treated with CY-conjugated anti-mouse antibody. A negative control was performed using IgG. One representative experiment is shown. (e) p-gp immunoprecipitation was performed by using 200 μg protein treated with 1 μg C219 mAb and caveolin-1 was identified by immunoblot, as described in Materials and methods. These results are representative of three separate experiments.

contrast, detection of pcav-1 indicates that phosphorylation of tyrosine-14 was increased by 1.6-fold upon Src overexpression.

The influence of tyrosine-14 phosphorylation in Cav-1 on the p-gp transport activity was also evaluated. [^{3}H]-Taxol and [^{3}H]-VBL accumulation were measured in Src-transfected RBE4 cells or control cells. As shown in Fig. 4a, [^{3}H]-VBL and [^{3}H]-Taxol accumulation were enhanced by 30% and 27%, respectively, in transfected cells as compared with control cells. The same result was obtained when cells were treated with Hoechst 33342, a substrate of p-gp (Fig. 4b). Consequently, these data are the first to show that phosphorylation of Cav-1 inhibits p-gp transport activity.

Cav-1 phosphorylation modulates p-gp/Cav-1 interaction and p-gp transport activity

The role of tyrosine-14 phosphorylation in Cav-1 on the Cav-1/p-gp interaction and on the inhibition of the p-gp transport activity was next examined. REB4 cells originally derived from brain capillary were transfected with plasmids expressing Cav-1 or a mutant Cav-1 where tyrosine-14 was replaced by phenylalanine. Cav-1, p-gp, and pcav-1 were detected by immunoblot at 48 h post-transfection. As shown in Fig. 5a, Cav-1 was overexpressed in cells transfected with WT Cav-1 and mutant Cav-1 (Y14F) by 1.9- and 1.8-fold, respectively, whereas p-gp expression was unaffected, which is clearly shown by densitometry analysis (Fig. 5b). As expected, Cav-1 overexpression was associated with an increase in pcav-1 by 1.5-fold compared with control cells. Phosphorylation of tyrosine-14 in mutant Cav-1 was unchanged compared with the control. We purified DRMs from cav-1 and cavY14F transfected cells and we could observe that p-gp localization was similar to the one obtain in the Fig. 1a (data not shown).

In order to determine the effect of tyrosine-14 phosphorylation of Cav-1 on p-gp/Cav-1 interaction, p-gp was immunoprecipitated from RBE4 cells transfected with either WT or mutant (Y14F) Cav-1. Cav-1 was detected by immunoblot. As shown in Fig. 5c, the level of Cav-1/p-gp interaction was

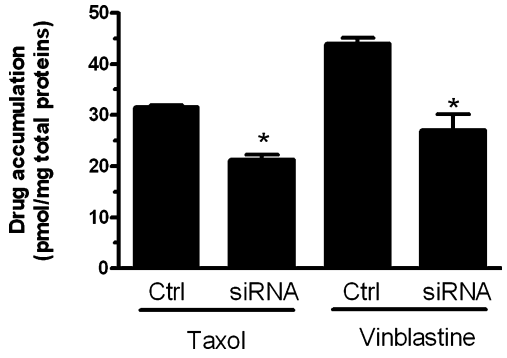

Fig. 2 Effect of caveolin-1 down-regulation on the accumulation of [^{3}H]-Taxol or [^{3}H]-Vinblastine, two p-glycoprotein substrates. Accumulation of 50 nmol/L [^{3}H]-Taxol or [^{3}H]-Vinblastine was measured after 1.5 h of treatment with those drugs into rat brain endothelial cell line cells transfected (or not) with siRNA against caveolin-1 as described in Materials and methods. Data represent the means ± SD of results obtained from three different experiments performed in triplicate. Asterisks denote statistical significance (p value < 0.05, Student's t-test).

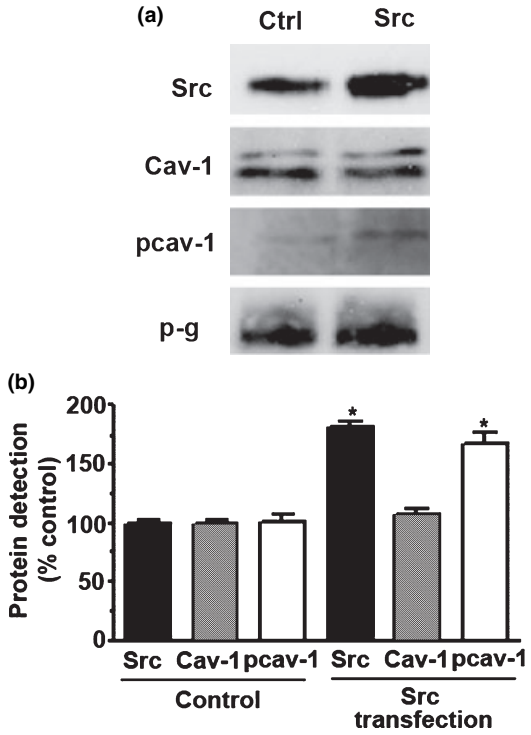

Fig. 3 Overexpression of Sarcoma (Src) enhances caveolin-1 phosphorylation. (a) Src, caveolin-1, tyrosine-14 phosphorylated caveolin-1 (pcav-1), and p-glycoprotein (p-gp) were detected in Src-transfected rat brain endothelial cell line cells by immunoblot against 10 μg/mL protein, as described in Materials and methods. These results are representative of three separate experiments. (b) Src, caveolin-1, and pcav-1 expression were evaluated by densitometry analysis. Asterisks indicate statistical significance of the three independent experiments (p < 0.05, Student's t-test).

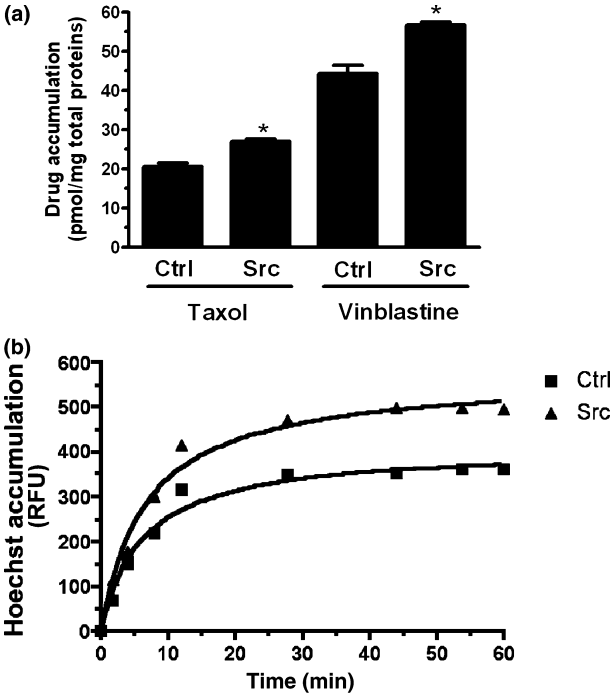

Fig. 4 Sarcoma (Src) overexpression increased accumulation of [^{3}H]-Vinblastine or [^{3}H]-Taxol p-glycoprotein substrates. (a) Accumulation of 50 nmol/L [3H]-Taxol or [3H]-Vinblastine into rat brain endothelial cell line cells transfected (or not) with Src was measured after 2 h as described in Materials and methods. (b) 48 h post-transfection, rat brain endothelial cell line wild-type and Src-transfected cells were incubated with 20 μmol/L Hoechst 33342 and the fluorescence was followed continuously for 1 h using 355 nm for excitation and 460 nm for emission as described in Materials and methods. These results correspond to three independent experiments. Asterisks denote statistical significance compared with the control (p < 0.05, Student's t-test).

increased in cells transfected with WT Cav-1 compared with their control. However, in RBE4 cells transfected with the mutant Cav-1 cavY14F, the interaction was decreased compared with transfection with the WT Cav-1. Thus, tyrosine-14 phosphorylation increased the interaction between Cav-1 and p-gp. We also measured the direct involvement of tyrosine-14 phosphorylation in the inhibition of p-gp transport activity. [^{3}H]-Taxol (Fig. 6a) and [^{3}H]-VBL (Fig. 6b) accumulation were measured in ECs transfected with WT or mutant Cav-1. As shown in Fig. 6(a and b), [^{3}H]-Taxol and [^{3}H]-VBL accumulation were increased by 29% and 46%, respectively, after WT Cav-1 transfection of RBE4 cells. In contrast, accumulation is reduced by 22% and 18%, respectively, for [^{3}H]-Taxol and [^{3}H]-VBL in cells transfected with mutant Cav-1 compared with the cells transfected with WT Cav-1. This indicates that Cav-1 interacts with p-gp when phosphorylated. Consequently, when Cav-1 is mutated at tyrosine-14, the level of p-gp/Cav-1 interaction decreases leading to the increase of the p-gp transport activity.

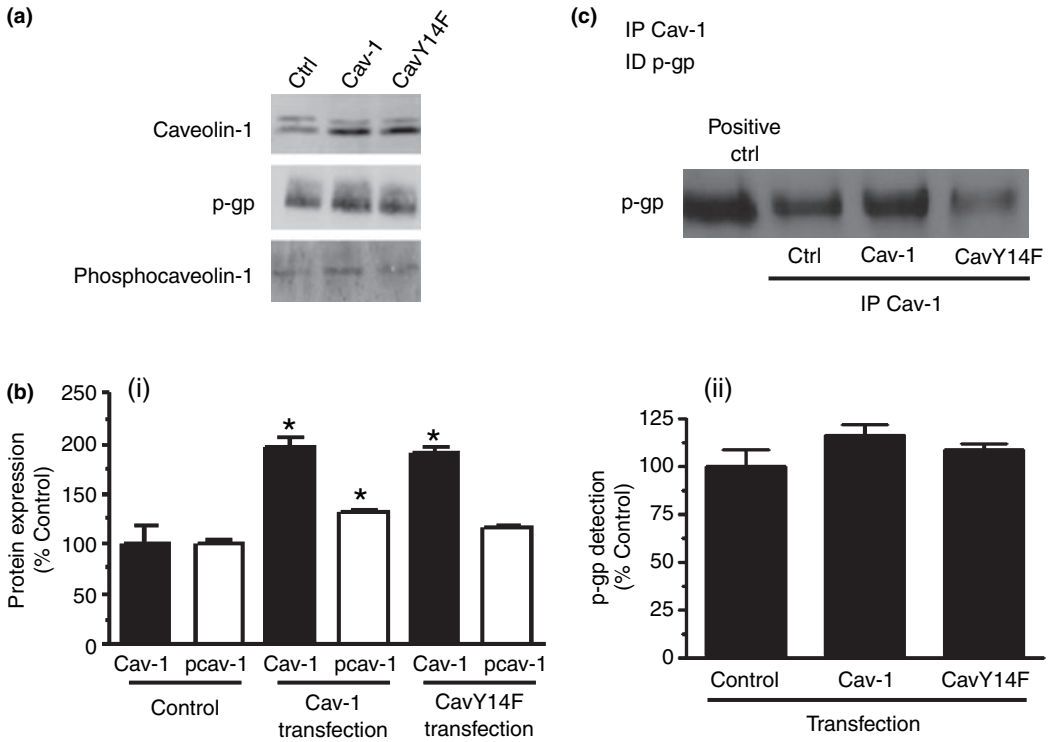

Fig. 5 Caveolin-1 (Cav-1) phosphorylation enhances p-glycoprotein (p-gp)/Cav-1 interaction. (a) Cav-1, phosphocaveolin-1 (pcav-1), and p-gp were detected in rat brain endothelial cell line cells transfected with Cav-1 or mutant cavY14F by immunoblot against 10 μg/mL protein from the cell types, as described in Materials and methods. Results are representative of three separate experiments. (b(i)) Cav-1 and pcav-1 expression were evaluated by densitometry analysis. (b(ii)) p-gp expression was evaluated by the same method. Asterisks indicate statistical significance of the three independent experiments ($p < 0.05$, Student's *t*-test). (c) p-gp immunoprecipitation was performed by using 200 μg protein treated with 1 μg C219 mAb; Cav-1 was identified by immunoblot, as described in Materials and methods. These results are representative of three separate experiments.

Discussion

Our previous work, demonstrating that p-gp interacts with Cav-1, used IP from the MDR-expressing cancer cell line CH^RC5 as well as MDR1 transfected COS-7 cells (Jodoin *et al.* 2003). The interaction between p-gp and Cav-1 was suggested for the first time by Lavie *et al.* 1998; Other studies showed that Cav-1 overexpression induces inhibition of p-gp transport activity by decreasing the plasma membrane cholesterol level (Cai and Chen 2004). In this study, we show that transfection of RBE4 with Cav-1 modulates p-gp transport activity in ECs from brain capillary. We have demonstrated for the first time by using siRNA that down-regulation of Cav-1 reduced the interaction between p-gp and Cav-1 and increased p-gp transport activity. As well, we demonstrated that Cav-1 overexpression inhibited p-gp activity. However, contradictory results have been published regarding p-gp/Cav-1 interaction. A previous study reported that different raft microdomains were isolated when Brij-96 and Triton X-100 non-ionic detergents were used with rat basophilic leukemia cells (Radeva and Sharom 2004). It was also suggested that p-gp was not localized in caveolae and that Cav-1/p-gp interaction was not detected (Belanger *et al.* 2004; Radeva *et al.* 2005). Using the same approach, Hinrichs and co-workers demonstrated that p-gp was localized in Lubrol-based detergent-insoluble glycosphingolipid-enriched membrane domains in 2780AD human ovarian carcinoma cells, which do not express Cav-1. In contrast, in $HT29^{col}$ cells expressing Cav-1, p-gp and Cav-1 were dissociated by using different detergents for DRM extraction (Hinrichs *et al.* 2004). A recent work suggested that during the process of caveolae purification using detergents, native caveolae could disappear and new DRM domains could be formed (Gaus *et al.* 2005). This indicates that p-gp/Cav-1 co-localization and interaction observed by IP could be lost during sonication-based methods of caveolae preparation using detergents.

Other studies have demonstrated by a biochemical approach that p-gp implicated in drugs efflux out of the ECs from the blood–brain barrier is localized at the luminal endothelial plasmalemma, where it is associated with the Cav-1 caveolar compartment (Beaulieu *et al.* 1997; Demeule *et al.* 2000; Virgintino *et al.* 2002).

We demonstrated in this study that the inhibition of p-gp transport activity is associated with Cav-1 phosphorylation. Activation of Src is responsible for the tyrosine-14 phos-

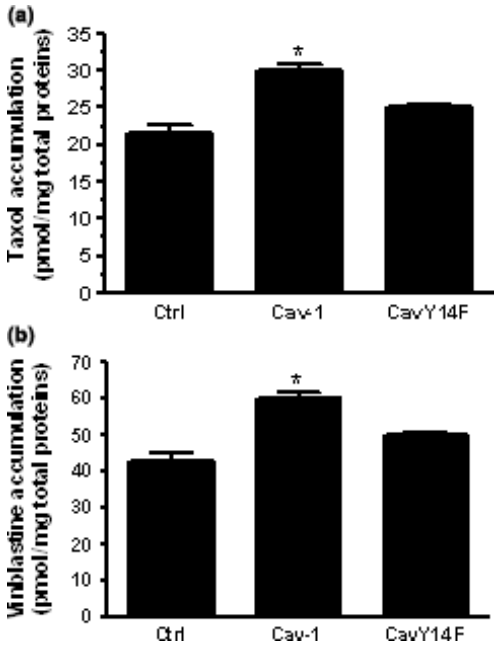

Fig. 6 Inhibition of caveolin-1 (Cav-1) tyrosine-14 phosphorylation reduces accumulation of the two p-glycoprotein substrates. Accumulation of 50 nmol/L [^{3}H]-Taxol (a) or [^{3}H]-Vinblastine (b) in rat brain endothelial cell line cells transfected (or not) with Cav-1 or with the mutant CavY14F was measured after 2 h as described in Materials and methods. These results correspond to three independent experiments. Asterisks denote statistical significance ($p < 0.05$, Student's *t*-test).

phorylation of Cav-1 (Labrecque *et al.* 2004). Here, we demonstrated that the transfection of RBE4 ECs with Src inhibits the transport of [^{3}H]-VBL or [^{3}H]-Taxol by p-gp. These are the first data suggesting that Cav-1 phosphorylation is involved in the modulation of p-gp transport activity. As described by other studies, Cav-1 interacts with many signaling proteins and inhibits their function, which may be why it has been identified as a candidate tumor suppressor (Li *et al.* 1996; Wiechen *et al.* 2001; Fiucci *et al.* 2002). However, the role of Cav-1 phosphorylation in regulating p-gp transport activity remains unknown.

To further examine Cav-1 phosphorylation, ECs were transfected with a plasmid encoding the WT Cav-1 gene or with a version mutated at tyrosine-14. p-gp transport activity was increased in cells transfected with the mutant Cav-1 compared with those transfected with the WT Cav-1. In addition, the interaction between p-gp and Cav-1 was decreased when tyrosine-14 was mutated. Thus, we demonstrated that Cav-1 regulates p-gp function and that p-gp interacts with the tyrosine-14 pcav-1. We also showed that this interaction seems to be regulated in part by Src, which is responsible for Cav-1 phosphorylation (Cao *et al.* 2002).

In summary, caveolae provides a favorable environment for the regulation of p-gp. It has already been demonstrated that cholesterol from caveolae is able to stimulate p-gp transport activity (Gayet *et al.* 2005). Here, we show that Cav-1 is also able to modulate p-gp function through phosphorylation of its tyrosine-14. This and previous studies suggest that a population of p-gp molecules can be localized in and out of caveolae depending on the Cav-1 phosphorylation status. Identification of new actors in the process of the regulation of p-gp transport activity in brain ECs could help to facilitate the penetration of anti-cancer agents into the brain.

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