Research Paper

Membrane Trafficking of the Human Organic Anion-Transporting Polypeptide C (hOATPC)

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Introduction. The human organic anion transporting polypeptide C (OATPC) is one of the major transport proteins involved in the enterohepatic circulation of bile salts and plays an important role in vectorial transport of organic anions and drugs across hepatocytes.

Materials and Methods. In this study, the effects of biological reagents on the membrane localization of OATPC were investigated by confocal microscopy and estrone-3-sulfate transport.

Results. Our results demonstrated that the functional membrane expression of fluorescent chimera OATPC-GFP was achieved in non-polarized (COS7 and HEK293) and polarized (MDCK) cells. Both brefeldin A (a Golgi complex disruptor) and bafilomycin A1 (an inhibitor of vacuolar H^+-ATPase) treatment significantly decreased the polarized membrane trafficking and markedly reduced the uptake of estrone-3-sulfate (~40–90%) in OATPC-GFP transfected cells, suggesting that membrane sorting of hOATPC-GFP was mediated by Golgi complex and vacuolar H^+-ATPase-related vesicle transport pathways. Treatment with 8-Br-cAMP (a cAMP analog) stimulated OATPC-GFP membrane localization and enhanced estrone-3-sulfate uptake by ~20%. The protein kinase A (PKA) inhibitors (H89 and KT5720), but not a PKG inhibitor, blocked the polarized membrane expression of OATPC-GFP and reduced estrone-3-sulfate transport activity. The simultaneous treatment of cells with PKA activator/inhibitor and bafilomycin A1 demonstrated that bafilomycin A1 did not change the effects of 8-Br-cAMP and H89 on the membrane localization of OATPC-GFP compared with the use of 8-Br-cAMP and H89 alone.

Discussion. These data suggest that a cAMP-PKA sensitive membrane sorting pathway for OATPC-GFP is independent of the vacuolar H^+-ATPase associated (bafilomycin A1 sensitive) vesicle mediated membrane sorting pathway. In contrast, with combined treatment with brefeldin A, neither the PKA-activator (8-Br-cAMP) nor the inhibitor (H89) further altered the plasma membrane expression and transport activity of OATPC-GFP compared with brefeldin A treatment alone. These data suggest that the cAMP-PKA regulation of OATPC membrane expression involves the Golgi complex. When the Golgi apparatus was disrupted by brefeldin A treatment, the effects of cAMP-PKA on the Golgi-to-basolateral surface sorting process of OATPC was also diminished. In summary, the plasma membrane localization of human OATPC is mediated by Golgi complex and vacuolar H^+-ATPase vesicle mediated membrane sorting pathways. cAMP-PKA regulates sorting process through the Golgi complex but not the vacuolar H^+-ATPase associated vesicular pathway.

KEY WORDS: human OATPC; membrane trafficking; organic anion transporter; protein kinase A.

INTRODUCTION

Asymmetrical distribution of transport proteins in the apical and basolateral membranes of polarized cells (such as intestine, kidney, and liver) is of critical importance to the

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ABBREVIATIONS: BA1, bafilomycin A1; BFA, brefeldin A; E3S, Estrone-3-sulfate; GFP, green fluorescent protein; H89, *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide; OATP-GFP, GFP-fused human OATPC; OATP, human organic anion transporting polypeptide-C; PKGi, protein kinase G inhibitor; 8-Br-cAMP, 8-bromoadenosine-3′,5′-cyclic monophosphate.

absorption and elimination of organic anions and cations (1). Previous studies have shown that polarized membrane expression of hepatic transporters is highly regulated by signaling systems including protein kinases and by vesicle-mediated retrieval or insertion of transport proteins into the canalicular (apical) and sinusoid (basolateral) domains, thus regulating their surface density (2). The mechanisms involved in the differential membrane trafficking of proteins into these structurally different domains, as well as their control mechanisms, are not completely understood.

Many organic anions and bulky organic cations are transported by the organic anion transporting polypeptide (OATP) family, which consists of ~20 members of OATPs in human and other species with different tissue distribution (1). Some members of OATP family (such as, Oatp1a1) contain PDZ consensus binding sites at their carboxyl-terminus, which have been identified as critical membrane sorting determinants for proper subcellular localization and function (1,3).

The human organic anion transporting polypeptide-C (hOATPC) (gene *SLC21A6*, also referred to as OATP2 (SLCO1B1) and LST1) is a liver-specific transporter involved in the hepatocellular uptake of a variety of organic anions, neutral steroids and bulky organic cations across the basolateral domain. The human OATPC is a glycoprotein of 80 kDa, composed of 691 amino acids, and possessing 12 transmembrane domains. Amino acid sequence analysis shows that OATPC contains several potential protein kinase phosphorylation sites including a potential PKA/PKG site. In contrast to other members of OATP family, hOATPC does not have a PDZ domain. Tirona *et al.* (4) and Michalski *et al.* (5) have reported the presence of multiple potentially important single-nucleotide polymorphisms (SNPs) of *OATP-C* in a population of African- and European-Americans. The genotypic frequencies of SNPs were dependent on race. Cell surface biotinylation experiments indicated that the altered transport activity of some of the OATPC variants was due, in part, to decreased plasma membrane expression (4,5). The single nucleotide polymorphisms in OATPC may affect membrane surface expression, and lead to an altered pharmacokinetic profile of drug delivery with implications for therapeutic efficacy or potential toxicity. So far, little is known about the mechanisms and regulation of the membrane sorting of hOATPC.

To understand the mechanisms underlying human OATPC membrane localization, the potential effects of biological reagents and protein kinases on OATPC plasma membrane expression and transport activity were investigated in this study by confocal microscopy and analysis of transport activity.

MATERIALS AND METHODS

Materials. [³H]Estrone-3-sulfate ([³H]E3S), (2.1–3.47 Ci/mmol) was purchased from DuPont NEN (Boston, MA). Unlabeled estrone-3-sulfate was purchased from Sigma Chemical Company (St. Louis, MO). KT5720, brefeldin A (BFA), and protein kinase G inhibitor (PKGi) were obtained from Calbiochem (La Jolla, CA). *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide (H-89), bafilomycin A1 (BA1), and 8-bromoadenosine-3′,5′-cyclic monophosphate (8-Br-cAMP) were acquired from Sigma Chemical Company (St. Louis, MO). Cell-culture supplies were obtained from Life Technologies, (Rockville, MD). Subcloning reagents, restriction enzymes and competent cells were obtained from Stratagene (La Jolla, CA), GIBCO BRL (Gaithersburg, MD), New England BioLabs (Beverly, MA), and Invitrogen (Carlsbad, CA).

Generation of GFP-fused human organic anion-transporting polypeptides C (hOATPC) cDNA. The full-length wild type human OATPC cDNA was inserted in-frame into the *Xho*I and *Xma*I sites of a enhanced green fluorescent protein (GFP) vector, pEGFPN2 (Clonetech, Palo Alto, CA) to produce the GFP-fused plasmid constructs as described previously (6).

Establishment of transiently and stably transfected cell lines. COS-7 (SV40 transformed monkey kidney fibroblast), HEK293 (human embryonic kidney cell), and MDCK II (Madin–Darby canine kidney) cells were used in this study. Cell culture and DNA plasmid transient transfection of COS-7 and HEK293 cells were done as described previously (7).

For stably transfected MDCK cells, human OATPC-GFP cDNA were stably expressed in MDCK cells as follows. On day 1, 60-mm plates were seeded with 5×10^4 MDCK cells. On day 2, the cells were transfected with 3 μg of hOATPC-GFP cDNA using the FuGENE 6 transfection reagent (Roche Applied Science). On day 3, the cells were split to two 100 mm dishes in culture medium containing 0.9 mg/ml of G418 (Invitrogen). After selection for about 15 days, the individual colonies were picked, expanded in 35-mm plates, and screened by Na⁺-independent [³H]estrone-3-Sulfate uptake, confocal image and immunoblotting for functional expression of hOATPC-GFP protein.

Biological reagent treatments. The effects of biological agents on cell surface expression of hOATPC-GFP were evaluated by estrone-3-sulfate transport assays and confocal fluorescence microscopy analysis. Brefeldin A (BFA) is a fungal metabolite, which inhibits protein secretion by specifically inhibiting the Golgi-associated guanine nucleotide exchange activity of the small GTP-binding protein (8). In our experiment, brefeldin A was added to a final concentration of 2 μM, as described previously for MDCK II cells (9). For bafilomycin A1 (BA1, a vacuolar H⁺-ATPases interrupting reagent) treatment, the transfected cells were incubated with 50 nM bafilomycin A1 in culture medium for 16 h at 37°C (10). Protein kinase A (PKA) specific inhibitors (KT5720 and H89) were evaluated at a final concentration of 10 μM (for KT5720) and 20 μM (for H89), as described previously (11,12). 8-Br-cAMP [a adenosine 3′,5′-cyclic monophosphate (cAMP) analog] was examined at a final concentration of 200 μM as described previously (11). The protein kinase G inhibitor (PKGi) was evaluated at a final concentration of 86 μM for 1 h at 37°C, as described previously (13). To examine the combined effects of biological reagents on the membrane localization of hOATPC, the transfected cells were treated in a manner based on that of Puri and Linstedt (14). Briefly, the cells were pretreated with bafilomycin A1 (50 nM) or brefeldin A (2 μM) for 16 h at 37°C before adding H89 or 8-Br-cAMP to the incubation medium. After addition of H89 (20 μM) or 8-Br-AMP (200 μM), cells were cultured at 37°C for additional 2 h.

Confocal fluorescence microscopy was carried out as described previously (6). Briefly, confocal microscopy was performed on a confluent monolayer of transfected cells cultured on glass coverslips. The cells were fixed and permeabilized for 7 min in 100% of methanol at −20°C, followed by rehydration in PBS. After being washed with PBS, the cells on coverslips were inverted onto a drop of Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Fluorescence was examined with a Leica TCS-SP (UV) 4-channel confocal laser-scanning microscope in the Imaging Core Facility Microscopy Center, Mount Sinai School of Medicine.

Estrone-3-sulfate influx assay. Na⁺-independent [⁺H]estrone-3-sulfate (E3S) influx assay was performed in 12 well plates or using a transwell filter culture system as described previously with some modification (15,16). Briefly, the confluent cell monolayer grown on 12-well plate was washed twice with warm PBS buffer with 2% BSA, and each well was incubated with uptake buffer (PBS with 1% BSA containing 0.67 μM [3H]estrone-3-sulfate at the final concentrations) for 5 min at 37°C. Following 5 min

incubation, the uptake of [+H]estrone-3-sulfate was terminated by aspirating the medium, and the cells were washed two times with ice-cold PBS buffer with 2% BSA and twice with ice-cold PBS buffer. The cells were lysed and harvested with 0.5 ml 0.5% Triton X-100 per well of 12 well plates. The cell-accumulated radioactivity was measured by scintillation counting. The level of protein expression of transporters in transfected cells was normalized by total protein concentrations. The protein concentration was determined with the BioRad protein assay kit. For the transwell filter system (Costar, Cambridge, MA), the stably transfected MDCK cells were plated on 24-well plates with 6.4-mm transwell filter inserts (Costar), and cultured in the culture medium containing 0.9 mg/ml of G418 (Invitrogen). After ~4–5 days, the cells were treated with 10 mM sodium butyrate for 16 h at 37°C to induce expression of transfected genes. [^{3}H]Estrone-3-sulfate uptake was performed at 37°C for 5 min. After a 5-min incubation, the uptake assay was terminated by aspirating the medium, and the filters were successively dipped into three beakers, each of which contained 100 ml of ice-cold PBS buffer with 2% BSA. The filters were excised from cups, and attached cells were solubilized in 0.2 ml of 1% SDS and transferred into scintillation vials with 4 ml Optifluor (DuPont NEN) for radioactivity assay. The protein concentration was determined with the BioRad protein assay kit. Preliminary experiments by our group and other laboratories indicate that 10 mM sodium butyrate does not significantly effects membrane localization of transporter proteins (data not shown) (17).

Statistics analysis. The results were expressed as mean value ± SEM and analyzed by using unpaired Student's *t* test for a difference in means between two groups that may have un-equal sizes. The *p* value <0.05 was considered statistically significant.

RESULTS

Cellular Localization of hOATPC-GFP and Transport of [^{3}H]estrone-3-sulfate (E3S) in Transfected Mammalian Cells

Mammalian cell lines (such as COS 7, HEK293, and MDCK cells) have been widely used to investigate cellular trafficking and transport function of organic anion transporters (5,7,18–20). In this study, a GFP-fused hOATPC construct was generated and its cellular distribution and transport activity were characterized in transiently and stably transfected mammalian cells. Fig. 1a demonstrated that the GFP-fused hOATPC (hOATPC-GFP) was located on the plasma membrane of transfected COS 7 and HEK293 cells (Fig. 1a). The [+H]estrone-3-sulfate uptake activity was increased more than sixfold in hOATPC-GFP transfected COS7 and HEK293 cells compared with non-transfected cells (Fig. 1b). In the polarized MDCK cells, the hOATPC-GFP proteins were expressed on the basolateral membrane domain of transfected MDCK cells (Fig. 2a). Estrone-3-sulfate uptake activity was increased about sixfold in hOATPC-GFP transfected MDCK cells compared with the non-transfected cells (Fig. 2b). These data indicate that GFP-fused hOATPC (hOATPC-GFP) can be functionally expressed on the cell membrane in non-polarized and polarized cell lines.
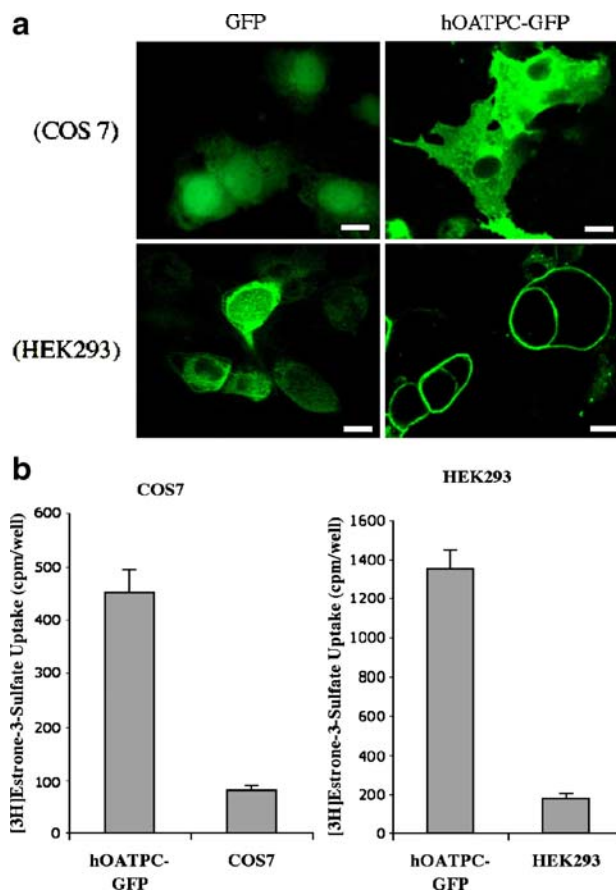

Fig. 1. Confocal microscopy and [+H]estrone-3-sulfate (E3S) uptake studies of human OATPC-GFP expressing in transfected COS-7 and HEK293 cells. The membrane expression and Na$^+$-independent (^{3}H)E3S uptake was performed on a confluent monolayer of COS7 and HEK293 cells transiently expressing GFP-fused human OATP-C (hOATPC-GFP) cDNA cultured on a glass coverslip or 12 well plates, respectively. **a** Confocal photomicrographs were obtained on a confluent monolayer of transfected COS 7 or HEK 293 cells cultured on glass coverslips. The glass coverslip-grown cells were fixed in 100% methanol and mounted with Vectashield mounting medium. The fluorescence was examined with a Leica TCS-SP (UV) 4-channel confocal laser scanning microscope (bar = 5 μm). **b** The Na$^+$-independent [+H]estrone-3-sulfate (E3S) influx was performed on transiently transfected COS-7 or HEK 293 cells grown on 12 well plates. GFP-fused OATP-C transfected cells were incubated in 0.67 μM (^{3}H)E3S at 37°C for 5 min. The data are presented as radioactivity (cpm) of total (^{3}H)E3S uptake per well of 12 well plates. All experiments were performed at least three times with triplicate samples and depicted as means ± SE.

Effects of Biological Reagents on Cellular Distribution and Transport Activity of hOATPC-GFP

To test the possibility that Golgi complex mediated and vacuolar H$^+$-ATPase (a vacuolar proton pump) related vesicular pathways may be involved in the polarized membrane localization of hOATPC, the effects of brefeldin A (BFA, a Golgi disrupting reagent which blocks movement of membrane proteins from an intracellular pool to the cell surface) and bafilomycin A1 (BA1, a specific inhibitor of vacuolar H$^+$-ATPase, which regulates the transit of vesicles in the secretory pathway) were evaluated. In Fig. 3a, the
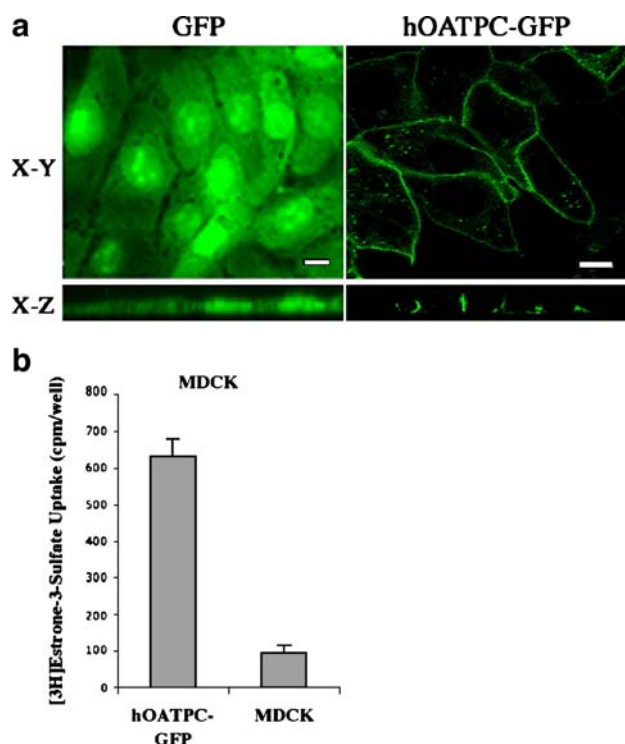
Fig. 2. Confocal microscopy and [³H]estrone-3-sulfate (E3S) uptake studies of human OATPC-GFP expressed in stably transfected MDCK cells. The polarity of membrane expression and Na⁺-independent (³H)E3S uptake were performed on a confluent monolayer of MDCK cells stably expressing GFP-fused human OATP-C (hOATPC-GFP) cultured on a glass coverslip or transwell culture system, respectively. The cells were treated with 10 mM Na⁺-butyrate overnight, at 37°C. **a** Confocal fluorescence photomicrographs of GFP-fused human OATP-C were obtained on a confluent monolayer of stably transfected MDCK cells cultured on glass coverslips. The glass coverslip-grown cells were fixed in 100% methanol and mounted with Vectashield mounting medium. The Confocal enface (X–Y) and X–Z cross-sectional photomicrographs were of the MDCK cells stably transfected with hOATPC-GFP. Fluorescence was examined with a Leica TCS-SP (UV) 4-channel confocal laser scanning microscope (bar = 5 μm). **b** [³H]Estrone-3-sulfate (E3S) influx assay of GFP-fused human OATP-C in stably transfected MDCK cells. MDCK cells transfected with hOATPC-GFP plasmid DNA were grown on 6-mm permeable transwell filter inserts for 5 days to ensure a polarized phenotype. The Na⁺-independent E3S influx was measured by incubating cells in 0.67 μM (³H)E3S at 37°C for 5 min. The data are presented as radioactivity (cpm) of total (³H)E3S uptake per transwell filter insert. All experiments were performed at least three times with triplicate samples and depicted as means ± SE.

confocal images showed a marked decrease of plasma membrane expression and significant increase of intracellular accumulation of hOATPC-GFP expressed in non-polarized (COS7 and HEK293) and polarized (MDCK) cells treated with 50 nM brefeldin A for 16 h at 37°C. This interruption of membrane sorting of hOATPC-GFP was associated with a significant reduction of estrone-3-sulfate uptake activity by 50% to 90% in the brefeldin A treated cells compared with untreated cells (Fig. 3b). The COS7 cells were more sensitive to the brefeldin A treatment. The estrone-3-sulfate uptake activity was decreased more than 90% compared with other two cell lines (a 75% decrease in MDCK cells and 50% in HEK293 cells). Similar to

experiments with brefeldin A, the confocal images demonstrated a significant increase in intracellular accumulation of hOATPC-GFP in non-polarized (COS7 and HEK293) and polarized (MDCK) cells after culture with 2 μM bafilomycin A1 for 16 h at 37°C (Fig. 4a). The estrone-3-sulfate uptake activity was decreased 50% to 60% in all of the three cell lines after treatment of 2 μM bafilomycin A1 compared with untreated cells (Fig. 4b). These results suggest that membrane sorting of hOATPC-GFP is mediated by Golgi complex and bafilomycin A1 sensitive vesicular pathways.

Effects of Protein Kinase on Cellular Localization and Transport Activity of hOATPC-GFP

Protein kinase (PK) phosphorylation may also be a factor regulating membrane localization of hOATPC. The protein sequence of human OATPC suggests that there is a potential protein kinase A (PKA) and/or protein kinase G (PKG) phosphorylation motif in the intracellular loop of the transport protein. To determine whether phosphorylation influences membrane trafficking of human OATPC, the effect of the PKA inhibitor (KT5720) and PKG inhibitor (PKGi) on membrane sorting of hOATPC-GFP was examined. Confocal imaging (Fig. 5a) demonstrated a significant decrease in plasma membrane expression and increased intracellular accumulation of hOATPC-GFP expressed in non-polarized (COS7 and HEK293) and polarized (MDCK) cells after treated with 10 μM KT5720 for 1 h at 37°C. This interruption of membrane sorting of hOATPC-GFP was associated with a significant reduction of estrone-3-sulfate uptake activity by ~50% to 70% in the KT5720 treated cells compared with untreated cells (Fig. 5b). In contrast, hOATPC-GFP localization by confocal microscopy and estrone-3-Sulfate uptake activity were not significantly changed in non-polarized (COS7 and HEK293) and polarized (MDCK) cells after treatment with 86 μM protein kinase G inhibitor for 1 h at 37°C (Fig. 6).

The cellular localization and transport activity of hOATPC-GFP were then assessed in cells treated with either 8-bromo-cAMP (8-br-cAMP, a cAMP analog which is able to activate PKA) and H89 (an inhibitor of cAMP-dependent protein kinase (PKA)). Confocal image analysis demonstrated a significantly increased intracellular accumulation of hOATPC-GFP in H89 treated cells (Fig. 7a). In contrast, the plasma membrane expression of hOATPC-GFP was enhanced after the stably transfected MDCK cells were cultured with 200 μM 8-Br-cAMP for 2 h at 37°C (Fig. 7a). Consistent with the results of confocal microscopy, estrone-3-sulfate transport activity was inhibited ~30% and enhanced ~20% after treatment with 20 μM H89 and 200 μM 8-br-cAMP for 2 hour at 37°C, respectively (Fig. 7b). These results suggest that PKA, but not PKG, regulated the membrane targeting of hOATPC-GFP.

Relationship of PKA Sensitive Pathway and Golgi/Vesicular Mediated Process

Previous studies have indicated that the effects of PKA on membrane protein trafficking may involve the Golgi complex and vesicle mediated pathways (2). To understand the possible interaction between these pathways, MDCK cells
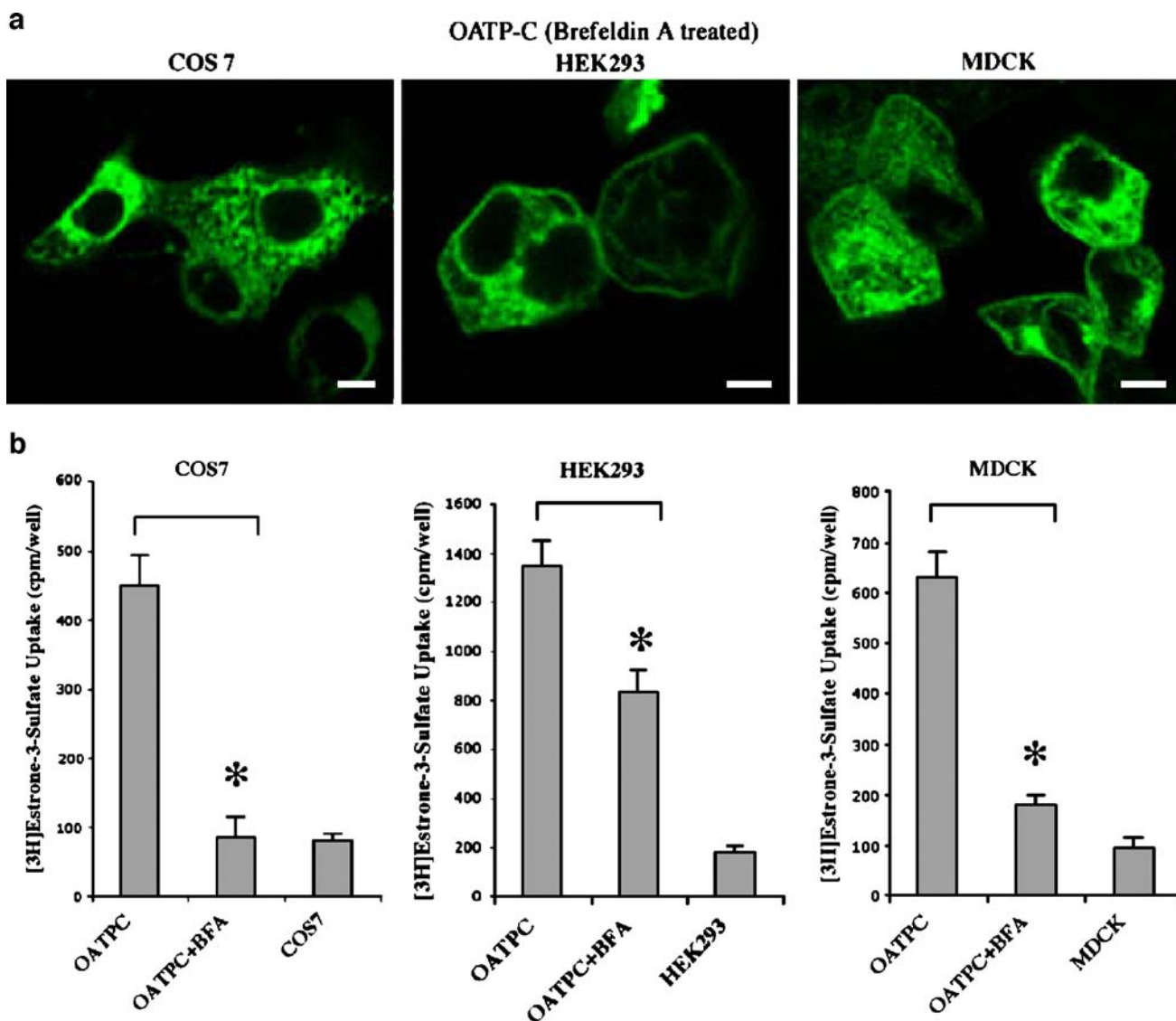
Fig. 3. The effects of brefeldin A on the cellular distribution and E3S uptake of GFP-fused human OATP-C in transfected cells. The cells transfected with hOATPC-GFP were pretreated with 2 μM brefeldin A (BFA, disrupts Golgi formation) for 16 h at 37°C. **a** Confocal fluorescence microscopy of transfected COS 7, HEK293, and MDCK cells expressing GFP-fused human OATP-C. Confocal photomicrographs of GFP-fused human OATP-C were obtained on a confluent monolayer of transfected cells cultured on glass coverslips. After 16 h culture with 2 μM brefeldin A, the glass coverslip-grown cells were fixed (bar = 5 μm). **b** [$^+$H]Estrone-3-sulfate (E3S) influx assay of GFP-fused human OATP-C in transfected cells. The Na$^+$-independent E3S influx was performed on transfected cells. After 16 h culture with 2 μM brefeldin A, the cells transfected with GFP-fused OATP-C were incubated in 0.67 μM (^{3}H)E3S at 37°C for 5 min. All experiments were performed at least twice with triplicate samples and depicted as means ± SE. *Asterisks* for each cell transfected with hOATPC-GFP indicate significant differences ($p < 0.05$) from untreated cells.

stably transfected with hOATPC-GFP were pretreated with 2 μM bafilomycin A1 or 50 nM brefeldin A for 16 h at 37°C before adding H89 or 8-Br-cAMP to the incubation medium. After addition of 20 μM H89 or 200 μM 8-Br-cAMP, cells were cultured at 37°C for additional 2 h. The cells treated with bafilomycin A1 alone showed a significantly increased intracellular accumulation of hOATPC-GFP (Fig. 8a, left panel). In cells exposed to the combined-treatment of brefeldin A and H89, the fluorescence intensities of hOATPC-GFP on the plasma membrane were further reduced (Fig. 8a, central panel) compared with the cells treated with bafilomycin A1 alone (Fig. 8a, left panel). In contrast, in cells exposed to combined treatment with bafilomycin A1 and 8-Br-cAMP, the fluorescence intensity

of hOATPC-GFP on the plasma membrane was enhanced (Fig. 8a, right panel) compared with cells treated with bafilomycin A1 alone (Fig. 8a, left panel). The results from estrone-3-sulfate uptake studies were consistent with confocal fluorescence microscopy. Fig. 8b demonstrated that the initial transport activity of the stably transfected MDCK cells treated with bafilomycin A1/H89 or bafilomycin A1/8-Br-cAMP was decreased more than 40% or increased ~25%, respectively compared with the cells treated with bafilomycin A1 alone. These data suggest that the effect of PKA on the membrane sorting of hOATPC-GFP is independent of the bafilomycin A1 sensitive vesicle mediated membrane-sorting pathway. In contrast, the membrane distribution and initial estrone-3-sulfate transport activity were not altered in the
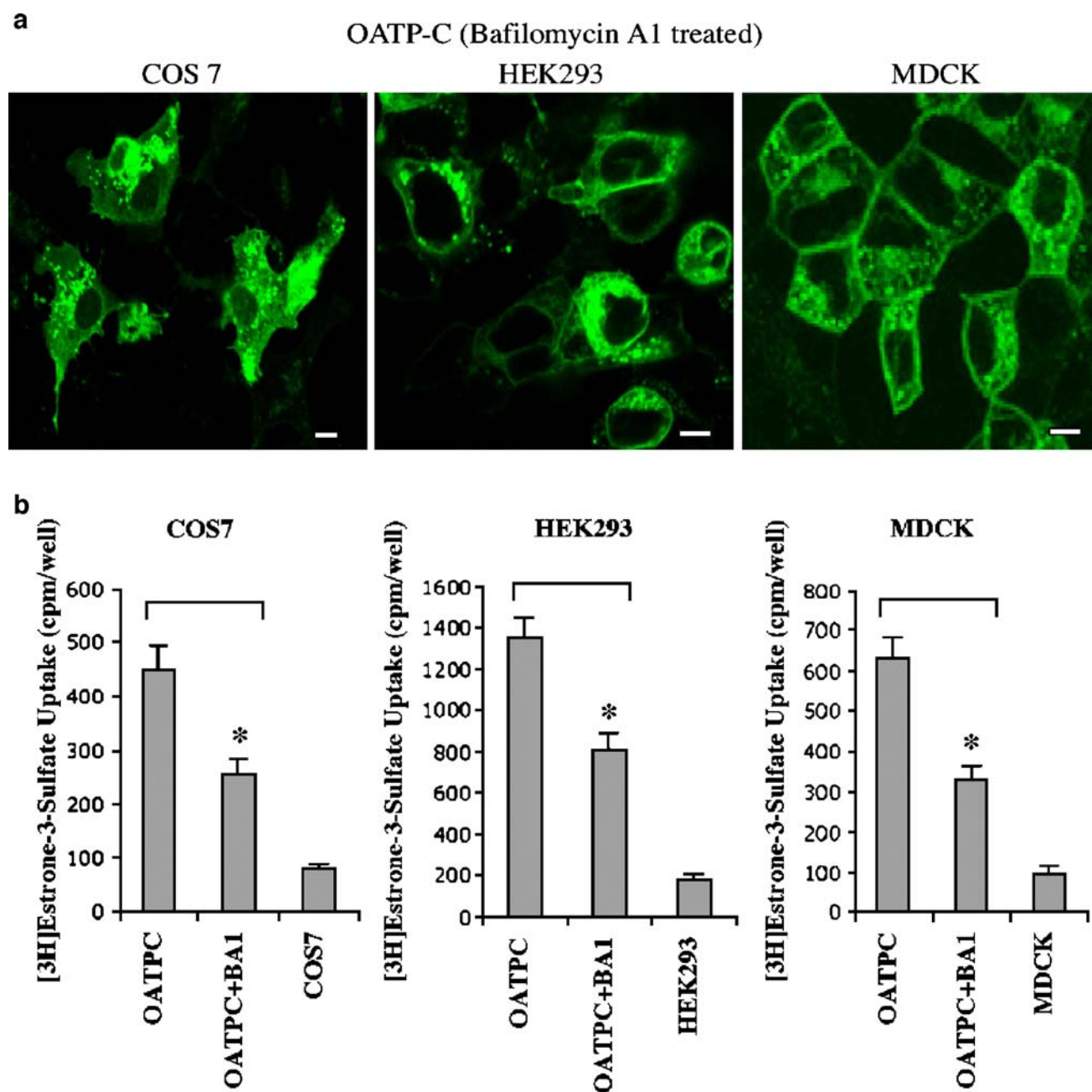
a

OATP-C (Bafilomycin A1 treated)

b

Fig. 4. The effects of bafilomycin A1 treatment on polarized membrane distribution and initial transport activity of GFP-fused human OATP-C in transfected cells. The transfected cells were pretreated with 50 nM bafilomycin A1 (an inhibitor of vacuolar H^+-ATPase) for 16 h at 37°C. **a** Confocal fluorescence microscopy of transfected COS 7, HEK293, and MDCK cells expressing GFP-fused human OATP-C. The cells were grown on glass coverslips and fixed with cold 100% MtOH after BA1 treatment. Confocal photomicrographs of GFP-fused human OATP-C were obtained on a confluent monolayer of transfected cells cultured on glass coverslips. The glass coverslip-grown cells were fixed in 100% methanol and mounted with Vectashield mounting medium (bar = 5 μm). **b** [$^+$H]Estrone-3-sulfate (E3S) influx assay of GFP-fused human OATP-C in transfected cells. The Na^+-independent E3S influx was performed on transfected cells after BA1 treatment. GFP-fused OATP-C transfected cells were incubated in 0.67 μM (^{3}H)E3S at 37°C for 5 min. *Asterisks* for each cell transfected with hOATPC-GFP indicate significant differences ($p < 0.05$) from untreated cells. All experiments were performed at least twice with triplicate samples and depicted as means ± SE.

cells treated with brefeldin A/H89 or brefeldin A/8-Br-cAMP compared to the effects of brefeldin A treatment alone (Fig. 9). These results suggest that the effect of cAMP-PKA on the membrane sorting of hOATPC-GFP is related to the Golgi complex membrane sorting pathway. After disruption of the Golgi complex by brefeldin A, the effects of PKA inhibitor and activator are completely abolished.

DISCUSSION

Plasma membrane transport proteins play a critical role in governing drug absorption, distribution, and elimination in the body. The human organic anion transporting polypeptides C (OATPC) is one of the major transport proteins involved in the enterohepatic circulation of bile salts and
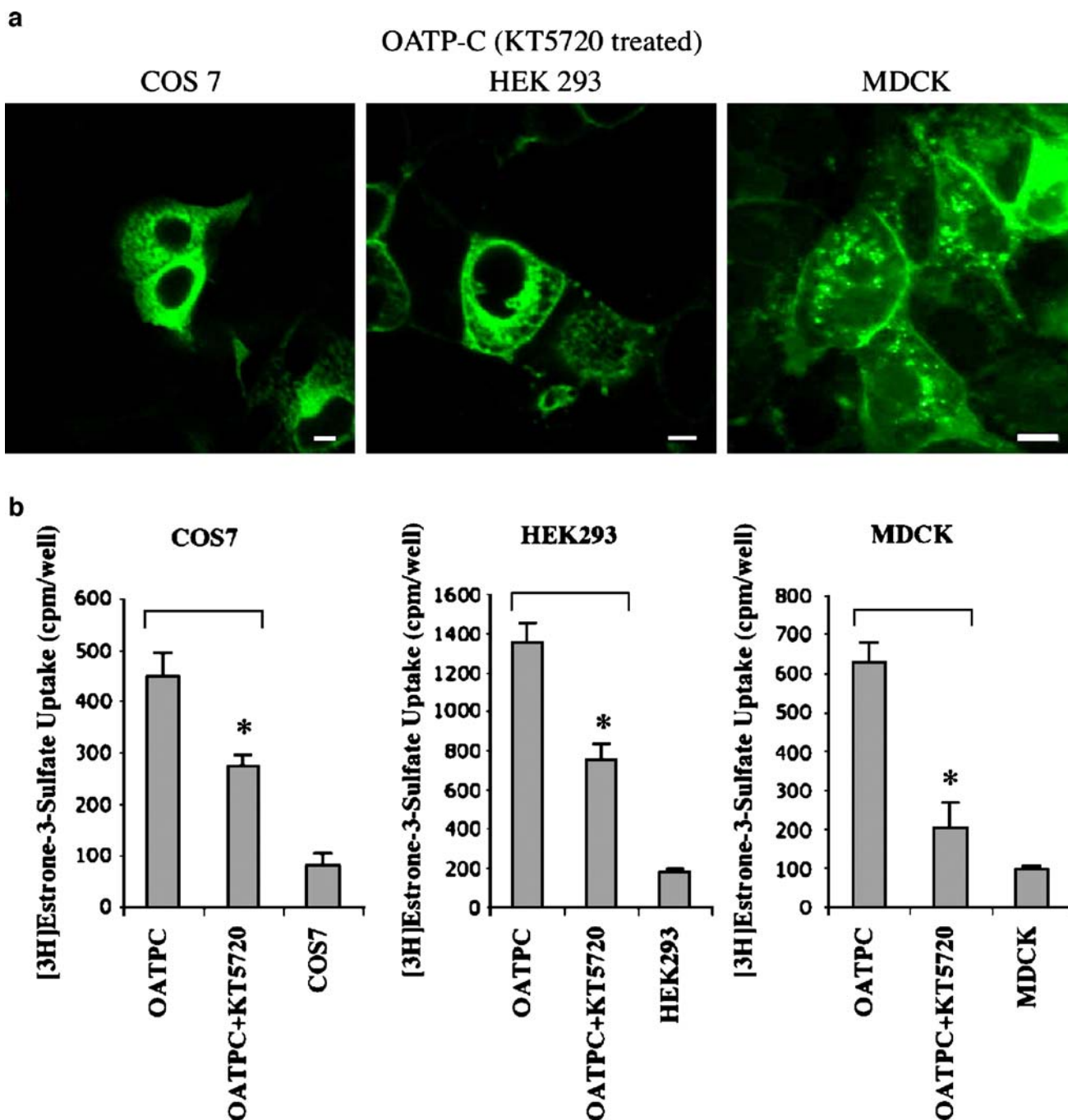
Fig. 5. Effects of KT5720 on the cellular distribution and the initial E3S transport activity of hOATPC proteins in transfected COS-7, HEK293, and MDCK cells. The transfected cells were pretreated with 10 μM KT5720 (a protein kinase A specific inhibitor) for 1 h at 37°C. **a** Confocal photomicrographs of GFP-fused human OATP-C were obtained on a confluent monolayer of transfected cells cultured on glass coverslips. The glass coverslip-grown cells were fixed in 100% methanol and mounted with Vectashield mounting medium after pretreated with 10 μM KT5720 for 1 h at 37°C (bar = 5 μm). **b** The Na^+-independent E3S influx was performed on a confluent monolayer of transfected cells after pretreated with 10 μM KT5720 for 1 h at 37°C. GFP-fused OATP-C transfected cells were incubated in 0.67 μM (^{3}H)E3S at 37°C for 5 min. *Asterisks* for each cell transfected with hOATPC-GFP indicate significant differences ($p < 0.05$) from untreated cells. All experiments were performed at least twice with triplicate samples and depicted as means ± SE.

plays an important role in vectorial transport of organic anions and drugs across hepatocytes [1,21,22].

Polarized membrane expression of transport proteins involves multiple protein sorting pathways. Drugs that block vesicle movement, such as brefeldin A, bafilomycin A1, and protein kinase inhibitors, have been widely used to map the trafficking pattern of transporters [18,20,23,24]. For example, the basolateral localization of human liver Na^+-taurocholate cotransporting polypeptide is mediated by a brefeldin A-sensitive vesicular-sorting pathway [20]. In addition, Wojtal et al. suggest that rerouting of Golgi-derived glycosphingolipids may underlie the delayed Golgi-to-apical surface transport of
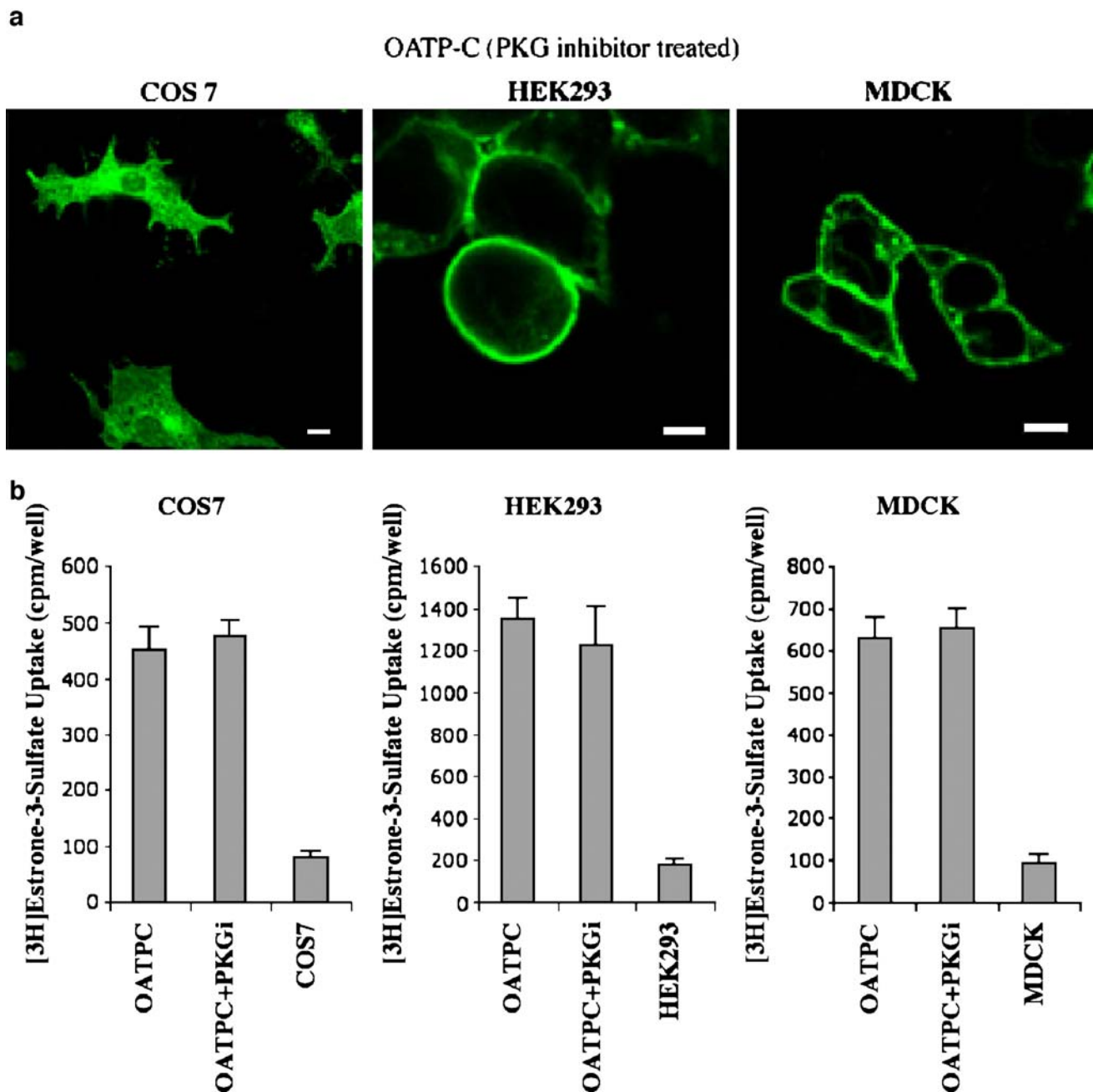
Fig. 6. Effects of protein kinase G inhibitor (PKGi) on the cellular distribution and the initial E3S transport activity of hOATPC proteins in transfected COS-7, HEK293, and MDCK cells. The cells transfected with hOATPC-GFP were pretreated with 86 μM PKGi for 1 h at 37°C. **a** Confocal photomicrographs of GFP-fused human OATPC were obtained on a confluent monolayer of transfected cells cultured on glass coverslips. Before fixed in 100% methanol, the glass coverslip-grown cells were pretreated with 86 μM PKGi for 1 h at 37°C (bar = 5 μm). **b** [$^{+}$H]Estrone-3-sulfate (E3S) influx assay of GFP-fused human OATP-C in transfected cells. The Na^{+}-independent E3S influx was performed on transfected cells. After pretreated with 86 μM PKGi for 1 h at 37°C, the cells transfected with GFP-fused OATP-C were incubated in 0.67 μM (^{3}H)E3S at 37°C for 5 min. The data are presented as radioactivity (cpm) of total (^{3}H)E3S uptake per well. All experiments were performed at least twice with triplicate samples and depicted as means ± SE.

MDR1 by the displacement of PKA-RIIalpha from the Golgi apparatus (23). The cholesterol accepting activity of exogenous apolipoprotein E was blocked by inhibitors of protein transport through the Golgi (24).

The vacuolar H^{+}-ATPases are a family of ATP-dependent proton pumps that reside predominantly within intracellular membrane compartments including cathrin-coated vesicles and the Golgi complex (25,26). The acidic luminal pH within these organelles and vesicles of the secretory pathway is established by vacuolar H^{+}-ATPases and is vital for processes such as intracellular membrane transport, receptor-mediated endocytosis, and intracellular targeting of lysosomal enzymes (25–27). Our previous studies demonstrated that the apical membrane localization of ileal
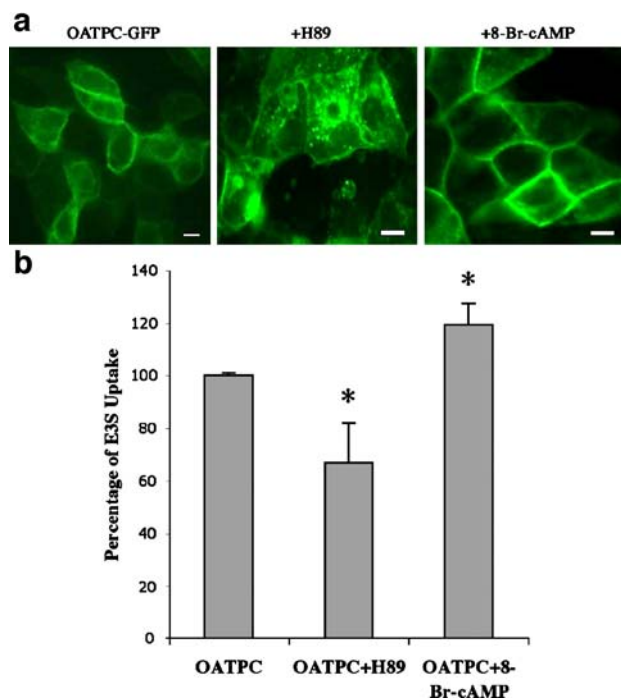
Fig. 7. Effects of PKA inhibitor (H89) and activator (8-Br-cAMP) on cellular distribution and E3S uptake of MDCK cells stably transfected with hOATPC-GFP. The MDCK cells stably transfected with hOATPC-GFP were pretreated with 20 μM H89 or 200 μM 8-Br-cAMP for 2 h at 37°C, respectively. **a** Confocal fluorescence microscopy of transfected cells expressing GFP-fused hOATPC. The confocal microscopy was performed on a confluent monolayer of MDCK cells stably expressing hOATPC-GFP grown on glass coverslips. The cells were incubated with 10 μM H89 or 200 μM 8-Br-cAMP for 2 h before fixed cells (bar = 5 μm). **b** [+H]Estrone-3-sulfate (E3S) influx assay of MDCK cells stably expression of hOATPC-GFP. The cells stably transfected with hOATPC-GFP were incubated with 10 μM H89 or 200 μM 8-Br-cAMP for 2 h before E3S uptake assay. The cells treated with H89 or 8-Br-cAMP were incubated in 0.67 μM (^{3}H)E3S at 37°C for 5 min. In this study, the E3S influx in untreated cells was set as 100%, and all values were graphed relative to this level. The data are presented as radioactivity (cpm) of total (^{3}H)E3S uptake per well. All experiments were performed at least twice with triplicate samples and depicted as means ± SE. *Asterisks* for each H89 or 8-Br-cAMP treated cells indicate significant differences ($p < 0.05$) from the cells chemically untreated.

apical sodium-dependent bile acid transporter is mediated by vacuolar H^+-ATPase associated apical sorting machinery (10).

Protein kinase mediated processes are also important in the regulation of activity and trafficking of membrane transporter proteins (28–30). cAMP has been generally recognized as a second messenger that operates via the activation of PKA, which catalyzes the phosphorylation of some key enzymes or cellular proteins involved in the control of many biological processes. The effects of PKA on different vesicular mediated trafficking pathways have been studied using cAMP analogs, such as 8-bromo cAMP (8-br-cAMP) (2). PKA activation can enhance membrane trafficking of transporter proteins in some epithelial cell types independent of their effects on transport activity (31,32). For example, the acute regulation of epithelial sodium channel function at the

apical surface of polarized kidney cortical collecting duct epithelial cells occurs in large part by changes in channel number, mediated by PKA responsive membrane vesicle trafficking (33,34). However, there are several studies showing that agents that increase cellular cAMP levels may also be able to activate PKG (35,36). Other studies have reported that 8-Br-cAMP is a much better activator of purified PKG than is db-cAMP or mb-cAMP (37,38). Golin-Bisello *et al.* (39) reported that both cAMP and cGMP-dependent phosphorylation regulates CFTR trafficking to the surface of enterocytes in rat jejunum.

The Golgi apparatus functions as an acceptor compartment in the secretory pathway. Previous studies have shown
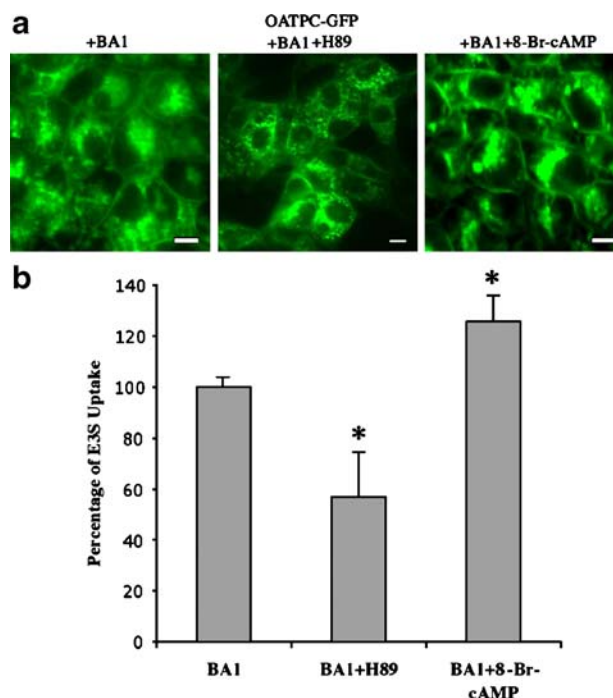

Fig. 8. Effects of PKA inhibitor (H89) and activator (8-Br-cAMP) on bafilomycin A1 (BA1) sensitive membrane localization and E3S uptake of hOATPC-GFP in stably transfected MDCK cells. The MDCK cells stably transfected with hOATPC-GFP were pretreated with 50 nM bafilomycin A1 for 16 h at 37°C, and then subsequently added 20 μM H89 or 200 μM 8-Br-cAMP for 2 h at 37°C, respectively. **a** Confocal fluorescence microscopy of transfected cells expressing GFP-fused hOATPC. The confocal microscopy was performed on a confluent monolayer of MDCK cells stably expressing hOATPC-GFP grown on glass coverslips. The cells were incubated with 50 nM BA1 for 16 h and subsequently 10 μM H89 or 200 μM 8-Br-cAMP was added to the cells for 2 h before fixed the cells (bar = 5 μm). **b** [+H]Estrone-3-sulfate (E3S) influx assay of MDCK cells stably expression of hOATPC-GFP. The cells stably transfected with hOATPC-GFP were pretreated with 50 nM BA1 for 16 h and then 10 μM H89 or 200 μM 8-Br-cAMP was added to the cells 2 h before E3S uptake assay. The cells treated with BA1/H89 or BA1/8-Br-cAMP were incubated in 0.67 μM (^{3}H)E3S at 37°C for 5 min. In this study, the E3S influx in the present of BA1 alone was set as 100%, and all values were graphed relative to this level. All experiments were performed at least twice with triplicate samples and depicted as means ± SE. *Asterisks* for each BA1/H89 or BA1/8-Br-cAMP treated cells indicate significant differences ($p < 0.05$) from the cells treated with BA1 alone. The data are presented as radioactivity (cpm) of total (^{3}H)E3S uptake per well.
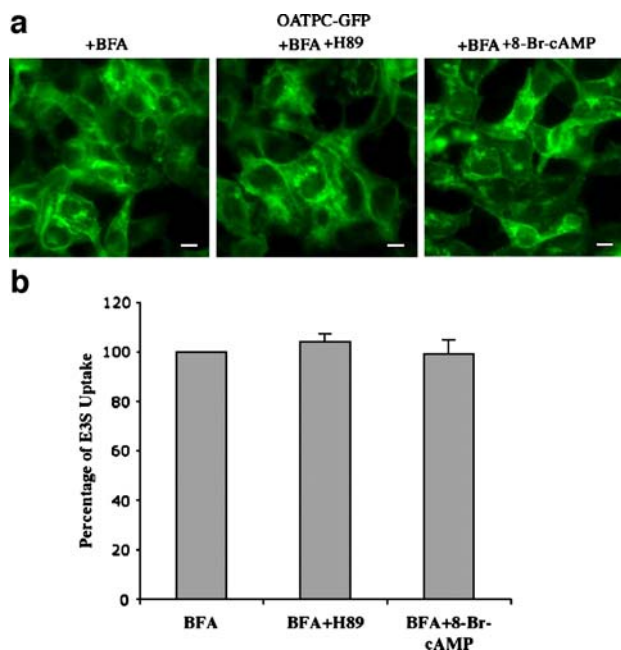
Fig. 9. Effects of PKA inhibitor (H89) and activator (8-Br-cAMP) on brefeldin A (BFA) sensitive membrane localization and E3S uptake of hOATPC-GFP in stably transfected MDCK cells. The MDCK cells stably transfected hOATPC-GFP were pretreated with 2 μM brefeldin A for 16 h at 37°C, and then 20 μM H89 or 200 μM 8-Br-cAMP was added to the cells and incubated for 2 h at 37°C, respectively. **a** Confocal fluorescence microscopy of transfected cells expressing GFP-fused hOATPC. The confocal microscopy was performed on a confluent monolayer of MDCK cells stably expressing hOATPC-GFP grown on glass coverslips. The cells were incubated with 2 μM BFA for 16 h and 10 μM H89 or 200 μM 8-Br-cAMP was subsequently added to the cells culture medium for 2 h before fixed cells (bar = 5 μm). **b** [³H]Estrone-3-sulfate (E3S) influx assay of MDCK cells stably expression of hOATPC-GFP. The cells stably transfected with hOATPC-GFP were incubated with 2 μM BFA for 16 h and 10 μM H89 or 200 μM 8-Br-cAMP was subsequently added to the cells 2 h before E3S uptake assay. The cells treated with BA1/H89 or BA1/8-Br-cAMP were incubated in 0.67 μM (³H)E3S at 37°C for 5 min. In this study, the E3S influx in the present of BFA alone was set as 100%, and all values were graphed relative to this level. All experiments were performed at least twice with triplicate samples and depicted as means ± SE.

that protein kinase A is stably associated with the Golgi complex during interphase, and plays an important role in the assembly and maintenance of a continuous Golgi ribbon from separated membrane stacks (40). Brefeldin A, a Golgi complex disruptor, inhibits protein processing through the Golgi, and blocked the 8-Br-cAMP stimulation of cholesterol efflux to exogenous apolipoprotein E (24). Thomas *et al.* reported that an early effect of cAMP on Na^+ transport is brefeldin A sensitive and is mediated via PKA, which increases trafficking of Na^+-channels to the apical cell surface (41). Marunaka *et al.* demonstrated that brefeldin A, an inhibitor of intracellular protein translocation, blocked the stimulatory action of H89 (an inhibitor of cAMP-activated protein kinase (protein kinase A) (42). Puri and Linstedt found that combined treatment of cultured cells with brefeldin A (to cause Golgi collapse) and the protein kinase inhibitor H89 (to block ER export) disperses golgins

and in particular disperses the *cis*-golgin GM130 to the ER (14). Unlike brefeldin A alone, which leaves matrix proteins as relatively large remnant structures outside the ER, the addition of H89 to BFA-treated cells caused ER accumulation of all Golgi markers tested.

In this study, the effects of individual and combined biological reagents (brefeldin A bafilomycin A1, PKA inhibitor/activator, PKGi) were investigated by confocal microscopy and estrone-3-sulfate transport activity. Our results demonstrated that the functional membrane expression of the fluorescent chimera hOATPC-GFP was achieved in non-polarized (COS7 and HEK293) and polarized (MDCK) cells. Confocal fluorescence microscopy showed that the hOATPC-GFP chimera was distributed mainly along the plasma membrane of COS7 and HEK293 cells and on the basolateral domain of MDCK cells. Estrone-3-sulfate transport activity by hOATPC-GFP in transfected COS7, HEK293, and MDCK cells was increased more then sixfold compared with that untransfected cells. Treatment with brefeldin A and bafilomycin A1 and protein kinase A (KT5720) and protein kinase G inhibitors have similar effects on the membrane localization of GFP-fused human OATPC proteins in non-polarized (COS7 and HEK293) and polarized (MDCK) cells. These results suggest that the membrane expression of hOATPC may utilize similar sorting pathway(s) in these non-polarized and polarized cells.

Studies of brefeldin A and bafilomycin A1 treatment demonstrated that both reagents significantly decreased the polarized membrane trafficking of hOATPC, suggesting that membrane sorting of hOATPC-GFP was mediated by Golgi complex and vacuolar H^+-ATPase related vesicle transport pathways. These findings indicated that stabilization of Golgi complex structure and vacuolar H^+-ATPase associated vesicles is an essential aspect of membrane trafficking events for basolateral membrane expression of hOATPC. To evaluate the role of protein kinase, we examined the effect of protein kinase activation and inhibition on membrane sorting of OATPC. The data show that 8-Br-cAMP (cyclic AMP analogues that mimic intracellular actions of cyclic AMP) stimulated hOATPC membrane localization and enhanced estrone-3-sulfate uptake activity. On the other hand, the PKA inhibitors (H89 and KT5720), but not a PKG inhibitor blocked polarized membrane expression of hOATPC and reduced estrone-3-sulfate transport activity. These results suggest a rise in intracellular cAMP and activation of protein kinase A (PKA) are important for the efficient Golgi-to-basolateral surface expression of human OATPC.

The simultaneous treatment of cells with a PKA activator or inhibitor and bafilomycin A1 demonstrated that bafilomycin A1 did not change the effects of 8-Br-cAMP and H89 on the membrane localization of hOATPC compared with use of 8-Br-cAMP and H89 alone. These data suggest that a cAMP-PKA sensitive membrane sorting pathway for hOATPC-GFP is independent of the vacuolar H^+-ATPase associated (bafilomycin A1 sensitive) vesicle mediated membrane sorting pathway. In contrast, when combined with brefeldin A (a Golgi complex disruptor), neither the PKA-activator (8-Br-cAMP) nor the inhibitor (H89) further altered the plasma membrane expression and transport activity of hOATPC compared with brefeldin A treatment alone. These data suggest that the cAMP-PKA regulation of

hOATPC membrane expression involved the Golgi complex. When the Golgi apparatus was disrupted by brefeldin A treatment, the effects (inhibition and stimulation) of cAMP-PKA on the Golgi-to-basolateral surface sorting process of hOATPC was also diminished.

In summary, the plasma membrane sorting and localization of human OATPC is regulated by cAMP-PKA independent of vacuolar H^+-ATPase secretory pathway but requiring the Golgi complex. Further studies are required to define the relationship between these important mechanisms.

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