Receptor for Activated C-Kinase 1 Regulates the Cell Surface Expression and Function of ATP Binding Cassette G2

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ABSTRACT:

In a previous report, we identified the receptor for activated C-kinase 1 (RACK1) as a positive regulator of the cellular localization and expression of ATP-binding cassette B4, a phosphatidylcholine translocator expressed on the bile canalicular membrane. In the present study, we focused on the role of RACK1 on ATP-binding cassette G2 (ABCG2), which is responsible for the cellular exclusion of compounds including antitumor drugs. Protein expression of ABCG2 was up-regulated by RACK1 overexpression, although mRNA expression of ABCG2 was not dependent on RACK1. The effect of RACK1 on the expression of ABCG2 on the cell surface was confirmed by the uptake of [3H]estrone sulfate, an ABCG2 substrate, into isolated membrane vesicles. The expression of RACK1 affected cellular resistance to mitoxantrone, an anticancer drug excreted by ABCG2, and this effect of RACK1 was abolished in the presence of fumitremorgin C, a selective ABCG2 inhibitor. These results suggest that RACK1 has functional significance as a regulatory cofactor of ABCG2 and is indispensable for the cell surface expression and excretion function of ABCG2. The precise mechanism for RACK1-dependent expression of ABCG2 remains to be clarified, because the results of N-benzoyloxy carbonyl (Z)-Leu-Leu-leucinal (MG132) and chloroquine treatment and those of metabolic labeling experiments did not give us clear evidence whether the reduction of ABCG2 expression in RACK1-knocked down cells may be caused by the suppression of ABCG2 protein synthesis or by acceleration of its degradation.

Introduction

Several lines of evidence suggest that the cellular localization and expression of ATP-binding cassette (ABC) transporters are regulated by several cofactors (Ortiz et al., 2004; Minami et al., 2009). In a previous report, we used yeast two-hybrid screening to show that the receptor for activated C-kinase 1 (RACK1) is a novel binding partner of ABCB4, which is responsible for the biliary excretion of phospholipids (Ikebuchi et al., 2009). We found that down-regulation of endogenous RACK1 expression in HeLa cells by small interfering RNA (siRNA) resulted in the localization of ABCB4 in the cytosolic compartment and reduced protein expression of ABCB4. Consequently, ABCB4-mediated phosphatidylcholine translocation activity decreased significantly when endogenous RACK1 expression was suppressed in HeLa cells. These results suggested that RACK1 has functional significance as a regulatory cofactor of ABCB4.

RACK1 is a scaffold protein with seven WD-40 repeats and was originally identified based on its ability to bind to the activated protein kinase C (PKC) isoforms, particularly PKC-βII (Mochly-Rosen et al., 1991). RACK1 stabilizes activated PKC-βII and facilitates its trafficking within the cells to regulate the phosphorylation state of target proteins. Previous studies have also shown that RACK1 interacts with a range of different cellular proteins and, consequently, may be involved in diverse cellular processes, including signal transduction, cell growth, differentiation, and protein synthesis (McCahill et al., 2002). In addition, it has been established that cell surface expression of several transmembrane proteins such as cystic fibrosis transmembrane conductance regulator (CFTR/ABCC7) may be positively regulated by RACK1 in a direct or an indirect manner (Liedtke et al., 2002). In the present study, we also examined whether the cellular localization and expression of other ABC transporters are affected by the suppression of endogenous RACK1. ABCG2 was identified as a candidate transporter.

ABCG2 is responsible for multidrug resistance to a broad range of anticancer drugs, including mitoxantrone, topotecan, and 7-ethyl-10-

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- ABBREVIATIONS: ABC, ATP-binding cassette; RACK1, receptor for activated C-kinase 1; siRNA, small interfering RNA; PKC, protein kinase C; SN-38, 7-ethyl-10-hydroxycamptothecin; E2S, estrone sulfate; FTC, fumitremorgin C; LAMP1, lysosomal-associated membrane protein; PCR, polymerase chain reaction; MEM, minimum essential medium; FBS, fetal bovine serum; EGFP, enhanced green fluorescent protein; PBS, phosphate-buffered saline; miRNA, microRNA; RIP, radioimmunoprecipitation assay; MG132, N-benzoyloxy carbonyl (Z)-Leu-Leu-leucinal; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.
hydroxyamphotericin (SN-38) (Doyle et al., 1998; Haimer et al., 2004). Knockout mice studies have shown that ABCG2 which is expressed in normal tissues, such as the gastrointestinal tract, liver, and mammary gland, and the blood-brain barrier, functions in the regulation of the disposition of exogenous compounds (Breedveld et al., 2005; Merino et al., 2005). In humans, ABCG2 gene expression is associated with a poor response to remission-induction therapy in patients with acute myeloid leukemia treated with mitoxantrone (Steinbach et al., 2002). In addition, single nucleotide polymorphisms in the ABCG2 gene affect its expression level and functional activity (Kondo et al., 2004) and, consequently, the clinical outcomes or the risk of adverse effects of treatment with anticancer drugs (Noguchi et al., 2005). Several factors involved in transcriptional regulation, including sex hormones (Imai et al., 2005), hypoxia (Krishnamurthy et al., 2009) and, consequently, the clinical outcomes or the risk of adverse effects of treatment with anticancer drugs (Noguchi et al., 2005; Merino et al., 2005). In humans, ABCG2 has not been established (Evseenko et al., 2007). Compared with transcriptional regulation, information on post-transcriptional regulation of ABCG2 has been reported to regulate the transcription of ABCG2 (Robey et al., 2009). In addition, cytokines such as tumor necrosis factor-α and growth factors have been reported to affect the transcription of ABCG2, although the precise molecular mechanism has not been established (Evseneko et al., 2007). Compared with transcriptional regulation, information on post-transcriptional regulation of ABCG2 is limited. Mogi et al. (2003) reported that Akt-1 is involved in the surface expression of ABCG2 in hematopoietic stem cells and contributes to the formation of a side population (Mogi et al., 2003). In addition, we reported that phosphorylation of ABCG2 mediated by the phosphatidylinositol 3-kinase-Akt/protein kinase B pathway increases the apical surface expression of ABCG2 in LLC-PK1 cells (Takada et al., 2005a). In the present study, we examined the role of RACK1 in the post-transcriptional regulation of ABCG2.

Materials and Methods

Materials. [35S]Methionine/cysteine cell labeling mix (NEQ-772) and [3H]Estrone sulfate (E,S) were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). Mitoxantrone dihydrochloride was purchased from LKT Laboratories (St. Paul, MN). Fumitremorgin C (FTC) was purchased from Calbiochem (Darmstadt, Germany). Anti-ABCG2 rat monoclonal antibody (BXP-53) and anti-α-tubulin rabbit polyclonal antibody (ab15246) were purchased from Abcam Inc. (Cambridge, MA). Anti-ABCG2 mouse monoclonal antibody (5D3) was purchased from eBioscience (San Diego, CA). Anti-RACK1 mouse monoclonal IgM was purchased from Transduction Laboratories (San Jose, CA). Anti-Na+/K+/ATPase α rabbit polyclonal antibody (H-300), anti-calnexin goat polyclonal antibody (C-20), and anti-Rab5a rabbit polyclonal antibody (S-19) were purchased from Santa Cruz Biotechnology Inc. (Heidelberg, Germany). Anti-LAMP1 rabbit monoclonal antibody was purchased from Sigma-Aldrich (St. Louis, MO). BODIPY TR-labeled ceramide and Alexa 633-conjugated transferrin were purchased from Invitrogen (Tokyo, Japan). All other chemicals used were commercially available and of reagent grade.

Plasmid Construction. The human ABCG2 cDNA (GenBank accession number NM_004827) was constructed as described previously (Takada et al., 2005b). The full-length coding sequence of RACK1 cDNA (GenBank accession number NM_006098) was amplified by polymerase chain reaction (PCR) and the myc tag sequence (EQKLISEEDL) was added to the 5′-end, followed by subcloning into a pcDNA3.1 vector plasmid (Invitrogen). The sequences of all constructs were confirmed using an automatic DNA sequencer (ABI PRISM 377 DNA Sequencer; Applied Biosystems, Tokyo, Japan). The sequences of all constructs were confirmed using an automatic DNA sequencer (ABI PRISM 377 DNA Sequencer; Applied Biosystems, Tokyo, Japan). The sequences of all constructs were confirmed using an automatic DNA sequencer (ABI PRISM 377 DNA Sequencer; Applied Biosystems, Tokyo, Japan).

Transient Suppression of RACK1 Expression via RNA Interference. Suppression of RACK1 expression was accomplished using an siRNA duplex targeted to RACK1 mRNA (siRACK1) sequences. The siRACK1 sequences were 5′-CACAUCAGGAAUGUAACTTG-3′ (sense) and 5′-GAUAAUCCAUAGCUGUAGGGTT-3′ (antisense) (Kiyely et al., 2006). The negative control siRNA (siPerfect Negative Control; siControl) was designed not to affect the expression of any genes. These siRNAs were purchased from Sigma-Aldrich.

To examine the effect of siRNAs on the expression of RACK1, HeLa cells were seeded at a density of 1.0 × 10⁵ cells per 12-well plates (0.25 × 10⁵ cells/cm²) and, simultaneously, siRACK1 or siControl was transfected into these cells using Lipofectamine RNAiMAX reagent (Invitrogen) according to the method recommended by the manufacturer. Seventy-two hours after siRNA transfection, RNA was isolated using RNA-Solv Reagent (Omega Bio-tek, Doraville, GA) according to the manufacturer’s instructions, and then reverse transcription was performed by ReverTra Ace-α (Toyobo, Tokyo, Japan). To quantify the mRNA expression levels, real-time quantitative PCR was performed using Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA) and the Chromo4 real-time PCR analysis system (Bio-Rad Laboratories, Hercules, CA) at 95°C for 10 min followed by 40 cycles at 95°C for 15 s, 50°C for 30 s, and 72°C for 40 s. The primers used for the quantification were as follows: 5′-GGAATTCACTTTGTGTTGGTGC-3′ (RACK1, sense) and 5′-GGTTGAATGCTCTAATACTGG-3′ (RACK1, antisense), 5′-GCATTCTCGATATGGTATT-3′ (ABCG2, sense) and 5′-TCAAGTGGGACGGATTTG-3′ (ABCG2, antisense), and 5′-TTCAACCCCCGAGCTTGGG-3′ (β-actin, sense) and 5′-GTTGAGTGGAAGCTTACGGC-3′ (β-actin, antisense). The mRNA expression levels of RACK1 and ABCG2 were normalized by that of β-actin.

Immunohistochemical Staining. To identify the cellular localization of exogenous ABCG2, HeLa cells were seeded on glass-bottom 35-mm dishes at a density of 2 × 10⁵ cells (0.22 × 10⁵ cells/cm²) and transfected with siRNAs against RACK1 using Lipofectamine RNAiMAX reagent. Then 24 h after siRNA transfection, these cells were transfected with enhanced green fluorescent protein (EGFP)-ABCG2 using FuGEN6 (Roche Applied Science, Indianapolis, IN). Cells were fixed with 100% methanol at −20°C for 10 min and washed three times with ice-cold phosphate-buffered saline (PBS). Each organellar marker was immunostained with the following primary antibodies: anti-calnexin antibody (100-fold diluted) as an endoplasmic reticulum marker, anti-LAMP1 antibody (200-fold diluted) as a lysosome marker, and anti-Rab5a antibody (100-fold diluted) as an early endosome marker. After cells were washed three times with PBS, they were incubated with the corresponding Alexa-conjugated secondary antibodies (Invitrogen) diluted 250-fold in PBS containing 0.1% bovine serum albumin at 37°C for 1 h. To stain the nucleus, cells were pretreated with 0.2 mg/ml RNaseA (Sigma-Aldrich) in PBS at room temperature for 10 min and incubated with TO-PRO-3 iodide (Invitrogen) diluted 250-fold in PBS at room temperature for 20 min. After cells were washed three times with PBS, they were mounted in Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA). In addition, the Golgi apparatus and recycling endosome were stained with BODIPY TR-labeled ceramide and Alexa 633-conjugated transferrin following the manufacturer’s instructions.

To examine the intracellular localization of endogenous ABCG2, cells were fixed with 4% paraformaldehyde at room temperature for 10 min with or without permeabilization treatment by 0.1% Triton X-100 at room temperature for 5 min. Endogenous ABCG2 was immunostained with two kinds of anti-ABCG2 primary antibodies, BXP-53 (50-fold diluted) and 5D3 (10-fold diluted), and the corresponding secondary antibodies. The cellular localization of the targeted proteins was visualized by confocal laser scanning microscopy (FV1000; Olympus, Tokyo, Japan).

Construction of Stable Cell Lines. Construction of stably knocked down ABCG2 cells was performed using lentivirus vectors as indicated by the manufacturer. In brief, lentiviruses carrying microRNA (miRNA) sequences were constructed using a BLOCK-it Pol II RNAi Expression Vector Kit (Invitrogen) and a ViraPower Promoterless Lentiviral Expression System (Invitrogen). The following sequences were used as the targeting miRNA sequence: 5′-GTCCTCAGCGCGACATTTT-3′ (miControl), 5′-CACTCCACCTTTGGGTGTTG-3′ (miRACK1*1), 5′-TCACGAGGCGCAAACACCTTT-3′ (miRACK1*2), and 5′-CCTCTTTGTCTGATCAATCG-3′ (miABCG2). Infected HeLa cells were selected using 3 μg/ml blasticidin (Invitrogen). The surviving cells were used as control cells (miControl cells),
stable RACK1-knocked down cells (miRACK1 cells) or stably ABCG2-knocked down cells (miABC2G cells).

HeLa cells were transfected with myc-tagged RACK1 cDNA or pcDNA3.1 using FuGENE6 to construct RACK1 stably expressing and control HeLa cells, respectively, and cultured for 48 h. The prepared cells were cultured in MEM medium supplemented with 10% FBS, 100 units/ml penicillin and streptomycin, 1% nonessential amino acids, and 1 mg/ml G418 sulfate (Nacalai Tesque) for 2 weeks. Selected cells were isolated and their expression levels of RACK1 were confirmed by Western blotting.

Western Blot Analysis. Cells were lysed with RIPA buffer (0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin, 1 µg/ml pepstatin, and 5 µg/ml aprotinin) at 4°C for 20 min. After centrifugation at 20,000g for 20 min, the supernatant was obtained, and the protein concentrations were determined by the method of Lowry et al. (1951). The specimens were subjected to Western blot analysis. The primary antibodies were anti-RACK1 antibody (500-fold diluted), anti-ABCG2 antibody (200-fold diluted), and anti-α-tubulin antibody (1000-fold diluted). Finally, the membranes were allowed to bind to horseradish peroxidase-labeled secondary antibodies (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and detected with an ECL Plus Western Blotting Detection System (GE Healthcare) using a luminescent image analyzer (Bio-Rad Laboratories). The data are expressed as the mean ± S.D. of triplicate preparations.

Cell Surface Biotinylation Assay. ABCG2 expression on the plasma membrane was assessed using a biotinylation method described previously (Hayashi et al., 2005). In brief, cells were seeded at a density of 2.0 × 10^6 cells per six-well plates (0.25 × 10^5 cells/cm^2) 72 h before the assay. The prepared cells were washed twice with ice-cold PBS containing 0.1 mM CaCl_2 and 1 mM MgCl_2 (PBS-Ca/Mg) and incubated with 1 mg/ml NHS-Ss-biotin (Pierce Biotechnology, Rockford, IL) at 4°C for 30 min. After removing the NHS-Ss-biotin, the cells were washed with PBS-Ca/Mg containing 100 mM glycine and incubated at 4°C for 15 min, then disrupted with 250 µl of RIPA buffer at 4°C for 30 min. After centrifugation at 20,000g for 20 min, the supernatant was obtained and streptavidin-agarose beads (Pierce Biotechnology) were added to the lysate, followed by incubation at 4°C for 2 h with continuous gentle shaking. The beads were washed five times with RIPA buffer. The biotinylated proteins were eluted with 20 µl of SDS loading buffer at 60°C for 15 min. The biotinylated proteins and whole-cell lysate were subjected to Western blot analysis.

ABCG2 Protein Stability. In each assessment, miControl cells and miRACK1 cells were seeded 48 h before the assay at a density of 2 × 10^6 cells per six-well plates (0.2 × 10^5 cells/cm^2). Cycloheximide (Sigma-Aldrich), an inhibitor of protein translation, was added (100 µM), and cells were collected after 0, 10, 30, 60, 120, and 240 min. N-Benzoyloxycarbonyl (Z)-Leu-Leu-Leu-leucinal (MG132) (Calbiochem), a proteasomal degradation inhibitor, was added (5 µM) 48 h after cell seeding, and the cells were cultured for an additional 24 h. Chloroquine diphosphate (Nacalai Tesque), a lysosomal degradation inhibitor, was added (250 µM) 48 h after cell seeding, and the cells were cultured for an additional 6 h. The protein expression levels of endogenous ABCG2 in each specimen were examined by Western blot analysis as described above.

Metabolic Labeling of ABCG2. miControl cells and miRACK1 cells were incubated in methionine/cysteine-free MEM (Invitrogen) for 1 h just before the experiment. The cells were incubated in the labeling medium containing 100 µCi/ml [35S]methionine/cysteine cell labeling mix and collected at 0, 10, 20, 30, 60, and 90 min. The cells were lysed with RIPA buffer and 0.5 µg of anti-ABCG2 monoclonal antibody (BXP-53) was added to immunoprecipitate endogenous ABCG2. The mixture was incubated overnight at 4°C with continuous gentle shaking. Immunoprecipitation was performed using magnetic beads coated with protein G (Ademtech, Pessac, France), and the immunoprecipitate was washed five times with RIPA buffer. Proteins bound to the protein G beads were eluted with 2× SDS loading buffer containing 50 mM dithiothreitol at 37°C for 1 h. The eluted specimens were separated by SDS-polyacrylamide gel electrophoresis and exposed to a Super Resolution PhosphorImager (PerkinElmer Life and Analytical Sciences). The radioactivity was detected using a Cyclone PhosphorImager (PerkinElmer Life and Analytical Sciences). ABCG2 expression was quantified and normalized by the expression level of total ABCG2 in miControl cells obtained at 1 h. The results are given as the mean ± S.D. of triplicate determinations.

Preparation of Membrane Vesicles. Membrane vesicles were prepared from HeLa cells according to a method described previously (Kondo et al., 2004). In brief, the harvested cells were diluted 40-fold with hypotonic buffer (1 mM Tris-HCl and 0.1 mM EDTA, pH 7.4, at 4°C) and stirred gently for 1 h on ice in the presence of 2 mM phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin, 1 µg/ml pepstatin, and 5 µg/ml aprotinin. The cell lysate was centrifuged at 100,000g for 30 min at 4°C, and the resulting pellet was suspended in 10 ml of isotonic TS buffer (10 mM Tris-HCl, pH 7.4, at 4°C, and 250 mM sucrose) and homogenized using a Dounce B homogenizer (glass/glass, tight pestle, 30 strokes). The crude membrane fraction was layered on top of a 38% (w/v) sucrose solution in 5 mM Tris-HEPES (pH 7.4 at 4°C) and centrifuged in a Beckman SW41 rotor at 280,000g for 45 min at 4°C. The turbid layer at the interface was collected, diluted to 23 ml with TS buffer, and centrifuged at 100,000g for 30 min at 4°C. The resulting pellet was suspended in 400 µl of TS buffer. Vesicles were formed by passing the suspension 30 times through a 25-gauge needle with a syringe. The membrane vesicles were finally frozen in liquid nitrogen and stored at −80°C until use.

Transport Study Using Membrane Vesicles. The transport study was performed using a rapid filtration technique, as described previously (Kondo et al., 2004). In brief, 16 µl of transport medium (10 mM Tris, 250 mM sucrose, 10 mM MgCl_2, 10 mM creatine phosphate, 100 µg/ml creatine phosphokinase, and 5 mM ATP or AMP, pH 7.4, at 37°C) containing a tracer concentration (550 nM) of [3H]E;S was preincubated at 37°C and then rapidly mixed with 4 µl of membrane vesicle suspension containing 5 µg of membrane protein. The transport reaction was stopped by the addition of 1 ml of ice-cold buffer containing 250 mM sucrose, 0.1 M NaCl, and 10 mM Tris-HCl (pH 7.4 at 4°C). The stopped reaction mixture was passed through a 0.45-µm HAWP filter (Millipore Corporation, Billerica, MA) and then washed twice with 5 ml of stop solution. The radioactivity retained on the filter was measured using a liquid scintillation counter.

Cytotoxicity Assay. Cytotoxicity was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method described previously (Lemos et al., 2008) with minor modifications. Cells were seeded at a density of 1.5 × 10^3 cells per 96-well plates (5.0 × 10^3 cells/cm^2) and incubated in antibiotic-free MEM medium supplemented with 10% FBS and 100 units/ml penicillin and streptomycin for 24 h. The cells were exposed to various concentrations of mitoxantrone dihydrochloride in the presence or absence of 5 µM FTC and cultured for additional 72 h. The drug-containing medium was discarded, 0.5 mg/ml MTT solution diluted by phenol red-free MEM medium (Invitrogen) was added, and the cells were incubated for an additional 4 h in the dark. The formazan crystals that had developed were dissolved in isopropyl alcohol supplemented with 0.4 N HCl at room temperature in the dark overnight. The absorbance at 540 nm was detected using a Multiskan JX microplate reader (Thermo Fisher Scientific, Waltham, MA).

Results

Effect of siRACK1 on the Cellular Localization of ABCG2. We first examined whether ABC transporters located on the bile canalicular membrane other than ABCB4 are regulated post-transcriptionally by RACK1. For this purpose, the cellular localization of exogenously expressed EGFP-tagged transporters in HeLa cells was examined. The expression of EGFP-ABC2G, which can be used to directly monitor ABCG2 expression and localization in living cells (Orbán et al., 2008), was affected by RACK1. As shown in the left panel of Fig. 1A, EGFP-fused ABCG2 localized predominantly on the plasma membrane of HeLa cells in the control conditions (Fig. 1A, left panel). In contrast, EGFP-ABC2G localized intracellularly in almost all cells transfected with siRNA against RACK1, as observed for ABCB4 as shown in the right panel of Fig. 1A. In RACK1-suppressed HeLa cells, EGFP-ABC2G localized intracellularly and colocalized partially with fluorescence-labeled ceramide, which is known to accumulate in the trans-Golgi region (Fig. 1B) (Lipsky and Pagano, 1985). In contrast, colocalization was not observed with...
calnexin, LAMP1, Rab5a, or transferrin, organellar markers of the endoplasmic reticulum, lysosomes, early endosomes, and recycling endosomes, respectively (Fig. 1, C–F). These results suggest that RACK1 plays an important role in the post-trans-Golgi modification and localization of ABCG2 on the plasma membrane in HeLa cells.

To confirm the effect of RACK1 suppression on the cell surface expression of EGFP-ABCG2, we performed a biotinylation assay and detected the amount of EGFP-ABCG2 on the plasma membrane (Fig. 1G). The efficacy of biotinylation with membrane proteins was validated by the expression levels of Na⁺/K⁺-ATPase and α-tubulin, which are marker proteins of cell surface and cytoplasm, respectively. As indicated in the upper panel of Fig. 1G, cell surface expression of EGFP-ABCG2 was reduced by siRACK1 treatment, which may be consistent with the observation of EGFP-ABCG2 localization by confocal microscopy (Fig. 1A).

Because endogenous expression of ABCG2 was readily detected and relatively high compared with other cell lines tested (Supplemental Fig. A), we also examined the effect of RACK1 suppression on the cellular localization of endogenous ABCG2 in HeLa cells using two kinds of anti-ABCG2 antibodies, BXP-53 and 5D3 (Fig. 2). BXP-53 reacts with an internal epitope of ABCG2, whereas 5D3 reacts with an external epitope of ABCG2. As shown in Fig. 2, endogenous ABCG2 on the plasma membrane could be detected by 5D3 in HeLa cells without permeabilization but not by BXP-53. On the other hand, both BXP-53 and 5D3 could react with endogenous ABCG2 after the permeabilization treatment. Under control conditions, endogenous ABCG2 localized on the plasma membrane in almost all cells, whereas it localized in intracellular compartments in HeLa cells transfected with siRNA against RACK1 (Fig. 2). In addition, the amount of cell surface ABCG2, which was confirmed by 5D3 without permeabilization, was decreased by siRACK1 treatment. These results suggest that RACK1 regulates the intracellular localization of ABCG2 on the plasma membrane, and RACK1 suppression leads to the decrease of cell surface expression of endogenous ABCG2 in HeLa cells.

**Positive Correlation between the Expression Levels of RACK1 and ABCG2.** As shown in Fig. 2, the fluorescence intensity of endogenous ABCG2 decreased in RACK1-knocked down HeLa cells. This result suggests that RACK1 also regulates the protein expression of ABCG2. To examine this possibility, we constructed two kinds of HeLa cell lines whose RACK1 is knocked down, referred to as miRACK1*1 and miRACK1*2, and HeLa cell lines stably overexpressing RACK1, referred to as RACK1*1 and RACK1*2. Compared with the miControl cells, endogenous RACK1 protein expression decreased to 54 and 56% in miRACK1*1 and miRACK1*2 cells, respectively, and endogenous ABCG2 protein expression also declined to approximately 50% (Fig. 3A). In contrast, in RACK1*1 and RACK1*2 cells, the increase in total expression of RACK1 protein, which is given as the sum of endogenous and exogenous myc-tagged RACK1 observed at 34 and 38 kDa, respectively, was associated with an increase in endogenous ABCG2 expression (Fig. 3A). These results indicate that the protein expression levels of ABCG2 and
RACK1 are positively related (Fig. 3B). ABCG2 mRNA expression was not affected by suppression or overexpression of RACK1 (Fig. 3C), suggesting that RACK1 regulates endogenous ABCG2 protein expression in a post-transcriptional manner.

We also used a biotinylation method to examine the cell surface expression level of endogenous ABCG2. As shown in Fig. 3D, compared with the expression level in the control cells, the cell surface expression of ABCG2 decreased in miRACK1 cells but increased in RACK1-overexpressing cells. These results are consistent with the results shown in Fig. 1.

**Effect of RACK1 on the Stability of ABCG2 Protein.** We have shown previously that RACK1 positively regulates the protein translation rate of ABCB4 but does not accelerate its degradation. Based on these findings, we examined whether the degradation of endogenous ABCG2 protein is affected by the suppression of endogenous RACK1. To examine this possibility, we tried to determine the half-lives of ABCG2 proteins in the presence of cycloheximide, a protein translation inhibitor. However, significant degradation of fully glycosylated ABCG2 (75 kDa) was not observed in either miControl or miRACK1 cells at 4 h, suggesting that the half-life of ABCG2 cannot be determined by this method (Fig. 4A). In addition, because of the very weak band density, we could not conclude whether the degradation rate of 60 kDa precursor protein, which may belong to immature forms of ABCG2, is different between miControl and miRACK1 cells. Furthermore, we also confirmed the effect of inhibitors (MG132, a proteasomal degradation inhibitor, and chloroquine, a lysosomal degradation inhibitor) for major protein degradation pathways. In many cases, protein degradation can be explained by these two degradation pathways. In the presence of MG132, the expression of immature ABCG2 (lower 60 kDa) was increased and showed almost the same level in miControl and miRACK1 cells, but this treatment did not affect the expression level of another immature (upper 60 kDa) and a mature fully glycosylated (75 kDa) forms of ABCG2 (Fig. 4B). It was considered that the two immature ABCG2 bands located around lower and upper 60 kDa represented the nonglycosylated form and the core glycosylated form, respectively (Mohrmann et al., 2005). On the other hand, treatment with chloroquine did not affect the expression level of endogenous ABCG2 (Fig. 4C). We reasoned that, if RACK1 suppression enhances the degradation of ABCG2 via the proteasomal or lysosomal degradation pathway and it results in the suppression of endogenous ABCG2 expression, the protein expression level of endogenous ABCG2 in miRACK1 cells should be restored to almost the same level as that in miControl cells by MG132 or chloroquine treatments. However, this was not the case. These results suggest that the reduced expression of ABCG2 protein under the RACK1-knocked down conditions is not caused by the accelerated degradation of ABCG2 protein.

We next examined the involvement of RACK1 in the translational regulation of ABCG2 by monitoring ABCG2 protein synthesis using [35S]methionine/cysteine. After initiation of metabolic labeling, two immature forms of ABCG2 appeared immediately, as observed previously (Imai et al., 2005). These nonglycosylated and core-glycosylated forms of ABCG2 shifted to the mature fully glycosylated form in both the miControl cells and miRACK1 cells in a time-dependent manner, although the proportion of mature form to immature form tend to be affected by RACK1 suppression (Fig. 4D, upper panel). In addition, the amount of newly synthesized ABCG2 in miRACK1 cells was not largely different from that in miControl cells. These results suggest that the reduction in the steady-state ABCG2 protein levels under RACK1-suppressed conditions (54–56% of miControl) (Fig. 3A) may not be absolutely ascribed to the suppressed protein synthesis.

**[3H]E1S Transport Activity.** To examine the functional activity of ABCG2 in RACK1-knocked down and -overexpressing cells, we prepared isolated membrane vesicles from these cells and assessed the uptake of [3H]E1S, a well known ABCG2 substrate. Reflecting the amount of ABCG2, the ATP-dependent transport activities differed between these cells (Fig. 5). [3H]E1S uptake was reduced to 63.1 ± 4.4 and 57.3 ± 8.1% of the control values in the isolated membrane vesicle prepared from miRACK1*1 and miRACK1*2 cells, respectively. In contrast, the transport activity in the isolated membrane vesicles from RACK1*1 and RACK1*2 cells increased to 134 ± 9 and 177 ± 3% of the control values, respectively. These results suggest that RACK1 regulates the cell surface expression of ABCG2 and consequently its functional activity.

**ABCG2-Mediated Drug Resistance.** We also used mitoxantrone to examine the effect of RACK1 expression on the anticancer drug resistance mediated by expression of ABCG2 (Scheffer et al., 2000). Although mitoxantrone is a dual substrate for ABCG2 and ABCB1, we assumed that we can examine the effect of RACK1 expression on the cellular function of ABCB2, because we have previously found that neither the expression level nor cellular localization of ABCB1 was affected by siRACK1 treatment in HeLa cells (Ikebuchi et al., 2009). HeLa cells whose RACK1 was knocked down stably and HeLa cells whose RACK1 was overexpressed stably were exposed to various concentrations of mitoxantrone and then subjected to the MTT assay (Fig. 6, A and B). We also constructed HeLa cells whose ABCG2 was knocked down stably, which are referred to as miABCG2 cells. The protein expression of endogenous ABCG2 in miABCG2 cells was almost the same as that in miRACK1 cells (data not shown). miRACK1*1, miRACK1*2, and miABCG2 cells showed higher
sensitivity to mitoxantrone than did miControl cells (Fig. 6A). In contrast, RACK1*1 and RACK1*2 cells showed lower sensitivity to mitoxantrone and survived in the presence of a higher concentration of mitoxantrone (Fig. 6B).

To test the hypothesis that the difference in sensitivity to mitoxantrone between these cells is dependent on the expression level of ABCG2, we examined the effect of FTC, a selective inhibitor of ABCG2 (Fig. 6, C and D); in fact, 5 μM FTC inhibited the function of ABCG2 but not that of ABCB1 (Rabindran et al., 2000). If the difference in sensitivity to mitoxantrone is explained mainly by the different expression levels of ABCG2, the difference in the sensitivity should disappear with inhibition of ABCG2. miControl cells and miRACK1*2 cells showed almost the same sensitivity to mitoxantrone in the presence of FTC (Fig. 6C). In the same manner, the difference in cell viability with mitoxantrone between pcDNA3.1 control cells and RACK1*2 cells disappeared in the presence of FTC (Fig. 6D). In addition, the suppression or overexpression of RACK1 did not affect the endogenous expression of ABCB1 (data not shown), which is that of a drug transporter with a capacity for mitoxantrone secretion. These results suggest that RACK1 regulates both the protein expression and cellular localization of ABCG2, which are accompanied by changes in transport function as well as resistance to mitoxantrone.

Discussion

It is well established that ABCG2 excretes many kinds of anticancer drugs and plays an important role in multidrug resistance (Doyle et al., 1998; Haimeur et al., 2004). ABCG2 is up-regulated in several tumor tissues and affects the prognosis of patients with acute myeloid leukemia (Steinbach et al., 2002), although the precise regulatory mechanism is not understood fully. The estrogen receptor regulates ABCG2 expression in a transcriptional manner (Imai et al., 2005), although there is little information on the post-transcriptional regulation of ABCG2.

In the present study, we examined whether RACK1 is involved in the regulation of the intracellular localization of ABCG2. We found that exogenous and endogenous ABCG2, which localized on the plasma membrane under basal conditions, localized intracellularly when the expression of RACK1 was suppressed by siRNA (Figs. 1A and 2). The effect of RACK1 suppression on ABCG2 localization was also confirmed by a biotinylation assay (Figs. 1G and 3D). ABCG2 is known to form a homodimer at the endoplasmic reticulum, and this dimer is translocated to the plasma membrane after glycosylation at the Golgi apparatus (Kage et al., 2002). It is possible that RACK1 mediates the dimerization or glycosylation of ABCG2 and consequently regulates the cell sur-
face expression of ABCG2. Under the RACK1-knocked down conditions, ABCG2 colocalized partially with the marker for the Golgi apparatus but not with that of the endoplasmic reticulum (Fig. 1, B and C). This observation suggested that the reduced RACK1 expression does not affect homodimer formation at the endoplasmic reticulum. Regarding the latter possibility, the pro-

![Fig. 4. Regulation of the expression of ABCG2 by RACK1. A, effect of RACK1 suppression on the degradation of ABCG2. Cycloheximide (100 μM) was added to the medium, and miControl cells and miRACK1*2 cells were collected at the times indicated. The protein levels of ABCG2 (top panels) and α-tubulin (bottom panels) were measured. B, effect of MG132 on the degradation of ABCG2. MG132 (5 μM) or vehicle (dimethyl sulfoxide) was added to the medium, and the cells were cultured for 24 h. The protein expression levels of ABCG2 and α-tubulin in each specimen were determined by Western blot analysis. C, effect of chloroquine on the degradation of ABCG2. Chloroquine (CQ) (250 μM) or vehicle (PBS) was added to the medium, and the cells were cultured for 6 h. The protein expression levels of ABCG2 and α-tubulin in each specimen were determined by Western blot analysis. D, effect of RACK1 suppression on protein synthesis of ABCG2. After precultivation in methionine/cysteine-free medium for 60 min, [35S]-labeled methionine/cysteine was added to the medium. The cells were harvested at the times indicated to chase the expression level of ABCG2. The cells were lysed, and ABCG2 was immunoprecipitated using anti-ABCG2 rat monoclonal antibody (BXP-53). The proteins were separated by SDS-polyacrylamide gel electrophoresis and detected using a PhosphorImager. The band densities (mature and immature form) were quantified and normalized by those in control (con) cells; [protein expression of ABCG2 in miControl (con) cells; [protein expression of ABCG2 in miRACK1 (mi) cells. The results are given as the mean ± S.D. of triplicate determinations. *, p < 0.05; **, p < 0.01, significantly different from the control cells by Student’s t test.]![Fig. 5. [3H]E1S transport assay. The uptake of [3H]E1S by membrane vesicles isolated from miControl, miRACK1, pcDNA3.1, and RACK1 cells was examined. Membrane vesicles (5 μg) were incubated in transport buffer (10 mM Tris, 250 mM sucrose, and 10 mM MgCl2, pH 7.4, at 37°C) containing 550 nM [3H]E1S, 10 mM creatine phosphate, 100 μM creatine phosphokinase, and 5 mM AMP or ATP at 37°C for 1 min. [amount of [3H]E1S taken up by the isolated membrane vesicles in the absence of ATP; [amount of [3H]E1S taken up by the isolated membrane vesicles in the presence of ATP. The results are given as the mean ± S.D. of triplicate determinations. ***, p < 0.01; ****, p < 0.001, significantly different from the control cells by Student’s t test.]
portion of the mature to immature form of ABCG2, which indicates the extent of glycosylation of ABCG2 at the Golgi apparatus, tends to be affected by RACK1 suppression (Fig. 4D). From this result, it could be indicated that RACK1 regulates the glycosylation and maturation of ABCG2 at the Golgi. As an alternative, it is also possible that RACK1 is involved in the trafficking process of ABCG2 from the Golgi apparatus to the plasma membrane.

Together with the altered intracellular localization of ABCG2, we found a positive correlation between the protein expression levels of RACK1 and ABCG2 (Fig. 3, A and B). The lack of effect of RACK1 suppression or overexpression (Fig. 3C) on mRNA expression of endogenous ABCG2 suggests that RACK1 regulates ABCG2 protein expression in a post-transcriptional manner. The homodimer of ABCG2 is stabilized by intra- and intermolecular disulfide bonding and N-glycosylation at several amino acid residues (Wakabayashi-Nakao et al., 2009). A defect in these post-transcriptional modifications causes protein misfolding and subsequent degradation by the endoplasmic reticulum-associated degradation pathway, which is sensitive to MG132. Another study showed that the expression level of the ABCG2 C592G/C608G mutant, which lacks the intramolecular disulfide bond, was increased by MG132 treatment, whereas that of the wild type was not affected (Wakabayashi et al., 2007). In addition, it has been indicated that RACK1 is involved in the degradation pathway of several proteins by modifying the ubiquitination state (Ruan et al., 2009). RACK1 has been recently identified as a novel interacting partner of CLEC-2, a C-type lectin-like receptor, and this interaction regulates the stability of CLEC-2 through modifying its ubiquitin-proteasome degradation pathway. Therefore, it is possible that RACK1 can be attributed to tumor genesis and the multidrug resistance phenotype through the positive regulation of ABCG2 expression such as protein synthesis, maturation, and other unidentified process(es).

We also examined the function of ABCG2 under RACK1-suppressed conditions. Our results indicated that RACK1 regulates the cell surface expression of ABCG2 and consequently affects drug resistance. It has been well established that ABCG2 excretes a wide range of anticancer drugs and its expression is significantly associated with response and progression-free survival in patients with small cell lung cancer treated with platinum-based chemotherapy (Kim et al., 2009). In addition, it has been recently shown that elevated RACK1 expression is not only closely related to in vitro cell proliferation and invasion (Berns et al., 2000) but also linked to in vivo growth and metastasis of pulmonary adenocarcinomas and breast carcinomas (Cao et al., 2010; Nagashio et al., 2010). From these observations, it is possible that RACK1 can be attributed to tumor genesis and the multidrug resistance phenotype through the positive regulation of ABCG2. In contrast to ABCG2, ABCB1 and ABCC1 expression was not affected by RACK1 suppression (data not shown).

RACK1 is also involved in the acquisition of cellular drug resistance by affecting other mechanisms. For example, RACK1-overexpressing MCF-7 cells exhibit resistance to paclitaxel (Zhang et al., 2008). We confirmed these observations by Zhang et al. (2008) using established HeLa cell lines; in our experiments, RACK1*1 and RACK1*2 cells showed reduced sensitivity against paclitaxel, doxorubicin, and vincristine, whereas miRACK1*1 and miRACK1*2 showed higher sensitivity to these anticancer drugs (data not shown). However, these phenomena may be independent of ABCG2 because the sensitivity to these drugs was not affected in miABCG2 cells (data not shown), and these drugs are poor substrates of ABCG2 (Litman et al., 2000). Although these drugs are substrates for ABCB1, the effect of RACK1 on drug resistance may not be mediated by ABCB1 because the localization and expression of ABCB1 were not affected.
by RACK1 suppression. It is possible that RACK1 is also involved in other unknown resistance mechanisms (Huang et al., 2008).

In conclusion, RACK1 regulated the expression and localization of ABCG2 in a post-transcriptional manner, suggesting that RACK1 affects the ABCG2 transport activity. Because RACK1 also regulates ABCB4 in a manner similar to that of ABCG2 but not ABCB1, it is possible that RACK1 plays a selective, functional role in the regulation of some ABC transporters.

References


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Fig. A  Endogenous expression of ABCG2 in cultured cell lines. (1) Messenger RNA were retrieved from each cell lines and ABCG2 expression on mRNA level was quantified by RT-PCR. Each amount of ABCG2 mRNA was normalized by that of β-actin. The results are expressed as the mean ± S.D. of triplicate determinations. (2) Cells were lysed with RIPA buffer and 15 µg of protein lysates were subjected to Western blot analysis. The primary antibodies were anti-ABCG2 antibody (BXP-53; 200-fold diluted) and anti-α-tubulin antibody (1,000-fold diluted).