ABC1 has been established to be required for the efflux of cholesterol and phospholipids to apolipoproteins such as apoA-I. At present, it is unclear whether ABCA1-mediated lipid exposure is specific with regard to lipid headgroups and whether it requires calcium activation and the presence of a lipid acceptor. In the present work, we found exofacial exposure of endogenous phosphatidylserine in the absence of apo-A-I to be enhanced in ABCA1-GFP expressing MDCKII and HeLa cells compared with control cells. By using C6-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) (NBD)-labeled phospholipid analogues, we observed elevated redistribution of phosphatidylethanolamine and phosphatidylserine analogues from the cytoplasmic to the exoplasmic leaflet of the plasma membrane of ABCA1-GFP expressing cells. Whereas glyburide affected neither the level of exofacial endogenous PS nor the outward movement of the amino phospholipid analogues, the latter was sensitive to intracellular Ca²⁺ in ABCA1-GFP expressing cells, further enhancing outward analogue redistribution with respect to control cells. Both receptor-mediated endocytosis and fluid-phase endocytosis were reduced in MDCKII cells expressing ABCA1-GFP. Glyburide raised the level of receptor-mediated endocytosis in the ABCA1-GFP expressing cell to the level of control cells in the absence of glyburide. In control cells, however, fluid-phase endocytosis but not receptor-mediated endocytosis was significantly reduced upon glyburide treatment.

ABC1, a member of the ATP-binding cassette (ABC) transporter superfamily, has been identified as the mutated gene in Tangier disease, an inherited plasma high density lipoprotein deficiency. The abnormal accumulation of cholesterol in macrophages of Tangier disease patients suggested that ABCA1 is involved in lipid efflux (1–3). Indeed, subsequent studies have shown that ABCA1 is required for cholesterol and phospholipids efflux to apolipoproteins such as apoA-I (4–7). Upon expression of functional ABCA1 in cultured cells, enhanced binding of apoA-I to the plasma membrane and increased cellular cholesterol and phospholipids efflux to apoA-I have been found. It has been suggested that ABCA1 serves as a receptor for docking of apoA-I to the plasma membrane. A chemical cross-linking approach revealed complex formation between both proteins (4, 5). The relevance of ABCA1 for binding of apoA-I is supported by the observation that Tangier patients typically fail to bind nascent apoA-I (8, 9).

An alternative model suggests that ABCA1 acts as a lipid transporter pumping the amino phospholipid phosphatidylserine (PS), which is typically sequestered to the cytoplasmic leaflet of mammalian plasma membranes to the exoplasmic leaflet (6, 10). Exposure of PS on the exoplasmic plasma membrane leaflet generates a microenvironment facilitating binding of apoA1 (7, 8). Another recent study also provided a clear indication that ABCA1 expression increases cell surface PS (11). Furthermore, the authors (11) found a strong enhancement of apoA-I binding to apoptotic cells with an elevated level of exofacial PS. However, this binding was not sufficient to trigger phospholipid and cholesterol efflux to apoA-I. Thus, apart from PS, other phospholipids such as phosphatidylcholine (PC) might also be transported by ABCA1 to enable lipid removal from the plasma membrane to apoA-I (12). This is supported by Wang et al. (13) who concluded a direct transport of both cholesterol and phospholipids via ABCA1.

The lipid transport activity of ABCA1 may also be involved in other cellular processes as endocytosis. An enhanced endocytic activity in Tangier disease cells has been associated with an impairment of PS exposure (14).

From these studies, it appears that ABCA1-mediated surface exposure of lipids is of rather low specificity. However, so far no systematic study on ABCA1-dependent lipid exposure has been done. Here we investigated the exposure of endogenous PS and various fluorescent lipid analogues in the plasma membrane of ABCA1-expressing MDCKII cells. In order to correlate directly the expression of ABCA1 and exposure of endogenous PS, ABCA1-GFP, which has been shown to be fully functional with respect to wild type ABCA1 (15), was expressed in MDCKII cells. We observed an ABCA1-mediated redistribution of amino phospholipid analogues of phosphatidylserine and phosphatidylethanolamine.

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The abbreviations used are: ABC, ATP-binding cassette; apoA-I, apolipoprotein A-I; GFP, green fluorescent protein; MDR1 F558, MDR1 F558P; BSA, bovine serum albumin; DFP, diisopropyl fluorophosphate; DMEM, Dulbecco’s modified Eagle’s medium; NBD, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl); PBS, Dulbecco’s phosphate-buffered saline; mPBS, modified Dulbecco’s phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; C6-NBD-PC, 1-palmitoyl-2-(6-NBD)-phosphatidic acid; C6-NBD-PE, 1-palmitoyl-2-(6-NBD)-PE; C6-NBD-PS, 1-palmitoyl-2-(6-NBD)-PS; FACs, fluorescence-activated cell sorter; HA, hemagglutinin; ANOVA, analysis of variance; FCS, fetal calf serum.

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dyeethanolamine from the cytoplasmic side to the exoplasmic plasma membrane leaflet. The enhanced surface exposure of the PS analogue is in line with the higher level of exofacial endogenous PS in comparison with control cells, which was confirmed for ABCA1-GFP expressing HeLa cells. In contrast to the amino phospholipid analogues, we did not find an enhanced surface exposure for the phosphatidylcholine analogue in ABCA1-GFP expressing cells. In parallel, we investigated the endocytotic activity observing that the down-regulated receptor-mediated and fluid-phase endocytosis in ABCA1-GFP expressing cells cannot solely be linked to the lipid transport activity of ABCA1.

EXPERIMENTAL PROCEDURES

Materials—Disopropyl fluorophosphate (DFP), fatty acid-free bovine serum albumin (BSA), and the calcium ionophore A23187 were purchased from Sigma. A23187 stocks were prepared in dimethyl sulfoxide. Dulbecco’s phosphate-buffered saline was supplemented with 24 mM glucose and 10 mM HEPES (mPBS). 1-Palmitoyl-2-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-phosphatidic acid (C6-NBD-PA), -PE (C6-NBD-PE), -PS (C6-NBD-PS) were purchased from Avanti Polar Lipids (Alabaster, AL), high performance TLC (2% BSA in Merck. APc-annexin V and binding buffer were bought from Pharmingen; propidium ioide from Sigma. Triton X-100 and trypsin/EDTA were from Fluka (Buchs, Switzerland) and Biochrom KG (Berlin, Germany), respectively. Tetramethylrhodamine-dextran (TMR-dextran) and Texas Red-transferrin (TR-transferrin) were obtained from Mobitec (Göttingen, Germany). Inhibitors of ABC transporters were glyburide (Sigma), PSC 833 (Novartis, Basel, Switzerland), and MK 571 (Merck). Glyburide and PSC 833 stocks were prepared in dimethyl sulfoxide or ethanol; MK 571 was prepared in double-distilled water. For all experiments, the effect of the solvent was determined.

Cells—Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum (FCS, Helvoort et al. (23)). Briefly, cells were incubated at 15 °C with 1 ml of 25 μM C6-NBD-PE in mPBS. After 150 min, analogues on the exoplasmic leaflet (C6-NBD-PA and -PE) were washed twice with cold mPBS. For t = 0, medium containing 2% (w/v) BSA and 5 mM DFP was added to the cell dish and incubated for 10 min on ice. To measure C6-NBD-PS or -PE outward transport, cultured (15 or 37 °C) mPBS with 2% (w/v) BSA and 5 mM DFP was added to the cell dish, and cells were incubated at 15 or 37 °C for 30 min. Removal of the BSA-containing mPBS was identical to the one described above, and the second BSA-containing mPBS was added to the CellQuest Pro software.

Lipid Analysis—After the incubations, the second BSA back-exchanged mPBS was removed from the first, and cells were scraped into mPBS. For C6-NBD-PS analysis, lipids from both scraped cells and media were extracted by isopropyl alcohol (5.5 ml per 1 ml of medium) to prevent substantial loss into the aqueous phase. Samples were centrifuged at 780 × g for 5 min, and the supernatant was transferred into new glass tubes and dried. For C6-NBD-PA and -PE analysis, lipids from both cells and cell media were extracted by the method of Bligh and Dyer (24) using 20 mM acetic acid in the aqueous phase. After two-dimensional separation (I, chloroform, methanol, 25% ammonium hydroxide (65:25:4, v/v); II, chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, v/v)) on TLC plates, fluorescent lipid spots were visualized under ultraviolet light, scraped, and quantified as described below (17, 18).

Expression of HA-GFP—MDCKII cells (50% confluent) were grown in DMEM supplemented with 10% FCS. Influenza virus hemagglutinin (strain X31) tagged with a GFP at the C terminus (HA-GFP) in vector pTM1 was expressed by using vaccinia virus T7 system (16). Cells were washed and infected with modified vaccinia virus (vTF7–3 virus) with a mixture of 20 μl of ExGen 500 in 0.15 M NaCl. After transfection, cells were trypsinized and seeded into 10-cm culture dishes. Transfection efficiency was monitored by flow cytometry (FACScan, BD Biosciences) before seeding. Hygromycin B selection (200 μg/ml) was performed for 14 days. Measurement was performed with a FACScanCalibur flow cytometer equipped with an Ar+ laser (488 nm) and a diode laser (630 nm) (BD Biosciences). Forward and side scatter were set at a linear scale. The following fluorescence channels (log scale) were used: FL1 (530/30, enhanced GFP), FL2 (585/42, propidium iodide, PE), FL3 (670LP, propidium iodide), and FL4 (661/16, APC-annexin V). Compensation parameters were as follows: FL1, 1.2% FL2; FL2, 23.9% FL1; FL2, 0.0% FL3; FL3, 14% FL2; FL3, 0.5% FL4; and FL4, 16.4% FL3. Threshold was set at 72 FCS-H. Data were analyzed by CellQuest Pro software.

Endocytosis—Cells grown on chambered cover glasses were washed with mPBS, 1 ml of 2 ml of BSA in DMEM containing 20 mM HEPES was added, and cells were incubated for 10 min at 37 °C. After washing with mPBS, 1 ml of 10 μM mPBS containing 5 min DFP (as in all following incubations) to prevent hydrolysis of analogues (19). After 30 min, culture dishes were transferred on ice, and cold mPBS containing BSA (final 2%, w/v) was added to extract NBD lipid analogues from the exoplasmic leaflet. C6-NBD-PS and -PE in the outer leaflet, not in the inner leaflet, of the plasma membrane have been shown to be selectively extracted by incubation with an excess of BSA (referred to as back exchange) (23). Phospholipids with two long fatty acid chains as endogenous phospholipids, for example, are not extracted by BSA. After 10 min, BSA-containing media were collected, and back exchange was repeated with cold mPBS plus 2% BSA for 10 min, followed by lipid analysis of cells and media.

Measurement of Outward Redistribution of C6-NBD-PS and -PE by Direct Labeling—Cells were labeled with 1 ml of 10 μM C6-NBD-PS or -PE on ice and incubated at 20 °C for 30 min to allow inward movement of the NBD lipid analogues as described above. Inhibitors (200 μM glyburide; 25 μM MK 571; 10 μM PSC833) and A23187 (0.5 μM) in Me2SO were added 20 min after the beginning of the incubation at 20 °C. NBD lipid analogues remaining on the cell surface were extracted twice with incubation by BSA (2% (w/v)) in mPBS for 10 min on ice. After removing the second BSA-containing mPBS, cells were washed twice with cold mPBS. For t = 0, medium containing 2% (w/v) BSA and 5 mM DFP was added to the cell dish and incubated for 10 min on ice. Measurement was performed with a FACScanCalibur flow cytometer equipped with an Ar+ laser (488 nm) and a diode laser (630 nm) (BD Biosciences). Forward and side scatter were set at a linear scale. The following fluorescence channels (log scale) were used: FL1 (530/30, enhanced GFP), FL2 (585/42, propidium iodide, PE), FL3 (670LP, propidium iodide), and FL4 (661/16, APC-annexin V). Compensation parameters were as follows: FL1, 1.2% FL2; FL2, 23.9% FL1; FL2, 0.0% FL3; FL3, 14% FL2; FL3, 0.5% FL4; and FL4, 16.4% FL3. Threshold was set at 72 FCS-H. Data were analyzed by CellQuest Pro software.
Expression and Localization of ABCA1-GFP—Expression of ABCA1-GFP in nonpolarized MDCKII cells was assessed by FACS analysis. Two populations of cells differing in their level of ABCA1-GFP expression were distinguished (Fig. 1, only shown for MDCKII cells). About 20% of MDCKII and 30% of HeLa cells expressed ABCA1-GFP at a high level (GFP-high, Table I), whereas the remaining cells showed only a low level or no expression of ABCA1-GFP (GFP-low). Staining of cells with the membrane-impermeable nucleic acid stain propidium iodide (PI) did not correlate with GFP fluorescence intensity (Fig. 1). Thus, viability of cells was not impaired by expression of ABCA1-GFP.

Expression of ABCA1-GFP was dependent on number of cell passages. For example, the number of highly ABCA1-GFP expressing MDCKII cells declined by about 50% and 70% after the first and second passage, respectively. A similar effect was observed for HeLa cells (data not shown).

ABCA1-GFP was localized to the plasma membrane in MDCKII and HeLa cells (Fig. 2). Cross-section images show that ABCA1-GFP is expressed in the plasma membrane with no difference between the side facing the dish and the opposite side. As revealed from co-labeling with TetramethylX-wheat germ agglutinin (not shown), ABCA1-GFP was also found in the Golgi apparatus. When MDCKII cells were allowed to reach confluency and to polarize, ABCA1-GFP distributed preferentially to the basolateral domain of the plasma membrane in line with previous observations (see Refs. 6 and 15 and data not shown). However, for our studies only nonpolarized cells were used. Both cell lines did not express endogenous ABCA1 as shown previously (6) and were verified by immunoblotting (data not shown).

C6-NBD-PS Outward Redistribution Is Enhanced in ABCA1-GFP Expressing MDCKII Cells—To study outward redistribution of C6-NBD-PS from the cytoplasmic to the exoplasmic leaflet of the plasma membrane, the cytoplasmic side was labeled following the approach of Pohl et al. (18). To this end, cells were incubated for 30 min at 20 °C upon incorporation of PS analogues into the exoplasmic leaflet (see “Experimental Procedures”). During this period, analogues rapidly redistributed to the cytoplasmic side mediated by an amino phospholipid translocase activity present in the plasma membrane of MDCKII cells (17). Analogues remaining on the exoplasmic leaflet were removed by repeated washing with BSA. About 50% of analogues became internalized in control cells. We observed a slightly reduced amount of intracellularly localized analogues for ABCA1-GFP expressing cells with respect to control cells after no passage or one passage of cells. The ratio of internalized analogues between ABCA1-GFP expressing and control cells was 0.83 ± 0.13 (mean ± S.E.; n = 3). However, the difference between ABCA1-GFP expressing cells and control cells disappeared when cells were used after three culturings passages correlating with the decline of ABCA1-GFP expressing cells (see above).

Subsequently, to follow outward redistribution of analogues, cells were continuously incubated at 37 or 15 °C in BSA-containing medium. Any analogue reaching the cell surface was extracted by BSA into the suspension medium. The kinetics of outward redistribution at 37 °C are shown in Fig. 3.

For control cells, about 50% of analogues originally located intracellularly became accessible to BSA within 30 min of incubation at 37 °C. At any time point measured, exposure of C6-NBD-PS to the exoplasmic leaflet was significantly higher (ANOVA, p < 0.05) for ABCA1-GFP expressing cells with respect to control cells (Fig. 3A).

When outward redistribution was measured for 30 min at 15 °C, only 30% of analogues could be extracted. However, exposure of C6-NBD-PS was also enhanced in ABCA1-GFP expressing cells and was even more pronounced in comparison to 37 °C (Fig. 4, passage 0, and Fig. 5). At this temperature (15 °C) vesicular transport is abolished (25), suggesting that the enhanced exposure of the PS analogue in ABCA1-GFP expressing cells is related to redistribution from the cytoplasmic leaflet. The differences between ABCA1-GFP expressing cells and control cells cannot be explained by

![Image](http://www.jbc.org/content/263/23/26323/F1.large.jpg)
cell-dependent hydrolysis of analogues, which was similar for \textit{ABCA1-GFP} expressing and control cells (data not shown).

Differences in outward redistribution between \textit{ABCA1-GFP} expressing and control cells depended on the number of passages. Usually, after two passages the exposure of C6-NBD-PS in \textit{ABCA1-GFP} expressing cells was reduced to the level of control cells (Fig. 4) mostly due to a reduction in the number of \textit{ABCA1-GFP} expressing cells (see above). Very likely, \textit{ABCA1-GFP} expressing cells grow at a slower rate than non-expressing cells.

After labeling of cells with C6-NBD-PS, inward redistribution and outward translocation of analogues was also followed by fluorescence microscopy (images not shown). Upon labeling of cells on ice, only the plasma membrane became fluorescent. NBD fluorescence was much more intense than GFP fluorescence. After incubation of cells at 20 °C for 30 min and subsequent BSA extraction of analogues from the exoplasmic leaflet, bright labeling of the cytoplasm was observed. Further incubation of cells for 30 min at 15 or 37 °C in the presence of BSA (see above) led to a significant reduction of intracellular fluorescence, in agreement with an outward movement of the analogues and their extraction onto BSA. Unfortunately, rapid bleaching of NBD fluorescence did not allow us to quantify and compare intracellular fluorescence between control and \textit{ABCA1-GFP} expressing cells.

Treatment of cells with 200 \textmu{M} glyburide affected exposure of the PS analogue neither in control cells nor in \textit{ABCA1-GFP} expressing cells. Similar observations were made in the presence of the ABCB1 (MDR1 Pgp) inhibitor PSC833 (10 \textmu{M}) and the ABCC1 (MRP1) inhibitor MK-571 (25 \textmu{M}) (not shown). Thus, in all cases the ratio of exposed C6-NBD-PS between \textit{ABCA1-GFP} expressing and control cells was not affected (Fig. 5; only shown for glyburide).

Upon pretreatment of cells with the calcium ionophore A23187, significantly enhanced (ANOVA, \textit{p} < 0.05) exposure of C6-NBD-PS in the presence of 2 \textmu{M} Ca\textsuperscript{2+} was found for \textit{ABCA1-GFP} expressing cells (Fig. 5). The ratio of exposed C6-NBD-PS between \textit{ABCA1-GFP} expressing and control cells increased from about 1.25 to 1.6, and was only slightly reduced in the presence of 200 \textmu{M} glyburide (Fig. 5). A23187 treatment in medium without Ca\textsuperscript{2+} did not affect the outward redistribution of C6-NBD-PS in \textit{ABCA1-GFP} expressing and in control cells (not shown). Similarly, neither addition of EGTA to

**Table I**

|                      | **MDCKII** |                   |                      | **HeLa** |                   |
|----------------------|------------|-------------------|----------------------|----------|-------------------|
|                      | GFP fluorescence intensity, mean ± S.D. | Fraction of cells |                      | GFP fluorescence intensity, mean ± S.D. | Fraction of cells |
| Control cells (no \textit{ABCA1-GFP}) background | 1 ± 0.08 | 100 % | 1.60 ± 0.09 | 100 % |
| Low \textit{ABCA1-GFP} expressing cells | 1.04 ± 0.08 | 74.08 ± 2.57 |                      | 1.60 ± 0.09 | 62.22 ± 1.17 |
| High \textit{ABCA1-GFP} expressing cells | 12.69 ± 0.59 | 25.92 ± 2.57 |                      | 9.47 ± 0.15 | 37.78 ± 1.17 |

**FIG. 2.** Localization of \textit{ABCA1-GFP} in the plasma membrane of MDCKII cells and HeLa cells. \textit{ABCA1-GFP} localizes to the plasma membrane in MDCKII cells (A and C) and in HeLa cells (B and D) but also to intracellular compartments (see “Results”). C and D, differential interference contrast images, DIC. Cross-sections of image stacks in an x and y direction are shown on the bottom and on the left side of images, respectively. For details see “Experimental Procedures.”
After removal of remaining PS analogues from the exoplasmic leaflet, cells were incubated in the presence of BSA at 37 °C. After various time points, the amount of extracted analogues was measured. For details see “Experimental Procedures.” Data represent mean ± range of a typical experiment in duplicate.

Enhanced C6-NBD-PS outward transport in ABCA1-GFP expressing MDCKII cells depends on the number of passages. With increasing number of passages, the enhanced exposure of C6-NBD-PS in ABCA1-GFP expressing cells declines. The ratio of the amount of extracted analogues between ABCA1-GFP expressing cells and control cells at 15 °C is shown (data not shown for 37 °C). Data represent means ± S.E. of at three independent experiments in duplicate. The total uptake of analogues for cells with no passage was 90 ± 18 pmol/10^5 cells and 72 ± 20 pmol/10^5 cells for control and ABCA1-GFP expressing cells, respectively. For details, see “Experimental Procedures” and legend to Fig. 3.

ABC1-GFP expressing cells with respect to control cells after continuous incubation of cells in BSA-containing medium for 30 min at 37 or 15 °C (Fig. 6A). The exposure was not affected by glyburide (200 μM), PSC833 (10 μM), or MK-571 (25 μM) (not shown). An enhanced but statistically not significant exposure of C6-NBD-PE in ABCA1-GFP expressing cells was observed in the presence of A23187 and 2 mM Ca^{2+} (not shown).

In a second approach, cells were labeled with C6-NBD-PE and incubated for 30 min at 20 °C following the technique used for C6-NBD-PS. It has been shown previously that the PE analogue is also rapidly transported to the cytoplasmic leaflet of the plasma membrane of MDCKII cells (17). After removal of analogues remaining on the exoplasmic leaflet, cells were incubated in BSA-containing medium for 30 min at 37 or 15 °C. The amount of accessible PE analogue was much higher in comparison to the previous situation where exposure of metabolically converted C6-NBD-PE was studied. About 80–90 and 50–70% of C6-NBD-PE was accessible to BSA at 37 and 15 °C, respectively. For both temperatures, we found an enhanced appearance (ANOVA, p < 0.05) of the PE analogue on the surface of ABCA1-GFP expressing cells with respect to control cells (Fig. 6B). However, the difference between both cell lines was less pronounced in comparison to that measured by exposure of C6-NBD-PE metabolically converted from the PS analogue. We surmise that the high fraction of accessible PE analogue may partly mask the difference between both cell lines.

Outward Redistribution of C6-NBD-PC Is Not Affected by Expression of ABCA1-GFP—Next, we examined the outward transport of C6-NBD-PC in ABCA1-GFP expressing and control MDCKII cells. To this end, cells were incubated with C6-NBD-PA. This lipid analogue is partially converted to C6-NBD-diacylglycerol, which rapidly crosses the plasma membrane and becomes available for intracellular synthesis of C6-NBD-PC (23). Transport of the newly synthesized analogues to the cell surface was measured at 15 °C where vesicular traffic is blocked (25). After incubation of cells (passage 1) with C6-NBD-PA for 120 min at 15 °C and removal of NBD lipid analogues from the exoplasmic leaflet (see “Experimental Procedures”), cells were incubated for a further 30 min at 15 °C in the presence of BSA. Less than 5% of C6-NBD-PC was extracted into the BSA medium with no significant difference between the ABCA1-GFP expressing cell line (3.62 ± 0.22%
We have also measured the amount of C6-NBD-PC extracted during a 3-h incubation of cells in the presence of BSA at 15 °C, following labeling with C6-NBD-PA in the presence of BSA. After this period, the amount of extracted PC analogue was on the order of about 50–60%. Again, no significant difference was seen between control and ABCA1-GFP expressing cells.

Notably, we detected only conversion to C6-NBD-PA into C6-NBD-PC and C6-NBD-PG. C6-NBD-PA may also be converted metabolically to other lipid analogues, e.g. to C6-NBD-PE. However, under our conditions synthesis of the PE analogue was beyond the level allowing quantitative detection.

C6-NBD-PS Outward Redistribution Is Also Enhanced in ABCA1-GFP Expressing HeLa Cells—
The outward redistribution of C6-NBD-PS was also studied in HeLa cells by BSA extraction as described (see above). Similar to MDCKII cells, we found an enhanced exposure of the fluorescent PS analogue in ABCA1-GFP expressing cells compared with control cells (transfected with irrelevant plasmid but the same vector) (Fig. 3B).

No enhanced outward redistribution was found for HeLa cells expressing the mutant form of ABCA1-GFP with impaired ATPase activity because of mutation of the lysine residue in the consensus motif in both ATP binding folds (for details of the mutant, see Ref. 6) (Fig. 3B). The fraction of cells expressing mutant ABCA1-GFP at a high level was similar to that of wild type ABCA1-GFP expressing HeLa cells (see above). Furthermore, mutation did not affect trafficking and localization of the protein as verified by confocal microscopy (data not shown).

ABC1A-expressing Cells Expose More Endogenous PS on the Cell Surface than Controls—Exposure of endogenous PS in MDCKII and HeLa cells (passage 0) was tested by flow cytometry. PS present on the exoplasmic plasma membrane leaflet of cells was detected by labeling with APC-annexin V, a high affinity PS-binding protein (27). To exclude damaged cells, co-labeling with propidium iodide was performed. Regions were set to exclude subcellular particles, and only single cells were counted (10,000 cells/sample). For propidium iodide staining, an additional gate was set. Usually, about 5–10% of cells were excluded because of staining with propidium iodide. No difference was observed between control and ABCA1-GFP expressing cells compared with propidium iodide staining.

### Fig. 5.

**Effect of Ca²⁺ and glyburide treatment (A) and temperature (B) on C6-NBD-PS outward redistribution.** Control and ABCA1-GFP expressing MDCKII cells were labeled with C6-NBD-PS on ice and incubated for 30 min at 20 °C to allow internalization of analogues. Glyburide (200 μM), A23817 (0.5 μM) and Ca²⁺ (2 mM) (final concentrations) were added 20 min after starting the incubation at 20 °C and were present during all further incubations. Control cells were incubated without treatment (A, no treatment). For details see "Experimental Procedures." The ratio of the amount of extracted analogues between ABCA1-GFP expressing cells and control cells is shown. All treatments did not affect exposure of PS in control cells compared with non-treated controls. Data represent means ± S.E. of at least three independent experiments in duplicate.

### Fig. 6.

**C6-NBD-PE outward transport in ABCA1-GFP expressing MDCKII cells.** Control and ABCA1-GFP expressing cells were labeled with C6-NBD-PS (A) or C6-NBD-PE (B) on ice and incubated for 30 min at 20 °C to allow internalization of analogues. After removal of remaining analogues on the exoplasmic leaflet, cells were incubated in the presence of BSA at 37 or 15 °C. After 30 min, the amount of extracted PE analogues was measured. During incubation intracellular C6-NBD-PS is partially converted to C6-NBD-PE (A). For details see "Experimental Procedures" and "Results." The ratio of the amount of extracted analogues between ABCA1-GFP expressing cells and control cells is shown. Data represent means ± S.E. of at least three independent experiments in duplicate.

(n = 2)) and control cells (3.38 ± 0.55% (n = 2)).

We have also measured the amount of C6-NBD-PC extracted during a 3-h incubation of cells in the presence of BSA at 15 °C, following labeling with C6-NBD-PA in the presence of BSA. After this period, the amount of extracted PC analogue was on the order of about 50–60%. Again, no significant difference was seen between control and ABCA1-GFP expressing cells.

Notably, we detected only conversion to C6-NBD-PA into C6-NBD-PC and C6-NBD-PG. C6-NBD-PA may also be converted metabolically to other lipid analogues, e.g. to C6-NBD-PE. However, under our conditions synthesis of the PE analogue was beyond the level allowing quantitative detection.

### C6-NBD-PS Outward Redistribution Is Also Enhanced in ABCA1-GFP Expressing HeLa Cells—
The outward redistribution of C6-NBD-PS was also studied in HeLa cells by BSA extraction as described (see above). Similar to MDCKII cells, we found an enhanced exposure of the fluorescent PS analogue in ABCA1-GFP expressing cells compared with control cells (transfected with irrelevant plasmid but the same vector) (Fig. 3B).

Apart from being converted into C6-NBD-PC, C6-NBD-diaclylglycerol has been shown to be metabolized to C6-NBD-PE (22). However, we observed that the amount of synthesized C6-NBD-PE was about 3–4 times lower with respect to the PC analogue, and we were not able to detect extraction C6-NBD-PE by BSA. If any extraction has been occurred, it was beyond the detection level of our approach.
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FIG. 7. ABCA1-GFP expressing cells expose elevated amounts of endogenous PS. Flow cytometric analysis of APC-annexin V binding to the cell surface of ABCA1-GFP expressing and control MDCKII and HeLa cells. A and B, the percentage of the APC-annexin V fluorescence intensity associated with control (100%, white bars), low or no ABCA1-GFP (gray bars), and high ABCA1-GFP expressing (black bars) MDCKII (A) and HeLa cells (B) is shown. Cells were co-labeled with APC-annexin V and the membrane-impermeable nucleic acid stain propidium iodide as described under “Experimental Procedures” and the legend to Fig. 1. Cells showing elevated propidium iodide staining were excluded. 10,000 cells were counted per sample. GFP fluorescence was measured simultaneously and revealed two populations, a high (GFP-high) and a low or no ABCA1-GFP expressing fraction (GFP-low) (see Fig. 1). C, no difference in APC-annexin V binding was observed between control (white bar) and HA-GFP expressing MDCKII cells (diagonal bar) (mean ± S.D. of two independent experiments).

FIG. 8. Receptor-mediated and fluid-phase endocytosis in ABCA1-GFP expressing MDCKII cells. Control and ABCA1-GFP expressing cells were incubated with TR-transferrin (A) or TMR-dextran (B) for 30 min at 37 °C with or without glyburide (200 μM). Cells were then fixed for fluorescence microscopy. Uptake of fluorescent markers was quantified by measuring intracellular fluorescence using the software Metamorph. For details see “Experimental Procedures.” The amount of internalized marker of control cells (no treatment) was set to 100% for each experiment. The means ± S.E. of at least three independent experiments is shown.

ABCA1-GFP expressing cells in comparison to control cells (Fig. 8A). Reduced endocytosis cannot be explained by a lower binding of fluorescent transferrin to the plasma membrane of ABCA1-GFP expressing cells. The amount of transferrin bound to the plasma membrane after incubation for 30 min on ice was similar to control and ABCA1-GFP expressing cells (not shown). In the presence of glyburide (200 μM), uptake of transferrin increased to the level of control cells, which was not affected by the inhibitor.

To assess fluid-phase endocytosis, cells were incubated with a tetramethylrhodamine-labeled high molecular weight dextran for 30 min at 37 °C. Similar to transferrin, uptake of dextran was significantly reduced (ANOVA, p < 0.05) in ABCA1-GFP expressing cells (Fig. 8B). Addition of glyburide raised the amount of internalized dextran to that of control cells in the absence of glyburide. However, in contrast to receptor-mediated endocytosis, glyburide caused a dramatic decrease of dextran uptake by about 50% for control cells in all experiments (n = 3). As a result, internalization of dextran in the presence of glyburide was about 1.5 times higher in ABCA1-GFP expressing cells than in controls (Fig. 8B).

**DISCUSSION**

Involvement of ABCA1 in Transport of Amino Phospholipids across the Plasma Membrane—In this study, we have found that expression of ABCA1-GFP promotes the exposure of fluorescent amino phospholipid analogues and endogenous PS in the plasma membrane of MDCKII cells and HeLa cells. Upon labeling of the cytoplasmic leaflet with C6-NBD-PS or -PE, ABCA1-expressing cells showed significantly increased outward transport of both lipid analogues compared with

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control cells at 37 °C. Likewise, exofacial C6-NBD-PE originating from intracellularly metabolized C6-NBD-PS was enhanced in ABCA1-GFP expressing cells. No influence of inhibitors of MDR1 Pgp and MRP1 was observed precluding any role of those ABC transporters in exposure of amino phospholipid analogues.

An elevated appearance of amino phospholipid analogues on the surface of ABCA1-GFP expressing cells was also observed at 15 °C when vesicular transport is inhibited in MDCKII cells (25). This confirms that the increased accessibility of amino phospholipid analogues to BSA in ABCA1-GFP expressing cells is associated with an enhanced outward redistribution of analogues in the plasma membrane. However, these data do not preclude that ABCA1 affects both intracellular vesicle transport (to the plasma membrane) and transbilayer organization of lipids in subcellular membranes (29) which may contribute to/interfere with the increased exposure of amino phospholipid analogues at the cell surface of ABCA1-GFP expressing cells at 37 °C and to differences in the temperature dependence of lipid exposure observed between C6-NBD-PE and C6-NBD-PS.

In agreement with our results on the surface exposure of C6-NBD-PS and consistent with a role of ABCA1 in the transport of endogenous PS, binding of APC-annexin V to the cell surface of ABCA1-expressing cells was significantly higher than binding to control cells. The dependence of PS exposure on ABCA1-GFP expression was confirmed by two independent observations. First, the amount of exofacial C6-NBD-PS and endogenous PS correlated with the expression of ABCA1-GFP. With increasing number of cell passages, the level of ABCA1-GFP expression decreased. This was accompanied by a reduction of C6-NBS-PS exposure almost to the level of control cells (6, 11, 14). For example, an enhanced binding of fluorescent annexin V was found in nontransfected RAW264.7 cells after induction of ABCA1 expression by cAMP analogues, indicating an increased exposure of endogenous PS on the exoplasmic leaflet of those cells (11).

The exposure neither of C6-NBD-PS nor of endogenous PS was reduced in ABCA1-expressing cells upon treatment of cells with glyburide. Glyburide has been found to inhibit ABCA1-mediated efflux of phospholipids and cholesterol to apoA-I even at a lower concentration (100 μM) as used in our study (6, 11, 13, 30). However, although several studies have shown that the level of exofacial PS is elevated in ABCA1-GFP expressing cells, it has not been reported to our knowledge that the exposure of PS is inhibited by glyburide. A reasonable explanation to this different influence of glyburide on lipid transfer may be provided by a recent study (26) that has shown that lipid transport activity and docking properties of ABCA1 are governed by topographically distinct domains. Glyburide might affect the docking side of ABCA1 and thereby the lipid efflux to apoA-I, but not its lipid transport activity.

Involvement of ABCA1 in Transport of PC across the Plasma Membrane—Precursor labeling of cells with C6-NBD-PA at 37 °C permitted us to investigate the transport of the newly synthesized C6-NBD-PC from the cytoplasmic to the exoplasmic plasma membrane leaflet. By using the same experimental approach, it has been shown that the ABCB1 (MDR1 Pgp) mediates a transport from the cytoplasmic to the exoplasmic leaflet of the C6-NBD-PC analogue (17, 20). For ABCA1-GFP expressing cells, we did not observe an enhanced outward redistribution of the PC analogue with respect to control cells. Thus, it is unlikely that ABCA1 functions as a PC transporter, at least of the PC analogue used. We cannot preclude that endogenous PC is transported by ABCA1. Recently, it has been suggested that ABCB1 and ABCB4 are involved in cell surface translocation of natural PC (31). However, these transporters have been shown to recognize NBD analogues of PC as well.

Furthermore, our results do not preclude an ABCA1-mediated transport of PC upon binding of apoA-I to ABCA1. This could be caused either by switching or by broadening of the lipid specificity of the transport activity upon apoA-I binding. An alternative hypothesis is that ABCA1 facilitates the transfer of PC from the exoplasmic leaflet to apoA-I. A similar function has been proposed for half-size ABC transporters ABCG5 and ABCG8. Small (32) suggested that the heterodimer ABCG5-ABCG8 may facilitate the exposure of membrane-bound cholesterol to bile salts. Thus, ABCA1 might present PC to apoA-I in an ATP-dependent manner. Once apoA-I complexes have been formed, cholesterol is taken up from the plasma membrane by these complexes. Previously, it has been shown that the transfer of phospholipids and cholesterol from cells to apoA-I represents different pathways. Although the transfer of phospholipids was dependent upon ABCA1 and sensitive to glyburide, cholesterol efflux was independent of functional ABCA1 (30). Furthermore, phospholipid transfer preceded the binding of cholesterol to apo-A-I.

Lipid-transporting ABC Transporters—Taken together, our results support the hypothesis that ABCA1 may function as a lipid transporter specifically recognizing amino phospholipids. Other members of the ABC transporter family have been identified in mammalian cells as mediators of phospholipid outward transport, differing in lipid specificity. ABCB4 (MRD3) and its mouse homologue (mdr2 Pgp) transport PC (31, 33–37). In contrast, ABCB1 (MDR1 Pgp), giving rise to a frequent form of pleiotropic resistance in tumor cells, transports various lipid analogues (18, 23, 36, 38) and probably also the endogenous lipids platelet-activating factor (a short-chain PC), phosphatidylcholine, phosphatidylserine, and glucosylceramide (18, 31, 39, 40) from the cytoplasmic to the exoplasmic plasma membrane leaflet. ABCC1 (MRP1), belonging to the ABCB family, was reported to mediate outward transport of C6-NBD-PC and -PS in murine erythrocytes (41, 42) and NBD-sphingolipids in epithelial cells (43). At the present state, ABCA1 seems to belong to a class of ABC lipid transporters of a rather high specificity, recognizing amino phospholipids. However, to provide convincing evidence for lipid transport activity of ABCA1 as well as for its putative lipid specificity, functional studies on reconstituted proteoliposomes are required. ABCA1 reconstituted into giant liposomes with a distinct lipid composition and a marginal degree of membrane curvature similar to that of a cell, avoiding the generation of high lateral pressure that could potentially inhibit lipid transport, might resemble the system of choice to adequately address its lipid transport activity (18, 44).

Role of ABCA1 in Endocytosis—Recently, Zha et al. (14) have shown that receptor-mediated endocytosis is up-regulated in homozygous Tangier fibroblasts in comparison to control cells. In addition, the authors (14) also observed enhanced receptor-mediated endocytosis upon glyburide treatment of control fibroblasts with functional wild type ABCA1 when studying uptake of fluorescent transferrin. These results were rationalized in the frame of a model originally developed by Devaux (45, 46) and supported by subsequent experimental studies (47–49). According to the model, invaginations of biological membranes can originate from transshlaver transport of lipids, leading to a difference of surface area between both membrane monolayers and, because of the very low compressibility of lipid membranes, finally to bending of the membrane. For example, such
a difference of surface area could be built up by the rapid and efficient transport of amino phospholipids, in particular of phosphatidylserine, from the exoplasmic to the cytoplasmic leaflet of the plasma membrane of eukaryotic cells by the amino phospholipid translocase (50). Thus, this directed transport could be an essential determinant of endocytosis. Indeed, knocking out of putative lipid transporters of the plasma membrane would reduce the surface area difference between monolayers and counteract invagination of the plasma membrane.

Here we have re-investigated the endocytic activity of control and ABCA1-GFP expressing cells. Following the protocol of Zha et al. (14), we observed a significantly reduced receptor-mediated endocytosis of fluorescent transferrin in ABCA1-GFP expressing cells in comparison with controls. Moreover, the level of endocytosis of ABCA1-GFP expressing cells increased to the level of control cells upon treatment with glyburide. Although these data are in agreement with those of Zha et al. (14), they cannot be explained straightforward in the frame of the model outlined above because we did not find an inhibition of PS transport in glyburide-treated ABCA1-GFP expressing cells. Our data do not necessarily contradict that such a model might be applicable to explain the influence of ABCA1 on endocytosis. Apart from its lipid transport activity, ABCA1 might modulate endocytosis by different yet unknown modes, at least one of them being sensitive to glyburide. Even though, at the present stage it cannot be excluded that glyburide affects endocytosis by various pathways as illustrated by our results on fluid-phase endocytosis. Again in agreement with the work of Zha et al. (14), we found that the reduced fluid-phase endocytosis in ABCA1-expressing cells increased upon treatment with glyburide to the level observed for control cells in the absence of glyburide. However, treatment of glyburide caused a significant decrease of this endocytic activity in control cells. Unfortunately, Zha et al. (14) did not report on the influence of glyburide on fluid-phase endocytosis in their cell systems. Nevertheless, our results clearly show that glyburide may differentially affect the various endocytic pathways of eukaryotic cells. Finally, this has to be emphasized that endocytosis phenotype and consequences of glyburide treatment may depend on the cell type and consequences of the ABCA1-dependent sides of the endocytotic machinery are sensitive to glyburide. Indeed, at the present stage we are inclined to explore how ABCA1 can influence endocytosis, as well as which ABCA1-dependent sides of the endocytotic machinery are sensitive to glyburide. This issue has not been studied by the authors (14), we found that the reduced fluid-phase endocytosis in ABCA1-GFP expressing cells. Following the protocol of Zha et al. (14) proposed that transport of PS by ABCA1 in the fibroblast cell system used by Zha et al. (14) would reduce the surface area difference between monolayers and counteract invagination of the plasma membrane.
