Phosphorylation by Protein Kinase Cassette A1 Transporter*

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In a previous characterization of the ABCA subfamily of the ATP-binding cassette (ABC) transporters, we identified potential protein kinase 2 (CK2) phosphorylation sites, which are conserved in eukaryotic and prokaryotic members of the ABCA transporters (Peelman, F., Labeur, C., Vanloo, B., Roosbeek, S., Devaud, C., Duverger, N., Denefle, P., Rosier, M., Vandekerckhove, J., and Rosseneu, M. (2003) J. Mol. Biol. 325, 259–274). These phosphorylation residues are located in the conserved cytoplasmic R1 and R2 domains, downstream of the nucleotide binding domains NBD1 and NBD2. To study the possible regulation of the ABCA1 transporter by CK2, we expressed the recombinant cytoplasmic domains of ABCA1, NBD1+R1 and NBD2+R2. We demonstrated that in vitro ABCA1 NBD1+R1, and not NBD2+R2, is phosphorylated by CK2, and we identified Thr-1242, Thr-1243, and Ser-1255 as the phosphorylated residues in the R1 domain by mass spectrometry. We further investigated the functional significance of the threonine and serine phosphorylation sites in NBD1 by site-directed mutagenesis of the entire ABCA1 followed by transfection into HeK-293 Tet-Off cells. The ABCA1 flippase activity, apolipoprotein AI and AII binding, and cellular phospholipid and cholesterol efflux were enhanced by mutations preventing CK2 phosphorylation of the threonine and serine residues. This was confirmed by the effect of specific protein kinase CK2 inhibitors upon the activity of wild type and mutant ABCA1 in transfected Hek-293 Tet-Off cells. The activities of the mutants mimicking threonine phosphorylation were close to that of wild type ABCA1. Our data, therefore, suggest that besides protein kinase A and C, protein kinase CK2 might play an important role in regulating the function and transport activity of ABCA1 and possibly of other members of the ABCA subfamily.

The role of the ABCA1 transporter, a member of the subfamily A of the ATP binding cassette transporters, in the efflux of cellular phospholipids and cholesterol has become well established (2). Several studies have linked ABCA1 mutations to impaired cellular cholesterol and phospholipid efflux characteristics of Tangier disease and high density lipoprotein-deficiency patients (3–5). Expression of WT and mutant ABCA1 in cultured cells demonstrated the correlation between the level of expression and activity of the ABCA1 transporter, the extent of binding to the apolipoprotein AI (apoAI) acceptor and the efflux of cellular lipid (6).

Human ABC transporters consist of a cytoplasmic nucleotide binding domain (NBD), which binds and hydrolyzes ATP, and of a membrane-spanning domain through which the substrate is translocated (7–8). Besides these elements, regulatory domains with putative phosphorylation sites were described in several human transporters (7). We carried out an extensive analysis of the subfamily A of the ABC transporters and showed that this subfamily consists of 13 human ABCA and of many eukaryotic and prokaryotic ABCA homologues (1). Multiple alignments of the subfamily A transporters demonstrated the high degree of sequence conservation and the particular topology of this subfamily. In the ABCA subfamily, the intracellular N-terminal residues are followed by a first transmembrane helix, by a long extracellular loop, and by a downstream cluster of five transmembrane helices separated by short loops. The transmembrane domain is followed by a cytoplasmic nucleotide binding domain NBD1 and by a downstream sequence of 80 conserved residues. In the full ABCA transporters, which have a high internal symmetry, these elements are repeated in the second half of the transporter (1). Among the human transporters of the ABCA subfamily, mutations in ABCA1 have been linked to Tangier disease, whereas mutations in ABCA2 impair the transport of retinylidene and cause retinal degeneration (9). In both transporters, mutations in the nucleotide binding domains decrease the ATPase activity and thereby impair the transport activity of the mutants. Point mutations in the ABCA1 and ABCA2 extracellular loops probably affect the three-dimensional structure of the transporters and decrease their association with acceptor proteins required for substrate export (10).

The 80-residue segments downstream of the NBDs are conserved in the eukaryotic ABCAs and in the prokaryotic subfamily 7 precursors (1). In ABCA1 none of the natural mutations causing either Tangier disease or familial hypo-α-

C, respectively; CK2, protein kinase 2; HeK, human embryonic kidney; PS, phosphatidylserine; PE, phosphatidylethanolamine; GFP, green fluorescent protein; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline.

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lipoproteinemia occur in these sequences, although one of the Tangier mutations is located close to this domain (11). The functional significance of the conserved sequences downstream of the NBDs is still unknown; they might have a regulatory function, as proposed for other transporters (12). Phosphorylation by protein kinase A or C has been demonstrated for several ABC transporters, including cystic fibrosis transmembrane conductance regulator (13). β-glycoprotein (14), and recently ABCA1 (15–17). According to See et al. (15) serines 1042 and 2054 in NBD are phosphorylated by PKA in vitro and may potentially regulate ABCA1 function (15). Yamauchi et al. (16) report that apoAI activates PKC, resulting in phosphorylation and stabilization of ABCA1 (16). According to Martinez et al. (17), the potential phosphorylation sites for protein kinase 2 (CK2) and PKA in the ABCA1-PEST sequence, threonine 1286 and 1305, respectively, are constitutively phosphorylated and protect ABCA1 against calpain-mediated degradation. PKA and CK2 inhibitors did not, however, alter phosphorylation of these residues.

Among the kinases, protein kinase 2 or CK2 is a constitutively active Ser/Thr protein kinase essential for cell viability (18). CK2 can target at least 200 proteins (19) and is implicated in a wide variety of cellular functions such as RNA and rRNA synthesis, apoptosis, and cell survival and transformation (20). The CK2 phosphoacceptor sites consist of clusters of acidic residues located mostly downstream from phospholipid aeryl or threonyl residues. Prediction of consensus sequence motives in human ABCA1 using PROSITE identified 39 potential CK2 phosphorylation sites in ABCA1. Among these, the 1242TTLEE and 2211TTLDQ motives within the 80-residue R1 and R2 domains, downstream of, respectively, NBD1 and NBD2, were conserved in most members of the human ABCA subfamily (1). Another potential CK2 phosphorylation site, 1525SGVD, is conserved in ABCA4, ABCA6–10, and ABCA12 but is absent in all NBD2s. Thus, it is conceivable that modulation of the ABCA1 transport activity might occur through serine or threonine phosphorylation by protein kinase CK2.

In this report we demonstrate that in vitro the NBD1-R1 and not the NBD2-R2 domain of ABCA1 is phosphorylated by CK2, and we used mass spectrometric methods to directly identify Thr-1243, Thr-1245, and Ser-1255 as the phosphorylated residues. We further investigated the functional significance of these CK2 phosphorylation sites in NBD1-R1 by site-directed mutagenesis of ABCA1. The ABCA1 flippase activity, the transporter ability to bind apolipoproteins AI and cholesterol, was enhanced by mutations preventing phosphorylation at these sites. The specific CK2 inhibitors 4,5,6,7-tetrabromobenzotriazole and apigenin also enhanced the activity of ABCA1 transfected into Hek-293 Tet-Off cells, thus confirming the effect of the mutations. Our data, therefore, suggest that besides PKA and PKC, protein kinase CK2 might play an important role in vivo in regulating the function and transport activity of ABCA1 and possibly other members of the ABCA subfamily.

**EXPERIMENTAL PROCEDURES**

**Materials**—Toylphenylalanine chloromethyl ketone-treated trypsin, barium hydroxide, and α-cyano-4-hydroxycinnamic acid were from Sigma. Other mass spectrometry reagents and solvents were of highest purity from Carlo Erba (Milan, Italy). NBD-labeled cholesterol, phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylcholine were purchased from Aventi Polar lipids (Alabaster, Alabama). Rhodamine hydrochloride and concanavalin A Alexa Fluor 488 were obtained from Molecular Probes (Leiden, the Netherlands). Doxycycline was purchased from Sigma. Imetyl-[3H]Choline and [7-3H]cholesterol were purchased from Amersham Biosciences. Hek-293 Tet-Off cells were purchased from Clontech (Erembodegem, Belgium). Recombinant apoAI was expressed in Escherichia coli (21), and plasma apoAII was prepared by ultrafiltration of isolation of high density lipoprotein, ultracentrifugation, and purification by DEAE chromatography as previously described (22). The CK2 inhibitor 4,5,6,7-tetrabromobenzotriazole was a gift of Dr. Shugur (Poland). Apigenin in Me2SO was purchased from Sigma.

**Construction of the Expression Vectors**—For expression of the His-NBD1-R1 domain between residues 861 and 1306 of His-NBD2-R2 between residues 1287 and 2222, vectors were constructed using the pET28α plasmid (Novagen, Madison, WI). DNA fragments encoding the protein sequences were PCR-amplified and cloned into the pET28α vector, and mutant plasmids were made by overlapping PCR. WT and mutant plasmids were checked by sequencing and by restriction analysis.

**In Vitro Phosphorylation Assays**—Recombinant CK2 and its α and β-subunits were expressed in E. coli and purified to homogeneity (25). For electrophoretic experiments, 2 or 8 μg of recombinant NBD1-R1, NBD2-R2, or mutants were incubated at 22 °C with either γ-[32P]ATP or 10 μM ATP and 10 μM MgCl2 in the presence of 0.3 μg of oligomeric CK2 or 1.0 μg of CK2α. The phosphorylated proteins were analyzed by SDS-PAGE and autoradiography after treatment with 5 M Ba(OH)2 for mass spectrometric analysis, recombinant NBD-R proteins were incubated with CK2 for 30 min at 30 °C in the presence of 0.2 μM ATP. The reaction was terminated by 5 min of boiling and drying the samples.

**Mass Spectrometric Analysis of the Phosphopeptides**—Purified NBD-R proteins before and after treatment with CK2 were subjected to reduction with an excess of dithiothreitol, alkylation using iodoacetamide, and digestion with trypsin. To obtain the corresponding alkene moiety from the phosphate esters, the β-elimination reaction was optimized and simplified by incubating the peptide mixture in 5 M barium hydroxide at 37 °C for 90 min under nitrogen. Solid carbon dioxide was then added to eliminate the precipitated barium carbonate, which was removed by centrifugation at 13,000 × g for 5 min (18).

MALDI mass spectra were recorded using a Applied Biosystems Voyager DE-PRO instrument operating in reflector mode. A mixture of the analyte and α-cyano-4-hydroxycinnamic acid (10 mg/ml) in acetonitrile, ethyl alcohol, 0.1% trifluoroacetic acid (1:1:1, v/v/v) was applied to the MALDI target plate in a well-defined pattern. Mass calibration was performed with insulin at 5734.5 Da and a matrix peak at 379.3 Da as internal standards. Raw data were analyzed by the software provided by the manufacturer and reported as average masses.

**Cell Cultures**—Hek-293 Tet-off cells were cultured in DMEM media plus 10% fetal bovine serum (FBS), 1-glutamine, and Geneticin on 12-well plates coated with poly-D-lysine. Cells were transfected for 24 h with the GFP-ABC1 constructs using a mixture of polyethylenimine and DNA at a 2/1 ratio, and total DNA charge, ratio and subsequently grown for 24 h. Cells were washed twice with phosphate-buffered saline (PBS) and incubated for 1 min with 500 μl of trypsin (Invitrogen). After the addition of 1 ml of PBS, 5% FBS and 0.05 mM EDTA, cells were filtered and kept on ice for flow cytometry. The percentage of transfected cells was determined by fluorescence-activated cell sorter.
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Analysis on a FACScalibur (BD Biosciences) with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

**Measurement of the ABCA1 Flippase Activity**—Mock- and ABCA1-transfected Hek-293 Tet-Off cells were incubated at 37 °C for 45 min with 5 μM NBD lipid, washed with PBS at 4 °C, and incubated with 64 μM sodium dithionite in a Tris-HCl buffer, pH 10, for 5 min at 4 °C. The fluorescence intensity of the NBD probe before (I_max) and after (I_q) dithionite quenching was measured on a Bio-Tek FLX 800 microplate reader with excitation and emission wavelengths of 485 and 528 nm, respectively. Percent quenching was calculated as \( \frac{I_{\text{max}} - I_q}{I_{\text{max}}} \times 100 \).

Similar measurements were performed in the presence of 50 μM 4,5,6,7-tetrabromobenzotriazole and apigenin CK2 inhibitors on Mock-transfected and Hek-293 cells transfected with WT ABCA1 and with the T1242A, T1243A, T1242A/T1243A, and T1242D/T1243D ABCA1 mutants.

**Monitoring of ApoAI and -AII Binding to Hek-293 Tet-Off Cells**—After transfection with WT ABCA1, cells were washed twice with a 10 mM HEPES buffer, pH 7.4, containing 1.8 mM CaCl_2, 1 mM MgCl_2, 5 mM KCl, 150 mM NaCl and incubated with carboxyrhodamine-labeled recombinant apoAI or -AII at concentrations between 0 and 50 μg/ml for 1 h at 4 °C to monitor extent of binding. In subsequent experiments cells transfected with WT and mutant ABCA1 were incubated with 20 μg/ml apoAI or -AII for 1 h at 4 °C. Cells were detached by mild trypsinization for 2 min at 37 °C using 0.05 g/liter trypsin in PBS and kept on ice, and flow cytometric recordings were performed. Cells were subdivided into three populations as a function of their level of ABCAI expression, reflected by the GFP fluorescence intensity. Apoprotein binding data were calculated for each cell population based upon the mean carboxyrhodamine fluorescence intensity (27).

**Phospholipid and Cholesterol Efflux to ApoAI and ApoAII Induced by ABCA1**—Phospholipid and cholesterol efflux were measured using both radioactivity and fluorescence. For radioactive measurements, Hek-293 Tet-Off cells transfected with WT and mutant ABCA1 were washed twice with DMEM and incubated either with 1 μCi/ml [methyl-3H]choline or [3H]cholesterol in DMEM plus 2.5% FBS. After 24 h cells were washed twice with DMEM and incubated with 1 ml of DMEM plus 0.2% bovine serum albumin for 16 h at 37 °C. Cells were washed twice with DMEM and incubated at 37 °C with 20 μg/ml recombinant apoAI for 4 h.

![Figure 1](image-url)  
**Figure 1.** Topology of the N-terminal half of an ABCA full-transporter. Six transmembrane helices are followed by the cytoplasmic NBD and the conserved regulatory R domain. The C-terminal half has an identical topology. The alignment of the R1 and R2 domains showing conservation of the potential CK2 phosphorylation sites at residues Thr-1242, Thr-1243 and Ser-1255 are shown for several species of ABCA1.
for [methyl-\(^{3}H\)]nicotinol efflux or 6 h for [\(^{3}H\)]cholate efflux. Supernatant was collected and centrifuged for 30 min at 810 \(\times g\). 500 \(\mu l\) of supernatant was transferred into 5 ml of scintillation liquid (Optiphase Hisafe 3). Cells were washed with PBS and lysed with 1 ml of NaOH (0.1 N). 500 \(\mu l\) of cell suspension was transferred into 5 ml of scintillation liquid (Optiphase Hisafe 3). 12 \(H\) counts per minute (cpm) were measured in a liquid scintillation counter (Wallace 1409). % lipid efflux was calculated as (cpm in supernatant - cpm in control) \times 100. For measurements using fluorescence, Hek-293 Tet-Off cells transfected with WT and mutant ABCA1 were labeled during NBD fluorescence intensity. Percentage efflux was calculated as (integrated fluorescence intensity at 37 \(\degree C\) for NBD phospholipids or after 6 h for NBD cholesterol by measuring NBD fluorescent intensity. Percentage efflux was calculated as \((I_{\text{control}} - I_{\text{apoAII}})/I_{\text{apoAII}}) \times 100\).

Confocal Microscopy—Cellular localization of the expressed ABCA1 WT and mutants was analyzed by confocal microscopy using GFP fluorescence at excitation and emission wavelengths of, respectively, 488 and 509 nm. Hek-293 Tet-Off cells were plated on poly-l-lysine-coated Lab-Tek® German borosilicate confocal microscopy slides at a density of 2.5 \(\times 10^{4}\) cells/chamber in 400 \(\mu l\) of DMEM plus 10% FBS, grown for 48 h in DMEM plus 10% FBS and washed twice with PBS. Cells were incubated with 25 \(\mu g/ml\) concanavalin A Alexa fluor 633, an endoplasmic reticulum-specific fluorescence dye, for 30 min. Cells were washed with PBS, and the Alexa fluor 633 fluorescence was measured using the excitation and emission wavelengths of, respectively, 632 and 647 nm.

ABCA1 Half-life Measurement—To determine the half-life of WT and mutant ABCA1, the transporter flipase activity was measured as a function of time in transfected Hek-293 Tet-Off cells, which were treated with 100 \(\mu g/ml\) doxycycline to stop transcription of the ABCA1 function of time in transfected Hek-293 Tet-Off cells, which were treated with 100 \(\mu g/ml\) doxycycline to stop transcription of the ABCA1 protein and in the entire ABCA1 construct. In both systems we engineered several mutants designed at preventing phosphorylation by mutation either of the target threonines and serine, T1242A, T1243A, T1242A/T1243A, T1242D, T1243D, S1255A, or of the downstream cluster of acidic residues, E1245Q, E1246Q, E1245Q/E1246Q. Mutants were also designed to mimic phosphorylated residues T1242D, T1243D, T1242D/T1243D.

All NBD1+R1 mutants were expressed as soluble proteins in E. coli and purified at yields comparable with WT NBD1+R1 (24). Using recombinant CK2, phosphorylation of the Thr-Ala mutants was not completely abolished, suggesting the presence of multiple CK2 phosphorylation sites within this domain (data not shown).

This was confirmed by MALDI mass spectrometric analysis of the native and phosphorylated recombinant NBD–R proteins (Table I). The phosphopeptides were identified by their mass differences due to the presence of phosphate moieties compared with the peptide masses expected from the protein sequence. In agreement with the phosphorylation assays, we observed no phosphorylation of NBD2+R2; in WT NBD1+R1 we observed that the signal at mz 4542.5 corresponds to the ABCA1 peptide 1229–1269, carrying three phosphate groups at Thr-1242, Thr-1243, and Ser-1255. The peptide mixture was then submitted to the \(\beta\)-elimination reaction followed by MALDI mass spectrometric analysis. Under strong alkaline conditions elimination of the phosphate moiety on Ser(P) and Thr(P) to form dehydroalanine (\(\Delta\)Ser) or dehydroalanine-2-butylcarboxylic acid (\(\Delta\)Thr) yields new signals corresponding to the \(\beta\)-eliminated peptides. The signal at mz 4249.5 was assigned to the 1229–1269 ABCA1 peptide, occurring 299 Da lower, thus confirming phosphorylation at the three positions. Analysis of the T1242A, T1243A, and T1242A/T1243A NBD1 mutants supports the loss of either one or two threonine phosphorylation sites in the mutated peptides, whereas the Ser-1255 peptide is preserved. Analysis of the S1255A mutant demonstrates the loss of one phosphorylation site in the Val-1251—Arg-1272 tryptic peptide (Table I). These data confirm that besides Thr-1242 and Thr-1243, Ser-1255 is another CK2 phosphorylation site in ABCA1. None of the predicted CK2 phosphorylation sites in the NBD1 (residues 866–995) or between NBD1 and the R1 domain was phosphorylated.

ABCA1 Activity Increases Linearly with Protein Expression—Hek-293 Tet-Off cells were transfected with varying amounts
of ABCA1-GFP DNA, between 1 and 10 μg. Extent of transfection was estimated as the product of the GFP fluorescence intensity and the number of transfected cells, both measured by fluorescence-activated cell sorter analysis (23). As a result of an increased flip-flop activity, the percentage of NBD PE in the plasma membrane outer leaflet increased linearly between 20 and 58% for a 5-fold increase of WT ABCA1 transfection (Fig. 3A). Parallel data were obtained for both apoAI binding to transfected cells (Fig. 3B) and phospholipid efflux (Fig. 3C). This linear relationship between ABCA1 expression and activity was used to normalize the activity of ABCA1 mutants compared with WT ABCA1.

**Mutation of the CK2 Phosphorylation Sites Increases ABCA1 Flipase Activity**—To assess the effect of CK2 phosphorylation on the ABCA1 flipase activity we measured phospholipid flip-flop by WT ABCA1 and the mutants. Incubation of ABCA1-transfected Hek-293 Tet-Off cells with NBD phospholipids at 37 °C and subsequent quenching of the fluorescence with sodium dithionite at 4 °C yielded the percentage of labeled phospholipid in the outer leaflet of the plasma membrane. In mock-transfected cells there was around 30 and 25% NBD PE and NBD PS, respectively, in the plasma membrane outer leaflet. After transfection with 3 μg of ABCA1-GFP DNA, these percentages increased to 38 and 35%, indicative of phospholipid flip-flop by ABCA1.

Compared with WT ABCA1, set as 100%, the PE and PS content in the outer leaflet of the plasma membrane increased up to 130 ± 14 and 123 ± 8% for the T1242A mutant, 154 ± 15 and 148 ± 11% for the T1243A mutant, 170 ± 15 and 207 ± 16% for the T1242A/T1243A mutant, 141 ± 11 and 136 ± 10% for the S1255A mutant, and 141 ± 13 and 120 ± 9% for the E1245Q/E1246Q mutant, respectively (Fig. 4). All measurements were performed in triplicate, and the differences were significant at p < 0.01 using a Student t test. In contrast, when the threonine residues were mutated to aspartic acid to mimic a phosphorylated residue, the phospholipid content in the membrane outer leaflet decreased compared with that of the Thr-Ala mutants and came closer to that of WT ABCA1 (Fig. 4).

The effect of the specific CK2 inhibitors 4,5,6,7-tetrabromobenzotriazole and apigenin on the flipase activity in Hek-293 Tet-Off cells with WT ABCA1 and with the T1242A, T1243A, T1242A/T1243A, S1255A, and T1242D/T1243D mutants was consistent with the above data. Incubation with 50 μM of either 4,5,6,7-tetrabromobenzotriazole or apigenin increased the flipase activity of WT ABCA1 by 30 ± 2.6 and 25 ± 3.4% (n = 3, p < 0.001), respectively, whereas the flipase activity increased only by around 5 ± 0.7% (n = 3, p < 0.01) in cells transfected with ABCA1 mutants.

**Mutations of the 1242TTLEE and 1255SGVD CK2 Phosphorylation Sites Modulate the Binding of ApoAI and ApoAII to ABCA1**—We compared the binding of carboxyrhodamine-labeled apoAI and apoAII to the Hek-293 Tet-Off cells transfected with WT and ABCA1 phosphorylation mutants by flow cytometric analysis (27). We analyzed apolipoprotein binding to three cell populations, with low, middle, and high levels of transfection, estimated from the GFP fluorescence, with cutoffs at >7 and <20 for population 1 (ABCA1+), >21 and <90 for population 2 (ABCA1++), and >91 for population 3 (ABCA1+++). Subtraction of the carboxyrhodamine fluorescence intensity of Mock-transfected cells yielded saturation curves, with a plateau above 1 μg/ml for apoAI/Rho and apoAII/Rho binding to WT ABCA1 (Fig. 5). Apparent binding constants to the three cell populations were 7, 30, and 24 × 10^{-8} M for apoAI binding compared with 4, 16, and 17 × 10^{-8} M for apoAII binding. These values are comparable with those measured for apoAI/Cy5 binding to WT ABCA1 (27).

The carboxyrhodamine fluorescence intensity due to specific binding of 20 μg/ml apoAI or apoAII to cells transfected with ABCA1 mutants was expressed relative to WT ABCA1 (Fig. 6). ApoAI- and apoAII-specific binding to the cells transfected with the single threonine and serine ABCA1 mutants increased...
compared with WT ABCA1. This increase was more pronounced for the T1243A mutation either alone or in combination with the T1242A mutation, whereas the effect of the T1242A and S1255A mutants was similar. The E1245Q/E1246Q mutation of the downstream acidic residues, which also impairs CK2 activity, similarly increased cellular apoAI and apoAII binding. The T1242D, T1243D, T1242D/T1243D mutations, which mimic CK2 phosphorylation, had an opposite effect to the Thr-Ala mutations, as apoprotein binding to cells transfected with these mutants amounted to around 110% of WT ABCA1.

**Mutation of the CK2 Phosphorylation Sites in ABCA1 Modulates Phospholipid and Cholesterol Efflux to ApoAI and ApoAII**—Phospholipid and cholesterol efflux to apoAI and apoAII together with phospholipid distribution across the plasma membrane were measured for Hek-293 Tet-Off cells transfected with WT ABCA1 and with the phosphorylation mutants. Data were normalized to account for differences in expression levels between WT ABCA1 and mutants. ABCA1 lipid efflux was measured using both radioactive-labeled and fluorescent-labeled lipids, yielding comparable data. Only the results for NBD-labeled lipids are given in Fig. 7, A and B.

Incubation of the transfected Hek-293 Tet-Off cells, labeled with NBD phospholipids or NBD cholesterol with either unlabeled apoAI or apoAII decreased the NBD lipid fluorescence intensity due to the efflux of labeled lipids to the unlabeled apoprotein acceptors. The saturation curves for the percentage of phospholipid efflux were similar to the apoprotein binding curves and reached a plateau around 20 μg/ml apoAI or apoAII. The percentages of NBD-labeled phosphatidylethanolamine and cholesterol efflux to apoAI and apoAI induced by WT ABCA1 were around 7 and 4%, respectively. Using radioactive-labeled PE and cholesterol, efflux percentages were, respectively, 8.5 and 4.7%, comparable with those measured using fluorescence. PE and PS efflux to apoAI and apoAII (Fig. 7A) and cholesterol efflux to apoAI (Fig. 7B) were expressed relative to WT ABCA1 for all mutants after normalization. The T1242A and T1243A mutations either alone or in combination and the E1245Q/E1246Q mutation, which all decrease CK2 activity, increased phospholipid and cholesterol efflux (Fig. 7, A and B). The effect
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**DISCUSSION**

In this paper we showed that besides PKA (15) and PKC (16–17), protein kinase CK2 is an important kinase to regulate ABCA1 activity. We further demonstrated that phosphorylation occurs at conserved threonine and serine residues in the R1 domain, downstream of NBD1. CK2-specific phosphorylation in the R1 domain affects the major functions of the ABCA1 transporter, including phospholipid flip-flop, ABCA1 binding to apoAI and apoAII, and cellular phospholipid and cholesterol efflux, in a coordinated manner. Mutations aimed at suppressing the phosphorylation sites increase these activities, whereas mutations of the CK2 phosphorylation sites have little effect upon the cellular trafficking of the ABCA1 transporter.

**ABCA1 WT and Mutants Have Similar Half-lives** — The decrease of ABCA1 flippase activity as a function of time was used to estimate the transporter half-life. In agreement with previous reports, we observed a short half-life of ~40 min for WT ABCA1 (30). All mutants had similar half-lives compared with wild type ABCA1, varying between 35 and 50 min (data not shown), indicating that mutations of the CK2 phosphorylation sites do not significantly affect the ABCA1 turnover.

**Cellular Localization of the ABCA1 WT and Mutants** — Using confocal microscopy, we observed the same cellular localization of WT and mutant ABCA1, which were mainly found at the plasma membrane of transfected Hek-293 Tet-Off cells. Incubation of the ABCA1-transfected cells with the endoplasmic reticulum-specific fluorescence dye concanavalin A Alexa fluor 633 showed that only a small percentage of ABCA1 WT and mutants is localized in the endoplasmic reticulum (Fig. 8). These results indicate that mutations of the CK2 phosphorylation sites do not significantly affect the ABCA1 turnover.

**Protein Kinase CK2 Phosphorylation Modulates ABCA1 Activity**

Using recombinant WT and mutant NBD1–R1 proteins, we further showed that they are in vitro substrates for CK2. Combined mutation of either the two threonines or the downstream acidic residues in the recombinant NBD1–R1 domain did not completely suppress protein phosphorylation. This is in agreement with the mass spectrometric identification of the CK2 phosphorylation sites in NBD1–R1, identifying residue Ser-1255 besides Thr-1242 and Thr-1243 as a putative ABCA1 phosphorylation site. This was then confirmed by the engineering of the S1255A mutant, whose activities are comparable with those of the T1242A mutant. Threonines 1242 and 1243 had been identified as some of the best conserved residues belonging to potential CK2 phosphorylation sites in cytoplasmic ABCA1 domains. These residues are conserved in most of the human and mammalian ABCA transporters and in several other species and even in family 7 bacterial transporters (1). The serine 1255 residue is not as highly conserved as the threonines among the different ABCA1 species or within the ABCA family. However, because serine is often the preferred substrate of CK2 (20), it might constitute a primary phosphorylation site in ABCA1, in analogy with the data obtained for PTEN phosphatidylinositol 3′-phosphatase (31). The alignment of the R1 and R2 domains shows the correspondence between the consensus CK2 phosphorylation sequence around residue Thr-1242 in R1 and residue Thr-2211 in R2. Threonine 2212 in NBD2 does not belong to a CK2 consensus sequence, whereas Ser-1255 in R1 has no counterpart in R2. As shown both by autoradiography and mass spectrometry, CK2 phosphorylation seems restricted to the N-terminal R1 domain of ABCA1. Because mutations of the CK2 phosphorylation sites and treatment with specific CK2 inhibitor both increase the ABCA1 flippase and cellular efflux activities as well as apoprotein binding, CK2 phosphorylation of the ABCA1 R1 domain might contribute to the in vivo regulation of ABCA1 functionality.

Although there is a high degree of sequence homology between the N- and C-terminal cytoplasmic domains of ABCA1, several reports suggest that NBD1 and NBD2 might have specific functions. There are differences between the NBDs ATPase activity, as we measured a 3-fold higher activity for recombinant ABCA1 NBD1 compared with NBD2 (24). Folding and association of the two NBD domains are not identical (32–33), and several reports suggest that NBD1 and NBD2 share a common interface in ABC full transporters (32–33). The activation cycle of the ABCA transporters consists of ATP binding and hydrolysis, ADP dissociation accompanied by con-
formational changes in the NBD domains, and finally, of substrate transport across the membrane. This process is not random but is probably initiated through ATP binding to the nucleotide binding domain with highest affinity (34). It is quite conceivable that phosphorylation of the R1 domain by CK2 could bring a conformational change to this domain and consequently to NBD1 to start the activation cycle.

The recent x-ray structure of the vitamin B12 transporter BtuCD (33) suggests that the two NBD domains interact with each other in an anti-parallel way and that these interactions are stabilized by interactions between the NBD downstream domains. The NBD downstream domains in the maltose transporter correspond to the conserved R1 and R2 domains identified in the ABCA subfamily. Interactions between R1 and R2 in the ABCA subfamily might enhance the cooperativity between the N- and C-terminal half of the ABCA transporters and, thus, contribute to a fine regulation of the transporter activity. These interactions are likely to be dependent upon the phosphorylation state of the protein domain. Serine and threonine phosphorylation by a cytoplasmic CK2 kinase might, thus, represent another mechanism for the intracellular regulation of the transporter activity.

Protein kinase CK2 is a constitutively active kinase that is implicated in a variety of cellular functions including cell proliferation, differentiation, and survival (19). Our experiments showed that the regulatory H9252 subunit of CK2 is required to achieve optimal phosphorylation of the ABCA1 substrate. Thus, this transporter belongs to the class I of the CK2 substrates whose phosphorylation is strictly dependent upon the presence of the CK2β subunit (35). On the other hand, we observed an increased functionality of the protein when its phosphorylation is abrogated, suggesting that CK2 acts as a down-regulator of ABCA1 function. A similar function of CK2 was previously described for Engrailed 2 secretion (36).

Regulation of ABCA1 might occur in different ways; phosphorylation might change the expression or cellular location of
PKA and PKC (13). Phosphorylation of the P-glycoprotein by transmembrane regulator, which is phosphorylated both by natural changes due to phosphorylation are, therefore, probably mutants of the NBD1 Trp exposure and the secondary structure of the various mu-

nine or by the glutamic acid to glutamine mutations (23). The and ATPase activity are not affected by the threonine to ala-

porter (16). PKC$_{\alpha}$ is activated by apoAI, and it subsequently phosphorylates and stabilizes ABCA1. According to Martinez et al. (17), a PEST sequence in ABCA1 is critical for apoAI protection against ABCA1 degradation by calpain. This sequence includes the casein kinase CK2 and PKA potential phosphorylation sites Thr-1286 and Thr-1305, respectively. These authors found little effect of the casein kinase inhibitors on ABCA1 phosphorylation, in contrast with our findings, and concluded that CK2 probably does not phosphorylate the Thr-1286 residue in the PEST sequence. Although this residue belongs to a potential CK2 phosphorylation site in ABCA1, it is localized in a low conservation sequence in the ABCA family and might not become phosphorylated, in contrast to the conserved Thr-1242, Thr-1243, and Ser-1255 residues tested in our study. Furthermore, we measured the effect of the CK2 inhibitors on the specific ABCA1 activity, whereas Martinez et al. (17) investigated the extent of total ABCA1 phosphorylation by autoradiography. Besides PKA and PKC, which increase ABCA1 activity, CK2 might play an opposite role and act as a down-regulator of ABCA1 in the cell.

ABCA1. This is, however, not likely as 1) our confocal micro-

scopic data showed similar locations at the plasma membrane, and 2) the expression of the Thr-Ala and Ser-Ala mutants was lower than of WT ABCA1. We also showed that ATP binding and ATPase activity are not affected by the threonine to alanine or by the glutamic acid to glutamine mutations (23). The Trp exposure and the secondary structure of the various mutants of the NBD1+R1 domain were quite similar. The structural changes due to phosphorylation are, therefore, probably minimal, and a likely effect of CK2 phosphorylation is, therefore, the modulation of the cooperativity between NBD1 and NBD2 in native ABCA1.

The activities of several ABC transporters are regulated by their phosphorylation, as demonstrated for the cystic fibrosis transmembrane regulator, which is phosphorylated both by PKA and PKC (13). Phosphorylation of the P-glycoprotein by PKA and PKC seems to modulate swelling-activated Cl$^-$ currents (14), not drug transport (37). PKA activation by cAMP analogues increased ABCA1 phosphorylation and cellular cholesterol efflux (15). Mutation of a PKA phosphorylation site in ABCA1 impaired apoAI-mediated release of cellular lipid (15). According to Yamauchi et al. (16) ABCA1 phosphorylation is, furthermore, critical for the stability and integrity of the trans-

FIG. 8. Cellular localization of GFP-tagged ABCA1 WT and mutants studied by confocal microscopy. Green fluorescence corresponds to the GFP fluorescence, and red fluorescence corresponds to the endoplasmic reticulum-specific probe concanavalin A Alexa fluor 633. Yellow fluorescence indicates the colocalization of the GFP-tagged ABCA1 and the endoplasmic reticulum. A, ABCA1 WT; B, T1243A mutant; C, T1242A/T1243A mutant; D, T1242D/T1243D mutant; E, S1255A mutant.

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