

ABCA1 mutants reveal an interdependency between lipid export function, apoA-I binding activity, and Janus kinase 2 activation

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Abstract ABCA1 exports cholesterol and phospholipids from cells by a multistep pathway that involves forming cell surface lipid domains, solubilizing these lipids by apolipoproteins, binding of apolipoproteins to ABCA1, and activating signaling processes. Here we used a mutational analysis approach to evaluate the relationship between these events. We prepared seven naturally occurring mutants and one artificial missense mutant of ABCA1 with varying degrees of impaired function, expressed them to similar levels as wild-type ABCA1 on the cell surface of BHK cells, and measured ABCA1-dependent lipid export, apolipoprotein A-I (apoA-I) binding, and signaling activities. Linear regression analyses showed that cholesterol and phospholipid efflux and cellular apoA-I binding correlated significantly with the ability of ABCA1 to form cell surface lipid domains. Lipid export and cellular apoA-I binding activities and formation of lipid domains also correlated with the amount of apoA-I that could be cross-linked to ABCA1. Moreover, each of these lipid export and apoA-I binding activities correlated with apoA-I-induced Janus kinase 2 (JAK2) activation. Thus, these missense mutations in ABCA1 impair lipid export, apoA-I binding, and apoA-I-stimulated JAK2 activities to similar extents, indicating that these processes are highly interactive components of a pathway that functions to export lipids from cells.—Vaughan, A. M., C. Tang, and J. F. Oram. ABCA1 mutants reveal an interdependency between lipid export function, apoA-I binding activity, and Janus kinase 2 activation. *J. Lipid Res.* 2009. 50: 285–292.

Supplementary key words ABCA1 mutations • apolipoprotein A-I • cholesterol efflux • phospholipid efflux • JAK2 activation

ABCA1 mediates the transport of excess cholesterol from cells to HDL apolipoproteins such as apoA-I (1). ABCA1 is induced by intracellular cholesterol through the liver X receptor transcription pathway (2, 3) and thus is highly expressed in cholesterol-loaded cells, including the foam cell

macrophages that populate early atherosclerotic lesions (4). Mutations that impair ABCA1 are associated with an increased risk of cardiovascular disease in humans (5), and ablation of ABCA1 in mouse macrophages promotes atherosclerosis in hypercholesterolemic mice (6, 7). Thus, the macrophage ABCA1 pathway is of central importance in the cardioprotective effects of HDL.

ABCA1 mediates the export of cellular cholesterol and phospholipids by a cascade of events. Induction of ABCA1 generates lipid domains on the cell surface that are solubilized by apoA-I to form nascent HDL particles (8–10). This lipid removal step appears to involve direct binding of apoA-I to ABCA1 (11) and activation of signaling molecules such as Janus kinase 2 (JAK2) (12, 13). It is still unclear, however, how these different ABCA1-dependent responses are related and whether they all depend on the same functional or structural properties of ABCA1.

Previous studies have shown that naturally occurring missense mutations in ABCA1 associated with Tangier disease or hypoalphalipoproteinemia cause various degrees of impairment in lipid export activity (5, 14). Here we generated transfected baby hamster kidney (BHK) cells expressing seven of these ABCA1 mutants and one artificial missense mutant to evaluate the relationship between the lipid export, apoA-I binding, and JAK2-stimulating activities of ABCA1. Results showed that all of these functions are highly correlated, suggesting that they form a tightly interactive pathway to modulate lipid export from cells.

EXPERIMENTAL PROCEDURES

ABCA1-transfected cells and apoA-I

BHK cells expressing mifepristone-inducible human ABCA1 were generated as described (8). Control (mock) BHK cells were

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Abbreviations: apoA-I, apolipoprotein A-I; BHK, baby hamster kidney; JAK2, Janus kinase 2; NBD, nucleotide binding domain.

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derived from the same clonal line transfected with plasmids lacking the ABCA1 cDNA insert. Site-directed mutagenesis of *ABCA1* was carried out using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene). HDL was prepared by sequential ultracentrifugation in the density range 1.125–1.21 g/ml and depleted of apoE and apoB by heparin-agarose chromatography, and apoA-I was purified from HDL as described previously (15).

Cholesterol and phospholipid efflux and cholesterol oxidase-reactive cholesterol

Cellular cholesterol was labeled with 1 $\mu\text{Ci/ml}$ [^3H]cholesterol (NEN Life Science Products) added to the growth medium 24 h prior to mifepristone incubations, and cellular phospholipids were labeled with 1 $\mu\text{Ci/ml}$ [^3H]choline chloride (Amersham Pharmacia Biotech) added during the mifepristone incubations. Washed cells were then incubated with DMEM/BSA minus or plus 10 $\mu\text{g/ml}$ apoA-I, and medium and cells were assayed for either free [^3H]cholesterol or [^3H]choline-labeled phospholipids (8, 13). ApoA-I-mediated lipid efflux was calculated as the percent total ^3H -lipid released into medium after subtraction of values obtained in the absence of apoA-I and was normalized to values for cells expressing wild-type ABCA1. To measure the fraction of cholesterol oxidase-reactive cellular cholesterol, [^3H]cholesterol-labeled cells were incubated for 4 h with mifepristone, treated for 15 min with 1 U/ml cholesterol oxidase, and assayed for conversion of [^3H]cholesterol to [^3H]cholestenone (8, 13, 16).

Cell surface and ABCA1 binding of apoA-I

For the whole-cell binding assay, cells were incubated for 2 h at 37°C with 2 $\mu\text{g/ml}$ ^{125}I -apoA-I \pm 200 $\mu\text{g/ml}$ unlabeled apoA-I, and cell-associated radioactivity and cell protein were measured in 0.2 N NaOH digests of washed cells (8, 13). Results were calculated as nanograms of apoA-I per milligram of cell protein after subtraction of values in the presence of unlabeled apoA-I and were normalized to values for BHK cells expressing wild-type ABCA1. For the ABCA1 binding studies, cells were incubated with 5 $\mu\text{g/ml}$ ^{125}I -apoA-I for 2 h, treated for 30 min at room temperature with PBS containing 1 mg/ml dithiobis (succinimidyl propionate) (cross-linking agent), and washed twice with cold PBS containing 20 mM glycine (13, 17, 18). ABCA1/apoA-I complexes

were isolated from detergent extracts by immunoprecipitation, apoA-I and ABCA1 were isolated by reduced SDS-PAGE, and ^{125}I -apoA-I was visualized by phosphorimaging and quantified using OptiQuant computer software (Packard Instruments).

Cellular ABCA1 and phosphorylated JAK2

To measure ABCA1 protein levels, cell proteins were solubilized in SDS buffer and resolved by SDS-PAGE, and ABCA1 was identified by immunoblot analysis (17, 19). Cell surface ABCA1 was measured by treating cells for 30 min at room temperature with 1 mg/ml sulfo-N-hydroxysulfosuccinimide-biotin, isolating ABCA1 by immunoprecipitation and SDS PAGE, and probing nitrocellulose blots with a streptavidin-HRP ECL assay (BioRad) (17, 18). To measure apoA-I-induced phosphorylation of JAK2 (P-JAK2), mifepristone-treated cells were incubated for 15 min without or with 10 $\mu\text{g/ml}$ apoA-I, and cellular contents of P-JAK2 and total JAK2 were measured by immunoblot analyses using antibodies to tyrosine-phosphorylated JAK2 (Biosource International) and JAK2 (Santa Cruz Biotech, Inc.) (19). Equal amounts of membrane or cell protein were added per gel lane. P-JAK2 levels were quantified from autoradiograph scans using OptiQuant computer software (Packard Instruments), and apoA-I-induced P-JAK2 was calculated as the difference between values without or with apoA-I normalized to values for cells expressing wild-type ABCA1.

Data analyses

Parameters of lipid efflux and cell surface apoA-I binding were calculated as mean \pm SD of three to six values, and statistical differences were calculated by Student's *t*-test. Linear regression analysis and correlation coefficients were obtained using Prism GraphPad (San Diego, CA). Differences or correlations were considered to be significant at $P < 0.05$.

RESULTS

We prepared seven naturally occurring mutants (5, 14) and one artificial missense mutant of *ABCA1* and transfected them into BHK cells under control of a mifepristone-inducible promoter. Of the natural mutations, four were in

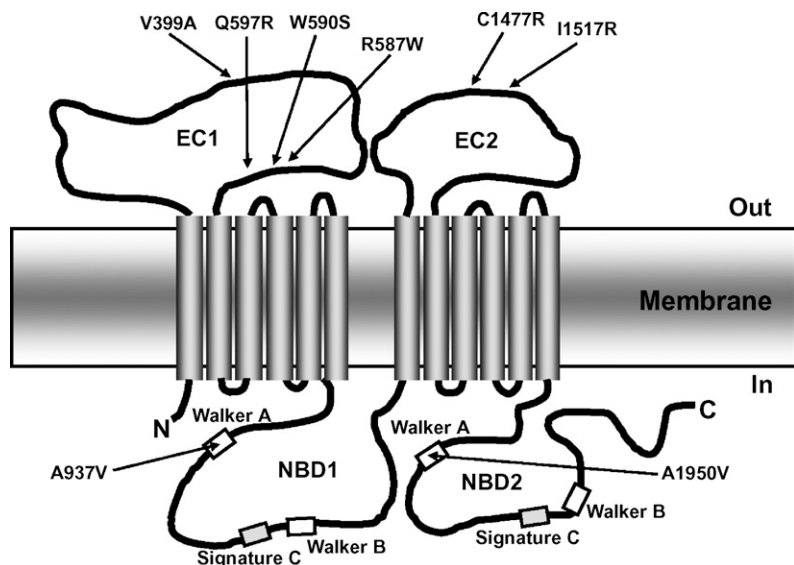


Fig. 1. Topological model of ABCA1 showing approximate locations of the missense mutations. EC, extracellular domain; NBD, nucleotide binding domain.

the first extracellular loop (V399A, R587W, W590S, and Q597R), two were in the second extracellular loop (C1477R and I1517R), and one was in the Walker A motif of the first nucleotide binding domain (A937V, NBD1) (Fig. 1). We also generated an artificial mutation in the Walker A motif of NBD2 (A1950V) that corresponds to the A937V mutation in NBD1. Immunoblot analyses indicated that all these mutant ABCA1s were expressed to levels similar to those of wild-type ABCA1 in mifepristone-treated BHK cells (not shown), and a cell surface biotinylation assay showed that wild-type and mutant ABCA1s were expressed to the same extent on the cell surface (Fig. 2F). We then used these cell lines to measure apoA-I-mediated cholesterol and phospholipid efflux (Fig. 2A, B), cell surface apoA-I binding (Fig. 2C), the fraction of cell surface cholesterol converted to cholestenone by the enzyme cholesterol oxidase (Fig. 2D), and the amount of apoA-I covalently cross-linked to ABCA1 (Fig. 2E).

All of these mutations impaired the different activities of ABCA1 to variable degrees (Fig. 2A–E). The rank order of severity in impaired apoA-I-mediated cholesterol efflux was Q597R>A937V>R587W>C1477R>V399A>I1517R>W590S>A1950V. This wide variability in ABCA1 activities allowed us to use linear regression analysis to evaluate correlations between these different ABCA1 responses.

ABCA1 forms lipid domains on the cell surface that are solubilized by apoA-I (10). The relative size of these lipid

domains can be assessed by treating cells with the enzyme cholesterol oxidase (8), which selectively reacts with cholesterol associated with disordered membrane phospholipids (20). Inducing wild-type ABCA1 in cells increased the fraction of oxidizable cholesterol 2.5-fold (Fig. 2D). This increase was significantly lower in all cells expressing ABCA1 mutants. To determine whether cholesterol and phospholipid efflux and cell surface apoA-I binding were functions of the size of the lipid domains generated by ABCA1, we compared values for these parameters with those for oxidizable cholesterol in each transfected cell line. Cholesterol and phospholipid efflux and cell surface apoA-I binding were all significantly correlated with the fraction of oxidizable cholesterol (Fig. 3A–C), implying that removal of cellular lipids by apoA-I is a function of the intrinsic ability of ABCA1 to form apolipoprotein-interacting lipid domains on the cell surface.

Cross-linking studies have shown that apoA-I binds directly to ABCA1 (9, 11, 17, 21–23), but it is still unclear whether this binding is required for removing lipids from cells. We therefore compared values for lipid efflux and cell surface apoA-I binding with values for the relative amount of apoA-I that could be cross-linked to ABCA1. Cholesterol and phospholipid efflux and apoA-I binding correlated significantly with apoA-I cross-linking to ABCA1 among the different cell lines expressing wild-type and mutant ABCA1

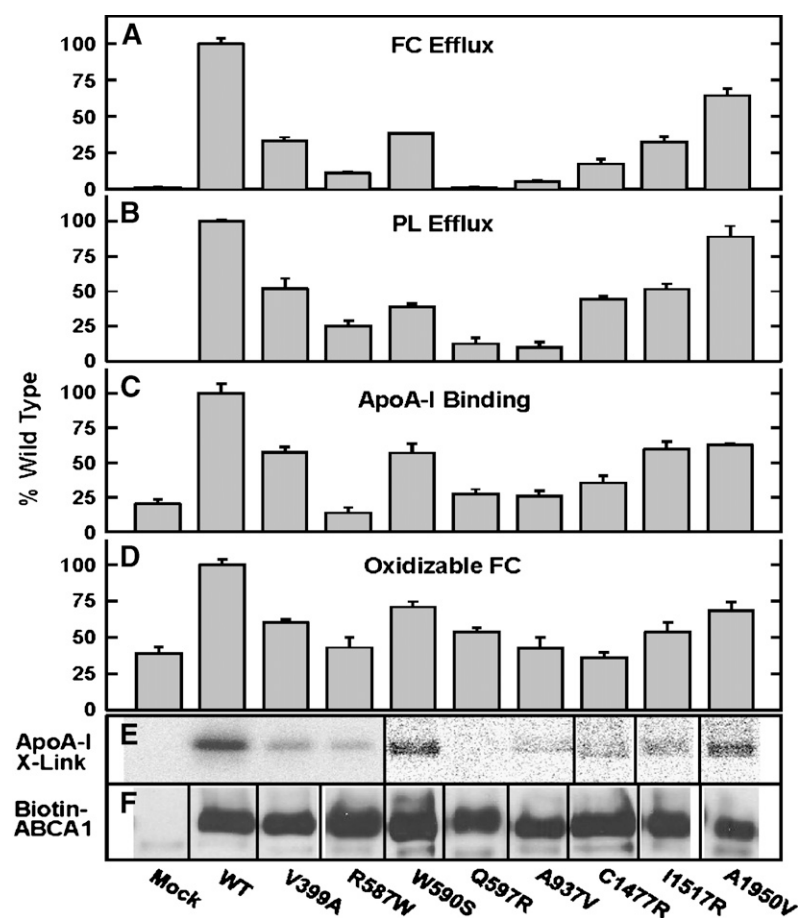


Fig. 2. Lipid export and apolipoprotein A-I (apoA-I) binding activities and cell surface expression of wild-type and mutant ABCA1. BHK cells were mock-transfected or transfected with wild-type (WT) or the indicated missense ABCA1 mutant, and ABCA1 expression was induced by incubating cells for 4 h (D) or 20 h (A–C, E, F) with 10 nM mifepristone. A, B: Cells were labeled with either [³H]cholesterol (A) or [³H]choline (B), and the fraction of labeled cholesterol (A) or phospholipid (B) released into the medium was measured after 2 h incubations with 10 μg/ml apoA-I. C: High-affinity binding of apoA-I to cells was measured after 2 h incubations at 37°C with 2 μg/ml [¹²⁵I]-apoA-I ± 200 μg/ml unlabeled protein. D: [³H]cholesterol-labeled cells were incubated for 4 h with 10 nM mifepristone, treated for 15 min with 1 U/ml cholesterol oxidase, and assayed for the fraction of labeled cholesterol converted to cholestenone. E: Cells were incubated for 2 h with 5 μg/ml [¹²⁵I]-apoA-I and treated with DSP, apoA-I/ABCA1 cross-linked protein was isolated by ABCA1 immunoprecipitation, and [¹²⁵I]-apoA-I was detected on SDS-PAGE gels by phosphorimaging. Bands shown are from two gels. F: Cell surface proteins were biotinylated, ABCA1 was isolated by immunoprecipitation and isolated by SDS-PAGE, and biotinylated ABCA1 was identified with a streptavidin assay. Bands shown are from one gel. Results in A–D are the mean ± SD of three to six values representative of two to three experiments for each parameter normalized to values for wild-type ABCA1 (100%). Blots in E or F are representative of two independent experiments. Vertical lines in E and F are sites of cropping done to reorder and align lanes. FC, free cholesterol; PL, phospholipid.

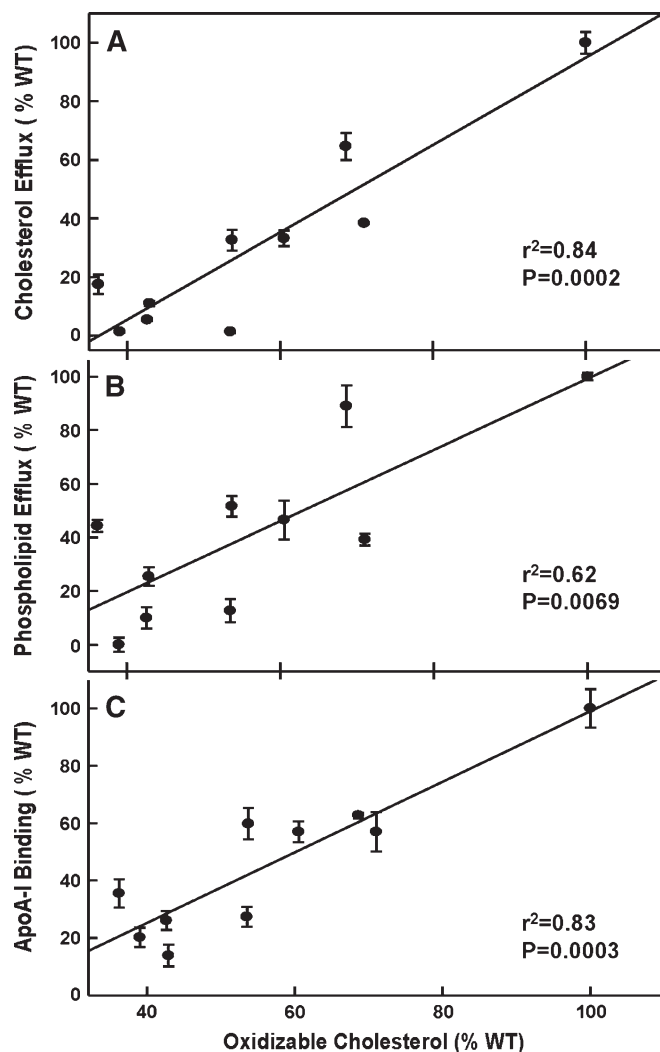


Fig. 3. Cholesterol and phospholipid export and cellular apoA-I binding correlate significantly with cholesterol oxidase-reactive cholesterol. Values from Fig. 2 for cholesterol efflux (A), phospholipid efflux (B), and apoA-I binding (C) were plotted against values for oxidizable cholesterol, and correlations were assessed using linear regression analysis. Values of 100% represent cells expressing wild-type ABCA1.

(Fig. 4A–D), consistent with the idea that direct binding of apoA-I to ABCA1 plays a role in lipid removal. Neither lipid efflux activity nor apoA-I cross-linking correlated significantly with the amount of cell surface ABCA1.

Previous studies have shown that the interaction of apoA-I with ABCA1-expressing cells rapidly activates JAK2 and that this enhances the binding of apoA-I to ABCA1 that promotes lipid removal (12, 13). Incubating cells expressing wild-type ABCA1 with apoA-I for 15 min markedly increased the phosphorylation and thus activation of JAK2 (Fig. 5). This activation was nearly abolished in cells expressing all mutants except two (W590S and A1950V), which are the same two mutants that have the highest ability to cross-link apoA-I (Fig. 2E). Total JAK2 levels were similar for all cell lines (Fig. 5).

We plotted parameters of lipid transport and apoA-I binding against the relative increase in JAK2 phosphorylation induced by apoA-I. Because most of the cell lines expressing mutant ABCA1 had a very low response to apoA-I, the values for phosphorylated JAK2 tended to cluster in two groups. Nevertheless, there was a significant correlation between all these lipid transport and apoA-I binding parameters with the amount of apoA-I-induced JAK2 phosphorylation (Fig. 6A–D), with apoA-I cross-linking having the strongest correlation (Fig. 6D). The results support the concept that activated JAK2 modulates the apolipoprotein binding and lipid export activities of ABCA1.

DISCUSSION

The export of cellular cholesterol and phospholipids by the ABCA1 pathway involves distinct cellular events, including intrinsic formation of cell surface lipid domains that interact with apolipoproteins, solubilization of these lipid domains, direct binding of apolipoproteins to ABCA1, and activation of signaling molecules (1, 10). Previous studies had shown that naturally occurring missense mutations in ABCA1 impair its function to varying degrees of severity (5, 14). We therefore used cell lines transfected with wild-type and ABCA1 mutants to examine the relationship between these events. Linear regression analyses showed that all of these activities are significantly correlated, implying that there is a strong interdependency between these diverse processes.

Of the seven naturally occurring mutations, six were in the two extracellular loops and one was in the intracellular ATP binding domain of NBD1 (Walker A motif). The two most severe mutations, which reduced apoA-I-mediated lipid efflux to less than 20% of normal, were located in the first extracellular loop (Q597R) and the ATP binding site (A937V). Interestingly, the equivalent mutation in the ATP binding site of NBD2 (A1950V) had only a modest impairment of ABCA1 function, suggesting that the ATP binding activity of NBD1 is more critical for function than that of NBD2 or that these mutations have different effects on protein conformation.

All ABCA1 mutants were expressed to levels comparable to wild-type ABCA1 in whole cells and on the cell surface. Fitzgerald et al. (24) reported similar cell surface localizations for mutants R587W, W590S, Q597R, and C1477R. In contrast, Singaraja et al. (14) and Tanaka et al. (25) reported that ABCA1 with the R587W or Q597R mutations had impaired translocation to the plasma membrane in transfected cells. These differences may reflect the cell type and the level of ABCA1 expression, which is robust in our transfected BHK cells. Tanaka et al. (26) showed that endoplasmic reticulum stress can promote translocation of the Q597R ABCA1 mutant to the plasma membrane, where it still has impaired function, indicating that decreased trafficking alone cannot explain the dysfunctional effects of this mutation.

The current study provides additional evidence that the apoA-I-mediated removal of lipids from cells depends

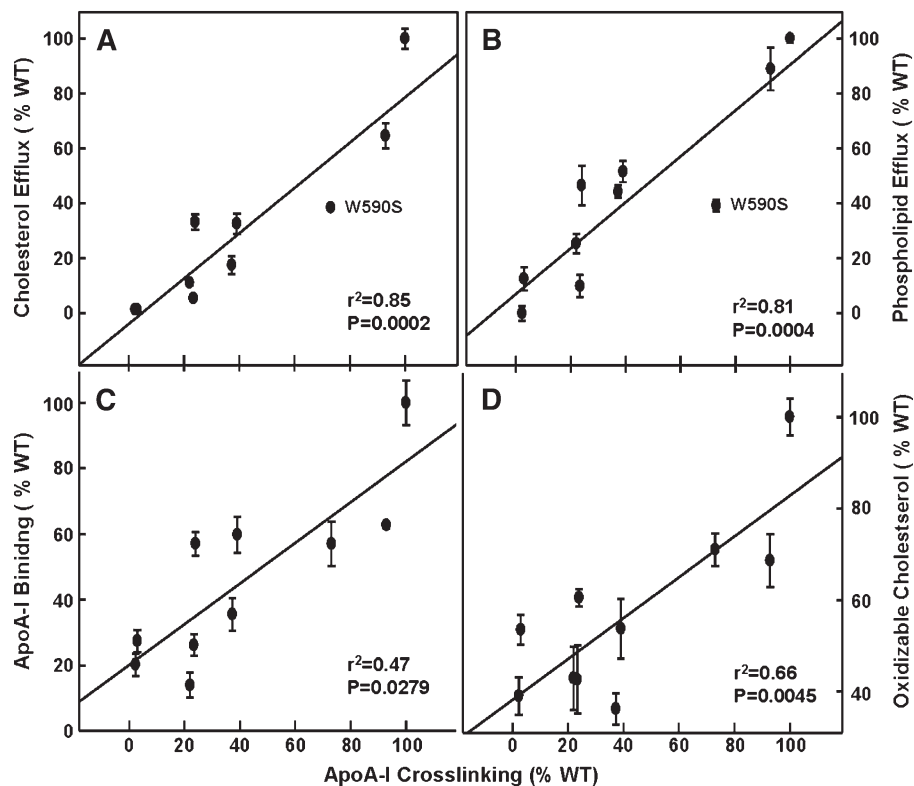


Fig. 4. Cholesterol and phospholipid export, cellular apoA-I binding, and cholesterol oxidase-reactive cholesterol correlate significantly with apoA-I binding to ABCA1. Values from Fig. 2 for cholesterol efflux (A), phospholipid efflux (B), apoA-I binding (C), and oxidizable cholesterol (D) were plotted against relative amounts of ^{125}I -apoA-I cross-linked to ABCA1, and correlations were assessed using linear regression analysis. Values for cross-linked apoA-I are the means of two quantified scans of phosphorimages from two independent experiments normalized to values for wild-type ABCA1 in each experiment. Cholesterol and phospholipid efflux values for mutant W590S are labeled in panels A and B. Values of 100% represent cells expressing wild-type ABCA1.

on the ability of ABCA1 to intrinsically form lipid domains on the cell surface. Inducing ABCA1 in cells more than doubles the fraction of cholesterol converted to cholestenone when cells are exposed to cholesterol oxidase (8) (Fig. 2D). The cholesterol removed from cells by apoA-I is exclusively from this oxidase-reactive pool (8). Cholesterol oxidase selectively interacts with cholesterol in disordered membrane lipid domains (20), which suggests that ABCA1 forms these domains on the cell surface and that they are targeted for removal by apolipoproteins. ABCA1-dependent formation of disordered lipid domains was supported by biochemical

assays (27) and by electron microscopy studies showing that ABCA1 generates bleb-like structures that protrude from the plasma membrane and bind apoA-I (10, 28). The strong correlation between lipid efflux and the fraction of oxidase-reactive cholesterol among the ABCA1 mutant cell lines supports the idea that the size of the lipid domains formed by ABCA1 determines the amount of cholesterol exported from cells.

Cross-linking studies have indicated that apolipoproteins bind to ABCA1 (11), but the consequences of this binding are poorly understood. Previous studies of ABCA1 and

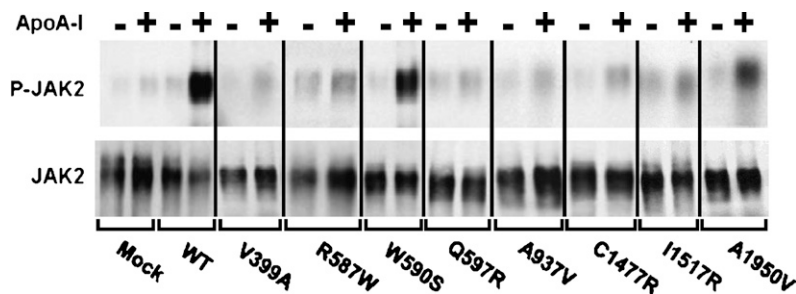


Fig. 5. ABCA1 mutations impair apoA-I-induced activation of JAK2. The indicated mifepristone-treated cell lines were incubated for 15 min without or with 10 $\mu\text{g}/\text{ml}$ apoA-I, and tyrosine-phosphorylated JAK2 (P-JAK2) was detected by immunoblot analysis. Blots were stripped of antibody and re probed with a total JAK2 antibody (JAK2). Results are representative of two independent experiments. Vertical lines are sites of cropping for reordering lanes.

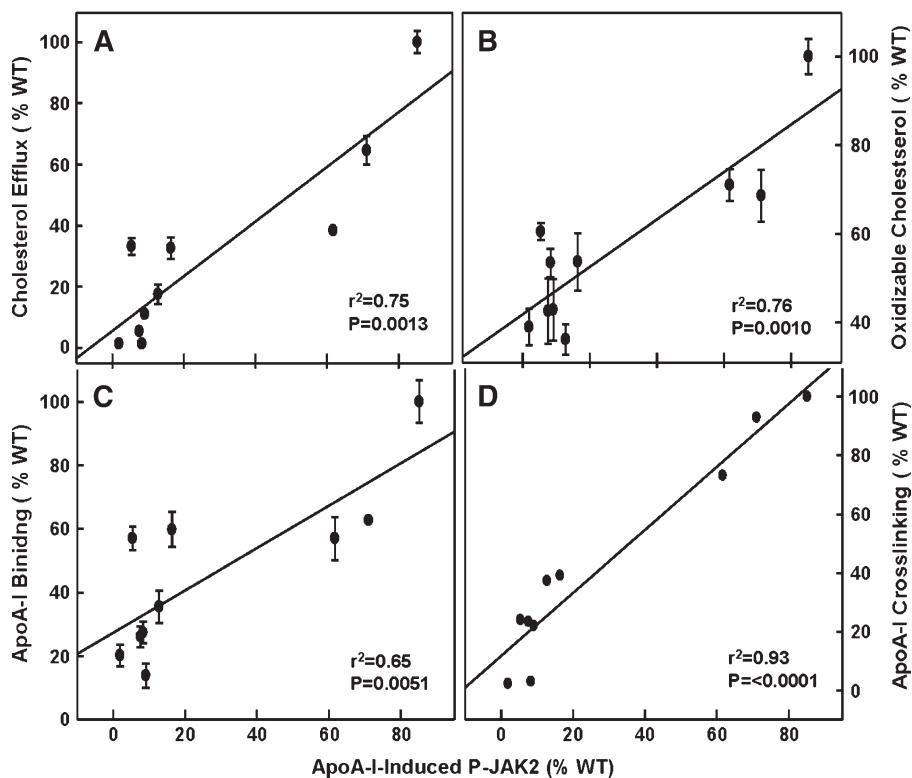


Fig. 6. Cholesterol export, cellular apoA-I binding, and cholesterol oxidase-reactive cholesterol and apoA-I binding to ABCA1 correlate significantly with apoA-I-induced P-JAK2. Values from Fig. 2 for cholesterol efflux (A), oxidizable cholesterol (B), and apoA-I binding (C), and from Fig. 4 for apoA-I cross-linking to ABCA1 (D) were plotted against the relative extent of JAK2 phosphorylation (P-JAK) induced by apoA-I, and correlations were assessed using linear regression analysis. Values for P-JAK2 levels were calculated from scans of autoradiographs. Values for apoA-I-induced P-JAK2 are the differences between cells incubated without or with apoA-I normalized to values for cells expressing wild-type ABCA1 and are the mean of duplicates from two independent experiments. Values of 100% on the Y axis represent cells expressing wild-type ABCA1.

apoA-I mutants showed an association between reduced apoA-I cross-linking to ABCA1 and impaired lipid efflux (9, 11, 21). Moreover, selectively inhibiting the apoA-I binding activity of ABCA1 reduces lipid efflux despite little change in the ability of ABCA1 to form apolipoprotein-interacting lipid domains (13). These observations strongly suggest that direct binding of apolipoproteins to ABCA1 plays a role in removing lipids from the cells. This assumption is supported by our results showing that apoA-I-mediated lipid efflux was significantly correlated with the amount of apoA-I that was cross-linked to ABCA1 among cell lines expressing the same surface concentrations of wild-type or mutant ABCA1. It is possible that binding of apoA-I to ABCA1 helps target apoA-I to lipid domains formed by ABCA1.

A previous study showed that the W590S ABCA1 mutation severely impaired lipid efflux without affecting apoA-I cross-linking to ABCA1 (24), suggesting that apoA-I binding to ABCA1 can be dissociated from lipid export activity. We also found that ABCA1 with this mutation had the highest apoA-I cross-linking activity of all the natural mutants examined, approaching 80% of wild-type values (Fig. 2E). This compared with lipid efflux values that were

only ~40% those of wild-type ABCA1 (Fig. 2A, B), consistent with a greater impairment of lipid export than apoA-I binding activity. The lipid export activity of the W590S ABCA1 mutant, however, was among the highest of the natural mutants, and thus values for cells expressing this mutant ABCA1 did not deviate far from the linear regression line when lipid transport activities were plotted against apoA-I/ABCA1 cross-linking (Fig. 4A, B).

The earliest biochemical event described for apolipoprotein interactions with ABCA1-expressing cells is phosphorylation of the tyrosine kinase JAK2, which occurs within 1 min of exposing cells to apoA-I and peaks by 10 min (12). Inhibiting or ablating JAK2 reduces apoA-I binding to ABCA1 and lipid export by ~70% without affecting the intrinsic ability of ABCA1 to form lipid domains (12, 13), indicating that JAK2 modulates the apolipoprotein binding to ABCA1 required for optimum removal of cellular lipids. Six of the eight ABCA1 mutants studied here had a severe impairment in the ability of apoA-I to stimulate JAK2 phosphorylation, which exceeded the extent of the impaired lipid transport functions for most of these mutants. The two ABCA1 mutants that had near-normal apoA-I-induced JAK2 activation were W590S

and A1950V, which also had near-normal apoA-I cross-linking activity.

Despite clustering of values at the low and high ends of the linear regression lines, there was a significant statistical correlation between parameters of lipid transport/apoA-I binding and the relative extent of JAK2 phosphorylation induced by 15 min incubations with apoA-I. The strongest correlation was with apoA-I cross-linking to ABCA1 (Fig. 6D), which is consistent with results showing that JAK2 modulates this activity (13). These findings provide additional evidence that activation of JAK2 plays a critical role in modulating ABCA1 lipid export function.

There are several possible reasons that most of these missense mutations in ABCA1 almost abolish the ability of apoA-I to activate JAK2 while retaining some residual lipid export activity. First, even low levels of acute transient activation of JAK2 could enhance ABCA1-dependent lipid efflux when measured during long-term incubations. Second, because JAK2 modulates most but not all of the lipid export activity of ABCA1, the residual lipid transport mediated by the ABCA1 mutants may occur by JAK2-independent processes. These observations raise the possibility that these missense mutations have the most profound effect on the specific interactions of apoA-I with ABCA1 that activate JAK2.

The current study and previous studies suggest the following sequential steps in the ABCA1 lipid export pathway. Inducing ABCA1 initiates formation of lipid domains on the cell surface. Within minutes, apolipoproteins interact with ABCA1 to activate JAK2. This activation enhances the interactions of apolipoproteins with ABCA1 that facilitate the solubilization and removal of the lipid domains formed by ABCA1. This multistep pathway generates nascent HDL particles that can promote export of more cholesterol from cells by other transport processes, particularly the ABCG1 pathway (29, 30).

In summary, the current study shows a strong correlation between lipid export, apoA-I binding, and apoA-I-dependent JAK2 activation among ABCA1 mutants with varying severities of functional impairment, implying that these different activities are highly interdependent. An important observation is that the apolipoprotein-independent formation of lipid domains by ABCA1 and apoA-I binding to ABCA1 appear to be reduced to similar extents by the same mutation, suggesting that single amino acid substitutions can alter ABCA1 structure so as to impair both of these activities. These mutational analyses also raise the possibility that the most severe of these mutations may selectively impair the early interactions of apoA-I with ABCA1 that activate JAK2, a feed forward mechanism for enhancing the binding of apoA-I to ABCA1 required for lipid removal.

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