

# Janus kinase 2 modulates the lipid-removing but not protein-stabilizing interactions of amphipathic helices with ABCA1

Chongren Tang,\* Ashley M. Vaughan,\* G. M. Anantharamaiah,<sup>†</sup> and John F. Oram<sup>1,\*</sup>

Division of Metabolism, Endocrinology, and Nutrition,\* Department of Medicine, University of Washington, Seattle, WA 98195, Department of Medicine, Biochemistry, and Molecular Genetics and Atherosclerotic Research Unit,<sup>†</sup> University of Alabama at Birmingham Medical Center, Birmingham, AL 35294

**Abstract** ABCA1 mediates the transport of cellular cholesterol and phospholipids to HDL apolipoproteins. Apolipoprotein A-I (apoA-I) interactions with ABCA1-expressing cells elicit several responses, including removing cellular lipids, stabilizing ABCA1 protein, and activating Janus kinase 2 (JAK2). Here, we used synthetic apolipoprotein-mimetic peptides to characterize the relationship between these responses. Peptides containing one amphipathic helix of L- or D-amino acids (2F, D-2F, or 4F) and a peptide containing two helices (37pA) all promoted ABCA1-dependent cholesterol efflux, competed for apoA-I binding to ABCA1-expressing cells, blocked covalent cross-linking of apoA-I to ABCA1, and inhibited ABCA1 degradation. 37pA was cross-linked to ABCA1, confirming the direct binding of amphipathic helices to ABCA1. 2F, 4F, 37pA, and D-37pA all stimulated JAK2 autophosphorylation. Inhibition of JAK2 greatly reduced peptide-mediated cholesterol efflux, peptide binding to ABCA1-expressing cells, and peptide cross-linking to ABCA1, indicating that these processes require an active JAK2. In contrast, apoA-I and peptides stabilized ABCA1 protein even in the absence of an active JAK2, implying that this process is independent of JAK2 and lipid efflux-promoting binding of amphipathic helices to ABCA1. These findings show that amphipathic helices coordinate the activity of ABCA1 by several distinct mechanisms that are likely to involve different cell surface binding sites.— Tang, C., A. M. Vaughan, G. M. Anantharamaiah, and J. F. Oram. Janus kinase 2 modulates the lipid-removing but not protein-stabilizing interactions of amphipathic helices with ABCA1. *J. Lipid Res.* 2006. 47: 107–114.

**Supplementary key words** ATP binding cassette transporter A1 • cholesterol efflux • atherosclerosis • HDL

ABCA1 mediates the transport of excess cholesterol from cells to HDL apolipoproteins (1, 2). ABCA1 mutations can cause Tangier disease (3, 4), a severe HDL de-

ciency syndrome characterized by cholesterol deposition in tissue macrophages and prevalent atherosclerosis (5–7). Genetic manipulations of ABCA1 expression in mice also affect plasma HDL levels and atherogenesis (8, 9). These observations indicate that ABCA1 activity is a major determinant of plasma HDL levels and cardiovascular disease.

The interaction of apolipoproteins with ABCA1-expressing cells elicits several responses. First, lipid-poor apolipoproteins remove cholesterol, phospholipids, and other lipophilic compounds that are translocated to cell surface domains by ABCA1 (1). Second, apolipoproteins stabilize ABCA1 protein (10, 11), which is rapidly degraded in the absence of apolipoproteins. Third, apolipoproteins modulate intracellular signaling pathways that influence ABCA1 expression and activity (12–15).

The biochemical and molecular properties of these diverse cellular responses are poorly understood. ABCA1 appears to intrinsically translocate cholesterol and phospholipids to the outer leaflet of the plasma membrane, where they become accessible for removal by apolipoproteins (16) or for interactions with other cells (17). Studies with synthetic peptides revealed that a nonstereoselective amphipathic helical motif is all that is required to remove cellular cholesterol and phospholipids by the ABCA1 pathway (18). Several possible mechanisms have been reported to be involved in stabilizing ABCA1 protein by apolipoproteins, including inhibiting the phosphorylation of an ABCA1 motif that is targeted for degradation by a calpain protease (19) and increasing ABCA1 phosphorylation by a protein kinase C pathway (20). Amphipathic helical peptides with opposite stereoselectivity (D-amino acids) also stabilize ABCA1 (21). Very little is known about the molecules and biochemical processes involved in eliciting ABCA1-modulating signals.

Covalent cross-linking and kinetic studies have shown that amphipathic helical motifs promote high-affinity reversible binding of apolipoproteins to ABCA1 and that

Manuscript received 10 June 2005 and in revised form 24 August 2005 and in re-revised form 5 October 2005.

Published, JLR Papers in Press, October 6, 2005.  
DOI 10.1194/jlr.M500240-JLR200

<sup>1</sup> To whom correspondence should be addressed.  
e-mail: joram@u.washington.edu

this is associated with cholesterol removal (22). We showed previously that inhibition or ablation of the tyrosine kinase Janus kinase 2 (JAK2) in ABCA1-expressing cells dramatically reduces apolipoprotein-mediated lipid efflux and apolipoprotein binding to ABCA1 without affecting the intrinsic cholesterol translocase of this protein (14). Exposing cells to apolipoprotein A-I (apoA-I) for only 15 min stimulates the autophosphorylation of JAK2 (14), suggesting that the interaction of apolipoproteins with cells activates a JAK2-dependent feed-forward mechanism that enhances the ABCA1 binding of apolipoproteins required for removing lipids.

The apolipoprotein motifs and cell surface binding sites responsible for activating the JAK2 pathway are unknown. Moreover, it is still unclear whether ABCA1 stabilization involves direct binding of apolipoproteins to ABCA1. Here, we used synthetic amphipathic helical peptides to assess the specificity of the JAK2 activation pathway, and we suppressed JAK2 activity to test the role of this pathway in stabilizing ABCA1. The results show that activation of JAK2 has the same broad specificity for class A amphipathic helices as ABCA1-dependent cholesterol efflux and ABCA1 stabilization, but ABCA1 stabilization is not modulated by JAK2. Thus, stabilizing ABCA1 does not appear to require the direct binding of amphipathic helices to ABCA1.

## EXPERIMENTAL PROCEDURES

### Lipoproteins, apoA-I, and peptides

LDL and HDL were prepared by sequential ultracentrifugation in the density ranges 1.019–1.063 and 1.125–1.21 g/ml, respectively, and HDL was depleted of apoE and apoB by heparin-agarose chromatography (23). ApoA-I was purified from HDL, and LDL was acetylated as described previously (23, 24). Peptides 2F (Ac-DWLKAFYDKVAEKLKEAF-NH<sub>2</sub>), D-2F (2F with all D-amino acids), 4F (Ac-DWFKAFYDKVAEKFKEAF-NH<sub>2</sub>), 37pA (two 2F peptides missing Ac and NH<sub>2</sub> joined by a P), and D-37pA (37pA with all D-amino acids) were synthesized as described previously (25, 26). Tyrosines in apoA-I and 37pA were radiolabeled with <sup>125</sup>I as described previously (27).

### Cell culture and cholesterol efflux

BHK cells expressing mifepristone-inducible human ABCA1 were generated as described (16). Mutant  $\gamma$ 2A cells lacking JAK2 (a gift from Dr. George R. Stark) were generated from wild-type 2C4 fibrosarcoma cells as described (28, 29). These cells and J774 macrophages were maintained in DMEM (Invitrogen) containing 10% fetal bovine serum or incubated in serum-free DMEM plus 1 mg/ml fatty acid-free BSA (DMEM/BSA). To radiolabel cellular cholesterol, 1  $\mu$ Ci/ml [<sup>3</sup>H]cholesterol (New England Nuclear Life Science Products) was added to the growth medium (BHK and fibrosarcoma cells) or to DMEM/BSA containing 50  $\mu$ g/ml acetylated LDL (J774 macrophages) 24 h immediately preceding treatments (16, 30). To induce ABCA1, cells were incubated for 16–20 h with DMEM/BSA containing 10 nM mifepristone (BHK transfectants), 10  $\mu$ M 22(R)-hydroxycholesterol plus 10  $\mu$ M 9-*cis*-retinoic acid (fibrosarcoma cells), or 0.5 mM 8-Br-cAMP (J774 macrophages) (16, 31).

To measure cholesterol efflux, cells were incubated with DMEM/BSA with or without the indicated peptides for 2 h at

37°C and chilled on ice, the medium was collected and centrifuged to remove detached cells, the supernatant was counted for <sup>3</sup>H, and the cells were assayed for free and esterified [<sup>3</sup>H]cholesterol after isolation by thin-layer chromatography (27). Peptide-mediated cholesterol efflux is expressed as the fraction of free [<sup>3</sup>H]cholesterol released into the medium after subtraction of values obtained in the absence of peptide.

### Metabolically labeled ABCA1 and immunoblot analyses

Macrophages were metabolically labeled by incubation for 15 min at 37°C with DMEM/BSA containing 100  $\mu$ Ci/ml [<sup>35</sup>S]methionine (Amersham Pharmacia Biotech, Inc.), and ABCA1 was isolated from 1% Triton X-100 digests of cells by immunoprecipitation and SDS-PAGE as described (30). Each gel lane received immunoprecipitated protein corresponding to equal amounts of cells. [<sup>35</sup>S]methionine-labeled ABCA1 was detected on gels by phosphorimaging (Cyclone; Packard Instrument Co.). To measure ABCA1 protein levels, microsomal membranes were isolated from homogenized cells by ultracentrifugation, membrane proteins were solubilized in SDS buffer and resolved by SDS-PAGE, and ABCA1 was identified by immunoblot analysis (14, 32). ABCA1 protein levels were quantified using OptiQuant computer software (Packard Instrument Co.). Cellular contents of JAK2 and phosphorylated JAK2 were measured by immunoblot analyses using antibodies to JAK2 (Santa Cruz Biotechnology, Inc.) and tyrosine-phosphorylated JAK2 (Biosource International) (14). Equal amounts of membrane or cell protein were added per gel lane.

### Cell surface and ABCA1 binding of apoA-I and 37pA

For the whole-cell binding assay, cells were incubated for 2 h at 37°C with 2  $\mu$ g/ml <sup>125</sup>I-37pA with or without 100  $\mu$ g/ml unlabeled 37pA (32). For the competitive sequential binding assay (18, 27, 33), BHK cells were first incubated for 4 h at 37°C with DMEM/BSA containing the indicated concentrations of peptides, chilled to 0°C, washed twice with ice-cold PBS/BSA, and then incubated for 2 h with ice-cold HEPES-buffered DMEM/BSA containing 2  $\mu$ g/ml <sup>125</sup>I-apoA-I with or without 200  $\mu$ g/ml unlabeled apoA-I. Cells were washed twice at 0°C with PBS/BSA and twice with PBS, and cell-associated radioactivity and cell protein were measured after digestion in 0.1 N NaOH. Results are expressed as nanograms of peptide per milligram of cell protein after subtraction of values in the presence of unlabeled 37pA or apoA-I. For the ABCA1 binding studies, cells were incubated for 2 h with 5  $\mu$ g/ml <sup>125</sup>I-apoA-I with or without unlabeled peptide or with 5  $\mu$ g/ml <sup>125</sup>I-37pA, treated for 30 min at room temperature with PBS containing 1 mg/ml Disuccinimidyl Suberate (a cross-linking agent), and washed twice with cold PBS containing 20 mM glycine (30, 32). ABCA1 was isolated from detergent extracts by immunoprecipitation and SDS-PAGE, and <sup>125</sup>I-labeled ABCA1 was visualized by phosphorimaging.

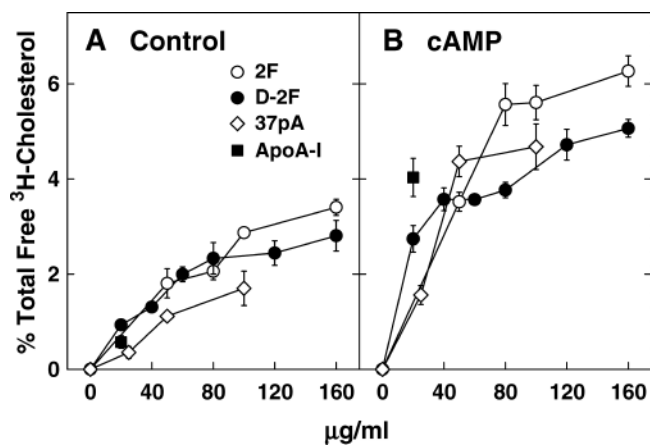
## RESULTS

We characterized apolipoprotein-mimetic peptides for their abilities to modulate different parameters of cellular ABCA1 metabolism. The prototype of these peptides is 2F (also called Ac-18A-NH<sub>2</sub>), an 18 amino acid analog of the class A amphipathic  $\alpha$ -helices found in HDL apolipoproteins (25). Other peptides studied were D-2F (2F containing all D-amino acids), 4F (2F containing two additional phenylalanines substituted for leucines), and

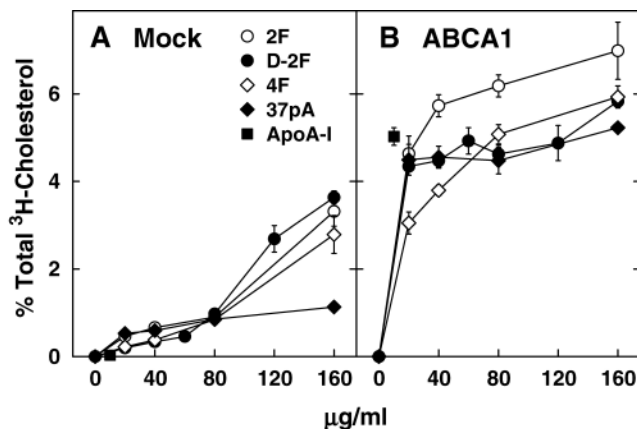
37pA (2F dimer joined covalently by a proline) (25, 26). We used two different cultured cell lines for most of these experiments: cholesterol-loaded murine J774 macrophages (32) and BHK cells stably transfected with a mifepristone-inducible ABCA1 cDNA (16). To induce ABCA1, J774 macrophages and transfected BHK cells were treated for 18–24 h with 8-Br-cAMP and mifepristone, respectively (16, 32).

To confirm that these peptides mimicked apoA-I in removing cellular lipids, we incubated cells lacking or expressing ABCA1 with different peptides and measured radiolabeled cholesterol efflux. For comparison, we also measured cholesterol efflux in the presence of purified apoA-I at a concentration above saturation of ABCA1-dependent cholesterol efflux (10  $\mu\text{g/ml}$ ). All peptides stimulated cholesterol efflux from both noninduced J774 macrophages (Fig. 1A) and mock-transfected BHK cells (Fig. 2A), despite a very low level of apoA-I-mediated cholesterol efflux. The 18 amino acid monomers were more active than the 37pA dimer, implying that the single amphipathic helices had a greater ability to remove cellular cholesterol by ABCA1-independent mechanisms compared with peptides containing two or more tandem helices. Induction of ABCA1 in macrophages (Fig. 1B) or transfected BHK cells (Fig. 2B) markedly increased the ability of all peptides to promote cholesterol efflux to levels comparable to that seen with apoA-I, indicating that these peptides mimicked apoA-I in removing cellular cholesterol by the ABCA1 pathway.

We used a sequential competitive binding assay to determine whether the synthetic peptides also mimic apoA-I in binding to ABCA1-expressing cells. We measured the amount of  $^{125}\text{I}$ -apoA-I (at 2  $\mu\text{g/ml}$ ) that binds to mock-and



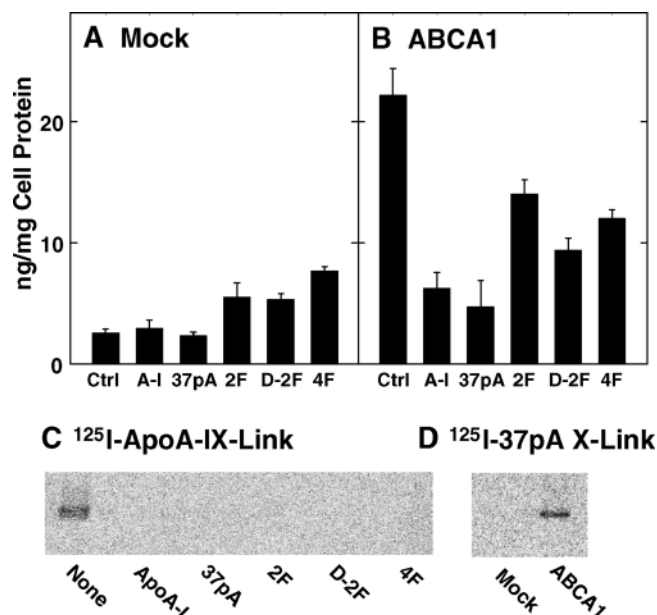
**Fig. 1.** Amphipathic helical peptides promote cholesterol efflux from J774 macrophages lacking (A) and expressing (B) ABCA1. J774 macrophages were incubated for 24 h with 50  $\mu\text{g/ml}$  acetylated LDL containing [ $^3\text{H}$ ]cholesterol followed by 18 h incubations without (A) or with (B) 0.5 mM 8-Br-cAMP, and [ $^3\text{H}$ ]cholesterol efflux was measured after 2 h incubations with medium containing the indicated concentrations of peptides 2F, D-2F, or 37pA or 10  $\mu\text{g/ml}$  apolipoprotein A-I (apoA-I). Values are percentage of total (medium plus cell) radiolabeled free cholesterol released into the medium. Each value is the mean  $\pm$  SD of three to nine incubations from three separate experiments.



**Fig. 2.** Amphipathic helical peptides promote cholesterol efflux from transfected BHK cells lacking (A) and expressing (B) ABCA1. [ $^3\text{H}$ ]cholesterol-labeled mock-transfected (A) and ABCA1-transfected (B) BHK cells were incubated for 20 h with 10 nM mifepristone, and [ $^3\text{H}$ ]cholesterol efflux was measured after 2 h incubations with medium containing the indicated concentrations of peptides 2F, D-2F, 4F, or 37pA or 10  $\mu\text{g/ml}$  apoA-I. Values are percentage of total (medium plus cell) radiolabeled cholesterol released into the medium. Each value is the mean  $\pm$  SD of 6–12 incubations from four separate experiments.

ABCA1-transfected BHK cells at 4°C after cells were first incubated at 37°C with no peptides or with unlabeled apoA-I (10  $\mu\text{g/ml}$ ) or peptides (20  $\mu\text{g/ml}$ ). With this assay, the peptides that bind to ABCA1 at 37°C dissociate slowly when cells are chilled to 4°C and thus block subsequent binding of apoA-I (18, 27, 33). Mock transfectants bound a small amount of  $^{125}\text{I}$ -apoA-I, and this was unaffected by pretreatment with either apoA-I or 37pA (Fig. 3A). Pretreatment with either the L- or D-isomer of the 18-mer peptides significantly increased  $^{125}\text{I}$ -apoA-I binding to mock-transfected cells ( $P < 0.05$  by ANOVA), perhaps as a result of the perturbation of membrane lipids suggested by the cholesterol efflux assay. Overexpression of ABCA1 led to a dramatic increase in  $^{125}\text{I}$ -apoA-I binding to cells, which was largely inhibited by pretreating cells with apoA-I or peptides ( $P < 0.05$ ) (Fig. 3B). The monomeric helical peptides were significantly ( $P < 0.05$ ) less effective in blocking  $^{125}\text{I}$ -apoA-I binding than were either apoA-I or the 37pA dimer. These results suggest that apoA-I and the synthetic peptides bind to common sites on ABCA1-expressing cells.

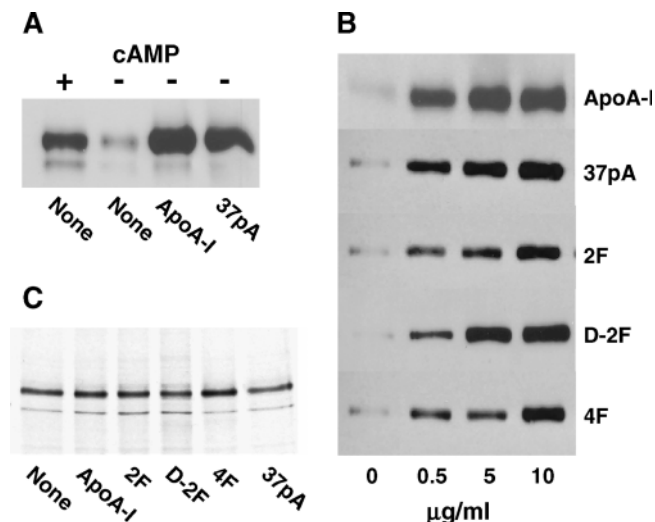
To test whether the synthetic peptides interfere with apoA-I binding to ABCA1, we incubated ABCA1-transfected BHK cells with  $^{125}\text{I}$ -apoA-I in the absence or presence of peptides, treated cells with the cross-linking agent DSS, and identified  $^{125}\text{I}$ -apoA-I-tagged ABCA1 by SDS-PAGE of immunoprecipitated protein. Results showed a single radiolabeled band in the absence of peptides that was reduced below detection in the presence of unlabeled apoA-I and each peptide (Fig. 3C), consistent with apolipoproteins and peptides binding to common sites on ABCA1. As a confirmation that peptides directly bind ABCA1, we performed the cross-linking experiments with radiolabeled 37pA, which contains two tyrosines accessible for iodination



**Fig. 3.** Amphipathic helical peptides bind to apolipoprotein binding sites on ABCA1-expressing cells and on ABCA1. A, B: Mifepristone-treated mock-transfected (A) and ABCA1-transfected (B) BHK cells were first incubated for 4 h at 37°C with either 10  $\mu\text{g/ml}$  apoA-I (A-I) or 20  $\mu\text{g/ml}$  of the indicated peptide, and high-affinity binding of  $^{125}\text{I}$ -apoA-I (at 2  $\mu\text{g/ml}$  with or without 200  $\mu\text{g/ml}$  unlabeled protein) was measured after subsequent 2 h incubations at 0°C. Each value is the mean  $\pm$  SD of quadruplicate incubations. C, D: ABCA1-transfected BHK cells were incubated for 2 h at 37°C with 2  $\mu\text{g/ml}$   $^{125}\text{I}$ -apoA-I minus (None) or plus 20  $\mu\text{g/ml}$  apoA-I or the indicated peptide (C), or mock- or ABCA1-transfected BHK cells were incubated for 2 h with 2  $\mu\text{g/ml}$   $^{125}\text{I}$ -37pA (D). Cells were treated with the cross-linker DSS, ABCA1 was isolated by immunoprecipitation and SDS-PAGE, and  $^{125}\text{I}$ -labeled ABCA1 was detected by phosphorimaging.

(27). Cross-linking of  $^{125}\text{I}$ -37pA to ABCA1 isolated from ABCA1-transfected BHK cells was readily detected, whereas no radiolabeled band was visible using mock-transfected cells (Fig. 3D).

We compared the abilities of apoA-I and synthetic peptides to stabilize ABCA1 protein. When 8-Br-cAMP-treated J774 macrophages were subsequently incubated without cAMP, most of the induced ABCA1 protein disappeared within 4 h (Fig. 4A), consistent with previous studies showing that ABCA1 is highly unstable in these cells (30, 32, 34). Including either apoA-I or 37pA in the 4 h chase medium completely prevented the ABCA1 disappearance and actually increased protein to levels above those in cells maintained on 8-Br-cAMP medium. ApoA-I, 37pA, 2F, D-2F, and 4F all progressively increased ABCA1 protein levels during the chase incubations as their concentrations were increased to 10  $\mu\text{g/ml}$ . At higher concentrations, no further increase in ABCA1 was observed (data not shown). When cells were incubated for 15 min with [ $^{35}\text{S}$ ]methionine after the chase incubations, neither apoA-I nor peptides had any effect on the incorporation of radiolabel into ABCA1 (Fig. 4C). Thus, the peptide-mediated increase in ABCA1 protein levels could not be attributed to enhanced synthesis. These results indicate that these



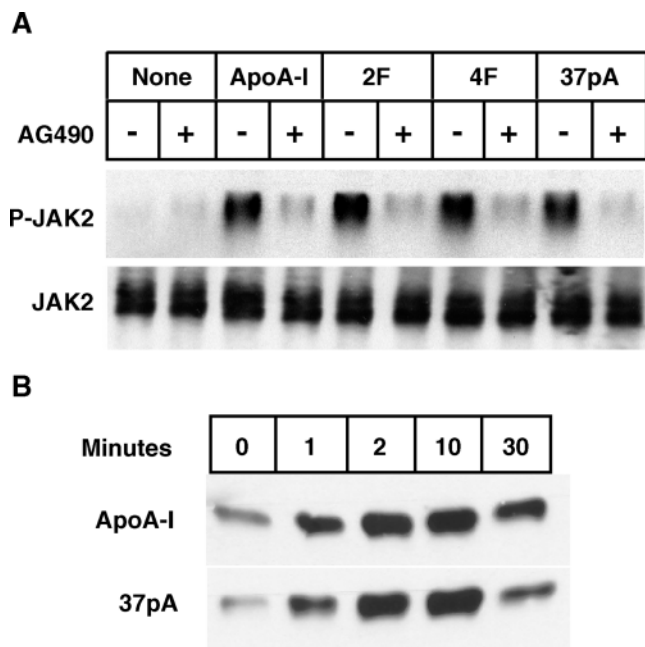
**Fig. 4.** Amphipathic helical peptides mimic apoA-I in stabilizing ABCA1 protein. A: Cholesterol-loaded macrophages were incubated for 18 h with 0.5 mM 8-Br-cAMP (cAMP) and then incubated for 4 h with (+) or without (-) 8-Br-cAMP minus (None) or plus 10  $\mu\text{g/ml}$  apoA-I or 20  $\mu\text{g/ml}$  37pA, and the membrane content of ABCA1 was measured by immunoblot analysis. B: Cholesterol-loaded, cAMP-treated macrophages were incubated for 4 h without 8-Br-cAMP plus the indicated concentrations of apoA-I or peptide before measuring ABCA1 levels. C: Cholesterol-loaded, cAMP-treated macrophages were incubated for 4 h without 8-Br-cAMP plus 10  $\mu\text{g/ml}$  apoA-I or 20  $\mu\text{g/ml}$  of the indicated peptide, cell proteins were radiolabeled with [ $^{35}\text{S}$ ]methionine (100  $\mu\text{Ci/ml}$ ) for 15 min, ABCA1 was isolated by immunoprecipitation and SDS-PAGE, and  $^{35}\text{S}$ -labeled ABCA1 was detected by phosphorimaging.

synthetic peptides mimic the ABCA1-stabilizing effects of apoA-I.

We showed previously that the interaction of apoA-I with ABCA1-expressing cells stimulates the autophosphorylation of JAK2 (14). Here, we examined whether synthetic peptides also have this effect. Immunoblot analyses with an antibody specific for phosphorylated JAK2 revealed that JAK2 was poorly phosphorylated in ABCA1-transfected BHK cells in the absence of apolipoprotein or peptide (Fig. 5A). Incubating cells with apoA-I, 2F, 4F, or 37pA for only 15 min dramatically increased the fraction of JAK2 that was phosphorylated. We observed the same stimulatory effect with 37pA that contained all D-amino acids (data not shown). Most of this increase in JAK2 phosphorylation was prevented by the JAK2-specific tyrosine kinase inhibitor AG490, indicating that this was an autophosphorylation event. Neither apoA-I, peptides, nor inhibitor had any effect on total JAK2 levels. Thus, these synthetic peptides mimic apoA-I in stimulating the autophosphorylation of JAK2.

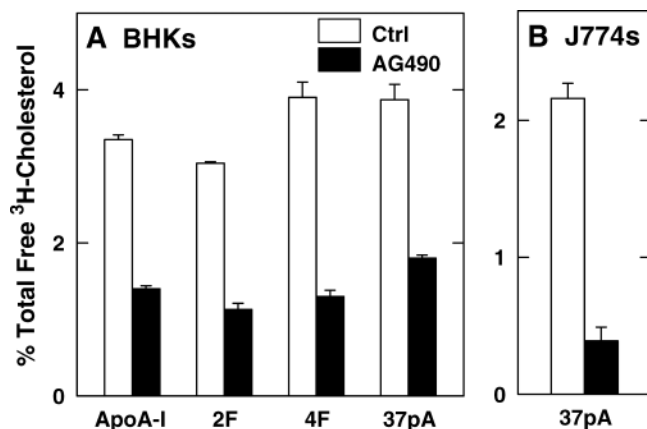
Time courses revealed that apoA-I and 37pA markedly increased the phosphorylation of JAK2 after only 1 min of exposure to cells (Fig. 5B), indicating that this was an acute response to the interaction of amphipathic helices with ABCA1-expressing cells. Maximum stimulation of JAK2 phosphorylation occurred at  $\sim 5$   $\mu\text{g/ml}$  for both apoA-I and 37pA (data not shown).

To determine whether this activated JAK2 has functional significance, we examined the effects of the JAK2



**Fig. 5.** Peptides stimulate Janus kinase 2 (JAK2) autophosphorylation. **A:** Mifepristone-treated ABCA1-transfected BHK cells were incubated for 1 h without (–) or with (+) 40  $\mu$ M AG490 followed by 15 min without (None) or with 10  $\mu$ g/ml apoA-I or 20  $\mu$ g/ml of the indicated peptide, and tyrosine-phosphorylated JAK2 (P-JAK2) was detected by immunoblot analysis. Blots were stripped of antibody and reprobed with a total JAK2 antibody (JAK2). **B:** ABCA1-transfected BHK cells were incubated for the indicated times with 10  $\mu$ g/ml apoA-I or 20  $\mu$ g/ml 37pA, and phosphorylated JAK2 was measured by immunoblot analysis.

inhibitor AG490 on peptide-mediated cholesterol efflux. AG490 substantially reduced the ability of apoA-I, 2F, 4F, and 37pA to remove [ $^3$ H]cholesterol from ABCA1-transfected BHK cells (**Fig. 6A**) and the ability of 37pA to



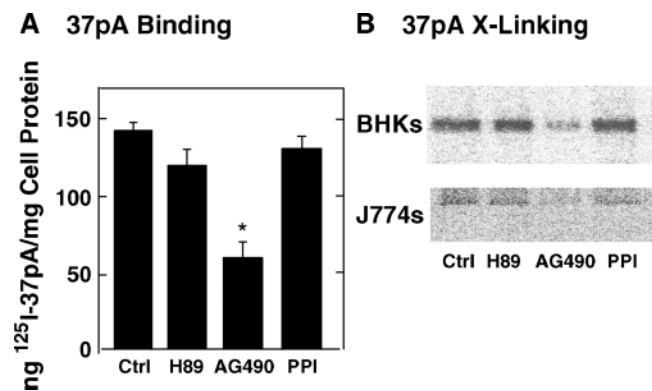
**Fig. 6.** JAK2 inhibition reduces amphipathic helical peptide-mediated cholesterol efflux from ABCA1-expressing cells. [ $^3$ H]cholesterol-labeled ABCA1-expressing BHK cells (**A**) or [ $^3$ H]cholesterol-loaded, cAMP-treated J774 macrophages (**B**) were incubated for 3 h without [control (Ctrl); open bars] or with (closed bars) 40  $\mu$ M AG490, and [ $^3$ H]cholesterol efflux was measured after subsequent 2 h incubations with 10  $\mu$ g/ml apoA-I (**A**) or 20  $\mu$ g/ml of the indicated peptide (**A, B**). Each value is the mean  $\pm$  SD of triplicate incubations.

remove [ $^3$ H]cholesterol from 8-Br-cAMP-treated J774 macrophages (**Fig. 6B**). Thus, the removal of cellular cholesterol by apoA-I and amphipathic helical peptides is largely dependent on an active JAK2.

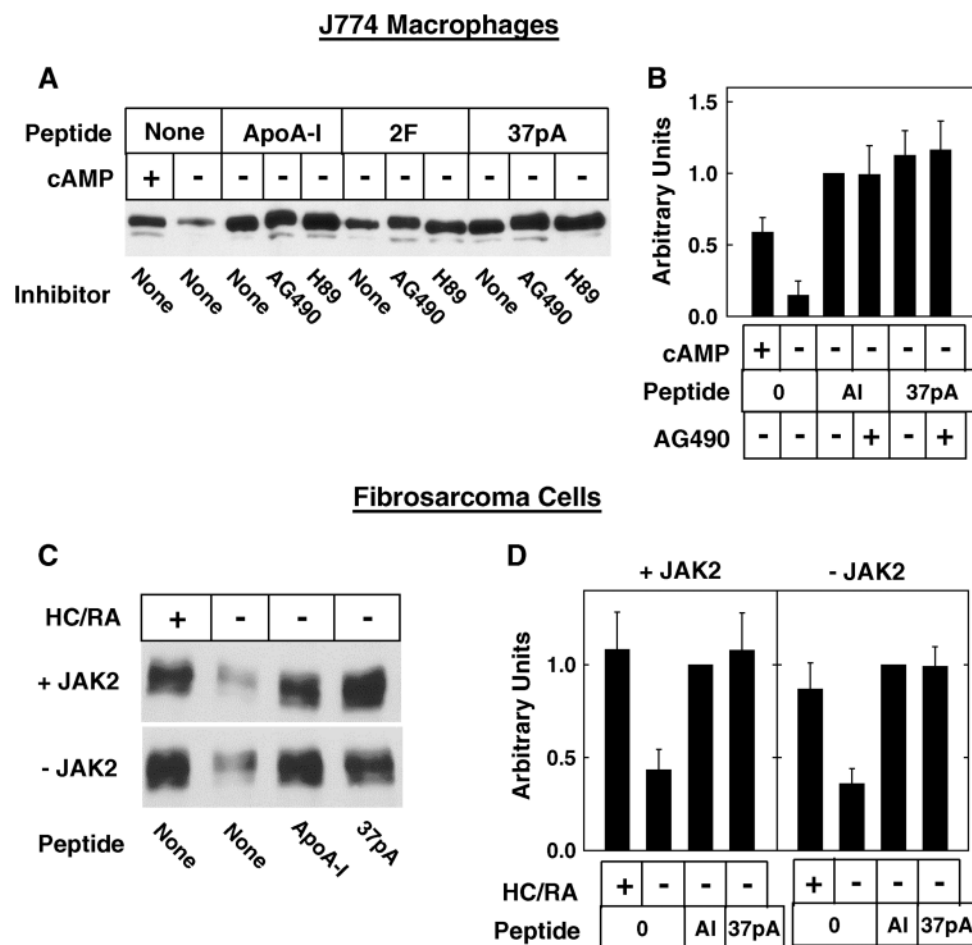
We showed previously that an active JAK2 is required for the ABCA1 binding of apoA-I that mediates the removal of cellular lipids (14). Therefore, we tested the effects of the JAK2 inhibitor AG490 on the binding of [ $^{125}$ I]-37pA to ABCA1-expressing BHK cells and on cross-linking of this peptide to ABCA1 in ABCA1-transfected BHK cells and 8-Br-cAMP-treated J774 cells. We used the protein kinase A inhibitor H89 and the Src family tyrosine kinase inhibitor PPI as controls (14). AG490, but not H89 or PPI, inhibited [ $^{125}$ I]-37pA binding to BHK cells (**Fig. 7A**) and almost abolished covalent cross-linking of [ $^{125}$ I]-37pA to ABCA1 in BHK cells and macrophages (**Fig. 7B**). Thus, as with apoA-I, optimum binding of 37pA to ABCA1 requires an active JAK2.

We investigated the possibility that JAK2 is involved in the ABCA1-stabilizing effects of apoA-I and peptides. As in **Fig. 4**, J774 macrophages were treated with 8-Br-cAMP to induce ABCA1 and then chased for 4 h in the absence of 8-Br-cAMP with or without apoA-I or peptides. To test the effects of kinase inhibitors, AG490 or H89 was added to the medium 2 h before and during the chase incubations. Neither AG490 nor H89 significantly interfered with the ability of apoA-I, 2F, or 37pA to prevent the degradation of ABCA1 that occurs after the removal of inducer (**Fig. 8A, B**). Thus, despite a large reduction in the peptide binding activity of ABCA1, JAK2 inhibition had no effect on peptide-mediated ABCA1 stabilization.

To confirm a lack of involvement of JAK2 in ABCA1 stabilization, we performed a similar pulse-chase protocol with a human fibrosarcoma cell line that expresses JAK2



**Fig. 7.** JAK2 inhibition reduces the binding of 37pA to ABCA1-expressing cells and to ABCA1. **A:** ABCA1-expressing BHK cells were incubated for 3 h without [control (Ctrl)] or with 40  $\mu$ M H89, 40  $\mu$ M AG490, or 10  $\mu$ M PPI and then incubated for an additional 2 h with the same medium containing 2  $\mu$ g/ml [ $^{125}$ I]-37pA with or without 100  $\mu$ g/ml unlabeled peptide, and the cell binding of [ $^{125}$ I]-37pA was measured. Each value is the mean  $\pm$  SD of triplicate incubations. Asterisk indicates  $P < 0.05$  compared to Control (Ctrl). **B:** ABCA1-expressing BHK cells or J774 macrophages were incubated without [control (Ctrl)] or with inhibitors and [ $^{125}$ I]-37pA (5  $\mu$ g/ml, no unlabeled peptide) as in **A**. After treating with the cross-linker DSS, ABCA1 was isolated by immunoprecipitation and SDS-PAGE, and [ $^{125}$ I]-labeled ABCA1 was detected by phosphorimaging.



**Fig. 8.** ApoA-I and amphipathic helical peptides stabilize ABCA1 independent of JAK2. **A:** Cholesterol-loaded macrophages were incubated for 16 h with 0.5 mM 8-Br-cAMP (cAMP) followed by 2 h incubations with 0.5 mM 8-Br-cAMP minus inhibitor (None) or plus 40  $\mu$ M AG490 or 40  $\mu$ M H89. Cells were then incubated for 4 h with (+) or without (-) 8-Br-cAMP minus peptide (None) or plus 10  $\mu$ g/ml apoA-I or 20  $\mu$ g/ml peptide without (None) or with the indicated inhibitor. The membrane content of ABCA1 was measured by immunoblot analysis. **C:** JAK2-expressing wild-type 2C4 cells (+JAK2) and mutant  $\gamma$ 2A cells lacking JAK2 (-JAK2) were incubated for 18 h with 22(*R*)-hydroxycholesterol plus 9-*cis*-retinoic acid (HC/RA) followed by 4 h incubations with (+) or without (-) HC/RA minus peptide (None) or plus 10  $\mu$ g/ml apoA-I or 20  $\mu$ g/ml 37pA, and ABCA1 levels were measured by immunoblot analysis. **B, D:** ABCA1 bands on immunoblots from three separate experiments with each cell line were quantitated (means  $\pm$  SD) by scanning computer analysis and normalized to values for cells incubated with apoA-I.

(2C4 cells) and a mutant line derived from these cells that lacks JAK2 ( $\gamma$ 2A cells) (14, 28, 29). Treating these cell lines with the liver X receptor ligand 22(*R*)-hydroxycholesterol plus the retinoid X receptor ligand 9-*cis*-retinoic acid induced ABCA1 to similar levels (Fig. 8C, D), as was shown previously (14). When these cells were then incubated for 4 h in the absence of these ligands, ABCA1 protein levels decreased in both cell lines, and including either apoA-I or 37pA in the chase medium prevented this disappearance (Fig. 8C, D). Thus, expression of JAK2 is not required for the ABCA1-stabilizing effects of amphipathic helical peptides.

## DISCUSSION

The current study shows that small synthetic peptide analogs of class A amphipathic  $\alpha$ -helices can mimic apoA-I

in promoting ABCA1-dependent cholesterol efflux, binding to ABCA1, stabilizing ABCA1 protein, and stimulating the autophosphorylation of JAK2 in ABCA1-expressing cells. Inhibition of JAK2 markedly reduced peptide-mediated cholesterol efflux from ABCA1-expressing cells and peptide binding to ABCA1, indicating that JAK2 plays a role in the interaction of amphipathic helices with ABCA1 required for removing cellular lipids. In contrast, these peptides were still capable of stabilizing ABCA1 protein when JAK2 was inhibited or absent. Thus, the ABCA1-stabilizing activity of amphipathic helices involves a JAK2-independent mechanism that is distinct from the lipid efflux-promoting interactions of helices with ABCA1.

Our results confirm a previous report showing that small apolipoprotein-mimetic peptides can remove cellular lipids by both ABCA1-independent and -dependent mechanisms (18), although the ABCA1 pathway domi-

nates in cells expressing high levels of this transporter. For the ABCA1-independent process, peptides comprising one 18 amino acid helix were more active than a peptide with two tandem helices, implying that monomeric helical peptides solubilize plasma membrane lipids to a greater extent than peptides containing multiple helices. Pre-treating cells with the monomers but not with the dimer increased nonspecific apoA-I binding to the cell surface, which is also consistent with a disruption of membrane lipids by the smaller peptides.

A previous study showed that the monomeric amphipathic helical peptide 2F could compete with cross-linking of apoA-I to ABCA1 (22), suggesting that this peptide binds directly to this transporter. Another study showed that the dimeric amphipathic helical peptides L-37pA and D-37pA could mimic apolipoproteins in stabilizing ABCA1 (21). Here, we show that the single helical peptides L-2F, D-2F, and 4F and the 37pA dimer all interact with ABCA1 and stabilize the protein. All of these peptides inhibited apoA-I binding to ABCA1-expressing cells and cross-linking of apoA-I to ABCA1, and the dimer was directly cross-linked to ABCA1. These results suggest that these peptides and full-length apolipoproteins interact with closely spaced or common sites on ABCA1. All of these peptides also inhibited ABCA1 degradation in J774 macrophages over similar concentration ranges.

We showed previously that the interaction of apoA-I with ABCA1-expressing cells acutely stimulates the autophosphorylation of the tyrosine kinase JAK2 and that an active JAK2 was required for ABCA1 binding of apoA-I and removing cellular lipids (14). Here, we show that the small synthetic amphipathic helical peptides also mimic apoA-I in activating JAK2 and that this occurs within 1 min of exposure of these helices to ABCA1-expressing cells. The JAK2-specific inhibitor AG490 inhibited both apoA-I- and peptide-mediated cholesterol efflux from ABCA1-expressing cells by >50%, indicating that JAK2 plays a role in modulating ABCA1-dependent lipid transport to small peptides as well as to apoA-I. This inhibitor also greatly reduced covalent cross-linking of 37pA to ABCA1 in ABCA1-transfected cells and ABCA1-expressing macrophages. Thus, the JAK2-regulated binding of amphipathic helices to ABCA1 appears to promote lipid removal from cells.

Together, the current findings indicate that ABCA1-dependent lipid removal, apolipoprotein binding sites on ABCA1, stabilization of ABCA1 protein, and ABCA1-dependent activation of JAK2 all have a broad specificity for amphipathic helices. Results showing that amphipathic helical peptides containing all D-amino acids have the same activities also indicate a lack of peptide stereoselectivity for all of these processes. These observations raised the possibility that amphipathic helices elicit these diverse cellular responses by a common mechanism. Although ABCA1 binding of amphipathic helices appears to be necessary for lipid removal, it was less clear whether other cellular responses to these helices also require ABCA1 binding. For example, there is evidence that the interaction of apolipoproteins with cellular lipids plays a role in stabilizing ABCA1 (20, 21).

The current study provides direct evidence that the ABCA1-stabilizing effect of amphipathic helices uses cellular binding sites distinct from those that mediate lipid removal. Inhibition or lack of expression of JAK2 had no effect on the ability of apoA-I or peptides to block the degradation of ABCA1, despite reducing peptide binding to ABCA1 by >70%. An active JAK2, therefore, was not required for stabilizing ABCA1. It appears unlikely that the residual peptide binding to ABCA1 in the absence of an active JAK2 could account for the stabilizing effects. Inhibiting JAK2 reduced peptide cross-linking to ABCA1 to levels observed when cells were incubated with <0.5 µg/ml apoA-I or peptide, which is <5% of the concentration needed to maximally inhibit ABCA1 degradation (~10 µg/ml). The best explanation for these findings is that direct binding of amphipathic helices to ABCA1 does not play a role in stabilizing ABCA1.

In summary, the current study shows that the amphipathic helix is the common structural motif that mediates multiple cellular responses to HDL apolipoproteins, including removal of lipids, direct binding to ABCA1, stabilization of ABCA1 protein, and activation of JAK2. Acute JAK2 activation appears to be a feed-forward mechanism for increasing the amphipathic helix binding activity of ABCA1 required for lipid removal. In contrast, ABCA1 stabilization by these helices is independent of JAK2, indicating that this process involves cellular interactions distinct from those that remove lipids. Thus, amphipathic helices coordinate the activity of the ABCA1 pathway through several distinct mechanisms that are likely to involve novel molecules. ■

This work was supported by National Institutes of Health Grants HL-18645, HL-55362, and DK-02456.

## REFERENCES

1. Oram, J. F. 2003. HDL apolipoproteins and ABCA1: partners in the removal of excess cellular cholesterol. *Arterioscler. Thromb. Vasc. Biol.* **23**: 720–727.
2. Wang, N., and A. R. Tall. 2003. Regulation and mechanisms of ATP-binding cassette transporter A1-mediated cellular cholesterol efflux. *Arterioscler. Thromb. Vasc. Biol.* **23**: 1178–1184.
3. Singaraja, R. R., L. R. Brunham, H. Visscher, J. J. Kastelein, and M. R. Hayden. 2003. Efflux and atherosclerosis: the clinical and biochemical impact of variations in the ABCA1 gene. *Arterioscler. Thromb. Vasc. Biol.* **23**: 1322–1332.
4. Wellington, C. L., L. R. Brunham, S. Zhou, R. R. Singaraja, H. Visscher, A. Gelfer, C. Ross, E. James, G. Liu, M. T. Huber, et al. 2003. Alterations of plasma lipids in mice via adenoviral-mediated hepatic overexpression of human ABCA1. *J. Lipid Res.* **44**: 1470–1480.
5. Assman, G., A. von Eckardstein, and H. B. Brewer. 1995. Familial high density lipoprotein deficiency: Tangier disease. In *The Metabolic and Molecular Bases of Inherited Disease*, C. R. Scriver et al., editors. McGraw-Hill, New York. 2053–2072.
6. Serfaty-Lacrosniere, C., F. Civeira, A. Lanzberg, P. Isaia, J. Berg, E. D. Janus, M. P. Smith, Jr., P. H. Pritchard, J. Frohlich, R. S. Lees, et al. 1994. Homozygous Tangier disease and cardiovascular disease. *Atherosclerosis*. **107**: 85–98.
7. Clee, S. M., J. J. Kastelein, M. van Dam, M. Marcil, K. Roomp, K. Y. Zwarts, J. A. Collins, R. Roelants, N. Tamasawa, T. Stulc, et al. 2000. Age and residual cholesterol efflux affect HDL cholesterol levels and coronary artery disease in ABCA1 heterozygotes. *J. Clin. Invest.* **106**: 1263–1270.

8. Joyce, C., L. Freeman, H. B. Brewer, Jr., and S. Santamarina-Fojo. 2003. Study of ABCA1 function in transgenic mice. *Arterioscler. Thromb. Vasc. Biol.* **23**: 965–971.
9. Aiello, R. J., D. Brees, and O. L. Francone. 2003. ABCA1-deficient mice: insights into the role of monocyte lipid efflux in HDL formation and inflammation. *Arterioscler. Thromb. Vasc. Biol.* **23**: 972–980.
10. Wang, C. S., P. Alaupovic, R. E. Gregg, and H. B. Brewer, Jr. 1987. Studies on the mechanism of hypertriglyceridemia in Tangier disease. Determination of plasma lipolytic activities,  $K_1$  values and apolipoprotein composition of the major lipoprotein density classes. *Biochim. Biophys. Acta.* **920**: 9–19.
11. Arakawa, R., and S. Yokoyama. 2002. Helical apolipoproteins stabilize ATP-binding cassette transporter A1 by protecting it from thiol protease-mediated degradation. *J. Biol. Chem.* **277**: 22426–22429.
12. See, R. H., R. A. Caday-Malcolm, R. R. Singaraja, S. Zhou, A. Silverston, M. T. Huber, J. Moran, E. R. James, R. Janoo, J. M. Savill, et al. 2002. PKA site-specific phosphorylation regulates ABCA1-mediated phospholipid efflux. *J. Biol. Chem.* **277**: 41835–41842.
13. Haidar, B., M. Denis, M. Marcil, L. Krimbou, and J. Genest, Jr. 2004. Apolipoprotein A-I activates cellular cAMP signaling through the ABCA1 transporter. *J. Biol. Chem.* **279**: 9963–9969.
14. Tang, C., A. M. Vaughan, and J. F. Oram. 2004. Janus kinase 2 modulates the apolipoprotein interactions with ABCA1 required for removing cellular cholesterol. *J. Biol. Chem.* **279**: 7622–7628.
15. Roosbeek, S., F. Peelman, A. Verhee, C. Labeur, H. Caster, M. F. Lensink, C. Cirulli, J. Grooten, C. Cochet, J. Vandekerckhove, et al. 2004. Phosphorylation by protein kinase CK2 modulates the activity of the ATP binding cassette A1 transporter. *J. Biol. Chem.* **279**: 37779–37788.
16. Vaughan, A. M., and J. F. Oram. 2003. ABCA1 redistributes membrane cholesterol independent of apolipoprotein interactions. *J. Lipid Res.* **44**: 1373–1380.
17. Hamon, Y., C. Broccardo, O. Chambenoit, M. F. Luciani, F. Toti, S. Chaslin, J. M. Freyssinet, P. F. Devaux, J. McNeish, D. Marguet, et al. 2000. ABC1 promotes engulfment of apoptotic cells and transbilayer redistribution of phosphatidylserine. *Nat. Cell Biol.* **2**: 399–406.
18. Remaley, A. T., F. Thomas, J. A. Stonik, S. J. Demosky, S. E. Bark, E. B. Neufeld, A. V. Bocharov, T. G. Vishnyakova, A. P. Patterson, T. L. Eggerman, et al. 2003. Synthetic amphipathic helical peptides promote lipid efflux from cells by an ABCA1-dependent and an ABCA1-independent pathway. *J. Lipid Res.* **44**: 828–836.
19. Wang, N., W. Chen, P. Linsel-Nitschke, L. O. Martinez, B. Agerholm-Larsen, D. L. Silver, and A. R. Tall. 2003. A PEST sequence in ABCA1 regulates degradation by calpain protease and stabilization of ABCA1 by apoA-I. *J. Clin. Invest.* **111**: 99–107.
20. Yamauchi, Y., M. Hayashi, S. Abe-Dohmae, and S. Yokoyama. 2003. ApoA-I activates PKC $\alpha$  signaling to phosphorylate and stabilize ABCA1 for the HDL assembly. *J. Biol. Chem.* **278**: 47890–47897.
21. Arakawa, R., M. Hayashi, A. T. Remaley, B. H. Brewer, Y. Yamauchi, and S. Yokoyama. 2004. Phosphorylation and stabilization of ATP binding cassette transporter A1 by synthetic amphiphilic helical peptides. *J. Biol. Chem.* **279**: 6217–6220.
22. Fitzgerald, M. L., A. L. Morris, A. Chroni, A. J. Mendez, V. I. Zannis, and M. W. Freeman. 2004. ABCA1 and amphipathic apolipoproteins form high-affinity molecular complexes required for cholesterol efflux. *J. Lipid Res.* **45**: 287–294.
23. Mendez, A. J., J. F. Oram, and E. L. Bierman. 1991. Protein kinase C as a mediator of high density lipoprotein receptor-dependent efflux of intracellular cholesterol. *J. Biol. Chem.* **266**: 10104–10111.
24. Goldstein, J. L., Y. K. Ho, S. K. Basu, and M. S. Brown. 1979. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc. Natl. Acad. Sci. USA.* **76**: 333–337.
25. Anantharamaiah, G. M., J. L. Jones, C. G. Brouillette, C. F. Schmidt, B. H. Chung, T. A. Hughes, A. S. Bhowan, and J. P. Segrest. 1985. Studies of synthetic peptide analogs of the amphipathic helix. Structure of complexes with dimyristoyl phosphatidylcholine. *J. Biol. Chem.* **260**: 10248–10255.
26. Datta, G., M. Chaddha, S. Hama, M. Navab, A. M. Fogelman, D. W. Garber, V. K. Mishra, R. M. Epanand, R. F. Epanand, S. Lund-Katz, et al. 2001. Effects of increasing hydrophobicity on the physical-chemical and biological properties of a class A amphipathic helical peptide. *J. Lipid Res.* **42**: 1096–1104.
27. Mendez, A. J., G. M. Anantharamaiah, J. P. Segrest, and J. F. Oram. 1994. Synthetic amphipathic helical peptides that mimic apolipoprotein A-I in clearing cellular cholesterol. *J. Clin. Invest.* **94**: 1698–1705.
28. Watling, D., D. Guschin, M. Muller, O. Silvennoinen, B. A. Witthuhn, F. W. Quelle, N. C. Rogers, C. Schindler, G. R. Stark, J. N. Ihle, et al. 1993. Complementation by the protein tyrosine kinase JAK2 of a mutant cell line defective in the interferon-gamma signal transduction pathway. *Nature.* **366**: 166–170.
29. Kohllhuber, F., N. C. Rogers, D. Watling, J. Feng, D. Guschin, J. Briscoe, B. A. Witthuhn, S. V. Kotenko, S. Pestka, G. R. Stark, et al. 1997. A JAK1/JAK2 chimera can sustain alpha and gamma interferon responses. *Mol. Cell. Biol.* **17**: 695–706.
30. Wang, Y., and J. F. Oram. 2002. Unsaturated fatty acids inhibit cholesterol efflux from macrophages by increasing degradation of ATP-binding cassette transporter A1. *J. Biol. Chem.* **277**: 5692–5697.
31. Wang, Y., B. Kurdi-Haidar, and J. F. Oram. 2004. LXR-mediated activation of macrophage stearyl-CoA desaturase generates unsaturated fatty acids that destabilize ABCA1. *J. Lipid Res.* **45**: 972–980.
32. Oram, J. F., R. M. Lawn, M. R. Garvin, and D. P. Wade. 2000. ABCA1 is the cAMP-inducible apolipoprotein receptor that mediates cholesterol secretion from macrophages. *J. Biol. Chem.* **275**: 34508–34511.
33. Mendez, A. J., and J. F. Oram. 1997. Limited proteolysis of high density lipoprotein abolishes its interaction with cell-surface binding sites that promote cholesterol efflux. *Biochim. Biophys. Acta.* **1346**: 285–299.
34. Natarajan, P., T. M. Forte, B. Chu, M. C. Phillips, J. F. Oram, and J. K. Bielicki. 2004. Identification of an apolipoprotein A-I structural element that mediates cellular cholesterol efflux and stabilizes ATP binding cassette transporter A1. *J. Biol. Chem.* **279**: 24044–24052.