Identification of an ABCA1-dependent phospholipid-rich plasma membrane apolipoprotein A-I binding site for nascent HDL formation: implications for current models of HDL biogenesis

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Abstract **It is well accepted that both apolipoprotein A-I (apoA-I) and ABCA1 play crucial roles in HDL biogenesis and in the human atheroprotective system. However, the nature and specifics of apoA-I/ABCA1 interactions remain poorly understood. Here, we present evidence for a new cellular apoA-I binding site having a 9-fold higher capacity to bind apoA-I compared with the ABCA1 site in fibroblasts stimulated with 22-(*R*)-hydroxycholesterol/9-*cis*-retinoic acid. This new cellular apoA-I binding site was designated "high-capacity binding site" (HCBS). Glyburide drastically reduced ^{125}I-apoA-I binding to the HCBS, whereas ^{125}I-apoA-I showed no significant binding to the HCBS in ABCA1 mutant (Q597R) fibroblasts. Furthermore, reconstituted HDL exhibited reduced affinity for the HCBS. Deletion of the C-terminal region of apoA-I (Δ187-243) drastically reduced the binding of apoA-I to the HCBS. Interestingly, overexpressing various levels of ABCA1 in BHK cells promoted the formation of the HCBS. The majority of the HCBS was localized to the plasma membrane (PM) and was not associated with membrane raft domains. Importantly, treatment of cells with phosphatidylcholine-specific phospholipase C, but not sphingomyelinase, concomitantly reduced the binding of ^{125}I-apoA-I to the HCBS, apoA-I-mediated cholesterol efflux, and the formation of nascent apoA-I-containing particles. Together, these data suggest that a functional ABCA1 leads to the formation of a major lipid-containing site for the binding and the lipidation of apoA-I at the PM. Our results provide a biochemical basis for the HDL biogenesis pathway that involves both ABCA1 and the HCBS, supporting a two binding site model for ABCA1-mediated nascent HDL genesis.**—Hassan, H. H., M. Denis, D-Y. D. Lee, I. Iatan, D. Nyholt, I. Ruel, L. Krimbou, and J. Genest. **Identification of an ABCA1-dependent phospholipid-rich plasma membrane apolipoprotein A-I binding site for nascent HDL formation: implications for current models of HDL biogenesis.** ***J. Lipid Res.*** **2007.** 48: **2428–2442.**

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A growing body of evidence indicates that both apolipoprotein A-I (apoA-I) and ABCA1 not only play a major role in HDL biogenesis and in the reverse cholesterol transport process but also have emerged as potential targets for therapies designed to inhibit the development of atherosclerotic vascular disease (1, 2). Therefore, there is a strong scientific rationale for a clear understanding of the mechanisms and molecular pathways underlying the apoA-I lipidation process, especially the mechanistic basis of lipid-free apoA-I interaction with ABCA1.

ApoA-I binding to the extracellular domain of ABCA1 results in the active removal of cellular cholesterol and phospholipids to lipid-poor apolipoproteins from a variety of cells (3, 4). This process plays crucial roles in both the formation and maintenance of HDL levels in plasma and is likely important for the first step of the reverse cholesterol transport process from peripheral tissues, including macrophages in the vessel wall (5).

Although the molecular basis for the interaction between ABCA1 and apoA-I has yet to be elucidated, there are two prevailing hypotheses describing the interaction. First, the direct association model proposes that ABCA1 acts as a receptor to which the apoA-I ligand binds directly. This direct interaction is proposed to stimulate ABCA1's

Abbreviations: apoA-I, apolipoprotein A-I; 9CRA, 9-*cis*-retinoic acid; 2D-PAGGE, two-dimensional polyacrylamide nondenaturing gradient gel electrophoresis; DSP, dithiobis(succinimidylpropionate); HCBS, high-capacity binding site; ICC, intracellular compartment; LpA-I, nascent apolipoprotein A-I-containing particle; 22OH, rLpA-I, reconstituted high density lipoprotein particle; 22-(*R*)-hydroxycholesterol; PC-PLC, phosphatidylcholine-specific phospholipase C; PM, plasma membrane; SMase, sphingomyelinase; WT, wild-type.

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cholesterol efflux activity, resulting in the transfer of cholesterol and phospholipids onto the acceptor apolipoprotein. Evidence for the direct association model comes from chemical cross-linking studies performed in our laboratory and by others, which indicate that apoA-I and ABCA1 are in very close proximity (<7 Å) (6–9). A second model has been proposed suggesting that ABCA1 acts by flipping phospholipids to the outer leaflet of the plasma bilayer. Subsequently, apoA-I is proposed to bind these translocated phospholipids and then extract both phospholipid and cholesterol in a process that requires no direct interaction between the apolipoprotein and ABCA1 (10, 11). This view gained support from studies showing that an intact ABCA1 ATPase activity was required for apoA-I binding to the cell surface (10). On the other hand, it has been suggested that the ABCA1-mediated apoA-I lipidation process may occur inside the cell as part of a retroendocytosis pathway (12, 13). These contrasting models demonstrate that the molecular mechanism by which ABCA1 mediates the lipidation of apoA-I has yet to be clarified.

In the present study, we investigate the structural characteristics of cellular binding sites for apoA-I and their functions related to the biogenesis of nascent HDL particles.

MATERIALS AND METHODS

Patient selection

For the present study, we selected fibroblasts from three normal control subjects and one patient with Tangier disease (homozygous for Q597R at the ABCA1 gene), as described previously (14). The protocol for the study was reviewed and accepted by the Research Ethics Board of the McGill University Health Center. Separate consent forms for blood sampling, DNA isolation, and skin biopsy were provided.

Cell culture

Human skin fibroblasts were obtained from 3.0 mm punch biopsies of the forearm of patients and healthy control subjects and were cultured in DMEM supplemented with 0.1% nonessential amino acids, penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% FBS. BHK cells stably transfected with an ABCA1 cDNA that is inducible by treating the cells with mifepristone and cells transfected with the same vectors lacking the ABCA1 cDNA insert (mock-transfected) were generously provided by Dr. John F. Oram from the Department of Medicine, University of Washington, and were characterized and cultured as described previously (15, 16). These BHK cells do not normally express ABCA1.

Purified apoA-I

Purified plasma apoA-I (Biodesign) was resolubilized in 4 M guanidine HCl and dialyzed extensively against PBS. Freshly resolubilized apoA-I was iodinated with ^{125}I by Iodo-Gen® (Pierce) to a specific activity of 3,000–3,500 cpm/ng apoA-I and used within 48 h. Expression of wild-type (WT) apoA-I and its mutants in a bacterial system, and the isolation and purification of these proteins, have been described previously by Marcel and colleagues (17). Deletion mutant apoA-I Δ(187-234) was provided by Dr. Yves L. Marcel.

Preparation of reconstituted HDL particles

Complexes comprising apoA-I and POPC were prepared using the sodium cholate dialysis method as described by Jonas, Steinmetz, and Churgay (18). An apoA-I/POPC molar ratio of 1:100 was used in this experiment. Reconstituted HDL particles (rLpA-I) were further concentrated by ultrafiltration (spiral ultrafiltration cartridge, molecular weight cut off 50,000; Amicon) to discard any lipid-free apoA-I or proteolytic peptides. ApoA-I-lipid complex formation was verified by analysis with two-dimensional polyacrylamide nondenaturing gradient gel electrophoresis (2D-PAGGE), as described previously (19).

Quantitative chemical cross-linking, and immunoprecipitation assay

Fibroblasts were grown to confluence in 100 mm diameter dishes and then stimulated with 2.5 μg/ml 22-(*R*)-hydroxycholesterol (22OH) and 10 μM 9-*cis*-retinoic acid (9CRA) for 20 h in DMEM/BSA. BHK cells stably expressing ABCA1 or mock-transfected cells were treated or not with mifepristone as described by Oram, Vaughan, and Stocker (15) and Vaughan and Oram (16). Cells were incubated in the presence of 10 μg/ml apoA-I for various periods of time or increasing concentrations of apoA-I. Cells were then washed three times with PBS. Dithiobis (succinimidylpropionate) (DSP) was dissolved immediately before use in DMSO. The cross-linking reaction was performed at room temperature in 6 ml/dish cross-linking solution for 30 min using 1 mM DSP, as described by Tall and colleagues (20). Cells were then washed twice with PBS and lysed at 4°C with immunoprecipitation buffer containing 20 mM Tris (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, and 0.5% dodecylmaltoside (Roche) in the presence of a protease inhibitor cocktail (Roche Diagnostics). Inactivation of the cross-linker was done by the addition of Tris (pH 7.5) to a final concentration of 20 mM. Samples containing ^{125}I-apoA-I cross-linked to ABCA1 (200 μg of total protein) were incubated with 10 μl of an affinity-purified human anti-ABCA1 antibody (Novus) for 20 h at 4°C, followed by the addition of Protein A bound to Sepharose (30 μl). The amount of bound iodinated apoA-I to ABCA1 (immunoprecipitates) or non-ABCA1-associated (supernatants) was determined by γ-counting, protein concentration was determined, and results were expressed as ng apoA-I/μg cell protein. However, both the incomplete cross-linking and immunoprecipitation of the apoA-I/ABCA1 complex may result in inaccurate quantification. Several controls were used to verify this important point. Different conditions of cross-linking and immunoprecipitation were used. Furthermore, we found that the cross-linking is effectively completed by 10 min, using 1 mM DSP.

Cellular cholesterol efflux

Cholesterol efflux was determined as described previously (7) with minor modifications. Briefly, 50,000 cells were seeded on 12-well plates. At midconfluence, the cells were labeled with 4–8 μCi/ml [^{3}H]cholesterol (Perkin-Elmer) for 24 h. At confluence, cells were cholesterol-loaded (20 μg/ml) for 24 h. During a 24 h equilibration period, cells were stimulated with 2.5 μg/ml 22OH and 10 μM 9CRA for 20 h. Cholesterol efflux were determined at either 2 h or 4 h with either 10 μg/ml apoA-I-HDL$_3$ or 3 μg/ml apoA-IΔ(187-243). Cellular cholesterol efflux was determined as follows: ^{3}H cpm in medium/(^{3}H cpm in medium + ^{3}H cpm in cells). The results are expressed as percentage of total radiolabeled cholesterol.

Cell surface biotinylation

Confluent fibroblasts were stimulated with 2.5 μg/ml 22OH and 10 μM 9CRA for 20 h. BHK cells stably expressing ABCA1 or

mock-transfected cells were treated or not with mifepristone. Cells were incubated in the presence of 10 μg/ml apoA-I for various time periods or for 45 min at 37°C with increasing concentrations of apoA-I. Cells were then washed three times with PBS, and surface proteins were biotinylated with 500 μg/ml sulfo-succinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate (Pierce) for 30 min at 4°C. The biotinylation reaction was quenched for 10 min at 4°C by the addition of 1 M Tris-HCl (pH 7.5) to the reaction mixture to a final concentration of 20 mM. Cells were washed twice with ice-cold PBS, lysed, and homogenized, and 200 μg of protein was added to 30 μl of streptavidin-Sepharose (Amersham Biosciences) beads and incubated overnight on a platform mixer at 4°C. The pellet [plasma membrane (PM)] or supernatant [intracellular compartment (ICC)] were washed two times with immunoprecipitation buffer and counted directly for radioactivity. To verify whether the biotinylation of surface protein is complete under the conditions used in the present experiment, the amount of biotin was increased up to 2 mg/ml. No significant additional association of ^{125}I-apoA-I with the PM or the ICC was observed.

Treatment with phospholipases

Confluent fibroblasts were incubated for 45 min in DMEM containing 10 μg/ml ^{125}I-apoA-I. After washing to remove unbound ^{125}I-apoA-I, cells were subjected to treatment with 2.5 U/ml phosphatidylcholine-specific phospholipase C (PC-PLC) or 0.4 U/ml sphingomyelinase (SMase) (Sigma) for 30 min at 37°C. After the washes, the cells were treated for cross-linking or cell surface biotinylation as described above. Cell membrane integrity and cellular toxicity under treatment with phospholipases were assessed by leakage of [^{3}H]adenine (21).

Triton X-100 cell solubilization

Cells were separated into Triton X-100-soluble and insoluble fractions as described previously (22). Briefly, confluent 22OH/9CRA-stimulated cells were washed in PBS and then scraped into MES-buffered saline (25 mM MES, pH 6.5, and 0.15 M NaCl) containing 1% Triton X-100 in the presence of a protease inhibitor cocktail. The suspension was homogenized, kept on ice for 20 min, and centrifuged at 14,000 *g* for 20 min at 4°C. The supernatant (containing the Triton X-100-soluble fraction) was removed, and the pellet was suspended in Triton X-100 buffer containing HEPES (pH 7.4) instead of MES and incubated at room temperature for 30 min to solubilize rafts. The amount of ^{125}I-apoA-I associated with the supernatants or pellets was determined by γ-counting. Aliquots of the supernatant and solubilized pellet were used for immunoblotting and ABCA1 immunoprecipitation.

Other methods

Removal of lipid-free apoA-I from nascent apolipoprotein A-I-containing particles (LpA-I) and separation of lipoproteins by 2D-PAGGE were performed as described previously (19, 23).

Statistical analysis

Results were compared statistically by the *t*-test. Two-tailed *P* values of <0.05 were considered significantly different.

RESULTS

Development of a quantitative binding assay for apoA-I

To examine the interaction between apoA-I and ABCA1 in a cell culture model, we first developed a quantitative assay based on optimal chemical cross-linking of ^{125}I-apoA-I to ABCA1 with the cell-permeable DSP followed by immunoprecipitation of the complex with an affinity-purified polyclonal anti-ABCA1, as described in Methods. The amount of bound iodinated apoA-I to ABCA1 (immunoprecipitates) or ^{125}I-apoA-I nonassociated with ABCA1 (supernatants) was determined by γ-counting. As shown in **Fig. 1B**, ^{125}I-apoA-I (10 μg/ml) was incubated with intact 22OH/9CRA-stimulated fibroblasts for 45 min at 37°C, followed by cross-linking and immunoprecipitation with an anti-ABCA1 antibody. Unexpectedly, the majority of ^{125}I-apoA-I was found nonassociated with ABCA1. We have assumed that the cross-liking reaction was complete based on results obtained with different concentration times of incubation with DSP (see Methods). Therefore, the concentration of 1 mM DSP and the incubation period of 30 min as well as 10 μl of ABCA1 antibody for the immunoprecipitation were used throughout the present study.

Importantly, a 45 min period of incubation of apoA-I with cells was chosen to permit sufficient time for the equilibration of apoA-I with different cellular compartments without interfering with the completion of the cross-linking reaction. It is possible, however, that the majority of ^{125}I-apoA-I found nonassociated with ABCA1 reflects the inefficiency of the cross-linking procedure. Several controls were used to verify this important point. Transferrin, a ligand that is known to specifically interact with the transferrin receptor, was used to verify the efficiency of the cross-linking reaction. ^{125}I-transferrin was incubated with HepG2 for 45 min at 37°C, followed by cross-linking with DSP (1 mM, 30 min) and immunoprecipitation of the cross-linked ^{125}I-transferrin/transferrin receptor complex using polyclonal anti-transferrin receptor antibody. We found that 92% of ^{125}I-transferrin coimmunoprecipitated with the transferrin receptor antibody (data not shown). This result supports the efficiency of the cross-linking reaction under similar conditions used for the cross-linking of apoA-I/ABCA1 described above.

The apoA-I/ABCA1 cross-linking assay permitted separation between the ABCA1 binding site and other eventual potential cellular sites for the binding of apoA-I, such as scavenger receptor class B type I, ABCG1, or specialized phospholipid membrane domains. However, there is no detectable presence of scavenger receptor class B type I or ABCG1 in human fibroblasts, as reported previously (24). To test the specificity of the cross-liking and immunoprecipitation methods, the presence of a 30-fold excess of unlabeled apoA-I during the incubation of ^{125}I-apoA-I with 22OH/9CRA-stimulated fibroblasts, the absence of 22OH/9CRA treatment, or ABCA1 mutant fibroblasts (Q597R) drastically reduced the larger fraction of ^{125}I-apoA-I nonassociated with ABCA1 (Fig. 1B). Furthermore, the minor fraction of ^{125}I-apoA-I associated with ABCA1 was also reduced. This result is consistent with the total binding of ^{125}I-apoA-I to the cell under different conditions (Fig. 1A). The observation that the presence of ABCA1 mutant Q597R abolished the association of ^{125}I-apoA-I to both ABCA1 associated and nonassociated

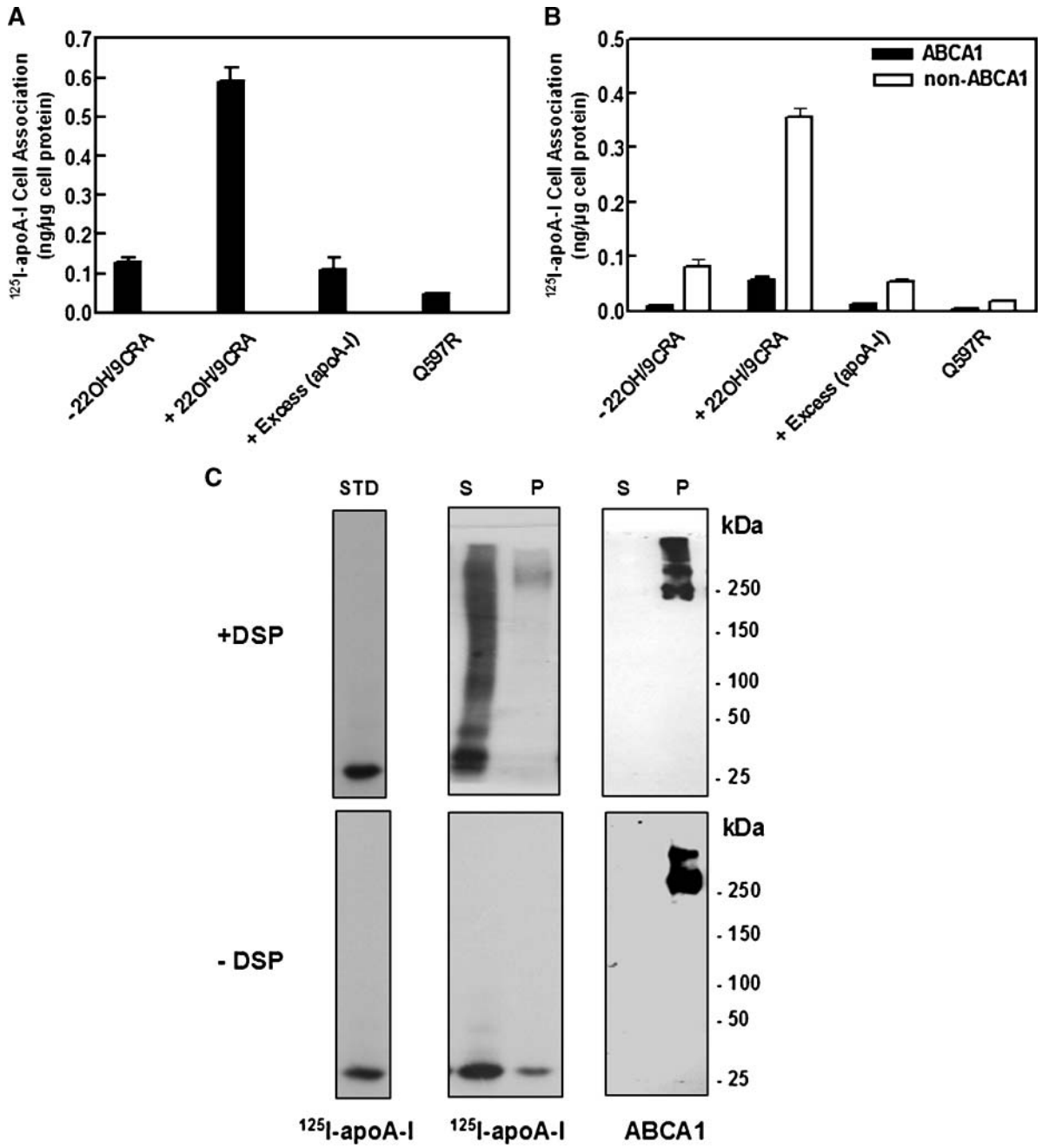

Fig. 1. Lipid-free apolipoprotein A-I (apoA-I) binding to ABCA1 and non-ABCA1 binding sites determined by quantitative cross-linking and immunoprecipitation assay. A: Confluent unstimulated, 22-(*R*)-hydroxycholesterol/9-*cis*-retinoic acid (22OH/9CRA)-stimulated fibroblasts and ABCA1 mutant (Q597R) fibroblasts were incubated with 10 μg/ml ^{125}I-apoA-I in the presence or absence of a 30-fold excess of unlabeled apoA-I (+ Excess) for 45 min at 37°C. After washing to remove unbound ^{125}I-apoA-I, cells were subjected to the dithiobis(succinimidylpropionate) (DSP) cross-linker (1 mM, 30 min). Before lysis, a fraction of cell lysate was counted directly for the determination of total ^{125}I-apoA-I binding to the cells. B: Samples containing ^{125}I-apoA-I cross-linked to ABCA1 (200 μg of total protein) were incubated with 10 μl of affinity-purified human anti-ABCA1 antibody. The radioactivity found in pellets (ABCA1-associated) and in supernatants (ABCA1-nonassociated) was determined by γ-counting as described in Methods. Values represent means ± SD from triplicate wells. Results shown are representative of four independent experiments. C (upper panel): Both the supernatants (S) and pellets (P) from ^{125}I-apoA-I incubated with 22OH/9CRA-stimulated normal fibroblasts, as described for B, as well as ^{125}I-apoA-I incubated in the presence of 1 mM DSP for 30 min without cells, used as a standard (STD), were loaded on a 4–22.5% SDS-PAGE gel and transferred onto a nitrocellulose membrane. ^{125}I-apoA-I was detected by direct autoradiography, and ABCA1 was revealed by an anti-ABCA1 antibody. C (lower panel): Experiments similar to those shown in the upper panel were conducted in the absence of DSP.

fractions indicates that a functional ABCA1 is required for the formation of the larger fraction of ^{125}I-apoA-I nonassociated with ABCA1.

To verify that the immunoprecipitates contained the majority of ABCA1, the immunoprecipitates and supernatants of 22OH/9CRA-stimulated fibroblasts in the presence and absence of DSP were analyzed by 4–22.5% SDS-PAGE under nonreducing conditions. ^{125}I-apoA-I was detected by direct autoradiography, and ABCA1 was revealed by an anti-ABCA1 antibody. As shown in Fig. 1C (upper panel), in the presence of DSP, the majority of ^{125}I-apoA-I was found in the supernatants as monomeric and multimeric forms, whereas a minor proportion of ^{125}I-apoA-I was found associated with ABCA1 in the immunoprecipitates. At the same time, no detectable ABCA1 was found in the supernatants, and the majority of the oligomeric ABCA1 complex was found in the immunoprecipitates, including the tetramers, as reported previously (25, 26). ^{125}I-apoA-I treated with DSP in the absence of the cell was used as a standard. On the other hand, ^{125}I-apoA-I associated or not with ABCA1 was found in its monomeric form in the absence of DSP (Fig. 1C, lower panel). This result is consistent with the quantitative analysis reported in Fig. 1B. The fact that the majority of ABCA1 was immunoprecipitated with the anti-ABCA1 antibody in the absence or presence of DSP indicates that the cross-linking with DSP did not affect the interaction of ABCA1 with the antibody. Together, these results support the specificity and the efficiency of both the cross-linking and immunoprecipitation methods and validate our quantitative assay.

Overexpressing various levels of ABCA1 in BHK cells promoted the formation of the putative non-ABCA1 binding site

Based on the experiments described above, we initially hypothesized that apoA-I nonassociated with ABCA1 may represent a new cellular apoA-I binding site. To further examine the role of ABCA1 in mediating the formation of this new, non-ABCA1 apoA-I binding site, different levels of ABCA1 were overexpressed in BHK cells with a mifepristone-inducible ABCA1 gene (15, 16). As shown in **Fig. 2A** (inset), in the absence of induction, BHK-ABCA1 cells had no detectable ABCA1 protein, whereas cells induced with 0.1 or 10 nM mifepristone for 20 h expressed increasing levels of ABCA1 in a dose-dependent manner. Concomitantly, cellular cholesterol efflux was increased significantly (2 ± 0.35, 8 ± 0.22, and 13 ± 0.68% at 0, 0.1, and 10 nM mifepristone, respectively). At the same time, the total binding of ^{125}I-apoA-I was also increased significantly by increasing the dose of mifepristone (Fig. 2A). On the other hand, the putative non-ABCA1 apoA-I binding site was increased significantly after induction with 10 nM mifepristone compared with 0.1 nM (Fig. 2B). Similarly, the smaller fraction of apoA-I associated with ABCA1 was also increased. These results support the data obtained with 22OH/9CRA-stimulated fibroblasts and indicate that ABCA1 is involved in the formation of the new putative non-ABCA1 apoA-I binding site.

Affinity binding of apoA-I to the putative non-ABCA1 binding site

To better characterize the new binding site for apoA-I, intact normal fibroblasts treated or not with 22OH/9CRA or ABCA1 mutant Q597R were incubated with increasing concentrations of ^{125}I-apoA-I for 45 min at 37°C. After washing to remove unbound ^{125}I-apoA-I, cross-linking, immunoprecipitation, and quantification of ^{125}I-apoA-I associated or not with ABCA1 were performed as described above. As shown in **Fig. 3A**, ^{125}I-apoA-I exhibited saturable binding to both ABCA1 and non-ABCA1 binding sites that was found to occur in a concentration-dependent manner in 22OH/9CRA-stimulated fibroblasts, whereas glyburide drastically reduced apoA-I binding to both sites (Fig. 3B). Furthermore, in the absence of 22OH/9CRA treatment, apoA-I exhibited reduced binding to both sites (Fig. 3C). At the same time, ABCA1 mutant fibroblasts (Q597R) showed no significant binding to both sites (Fig. 3D). We reported previously that the present ABCA1 mutant did not bind apoA-I but was expressed normally and localized to the cell surface (7, 27). Interestingly, kinetic analysis revealed that the apparent binding capacity of ^{125}I-apoA-I to the non-ABCA1 binding site in stimulated fibroblasts (Fig. 3A) was at least 9 orders of magnitude higher than that of the ABCA1 binding site, whereas apoA-I exhibited a nearly 2-fold higher affinity for ABCA1 than the non-ABCA1 binding site (**Table 1**). These results suggest that ABCA1 creates a new cellular "high-capacity binding site" (HCBS) for the binding of apoA-I.

Affinity binding of rLpA-I to the HCBS

We have shown previously that lipid association with apoA-I or apoE3 reduced their ability to interact with ABCA1 (7, 23, 28). In the current study, we tested the role of lipid association on the ligand binding affinity of apoA-I to both ABCA1 and non-ABCA1 binding sites (HCBS). ^{125}I-apoA-I was used for the preparation of rLpA-I with POPC, as described in Methods. Most of the discoidal rLpA-I had diameters of 13 nm (data not shown). **Figure 4A, B** shows that ^{125}I-rLpA-I exhibited a 6-fold decreased capacity and a 2-fold decreased affinity for the HCBS compared with lipid-free ^{125}I-apoA-I (Table 1). At the same time, ^{125}I-rLpA-I showed a nearly complete absence of binding to the ABCA1 binding site. This is consistent with our previous results showing that either rLpA-I or HDL_3 competed poorly for the binding of ^{125}I-apoA-I to ABCA1 (7). This result indicates that the association of apoA-I with lipid decreased apoA-I affinity for both ABCA1 and the HCBS. It is possible that apoA-I lipidation weakened the formation of a high-affinity complex between apoA-I and ABCA1 that resulted in impaired binding of apoA-I to the HCBS.

Effect of the C-terminal deletion of apoA-I on the ability of apoA-I to bind to the HCBS

It is well documented that the C-terminal region of apoA-I is important in the ABCA1-mediated lipid efflux pathway. Indeed, previous studies have documented that the apoA-I deletion mutant lacking residues 187–243 of the C-terminal domain [Δ(187-243)] exhibits both reduced

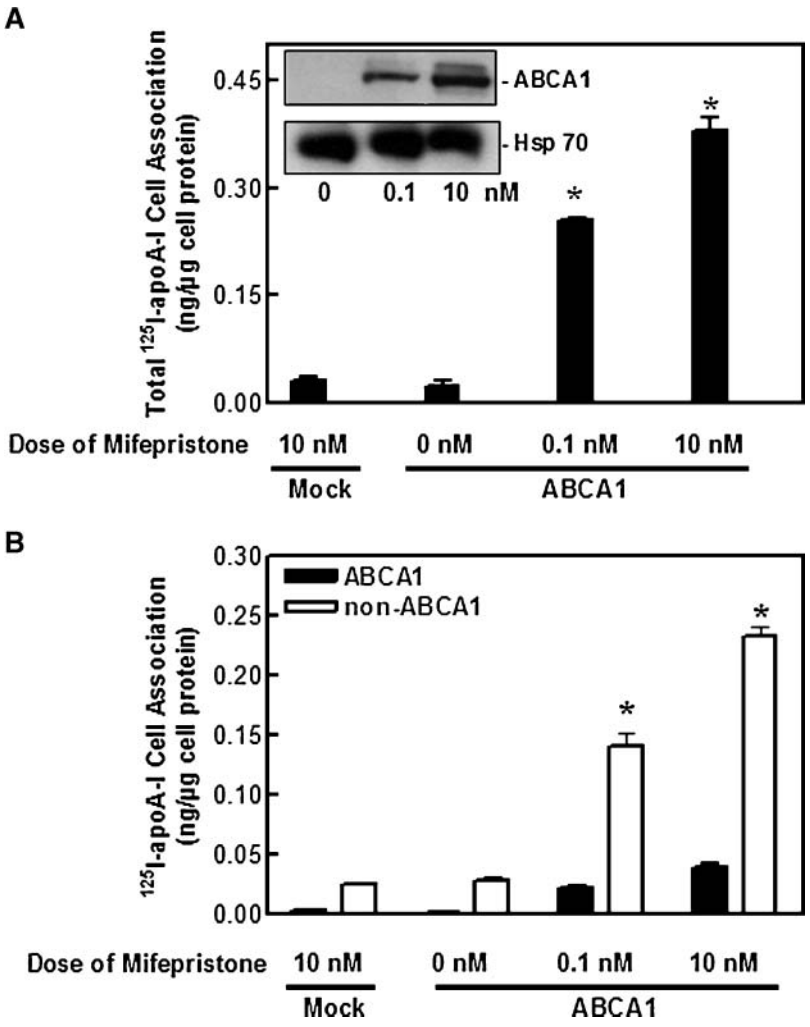

Fig. 2. ABCA1 expression promoted the formation of a non-ABCA1 apoA-I binding site. A: Mock-transfected or transfected BHK cells were treated or not with 0.1 or 10 nM mifepristone for 20 h. Cells were incubated with 10 μg/ml ^{125}I-apoA-I for 45 min at 37°C. After washing to remove unbound ^{125}I-apoA-I, cells were subjected to DSP (1 mM, 30 min). Before lysis, a fraction of cell lysate was counted directly for the determination of total ^{125}I-apoA-I binding to the cells. The inset shows separation by SDS-PAGE of equal amounts of cell protein (40 μg) in ABCA1-transfected cells treated or not with 0.1 or 10 nM mifepristone for 20 h. ABCA1 was detected with an ABCA1 antibody. Cytosolic protein Hsp-70 served as a loading control. B: Samples containing ^{125}I-apoA-I cross-linked to ABCA1 (200 μg of total protein) were incubated with 10 μl of affinity-purified human anti-ABCA1 antibody. The radioactivity found in pellets (ABCA1-associated) and in supernatants (ABCA1-nonassociated) was determined by γ-counting. Values represent means ± SD from triplicate wells. Results shown are representative of two independent experiments. * $P < 0.001$.

cell surface binding and reduced ability to promote lipid efflux (29, 30). To further examine the role of the C-terminal domain of apoA-I in binding to the HCBS and in promoting ABCA1-mediated cholesterol efflux, iodinated WT apoA-I and the Δ(187-243) mutant (3 μg/ml) were incubated with stimulated fibroblasts for 45 min at 37°C. After washing to remove unbound ^{125}I-apoA-I, cross-linking, immunoprecipitation, and quantification of ^{125}I-apoA-I associated or not with ABCA1 were performed as described above. As shown in Fig. 4C, apoA-I mutant Δ(187-243) exhibited a drastically reduced binding to both ABCA1 and the non-ABCA1 binding site (HCBS). As expected, the presence of apoA-I mutant Δ(187-243) greatly reduced cholesterol efflux from 22OH/9CRA-stimulated fibroblasts compared with WT apoA-I (Fig. 4D). In a parallel experiment, we confirmed that apoA-I Δ(187-243) exhibited reduced cross-linking to ABCA1 compared with WT apoA-I, as assessed by SDS-PAGE followed by detection of the apoA-I/ABCA1 complex with apoA-I antibody (data not shown). These results indicate that the C-terminal domain of apoA-I is essential for the binding of apoA-I to both ABCA1 and the HCBS.

Localization of the HCBS to the PM

The finding that ABCA1 mediated the formation of a new apoA-I binding site (HCBS) raised the question of whether this binding site is localized to the PM and/or ICCs. To address this question, we used a well-established biochemical assay based on a cell surface biotinylation approach to quantify the amount of apoA-I associated with the PM and ICCs. Stimulated intact normal or ABCA1 mutant (Q597R) fibroblasts were incubated with 10 μg/ml ^{125}I-apoA-I for 45 min at 37°C. Biotinylation was performed, and ^{125}I-apoA-I associated with PM (pellets) or ICCs (supernatants) was determined by γ-counting. As shown in **Fig. 5A**, PM was found to have a 2-fold higher capacity to accommodate apoA-I compared with ICCs. In separate experiments, we found that ^{125}I-apoA-I exhibited saturable binding that occurred in a concentration-dependent manner to both PM and ICC fractions (data not shown). Furthermore, no significant apoA-I binding was detected in both PM and ICC fractions in ABCA1 mutant Q597R, used as a negative control in the present experiment (Fig. 5A). The integrity of the PM and ICC fractions was assessed by separation of both isolated PM and ICCs by SDS-PAGE and detection of different cellular markers that colocalized specifically with the PM and ICCs by appropriate antibodies. No significant contamination of the PM with ICCs was observed, as assessed by the absence of Alpha-2 integrin subunit from ICCs, which is considered a marker for the PM. At the same time, heat shock

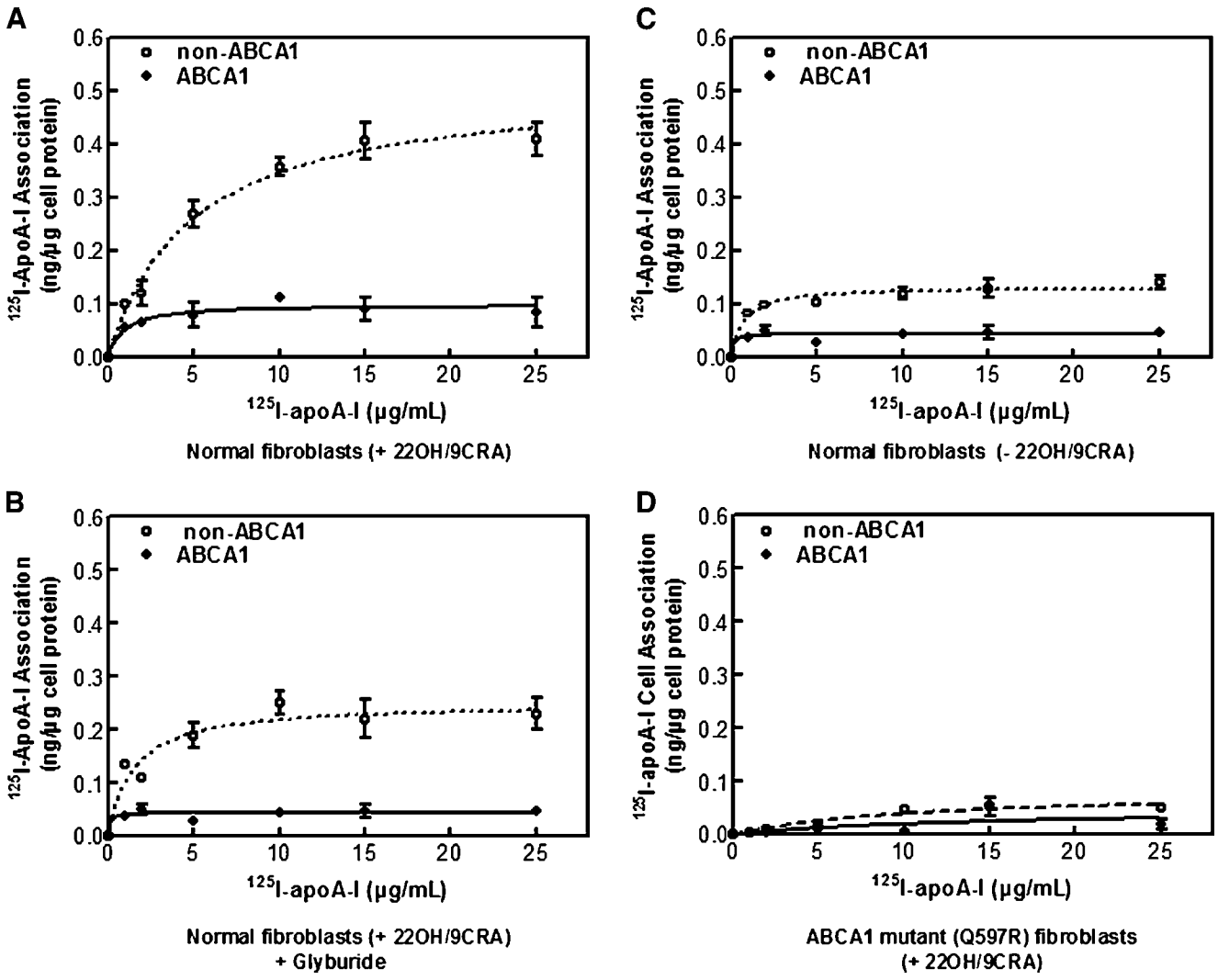

Fig. 3. Effects of 22OH/9CRA and glyburide on the association of apoA-I with ABCA1 and the non-ABCA1 binding site ["high-capacity binding site" (HCBS)]. Confluent normal (A–C) and ABCA1 mutant (Q597R) (D) fibroblasts were incubated with increasing concentrations of ^{125}I-apoA-I for 45 min at 37°C in the presence (A, B, D) or absence (C) of 22OH/9CRA or 300 µM glyburide (B). After washing to remove unbound ^{125}I-apoA-I, cells were subjected to DSP (1 mM for 30 min). Samples (200 µg of total protein) were incubated with 10 µl of affinity-purified human anti-ABCA1 antibody. The radioactivity found in pellets (ABCA1-associated) and in supernatants (ABCA1-nonassociated) was determined by γ-counting. Values represent means ± SD from triplicate wells. Results shown are representative of four different independent experiments. Binding parameters of ^{125}I-apoA-I to both ABCA1 and non-ABCA1 binding sites (dissociation constant and binding capacity) were obtained using GraphPad Prism 4.00 software.

protein-70 and tubulin were found exclusively associated with ICCs (data not shown). This finding indicates that the majority of the HCBS was associated with the PM domain.

Structural characterization of the HCBS

Given that ABCA1 is a major protein that binds apoA-I in fibroblasts, as assessed by cross-linking and SDS-PAGE (7, 25), the question was raised whether the HCBS is constituted of phospholipids. We reasoned that treating the cells with specific phospholipases would severely disrupt the lipid environment, structure, or composition of the HCBS, which could subsequently decrease the binding of apoA-I. As shown in Fig. 5B, treatment of intact fibroblasts with PC-PLC, but not SMase, as described in Methods, drastically reduced the association of ^{125}I-apoA-I with the HCBS. To further assess the specific effect of PC-PLC and SMase on ^{125}I-apoA-I associated with both the PM and ICC domains, after ^{125}I-apoA-I binding and treatment or not with phospholipases, the cells were biotinylated for 30 min at 4°C. Then, the amount of ^{125}I-apoA-I associated with the PM and ICCs was determined by direct γ-counting. As shown in Fig. 5C, PC-PLC treatment decreased by 70% the amount of apoA-I associated with the PM, whereas SMase treatment induced a modest reduction in apoA-I associated with PM (10%). As expected, phospholipase treatment did not significantly affect the amount of apoA-I associated with ICCs.

To assess how effectively phospholipids were removed by phospholipase treatment, the cells were labeled with

TABLE 1. Binding parameters of the interaction of lipid-free apoA-I and rLpA-I with ABCA1 and non-ABCA1 binding sites ("high-capacity binding site")

Binding Affinity Parameters	Binding Capacity	Dissociation Constant
	ng/μg cell protein	*μg/ml*
^{125}I-apoA-I		
ABCA1-associated	0.04 ± 0.01[a]	1.80 ± 0.10[b]
ABCA1-nonassociated	0.36 ± 0.03	3.50 ± 0.20
^{125}I-rLpA-I		
ABCA1-associated	0.01 ± 0.01[a]	9.25 ± 0.80[b]
ABCA1-nonassociated	0.06 ± 0.01	7.50 ± 1.45

ApoA-I, apolipoprotein A-I; rLpA-I, reconstituted high density lipoprotein particle. rLpA-I was prepared as described in Methods. The values shown are means ± SD of triplicate measures. Kinetic data were obtained using GraphPad Prism 4.0 software. *P* values are for ABCA-associated compared with ABCA1-nonassociated for lipid-free apoA-I and for comparison between lipid-free apoA-I and rLpA-I, respectively.

[a] $P < 0.001$.

[b] $P < 0.05$.

[^{3}H]choline and the lipids were separated by TLC and counted. PC-PLC and SMase treatment digested >65% of phosphatidyl [^{3}H]choline and 80% of [^{3}H]sphingomyelin (Fig. 5D). [^{3}H]free cholesterol was used as a control for the present experiment. On the other hand, cell membrane integrity and cellular toxicity after phospholipase treatments were assessed by [^{3}H]adenine leakage. No significant increase of [^{3}H]adenine release was observed with both PC-PLC and SMase treatments compared with untreated cells (data not shown). This result suggests that the HCBS is a phosphatidylcholine-containing site that binds apoA-I. The lack of SMase effect on the binding of ^{125}I-apoA-I to the HCBS indicates that the present binding site is not associated with sphingomyelin-rich membrane raft domains. To further verify that the HCBS was not localized to membrane rafts, the distribution of ^{125}I-apoA-I associated with the HCBS between Triton X-100-soluble and insoluble fractions was examined. Detergent insolubility has been used as a tool to identify lipid rafts and associated proteins (31).

After incubation with ^{125}I-apoA-I, stimulated cells were subjected or not to DSP and homogenized with Triton X-100 buffer, and the detergent-soluble and insoluble fractions were separated as described in Methods. As shown in **Fig. 6A**, the majority of ^{125}I-apoA-I associated with the HCBS was found in the Triton X-100-soluble fraction. Furthermore, ^{125}I-apoA-I associated with the HCBS was not coimmunoprecipitated by an anti-caveolin-1 antibody (Fig. 6B, upper panel). This result indicates that the HCBS does not colocalize with caveolin-1, a marker for raft domains (Fig. 6B). Coimmunoprecipitation of the HCBS with an anti-clathrin antibody was used as a control for the present experiment and showed no significant association of the HCBS with clathrin, documented to be localized with membranes distinct from membrane rafts (32). Furthermore, a small proportion of ^{125}I-apoA-I was coimmunoprecipitated with anti-ABCA1 antibody, as described above. The immunoprecipitation of ABCA1, caveolin-1, or clathrin was complete, as assessed by the absence of these proteins from the supernatants, which contain the majority of ^{125}I-apoA-I associated with the HCBS (Fig. 6B, lower panel). At the same time, we confirmed that ABCA1 was found in the Triton X-100-soluble fraction and does not colocalize with caveolin-1, as documented previously (33, 34). These data indicate that both the HCBS and ABCA1 were not associated with membrane raft domains.

The HCBS is essential for the biogenesis of nascent apoA-I-containing particles

Having determined that treating cells with PC-PLC strongly decreased the amount ^{125}I-apoA-I associated with the HCBS, the question was raised whether the HCBS is involved in the lipidation of apoA-I. To further assess the role of the HCBS in promoting cholesterol efflux mediated by apoA-I, we incubated [^{3}H]cholesterol-labeled and cholesterol-enriched fibroblasts with lipid-free apoA-I or HDL_3 for 90 min, after treatment of fibroblasts with PC-PLC or SMase. As shown in **Fig. 7A**, PC-PLC treatment drastically reduced apoA-I-mediated cholesterol efflux from ABCA1-stimulated fibroblasts, but cholesterol efflux to HDL_3 was not affected significantly. In contrast, SMase treatment did not impair apoA-I-mediated cholesterol efflux, but efflux of cholesterol to HDL_3 was decreased. At the same time, lipid-free ^{125}I-apoA-I incubation with stimulated normal fibroblasts for 90 min at 37°C generated nascent-apoA-I-containing particles (LpA-I) having α-electrophoretic mobility with diameters of 9–20 nm, as we have documented previously (7, 23). In contrast, treating cells with PC-PLC, but not SMase, inhibited the formation of larger LpA-I particles (Fig. 7B).

Because PC-PLC and SMase were absent during ^{125}I-apoA-I incubation with cells, we ruled out the possibility that the inhibition of nascent LpA-I formation was attributable to a direct effect of PC-PLC on LpA-I particles. In parallel experiments, we documented that apoA-I-mediated cholesterol efflux, as well as the formation of nascent LpA-I particles, after phospholipase treatments were absent in ABCA1 mutant (Q597R) fibroblasts under conditions similar to those used in the present experiments (data not shown). On the other hand, there is no evidence for lipid-free ^{125}I-apoA-I aggregation, as assessed by the complete removal of lipid-free ^{125}I-apoA-I incubated with ABCA1 mutant (Q597R) fibroblasts, under treatment or not with phospholipases by filtration with a 50,000 molecular weight cut off filter system (23). The inhibition of both apoA-I-mediated cholesterol efflux and the formation of larger nascent LpA-I after the disruption of the HCBS with PC-PLC treatment indicate that the HCBS provides lipid for efflux promoted by apoA-I through the ABCA1 pathway, which is essential for the formation of nascent LpA-I particles.

DISCUSSION

In this study, we sought to better understand the molecular interaction between apoA-I and ABCA1 that leads to the formation of nascent HDL particles. Analysis of saturation binding data identified two distinct sites that bound

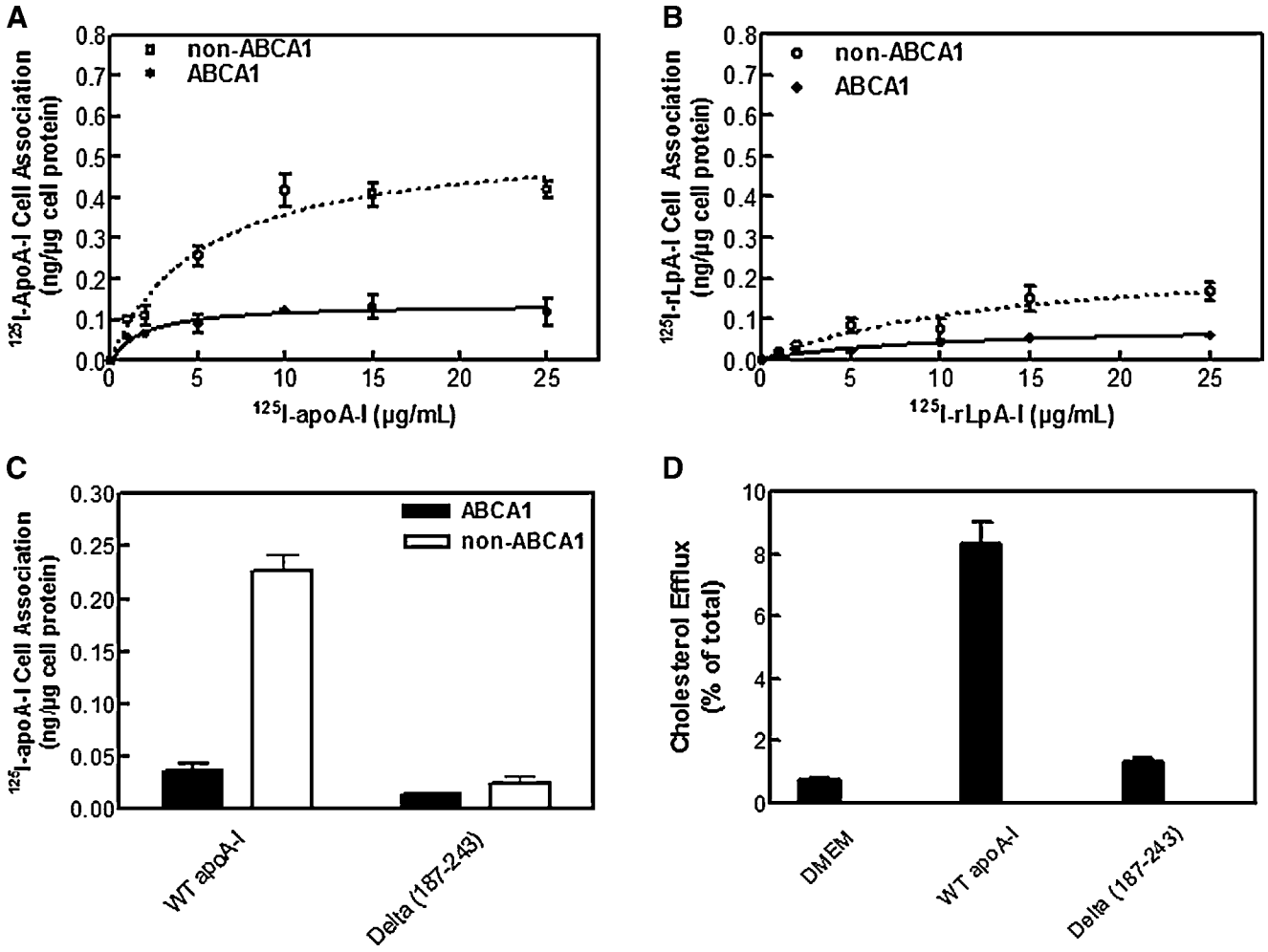

Fig. 4. Effects of apoA-I lipidation and deletion of the C-terminal domain of apoA-I on affinity binding to ABCA1 and the non-ABCA1 binding site (HCBS). A, B: Confluent stimulated normal fibroblasts were incubated with increasing concentrations of ^{125}I-apoA-I (A) or reconstituted HDL particles (^{125}I-rLpA-I) (B). After washing to remove unbound ligands, cells were subjected to DSP and the radioactivity associated with both ABCA1 and non-ABCA1 binding sites was determined. C: Iodinated wild-type (WT) apoA-I or apoA-I Δ(187-243) (3 μg/ml, specific activity = 3,500–4,000 cpm/ng) were incubated for 45 min at 37°C. After washing to remove unbound ligands, cells were subjected to DSP and the radioactivity associated with both ABCA1 and non-ABCA1 binding sites was determined. Binding parameters of ^{125}I-apoA-I and ^{125}I-rLpA-I to both ABCA1 and non-ABCA1 binding sites (dissociation constant and binding capacity) were obtained using GraphPad Prism 4.00 software. D: Confluent normal fibroblasts were labeled with 4 μCi/ml [^{3}H]cholesterol and then enriched with 20 μg/ml cholesterol. Cells were stimulated with 22OH/9CRA, and efflux was initiated by the addition of 3 μg/ml either WT apoA-I or apoA-I Δ(187-243). After a 4 h incubation, cholesterol efflux was determined as described in Methods. Values represent means ± SD from triplicate wells. * $P < 0.001$.

apoA-I in fibroblasts stimulated with 22OH/9CRA: a low-affinity site, which has a relatively high binding capacity (HCBS, nonassociated with ABCA1); and a high-affinity site, with a relatively low binding capacity (ABCA1 site). Importantly, the HCBS exhibited a 9-fold higher binding capacity to accommodate apoA-I compared with the ABCA1 binding site (Table 1). This is consistent with the results showing that overexpressing ABCA1 in BHK cells promoted the formation of the HCBS, whereas the inhibition of ABCA1 activity by glyburide or the presence of ABCA1 mutant fibroblasts (Q597R) drastically reduced the binding of apoA-I to the HCBS (Figs. 2, 3). These findings indicate that a functional ABCA1 is required for the formation of this novel apoA-I binding site. Interestingly, a previous study by Marcel and colleagues (11) has documented the existence of an apoA-I binding site in the extracellular matrix of macrophages (but not fibroblasts) that was partly ABCA1-dependent and that was inhibited by glyburide and by trypsin. The cause of this disparity is unknown, but it may be attributable to ABCA1 stimulation with 22OH/9CRA in our experimental cell culture model, consistent with our finding that treatment of fibroblasts with 22OH/9CRA or overexpression of ABCA1 in BHK cells strongly increased the binding of apoA-I to the HCBS (Figs. 2, 3). Importantly, after submission of this paper, Phillips and colleagues (35) confirmed the existence of the HCBS and its involvement in the assembly of nascent HDL particles, based on our previous reports on the HCBS (4).

The structural characteristics of the HCBS have not yet been determined. In the present study, we obtained evi-

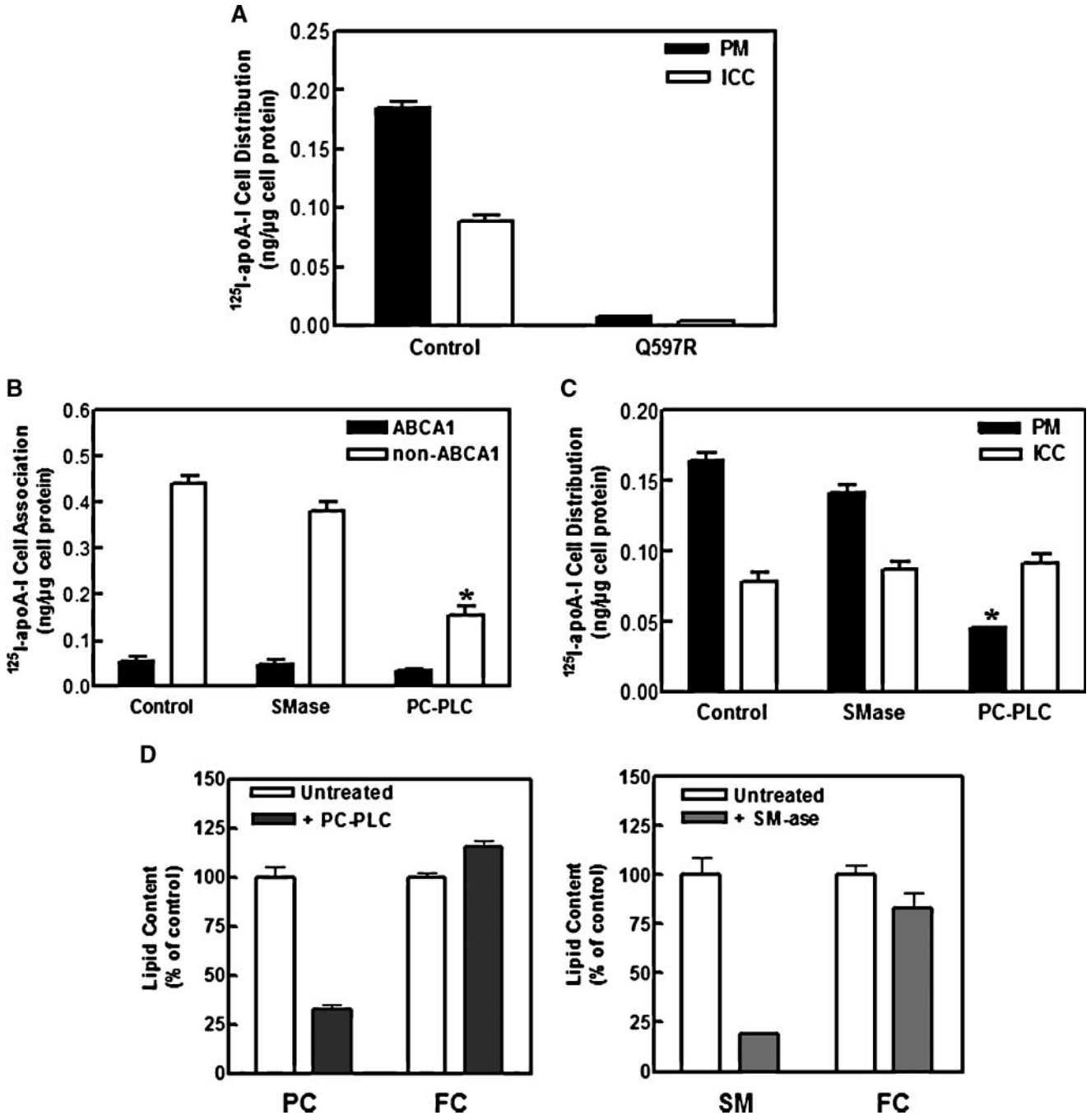

Fig. 5. Distribution of apoA-I between plasma membrane (PM) and intracellular compartments (ICCs) and effect of phospholipase treatment on the binding of apoA-I to the non-ABCA1 binding site (HCBS). A: Confluent stimulated normal and ABCA1 mutant (Q597R) fibroblasts were incubated with 10 μg/ml ^{125}I-apoA-I for 45 min at 37°C. Cells were then washed three times with PBS, and surface proteins were biotinylated as described in Methods. Cells were washed twice with ice-cold PBS, lysed, and homogenized, and 200 μg of protein was added to 45 μl of streptavidin-Sepharose beads and incubated overnight on a platform mixer at 4°C. Recovered supernatants (ICC) and pellets (PM) washed with lysis buffer were counted directly for radioactivity. B: Confluent stimulated normal fibroblasts were incubated with 10 μg/ml ^{125}I-apoA-I for 45 min at 37°C. After washing to remove unbound ^{125}I-apoA-I, cells were treated or not with either 2.5 U/ml phosphatidylcholine-specific phospholipase C (PC-PLC) or 0.4 U/ml sphingomyelinase (SMase) for 30 min at 37°C. Cells were subjected to DSP, and the radioactivity associated with both ABCA1 and non-ABCA1 binding sites was determined. C: Confluent stimulated normal fibroblasts were incubated with ^{125}I-apoA-I and treated with phospholipases as described for B. Cells were then washed three times with PBS, and surface proteins were biotinylated as described in Methods. Recovered supernatants (ICC) and pellets (PM) washed with lysis buffer were counted directly for radioactivity. D: To assess how effectively phospholipids were removed by phospholipase treatment, cells were labeled with [^{3}H]choline and [^{3}H]cholesterol and the lipids were separated by TLC and counted. PC-PLC and SMase treatment digested >65% of phosphatidyl [^{3}H]choline (PC) and 80% of [^{3}H]sphingomyelin (SM). [^{3}H]free cholesterol (FC) was used as a control. Values represent means ± SD from triplicate wells. Results shown are representative of two different independent experiments. * $P < 0.001$ by Student's *t*-test.

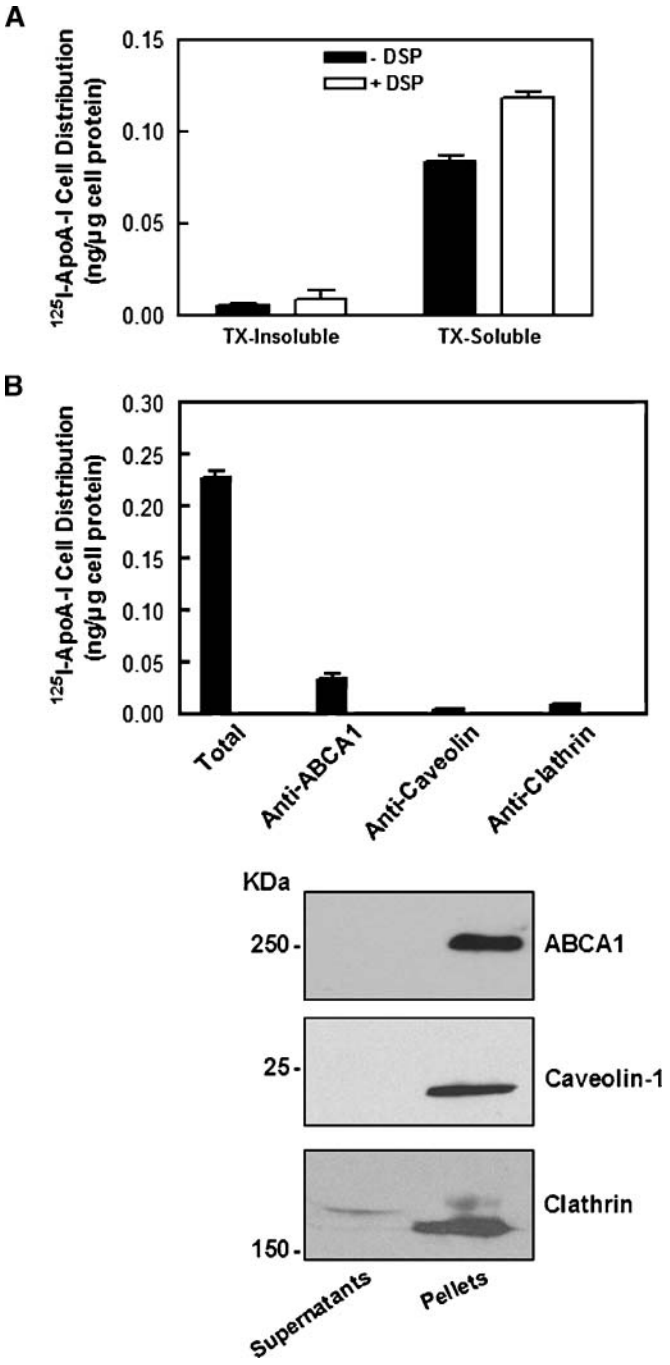

Fig. 6. Localization of the HCBS to nonraft membrane domains. A: Confluent stimulated normal fibroblasts were incubated with 10 μg/ml ^{125}I-apoA-I for 45 min at 37°C. After washing to remove unbound ^{125}I-apoA-I, cells were subjected or not to DSP and then subjected to Triton X-100 (TX) solubilization as described in Methods. The radioactivity associated with both soluble and insoluble fractions was determined. B (upper panel): Confluent stimulated normal fibroblasts were incubated with 10 μg/ml ^{125}I-apoA-I for 45 min at 37°C. After washing to remove unbound ^{125}I-apoA-I, cells were subjected to DSP. Samples containing ^{125}I-apoA-I (200 μg of total protein) were incubated with anti-ABCA1, anti-caveolin-1, or anti-clathrin antibody (Santa Cruz Biotechnology). The radioactivity found in pellets and supernatants was determined by γ-counting. B (lower panel): Equal amounts of protein from the supernatants and pellets were separated by SDS-PAGE, and ABCA1, caveolin-1, or clathrin was detected by appropriate antibodies. Values represent the mean ± SD from triplicate wells.

dence that PC-PLC treatment of cells, but not SMase, causes a dramatic decrease in apoA-I binding to the HCBS (Fig. 7), suggesting that phosphatidylcholine is the major phospholipid component of the HCBS. We propose that the rapid transient apoA-I binding to the ABCA1 site allows secondary interactions with the HCBS. Therefore, the HCBS could provide a stable environment for apoA-I in close proximity to the PM that permits the extraction of lipids and the subsequent formation of nascent HDL particles. Although evidence has been presented demonstrating molecular interactions between ABCA1 and apoA-I on the basis of chemical cross-linking performed in our laboratory and by other groups, indicating that apoA-I and ABCA1 are in very close proximity (<7 Å) (7, 36, 37), it remains controversial whether there is "molecule-to-molecule contact" between apoA-I and ABCA1. Several competing models have been proposed for this interaction (10, 11, 38).

The present study supports the concept that the generation of the HCBS is dependent on the formation of a high-affinity complex between apoA-I and ABCA1. However, it is possible that the low-capacity site involving direct apoA-I/ABCA1 interaction serves a regulatory function and stabilizes ABCA1, as reported recently by Phillips and colleagues (35). In the present study, we obtained evidence that increasing apoA-I binding to ABCA1 by 22OH/9CRA stimulation or overexpression of ABCA1 promoted the binding of apoA-I to the HCBS (Figs. 2, 3). Conversely, inhibiting the binding of apoA-I to ABCA1 also inhibits its binding to the HCBS. For example: *1*) inhibition of ABCA1 activity by glyburide decreased the binding of apoA-I to both ABCA1 and the HCBS; *2*) rLpA-I exhibited reduced affinity for ABCA1 and also for the HCBS (Fig. 4A); and *3*) ABCA1 mutants (Q597R and C1447R) that failed to bind apoA-I also failed to mediate the formation of the HCBS. Together, our data indicate that the initial interaction of apoA-I with the ABCA1 site is required for the subsequent association of apoA-I with the HCBS.

The finding that the majority of apoA-I was nonassociated with ABCA1 after a 45 min incubation period with either stimulated fibroblasts or ABCA1-overexpressing BHK cells suggests that under conditions of continuous exposure to an excess of apoA-I, apoA-I/ABCA1 complexes must dissociate rapidly enough to allow replenishment of the free ABCA1 binding site. This productive binding event targeted apoA-I to the adjacent phosphatidylcholine-containing HCBS, which exhibited a nearly 9-fold higher capacity to bind apoA-I compared with the ABCA1 site. This is consistent with the concept that ABCA1 may be reused for a large number of apoA-I lipidation cycles, which may result in the lipidation of many apoA-I molecules by the same oligomeric ABCA1 complex (4, 7). Thus, the present tandem two binding site model seems to be an efficient pathway for the lipidation of apoA-I. On the other hand, the rapid interaction of apoA-I/ABCA1 may have important implications for the activation of ABCA1 itself, consistent with our previous observations that treatment of either stimulated fibroblasts or CHO cells overexpressing ABCA1 with apoA-I for short periods of time

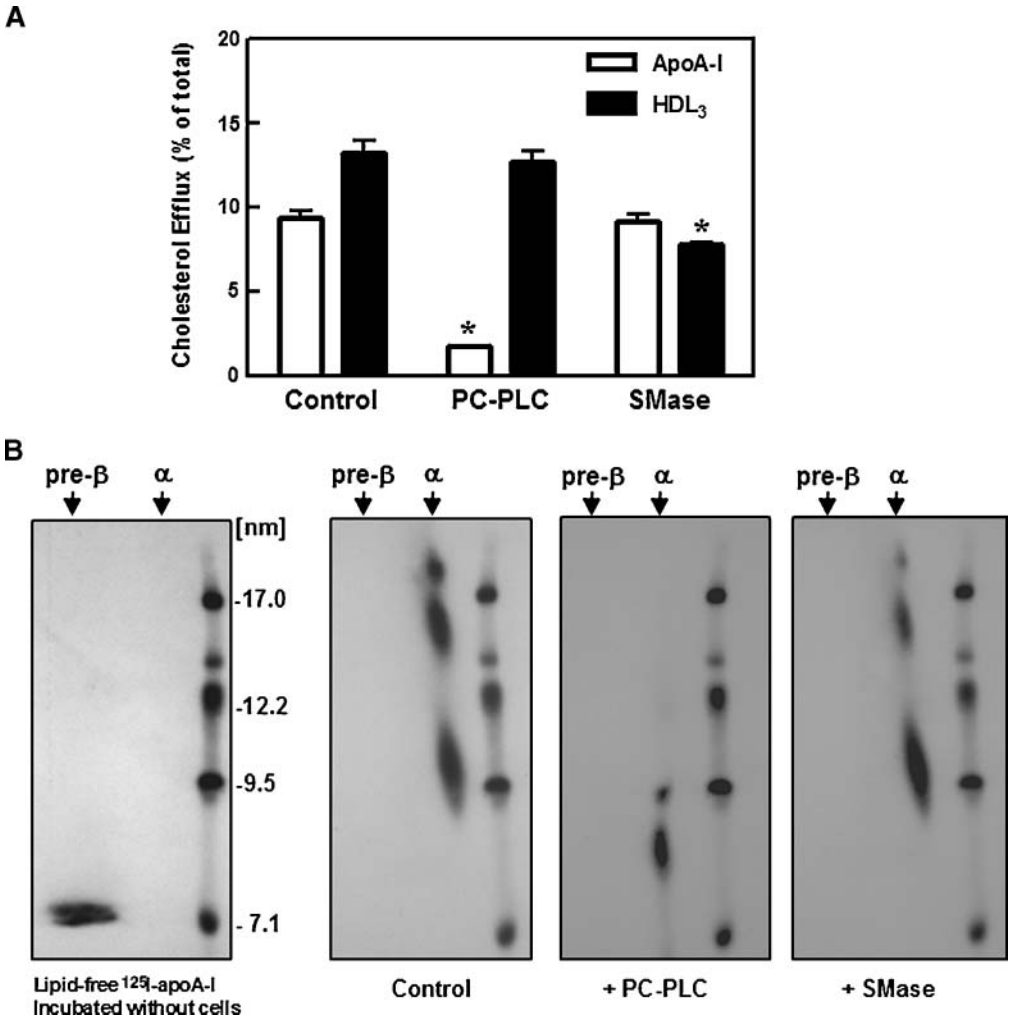

Fig. 7. Disruption of the HCBS by PC-PLC treatment inhibited apoA-I-mediated cholesterol efflux and the formation of nascent apoA-I-containing particles. A: Confluent normal fibroblasts were labeled with 8 μCi/ml [^{3}H]cholesterol and then enriched with 20 μg/ml cholesterol. Cells were stimulated with 22OH/9CRA for 20 h. Cells were subjected to treatment with 2.5 U/ml PC-PLC or 0.4 U/ml SMase for 30 min at 37°C. After extensive washes, efflux was initiated by the addition of 10 μg/ml either apoA-I or HDL_3. After 90 min of incubation, cholesterol efflux was determined as described in Methods. Values represent the mean ± SD from triplicate wells. * $P < 0.001$. B: Confluent stimulated normal fibroblasts were treated or not with phospholipases as described for A. Cells were then washed three time with PBS to remove phospholipases and incubated with 10 μg/ml ^{125}I-apoA-I for 90 min. Lipid-free ^{125}I-apoA-I was removed using both ultrafiltration (molecular weight cut off 50,000) and dialysis membrane (molecular weight cut off 50,000) as described in Methods. Samples were separated by two-dimensional polyacrylamide nondenaturing gradient gel electrophoresis, and ^{125}I-apoA-I was detected directly by autoradiography. Molecular size markers are indicated on the right side of each gel.

triggered cAMP production and consequently induced ABCA1 phosphorylation via a protein kinase A-dependent mechanism (27, 39). This is consistent with the finding of Oram and Heinecke (40) that ABCA1 activity could be regulated by different signaling processes, including the JAK2 pathway.

Defining the structural characteristics of cellular apoA-I binding sites is key for understanding how ABCA1 regulates the associations of apoA-I with different cellular compartments. The present study shows that approximately two-thirds of total cell apoA-I was found associated with PM and the remainder with ICCs (Fig. 5A). On the other hand, we documented that the majority of cell-associated apoA-I was found not bound to ABCA1 after short incubation periods. It is most likely that the HCBS is a PM lipid binding domain, consistent with our result showing that PC-PLC treatment of the cells drastically reduced apoA-I binding to the HCBS and specifically apoA-I associated with PM, but not ICCs (Fig. 5B, C). Importantly, the HCBS was found not to be associated with sphingomyelin-rich membrane raft domains, based on detergent solubility and lack of colocalization with marker proteins associated with raft domains, such as caveolin (Fig. 6), in agreement with the observation that the majority of phosphatidylcholine-

containing PMs was found in nonraft domains (32). Similarly, previous studies have shown that ABCA1 itself was associated with a membrane fraction distinct from cholesterol and sphingomyelin-rich rafts (33, 34).

This result was further strengthened by the demonstration that disruption of the HCBS by PC-PLC treatment drastically reduced apoA-I-mediated cholesterol efflux from cells and inhibited the formation of nascent apoA-I-containing particles (Fig. 7A, B) but cholesterol efflux to HDL_3 was not significantly affected. In contrast, SMase treatment did not impair apoA-I-mediated cholesterol efflux, but efflux of cholesterol to HDL_3 was decreased significantly (Fig. 7A). This is in agreement with the finding of Mendez et al. (33) that sphingomyelin-rich membrane rafts do not provide lipid for efflux promoted by apoA-I through the ABCA1-mediated lipid secretory pathway. On the other hand, it is well documented that caveolae, a specialized type of membrane raft domain, play a role in cellular cholesterol transport and lipid efflux promoted by HDL or whole plasma (41, 42), consistent with our result showing that disruption of sphingomyelin-rich membrane raft domains by SMase treatment significantly decreased HDL_3-mediated cholesterol efflux (Fig. 7A). The finding that both ABCA1 and the HCBS were found in nonraft domains suggests that the two binding sites are in close proximity, but more detailed analysis will be needed before this conclusion can be stated with certainty.

The structural requirements for apoA-I to associate with both ABCA1 and the HCBS are as yet unknown. In the present report, we obtained evidence that deletion of the C-terminal region of apoA-I, residues 187–243, blocked the binding of apoA-I to both ABCA1 and the HCBS. At the same time, apoA-I-mediated cholesterol efflux was almost completely inhibited in the presence of apoA-I Δ(185-243) (Fig. 4C, D), consistent with a previous study by Chroni and colleagues (6) documenting that direct cross-linking of apoA-I Δ(185-243) and Δ(220-243) to ABCA1 revealed that the dissociation constant values for these mutants were increased by 3-fold compared with that for WT apoA-I. In contrast, a recent study by Phillips and colleagues (35) reported that the C-terminal deletion mutants Δ(190-243) and Δ(223-243) exhibited marked reductions in their abilities to bind to the surface of ABCA1-upregulated cells, but these C-terminal deletion mutants cross-linked to ABCA1 as effectively as WT apoA-I. The cause of this disparity is unknown, but it may be attributable to the concentrations of apoA-I used in these experiments. Indeed, it was reported that significant differences for cell binding and cholesterol efflux between WT apoA-I and apoA-I mutants were seen when the cells were exposed to low concentrations of apoA-I (30). It is well established that the C-terminal α-helices are important for effective cellular lipid efflux (29, 30). Interestingly, we reported previously that apoA-I Δ(185-243) incubated with HepG2 failed to form larger nascent LpA-I particles compared with WT apoA-I (4). It is possible that the C-terminal region of apoA-I is required for the formation of a productive complex with ABCA1 that leads to the creation of the HCBS, or, alternatively, that apoA-I Δ(185-243) was unable to bind directly to a phosphatidylcholine-containing HCBS (35).

Previous studies by Takahashi and Smith (12) and Neufeld and colleagues (13) have proposed that the entire lipid transfer reaction may occur inside the cell as part of a retroendocytosis pathway. We obtained evidence that two-thirds of total cell apoA-I was found associated with the PM and the remainder with ICCs (Fig. 5A). Furthermore, the presence of ABCA1 mutant Q597R abolished the association of apoA-I with both compartments, consistent with the idea that ABCA1 seems to be required for the association of apoA-I with both the PM and ICCs. These findings provide strong support for the existence of an intracellular lipidation pathway. This is consistent with the findings of Chen, Wang, and Tall (43) that deletion of the PEST sequence leads to decreased internalization of ABCA1 and decreased cholesterol efflux from late endosomal cholesterol pools. Furthermore, a recent study by Cavelier, Rohrer, and von Eckardstein (44) has documented that ABCA1 modulates apoA-I transcytosis in endothelial cells. These results suggest that the internalization of ABCA1 is functionally important in mediating the intracellular lipidation of apoA-I. We are currently investigating the cellular compartmentalization and trafficking of apoA-I/ABCA1 and its relationship to the formation of nascent apoA-I-containing particles.

The concept that ABCA1 creates the putative phosphatidylcholine-containing HCBS may have important implications for the understanding of the ABCA1-mediated HDL genesis pathway. Our results support the two-step model for ABCA1-mediated lipid efflux proposed by Freeman and colleagues (6, 8). The initial rapid and transient productive binding of apoA-I molecules to the homotetrameric ABCA1 complex (25), likely through the α-helices in the C-terminal-region of apoA-I (Fig. 4C, D), permits apoA-I insertion into the adjacent phosphatidylcholine-containing HCBS, having nearly 10-fold higher capacity to bind apoA-I, most likely created by the phospholipid translocase activity of ABCA1. The interaction of apoA-I with the HCBS allows phospholipid and free cholesterol extraction and subsequent dissociation of the lipidated products (7, 25), thereby allowing the formation of nascent HDL particles. Although the structural details of the two-step site model have not yet been elucidated, the present study demonstrates that ABCA1 activity creates a new high HCBS required for the formation of nascent apoA-I-containing particles (Fig. 7B), emphasizing the importance of ABCA1 in the HDL biogenesis pathway. However, it remains unknown whether ABCA1 modifies adjacent phospholipid bilayer domain(s) that permit the association of apoA-I with the HCBS and extraction of phospholipid and cholesterol, or alternatively, whether ABCA1 may alter apoA-I structure to increase its ability to bind to a phospholipid bilayer within the HCBS. Further elucidation of the molecular interactions between apoA-I/ABCA1 and the HCBS should clarify the mechanism by which ABCA1 is involved in the biogenesis of HDL particles.

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