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apoA-IV tagged with the ER retention signal KDEL perturbs the intracellular trafficking and secretion of apoB

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Abstract To examine the role of apolipoprotein A-IV (apoA-IV) in the intracellular trafficking and secretion of apoB, COS cells were cotransfected with microsomal triglyceride transfer protein (MTP), apoB-41 (amino terminal 41% of apoB), and either native apoA-IV or apoA-IV modified with the carboxy-terminal endoplasmic reticulum (ER) retention signal, KDEL (apoA-IV-KDEL). As expected, apoA-IV-KDEL was inefficiently secreted relative to native apoA-IV. Coexpression of apoB-41 with apoA-IV-KDEL reduced the secretion of apoB-41 by ~80%. The apoA-IV-KDEL effect was specific, as neither KDEL-modified forms of human serum albumin or apoA-I affected apoB-41 secretion. Similar results were observed in McA-RH7777 rat hepatoma cells, which express endogenous MTP. The full inhibitory effect of apoA-IV-KDEL on apoB secretion was observed only for forms of apoB containing a minimum of the amino-terminal 25% of the protein (apoB-25). However, apoA-IV-KDEL inhibited the secretion of both lipid-associated and lipid-poor forms of apoB-25. Dual-label immunofluorescence microscopy of cells transfected with native apoA-IV and apoB-25 revealed that both apolipoproteins were localized to the ER and Golgi, as expected. However, when apoA-IV-KDEL was cotransfected with apoB-25, both proteins localized primarily to the ER. These data suggest that apoA-IV may physically interact with apoB in the secretory pathway, perhaps reflecting a role in modulating the process of triglyceride-rich lipoprotein assembly and secretion.—Gallagher, J. W., R. B. Weinberg, and G. S. Shelness. **apoA-IV tagged with the ER retention signal KDEL perturbs the intracellular trafficking and secretion of apoB. *J. Lipid Res.* 2004.** 45: **1826–1834.**

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Apolipoprotein A-IV (apoA-IV) is a 46 kDa plasma glycoprotein (1) that is synthesized by the mammalian intestine (2) during lipid absorption, incorporated into nascent chylomicrons (3), and secreted into the circulation on the surface of lymph chylomicrons (4). Although a broad spectrum of physiological functions have been proposed for apoA-IV (5, 6), the preponderance of evidence suggests that its primary biological function is related to intestinal lipid absorption. In humans, apoA-IV expression is restricted to the intestine and is specifically stimulated by triglyceride absorption (7–10). The secretion of apoA-IV into mesenteric lymph rapidly increases during fat absorption in parallel with lymph triglycerides (11). Plasma apoA-IV levels increase after fat feeding (11–13) and decrease during fasting (14). Moreover, plasma apoA-IV levels are correlated with dietary fat intake (15) and are depressed in digestive disorders that cause fat malabsorption (16). Finally, the absence of the entire apoA-I/apoC-III/apoA-IV gene complex (17), but not isolated absence of the apoA-I and apoC-III genes (18), is associated with fat-soluble vitamin malabsorption.

Chylomicron assembly is the final, essential step in intestinal lipid absorption (19), and several additional lines of evidence specifically implicate apoA-IV in this process. The hydrophobic surfactant Pluronic L-81 simultaneously and selectively blocks both chylomicron assembly and intestinal apoA-IV synthesis but not the absorption of luminal fatty acids and their intracellular esterification (20). Enterocyte apoA-IV mRNA and protein levels do not increase during absorption of short-chain fatty acids, which, unlike long-chain fatty acids, are absorbed directly into the portal blood and do not require chylomicron assembly (21). Plasma apoA-IV levels are decreased in subjects with abetalipoproteinemia (4, 12) and hypobetalipoproteinemia (1), genetic disorders in which chylomicron assembly and secretion are impaired. Nonetheless, lipid absorption is grossly normal in apoA-IV knockout mice (22),

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Abbreviations: apoA-IV, apolipoprotein A-IV; DSP, dithiobis(succinimidyl propionate); ER, endoplasmic reticulum; HSA, human serum albumin; MTP, microsomal triglyceride transfer protein.

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suggesting that apoA-IV may play a facilitating or regulatory role in chylomicron assembly and intestinal lipid transport. Recent studies by Lu et al. (23) support this hypothesis by demonstrating that apoA-IV expression stimulates transcellular triglyceride transport in neonatal pig intestinal epithelial cells.

Although many lines of evidence support a role of apoA-IV in intestinal lipid absorption and perhaps chylomicron assembly, the mechanism underlying its intracellular function is unknown. We have proposed that the interfacial properties of apoA-IV enable it to regulate particle expansion in the second stage of triglyceride-rich particle assembly (24), during which small, HDL-sized nascent particles acquire large amounts of additional triglyceride (19, 25). To test the hypothesis that apoA-IV interacts with apoB within the secretory pathway, we explored the consequences of altering apoA-IV intracellular trafficking by modifying it with the carboxy-terminal endoplasmic reticulum (ER) retention signal, KDEL (26). This approach has been shown previously to be useful for assessing intracellular protein-protein interactions (27–29). The present studies have revealed that ER retention of apoA-IV specifically inhibits the trafficking of apoB. Hence, apoA-IV may interact either directly or indirectly with nascent apoB-containing lipoprotein particles early in the triglyceride-rich particle assembly process.

MATERIALS AND METHODS

Expression plasmids

Human apoA-IV cDNA was produced by reverse transcriptase-coupled PCR using human small intestine total RNA (Clontech) as a template and 5′ and 3′ apoA-IV flanking oligonucleotides as primers. Human serum albumin (HSA) cloned into the human cytomegalovirus (CMV) immediate early promoter-based expression plasmid pBAT14 was obtained from Dr. Peter Arvan (University of Michigan). Human apoA-I cDNA cloned into expression plasmid pCMV5 was obtained from Dr. Mary Sorci-Thomas (Wake Forest University School of Medicine). Addition of the tetrapeptide Lys-Asp-Glu-Leu (KDEL) to the carboxy-terminal ends of apoA-IV, HSA, and apoA-I was achieved by standard PCR-based cloning techniques. Briefly, antisense PCR primers were designed that hybridized to the carboxy-terminal 7–10 amino acids of each cDNA and also contained sequences encoding the KDEL tetrapeptide followed by a termination codon (26). These were used in combination with specific 5′ sense strand primers to produce the KDEL-modified forms of each open reading frame. PCR products were cloned into the expression vector pCMV5 (30). The validity of each construct was confirmed by DNA sequence analysis. All apoB truncation mutants contained a carboxy-terminal FLAG (DYKDDDDK) epitope (31, 32), with the exception, as noted, where carboxy-terminal 6× His-tagged constructs were used (33).

Transfection, metabolic labeling, and immunoprecipitation

COS-1 cells in 100 mm dishes were transfected at 50–60% confluence with equal mass quantities of the indicated DNA plasmids (6 μg total DNA) using the DEAE-Dextran method (34). McA-RH7777 cells in 100 mm dishes were transfected with a total of 10 μg of plasmid DNA using Fugene-6 transfection reagent (Roche Applied Science) (31). Transfected cells were metabolically radiolabeled with 100 μCi/ml [^{35}S]Met/Cys (EasyTag Express Protein Labeling Mix; Perkin Elmer Life Sciences) in Met- and Cys-deficient DMEM (ICN) for the times indicated. Protein from media and cell lysates was immunoprecipitated with goat anti-human apoB (Academy Biomedical, Houston, TX), rabbit anti-HSA (Roche Applied Science), goat anti-human apoA-I (Academy Biomedical), rabbit anti-human apoA-IV (1), or mouse anti-FLAG M2 antibody (Sigma), as indicated. Immunoprecipitations and SDS-PAGE were performed as described (31). Dried gels were exposed to BioMax MS film in combination with a BioMax TransScreen-LE intensifying screen (Kodak) at −70°C. For pulse-chase studies, COS-1 cells in 150 mm dishes were cotransfected with equal mass quantities of the indicated DNAs (15 μg total). Twenty-four hours after transfection, cells were trypsinized and replated at ∼50% confluence in 60 mm dishes. Twenty-four hours after replating, cells were pulse radiolabeled with [^{35}S]Met/Cys for 10 min and then chased with media containing an excess of cold Met and Cys for the times indicated (35). apoB from media and cell lysates was immunoprecipitated and fractionated by SDS-PAGE, and bands were quantified using a Molecular Dynamics 445 SI Phosphorimager (36).

In situ cross-linking of apoA-IV and apoB-25

COS-1 cells in 100 mm dishes were transfected, using Fugene-6, with equal mass quantities of either apoB-25 and apoA-IV or apoB-25 and HSA-KDEL. Twenty-four hours after transfection, cells were metabolically radiolabeled with [^{35}S]Met/Cys for 2 h. After washing cell monolayers with PBS, cells were incubated on ice for 30 min with either 10 ml of PBS or 10 ml of PBS containing 200 μM dithiobis(succinimidyl propionate) (DSP; Pierce) (37). After adjusting cells to 50 mM Tris-HCl, pH 8.2, to inactivate unreacted DSP, monolayers were washed with PBS and the cells were lysed as described above. Lysates were divided into equal aliquots and immunoprecipitated with anti-apoA-IV or anti-HSA antibodies, as indicated. Before gel loading, samples were boiled in SDS-PAGE sample buffer containing 100 mm DTT.

Immunofluorescence of intracellular apoA-IV and apoB

COS-1 cells in 3 cm dishes were transfected with equal mass quantities of apoB-25 and either apoA-IV-KDEL or HSA-KDEL (1.5 μg of total DNA) using Fugene-6. Twenty-four hours after transfection, cells were fixed in 3.7% formaldehyde in PBS for 10 min and permeabilized with 0.1% Saponin in PBS (PBS-Saponin). Fixed cells were incubated with 1% BSA in PBS-Saponin for 30 min, followed by 30 min with primary antibodies in the same buffer at the following dilutions: mouse anti-FLAG monoclonal antibody M2, 12.5 μg/ml; rabbit anti-HSA, 1:400; rabbit anti-apoA-IV, 1:300. Cells were then incubated with rhodamine-conjugated goat anti-mouse IgG and FITC-conjugated goat anti-rabbit IgG secondary antibodies (Jackson ImmunoResearch) at concentrations of 25 μg/ml in PBS-Saponin containing 1% BSA. Cells were postfixed, mounted in 90% glycerol, and viewed using a Zeiss Axioplan 2 microscope with a 63× oil objective. Images were captured with a Zeiss Axiocam using a gain setting of 3.

RESULTS

apoA-IV-KDEL selectively reduces the secretion of apoB-41

COS cells were cotransfected with apoB-41 and microsomal triglyceride transfer protein (MTP) and one of the following: HSA, HSA-KDEL, apoA-IV, or apoA-IV-KDEL.

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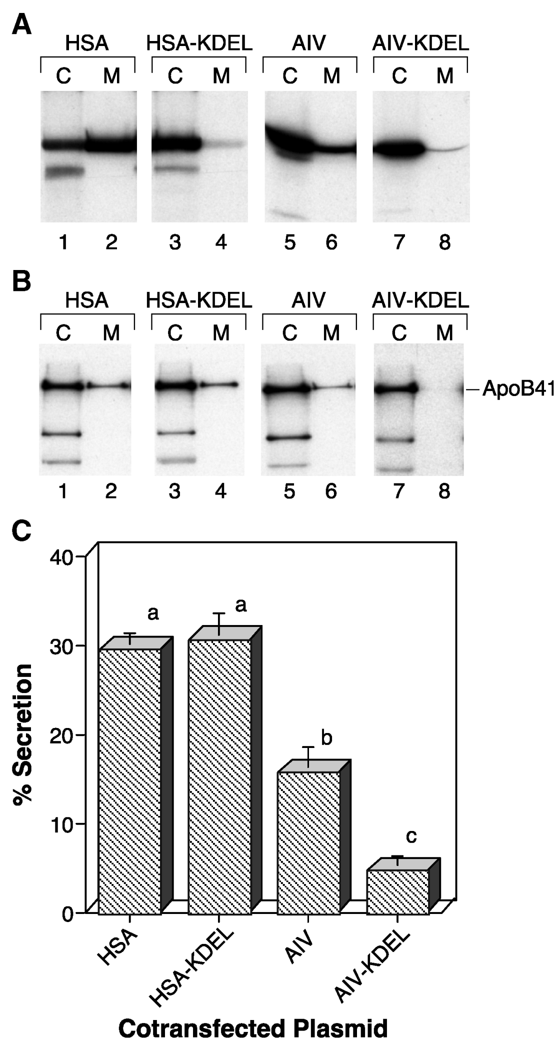

Fig. 1. KDEL-modified apolipoprotein A-IV (apoA-IV) inhibits apoB-41 secretion. A and B: COS cells were cotransfected with 2 μg each of apoB-41 and microsomal triglyceride transfer protein (MTP) and one of the following: human serum albumin (HSA), HSA-KDEL, apoA-IV (AIV), or apoA-IV-KDEL (AIV-KDEL), as indicated. Cells were radiolabeled with [^{35}S]Met/Cys for 3 h, and equal aliquots of cell lysates (C) and media (M) were subjected to immunoprecipitation with anti-HSA (A, lanes 1–4), anti-apoA-IV (A, lanes 5–8), or anti-apoB (B, lanes 1–8) antibodies. Immune complexes were analyzed by SDS-PAGE and fluorography. C: Parallel dishes of cotransfected cells were pulse radiolabeled with [^{35}S]Met/Cys for 10 min and then chased with medium containing excess cold Met and Cys for 0 or 120 min. After immunoprecipitation with anti-apoB antibodies, the mean percentage of newly synthesized apoB (cell-associated protein after the 10 min pulse) secreted into medium during the 120 min chase (±SD) was calculated based on Phosphorimager analysis of dried gels. Statistically significant differences in secretion efficiencies are indicated by different lower case letters (ANOVA, $P < 0.0001$; Tukey/Kramer posthoc analysis; n = 3).

As expected, KDEL modification of both HSA and apoA-IV markedly inhibited their secretion (**Fig. 1A**, compare lanes 2 and 4 and lanes 6 and 8). HSA-KDEL and apoA-IV had little impact on apoB-41 secretion relative to the HSA control (Fig. 1B, lanes 1–6). In contrast, apoA-IV-KDEL virtually eliminated apoB-41 secretion (Fig. 1B, lane 8). To quantitate the impact of the KDEL-modified proteins on apoB secretion, cotransfected COS cells were pulse radiolabeled with medium containing [^{35}S]Met/Cys for 10 min and then chased with medium containing an excess of cold Met and Cys for 0 or 120 min. Native apoA-IV reduced apoB-41 secretion by ~45% relative to both HSA and HSA-KDEL, whereas apoA-IV-KDEL inhibited apoB-41 secretion by greater than 80% (Fig. 1C).

The perturbing effect of apoA-IV-KDEL was specific for assembly-competent forms of apoB, as apoB-6.6, a highly truncated apoB that lacks the capacity to form nascent lipoproteins (31, 33), was unaffected by either apoA-IV or apoA-IV-KDEL coexpression (**Fig. 2A**). We also tested whether KDEL modification of another lipid binding protein, apoA-I, could similarly affect apoB secretion. As observed in Fig. 2B, KDEL modification of apoA-I markedly reduced its secretion; however, there was no corresponding effect on apoB-41 secretion (Fig. 2C). Finally, to address the possibility that apoA-IV-KDEL artifactually reduced the expression of MTP in the cotransfected COS cells, we tested whether the apoA-IV-KDEL-mediated inhibition of apoB secretion could be reproduced in McA-RH7777 rat hepatoma cells, which express endogenous MTP. Results of cotransfection of apoB-34 (another assembly-competent form of apoB) (33) with native or KDEL-modified apoA-IV are displayed in Fig. 2D. As observed previously for apoB-41 in COS cells (Fig. 1), apoA-IV-KDEL severely reduced the secretion of apoB-34 in McA-RH7777 cells (Fig. 2D, compare lanes 2, 4, and 6 with lane 8). Although it might be expected that the transfected apoA-IV-KDEL would also reduce the secretion of endogenous apoB-48 and perhaps apoB-100 in McA-RH7777 cells, this was not observed (data not shown). This may be because the relatively low transfection efficiency (~20% of cells) masks the impact of the transfected apoA-IV-KDEL. Alternatively, it is possible that the observed interaction between human apoA-IV and human apoB is species specific, an issue currently under study.

apoA-IV-KDEL inhibits apoB secretion independently of its lipidation state

Approximately 75% of apoB-25 coexpressed with MTP in COS cells is assembled into a buoyant lipoprotein particle that floats at d < 1.25 g/ml (33). To determine whether the ability of apoA-IV-KDEL to perturb apoB secretion is dependent on apoB's lipidation state, apoB-25 and MTP were cotransfected along with apoA-IV or apoA-IV-KDEL into COS cells. Density gradient centrifugation was used to separate cell medium into lipoprotein-containing (d < 1.25 g/ml) and lipid-poor (d > 1.25 g/ml) fractions, as described (33). Relative to HSA-KDEL, apoA-IV-KDEL abolished the secretion of apoB-25 into both the lipoprotein and lipid-poor density fractions (**Fig. 3**, compare lanes 2 and 3 with lanes 5 and 6). This result favors the interpretation that apoA-IV-KDEL can interact directly with apoB. To confirm this finding, the experiment was repeated in the absence of MTP coexpression. Even without the expression of MTP to induce lipoprotein assembly, both apoA-IV and apoA-IV-KDEL reduced the efficiency

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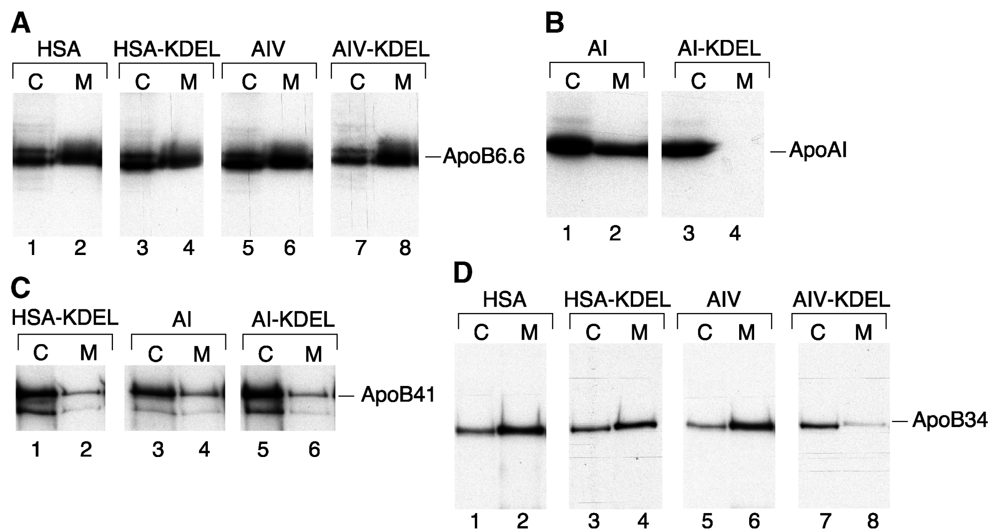

Fig. 2. Specificity of apoA-IV-KDEL-mediated inhibition of apoB secretion. A: COS cells were cotransfected with apoB-6.6 and one of the following: HSA, HSA-KDEL, apoA-IV, or apoA-IV-KDEL, as indicated. Cells were radiolabeled with [^{35}S]Met/Cys for 3 h, and cell lysate (C) and media (M) samples were subjected to immunoprecipitation with anti-apoB antibodies followed by SDS-PAGE and fluorography. B and C: COS cells were cotransfected with apoB-41 and MTP and one of the following: HSA-KDEL, apoA-I (AI), or apoA-I-KDEL (AI-KDEL), as indicated. Cells were radiolabeled with [^{35}S]Met/Cys for 3 h, and equal aliquots of cell lysate and media samples were subjected to immunoprecipitation with anti-apoA-I (B) or anti-apoB (C) antibodies. D: McA-RH7777 cells were cotransfected with apoB-34 and one of the following: HSA, HSA-KDEL, apoA-IV, or apoA-IV-KDEL, as indicated. Twenty-four hours after transfection, cells were radiolabeled with [^{35}S]Met/Cys for 3 h, and cell media and lysates were subjected to immunoprecipitation with anti-FLAG antibody.

of apoB-25 secretion to a level similar to that observed for apoB-41 (**Fig. 4**; see also Fig. 1).

Effect of apoB carboxy-terminal truncation on apoA-IV-KDEL-mediated inhibition of apoB secretion

We previously established that a very narrow interval in the $\beta\alpha_1$ domain (38) of apoB, between residues 884 (apoB-19.5) and 912 (apoB-20.1), completes a sequence fully capable of initiating the assembly of small emulsion-like triglyceride-rich lipoproteins (33). To examine the relationship between the structural requirements for particle assembly and the ability of apoA-IV-KDEL to perturb apoB secretion, a panel of carboxy-terminal truncated apoB constructs ranging from apoB-19 to apoB-25 was cotransfected into COS cells along with either apoA-IV-KDEL or HSA-KDEL. The secretion efficiency of each construct in the presence of apoA-IV-KDEL was compared with the efficiency in the presence of HSA-KDEL. Neither apoB-19 nor apoB-21 secretion was affected by apoA-IV-KDEL (**Fig. 5**); apoA-IV-KDEL caused an ~75% inhibition of apoB-23 secretion, whereas apoB-25 was inhibited to the same extent observed previously (Fig. 4). These data indicate that apoA-IV may interact directly with apoB at a site that includes a domain positioned between apoB-21 and apoB-25.

apoA-IV-KDEL alters the cellular distribution of apoB

To examine whether apoA-IV-KDEL causes a redistribution of apoB within the secretory pathway, the intracellular localization of apoB-25 in apoA-IV- or apoA-IV-KDEL-transfected COS cells was examined by dual-label immunofluorescence microscopy. When transfected together, both apoB-25 (**Fig. 6A**) and apoA-IV (Fig. 6B) displayed diffuse cytoplasmic and prominent heminuclear Golgi staining (arrows), suggesting that both are colocalized to the ER and Golgi compartments (Fig. 6C). In contrast,

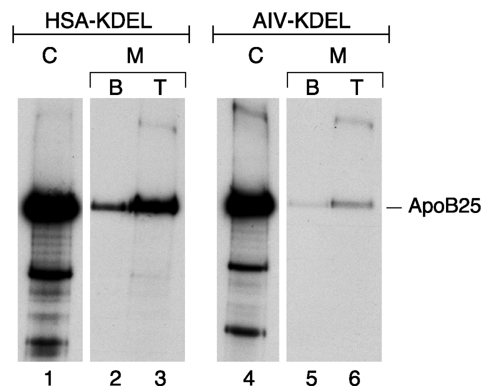

Fig. 3. apoA-IV-KDEL inhibits the secretion of buoyant and lipid-poor forms of apoB-25. COS cells were cotransfected with apoB-25 and MTP and either HSA-KDEL or apoA-IV KDEL, as indicated. Cells were metabolically radiolabeled with [^{35}S]Met/Cys for 3 h, and media (M) samples were subjected to density gradient centrifugation to obtain d $<$ 1.25 g/ml buoyant lipoprotein top (T) and d $>$ 1.25 g/ml lipid-poor bottom (B) fractions (33). apoB-25 from gradient fractions was recovered by immunoprecipitation with anti-apoB antibodies and analyzed by SDS-PAGE and fluorography. C, cell lysate.

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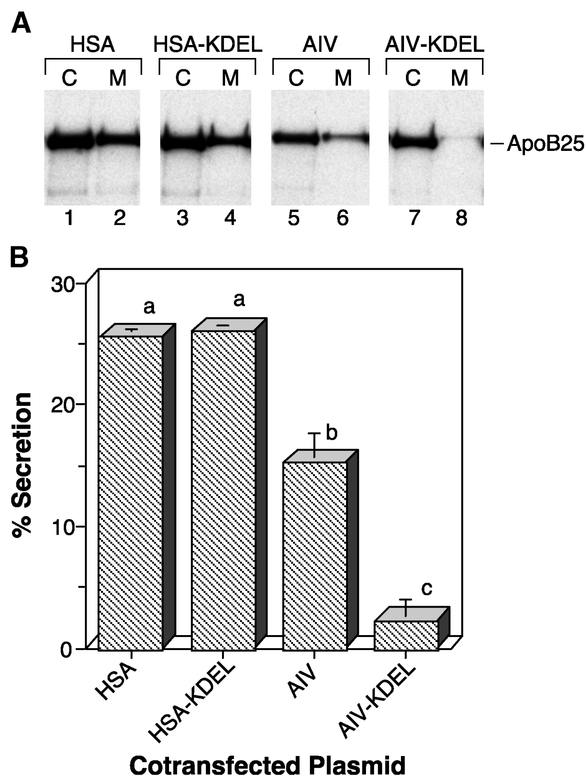

Fig. 4. apoA-IV-KDEL inhibits the secretion of apoB-25 produced in the absence of MTP. A: COS cells were transfected with apoB-25 and one of the following: HSA, HSA-KDEL, apoA-IV, or apoA-IV-KDEL, as indicated. Cells were radiolabeled with [^{35}S]Met/Cys for 3 h, and cell lysates (C) and media (M) were subjected to immunoprecipitation with anti-apoB antibodies followed by SDS-PAGE and fluorography. B: Parallel dishes of cotransfected cells were subjected to pulse-chase analysis as described for Fig. 1C. The mean percentage of newly synthesized apoB-25 secreted into media during the 120 min chase (±SD) under each condition was calculated. Statistically significant differences in secretion efficiencies are indicated by different lower case letters (ANOVA, $P < 0.0001$; Tukey/Kramer posthoc analysis; n = 3).

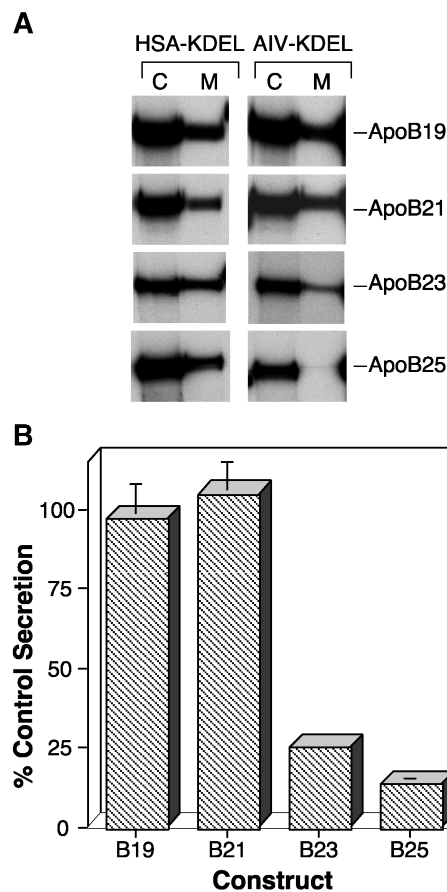

Fig. 5. Effect of carboxy-terminal truncation of apoB on apoA-IV-KDEL-mediated inhibition of apoB secretion. COS cells were cotransfected with the indicated apoB carboxy-terminal 6× His-tagged truncation mutant and either HSA-KDEL or apoA-IV-KDEL. A: Cells were radiolabeled for 3 h with [^{35}S]Met/Cys, followed by immunoprecipitation of media (M) and cell lysates (C) with anti-apoB antibodies. B: Parallel dishes of transfected cells were pulse radiolabeled for 10 min and chased for 120 min. The percentage of each construct secreted during the 120 min chase in the presence of apoA-IV-KDEL was expressed as a mean percentage (±SD; n = 3) of the secretion observed in HSA-KDEL-transfected cells.

apoA-IV-KDEL displayed only diffuse cytoplasmic and perinuclear staining, consistent with predominant ER localization (Fig. 6E) (39). As predicted by the secretion results, apoA-IV-KDEL changed the intracellular distribution of cotransfected apoB-25 from the ER-Golgi distribution observed in Fig. 6A to the predominantly ER localization observed in Fig. 6D, Hence, it appears that apoA-IV-KDEL alters the distribution of apoB within the secretory pathway. Control studies revealed that HSA-KDEL did not cause a redistribution of apoB-25 to the ER (Fig. 6G).

In situ cross-linking of apoA-IV and apoB-25

As another means of demonstrating an interaction between apoA-IV and apoB, in situ cross-linking was performed. COS cells were cotransfected with apoB-25 and either apoA-IV or HSA-KDEL. After metabolic radiolabeling, cell monolayers were incubated with PBS or PBS containing 200 μM of the reversible cross-linker DSP, as described (37). After inactivation of DSP, cells were lysed and subjected to immunoprecipitation with antibodies to either apoA-IV or HSA. As observed in **Fig. 7**, lane 1, only a small amount of apoB-25 was coimmunoprecipitated with apoA-IV antibodies in the absence of cross-linker. This may be attributable to the weak and/or transient nature of the intermolecular interaction between the proteins, which is destabilized by detergent lysis. However, the addition of the cross-linking reagent DSP before cell lysis resulted in considerable coimmunoprecipitation of apoB-25 with anti-apoA-IV antibody (Fig. 7, lane 2). When apoA-IV was replaced with the control protein HSA-KDEL, only background levels of cross-linking to apoB-25 were observed (Fig. 7, lane 4). These experiments provide additional evidence that apoA-IV and apoB can interact intracellularly.

Secretion kinetics of apoA-IV

KDEL modification of apoA-IV induced an exaggerated redistribution of both apoA-IV and cotransfected apoB.

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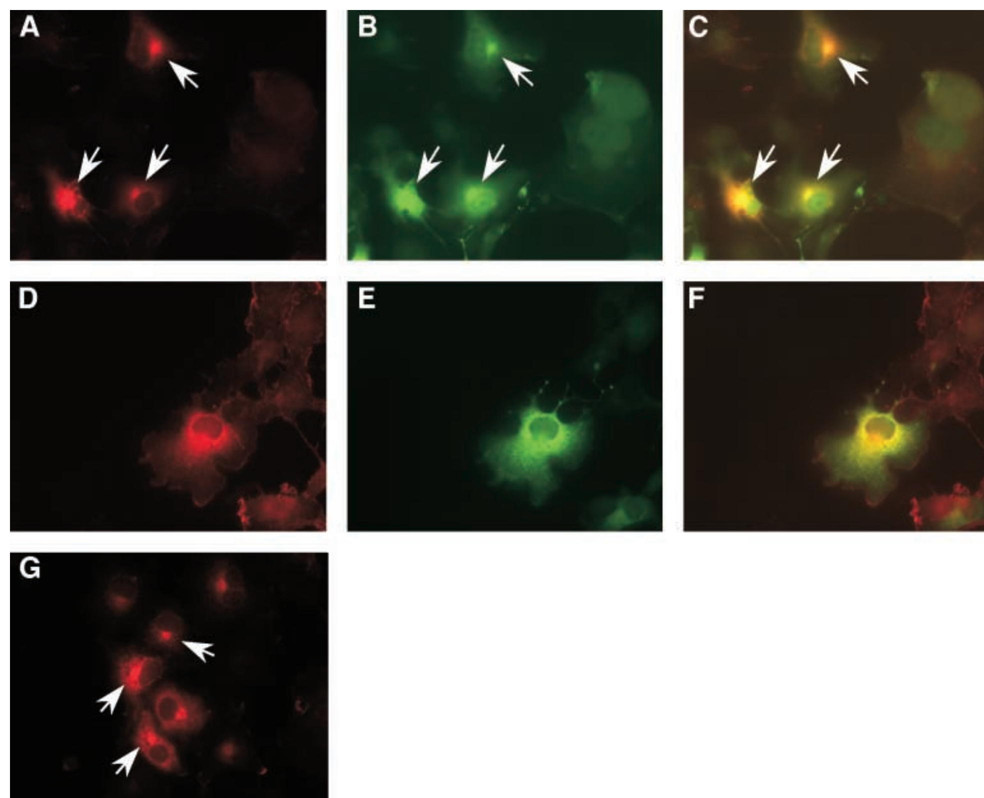

Fig. 6. apoA-IV-KDEL alters the intracellular distribution of apoB-25. COS cells were cotransfected with apoB-25 and one of the following: apoA-IV (A–C), apoA-IV-KDEL (D–F), or HSA-KDEL (G). Cells were fixed and immunostained with goat anti-apoB and rabbit anti-apoA-IV (A–F) or goat anti-apoB and rabbit anti-HSA (G) antibodies. Cells were then stained with rhodamine-conjugated anti-goat IgG and fluorescein-conjugated anti-rabbit IgG. A, D, and G show rhodamine fluorescence (red); B and E show fluorescein fluorescence (green); C and F show the overlap (yellow) between rhodamine and fluorescein fluorescence. Arrows indicate Golgi staining.

However, we also observed that native apoA-IV had a partial inhibitory effect on apoB secretion (Figs. 1, 4), perhaps because of apoA-IV's inherently slow secretion rate. To examine the secretion kinetics of apoA-IV relative to the more generic secretory protein HSA, transfected COS cells were pulse radiolabeled for 10 min and chased for 0–240 min. For HSA, ~50% of the newly synthesized protein was secreted within the first 30 min of chase, and the overall secretion efficiency approached 90% (**Fig. 8**, closed circles). However, native apoA-IV displayed a much slower rate of secretion, with less than 20% secreted after the 240 min chase (Fig. 8, open circles).

DISCUSSION

Considerable correlative and some direct evidence supports a role of apoA-IV in intestinal lipid transport. Most recently, Lu et al. (23) demonstrated that expression of apoA-IV in IPEC-1 newborn swine intestinal epithelia cells markedly stimulated triglyceride transport in chylomicron particles without affecting the expression of other proteins implicated in lipid transport or metabolism. In the current report, we explored the hypothesis that the effect of apoA-IV on intestinal lipid transport involves a direct or indirect intracellular interaction between apoA-IV and apoB. To test this hypothesis, apoA-IV was modified with the carboxy-terminal ER retention signal KDEL (26) and the potential impact on apoB trafficking was examined. These studies revealed that intracellular retention of apoA-IV caused by KDEL modification resulted in a specific ~80% reduction in the secretion of apoB-41. Native apoA-IV, which displayed relatively slow secretion kinetics, even without KDEL modification, also delayed apoB secretion. Truncation analysis demonstrated that a minimum of the amino-terminal 25% of apoB is required for apoA-IV-KDEL to inhibit secretion. Finally, the effects of apoA-IV-KDEL on apoB trafficking occurred even in the absence of MTP, suggesting that apoA-IV may have the capacity to interact directly with apoB, as demonstrated by cross-linking analysis.

The ability to interact with apoB and perturb its movement within the secretory pathway suggests a possible mechanism by which apoA-IV may enhance intestinal lipid transport. In the first stage of intestinal triglyceride-rich particle assembly, apoB-48 is cotranslationally lipidated with a small amount of phospholipid and triglyceride by MTP to form small, HDL-sized nascent particles

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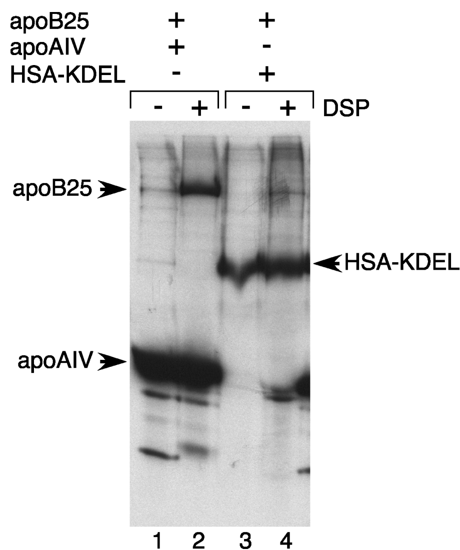

Fig. 7. In situ cross-linking of apoA-IV and apoB-25. COS cells were cotransfected with apoB-25 and either apoA-IV or HSA-KDEL, as indicated. After metabolic radiolabeling, cell monolayers were treated without (−) or with (+) dithiobis(succinimidyl propionate) (DSP), followed by detergent lysis and immunoprecipitation with anti-apoA-IV (lanes 1 and 2) or anti-HSA (lanes 3 and 4) antibodies. Samples were then heated in the presence of reducing agent to break cross-links and analyzed by SDS-PAGE and fluorography.

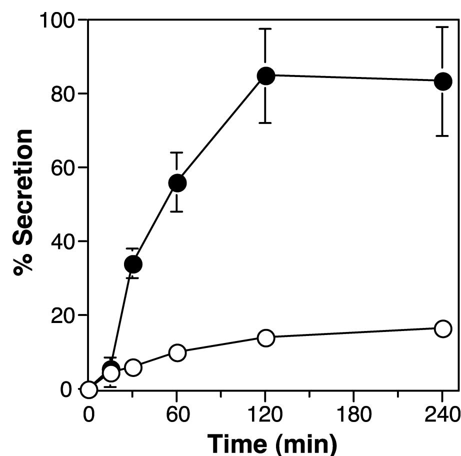

Fig. 8. Kinetics of apoA-IV and HSA secretion. COS cells transfected with apoA-IV (open circles) or HSA (closed circles) were pulse radiolabeled with [^{35}S]Met/Cys for 10 min and chased for the times indicated. After each chase time, the percentage of protein recovered in cells and media was quantified by immunoprecipitation, SDS-PAGE, and Phosphorimager analysis. The mean percentage of newly synthesized protein that was recovered in the media after each time point was plotted ± SD (n = 3).

(40). The absence or inhibition of MTP blocks this first stage of assembly (41). In the second stage of assembly, nascent chylomicron particles, which already have apoA-IV on their surface (42), acquire large amounts of additional triglyceride and expand to diameters of 500–1,000 nm before being secreted from the enterocyte basolateral membrane. Although MTP is believed responsible for the trafficking of lipid into the secretory pathway for second step expansion (43–45), the mechanism by which the resulting lipid droplets are incorporated into nascent apoB-containing particles is unknown. Our studies raise the possibility that apoA-IV may function as a modulatory cofactor to reduce apoB's rate of intracellular transport, thereby increasing its residence time within a lipoprotein expansion compartment. The exact intracellular compartments involved in second step expansion are under active investigation but appear to involve the ER (25, 46) and/or Golgi (47–49). Because the capacity to enlarge nascent lipoproteins is the predominant means by which the intestine accommodates increased lipid flux, apoA-IV, via its trafficking effects on apoB, may play an important role in this process, particularly under conditions of high dietary fat intake.

The validity of the hypothesis that apoA-IV may modulate the trafficking of apoB within the secretory pathway requires that the intracellular apoA-IV/apoB molar ratio be sufficiently high to ensure that each nascent apoB-containing lipoprotein interact with one or more apoA-IV molecule. Indeed, studies in rat and swine enterocytes reveal that the intracellular apoA-IV/apoB-48 ratio ranges from 12 to 19 (50–52). Importantly, this ratio is also observed in the cotransfection studies performed here. Inspection of the electrophoretic band intensities corresponding to apoA-IV and apoB indicate that, at steady state, there is ∼2- to 3-fold more radioactivity incorporated into apoA-IV or apoA-IV-KDEL than there is into apoB-41 (e.g., compare the band intensities in Fig. 1A, B, lanes 5 and 7). Taking into account the different Met content of each protein, the apoA-IV/apoB ratio in the transfected cells is on the order of 16, a value within the range observed in enterocytes. Hence, we propose that the relative levels of apoB and apoA-IV expression achieved in our transfected cell system are comparable to those achieved in vivo and are consistent with the ability of apoA-IV to modulate apoB trafficking. Furthermore, it appears that the observed effects of apoA-IV on apoB trafficking cannot be attributed to overexpression per se, as comparable expression levels of control proteins, including HSA, HSA-KDEL (Fig. 1), and the lipid binding proteins apoA-I and apoA-I-KDEL (Fig. 2), had no impact on the trafficking of cotransfected apoB.

The underlying basis for the observed apoB-apoA-IV interactions observed in the present report appears to be mediated, at least in part, by protein-protein interactions. However, the possibility that an interaction can also arise by a hydrophobic interaction between apoA-IV and the lipid interface of nascent apoB-containing lipoprotein particles cannot be ruled out. The latter theory arose from studies of the dynamic interfacial properties of apoA-IV, which noted that the ability of apoA-IV to decrease surface tension while increasing interfacial elasticity is ideally suited to meet the thermodynamic requisites of expanding lipid emulsion particles in an aqueous substrate (24, 53). In the present study, this mechanism is favored by the finding that the apoA-IV-KDEL inhibition effect was seen

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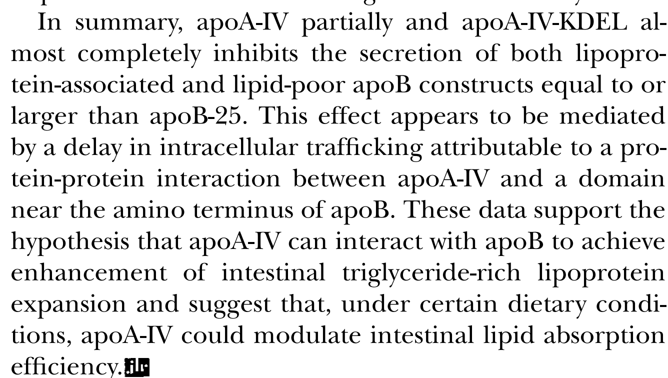
primarily with the apoB truncations that undergo substantial lipidation, i.e., apoB-25 and higher (33). Conversely, the findings that apoA-IV-KDEL attenuated the secretion of both lipid-associated and lipid-poor apoB-25 in the presence MTP and lipid-poor apoB-25 in the absence of MTP strongly argue for a direct protein-protein interaction with apoB at some site that includes residues between amino acids 953 (apoB-21) and 1,134 (apoB-25).

A role of apoA-IV in modulating intestinal lipid absorption would at first appear inconsistent with two previous studies in apoA-IV knockout (22) and human apoA-IV transgenic (54) mice, which found no effect of apoA-IV expression on postprandial triglyceride-rich lipoprotein kinetics or fat-soluble vitamin absorption. However, it is critical to note that those studies measured these parameters after a single fat bolus in animals that had been maintained on a chow diet. Thus, the maximal triglyceride absorptive capacity of these animals was not achieved, and the ability of apoA-IV to modulate absorption of higher dietary fat loads could not be ascertained. Indeed, demonstration of the physiological impact of apoA-IV expression on the efficiency of intestinal lipid absorption will likely require fat balance studies, which integrate fat absorption over a longer time period. The need for this approach is exemplified by a study in the Mdr2 knockout mouse, in which biliary lipid secretion is impaired: no difference in single-bolus plasma triglyceride kinetics was found between control and $Mdr^{+/-}$ mice, whereas fat balance studies demonstrated a significant decrease in fat absorption in $Mdr^{+/-}$ mice, but only on a high-fat diet (55).

Given that apoA-IV might modulate intestinal lipid absorption efficiency under conditions of high dietary fat intake, it is interesting that the apoA-IV T347S and Q360H polymorphisms, which are known to have an impact upon protein structure (56), postprandial triglyceride metabolism (57), and cholesterol absorption (58), have been found in epidemiological studies to be associated with a lower body mass index (Q360H) (5, 59) or increased body mass index and adiposity (T347S) (5). This raises the possibility that these genetic polymorphisms might affect intestinal lipid absorption and thus could have important implications for the functional genomics of obesity.



In summary, apoA-IV partially and apoA-IV-KDEL almost completely inhibits the secretion of both lipoprotein-associated and lipid-poor apoB constructs equal to or larger than apoB-25. This effect appears to be mediated by a delay in intracellular trafficking attributable to a protein-protein interaction between apoA-IV and a domain near the amino terminus of apoB. These data support the hypothesis that apoA-IV can interact with apoB to achieve enhancement of intestinal triglyceride-rich lipoprotein expansion and suggest that, under certain dietary conditions, apoA-IV could modulate intestinal lipid absorption efficiency.

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