Calcium-Dependent Binding of the Plasma Protein Apolipoprotein A-I to Two Members of the Annexin Family†

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ABSTRACT: Affinity chromatography with purified annexins coupled to CNBr-activated Sepharose 4B was used to determine the capacity of proteins found in cytosolic fractions of the bovine adrenal medulla to bind to an immobilized annexin in a Ca^{2+}-dependent manner. Several proteins were eluted from a recombinant annexin I column in the presence of 2 mM EGTA, including protein kinase C (PKC), members of the annexin family, and a 26 kDa protein that appeared as the most prominent band on SDS−PAGE. The identities of PKC, annexin I, annexin IV, annexin VI, and annexin VII were confirmed by Western blotting. The 26 kDa protein was purified by anion exchange chromatography on a Poros Q column and determined to be apolipoprotein A-I (apoA-I) by peptide sequencing. Comigration of apoA-I and chromobindin 2 on two-dimensional gels identified apoA-I as chromobindin 2. Overlay assays were performed to verify the apoA-I−annexin I interaction using apoA-I immobilized on nitrocellulose and annexin I in solution with binding detected using anti-annexin I antiserum. Additionally, the ability of biotin-labeled apoA-I in solution to bind to several purified annexins immobilized on nitrocellulose was determined by detection with horseradish peroxidase-conjugated avidin. Using these methods, it was shown that both annexin I and annexin VII bind to bovine apoA-I in a Ca^{2+}-dependent manner. Other annexins, such as annexin IV and annexin VI, do not exhibit this binding. The results suggest that certain annexins may function as extracellular binding sites for plasma proteins.

The annexins are a family of proteins that are able to bind lipid membranes in a Ca^{2+}-dependent manner. Synexin (annexin VII) was the first to be isolated (Creutz et al., 1978) and demonstrated the ability to cause adrenal medullary chromaffin granule aggregation *in vitro*. Chromaffin granules aggregated by synexin or other annexins are relatively stable with little vesicle fusion. As a result, it has been proposed that their role is primarily to initiate membrane contact in that they do not act as a fusogen between membranes (Creutz et al., 1978). However, when cis-unsaturated fatty acids are added to annexin-aggregated chromaffin granules, the rate of fusion increases (Creutz, 1981). It has been suggested that the annexins may be involved in a variety of cellular functions, including the antiinflammatory response (Flower, 1988; Whitehouse, 1989), exocytosis (Creutz, 1992), ion permeation (Pollard et al., 1990; Rojas et al., 1990), the regulation of blood coagulation (Tait et al., 1988), and bone mineralization (Genge et al., 1990).

Ten mammalian members of the annexin family have been identified. Annexins have also been identified in lower eukaryotes including *Dictyostelium*, *C. elegans*, and *Hydra*, as well as green plants. Structurally, the annexins are composed of a conserved C-terminal core region, containing 4 or 8 repeats of a 70 amino acid sequence that is 40−70% conserved, and a unique N-terminus. The conserved core region is thought to be responsible for the Ca^{2+} and lipid binding properties of the annexins. Although the annexins are calcium binding proteins, they differ structurally from the calmodulin and troponin C family of calcium binding proteins in that they lack the "EF" hand helix−loop−helix structure (Kretsinger & Creutz, 1986). The sequences of the N-termini of this family are highly variable, leading to the hypothesis that the differences at the N-termini may contribute to the specific cellular function of each class of annexin.

Members of the annexin family have been shown to bind proteins of the S100 family. Annexin I has been shown to bind S100C (Naka et al., 1994), annexin II binds p11 (Glenney et al., 1986), and annexin XI binds calcyclin (Watanabe et al., 1993). The annexin I−S100C and annexin XI−calcyclin interactions are Ca^{2+}-dependent, while the annexin II−p11 interaction is Ca^{2+}-independent. It has been proposed that p11 binding to annexin II not only modulates the physical state of annexin II but also regulates at least some of its biochemical properties. Recently, it has also been demonstrated that annexin IV binds to lung surfactant protein A in a Ca^{2+}-dependent manner (Sohma et al., 1995). Together these data provide a precedent that additional annexins may be capable of forming stable protein−protein interactions. Annexin I and II also exhibit enzyme−substrate interactions in that they can be phosphorylated at their N-termini on serine residues by protein kinase C (PKC)[1] (Summers & Creutz, 1985; Khanna, 1986) and on tyrosine residues by EGF and IGF receptor kinases (annexin I) (Fava

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[1] Abbreviations: apoA-I, apolipoprotein A-I; DTT, DL-dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HDL, high-density lipoprotein; HRP, horseradish peroxidase; PKC, protein kinase C; PMSF, phenylmethanesulfonyl fluoride; SDS−PAGE, sodium docecyl sulfate−polyacrylamide gel electrophoresis; t-PA, tissue plasminogen activator.

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& Cohen, 1984; Karasik et al., 1988) and *src* kinase (annexin II) (Glenney, 1985; Glenney & Tack, 1985), respectively.

The binding of proteins to members of the annexin family may play a role in the *in vivo* regulation of the annexins. Further knowledge of these interactions may help to clarify the functions of the annexins in various cellular processes. The present study was undertaken to determine whether additional cytosolic adrenal medullary proteins bind to annexins in a Ca^{2+}-dependent manner.

EXPERIMENTAL PROCEDURES

Isolation of Recombinant Annexin I. Recombinant human annexin I was isolated from extracts of the protease-deficient yeast strain dby334 (*MAT*α, leu2, ma3, gal1, reg1, pep4, prb1, cam1) transformed with the expression vector PDB60 (Wang & Creutz, 1994) incorporating human annexin I cDNA, by Ca^{2+}-dependent binding to acidic phospholipid vesicles following the procedures described previously (Creutz et al., 1992).

Preparation of Postmicrosomal Supernatants and Affinity Chromatography. Postmicrosomal supernatants (cytosol fraction) were prepared from adrenal medullary tissue as previously described (Creutz et al., 1983). The affinity matrix was prepared as follows: 1 mg of recombinant annexin I prepared as described above was suspended in 20 mL of coupling buffer (0.5 M NaCl, 0.1 M $NaHCO_3$) with 1 mM PMSF and 1 mM DTT added and mixed with a slurry of CNBr-activated Sepharose 4B (Pharmacia) prepared from 5 g of freeze-dried powder according to the manufacturer's recommendations. Coupling was allowed to proceed overnight at 4 °C on a rocking table. The gel was then washed once with coupling buffer and incubated for 1 h at room temperature with 250 mL of 1 M ethanolamine chloride, pH 8.0.

Chromatography using the postmicrosomal supernatants was performed as previously described (Creutz et al., 1983). Briefly, the coupled Sepharose was washed with 50 mL of column buffer (240 mM sucrose, 30 mM KCl, 2 mM EGTA, 1 mM $MgCl_2$, and 25 mM HEPES−NaOH, adjusted to pH 7.3 at 37 °C) and packed into a water-jacketed column (1.6 cm × 7.5 cm), giving a bed volume of approximately 15 mL. The column temperature was maintained at 37 °C during chromatography. All column buffers were pumped with an LKB Microperpex peristaltic pump operated at maximum speed (2 mL/min). The packed column was washed with 50 mL of column buffer and was then equilibrated with 50 mL of column buffer to which 4 mM $CaCl_2$ had been added such that the final free Ca^{2+} concentration equaled 2 mM. Solutions of KCl, HEPES, $MgCl_2$, EGTA, and $CaCl_2$ were added to 40 mL of the cytosol fraction so that the final concentrations equaled those of the Ca^{2+}-containing column buffer. The cytosol fraction, containing 200−300 mg of protein, was then applied to the column, and the column was subsequently washed with 50 mL of column buffer containing 2 mM free Ca^{2+}. Ca^{2+}-dependent binding proteins were eluted from the column with 50 mL of column buffer (containing 2 mM EGTA) to which no $CaCl_2$ had been added. Following the elution of Ca^{2+}-dependent binding proteins, 50 mL of 1 M NaCl, pH 7.3, was used to wash the column. Chromatography was carried out either in the presence or in the absence of 1 mM ATP added to the cytosol and column buffer.

Purification and Sequencing of p26. p26 was separated from the other proteins found in the peak protein fractions of the annexin I affinity column by performing FPLC using a Poros Q Strong Anion Exchanger (PerSeptive Biosystems, Cambridge, MA) as the column packing. The column was run at a flow rate of 1 mL/min with 20 mM Tris-HCl, pH 7.5, as buffer and a gradient of 0−1.0 M NaCl in 50 min. Purified p26 was digested with lysyl peptidase purified from *Achromobacter lyticus* (Waco Bioproducts, Richmond, VA) followed by separation of the peptides using reverse-phase HPLC on a C18 column (buffer A, 0.1% trifluoroacetic acid; buffer B, 0.09% trifluoroacetic acid in acetonitrile). The amino acid sequence of peptides was determined by Edman degradation on an Applied Biosystems Model 470A gas phase sequencer with a 120A PTH analyzer.

Annexin I Overlay Assays. Fifty micrograms of annexin I affinity column fractions or 20 μg of purified p26 was separated by one-dimensional gel electrophoresis and then transferred to nitrocellulose. The nitrocellulose filter was blocked for 2 h in blocking buffer [150 mM NaCl, 8 mM Na_2HPO_4, 2 mM NaH_2PO_4 (pH 7.3), and 5% nonfat dry milk with addition of 2 mM $CaCl_2$ or 2 mM EGTA]. Individual strips were then incubated with 5 μg/mL (140 nM) recombinant annexin I in blocking buffer for 1.5 h. All wash steps consisted of three 5 min incubations with blocking buffer. The strips were washed, incubated with rabbit antiserum raised against annexin I at 1:500 dilution in blocking buffer for 2.5 h, were washed again, and then incubated with a goat anti-rabbit HRP-conjugated antibody (Hyclone Laboratories Inc., Logan, UT) at 1:500 dilution in blocking buffer for 1.5 h. Following a final wash, the strips were developed using 4-chloro-1-naphthol. All steps were performed at room temperature.

Biotin-Labeled Apolipoprotein A-I Overlay Assays. Either 50 μg of annexin I or 25 μg of other annexins was run on a SDS−polyacrylamide gel, and the proteins were transferred to nitrocellulose. ApoA-I purified by FPLC on a Poros Q anion exchange column as described above or commercially-available, purified bovine apoA-I (Sigma) was biotinylated with *N*-hydroxysulfosuccinimide-LC-biotin (NHS-LC-Biotin) (Pierce) according to the manufacturer's recommendations. The nitrocellulose filter with bound annexin protein was blocked with buffer A [50 mM Tris-HCl (pH 8.0), 90 mM NaCl, and 5% BSA with 2 mM $CaCl_2$ or 2 mM EGTA] for 1 h and was then incubated with 4 μg/mL (150 nM) biotinylated apoA-I in buffer A, pH 8.0, for 1.5 h. The biotinylated apoA-I titration experiment was carried out as outlined in this section except that nitrocellulose filters with 5 μg of annexin I bound per lane were incubated with varying concentrations of apoA-I (Sigma) that had been biotinylated: 26 ng/mL (1 mM), 130 ng/mL (5 mM), 260 ng/mL (10 mM), 650 ng/mL (25 nM), and 1.3 μg/mL (50 nM). The nitrocellulose was washed 3 times for 10 min each with buffer A and was then incubated with 2 μg/mL HRP-conjugated avidin (Pierce) in buffer A, pH 7.4, for 30 min. Following three 10 min washes with buffer B [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 5% BSA with either 2 mM $CaCl_2$ or 2 mM EGTA], binding was detected using 4-chloro-1-naphthol. All steps were performed at room temperature.

Analytical Methods. Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as standard. One-dimensional gel electrophoresis

was performed as described by Laemmli (1970) using 10% gels. The following protein standards were used for molecular mass determination: phosphorylase, 97 kDa; serum albumin, 67 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; trypsinogen, 21 kDa; ribonuclease, 14 kDa. Two-dimensional gel electrophoresis was carried out as described by O'Farell (1975). Gels were stained with Coomassie blue or with silver following the procedure of Morrissey (1981). Western immunoblots were performed as described by Burnette (1981), using horseradish peroxidase-coupled secondary antibodies and 4-chloro-1-naphthol for detection. Antiserum for annexin I (Drust & Creutz, 1991) was prepared as described previously.

RESULTS

Annexin I Affinity Chromatography. Affinity chromatography with recombinant annexin I bound to CNBr-activated Sepharose 4B was used to determine whether cytosolic proteins of the bovine adrenal medulla were able to bind annexin I in a Ca^{2+}-dependent manner. Adrenal medullary cytosol with 2 mM $CaCl_2$ added was applied to the annexin I affinity column in the presence or absence of 1 mM ATP. When this column was subsequently eluted with buffer containing 2 mM EGTA, a distinct protein peak was observed (Figure 1A). A smaller peak was observed on washing the column with 1 M NaCl (Figure 1A). Several of the major proteins were identified in the fractions eluted by EGTA by Western blotting (data not shown) including PKC (85 kDa), annexin I, annexin IV, annexin VI, and annexin VII. However, the identity of the major protein at 26 kDa (p26) was unknown (Figure 1B). ATP was used as a variable for determining protein binding in addition to Ca^{2+} because a group of seven cytosolic adrenal medullary proteins, known as chromobindin A and recently identified as subunits of the cytosolic chaperonin, TCP-1 (Creutz et al., 1994), bind chromaffin granule membranes in the presence of Ca^{2+}, but are not released from membranes by the removal of Ca^{2+} unless ATP is present (Martin & Creutz, 1987). Therefore, ATP was used in the column buffers and cytosol fractions of the current experiments to determine whether other cytosolic proteins were capable of binding annexin I in a similar manner. It was noted that PKC was found in the 1 M NaCl wash when the column was run in the absence of 1 mM added ATP, possibly because it bound to annexin I which can serve as a PKC substrate and was unable to be released due to low ATP levels in the cytosol and the absence of ATP in the buffers.

Identification of p26 as Apolipoprotein A-I. Separation of p26 from the other proteins found in the peak fractions of the affinity column was performed by FPLC utilizing a Poros Q strong anion exchange column. p26 eluted from the column as a broad peak between 0.25 and 0.30 M NaCl, suggesting that it might have charge isoforms. Purified p26 was then digested with a lysyl peptidase, and the resulting peptides were separated using reverse-phase HPLC. A 15 amino acid peptide (VAPLGEEFREGARQK) was sequenced by Edman degradation and the sequence of this peptide exactly matched that of a portion of bovine apolipoprotein A-I (apoA-I) (O'Huigin et al., 1990).

Identification of ApoA-I as Chromobindin 2. The chromobindins are a group of proteins that are able to bind chromaffin granule membranes in a Ca^{2+}-dependent manner

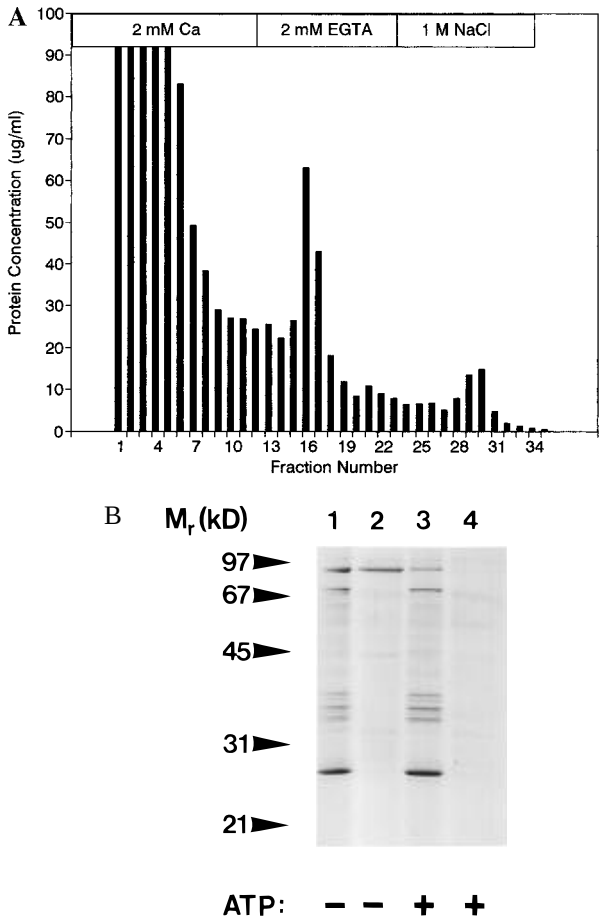

FIGURE 1: Protein content and SDS gels of annexin I affinity column peak fractions. (A) Protein concentration of fractions obtained from the annexin I affinity column. Cytosol was applied to the column prior to treatment with buffers containing Ca^{2+}, EGTA, or NaCl as indicated at the top of the graph. Ca^{2+}-containing buffer was applied at fraction 1. The maximum of protein concentration observed at the far left of the graph represents cytosolic proteins not bound to the immobilized annexin I being washed off the column by buffer containing 2 mM $CaCl_2$. Column volume was approximately 15 mL, and fraction size was 4 mL. (B) 25 μg of the peak fractions was run per lane on an SDS−polyacrylamide gel, and the gel was then stained with Coomassie blue. The gel shows the peak fractions of the 2 mM EGTA elutions when the column was run in the absence (lane 1) or presence (lane 3) of an added 1 mM ATP and the peak fractions of the 1 M NaCl wash in the absence (lane 2) or presence (lane 4) of an added 1 mM ATP.

(Creutz et al., 1983). Several now well-characterized proteins are included among the chromobindins, including annexins (Geisow & Walker, 1986; Creutz, 1992), PKC (Creutz et al., 1983), calmodulin (Creutz et al., 1983), and phosphatidylinositol-specific phospholipase C (Creutz et al., 1985). Chromobindin 2 was previously described as a protein having a molecular mass of 26 kDa with a p*I* of 5.7 that did not require ATP to bind to granule membranes and was not capable of binding lipids (neither PS nor chromaffin granule lipids) (Creutz et al., 1983). Two-dimensional gel electrophoresis of the chromobindins (Figure 2A), apoA-I alone (Figure 2B), and apoA-I coelectrophoresed with the chromobindins (Figure 2C) showed that the major charge isoform of apoA-I found in blood plasma, previously determined to have a p*I* of 5.64 (Zannis et al., 1980), comigrates with chromobindin 2, thus identifying bovine apoA-I as chromobindin 2.

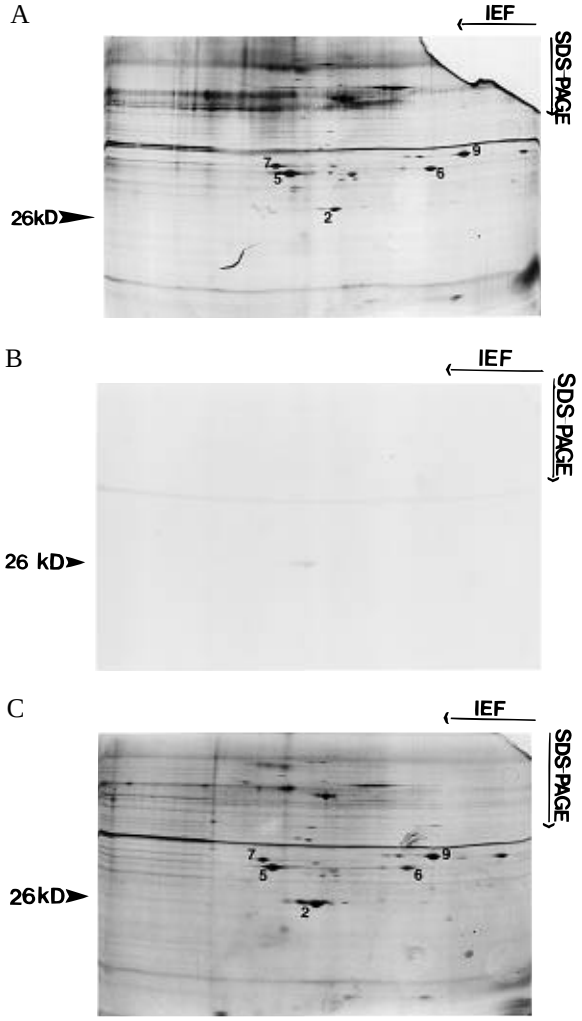

FIGURE 2: Identification of apoA-I as chromobindin 2 by two-dimensional gel electrophoresis. Two-dimensional gels of (A) the chromobindins (50 μg loaded), (B) apoA-I purified from the annexin I affinity column eluate by FPLC (15 μg loaded), and (C) the coelectrophoresis of the chromobindins (50 μg loaded) and apoA-I (15 μg loaded). The numerical labels on the gels in (A) and (C) indicate the positions of chromobindins 2, 5, 6, 7, and 9. All gels were stained with silver (Morrissey, 1981).

Verification of the Interaction between Annexin I and ApoA-I. Since the annexins are capable of binding to lipids in a Ca^{2+}-dependent manner and the cytosol fraction used in the experiments may contain small amounts of endogenous lipids, it was of interest to determine if apoA-I interacted directly with annexin I or associated with it through a lipid-dependent association. Therefore, apoA-I was transferred to nitrocellulose after electrophoresis on an SDS−polyacrylamide gel and was incubated with annexin I in solution. Annexin I bound to apoA-I was then detected using anti-annexin I antiserum to verify the apoA-I−annexin I interaction. The interaction was confirmed by these experiments, and, as is shown in Figure 3, a greater amount of annexin I was able to interact with apoA-I in the presence of 2 mM $CaCl_2$ (lane 2) than in the presence of 2 mM EGTA (lane 3).

To further verify the interaction, an overlay protocol was devised to test the ability of apoA-I in solution to bind to immobilized annexin I. Recombinant human annexin I was transferred to a nitrocellulose filter following SDS−PAGE. This nitrocellulose filter was then incubated with apoA-I purified by anion exchange chromatography and subsequently labeled with biotin (NHS-LC-biotin, Pierce). Bound apoA-I was then detected with HRP-labeled avidin (Pierce) (Figure 4A). While the control lane (C in Figure 4A) containing annexin I incubated only with HRP-conjugated avidin has no band, the lane containing annexin I (ApoAI in Figure 4A) incubated with both biotinylated apoA-I and HRP-conjugated avidin has a distinct band running at 35 kDa, the molecular mass of annexin I. Further confirmation of this interaction was demonstrated when commercially-

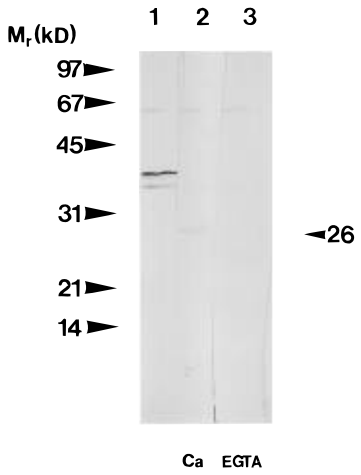

FIGURE 3: Detection of an interaction between annexin I and immobilized apoA-I by overlay assay. 20 μg of apoA-I from the annexin I affinity column eluate was run on an SDS−polyacrylamide gel and was then transferred to nitrocellulose. The nitrocellulose was incubated with 5 μg/mL (140 nM) recombinant annexin I. Bound annexin I was then detected by sequentially using rabbit anti-annexin I antiserum, a goat anti-rabbit HRP-conjugated antibody, and 4-chloro-1-naphthol as the chromogenic substrate. Lane 1, containing annexin I and an annexin I degradation product, is a positive control for the antibody. The last two lanes represent the overlay experiment performed in the presence of 2 mM added $CaCl_2$ (lane 2) or in the presence of 2 mM added EGTA (lane 3) in all buffers used.

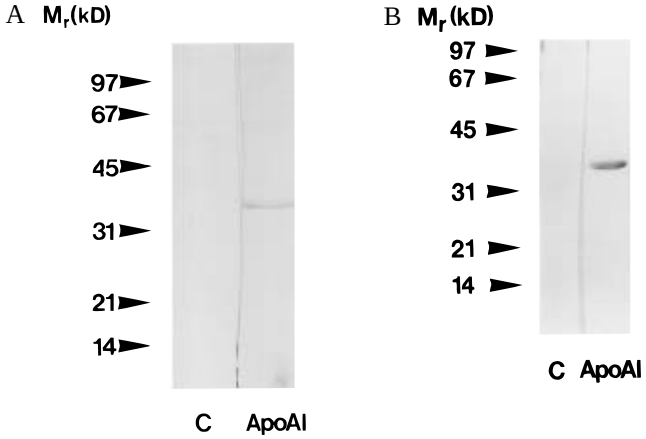

FIGURE 4: Confirmation of the interaction between annexin I and apoA-I in overlay assays using biotin-labeled apoA-I. 50 μg/lane of annexin I was run on an SDS−polyacrylamide gel, and the gel was then transferred to nitrocellulose. Either (A) apoA-I purified from the affinity column fractions or (B) purified apoA-I obtained from Sigma was biotinylated. The blocked blot was incubated with 4 μg/mL (150 nM) biotinylated apoA-I. The blot was then washed and incubated with 2 μg/mL HRP-conjugated avidin (Pierce). Following a final wash, binding was detected using 4-chloro-1-naphthol. The overlay assay was performed with all buffers containing 2 mM $CaCl_2$. The control lanes (C) contain annexin I incubated with only HRP-conjugated avidin. The lanes labeled ApoAI contain annexin I incubated with both biotinylated apoA-I and HRP-conjugated avidin.

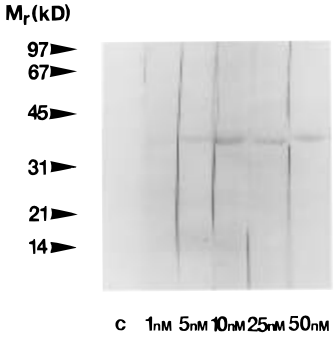

FIGURE 5: Concentration dependence of the binding of apoA-I to annexin I. Overlay assays using biotinylated apoA-I prepared from purified apoA-I obtained from Sigma were performed as described in Figure 4 with 5 μg of annexin I run per lane. The first lane is a control containing annexin I incubated with only HRP-conjugated avidin. The subsequent lanes were incubated with varying amounts of biotinylated apoA-I at the indicated concentrations (1 nM, 5 nM, 10 nM, 25 nM, 50 nM) followed by incubation with HRP-conjugated avidin.

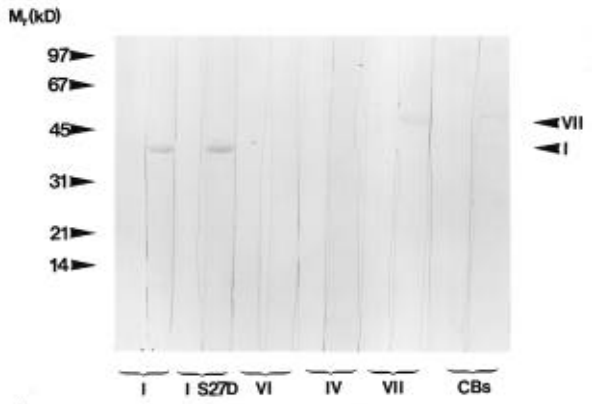

FIGURE 6: Binding of biotinylated apoA-I to members of the annexin family. Overlay assays using biotinylated apoA-I were performed as described in Figure 4 using various members of the annexin family bound to nitrocellulose. 25 μg of annexin was run per lane. Blots were performed with all buffers containing 2 mM $CaCl_2$. The first lane of every pair is a control containing the indicated annexin incubated only with HRP-conjugated avidin. The second lane of every pair represents the annexin incubated sequentially with biotinylated apoA-I and HRP-conjugated avidin. The lanes are as follows: lanes 1 and 2, annexin I; lanes 3 and 4, annexin I mutant with S27D mutation at its N-terminus; lanes 5 and 6, annexin VI; lanes 7 and 8, annexin IV; lanes 9 and 10, annexin VII; lanes 11 and 12, chromobindins.

purified apoA-I (Sigma) that was biotin-labeled also bound to nitrocellulose-immobilized annexin I (Figure 4B).

In an attempt to determine a lower range of the concentration dependence of the binding of apoA-I to annexin I, nitrocellulose-immobilized annexin I was incubated with varying concentrations (1−50 nM) of commercially-purified apoA-I that had been biotin-labeled (Figure 5). Binding was detected at all concentrations, including 1 nM apoA-I, which suggests a relatively strong protein−protein interaction. In a similar experiment, the binding of 35 ng/mL or 1 nM biotin-labeled annexin I to apoA-I was observed.

Interaction of ApoA-I with Other Annexins. To determine whether other members of the annexin family were able to bind to apoA-I in a Ca^{2+}-dependent manner, overlay assays using biotinylated apoA-I were performed as described above using various members of the annexin family (Figure 6). ApoA-I was able to bind to annexin I as was shown in Figure 4, but it was also capable of binding a mutant annexin I with serine 27, the major PKC phosphorylation site, changed to aspartic acid (Wang & Creutz, 1994). Additionally, cleavage within the N-terminal domain of the annexin I S27D mutant produced a proteolytic product of annexin I composed of the C-terminal core. ApoA-I was able to bind this core region that can be observed in Figure 5 as a faint band below the full-length annexin I S27D band. Therefore, the apoA-I−annexin I interaction appears to be insensitive to changes in the N-terminal domain of annexin I and may, therefore, be mediated by the C-terminal core of annexin I. These experiments also demonstrated that apoA-I is able to bind annexin VII (Figure 6). Faint bands corresponding to annexin I and annexin VII were observed in the chromobindin fraction as well. ApoA-I was unable to bind recombinant annexin IV or annexin VI nor was it able to bind annexin V which was present in the chromobindin fraction (Figure 6). When this blot was performed with all buffers containing 2 mM EGTA, no binding was observed (data not shown).

DISCUSSION

The results demonstrate that chromobindin 2 is apolipoprotein A-I and that two members of the annexin family, annexin I and annexin VII, bind to bovine apoA-I in a Ca^{2+}-dependent manner. The identity of apoA-I has been confirmed by sequencing peptide fragments and by determining its isoelectric point and electrophoretic mobility using two-dimensional gel electrophoresis. The apoA-I−annexin I interaction has been demonstrated using multiple methods that include affinity chromatography with annexin I coupled to CNBr-activated Sepharose 4B, overlay assays utilizing anti-annexin I antiserum, and overlay assays performed with biotin-labeled apoA-I. Additionally, further confirmation of the interaction was demonstrated when bona fide apoA-I, obtained from a commercial source, was able to bind annexin I.

The overlay assays have shown that apoA-I binds not only to full-length annexin I but also to a C-terminal proteolytic product of annexin I. Since annexin I and annexin VII have very different N-terminal sequences, but similar C-terminal core regions, these observations suggest that apoA-I is binding to the conserved C-terminal core region of these annexins and not their unique N-termini. The binding site of p11 on annexin II has been mapped to the unique N-terminus of the protein (Glenney et al., 1986). However, in experiments using calcyclin affinity chromatography, the conserved C-terminal core region of annexin II was shown to interact with calcyclin in a Ca^{2+}-dependent manner (Filipek et al., 1991). These experiments demonstrate that p11 and calcyclin have different binding sites on the annexin II polypeptide chain and provide a precedent for the ability of additional proteins to bind to the conserved C-terminal core domains of other members of the annexin family.

The results presented in this paper raise the question of the physiological significance or relevance of an interaction between annexin I and apoA-I. ApoA-I that was found as a component of the postmicrosomal supernatant of bovine adrenal medullary tissue probably originated from blood plasma, since apoA-I has not otherwise been established to be a protein component of the adrenal medulla. ApoA-I is a plasma protein and is both the major protein constituent and an important structural component of plasma high-

density lipoprotein (HDL). ApoA-I is synthesized and secreted by the liver and intestine (Imaizumi et al., 1978; Wu & Windmueller, 1979) and is present in human plasma in circulating concentrations of approximately 1.30−1.50 mg/mL in normal fasting humans, most of which is HDL-bound. However, approximately 4% of plasma apoA-I (62 μg/mL) is not HDL-bound and is found as the free protein in the plasma (Ishida et al., 1987). Additionally, mature apoA-I is polymorphic in plasma and has five charge isoforms that can be observed when two-dimensional gel electrophoresis of isolated apoA-I is performed with the major isoform unsialylated and the other isoforms deviating by unit charge differences from the major isoform (Zannis et al., 1980).

Although the annexins in general do not have characteristics of secreted proteins, e.g., they do not have an obvious signal sequence for secretion by a classical pathway, some annexins have been unequivocally demonstrated to be present in extracellular fluid (Haigler & Christmas, 1990). Prostatic fluid contains concentrations of >50 μg/mL annexin I (Christmas et al., 1991) while blood plasma contains levels that are approximately 50 ng/mL (Uemura et al., 1992). Additionally, there is evidence that annexin I is able to bind to different types of cell surfaces since the presence of high-affinity, saturable binding sites for annexin I has been described on the surface of circulating monocytes and neutrophils (Goulding et al., 1992; Goulding & Guyre, 1993). Therefore, interactions between annexins and secreted proteins may occur extracellularly.

The annexin I affinity column experiments presented in this paper suggest that free apoA-I (non-HDL-bound) was bound to the immobilized annexin I, since other protein components of HDL such as apoA-II (17 kDa), apoC-I (6 kDa), apoC-II (9 kDa), apoC-III (9 kDa), and apoD (22 kDa) did not appear to be present in the affinity column fractions when the proteins were separated by 12% SDS−polyacrylamide gels. The concentration of free apoA-I (non-HDL-bound) in blood plasma is 62 μg/mL or 2.4 μM (Ishida et al., 1987). The data from Figure 5 show that binding of 26 ng/mL or 1 nM free apoA-I to annexin I can be detected using the methods outlined in this paper. Therefore, the concentration of apoA-I is high enough, relative to its affinity for annexin I, that binding should occur under physiological conditions. Additionally, as observed in this study, 35 ng/mL or 1 nM biotin-labeled annexin I is able to bind to apoA-I. Since the concentration of annexin I in plasma is approximately 50 ng/mL (Uemea et al., 1992), this experimentally observed binding at 35 ng/mL annexin I along with the high concentrations of apoA-I found in plasma make an apoA-I−annexin I interaction physiologically possible. Finally, apoA-I and annexin I should be able to interact under extracellular physiological conditions, since the concentration of Ca^{2+} in extracellular fluid and plasma is of the order of 2 mM, a concentration at which binding was observed in our experiments.

Recently, annexin II was identified as a receptor for tissue plasminogen activator (t-PA) on human endothelial cells. Additionally, annexin II was shown to bind the t-PA substrate, plasminogen (Hajjar et al., 1994), possibly via a proteolytically derived carboxyl-terminal residue (Lys307) of annexin II. These data suggest a novel role for annexin II on the surface of the blood vessel wall, and these findings along with the annexin I and annexin VII interactions with apoA-I discussed in this paper suggest that certain annexins may function as extracellular binding sites for plasma proteins.

It has been proposed that apoA-I is involved in the interaction of HDL with a specific cell surface receptor and participates in the HDL-mediated removal of cholesterol from cells (Fidge & Nestel, 1985). However, additional functions of apoA-I have been discovered, including the ability to promote the *in vitro* growth of T-lymphocytes (Jurgerns et al., 1989) and to decrease the degranulation and superoxide production of neutrophils, thus playing a role in the modulation of immune and inflammatory responses (Blackburn et al., 1991).

During the acute phase of the inflammatory response, there are profound changes in the structure and metabolism of HDL (Cabana et al., 1989). The composition of circulating acute phase HDL is altered such that serum amyloid A is incorporated into the HDL particle and apoA-I is depleted (Shepard et al., 1987). The fate of apoA-I is unknown, but incubation of acute phase HDL with neutrophils results in the association of apoA-I with the neutrophil membrane (Shepard et al., 1987). Competitive binding studies have indicated that resting neutrophils have approximately 190 000 ($K_d = 1.7 \times 10^{-7}$) binding sites per cell for apoA-I, suggesting a ligand−receptor interaction (Blackburn et al., 1991). Possibly annexin I associated with the extracellular surface of neutrophils could provide such a binding site for apoA-I. Although less is known about the disposition or localization of annexin VII, its ability to bind apoA-I suggests it may play a similar role to that of annexin I.

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