Schellman, J. A. (1978) *Biopolymers 17*, 1305-1322.
Schmid, F. X., & Baldwin, R. L. (1979) *J. Mol. Biol. 135*, 199-215.
Segawa, T., & Sugai, S. (1983) *J. Biochem. (Tokyo) 93*, 1321-1328.
Sillén, L. G., & Martell, A. E. (1964) *Stability Constants of Metal-Ion Complexes*, Special Publication No. 17, The Chemical Society, London.
Sillén, L. G., & Martell, A. E. (1971) *Stability Constants of Metal-Ion Complexes Supplement No. 1*, Special Publication No. 25, The Chemical Society, London.
Svärd, M., & Drakenberg, T. (1986) *Acta Chem. Scand., Ser. B B40*, 689-693.
Williams, T. C., Corson, D. C., Oikawa, K., McCubbin, W. D., Kay, C. M., & Sykes, B. D. (1986) *Biochemistry 25*, 1835-1846.
Yagi, K., Matsuda, S., Nagamoto, H., Mikuni, T., & Yazawa, M. (1982) in *Calmodulin and Intracellular Ca^{++} Receptors* (Kakiuchi, S., Hidaka, H., & Means, A. R., Eds.) pp 75-91, Plenum, New York.

Alterations of the Glutamine Residues of Human Apolipoprotein AI Propeptide by in Vitro Mutagenesis. Characterization of the Normal and Mutant Protein Forms†

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ABSTRACT: We have used site-directed mutagenesis to independently alter the Gln residues at positions −1 and −2 of the human apoAI propeptide to Arg residues. The normal and mutated genes were placed under the control of the mouse metallothionein 1 promoter in a bovine papilloma virus (BPV) vector which also carries a copy of the human metallothionein 1A gene. Following transfection of mouse fibroblast C127 cells with the vectors, cell clones resistant to $CdCl_2$ were selected and analyzed for production of apoAI mRNAs and protein. The RNA blotting analysis showed that the steady-state apoAI mRNA levels of cell clones expressing either the normal or the mutant apoAI gene are 3-5-fold higher than that of the liver or HepG2 cells. Two-dimensional gel electrophoresis of radiolabeled apoAI showed that the apoAI-expressing clones secreted mainly the proapoAI form. Furthermore, both mutant proapoAI's differed by one positive charge from the normal apoAI. Secretion of apoAI into the culture medium follows apparent first-order kinetics and gives similar rate constants for the normal and mutant apoAI forms. Separation of secreted apoAI by density gradient ultracentrifugation in the presence of human plasma or HDL shows identical distribution of plasma and nascent (normal and mutant) apoAI. The findings indicate that in the cell system used the modification of either of the two glutamines of the apoAI prosegment does not affect the intracellular transport and secretion of apoAI, and its ability to associate with HDL.

Apolipoprotein AI (apoAI)[1] is the major protein component of HDL and serves as a cofactor for the enzyme lecithin-cholesterol acyltransferase (LCAT) (Fielding et al., 1972; Soutar et al., 1975). In tissue culture, apoAI is a ligand for the recognition of HDL by a putative receptor (Fidge & Nestel, 1985; Hwang & Menon, 1985; Schmitz et al., 1985a,b) and promotes efflux of cholesterol from cells (Jackson et al., 1975).

Studies of normal human apoAI synthesis and secretion (Zannis et al., 1980, 1983; Gordon et al., 1983) and DNA sequence analysis of the human apoAI gene (Karathanasis et al., 1983; Shoulders et al., 1983; Cheung & Chan, 1983) have established that newly secreted apoAI has a six amino acid long NH_2-terminal extension (prosegment) with the sequence Arg-His-Phe-Trp-Gln-Gln. It is interesting that both the human (Zannis et al., 1983) and rat (Gordon et al., 1982) apoAI prosegments terminate in a pair of Gln residues. In contrast, the chicken apoAI propeptide contains Gln-His (Rajavashisth et al., 1987), and most other known propeptides contain a pair of basic amino acids at this region (Chance et al., 1968; Hamilton et al., 1974; Russell & Geller, 1975; Nakanishi et al., 1979; Kangawa & Matsuo, 1979; Patzelt et al., 1979; Steiner et al., 1980). The latter configuration allows these propeptides to be cleaved in secretory vesicles by trypsin or carboxypeptidase B type proteases, whereas the apoAI prosegment is removed in plasma by a specific calcium-dependent metalloprotease which cleaves the −1 Gln−+1 Asp bond of proapoAI (Edelstein et al., 1984; Sliwkowski & Windmueller, 1984; Bojanovski et al., 1984).

Recent studies have suggested important roles of protein pre- and prosegments for the structure, function, and intracellular processing and transport of proteins (Folz & Gordon, 1986, 1987; Wu et al., 1986; Suttie et al., 1987; Jorgensen et al., 1987; Blachly-Dyson & Stevens, 1987; Johnson et al., 1987; Valls et al., 1987; Wise et al., 1988).

This study was undertaken in order to investigate the biological significance of the two Gln residues of apoAI propeptide. The results indicate that in the cell system used alteration of either of the Gln residues of the human apoAI

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[1] Abbreviations: apoAI, apolipoprotein AI; HDL, high-density lipoprotein; LDL, low-density lipoprotein; IDL, intermediate-density lipoprotein; VLDL, very low density lipoprotein; SDS, sodium dodecyl sulfate; BPV, bovine papilloma virus; mMT-1, mouse metallothionein 1; bp, base pair; kb, kilobase.

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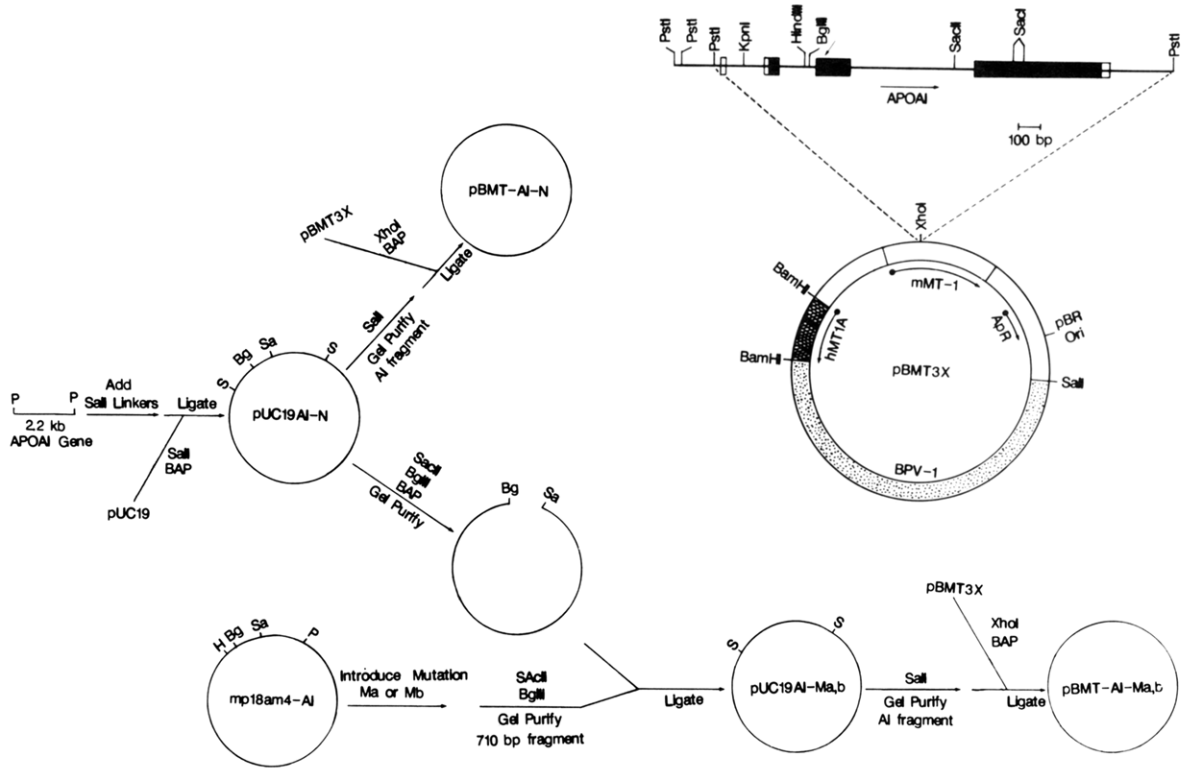

FIGURE 1: Schematic representation of the various steps leading to the construction of recombinant BPV expression vectors containing the normal (AI-N) and mutant (AI-Ma and AI-Mb) human apoAI genes. See Methods for details. The final constructs (designated pBMT-AI-N and pBMT-AI-Ma or -Mb) also carry a copy each of the mMT-1 gene and human metallothionein 1A gene, a copy of the ampicillin-resistant gene (ApR), and a pBR322 origin of replication (see insert). The direction of the apoAI transcription is indicated by an arrow under the restriction map. The location of the point mutations which altered the Gln residues of the apoAI prosegment to Arg residues is indicated by an arrow drawn on the map of the apoAI gene fragment.

prosegment does not affect the intracellular transport and secretion of apoAI and its affinity for HDL.

Materials and Methods

Materials

The Klenow fragment of DNA polymerase I, T4 DNA ligase, and *Sal*I linkers were from New England Biolabs. Restriction enzymes were purchased either from New England Biolabs or from Bethesda Research Laboratories (BRL). Bacterial alkaline phosphatase was obtained from BRL. Nitrocellulose filters were purchased from Schleicher & Schuell. [^{35}S]Methionine (1200 Ci/mmol), [γ-^{32}P]ATP, and [α-^{32}P]dCTP (3000 Ci/mmol) were acquired from New England Nuclear. Dulbecco's modified Eagle's medium (D-MEM) and Dulbecco's Eagle's medium without methionine were obtained from GIBCO. Calcium chloride (analar) was from British Drug House (Gallard Schlessinger). The materials necessary for two-dimensional polyacrylamide gel electrophoresis have been described previously (Zannis et al., 1982; Zannis, 1986). Goat anti-human apoAI was a gift from P. Herbert (Miriam Hospital, Providence, RI). IgGSorb was purchased from the Enzyme Center (Boston, MA). The pBMT3X vector (Krystal et al., 1986) was a gift from Dr. G. Pavlakis of the National Cancer Institute, Frederick Cancer Research Facility, Bethesda, MD.

Methods

Plasmid Construction and Mutagenesis. The plasmids used in this study were constructed by standard techniques (Maniatis et al., 1982). The 2.2-kb *Pst*I–*Pst*I fragment (Figure 1) containing the entire apoAI coding sequence as well as approximately 270 bp of sequences upstream of the first nucleotide of the initiation codon of apoAI and 950 bp of sequences downstream of its poly(A) site (Karathanasis et al., 1983; Shoulders et al., 1983) was gel purified, and the protruding 3′ ends were digested by T4 DNA polymerase. Following ligation of the fragment with *Sal*I linkers, the resultant mixture was subcloned into the *Sal*I site of the pUC19 vector to produce the pUC19AI-N vector. In addition, the 1.7-kb *Hin*dIII–*Pst*I fragment of the apoAI gene (Figure 1) was subcloned into vector M13mp18am4 which contains an amber mutation at position 5327 in the M13 gene 4 (Carter et al., 1985). The single-stranded phage obtained from the recombinant vector was used for the site-directed mutagenesis. The oligonucleotide site-directed mutagenesis was performed by a single-priming method as described (Carter et al., 1984, 1985) using 25-mers [dATTTCTGGCAGC*G*AGATGAACCCCC and dGGCATTTCTGGC*G*GCAAGATGAACC] to create mutant a (Ma) and mutant b (Mb), respectively. Each oligomer contained a single base mismatch (italicized). The primer was synthesized by the solid-phase phosphite triester method (Caruthers, 1985) using an automated oligonucleotide synthesizer (Applied Biosystems 380-A) and purified by two consecutive purification steps by HPLC through a C18 column. After overnight primer extension and ligation, the mixture was used to transform competent JM101 cells. The cells were plated on 2XYT plates to generate progeny plaques. Mutant phages were selected by colony hybridization using the mutagenic oligomers as hybridization probes. The nitrocellulose filters were baked for 2 h at 80 °C under vacuum. After prehybridization (1 h at 67 °C) and hybridization (1 h at 67 °C, followed by a gradual decrease in temperature to 25 °C over a 2–3-h period) in petri dishes, the filters were washed 3 times, each time in 100 mL of fresh 6× standard saline citrate (SSC) at 25 °C for a total of 10 min. Following autoradiography at room temperature for 3–5

h, the filters were washed twice more at 63 and 73 °C, respectively, in 100 mL of 6× SSC for 4–5 min. Between each wash, the filters were air-dried and subjected to autoradiography for 5–20 h. For each mutation, 10 putative mutant phages were plaque-purified as reported (Carter et al., 1985), and subsequently approximately 200 resultant plaques were screened by hybridization as described above. A total of six putative mutant phages were chosen and sequenced by the dideoxy chain termination method (Sanger et al. 1977). Following mutagenesis, the 0.71-kb *Bgl*II–*Sac*II fragments (containing the mutations) were excised from the replicative form of the recombinant M13 phages and substituted for the corresponding normal fragment of pUC19AI-N vector (described above) to produce pUC19AI-Ma and pUC19AI-Mb vectors (Figure 1).

Expression of Normal and Mutant ApoAI Genes. (*A*) *Construction of Recombinant Expression Vectors.* The promoterless normal and mutant apoAI genes were placed under the control of the mMT-1 promoter in the BPV expression vector pBMT3X described previously (Krystal et al., 1986). This vector carriers the entire human MT1A and mouse MT-1 genes plus a genetically engineered unique *Xho*I restriction site (created in place of a *Bgl*II site). This restriction site is positioned 92 bp downstream of the TATA box of mMT-1 promoter and 70 bp downstream of the transcription initiation site of the mMT-1 gene (Glanville et al., 1981). To construct the expression vector containing the normal apoAI gene (designated pBMT-AI-N), the 2.2-kb fragment of apoAI (flanked by *Sal*I linkers) was cleaved from the pUC19AI-N vector and ligated into the *Xho*I site of the pBMT3X vector (Figure 1). Similarly, the 2.2-kb fragments of apoAI containing the mutations Ma and Mb were cleaved from the corresponding pUC19AI-Ma and pUC19AI-Mb vectors and subcloned into the pBMT3X vector to generate expression vectors designated pBMT-AI-Ma and pBMT-AI-Mb, respectively (Figure 1).

(*B*) *Cell Transfection and Selection of Stable Cell Lines.* The method of phosphate–DNA coprecipitation was utilized to introduce the normal and mutant apoAI into the murine fibroblast cell line C127 to produce permanent cell lines (Lowy et al., 1978). The cells were maintained in log phase as monolayer with Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% fetal bovine serum in 100-mm-diameter petri dishes. The day before the transfection, the C127 cells were split at 1×10^6 per 100-mm dish. The purified plasmid DNA (35 μg) and calcium chloride (0.248 M) were mixed to prepare the transfection mixture (calcium phosphate–DNA precipitate). The mixture was added dropwise with air stream to an equal volume of 2× Hepes-buffered saline (42 mM Hepex, pH 7.1, 0.2 M NaCl, 1 mM KCl, 0.14 mM Na_2HPO_4, and 0.2% dextrose). Following incubation at room temperature for 20 min, the transfection mixture was added to the cell cultures, and the culture dishes were kept at 37 °C for 3.5–4 h. The cell monolayers were washed with serum-free medium and then shocked for 1 min with 1× Hepes-buffered saline solution containing 15% glycerol. Subsequently, the cells were quickly rinsed twice with serum-free media and refed with D-MEM containing 10% fetal bovine serum. After 48-h incubation at 37 °C, the cells were split 1:5 and plated in D-MEM medium supplemented with 10% fetal bovine serum and 10 μM $CdCl_2$. The cells were refed every 3 days until discrete isolated colonies were generated, and the colonies were collected using cloning cylinders.

Northern Blotting Analysis. Nearly confluent cultures of transfected C127 cell clones were scraped off plates and solubilized in guanidine isothiocyanate. Total cellular RNA from the cells was purified by cesium chloride gradient centrifugation (Chirgwin et al., 1979). RNA was subjected to electrophoresis on 1% agarose–formaldehyde gels (Thomas, 1983), transferred onto nitrocellulose filters, and subsequently hybridized with a radiolabeled human apoAI gene probe (Karathanasis et al., 1983). The probe used was the 1.7-kb *Hin*dIII–*Pst*I fragment of apoAI gene labeled by the multiprime DNA labeling system (Feinberg & Vogelskin, 1984). The autoradiography was carried out for 15 h at −70 °C using Kodak X-Omat AR film and Lightning Plus intensifying screens (Du Pont).

Analysis of C127 Cell Clones for ApoAI Synthesis. C127 cell clones expressing the normal and mutant apoAI genes and HepG2 cells were grown to approximately 95% confluency in 60-mm-diameter petri dishes in D-MEM containing 10% fetal bovine serum, 10 μM $CdCl_2$, and 2 mM glutamine. The cell monolayer was rinsed twice with methionine-free D-MEM (GIBCO) supplemented with 10 μM $CdCl_2$ and 2 mM glutamine and preincubated in 2 mL of this medium for 1–1.5 h at 37 °C. The cells were then incubated with 2 mL of the same medium containing 0.25 mCi of [^{35}S]methionine for 40 min. The cell medium and the cell lysate were collected separately, immunoprecipitated with goat anti-human apoAI antibody, and analyzed by one- and two-dimensional polyacrylamide gel electrophoresis and autoradiography as described previously (Zannis et al., 1982, 1986). In a few instances following immunoprecipitation, equal amounts (cpm) of various combinations of normal or mutant apoAI were mixed and analyzed similarly as described above.

Pulse–Chase Experiments: Kinetics of Secretion of Normal and Mutant ApoAI Forms. Monolayers of C127 cell clones expressing the normal and mutant apoAI genes grown in 35-mm-diameter dishes were pulsed for 30 min as described above and then chased for various times with D-MEM containing 10 mM methionine and 10 μM $CdCl_2$. The cell lysate and the culture medium were immunoprecipitated and analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography as described. The radioactivity bands corresponding to various chase times were excised from the gels and solubilized in 2 mL of 30% hydrogen peroxide overnight at 60 °C in scintillation vials. Following addition of 15 mL of scintillation fluid, the samples were counted, and the data were subjected to first-order kinetic analysis.

Density Gradient Ultracentrifugation of Nascent ApoAI. C127 cell clones expressing the normal or mutant apoAI gene were grown to 90% confluency in 60-mm-diameter petri dishes and labeled without any preincubation step with 0.4 mCi of [^{35}S]methionine for 5 h as described above. The cell culture medium (2 mL) was collected and analyzed by discontinuous KBr density gradient ultracentrifugation (Redgrave et al., 1975; Zannis et al., 1982). For this analysis, a 2.0-mL aliquot of the culture medium was mixed either with 250 μL of human serum or with 50 μL of HDL (18 mg/mL). The mixture was adjusted to a density of 1.21 g/mL with 0.73 g of potassium bromide, placed in a cellulose nitrate tube, and overlaid sequentially with 1.75 mL of potassium bromide solution of d = 1.15 g/mL and 3 mL each of potassium bromide solutions of d = 1.063 g/mL and d = 1.019 g/mL followed by normal saline. The tubes were then centrifuged in a Beckman SW41 rotor at 40 000 rpm for 19 h at 4 °C.

Following ultracentrifugation, 12 fractions (0.95 mL each) were collected from the top of each gradient tube using a Haake-Buchler Auto Densi-flow IIC pump and a Pharmacia Frac-100 fraction collector. A refractometer was used to

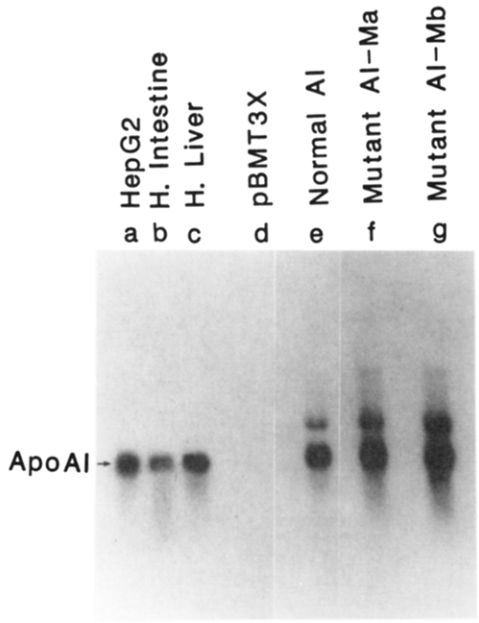

FIGURE 2: Autoradiogram of Northern blotting analysis of RNA isolated from fetal human tissues, HepG2 cells, and C127 cell clones expressing either the normal or the mutant apoAI genes. Lanes a–g contain 30 μg of total RNA isolated from (a) HepG2 cells, (b) fetal human intestine, (c) fetal human liver, (d) C127 cell clones transfected with the pBMT3X vector, (e) C127 cell clones expressing the normal apoAI gene, and (f and g) C127 cell clones expressing the mutant AI-Ma and AI-Mb genes, respectively.

determine the density of 12 fractions similarly collected from a blank tube. After extensive dialysis against 1 mM methionine, the samples were lyophilized, dissolved in 150 μL of SDS–polyacrylamide gel electrophoresis sample buffer (Zannis, 1986), and analyzed by one-dimensional SDS–polyacrylamide gel electrophoresis and autoradiography.

RESULTS

Site-Directed Mutagenesis and Expression of Normal and Mutant ProapoAI Genes. To study the biological significance of the apoAI prosegment, the two Gln residues in the apoAI propeptide were independently altered to Arg residues by site-directed mutagenesis. In the mutagenesis, the CAA codon specifying the first Gln residue nearest to the propeptide cleavage site and the CAG codon of the second Gln residue were altered to CGA (mutant a = Ma) and CGG (mutant b = Mb) codons, respectively. The presence of the mutations was confirmed by DNA sequencing analyses (data not shown).

To express the normal and mutated apoAI genes, the 2.2-kb *Pst*I–*Pst*I fragment containing the promoterless normal and mutant apoAI genes was inserted into the unique *Xho*I site of the pBMT3X vector (Figure 1) to generate the recombinant expression vectors pBMT-AI-N, pBMT-AI-Ma, and pBMT-AI-Mb, respectively. Transfection of mouse C127 cells with these vectors permitted selection of several cell clones resistant to 10 μM $CdCl_2$. These cell clones were found to produce apoAI mRNA and protein. A representative RNA blotting analysis of a few such clones as well as the proper controls is shown in Figure 2. The steady-state apoAI mRNA levels of cell clones expressing either the normal or the mutant apoAI genes, determined by scanning densitometry, are 3–5-fold higher than that of liver or HepG2 cells.

Synthesis and Secretion of Human ProapoAI. Effect of Mutations at the Cleavage Site of the ApoAI Prosegment. C127 cells expressing the normal and mutant apoAI genes were labeled for 40 min, and the newly synthesized apoAI proteins were immunoprecipitated with goat anti-human apoAI

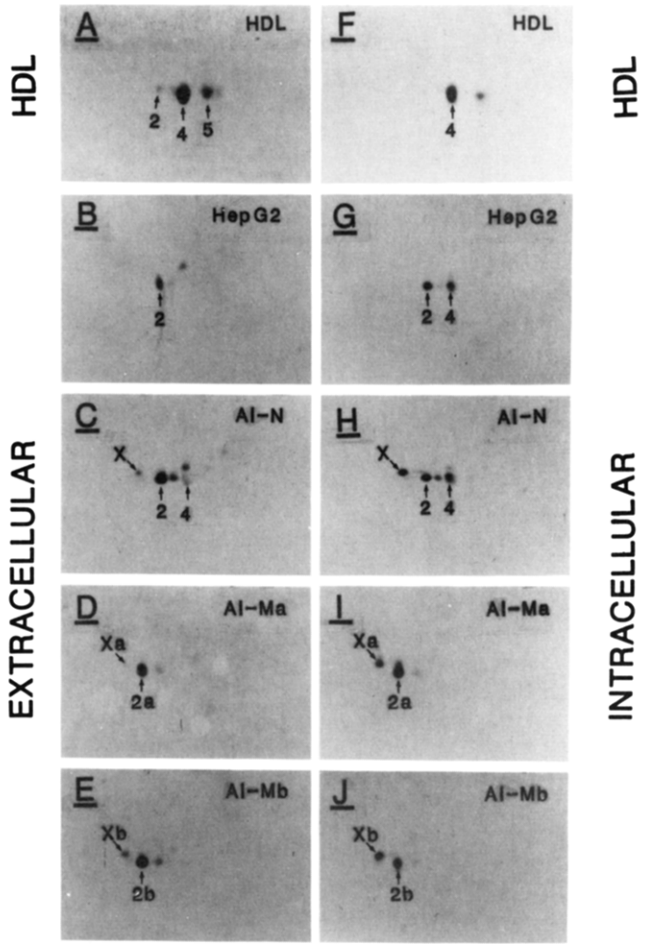

FIGURE 3: Comparison of plasma with intracellular and secreted forms of apoAI produced by HepG2 cells and C127 cell clones expressing the human apoAI gene. All panels except A and F show portions of the autoradiogram obtained following two-dimensional gel electrophoretic analyses of the samples. Panels A and F are Coomassie brilliant blue stained two-dimensional gels of HDL samples. Panels B–E and panels G–J represent the secreted and the intracellular forms of apoAI, respectively. Panels B and G show secreted and intracellular forms of apoAI, respectively, produced by HepG2 cells. Similarly, panels C and H show the secreted and intracellular forms of normal apoAI, respectively, produced by the C127 cell clones. Panels D, E and I, J show secreted and intracellular forms, respectively, of apoAI produced by C127 cell clones expressing the mutant genes. The normal and mutant (Ma and Mb) proapoAI forms are designated as forms 2, 2a, and 2b, respectively. In addition, forms 4 and 5 represent the mature apoAI protein. Forms X, Xa, and Xb are tentatively identified as the preproapoAI forms of the normal apoAI and that of the mutant AI-Ma and AI-Mb, respectively.

antibody. The resultant protein samples were analyzed by one- and two-dimensional SDS–polyacrylamide gel electrophoresis and autoradiography as described under Methods. Figure 3 shows the isoprotein composition of intracellular and secreted apoAI produced by cell lines expressing the normal (panels B, C, G, and H) and mutant (panels D, E, I, and J) apoAI. Panels B–E represent extracellular and panels G–J intracellular forms of apoAI. Panels A and F are Coomassie brilliant blue stained two-dimensional gels of purified HDL samples to allow comparison of the two-dimensional pattern of the plasma and nascent apoAI isoprotein forms. Panels B and G represent the extracellular and intracellular forms of apoAI, respectively, synthesized by the human hepatoma (HepG2) cells. It is interesting that the intracellular apoAI contains both pro (AI_2) and mature (AI_4) forms (panel G) whereas the secreted apoAI consists primarily of the proapoAI form (panel B). Similarly, the C127 cells expressing the normal or mutant apoAI genes

produce both proapoAI and the mature apoAI form intracellularly (panels H–J) and secrete mostly the proAI form (panels C–E). However, the relative amounts of the mature apoAI detected intracellularly and in the media increase significantly if the HepG2 cells or C127 cell clones are labeled overnight, suggesting a limited conversion of proapoAI to the mature form both intracellularly and extracellularly (data not shown). The normal and mutant C127 cell clones also produce another form of apoAI (designated X, Xa, or Xb) which is slightly larger in size and contains a single additional positive charge as compared to the proapoAI form (panels C–E and H–J). Protein X overlaps on two-dimensional gels with the preproapoAI described previously (Zannis et al., 1983). The X, Xa, and Xb species are detected in higher quantities intracellularly than in the media (compare panels C–E with panels H–J, respectively). The mutant forms of apoAI, Ma (panels D and I) and Mb (panels E and J), differ from their normal counterparts by one positive charge. This charge difference is consistent with substitution by in vitro mutagenesis of the Gln −2 or Gln −1 residues of apoAI for an Arg residue. The putative mutant preproapoAI forms Xa and Xb also differ by one positive charge from the normal preproapoAI form (protein X).

To compare further the normal and mutant apoAI synthesized and secreted by C127 cell clones with that of the HepG2 cells, we analyzed equal amounts of various mixtures of the secreted forms of these proteins by two-dimensional polyacrylamide gel electrophoresis and autoradiography (Figure 4). Comparison of panel A with panel B shows that the proapoAI produced by C127 cells expressing the normal apoAI gene overlaps on two-dimensional gels with that of HepG2 cells. Panels C and D confirm that the apoAI produced by cell clones expressing the mutant (Ma and Mb) genes differs by one positive charge from the normal proapoAI produced by HepG2 cells. Finally, panel E shows that both mutant apoAI forms (Ma and Mb) are indistinguishable by two-dimensional polyacrylamide gel electrophoresis analysis.

Rates of Secretion of Normal and Mutant ApoAI Forms. Kinetic analysis showed that apoAI secretion follows apparent first-order kinetics (Figure 5A,B). The $t_{1/2}$ values obtained were 22.7, 21.7, and 21 min for the normal AI-N, mutant AI-Ma, and mutant AI-Mb, respectively. The corresponding apparent first-order rate constants (k) were 3.05×10^{-2} min^{-1} (AI-N), 3.19×10^{-2} min^{-1} (AI-Ma), and 3.30×10^{-2} min^{-1} (AI-Mb).

Flotation Properties of Normal and Mutant ApoAI Forms. Density gradient ultracentrifugation of the secreted apoAI in the presence of human serum or HDL showed that the distribution of apoAI radioactivity determined by autoradiography is identical with the distribution of plasma apoAI determined by staining with Coomassie brilliant blue for both normal and mutant apoAI (compare Figure 6A–F with Figure 6A′-F′). These findings suggest that the apoAI mutations did not have a gross effect on the affinity of apoAI for plasma HDL.

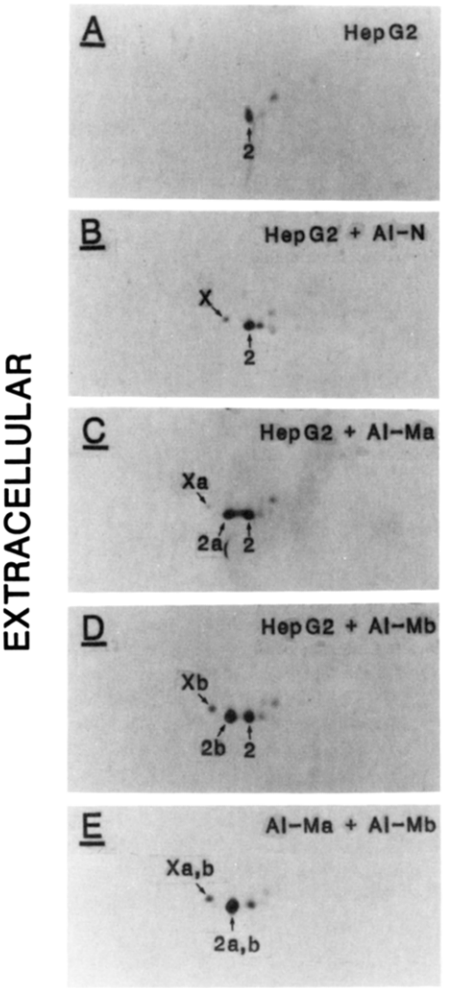

FIGURE 4: Comparison of secreted apoAI produced by HepG2 cells and C127 cell clones expressing either the normal or the mutant apoAI gene. The comparison was made by two-dimensional gel electrophoresis and autoradiography as explained in Figure 3. (Panel A) ApoAI secreted by HepG2 cells; panels B, C, and D show the analysis of a mixture of equal counts (10 000–15 000 cpm) of apoAI immunoprecipitated from the culture media of HepG2 cells and cell clones expressing the normal and the two mutant (Ma, Mb) apoAI genes, respectively. Panel E shows the analysis of equal counts of apoAI secreted by cell clones expressing the two mutant (AI-Ma and AI-Mb) apoAI genes. The forms 2, 2a, 2b, X, Xa, and Xb are all defined in the legend to Figure 3. The forms 2a,b and Xa,b represent the mixture of mutant isoproteins produced by cell clones expressing the corresponding genes (panel E).

Discussion

Background. A distinct feature of human apoAI is that it contains an N-terminal hexapeptide with the sequence Arg-His-Phe-Trp-Gln-Gln (Cheung & Chan, 1983; Gordon et al., 1983; Karathanasis et al., 1983; Shoulders et al., 1983; Zannis et al., 1983). Several laboratories have described a proteolytic activity in plasma and lymph which cleaves the −1 Gln−+1 Asp peptide bond (Bojanovski et al., 1984; Edelstein et al., 1984; Sliwkowski & Windmueller, 1984). The in vitro cleavage of the apoAI prosegment is slow and partial and raises questions on the importance of this cleavage for the extracellular function(s) of apoAI.

Recently, research has focused on the importance of the pre- and prosegments of several proteins for their intracellular co- and posttranslational modifications as well as their intracellular transport. Thus, the propeptides of preprofactor IX and vitamin K dependent carboxylase may be signals which direct the posttranslational γ-carboxylation of the mature proteins (Jorgensen et al., 1987; Suttie et al., 1987). The amino-terminal presegment of yeast carboxypeptidase Y is important for efficient cotranslational translocation across the endoplasmic reticulum as well as for its efficient glycosylation (Blachly-Dyson & Stevens, 1987). Furthermore, the propeptide of the yeast carboxypeptidase Y contains the signal for targeting the enzyme to vacuoles (Johnson et al., 1987) and for controlling the proteolytic activity during intracellular transport (Valls et al., 1987). The amino- and carboxy-terminal peptides of type I procollagen affect the steady-state collagen mRNA levels (Wu et al., 1986). Finally, the pro-

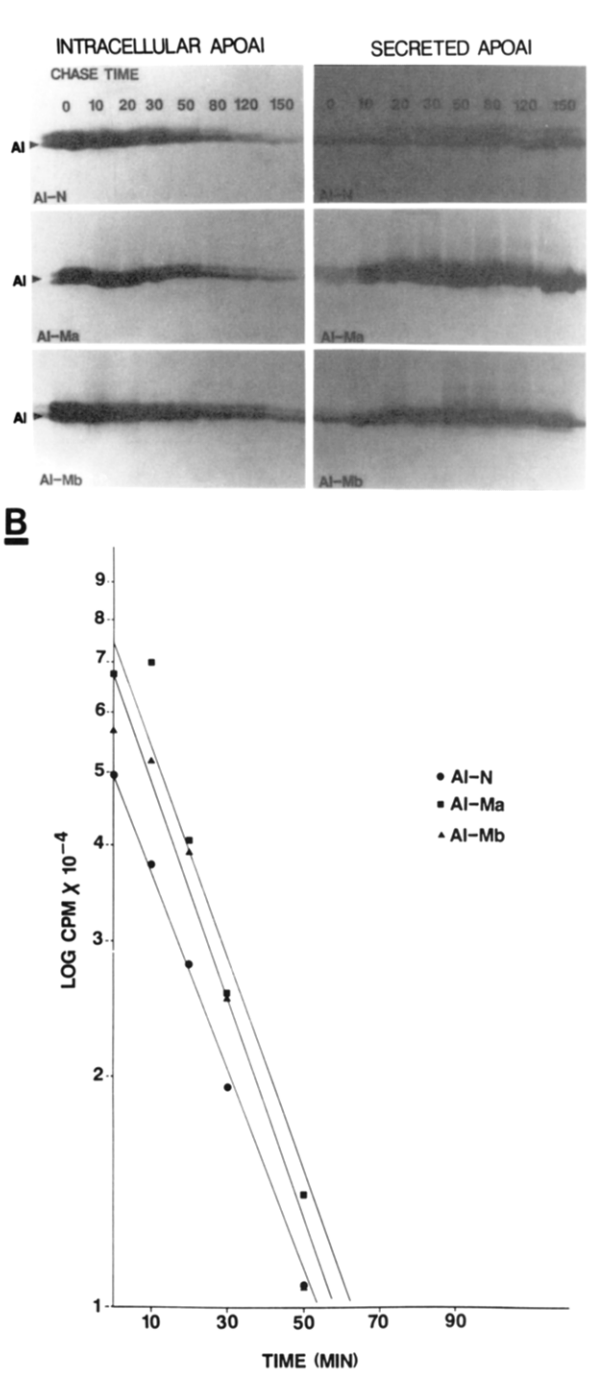

FIGURE 5: Kinetics of apoAI secretion. (Panel A) Time course of apoAI secretion. Monolayer cultures of C127 cell clones expressing either the normal or the mutant apoAI gene were labeled with [^{35}S]methionine, immunoprecipitated, and analyzed by one-dimensional SDS–polyacrylamide gel electrophoresis and autoradiography as described under Methods. The figure shows the autoradiogram obtained from this analysis. (Panel B) Kinetics of apoAI secretion. For this analysis, protein bands shown in panel A corresponding to chase times of 0–50 min were excised from the gel, solubilized, and counted, and the data were analyzed in a semilogarithmic plot. The closed circles (●), closed squares (■), and closed triangles (▲) represent ^{35}S counts for normal (AI-N) and mutant (AI-Ma and AI-Mb) apoAI forms, respectively.

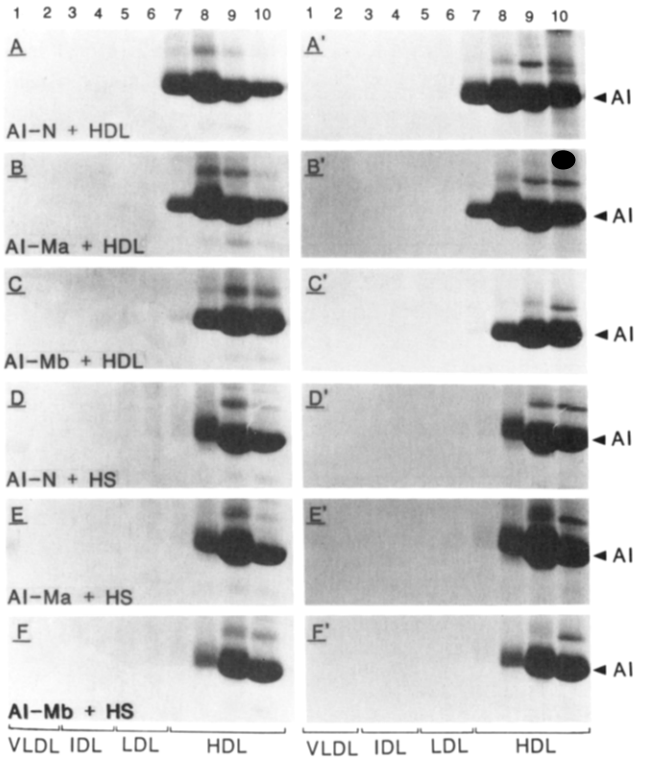

FIGURE 6: One-dimensional SDS–polyacrylamide gel electrophoretic analysis of proteins secreted by mouse C127 cells expressing the human apoAI gene following purification by density gradient ultracentrifugation. Density gradient ultracentrifugation of the culture medium in the presence or absence of human serum or HDL was performed as explained under Materials and Methods. The average density (g/mL) of the fractions analyzed was as follows: 1 = 1.010; 2 = 1.014; 3 = 1.018; 4 = 1.032; 5 = 1.048; 6 = 1.072; 7 = 1.10; 8 = 1.13; 9 = 1.162; 10 = 1.186. Following staining with Coomassie brilliant blue, the gels obtained from this analysis showed the position of the plasma apoAI (panels A–F). The corresponding autoradiograms show the position of the nascent ^{35}S-labeled apoAI (panels A′-F′). Panels A–C and A′-C′ show analysis in the presence of human HDL, and panels D–F and D′-F′ show analysis in the presence of human serum. Note the distribution of plasma apoAI (panels A–F) is identical with that of ^{35}S-labeled nascent apoAI (panels A′-F′).

peptide of von Willebrand factor (vWF) contains the determinants which mediate the assembly of vWF multimers (Wise et al., 1988).

Folz and Gordon (1986) have recently reported that removal of the prosegment of human apoAII affects the site of cotranslational signal peptide cleavage in vitro, whereas removal of the apoAI prosegment affects only the rate of the signal peptide cleavage (Folz & Gordon, 1987).

The unusual feature of the prosegments of human and rat apoAI is that they terminate with two Gln residues (Cheung & Chan, 1983; Gordon et al., 1982, 1983; Karathanasis et al., 1983; Shoulders et al., 1983; Zannis et al., 1983) whereas the chicken apoAI terminates with Gln-His (Rajavashisth et al., 1987) and the prosegments of most other secretory proteins terminate with a pair of basic amino acids (Chance et al., 1968; Hamilton et al., 1974; Russell & Geller, 1975; Nakanishi et al., 1979; Kangawa & Matsuo, 1979; Patzelt et al., 1979; Steiner et al., 1980). To investigate the significance of the Gln residues of the apoAI prosegment on the intracellular transport and secretion of apoAI and its association with HDL, we have altered by in vitro mutagenesis the codons specifying residues −1 Gln and −2 Gln to codons specifying Arg residues.

Synthesis and Secretion of Normal and Mutant ApoAI by C127 Cell Clones Expressing the Corresponding Genes. To produce large quantities of normal and mutant apoAI, we placed the corresponding genes under the control of the mouse metallothionein 1 gene in a bovine papilloma virus vector which also contains a copy of the human metallothionein 1A gene. Following transfection with the recombinant vectors, cell clones were selected for resistance to 10 μM Cd^{2+} and were analyzed for synthesis of apoAI mRNA and protein. The analysis showed that the apoAI mRNA levels of the cell clones expressing either the normal or the mutant apoAI gene were 3–5

times higher than the mRNA levels produced by liver or HepG2 cells. These data indicate that the C127 clones overexpress the human apoAI gene. The size of the major apoAI mRNA produced by C127 cell clones is slightly larger than that of human fetal liver and HepG2 cells (Figure 2). This size mRNA is consistent with utilization of the transcription initiation site of the mMT-1 promoter located approximately 70 bp upstream from the *Xho*I site where the apoAI gene was inserted (Figure 1). Utilization of this transcription initiation site will imply that the mRNA synthesized by C127 cell clones contains 70 bp of the 5′-untranslated region of the mMT-1 gene (Glanville et al., 1981) as well as the entire sequence of the human apoAI gene. Expression of pBMT-AI constructs in C127 cells also produces some higher size mRNA species which cross-hybridize with the human apoAI probe. The most abundant of these mRNA species may represent a fusion transcript containing the human apoAI and the mMT-1 mRNAs. Such a transcript can arise by utilization of the polyadenylation signal of the mouse metallothionein 1 gene which is located approximately 2 kb downstream of the *Xho*I site of the pBMT3X vector. The less abundant of these species may represent nuclear apoAI mRNA forms which may accumulate due to the enhanced transcriptional activity of the apoAI gene in these cells. Similar mRNA species have been observed in cell clones expressing the human apoE gene (Cladaras et al., 1987).

Protein analysis shows that despite the mRNA heterogeneity the protein synthesized by the apoAI-producing clones consists mainly of the proapoAI form. As expected, both mutant apoAI forms differ from the normal apoAI by one positive charge. An unexpected finding in these studies is that both normal and mutant apoAI synthesize and secrete a protein form which coincides on two-dimensional gels with the preproapoAI. It is possible that, due to the overproduction of apoAI mRNA in C127 cells as compared to HepG2 cells, either the attachment of ribosomes (translating apoAI mRNA) to the endoplasmic reticulum or the cleavage of the signal peptide becomes rate limiting (Garoff et al., 1978). This will result in intracellular accumulation of preproapoAI. It is interesting that small quantities of the protein X are also secreted into the media, suggesting that at least a small portion of preproapoAI enters the secretory pathway. This finding also suggests that cleavage of the signal peptide is not a prerequisite for apoAI secretion by the C127 cells.

Substitution of Gln Residues of the Prosegment by Arg Has No Effect on ApoAI Secretion and HDL Binding. The kinetics of apoAI secretion were determined by pulse–chase labeling as explained under Materials and Methods. This analysis showed that in the cell system used mutations in the two Gln residues of apoAI did not affect the intracellular transport and rate of secretion of this protein. Nevertheless, the possibility still exists that these mutations may have different effects on an apoAI-producing cell line such as HepG2 cells. Secretion of both normal and mutant apoAI forms follows apparent first-order kinetics with half-lives of 22.7 min (AI-N), 21.7 min (AI-Ma), and 21 min (AI-Mb). Furthermore, flotation of apoAI in the presence of human serum or purified HDL showed that the distribution of the radiolabeled apoAI was similar to that of plasma apoAI. This finding indicates that the two Gln residues probably are not critical for the association of nascent apoAI with HDL.

It has been suggested that the two Gln residues may serve as recognition sites for the extracellular protease which removes the apoAI prosegment (Gordon et al., 1982). Studies are under way to determine the effects of these mutations on the fidelity, efficiency, and specificity of the extracellular protease which removes the apoAI prosegment.

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REFERENCES

Blachly-Dyson, E., & Stevens, T. H. (1987) *J. Cell Biol. 104*, 1183–1191.

Bojanovski, D., Gregg, R. E., & Brewer, H. B., Jr. (1984) *J. Biol. Chem. 259*, 6049–6051.

Carter, P., Winter, G., Wilkinson, A. J., & Fersht, A. (1984) *Cell (Cambridge, Mass.) 38*, 835–840.

Carter, P., Bedouelle, H., & Winter, G. (1985) *Nucleic Acids Res. 13*, 4431–4443.

Caruthers, M. H. (1985) *Science (Washington, D.C.) 230*, 281–285.

Chance, R. E., Ellis, R. M., & Bromer, W. W. (1968) *Science (Washington, D.C.) 161*, 165–167.

Cheung, P., & Chan, L. (1983) *Nucleic Acids Res. 11*, 3703–3715.

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., & Rutter, W. J. (1979) *Biochemistry 18*, 5294–5299.

Cladaras, C., Hadzopoulou-Cladaras, M., Felber, B. K., Pavlakis, G., & Zannis, V. I. (1987) *J. Biol. Chem. 262*, 2310–2315.

Edelstein, C., Gordon, J. I., Toscas, K., Sims, H. F., Stauss, A. W., & Scanu, A. M. (1984) *J. Biol. Chem. 258*, 11430–11433.

Feinberg, A. P., & Vogelstein, B. (1984) *Anal. Biochem. 136*, 266–267.

Fidge, N. H., & Nestel, P. J. (1985) *J. Biol. Chem. 260*, 3570–3575.

Fielding, C. J., Shore, V. G., & Fielding, P. D. (1972) *Biochem. Biophys. Res. Commun. 46*, 1943–1949.

Folz, R. J., & Gordon, J. I. (1986) *J. Biol. Chem. 261*, 14752–14759.

Folz, R. J., & Gordon, J. I. (1987) *J. Biol. Chem. 262*, 17221–17230.

Garoff, H., Simons, K., & Dobberstein, B. (1978) *J. Mol. Biol. 124*, 587–600.

Glanville, N., Durnam, D. M., & Palmiter, R. (1981) *Nature (London) 292*, 267–269.

Gordon, J. I., Smith, D. P., Andy, R., Alpers, D. H., Schonfeld, G., & Strauss, A. W. (1982) *J. Biol. Chem. 257*, 971–978.

Gordon, J. I., Sims, H. F., Lentz, S. R., Edelstein, C., Scanu, A. M., & Strauss, A. W. (1983) *J. Biol. Chem. 258*, 4037–4044.

Hamilton, J. W., Niall, H. D., Jacobs, J. W., Keutmann, H. J., Potts, J. T., Jr., & Cohen, D. V. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 653–656.

Hwang, J., & Menon, K. M. J. (1985) *J. Biol. Chem. 260*, 5660–5668.

Jackson, R. L., Gotto, A. M., Stein, O., & Stein, Y. (1975) *J. Biol. Chem. 250*, 7204–7209.

Johnson, L. M., Bankaitis, V. A., & Emr, S. D. (1987) *Cell (Cambridge, Mass.) 48*, 875–885.

Jorgensen, M. J., Cantor, A. B., Furie, B. C., Brown, C. L., Shoemaker, C. B., & Furie, B. (1987) *Cell (Cambridge, Mass.) 48*, 185–191.

Kangawa, K., & Matsuo, H. (1979) *Biochem. Biophys. Res. Commun. 86*, 153–160.

Karathanasis, S. K., Zannis, V. I., & Breslow, J. L. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 6147–6151.

Krystal, M., Li, R., Lyles, D., Pavlakis, G., & Palese, P. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2709–2713.
Lowy, D. R., Rand, E., & Scolnick, E. M. (1978) *J. Virol. 26*, 291–298.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) in *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Nakanishi, S., Inoue, A., Kita, T., Nakamura, M., Chang, A. C. Y., Cohen, S. N., & Numa, S. (1979) *Nature (London) 278*, 423–427.
Patzelt, C., Tager, H. S., Carroll, R. J., & Steiner, D. F. (1979) *Nature (London) 282*, 260–266.
Rajavashisth, T. B., Dawson, P. A., Williams, D. L., Shackelford, J. E., Lebherz, H., & Lusis, A. J. (1987) *J. Biol. Chem. 262*, 7058–7065.
Redgrave, T. G., Roberts, D. C. K., & West, C. F. (1975) *Anal. Biochem. 64*, 42–49.
Russell, J. H., & Geller, D. B. (1975) *J. Biol. Chem. 250*, 3409–3413.
Sanger, R., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.
Schmitz, G., Niemann, R., Brennhausen, B., Krause, R., & Assmann, G. (1985a) *EMBO J. 4*, 2773–2779.
Schmitz, G., Robenek, H., Lohmann, U., & Assmann, G. (1985b) *EMBO J. 4*, 613–622.
Shoulders, C. C., Kornblihtt, A. R., Munro, B. S., & Baralle, F. E. (1983) *Nucleic Acids Res. 11*, 2827–2837.
Sliwkowski, M. B., & Windmueller, H. G. (1984) *J. Biol. Chem. 259*, 6459–6465.
Soutar, A., Garner, C., Baker, H. N., Sparrow, J. T., Jackson, R. L., Gotto, A. M., & Smith, L. C. (1975) *Biochemistry 14*, 3057–3064.
Steiner, D. F., Quinn, P. S., Chan, S. J., Marsh, J., & Tager, H. S. (1980) *Ann. N.Y. Acad. Sci. 343*, 1–15.
Suttie, J. W., Hoskins, J. A., Engelke, J., Hopfgartner, A., Ehrlich, H., Bang, N. U., Belagaje, R. M., Schoner, B., & Long, G. L. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 634–637.
Thomas, P. (1983) *Methods Enzymol. 100*, 255–266.
Valls, L. A., Hunter, C. P., Rothman, J. H., & Stevens, T. H. (1987) *Cell (Cambridge, Mass.) 48*, 887–897.
Wise, R. J., Pittman, D. D., Handin, R. I., Kaufman, R. J., & Orkin, S. H. (1988) *Cell (Cambridge, Mass.) 49*, 229–236.
Wu, C. H., Donovan, C. B., & Wu, G. Y. (1986) *J. Biol. Chem. 261*, 10482–10484.
Zannis, V. I. (1986) *Methods Enzymol. 128*, 823–851.
Zannis, V. I., Breslow, J. L., & Katz, A. J. (1980) *J. Biol. Chem. 255*, 8612–8617.
Zannis, V. I., Kurnit, D. M., & Breslow, J. L. (1982) *J. Biol. Chem. 257*, 536–544.
Zannis, V. I., Karathanasis, S. K., Keutmann, H. T., Goldberger, G., & Breslow, J. L. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2574–2578.

Adsorption of Cations to Phosphatidylinositol 4,5-Bisphosphate†

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ABSTRACT: We investigated the binding of physiologically and pharmacologically relevant ions to the phosphoinositides by making ^{31}P NMR, electrophoretic mobility, surface potential, and calcium activity measurements. We studied the binding of protons to phosphatidylinositol 4,5-bisphosphate (PIP_2) by measuring the effect of pH on the chemical shifts of the ^{31}P NMR signals from the two monoester phosphate groups of PIP_2. We studied the binding of potassium, calcium, magnesium, spermine, and gentamicin ions to the phosphoinositides by measuring the effect of these cations on the electrophoretic mobility of multilamellar vesicles formed from mixtures of phosphatidylcholine (PC) and either phosphatidylinositol, phosphatidylinositol 4-phosphate, or PIP_2; the adsorption of these cations depends on the surface potential of the membrane and can be described qualitatively by combining the Gouy–Chapman theory with Langmuir adsorption isotherms. Monovalent anionic phospholipids, such as phosphatidylserine and phosphatidylinositol, produce a negative electrostatic potential at the cytoplasmic surface of plasma membranes of erythrocytes, platelets, and other cells. When the electrostatic potential at the surface of a PC/PIP_2 bilayer membrane is −30 mV and the aqueous phase contains 0.1 M KCl at pH 7.0, PIP_2 binds about one hydrogen and one potassium ion and has a net charge of about −3. Our mobility, surface potential, and electrode measurements suggest that a negligible fraction of the PIP_2 molecules in a cell bind calcium ions, but a significant fraction may bind magnesium and spermine ions.

A number of hormones, neurotransmitters, chemoattractants, and growth factors bind to receptors in the cell membrane and activate, probably through a G protein (Litosh & Fain, 1986; Williamson, 1986; Cockcroft, 1987), a specific phospholipase C. This enzyme hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2)[1] into the second messengers inositol

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[1] Abbreviations: G_{M1}, galactosyl-*N*-acetylgalactosaminyl(*N*-acetylneuraminyl)galactosylglucosylceramide; IP_3, inositol 1,4,5-trisphosphate; MOPS, 4-morpholinepropanesulfonate; PA, phosphatidic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine.

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