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GENETIC EVIDENCE THAT THE MULTIPLE APOLIPOPROTEIN A-1 ISOFORMS ARE ENCODED BY A COMMON STRUCTURAL GENE

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SUMMARY: We have used a genetic structural variation of apolipoprotein A-I in mice to examine the origin of the multiple charge isoforms of the plasma protein. Apolipoprotein A-I translated in vitro from hepatic or enteric mRNA revealed that the genetic variation simultaneously alters the charge of the protein produced by both tissues. The variation also shifted the charge of the entire family of isoforms found in plasma or translated in vitro. These results indicate that the protein produced by different tissues as well as the multiple isoforms are all derived from a common structural gene by processing.

Apolipoprotein A-I[1] is the major protein in mammalian high density lipoproteins (1). Apo-A-I participates in cholesterol ester formation by acting as a cofactor for lecithin-cholesterol acyltransferase (2) and in vitro studies suggest that it functions in the removal of cholesterol from plasma membranes (3). The principle sites of apo-A-I synthesis appear to be liver and intestine (4-8).

Plasma apo-A-I in mammals that have been examined is present as a series of charge isoforms with similar molecular weights (9-13). The origin of the forms is unclear. In human plasma up to six isoforms can be resolved by isoelectric focusing; these have similar antigenicity, amino acid composition and peptide maps (9,10). Biochemical studies have suggested that the multiple forms do not result from variable phosphate or carbohydrate content (9, 12). Recent in vitro translation studies of rat enteric apo-A-I indicate that the protein is synthesized as preproprotein containing a 24 amino acid extension at the amino terminus (14), raising the possibility that proteolytic processing accounts for some of the heterogeneity.

[1]Abbreviations: apolipoprotein A-I (apo-A-I); sodium dodecyl sulfate (SDS).

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In this report we have examined whether the apo-A-I isoforms are derived from multiple structural genes, perhaps differentially expressed in various tissues, or whether they originate from a single gene by modification. For these studies we have utilized a genetic structural variation in mice that alters the charge of the protein (15). We have previously shown that mouse apo-A-I resembles human and rat apo-A-I in that it consists of a series of charge isoforms of similar molecular weight (13, 15).

METHODS

Animals and reagents. Mice (females, 2-4 months of age) were purchased from Jackson Laboratories, Bar Harbor, Maine; oligo(dT)-cellulose was from Bethesda Research Laboratories; Pansorbin, consisting of a slurry of fixed Staphylococcus aureus cells, was from Calbiochem-Behring; [^{35}S]methionine, 1120 Ci/mmole, was from New England Nuclear. Rabbit reticulocyte lysate was prepared and treated with nuclease as described (16).

RNA isolation. Mouse tissues were homogenized in guanidine thiocyanate (Eastman Kodak), and RNA from the homogenate was isolated by centrifugation through a cesium chloride cushion as previously described (17). The poly(A) containing RNA was enriched by oligo(dT)-cellulose chromatography (18).

In vitro translation. [^{35}S]methionine was added to rabbit reticulocyte lysate (3-4 µCi/µl lysate), and the mRNA was translated in a ratio of 0.2 µg mRNA to 10 µl of the lysate-methionine mixture for 60 min at 30°C. The total radioactivity incorporated was determined by boiling aliquots in 1 ml 10% trichloracetic acid for 10 min, cooling on ice and collecting the precipitate on glass fiber filters. The filters were then incubated at 50°C in 1 ml 30% hydrogen peroxide overnight. Ten ml of scintillation fluid were added and radioactivity determined.

Immunoprecipitation. The in vitro translation lysates were diluted 5-fold with 0.02 M sodium phosphate, 0.15 M NaCl, pH 7.4, containing 0.1% sodium lauroyl sarcosine, 6.5 mg/ml bovine serum albumin and 1 mg/ml methionine. The lysates were precleared by air-fuge centrifugation at 100,000 x g for 5 min. The supernate was then incubated with 2 µl of 10% (w/v) slurry of Pansorbin/ µl of original lysate. After 15 min at 4°C , the cells were removed by centrifugation at 15,000 x g for 2 min. Antiserum to mouse apo-A-I was obtained from rabbits injected with purified mouse apo-A-I (13). The apo-A-I antiserum was added in a 1:50 dilution to the diluted lysates and incubated overnight at 4° C . A 10% slurry of Pansorbin (2.5 µl/µl original lysate) was then added, and the mixture was incubated at 4° C for 15 min with occasional agitation. The cells were sedimented by centrifugation at 15,000 x g for 2 min and washed 3 times with 0.02 M sodium phosphate, 0.15 M NaCl, pH 7.4, containing 0.1% sodium sarcosine. The immune complexes were dissociated from the cells by boiling for 3 min in SDS sample buffer, followed by low speed centrifugation to remove cells. For isoelectric focusing, the pellet was resuspended in sample buffer (see below), incubated at room temperature for 15 min, sedimented and reextracted in the same manner.

Electrophoresis. SDS polyacrylamide gel electrophoresis was done according to Laemmli (19). Slab gel isoelectric focusing was done as previously described (20). Urea solutions were always freshly prepared from ultrapure urea (Schwartz-Mann) to minimize carbamylation. The isoelectric focusing sample buffer contained 9.5 M urea, 2% Triton X-100, 1% pH 3.5-10 ampholines (LKB), 1% pH 4-6 ampholines and 5% 2-mercaptoethanol. Gels were prepared

for fluorography with ENHANCE (New England Nuclear) according to the manufacturer's instructions.

Quantitation of mRNA activity. Consecutive 2 mm slices were cut with a razor blade from the dried gel around the band after superimposing on the autoradiogram. Each slice was solubilized and counted as described (21). The radioactivity from the peak fractions was then added together.

Cotranslational processing. Dog pancreas microsomes (22, 23) were a gift from William Wickner (University of California, Los Angeles). Reactions were carried out with 2.8 A_{280} units of microsomes per ml lysate, followed by quenching with 6 volumes of lysis buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.01% NaN_3, 0.1 M NaCl, 10 mM sodium phosphate, pH 7.5). In some experiments, 200 µg/ml proteinase K plus and minus 1% Triton X-100 were added prior to dilution with lysis buffer. Proteinase digestion proceeded for 60 min at 4°C and was terminated with 4 mM phenylmethylsulfonylfloride.

RESULTS AND DISCUSSION

Translation of apo-A-I mRNA and cotranslational processing. Poly(A) containing RNA was isolated from mouse liver and translated in a reticulocyte lysate system in the presence of [^{35}S]methionine. The apo-A-I was precipitated from the lysate with rabbit antiserum to mouse apo-A-I. The resulting precipitate gave a single band of radioactivity when subjected to SDS gel electrophoresis (Fig. 1), but at least three bands were obtained after isoelectric focusing (Fig. 2). The apo-A-I identity of the labeled bands was confirmed by showing that they could be displaced from antibody by the inclusion of 10 µg purified mouse apo-A-I (data not shown). As judged by SDS gel electrophoresis, the *in vitro* translated protein is larger than the protein isolated from plasma, with apparent molecular weights of 26,500 and 25,000, respectively. The *in vitro* product is also more basic than the plasma apo-A-I (Fig. 2). Apo-A-I translated *in vitro* from intestine mRNA was similar in both size and charge to the liver mRNA product.

When microsomes were included in the translation, a lower molecular weight, more acidic form of apo-A-I was produced (Fig. 1). This processed protein was resistant to proteinase K digestion, indicating that it was located on the inside of the microsomes. It became sensitive to the protease when the microsomes were disrupted by the addition of detergent. After cotranslational processing the *in vitro* translated apo-A-I was not discernibly different in either size or isoelectric point from the mature plasma protein (data not

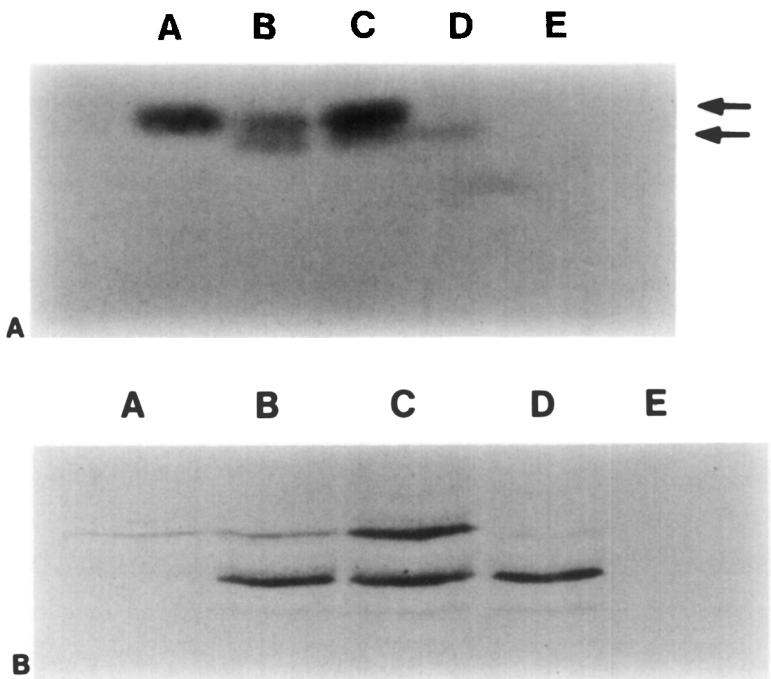

Figure 1: Cotranslational Processing of Apo-A-I. C57BL/6J liver mRNA was translated in vitro in the presence of [^{35}S]methionine, and the immunoprecipitated apo-A-I was analyzed by fluorography after SDS polyacrylamide gel electrophoresis (Panel A) or isoelectric focusing (Panel B). The mRNA was translated in the absence (Well A) or presence (Well B) of dog pancreas microsomes. Well (C) contains a mixture of products translated in the absence or presence of microsomes. In other experiments the products translated in the presence of microsomes were treated with proteinase K (Well D) or proteinase K plus Triton X-100 (Well E) prior to immunoprecipitation. The two major bands observed after SDS gel electrophoresis (shown by arrows) had apparent molecular weights of about 26,500 (top) and 25,000 (bottom). The two major bands observed after isoelectric focusing had isoelectric points of about 5.4 (top) and 5.3 (bottom).

shown). Our studies do not address the possibility of a propeptide following the "signal" peptide of mouse apo-A-I; if present, its net charge is presumably zero.

Tissue distribution: RNA from various tissues of strain C57BL/6 mice was translated in vitro in the presence of [^{35}S]methionine and the radioactivity incorporated into apo-A-I determined. The relative mRNA activity was expressed as a percentage of the radioactivity incorporated into apo-A-I compared to total trichloracetic acid precipitable radioactivity. Liver and intestine had similar relative rates of apo-A-I synthesis (1.0 ± 0.1% and 0.9 ± 0.1%, respectively) while lung, spleen, heart and kidney had

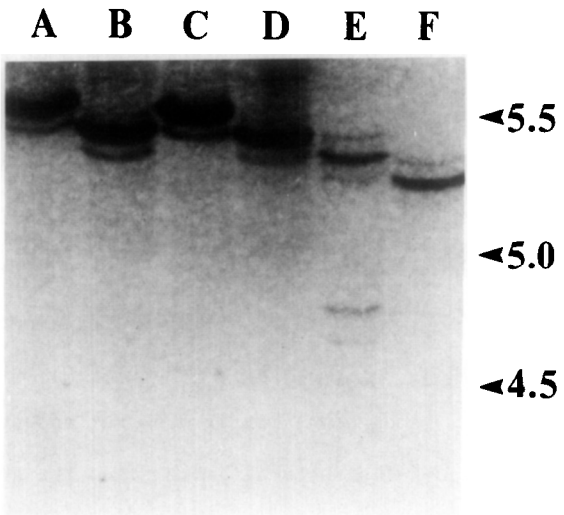

Figure 2: Isoelectric Focusing of Apo-A-I Derived From Liver and Intestine: Structural Mutation Simultaneously Alters Charge of Protein in Both Tissues. mRNA was translated in vitro in 10 µl reaction mixtures in the presence of [^{35}S]methionine, and the products were immunoprecipitated, subjected to isoelectric focusing in polyacrylamide gels, and fluorographed. The mRNA for the reactions was isolated from BALB/cJ liver (Well A) C57BL/6J liver (Well B) BALB/cJ intestine (Well C), or C57BL/6J intestine (Well D). Shown for comparison are the patterns of plasma apo-A-I from strains of BALB/cJ (Well E) and C57BL/6J (Well F) stained with Coomassie G-250. The approximate pH gradient, determined from eluates of gel slices, is indicated by arrows.

negligible mRNA activity. We conclude that, unlike apolipoprotein E (24), little plasma apo-A-I is derived from tissues other than liver and intestine.

A common structural gene for apo-A-I is expresed in liver and intestine. We previously characterized a genetic structural variation for apo-A-I that alters the charge of the protein as determined by isoelectric focusing under denaturing conditions. The variation is inherited as a single Mendelian gene residing on mouse chromosome 9 and exhibits codominant expression (15).

The availability of this variation allowed us to test whether the same structural gene for apo-A-I is expressed in liver and intestine. Hepatic and enteric mRNA were isolated from two strains of mice, C57BL/6J and BALB/cJ, carrying different structural alleles of apo-A-I. C57BL/6J mice carry the acidic alloform of apo-A-I while BALB/cJ mice carry the less acidic form (Fig. 2). The mRNA from each tissue was translated in vitro, and the apo-A-I product was immunoprecipitated and subjected to isoelectric focusing (Fig. 2). The allelic charge variation in apo-A-I was seen in both liver

(lanes A and B) and intestine (lanes C and D) at the level of mRNA, but the charge of apo-A-I from the two tissues of the same strain of mouse was identical. The fact that a single mutation simultaneously alters the charge of apo-A-I in liver and intestine indicates that the protein in both tissues is derived from a common gene.

Charge isoforms. Mouse plasma apo-A-I consists of a series of charge isoforms separable by isoelectric focusing (12, Fig. 2). Similar heterogeneity exists in the protein translated from hepatic and enteric mRNA (Fig. 2). The nature of the heterogeneity is unknown, but it does not appear to result from the presence of multiple apo-A-I structural genes since the genetic variation between BALB/cJ and C57BL/6J shifts the charge of the entire family of isoforms (Fig. 2). This conclusion is supported by recent studies involving hybridization of apo-A-I cDNA to RNA(25) or genomic DNA(26).

The chemical nature of the modifications involved in generating the apo-A-I isoforms is still unknown. The studies of Zannis, et al. (9) involving intestinal organ culture suggest that the modification of apo-A-I occurs in part after secretion into the circulation. On the other hand, our observation that at least two major apo-A-I isoforms are produced upon cell-free translation of mRNA in the absence of microsomes raises the possibility that some heterogeneity may be present prior to translation. Two apo-A-I translation products were also observed after cell-free translation of human fetal liver apo-A-I (27).

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