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Biosynthetic relationships between three rat apolipoprotein B peptides

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Abstract Rat liver is unique in secreting very low density lipoproteins (VLDL) with three size-isoforms of apolipoprotein B: PI and PIII correspond to B-100 and B-48, respectively, while PII is slightly smaller than PI and has no counterpart in other species. Antibodies against a fusion protein corresponding to the extreme C-terminal region of PI fail to react with PII, suggesting that the latter lacks this moiety. [35S]Methionine-labeled perfused rat liver and isolated hepatocytes secrete labeled PII, but intracellular apoB contains only PI and PIII. The absence of labeled PII from Golgi VLDL, and the absence of continued PII production within the plasma compartment, strongly suggest that PIII-containing VLDL are formed by a one-time proteolytic processing of a certain proportion of PI-containing VLDL at the time of secretion. In contrast, polysome run-off translation experiments and analysis of polysome-bound nascent apoB chains show that both rat liver and intestinal polysomes release PIII-sized peptides directly at the appropriate point of elongation, in a manner incompatible with their formation by posttranslational processing. M These results strongly suggest that the large (PI, B-100) and small (PIII, B-48) apoB peptides are translated from separate mRNAs. Thus, although both PII and PIII are C-terminally truncated products of PI, the mechanisms involved are entirely different. - Reuben, M. A., K. L. Svenson, M. H. Doolittle, D. F. Johnson, A. J. Lusis, and J. Elovson. Biosynthetic relationships between three rat apolipoprotein B peptides. J. Lipid Res. 1988. 29: 1337-1347.

Supplementary key words rat apoB C-terminal sequence • proteolytic processing of PI • separate PI and PIII mRNA

Mammalian triglyceride-rich lipoproteins contain apoB, which appears to be obligatory for their assembly and/or secretion, since VLDL and chylomicrons are exclusively made in liver and intestine, the only adult tissues capable of expressing apoB (1, 2). Several years ago a number of laboratories independently reported the existence of two major size-isoforms for apoB (3-7). In humans and most other mammals, the larger (B-100, PI, or B_h) and smaller (B-48, PIII, or B_l) forms are produced almost exclusively by the adult liver and intestine, respective (8-10). However, unlike other rodents such as guinea pig and hamster (Elovson, J., and R. Kannan, unpublished results), the rat and mouse synthesize comparable amounts of both larger and smaller apoB peptides in their livers (2, 4, 6, 7, 11, 12). Whether or not there is an identity between the hepatic and intestinal forms remains to be established by direct sequencing of their respective N-terminal and C-terminal amino acids. In addition, samples of human plasma LDL may contain two other apoB peptides, referred to as B-74 and B-26 in the centile nomenclature (3, 8); however, these appear to be artifacts of kallikrein and/or thrombin cleavage of B-100 during LDL isolation (13). In contrast, rat plasma apoB differs further from that of all other species examined so far (Elovson, J., and R. Kannan, unpublished results) in that its large isoform invariably runs as a doublet on SDS-PAGE, with the slower component comigrating with human B-100. We have called the faster-migrating component rat apoB P(eptide)II, with rat PI and PIII corresponding to human B-100 and B-48, respectively, and also refer to the larger isoforms collectively as PI,II (4). PII has been referred to as B-95 by others (14); however, the rat nomenclature is retained here.

The structural and biosynthetic relationships between the various apoB peptides and their functional correlates remained unsettled even after the recent cloning and sequencing of human B-100 (15-19). Previously available evidence strongly suggested that B-48 was coextensive with the N-terminal moiety of B-100 (18, 20-22). However, as discussed below, neither posttranslational cleavage nor differential splicing of the transcript from the single apoB gene seemed able to account for B-48 synthesis. This impasse has now been resolved: Powell et al. (23)

Abbreviations: VLDL, very low density lipoproteins; LDL, low density lipoproteins; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco's minimal essential medium.

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and Chen et al. (24) have independently demonstrated a single C to U change in B-48 mRNA, which converts a glutamine₂₁₅₃ codon in the B-100 sequence into an inframe stop codon, presumably through a novel posttranscriptional mechanism. We report here some findings concerning translational and posttranslational events that produce three distinct apoB peptides in the rat. The results in both rat liver and intestine are consistent with the mechanism proposed by Powell et al. (23) and Chen et al. (24) for the generation of human and rat intestinal B-48/PIII; in contrast, they show rat PII to be a posttranslational cleavage product of rat PI. Some of these results have been previously presented in abstract form (25).

MATERIALS AND METHODS

Cloning

Rat apoB clone2, originally isolated by antibody screening of a rat liver cDNA library in lambda gt11 (21), was subcloned into the TrypE expression vector (26) and transformants were isolated by antibody screening. The larger rat apoB clone rb9e, isolated by rescreening the original lambda gt11 library with clone2, was sequenced by the dideoxy method of Sanger, Nicklen, and Coulson (27) after subcloning of restriction fragments into mp18 or -19; both universal and sequence-specific oligonucleotide primers were used. cDNA sequences were assembled and analyzed using the University of Wisconsin Genetic Computer Group software package (28, 29).

Antibodies

Rat PI,II and PIII were prepared from delipidated Triton-VLDL, by preparative SDS-PAGE as previously described (30). The TrypE-clone2 fusion protein was similarly purified from the insoluble fraction of bacterial lysates.

Rabbit antibodies raised against rat PI,II were affinitypurified on Sepharose-coupled rat PI,II. A portion of these antibodies, which recognize all three apoB peptides, was rendered specific for the epitopes present only on PI,II by exhaustive absorption with Sepharose-PIII. A second portion was rendered specific for epitopes present only on PI by absorption to and elution from Sepharoseclone2 fusion protein (see below). Similar antibodies were also raised directly against clone2 fusion protein, and were affinity purified in the same manner.

Tissue labeling experiments

Liver perfusion experiments. Rat livers were pulse-labeled by recirculating perfusion for 15 min with 1 mCi/g [³⁵S]methionine, as described elsewhere (31). In pulse/chase experiments, this was followed by single pass perfusion with DMEM supplemented with 1 mM methionine at a flow rate of 2 ml/min per g liver. At selected times, chase effluent from the liver was collected for analysis of VLDL apoB (see below). Perfusion media were continuously gassed with 95:5 O_2 - CO_2 at 37°C.

Isolated rat hepatocytes. Isolated rat hepatocytes obtained by collagenase perfusion (32) were resuspended in 10 mM HEPES in Krebs-Ringer bicarbonate and preincubated in 95:5 O_2 - CO_2 for 5 min at 37°C, using a Dubnoff shaker. Aliquots of about 10⁷ cells in 1 ml were pulsed with 0.2 mCi [³⁵S]methionine; after 20 min, cells were chased by washing once in DMEM, followed by another hour's incubation in DMEM supplemented with 1 mM methionine. Cells and medium were processed separately for analysis of intra- and extracellular VLDL apoB, as described below.

Rat intestinal slices. Slices were prepared and labeled as described (2).

Subcellular fractionation. All solutions contained 2 mM phenylmethylsulfonyl fluoride, 0.01% soybean trypsin inhibitor, and 0.001% each leupeptin, pepstatin, antipain, and chymostatin. Tissues and cells were homogenized in 10 volumes of ice-cold 0.25 M sucrose, using a Polytron operated at half-maximum speed. Total microsomes (33) and a light Golgi fraction (34) were prepared as described. Where indicated, the intracisternal contents of microsome/Golgi fractions (perfused liver) or total homogenate (isolated hepatocytes) were released by treatment with sodium carbonate (35), followed by ultracentrifugal flotation at saline density to isolate nascent VLDL particles.

Polysome-bound peptides. To obtain nascent polysomebound apoB peptides, total polysomes were isolated from methionine-labeled perfused livers and intestinal slices by the magnesium precipitation method (36). The labeled nascent chains were released by boiling in 1% SDS in 0.1 M Tris-HCl, pH 9, followed by quenching with Triton X-100 and sodium sarcosylate to final concentration of 3, 1, and 0.3% of Triton, sarcosylate, and SDS, respectively.

Polysome run-off translation in vitro

Unlabeled rat liver and intestinal polysomes (4 A_{260} units) were incubated with 70 μ Ci [³⁵S]methionine (Amersham) for 60 min at room temperature in 0.135 ml wheat germ lysate reaction mixtures, as suggested by the supplier (BRL), with addition of 0.002% RNasin, 50 KIU Trasylol, and 0.0025% each of pepstatin, leupeptin, chymostatin, and antipain.

Immunoprecipitations

All samples were made to a final concentration of 3, 1, and 0.3% of Triton X-100, sodium sarcosylate, and SDS, respectively, in the presence of protease inhibitors as above, followed by removal of insoluble residues as needed at 100,000 g, using thick-walled polycarbonate tubes in the Beckman 40.3 rotor. Lysate aliquots were treated with excess antibodies on ice for 1 hr as indicated, followed by an excess of SDS-washed *Staphylococcus aureus* cells for another 15 min. After washing, the precipitated proteins were released by boiling in 1% SDS, 50 mM Tris-sulfate, pH 6.5, 5 mM dithiothreitol, and analyzed by SDS-PAGE/fluorography (Enhance, NEN) as indicated in figure legends.

ApoB secretion in vivo

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Adult male rats received a bolus of 600 mg Triton WR-1339/kg body weight plus 1 mCi tritiated leucine through jugular cannulas. At the indicated times, 0.5-ml samples of blood were withdrawn into syringes containing 1 ml 2 mM EDTA, pH 7.4, with the above cocktail of protease inhibitors, and stored on ice until completion of the experiment. Blood cells were removed by low-speed centrifugation in the cold, and the VLDL fractions were collected by overnight centrifugation at 100,000 g in the Beckman 40.3 rotor, using thick-walled polycarbonate tubes. Aliquots containing 5 to 20 μ g apoB were electrophoresed on SDS-PAGE tube gels, and stained, scanned, sliced, and counted for ³H as described (4, 34).

RESULTS

Relationship between rat apoB PI and PII

Our original Western blot analysis of rat apoB clone2 (21), showed that its fusion protein product was recognized by antibodies specific for the C-terminal moiety of rat PI,II, which is absent from rat PIII. To confirm this assignment, the clone2 sequence was subcloned into the TrypE vector for more efficient expression. Fusion protein purified by preparative SDS-PAGE was used to raise antibodies in rabbits, and to prepare an immunoabsorbent by coupling to Sepharose. The latter was used to immunoselect those antibodies in the original antiserum against rat PI,II which recognized the clone2 epitopes. Fig. 1 shows a Western blot of standard rat VLDL apoB (prepared as described in reference 4) stained with these various antisera. All three peptides react with antibodies against rat PI,II (lane 1), while the subclass of antibodies immunoselected on clone2 fusion protein shows the expected lack of reactivity with PIII (lane 2). In addition, however, these immunoselected antibodies also fail to react with PII (lane 2). Furthermore, the antiserum raised against the fusion protein itself shows the same specificity (lane 3). Thus, clone2 encodes a region of rat PI that is absent from PII, as well as from PIII.

As mentioned above, the mobility of PII on SDS-PAGE corresponds to that of a peptide about 5% smaller than PI/B-100. Differences in carbohydrate content between PI and PII cannot be excluded, but the finding that PII also is slightly retarded compared to PI on gel filtration in 6



DIII

Fig. 1. Epitopes expressed on rat apoB clone2-lacZ fusion protein are absent from both rat apoB PII and PIII. Western blot of rat VLDL apoB stained with: antiserum against rat PI,II (lane 1); the same antibody affinity-purified on immobilized apoB clone2-lacZ fusion protein (lane 2); and antibodies raised against the apoB clone2-lacZ fusion protein (lane 3).

M guanidine hydrochloride (37) suggests that the PII peptide in fact is smaller than PI. The location of clone2 in the 3' moiety of the rat apoB cDNA sequence was therefore examined, to determine whether loss of the corresponding epitopes from PII could be accounted for by a simple truncation of PI to form PII. For this purpose the original rat liver library was rescreened with the clone2 insert, and a larger clone, rb9e, which contained 3kb of the 3'-most region of rat apoB mRNA, was selected for sequencing. As shown in **Fig. 2A**, this places the epitopes coded for by clone2 between 381 and 205 amino acid residues from the C-terminus of rat PI. Assuming PI to be the same size as human B-100, these positions correspond to the C-termini of rat B-91 and B-95, respectively, i.e., in range of the B-95 estimate for PII.

The rat 3' sequence was also compared to the human counterpart, as well as to the recently reported chicken sequence in this region (38). As shown in **Fig. 2B**, the Cterminal 230 amino acids in the rat are about 80% identical to the human sequence. However, the preceding 80 JOURNAL OF LIPID RESEARCH

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151	301	451	601	151	106	1051	1201	1351	1501	1651	1801	1951	2101	2251	2401	2551	2701	2851	1006	
r Arg Ser Ser Val Arg lev Gin Gly Ala Ser Aen Phe Ala Gly Ile Trp Aen Phe Glu Val Gly Glu Ann Phe Ala Gly Glu Ala Thr lev Arg He Tyr Gly Thr Trp Glu Ele Aen Met Ile Aen Het Aen Aet Tra Glu Fit Glu Fit Aen Het Ile Ae	T Phe Asp Thr Lys Gly Lys Gln Thr Cys Arg Ala Thr Lev Glu Lev Grz Thr Het Ser Thr Lev Lev Gln Val Ser Gln Fro Ser Fro Lev Phe Asp Eau Sile Ang Gln Glu Val Lie 6 TTT GAC AGA GAA AAA GAA AAA TOC AGA GOC ACE OTA GAG TOC ATG TOC AGE GTT CTA CAG GTT CAAT COC CAC TAT GAT CAC CAT CAC CAT GAC CAG GAA GTG AAC 301	r lys Ann Gln lys Val Ser Trp lys Ser Glu Val Glu Val Glu Ser Gln Val Lau Gln Bis Ann Ala Bis Phe Ser Ann Ang Gln Glu Glu Val Ang Law Ala Gly Ser Lau Glu Gly Gln Lau Trp Ang T And Anc Cho And Gho Mor Gho And Gho Gho Ghi Ghu San Cho Gho Gho Gho Gho Cho Gho Gho Gho Gho Gho Gho Gho Gho Gho	e leu foo als the GLY Lys set leu Arg GLU leu Leu GLU 114 Aug GLY Lys Arg GLU Tyr Leu GLA Als Ser Thr Ser leu His Tyr Thr Lys Ann Fro Ann GLY Tyr leu Leu Ber Leu Fro Val GLN GLU Leu T CTA 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r Ser Se TCT TC	GAC AC	Ann GI	Pro Al	Pro CCA GG	CTC CC	Pro GI	Bis Va CAT GT	TYF AS TAT AN	Thr Va ACT GT	Trp GU Too GN	ALA ILA GOC AT	The GL	Ser GL TCT CM	Leu Asi TTA AN	TI- Asi ATC AM	Ala Phi GCC TT	Ala Sei GCT TCC	Ala Val GCC CTC	AFG TYI	TTT CM
Thr Arg ACT CGG	TYE Phe TAC TTT	The Lys Act AAG	Phe Leu TTT CTA	II- II- ATT ATA	Ser Ser TCC TCT	Thr Leu Acc CTG	Ala Asp scA GAC	Val Lys STG AMG	IIe GLY ATA GOC	Ile Asn NTT AMC	zlu His ZAA CAC	NEG IL-	rta crc	alu Ang ang GNC	MP BIS INT CAT	tet Lys VTC AMC	Vep Phe LAT THE	ler The fcc ACT	the Leu	KEN CTT
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2	152	302	452	602	752	902	1052	1202	1352	1502	1652	1802	1952	2102	2252	2402	2552	2702	2852	3002

Fig. 2A. Nucleic acid sequence and protein translation of rat apoB clone rb9E. Translation begins at nucleotide 2 of the clone, corresponding to the first in frame triplet. The first nucleotide, G, is not shown. The 3129 bases of this clone correspond to bases 10691 to 13903 of the human apoB cDNA. Previous (21) rat clones 49a (1454 to 1683) and 2 (1825 to 2356) are underlined. The rat clone does not contain a polyadenylation signal, which is consistent with it falling short of the position of the human polyadenylation signal.

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Human Rat	1 IWNLEVKENF IWNFEVGENF	AGEATLORIY	SLWEHSTKNH GTWEHNMINH	LQLEGLFFTN LQVFSYFDTK	50 GEHTSKATLE GKQTCRATLE) LSPWOMSALV 1 LSPWIMSTLL	QVHASQPSSF QVHVSQPSPL	HDFPDLGQEV FDLHHFDQEV	Alnantknok lkastknok	10 IRWKNEVRIH 11 11 VSWKSEVQVE	0 SGSFQSQVEL SQVLQHNAHF	SNDQEKAHLD
Human Rat	121 IAGSLEGHLR IIIIII IAGSLEGQLW	FLKNIILPVY DLENFFLPAF	DKSLWDFLKL GKSLRELLQI	DVTTSIGRRQ DGKRQ	17 HLRVSTAFVY YLQASTSLHY	0 TKNPNGYSFS TKNPNGYLLS	IPVKVLADKF	ITPGLKLNDL IIPGLKLN	NSVLVMPTFH	22 VPFTDLQVPS	0 CKLDFREIQI II DFSGIKI	YKKLRTSSFA YKKLSTSPFA
Human Rat	241 LNLPTLPEVK LNLTMLPKVK	FPEVDVLTKY FPGVDLLTQY	SOPEDSLIPF SKPEGSSVPT	FEITVPESOL FETTIPBIOL	29 TVSQFTLPKS TVSQFTLPKS	90 VSDGIAALDL FPVGNTVFDL	NAVANKIADF NKLTNLIADV	ELPTIIVPEQ DLPSITLPEQ	TIEIPSIKFS TIEIPSLEFS	34 VPAGIVIPSF VPAGIFIPFF	0 QALTARFEVD GELTAHVGMA	SPVYNATWSA SPLYNVTWST
Human Rat	361 SLKNKADYVE GWKNKADHVE	TVLDSTCSST TFLDSTCSST	VQFLEYELNV LQFLEYALKV	LGTHKIEDGT VGTHRIENDK	4) LASKTKGTLA FIYKIKGTLQ	10 HRDFSAEYEE HCDFNVKYNE	DGKFEGLQEW DGIFEGLWDL	EGKAHLNIKS EGEAHLDITS	PAFTDLHLRY PALTDFHLHY	46 QKDKKGISTS KEDKTSVSAS	0 AASPAVGTVG AASPAIGTVS	Momordodfs LDASTDDOSV
Human Rat	481 KWNFYYSPQS RLHVYFRPQS	SPDKKLTIFK PPDNKLSIFK	TELRVRESDE	ETQIKVNWEE	5: EAASGLLTSL EAAFRLLDSL	30 KDNVPKATGV KSNVPKASEA	LYDYVNKYHW VYDYVKKYH.	EHTGLTLREV LGHA	SSKLRRNLON SSELRKSLON	58 NAEWVYQGAI 11 11 DAEHAI	0 RQIDDIDVRF RMVDEMNVNA	QKAASGTTGT Q
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Human Rat	721 KLIDVISMYR FKCPFSPTPC	ELLKDLSKEA KLKDVLLIFR	QEVFKAIQSL EDLNILSNLG	KTTEVLRNLQ QQDINFTTIL	7 DLLQF IFQLI SDFQSFLERL	70 EDNIKQLKEM 1 ; i LDIIEEKIEC	KFTYLINYIQ LKNNESTCVP	Deintifndy Dhinmffkth	IPYVFKLLKE IPFAFKSLRE	82 NLCLNLHKFN NIYSVFSEFN	0 EFIQNELQEA DFVQSILQEG	SQELQQIHQY 1 SYKLQQVHQY
Chicken	RGRNVLDQIK	 Emlkhloeki	ROTEVTLOEA	DFAGKLNRLK	I OVVORTFORA	GNMVRSLQSK	NFEDIKVOMO	QLYKDAMASD	i Yahklrslae	I I NVKKYISQIK	IIII NFSQKTLQKL	I III I SENLQQLVLY
Human Rat	841 IMALREEYFD I IIIII MKAFREEYFD II IIIII	PSIVGWTVKY PSVVGWTVKY 	YELEEKIVSL YEIEEKMVDL 	IKNLLVALKD IKTLLAPLRD 	8 FHSEYIVSAS 1 111 1 FYSEYSVTAA 1 11 1 11	90 NFTSQLSSQV DFASKMSTQV 	EQFLHRNIQE	YLSILTDPDG YLSMLADING 	KGREKIAELS	94 ATAQEIIKSQ IVVKERIKSW SANGEVIDVN	IO AIATKKIISD I III STAVAEITSD I SAVAWDRINE	YHQQFRYKLQ YLRQLHSKLQ
CUICKEN	961	FILGWSVKI	IEVEDKVLGL	LINNLEDILYI	WINDIAKOLS	010	REDVENINGE	TIDETIDARG	VONAVAUETS	JANYONIKIW	SUAUKUINE	THING VINING
Human Rat	DFSDQLSDYY 111111111 DFSDQLSGYY 111	EKFIAESKRL	IDLSIQNYHT IIIIIIII IDLSIQNYHM	FLIYITELLK FLRYIAELLK 	KLQSTTVMN. KLQVATANNV 	.PYMKLAPGE SPYLRFAQGE 	LTIIL* LIITF* 					
Chicken	EIYGQLSDSQ	EKLINVARML	1DLTVERYST	FMATIFELLR	WFEQATADSI	KP Y 1AVREGE	TELDALLOADE					

Fig. 2B. Comparison of C-terminal apoB amino acid sequences from rat, human, and chicken. Computer alignment was done using the University of Wisconsin Genetic Analysis software programs (28). Homologies are indicated between human and rat as well as chicken and rat sequences. There are an additional 26 identities between the chicken and human sequences which are not marked. The human and rat share the same termination codon while the chicken sequence goes beyong this termination site by 72 amino acids (remaining chicken amino acids are not shown). Note that numbering refers to residue positions compared; thus, the C-terminal phenylalanine in the rat sequence is numbered 1025, corresponding to the 989 amino acids coded for by clone rb9e plus the 36 residues worth of gaps introduced by the sequence alignment.



Fig. 2C. Kyte-Doolittle hydrophobicity plots for rat, human and chicken apoB sequences. Hydrophobic plots were first run on the entire human and rat sequences, and lined up by introducing gaps in the rat hydropathy plot to correspond to gaps shown in the human:rat sequence comparison (see Fig. 2B).

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amino acids show only about 10% identity, and contain 4 cysteines without human counterparts. The remaining 650 amino acids towards the N-terminus are about 70% identical to the human sequence, not counting a 25 amino acid deletion in the rat. Altogether, including conservative substitutions, and excluding the deletions and the highly divergent region, the rat sequence shows about 80% homology with the human, but retains only 1 of 4 human cysteines and introduces 6 new ones. Specifically, the overall picture is one of near-perfect retention of the amphipathic motifs throughout, as shown by the Kyte-Doolittle plots (**Fig. 2C**). Although lower overall, the degree of homology between the rat and chicken sequences is very similar to that between rat and human (Fig. 2B), as is the overall amphipathic motif (Fig. 2C).

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Loss of a C-terminal region from rat PII could occur either at the nucleotide or protein level, most likely by alternative splicing or proteolysis, respectively. To distinguish between these alternatives, the labeling of three rat apoB peptides in rat liver was next examined. **Fig. 3A**, left panel, shows the results of immunoprecipitations from a lysate of total liver homogenate after a 15-min perfusion with [³⁵S]methionine. As expected from their specificity (Fig. 1), antibodies against clone2 fusion proteins precipitate only labeled PI (lane 2). However, although antibodies against PI,II are capable of reacting with all three rat peptides (Fig. 1, lane 1), the corresponding immunoprecipitate contains only labeled PI and PIII, but no labeled PII (Fig. 3A, lane 1). Thus, little or no PII was synthesized during the 15-min pulse. This would immedi-



Fig. 3. Biosynthesis of rat apoB PII. A: PII production by perfused rat liver and isolated hepatocytes. SDS-PAGE fluorograms of immunoprecipitated apoB peptides separated on 5% acrylamide SDS minigels. Perfused livers (left panel) were pulsed by recirculation with [³⁵S]methionine for 15 min and one such liver was used to prepare the homogenate sample for lanes 1 and 2. A second liver was chased by single-pass perfusion with unlabeled medium for another 30 min, with collection of perfusate samples for lanes 3 and 4 during the final 5 min; this liver was then used to prepare the sample of Golgi VLDL for lane 5. Isolated hepatocytes (right panel) were pulsed for 2 hr with [³⁵S]methionine. Medium and cells were processed as described in Materials and Methods to analyze labeled apoB peptides on nascent VLDL particles. Lane 6: medium VLDL. Lane 7: cellular VLDL. Immunoprecipitations were performed with affinity-purified antibodies against rat apoB (lanes 1, 3, 5, 6, 7) and rat apoB clone2-lacZ fusion protein (lanes 2 and 4). B: ApoB PI and PII accumulate in parallel in plasma VLDL of Triton-treated rats, without further processing in the circulation. Closed symbols: PI; open symbols: PII. Abscissa: time after intravenous injection of Triton WR-1339. Ordinate: μg VLDL PI and PII per ml blood.



ately exclude synthesis of PII from a separate mRNA; rather, it suggests that PII is formed by late posttranslational processing of PI. This was confirmed by analyzing the labeled apoB peptides of nascent VLDL in the Golgi fraction isolated from a perfused liver after a 15-min pulse followed by a 30-min chase period. As seen in Fig. 3A, middle panel, apoB secreted into the single-pass perfusate during the last 5 min of the chase contains labeled PII as well as PI and PIII (lane 3); however, the Golgi VLDL contains only the latter two (lane 5). Thus, it appears that processing of PI to PII occurs after passage of PI-containing VLDL through the Golgi secretory vesicles, i.e., during or after their externalization from the hepatocytes. The actual locus and enzymatic machinery responsible for processing is unknown. However, it does not appear to require the presence of either liver Kupffer or endothelial cells, since processing also is performed by isolated hepatocytes (Fig. 3A, lanes 6, 7). Nor does it require contact with serum or plasma, since it occurs during single-pass perfusion with serum-free perfusate. Furthermore, as shown in Fig. 3B, when the removal of newly secreted rat VLDL is blocked with Triton WR-1339 in vivo PI- and PII-containing VLDL accumulate in parallel in plasma for at least 2 to 3 hr. It is interesting to note that conversion of PI to PII is greater in fasted as compared to fed animals (Fig. 3B); however, in neither case does processing continue once the particles have been secreted. In keeping with this, the [³H]leucine specific activities of the two peptides are also equal at each timepoint (data not shown).

Relationship between rat apoB PI and PIII

As mentioned above, the rat is unusual, in that a PIII/B-48-sized protein is produced in the liver as well as in the intestine. In this regard we (2) and others (39) have shown that intracellular apoB of amino acid-labeled adult rat intestine contains only labeled PIII, while that of rat liver contains both PI and PIII (2, and Fig. 3A, above). We first approached the question of whether the same mechanism was responsible for PIII formation in the two tissues by analyzing labeled apoB peptides produced by polysome run-off translation in vitro. As seen in Fig. 4, the total apoB products from liver and intestinal polysomes show qualitatively very similar patterns of peptides smaller than PIII. We interpret the ladders of discrete bands as arising by stepwise, partial elongations of polysome nascent chains, caused by secondary structures in the apoB mRNA. Note, however, that intestine shows no product larger than PIII, while the liver pattern extends up to the position for full-length PI. This is clearly inconsistent with posttranslational cleavage of PI to form PIII, and instead suggests that PIII is translated from a separate mRNA, which terminates elongation of nascent apoB peptides once they reach PIII size. Conversely, in the liver, but not in the intestine, a separate message codes only for full-length PI/B-100.

It may be noted in Fig. 4 that the PI,II-specific antibodies (lane 4) only precipitate apoB products larger than PIII, as expected from the fact that PIII corresponds to the N-terminal moiety of PI. Thus, only nascent peptides larger than PIII will express PI-specific epitopes.

The patterns of newly synthesized and nascent polysome-bound apoB peptides were also examined after [³⁵S]methionine labeling of intact hepatocytes and intestinal slices in culture. This experiment incorporates mixed extract controls to exclude the possibility that the absence of intestinal apoB peptides larger than PIII is an artifact of high proteolytic activity even in the presence of protease inhibitors. **Fig. 5** demonstrates that this is not the case, since mixing the labeled hepatocytes with an equal weight of unlabeled intestinal slices prior to homogenization does not reduce the ratio of labeled PI to PIII in the



Fig. 4. Run-off translations of rat liver and intestinal polysomes in wheat germ lysates. Translation products were immunoprecipitated and analyzed by SDS-PAGE/fluorography on 3 to 15% gradient gels. Lanes 1 and 2 show liver and intestinal products, respectively, immunoprecipitated with antibodies against rat PI,II. In a separate experiment liver products were again immunoprecipitated with antibodies against rat PI,II is a separate experiment liver products were again immunoprecipitated with antibodies against rat PI,II (lane 3), or with the same antibodies rendered specific for PI,II by passage over a PIII-Sepharose column (lane 4). Note that the intestinal polysomes produce no peptides larger than PIII (lane 2). Also note that PI,II-specific antibodies only recognize epitopes on peptides larger than PIII (lane 4), consistent with the fact that PIII constitutes the amino terminal half of PI.

INTESTINE

2

3



Fig. 5. Absence of detectable newly synthesized apoB PI in rat intestine is not due to artifactural proteolytic degradation. Samples of [³⁵S]methionine-labeled rat hepatocytes, either alone (panel A) or mixed with unlabeled intestinal rings (panel B), as well as a sample of [35S]methionine-labeled rat intestinal rings mixed with unlabeled rat hepatocytes (panel C), were identically lysed and prepared for immunoprecipitation as described in Materials and Methods, using nonimmune serum (lanes 1), antibodies against rat PI,II (lanes 2), and PI-specific antibodies raised against rat apoB clone2 fusion protein (lanes 3).

polysome-free lysate immunoprecipitate (panels A and B). Similarly, mixing the labeled intestinal slices with an equal weight of unlabeled hepatocytes prior to homogenization does not cause the appearance of labeled PI in the immunoprecipitate from the polysome-free lysate (panel C). It appears, therefore, that neither the action of intestinal proteases nor of hepatic protease inhibitors can account for the presence of PI in liver, but not in intestine. Furthermore, when isolated from such mixed homogenates, only liver polysomes contain nascent labeled PI peptides (Fig. 6). Thus, synthesis of intestinal PIII proceeds by direct release of nascent apoB peptides at the appropriate point of elongation, as expected for polysomes containing a PIII-specific mRNA. Furthermore, PIIIsized peptides appear to be released from the rat liver polysomes, consistent with the presence of two hepatic apoB mRNA populations, one coding for PI and the other for PIII. Thus, this mechanism can also account for PIII synthesis in rat liver.

DISCUSSION

ApoB is the largest mammalian peptide characterized to date; however, very little is known about the relationships between its functions and primary structure, including the reason for the existence of the two major sizeisoforms. It is generally agreed that B-100, but not B-48, binds to the LDL receptor (40), and sequence homologies to apoE as well as differential effects of monoclonal antibodies (15, 16, 20) strongly suggest that the receptor binding site in fact is located to the C-terminal half of B-100, which is absent from B-48. In regard to the other major function for apoB, we have recently shown that PIIIcontaining rat VLDL carry a single PIII peptide per particle (30). Thus, a single copy of the N-terminal half of the PI/B-100 sequence appears to contain all the structural information required for the assembly of a rat VLDL particle, and presumably for rat and human chylomicrons as well.

The significance of the PII isoform is obscure, and appears to be unrelated to either of the above apoB functions, since so far it has only been observed in the rat (Elovson, J., and R. Kannan, unpublished results). The actual C-terminal truncation involved in its formation is

LIVER

2

1

PIII-

3



-PI

PIII-

[³⁵S]methionine-labeled polysome-bound apoB peptides from rat hepatocytes (left panel) or intestinal rings (right panel) were immunoprecipitated as described in Materials and Methods. Antibodies in lanes 1, 2, and 3 as in Fig. 5.

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unknown. The clone2 sequence, which codes for epitopes missing from PII, lies between the C-termini of the equivalents of B-91 and B-95, suggesting either that "B-95" (14) is an SDS-PAGE overestimate of the actual size of PII, or that the missing epitopes all are clustered at the C-terminus of clone2. The latter contains the above mentioned region which has diverged greatly between rat, human, and chicken (Fig. 2A). Therefore it is quite possible, although not yet determined, that the rabbit sequence is also not conserved in this region, suggesting that this particular rat sequence may in fact be a better than average immunogen in the rabbit. The size consideration also argues against substantial additional proteolytic processing at the N-terminus; however, the precise portion of PI retained in PII can only be determined by direct N- and C-terminal amino acid sequencing of the latter, an impractical proposal at this time. In any event, it is interesting that Windmueller and Spaeth (14) found that PII-containing rat LDL were removed somewhat more slowly from plasma than were those which contain PI; the relationship, if any, of this finding to the fact that the C-terminal sequence lost from PII is about 1000 residues away from the proposed LDL-receptor binding site, is unknown.

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The precise locus and mechanism of PII formation is unknown. However, we have shown that PII is absent from a Golgi secretory fraction at the time when it is found in a single-pass liver perfusate, that it is produced by isolated hepatocytes, and that no measurable further conversion occurs in the plasma of Triton WR-1339treated rats. Taken together, these findings point to a single-hit mechanism, with a certain one-time probability for a nascent VLDL particle to have its PI peptide processed to PII at the time of externalization from the hepatocyte. In all these regards the PII processing appears to be completely unrelated to the removal of the short Nterminal peptides of apolipoproteins A-I and A-II (41, and references therein), which, although relatively slow, progresses essentially to completion and occurs almost entirely after secretion into the plasma. In contrast, PII is formed by one-time removal of at least a 20 kDa Cterminal peptide from at most about one-half of PI at the time of secretion from the hepatocyte, with no further conversion in plasma.

The mechanism responsible for the formation of the two major size-isoforms of apoB has long been elusive. Our initial chymotryptic fingerprints of the rat peptides suggested that PIII could be very similar or perhaps even identical to a major portion of PI,II (4). Although supported by the specificities of monoclonal antibodies for B-100 and B-48 (42), progress in this area was first slowed by the lack of sequence information for the two entities. However, even successful cloning of rat and human apoB, with determination of the full cDNA sequence of the latter (15-19), left this issue unresolved until very recently. Thus, on the one hand, the major component of intestinal apoB mRNA was found to have the same size as the 14 kb liver B-100 mRNA (9, 21, 43). However, while this suggested posttranslational processing of B-100 to B-48, pulse chase experiments and our polysome run-off translations argued against this mechanism (2, 9, 25). On the other hand, reports of the presence of smaller and variable amounts of a shorter intestinal B-48 message (19, 43, 44) were difficult to evaluate, since the sequence of the single apoB gene (45) did not provide for the alternative splicing needed for its synthesis. This impasse has recently been resolved by the finding (23, 24) of a single C to U change in B-48 mRNA, which converts a glutamine₂₁₅₃ codon in the B-100 sequence into an in-frame stop codon, presumably through a novel posttranscriptional mechanism. It is shown here that rat intestinal polysomes contain no peptides larger than PIII/B-48, while liver polysomes contain nascent apoB peptides of all lengths up to full-length PI/B-100, and that this difference does not reflect greater proteolytic degradation in the intestinal sample. These results are incompatible with posttranslational cleavage of PI/B-100 to form PII/B-48, and strongly suggest that the two apoB peptides are the products of separate messages. Thus, they provide independent support at the translational level for the unprecedented mechanism proposed by Powell et al. (23) and Chen et al. (24). How this potentially lethal mechanism may have evolved, and how B-48/PIII-producing cells are able to control it, is completely unknown at this time.

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