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Membrane-bound apolipoprotein B is exposed at the cytosolic surface of liver microsomes

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We have used a competitive enzyme-linked immunoassay with a panel of monoclonal antibodies to probe the topography of the membrane-bound form of apolipoprotein B (apo B) in rabbit microsomes. All epitopes investigated were found to be expressed at the cytosolic side of the microsomal membrane under conditions in which the vesicles remained sealed. These results indicate that the membrane-associated form of apolipoprotein B is either at the cytosolic side of the endoplasmic reticulum membrane or integrated into the membrane. From this site apo B may be translocated to the lumen for assembly into VLDL or may be degraded.

Apolipoprotein B; Rabbit; Liver; Endoplasmic reticulum membrane; Topography; Monoclonal antibody

1. INTRODUCTION

Apolipoprotein B (apo B) is essential for the secretion of very low density lipoproteins (VLDL) from liver and plays an important role in determining the plasma cholesterol levels. Translocation of apo B across the endoplasmic reticulum membrane and its assembly with lipid is of special interest as this protein is very large (512 kDa) and hydrophobic. Moreover, apo B does not behave as a typical secreted protein but exists in membrane-bound and cisternal forms in both the endoplasmic reticulum and the Golgi region [1–8]. Studies of HEP-G2 cells have suggested that the membrane-bound form of apo B is a precursor of the secreted form [9–11]. In this investigation we have used a panel of monoclonal antibodies against rabbit apo B to probe the topography of membrane-bound apo B in rabbit microsomal membranes.

2. MATERIALS AND METHODS

2.1. *Animals*

Dwarf lop-ear or small New Zealand white rabbits (approximately 2 kg) were used for all studies.

2.2. *Antibodies*

Monoclonal antibodies specific for rabbit apo-B (MAC 22, 27, 28, 29 and 31) and sheep anti-rabbit apo B antiserum were prepared as described previously [13].

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2.3. *Subcellular fractions*

Total microsomes (endoplasmic reticulum) were prepared as described previously for rat liver [14] with the addition of a cocktail of protease inhibitors to the homogenate [6]. In some experiments the secretory proteins in the lumen were labelled by injection of [^{3}H]leucine (100 μCi) into the portal vein 15–30 min before removal of the liver [15].

2.4. *ELISA*

LDL prepared from rabbit serum (d 1.019-d 1.063 fraction–0.53 μg apo B per well) were placed in ELISA plates (Nunc) which were sealed with clingfilm and refrigerated overnight. The wells were washed four times with TBS (50 mM Tris, 200 mM NaCl, pH 7.4) containing 0.05% (v/v) Tween 20 and 0.5% (w/v) RIA grade bovine serum albumin (BSA) and blocked for 2 h with TBS containing 3% BSA. The wells were aspirated and 100 μl of antibody (dilutions up to 1:200,000) in TBS containing 1% BSA were added together with 50 μl of buffer or 50 μl of LDL (0.1–200 μg apo B/ml) or 50 μl of microsomes and incubated overnight with shaking. The wells were washed four times as above and 150 μl of secondary antibody (goat anti-rat IgG coupled to alkaline phosphatase (Sigma) for monoclonals or donkey anti-sheep coupled to alkaline phosphatase for polyclonals (Sigma)) diluted 1:1000 in TBS containing 1% BSA were added. Plates were incubated for 4 h and washed four times as above followed by a wash with 0.1 M glycine buffer, pH 10.4, containing 0.001 M $MgCl_2$ and ZnCl. Alkaline phosphatase bound was measured using *p*-nitrophenyl-phosphate (Sigma); terminated by addition of 50 μl of 0.1 M EDTA after 30 min, and read at 405 nm in an LKB plate-reader. In some experiments 0.4% taurocholate was added to open the microsomal vesicles [16].

2.5. *Integrity of microsomal vesicles*

Integrity was determined by (i) measurement of the leakage of radiolabelled secretory proteins during ELISA assays, and (ii) by determination of the availability of albumin, a lumen protein, to anti-albumin antibody in the incubation medium. For (i), after incubation, the microsomes from triplicate wells were removed, diluted to 10 ml with 0.25 M sucrose and centrifuged at 105,000 × *g* for 45 min. Aliquots of the supernatants and the resuspended microsomes were precipitated by addition of an equal volume of 15% trichloracetic acid.

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The denatured protein was isolated by centrifugation, solubilised in Beckman tissue solubiliser and counted. For (ii), competition curves with and without taurocholate were prepared for albumin as above except that crystalline human albumin (fraction V, Sigma) replaced LDL; gelatin replaced BSA in the blocking and wash solutions; and the secondary antibody was goat anti-human albumin (Sigma). In preliminary experiments it was demonstrated by immunoblotting that the anti-human albumin antibody cross-reacts with rabbit serum albumin.

3. RESULTS AND DISCUSSION

The ability of LDL-apo B to compete with a fixed amount of immobilised LDL-apo B varied for each monoclonal antibody. 50% inhibition of antibody binding to the immobilised LDL occurred at 1.58 μg apo B/well for MAC 22, 0.63 μg apo B/well for MAC 27, 0.035 μg apo B/well for MAC 28, 0.99 μg apo B/well for MAC 29, and 0.25 μg apo B/well for MAC 31 compared with 0.63 μg apo B/well for sheep anti-rabbit apo B antiserum. When the assays were performed in the presence of taurocholate the competition curve obtained for each antibody was unchanged.

The expression of epitopes of apo B at the surface of microsomal vesicles was assessed by determining their ability to compete with immobilised LDL-apo B for the range of monoclonal antibodies (Table I). The apo B content of the microsomes was calculated from RIA [7,8] and ELISA using sheep anti-rabbit apo B antiserum and the distribution of apo B between the membrane and cisternal contents determined by RIA [7,8].

Table I

Expression of epitopes of apo B in microsomal vesicles compared with LDL-apo B

Monoclonal antibody	% Inhibition	% Expected inhibition[a]	
Unopened microsomes			
MAC 22	47.03 ± 5.39 (9)	43.2	$P > 0.05$
MAC 27	64.43 ± 3.45 (8)	78.4	$P < 0.002$
MAC 28	57.53 ± 2.38 (3)	92.3	$P < 0.002$
MAC 29	52.80 ± 6.02 (9)	73.4	$P < 0.002$
MAC 31	56.37 ± 2.95 (3)	81.3	$P < 0.002$
Opened vesicles[b]			
MAC 22	82.67 ± 3.6 (3)	80.1	$P > 0.1$
MAC 27	90.43 ± 6.43 (3)	94.3	$P > 0.1$
MAC 28	82.25 ± 5.26 (5)	96.3	$P < 0.01$
MAC 29	81.55 ± 0.62 (3)	88.6	$P > 0.05$
MAC 31	94.00 ± 0.62 (3)	96.6	$P > 0.1$

Results are expressed as mean ± SD (number of observations).

[a] The expected inhibition was determined from the standard curve for LDL performed simultaneously. The % expected inhibition for unopened vesicles is calculated from the apo B of the membrane fraction and that for opened vesicles from the total apo B of the microsomal fraction.

[b] Microsomes were opened by addition of 0.4% taurocholate [16] to the incubation. The LDL competition curve was performed simultaneously in the presence of the same detergent.

From these values we calculated the % inhibition which would be expected if all of the membrane-bound pool of apo B is exposed at the vesicle surface in a similar way to that in LDL. This was compared with the actual % inhibition by the subcellular fracations. Closed microsomal vesicles competed with immobilised LDL for all monoclonal antibodies. However, only the epitope recognized by MAC 22 competed on an equal basis with LDL apo B, while those for MAC 27, 28, 29 and 31 were less effective competitors indicating that these epitopes in membrane-bound apo B are partly shielded compared with LDL apo B. When the microsomal vesicles were opened with taurocholate to expose both the cisternal surface of the membrane and apo B in the cisternal contents the % inhibition of antibody binding was similar to that which would be expected if the total apo B of the microsomes behaved as LDL-apo B (Table I).

Two control experiments indicated that microsomal vesicles remained sealed during the ELISA (Table II). It was demonstrated that there was no loss of radiolabelled protein over the incubated control into the medium during ELISA. Furthermore albumin, a secretory protein in the microsomal vesicles, could not be detected by ELISA using an anti-albumin antibody unless the vesicles were opened with taurocholate.

The epitope for MAC 31 is in the N-terminal 48% (recognises apo B48); that for MAC 22 is in the N-terminal 48–74% (recognises apo B74) and those for MAC 27, 28 and 29 are located in the C-terminal 26%

Table II

Investigation of the integrity of microsomal vesicles during ELISA

(A) Leakage of [^{3}H]leucine labelled secretory proteins

Treatment before centrifugation	% loss of labelled protein into supernatant
Untreated microsomes held on ice in 0.25 M sucrose	11.10 ± 3.86
Microsomes incubated on ELISA plates with TBS-BSA	13.70 ± 2.88
Microsomes incubated on ELISA plates in complete assay medium	14.44 ± 4.36
Microsomes incubated on ELISA plates in complete assay medium plus 0.4% taurocholate	76.80 ± 1.56

Values given are the average of four separate assays ± SD.

(B) Availability of albumin in microsomes for probing by anti-albumin

	% Inhibition	Albumin detected (ng/mg microsomal protein)
No competing albumin	0%	0
Microsomes	0%	0
Microsomes + 0.4% taurocholate	50–60%	15.49 ± 3.55

Values are the average of 8 experiments performed in duplicate.

(recognises apo B100) [13]. Our observations therefore suggest that multiple regions throughout the length of the apo B molecule are exposed at the cytosolic surface of the microsomal membrane. We have not directly addressed the question whether apo B has transmembrane domains although our results are consistent with this possibility. Apo B lacks hydrophobic regions of sufficient length to provide conventional transmembrane α-helical domains. However, recent studies of bacterial porins have demonstrated that shorter hydrophobic β-sheets, which are found in apo B, may form transbilayer domains in membrane proteins [17].

Kinetic studies in HEP-G2 cells have indicated that the membrane-bound apo B is a precursor of the cisternal apo B, which is packaged with lipid for secretion [9,10] and that apo B which is not assembled with lipid is degraded [11]. The possibility that apo B is inserted into the endoplasmic reticulum is of interest in view of the recent observations that charged amino acids in transmembrane regions may be the structural motif necessary for targetting membrane-bound proteins for degradation [18]. Similar mechanisms may be involved in the degradation of membrane-bound apo B in liver cells.

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REFERENCES

[1] Davis, R.A., Thrift, R.N., Wu, C.C. and Howell, K.E. (1990) J. Biol. Chem. 265, 10005–10011.
[2] Bamberger, M.J. and Lane, M.D. (1988) J. Biol. Chem. 263, 11868–11876.
[3] Borchardt, R.A. and Davis, R.A. (1987) J. Biol. Chem. 262, 16394–16402.
[4] Wong, L. and Pino (1987) Eur. J. Biochem. 164, 357–367.
[5] Cartwright, I.J. and Higgins, J.A. (1990) Biochem. Soc. Trans. 18, 1176–1177.
[6] Cartwright, I.J. and Higgins, J.A. (1992) Biochem. J. (in press).
[7] Wilkinson, J., Higgins, J.A., Groot, P.H.E., Gherardi, E. and Bowyer, D.E. (1990) Biochem. Soc. Trans. 18, 1181.
[8] Wilkinson, J., Higgins, J.A., Groot, P.H.E., Gherardi, E. and Bowyer, D.E. (1992) (submitted).
[9] Bostrom, E., Boren, J., Wettesten, M., Sjoberg, A., Bondjers, G., Wiklund, O., Carlsson, P. and Olofsson, S.-O. (1988) J. Biol. Chem. 263, 4434–4442.
[10] Boren, J., Wettesten, M., Sjoberg, A., Thorlin, T., Bondjers, G., Wiklund, O. and Olofsson, S.-O. (1990) J. Biol. Chem. 265, 10556–10564.
[11] Dixon, J.L., Furukawa, S. and Ginsberg, H.N. (1991) J. Biol. Chem. 266, 5080–5086.
[12] Sato, R., Imanaka, T., Takastuki, A. and Takano, T. (1990) J. Biol. Chem. 265, 11880–11884.
[13] Gherardi, E., Hutchings, A., Galfre, G. and Bowyer, D.E. (1988) Biochem. J. 252, 237–245.
[14] Higgins, J.A. and Fieldsend, J.K. (1987) J. Lipid Res. 28, 268–278.
[15] Kreibich, G., Debey, P. and Sabatinni, D.D. (1973) J. Cell Biol. 58, 436–462.
[16] Arion, W.J., Ballas, L.M., Lange, A.J. and Wallin, B.K. (1976) J. Biol. Chem. 251, 4901–4907.
[17] Weiss, M.S., Weckesser, A.J., Welte, W., Schilz, E. and Schulz, G.E. (1991) Science 254, 1627–1630.
[18] Bonifacio, J.S. and Lippincott-Schwartz, J. (1991) Current Opinion in Cell Biology 3, 592–600.