Intracellular degradation in the regulation of secretion of apolipoprotein B-100 by rabbit hepatocytes

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Isolated rabbit hepatocytes were incubated with [^{35}S]methionine to label intracellular pools of apolipoprotein B (apo-B). The cells were then reincubated with an excess of unlabelled methionine in the presence of oleate or protease inhibitors and the intracellular sites of accumulation of radiolabelled apo-B and the mass of apo-B were determined by isolation and analysis of subcellular fractions. Oleate or inhibitors of metalloproteases (*o*-phenanthroline), serine proteases (aprotinin), serine/cysteine proteases (leupeptin) or cysteine proteases (calpain inhibitor I; ALLN) but not aspartate proteases (pepstatin) resulted in inhibition of the cellular degradation of apo-B. The effect of *o*-phenanthroline was reversed by the addition of zinc ions. Oleate, *o*-phenanthroline and leupeptin also stimulated secretion of radiolabelled apo-B; the effects of the inhibitors and oleate were additive, suggesting that they could act via different mechanisms. *o*-Phenanthroline caused accumulation of apo-B in the rough endoplasmic reticulum (RER) and smooth endoplasmic reticulum (SER) membranes; leupeptin caused accumulation of apo-B in the SER and *cis*-Golgi membranes, and ALLN and aprotinin caused accumulation of apo-B in the *trans*-Golgi membranes. These results suggest that intracellular degradation of apo-B occurs in the endoplasmic reticulum and in the *trans*-Golgi membranes and involves different proteases. Apo-B that accumulates in the ER membrane can be diverted into the lumen for secretion; however, apo-B that accumulates in the *trans*-Golgi membrane is irretrievably diverted from secretion.

INTRODUCTION

Apolipoprotein B100 (apo-B) is the major and essential apolipoprotein of very-low-density lipoproteins (VLDL), the vehicle of transport of endogenous lipid from the liver. Increased rates of secretion of VLDL from the liver are associated with hyperlipaemia. It is therefore important to elucidate the mechanisms involved in regulation of the synthesis, intracellular transit and assembly of apo-B and VLDL. Rapid changes in the secretion of apo-B-VLDL are not accompanied by changes in the mRNA for apo-B and it is generally considered that the acute regulation of apo-B secretion is at the post-translational level [1–3]. A number of studies of rat hepatocytes, rabbit hepatocytes and hepatoma cell lines have indicated that apo-B is synthesized in excess over that secreted and that the excess protein is degraded intracellularly [4–15]. This has led to the idea that intracellular proteolytic digestion of apo-B may be one mechanism by which VLDL secretion is regulated. Thus, degradation of apo-B could be an appropriate target for therapeutic regulation of VLDL secretion. To exploit this, however, it is necessary to determine the intracellular sites of degradation, the nature of the proteases involved and the way in which degradation of apo-B is linked to assembly and secretion of VLDL.

Most studies of intracellular degradation of apo-B have used Hep-G2 cells as a model system [5,6,10–15]. A common protocol has been one in which the cells are pulse-labelled with [^{35}S]methionine, chased in the presence of excess unlabelled methionine, the apo-B is immunoprecipitated, separated by SDS/PAGE and the decrease in the radioactivity in apo-B estimated by counting or fluorography. The loss of radioactivity from apo-B is then used as an indicator of intracellular proteolysis. Although such studies have provided valuable insights into the characteristics of the proteolytic process, the total cellular apo-B is pooled in the procedures used and the mass of apo-B has not been measured. Moreover, although these cells synthesize and secrete apo-B, this is in particles with a low triacylglycerol content that have the density of low-density lipoproteins [15–17]. Recent studies by Gibbons et al. [18] suggest that Hep-G2 cells lack the ability to mobilize and secrete cytosolic triacylglycerol, which in rat hepatocytes is the direct source of VLDL triacylglycerol. It has also been suggested that VLDL assembly is a two-step process the second step of which, fusion with triacylglycerol-rich particles, is absent from Hep-G2 [19]. As our investigations are intended to elucidate events in normal liver with a view to extrapolation to human we have performed our studies on adult rabbit hepatocytes. We have previously shown that freshly isolated rabbit hepatocytes secrete apo-B100, dependent on added oleate, at a rate of approx. 1 μg/h per mg of cell protein [20]. This compares favourably with the secretion of apo-B by Hep-G2 cells or rat hepatocytes in culture [1,9].

Our studies have shown that in rabbit liver apo-B exists in both membrane-bound and luminal pools in the endoplasmic reticulum (ER) and the Golgi [20–25]. The membrane-bound form of apo-B in the rough ER (RER) and smooth ER (SERR) is in at least two pools, which are differentially available to monoclonal antibody probes; one at the luminal surface, which is masked in closed vesicles and exposed in open vesicles, and a second pool exposed at the cytosolic side of the membrane. In the Golgi, all of the membrane-bound form of apo-B is exposed at the cytosolic side of the membrane [22–24]. Investigations of the kinetics of transit of apo-B have shown that in isolated rat or rabbit hepatocytes incubated with [^{35}S]methionine the apo-B pools in RER, SER, *cis*-Golgi and *trans*-Golgi membranes and luminal contents are not uniformly labelled and turn over at

Abbreviations used: ALLN, calpain inhibitor I; apo-B, apolipoprotein B; ER, endoplasmic reticulum; RER, rough ER; SER, smooth ER; VLDL, very-low-density lipoproteins.

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different rates [20,21]. Newly synthesized apo-B is preferentially retained in the RER membrane and is preferentially transferred to the lumen. The main site of degradation of newly synthesized apo-B is the RER. However, apo-B also moves to the *trans*-Golgi membrane and is subsequently degraded: this pool turns over more slowly than that in the ER.

In the present study we investigated the nature of the proteases involved in degradation of apo-B and the intracellular sites of its accumulation in hepatocytes, in which degradation is inhibited. Freshly isolated rabbit hepatocytes were incubated with [^{35}S]methionine to label the intracellular apo-B. The cells were then isolated by centrifugation and reincubated in the presence of excess unlabelled methionine in the presence and absence of different protease inhibitors to block degradation of apo-B. Subcellular fractions were prepared at intervals during the reincubation step and their content of both newly synthesized (radiolabelled) and total apo-B determined. Our observations suggest that different proteases acting in the ER and the Golgi may be involved in degradation and that apo-B protected from degradation in the ER is apparently available for secretion, whereas apo-B in the *trans*-Golgi is committed to the degradation pathway.

METHODS

Materials

Proteolytic inhibitors, *o*-phenanthroline, leupeptin, aprotinin and pepstatin were purchased from Sigma, and ALLN (calpain inhibitor I) from Calbiochem. PHM Liposorb was from Calbiochem. All other materials and radiolabelled chemicals were as described previously [20–25].

Hepatocyte and subcellular organelle preparation

Rabbit hepatocytes were isolated and characterized by using methods previously described [20] and subcellular fractions (RER, SER, *cis*-Golgi and *trans*-Golgi) were prepared from hepatocytes and characterized as described previously [20,21]. Membrane and content subfractions were prepared from the intact subcellular fractions by suspension (less than 1 mg/ml protein) in sodium carbonate (100 mM) followed by centrifugation as described previously [26].

Incubation of hepatocytes with [^{35}S]methionine

Hepatocytes were incubated as described previously [20,21]. Briefly, freshly isolated rabbit hepatocytes were resuspended (50 %, w/v) in modified methionine-free Eagles' minimum essential medium, which had been gassed with O_2/CO_2 (19:1). Hepatocytes (1 or 2 g) were incubated in 50 ml siliconized flasks, which were gassed at intervals before and during the incubation period. [^{35}S]Methionine (approx. 70–150 μCi) was added and the flasks were incubated for 30 min. The cells were isolated by centrifugation at 1000 ***g*** for 2 min at ambient temperature, suspended (50 %, w/v) in minimum essential medium containing 45 mM unlabelled methionine and reincubated for up to 180 min. Depending on the experiment, albumin (fatty acid-free), oleate [bound to 1 % (w/v) fatty acid-free albumin], or protease inhibitors were added at the beginning of the reincubation period to give final concentrations as follows: fatty acid-free albumin (1 mg/ml), oleate (1 mM), aprotinin (2 μg/ml), ALLN (42 μg/ml), pepstatin (0.68 μg/ml), leupeptin (24 μg/ml), *o*-phenanthroline (0.2 mg/ml). After incubation for the selected time, the hepatocytes were isolated by centrifugation at 1000 ***g*** for 5 min at ambient temperature. In some experiments, cells and supernatants (media) were immediately processed for analysis of apo-B, otherwise subcellular fractions were prepared from the cell pellets [20,21]. As we have found that preparation of all subcellular fractions from a single homogenate does not yield satisfactory results, RER and SER or *cis*-Golgi and *trans*-Golgi were prepared from duplicate incubations of hepatocytes at each time point investigated. The viability of the hepatocytes was checked by Trypan Blue exclusion and also by the ability of the cells to incorporate [^{35}S]methionine into total and secreted proteins [21].

Time-course experiments involving subcellular fractionation required centrifugation of a large number of samples, so it was not possible to perform sufficient repeated individual time points for statistical analysis. In repeated experiments the radiolabel added, but not the amount of methionine, varied owing to decay. Therefore results of single experiments are presented; however, the patterns of incorporation of radiolabel were similar in repeated experiments.

Determination of secretion of radiolabelled apo-B

The incubation media were centrifuged at 17500 ***g*** for 30 min at 4 °C to remove any cell debris and the secreted lipoproteins in the total incubation media were adsorbed onto PHM-L Liposorb (10 mg). Preliminary experiments established that this resulted in complete removal of apo-B from the media as detected by SDS/PAGE and immunoblotting. The Liposorb was isolated by centrifugation, the proteins were solubilized in sample buffer, separated by SDS/PAGE and the apo-B-containing band was excised and the radioactivity determined as described previously [20,21,25].

Determination of radiolabelled apo-B in hepatocytes and subcellular fractions

In some experiments the rate of degradation of apo-B in unfractionated hepatocytes was investigated. The hepatocyte pellets were resuspended and protein was determined. Aliquots equivalent to 1 mg of protein were isolated by centrifugation, dissolved in sample buffer and separated by SDS/PAGE. The apo-B-containing bands were excised, solubilized and counted as described previously [20,21,25]. In other experiments subcellular fractions were prepared from hepatocytes. The radioactivity in apo-B in each fraction was determined after SDS/PAGE. In most experiments the amount of apo-B was also determined in parallel SDS/PAGE gels by quantitative immunoblotting as described previously [25]. For this assay, a standard curve of apo-B prepared from low-density lipoproteins was applied to each gel with the unknown samples. After SDS/PAGE the proteins were electrotransferred to nitrocellulose and stained with Amido Black to locate the apo-B-containing bands by comparison with the standards. The membrane was destained, blocked and incubated sequentially with the primary antibody, secondary antibody and ^{125}I-labelled Protein A with washes between steps [25]. The apo-B-containing bands were cut from the membrane, the radioactivity was determined and the apo-B content of each band calculated from the standard curve as described previously [25]. Thus only intact apo-B (molecular mass approx. 500 kDa) was determined.

RESULTS

As previously described, incorporation of [^{35}S]methionine into apo-B of rabbit hepatocytes was rapid [20], so that within minutes of incubation radiolabelled apo-B was present in each subcellular fraction. Therefore, as in previous studies, we selected 30 min for the first incubation step because this resulted in

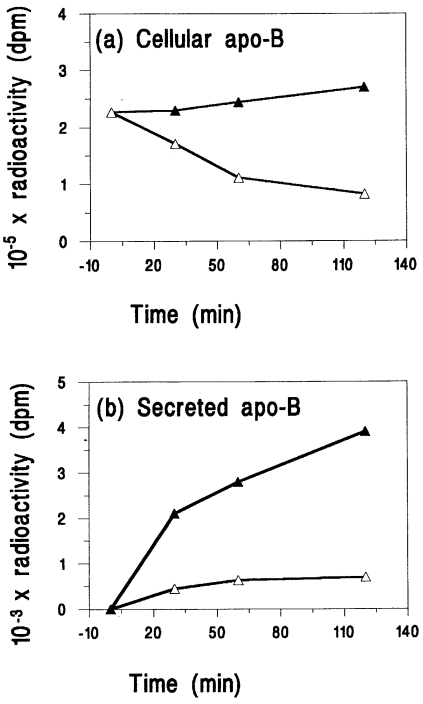

Figure 1 Effect of oleate on the degradation and secretion of newly synthesized apo-B by isolated rabbit hepatocytes

Isolated hepatocytes were pulse-labelled with [^{35}S]methionine for 30 min, isolated by centrifugation and reincubated with and without addition of oleate; at intervals the cells were pelleted by centrifugation and the radiolabel in cells and secreted apo-B determined as described in the Methods section. Symbols: ▲, with oleate; △, without oleate. Plotted results are d.p.m. in apo-B per g of hepatocytes (**a**) and total d.p.m. secreted by 1 g of hepatocytes (**b**); 1 g of hepatocytes contains 40 mg of protein.

sufficient incorporation of [^{35}S]methionine to allow measurement of the radiolabelled apo-B in subcellular fractions prepared from 1 g of hepatocytes [20,21]. In the second incubation the fates of the different pools of apo-B were followed.

Effect of oleate on the degradation and secretion of apo-B

Hepatocytes

Newly synthesized apo-B was lost from hepatocytes in the second incubation step, so that after 120 min $44.25 \pm 7.5\%$ (S.D.; $n = 4$) of the radioactivity in apo-B had been lost (Figure 1). Addition of oleate to the incubation medium prevented this degradation and also stimulated secretion of apo-B more than 6-fold compared with that in the absence of oleate (Figure 1).

Subcellular fractions

After 30 min of incubation the radiolabel in apo-B in the RER fractions was considerably higher than that in the other fractions (Figure 2) and the specific activity of apo-B in the RER luminal contents (415617 d.p.m./μg) was approximately three times that in the membrane (136629 d.p.m./μg), indicating that newly synthesized apo-B does not mix with the membrane-bound pool and is preferentially transferred to the lumen of the RER. The amount of apo-B (μg/mg of fraction protein) in the subcellular fractions was similar to that reported previously with the highest level in the *trans*-Golgi lumen and membranes (Table 1). The radiolabelled (newly synthesized) apo-B therefore does not reflect the mass of intracellular pools of apo-B, and the SER, *cis*-Golgi and *trans*-Golgi pools apparently turn over more slowly than those of the RER.

When hepatocytes were reincubated in the presence of excess unlabelled methionine the radiolabel in apo-B in the RER, SER and *cis*-Golgi membrane fell by between 70 and 90%, whereas that in the *trans*-Golgi membrane remained level (Figure 2). The radiolabel in apo-B in the luminal contents of the RER and SER also fell, while that in the *trans*-Golgi and the *cis*-Golgi lumen rose and fell. These observations are consistent with previous findings and indicate that there is considerable degradation of newly synthesized apo-B and that the RER membranes and lumen are the major site of this degradation [20,21].

In intracellular fractions the major effect of addition of oleate to the incubation medium oleate was apparently to protect the mass of apo-B from degradation in the membranes and to stimulate its transfer into and through the lumen of the secretory pathway (Figure 2). The pattern of change in radiolabelled apo-B differed from that of the mass of apo-B, suggesting that newly synthesized apo-B does not mix with unlabelled apo-B that is presumably present in the hepatocytes before incubation and synthesized during incubation. This pool of unlabelled apo-B is apparently protected from degradation by the addition of oleate.

Effect of protease inhibitors on the degradation and secretion of apo-B

Hepatocytes

Addition of inhibitors of metalloproteases (*o*-phenanthroline), serine proteases (aprotinin), serine/cysteine proteases (leupeptin) or cysteine proteases (ALLN) to the second incubation step inhibited cellular degradation of apo-B (Figure 3). Both ALLN and EDTA exhibited a lag in their effect, suggesting that these inhibitors did not gain access to the proteases immediately. Addition of 1 mM zinc chloride with *o*-phenanthroline completely reversed the inhibition, confirming the involvement of a zinc-dependent protease (Table 2). Protein synthesis from [^{35}S]methionine was not reduced in the presence of any of the inhibitors compared with controls, indicating that the hepatocytes remain viable and that the effects of the inhibitors are not a result of inhibition of protein synthesis (Figure 4). We also tested the effect of the aspartate protease inhibitor, pepstatin: this had no effect on apo-B degradation or secretion.

Leupeptin and *o*-phenanthroline both increased apo-B secretion about 4-fold compared with the control, to about 60% of that stimulated by oleate. The other inhibitors investigated had no significant effect on secretion. One explanation for this observation is that *o*-phenanthroline and leupeptin might inhibit degradation early in the secretory pathway so that the protected apo-B is secreted, whereas the other inhibitors act later in the pathway when apo-B is irretrievably diverted from secretion. To test this possibility the intracellular sites of accumulation of apo-B were investigated.

Subcellular fractions

When *o*-phenanthroline was added to the second incubation step, its main effects were to cause accumulation of the radiolabel in apo-B and to increase the mass of apo-B in the RER membrane and the SER membrane, with little effect on the luminal content fractions (Figure 5). There was no effect on the disappearance of newly synthesized apo-B from the *cis*-Golgi or *trans*-Golgi membranes, although the mass of apo-B decreased more slowly

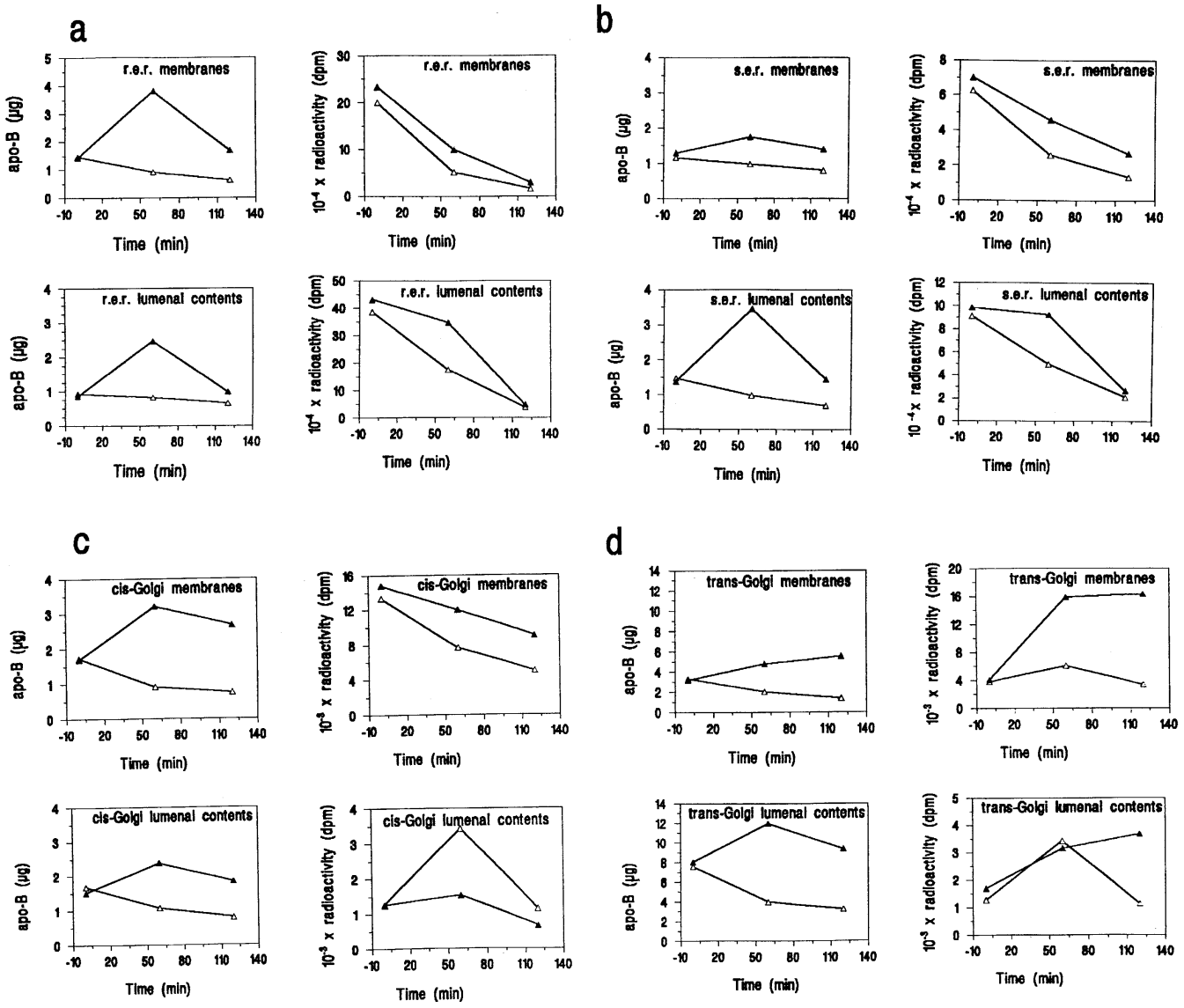

Figure 2 Effect of oleate on the intracellular degradation of apo-B in pulse-labelled hepatocytes

Isolated hepatocytes were pulse-labelled with [^{35}S]methionine for 30 min, isolated by centrifugation and reincubated with and without addition of oleate as described in the Methods section. At intervals the cells were pelleted by centrifugation, subcellular fractions prepared and radiolabel and mass of apo-B determined as described in the Methods section. Symbols: ▲, with oleate; △, without oleate. The results plotted are d.p.m. in apo-B or μg of apo-B per mg of subcellular fraction protein. Data are from a single experiment. In repeated experiments the amounts of apo-B present in each subcellular fractions were within 10% (see Table 1). (**a**) RER (r.e.r.) fractions; (**b**) SER (s.e.r.) fractions; (**c**) *cis*-Golgi fractions; (**d**) *trans*-Golgi fractions. In each group the upper two panels are results from membranes and the lower two panels are from luminal contents.

than in the absence of the inhibitor. This may be apo-B that has been protected from degradation in the RER moving to the Golgi membranes. The newly synthesized apo-B in the *cis*-Golgi and *trans*-Golgi luminal contents increased and subsequently fell as the apo-B was secreted. *o*-Phenanthroline thus seems to block degradation of apo-B in the ER membranes and to divert the newly synthesized apo-B into the lumen and through the secretory pathway.

Addition of leupeptin to the second incubation medium inhibited loss of both radiolabel in apo-B and the mass of apo-B in the SER membrane and the *cis*-Golgi membrane (Figure 5). As with *o*-phenanthroline, the newly synthesized apo-B in the *cis*-Golgi and *trans*-Golgi luminal contents increased and subsequently fell as the apo-B was secreted. However, in contrast with *o*-phenanthroline, addition of leupeptin had no effect on the apo-B in the RER membrane or luminal content. Leupeptin thus seems to inhibit degradation of apo-B in the SER and *cis*-Golgi membranes. The newly synthesized apo-B protected from degradation is apparently still able to be secreted.

Both ALLN and aprotinin were similar in their effects and differed from those of *o*-phenanthroline or leupeptin (Figure 5). Both reagents inhibited loss of both radiolabel and mass of apo-B in the *trans*-Golgi membrane and had no effect on degradation of apo-B in the RER, the SER or the *cis*-Golgi membranes or luminal content fractions.

Time-course experiments involving subcellular fractionation

Table 1 Apo-B content of subcellular fractions isolated from rabbit hepatocytes

Subcellular fractions were prepared from isolated rabbit hepatocytes as described in the Methods section. The apo-B content was determined by quantitative immunoblotting and expressed as μg/mg protein of each subcellular fraction before separation into membranes and luminal content subfractions. The results are the means ± S.D. of determinations of apo-B in subcellular fractions prepared from six separate hepatocyte preparations.

	Apo-B (μg/mg protein of original fraction)	
Fraction	Membrane	Luminal content
RER	1.40 ± 0.05	0.95 ± 0.05
SER	1.22 ± 0.05	1.39 ± 0.07
cis-Golgi	1.70 ± 0.02	1.65 ± 0.07
trans-Golgi	3.07 ± 0.16	7.40 ± 0.61

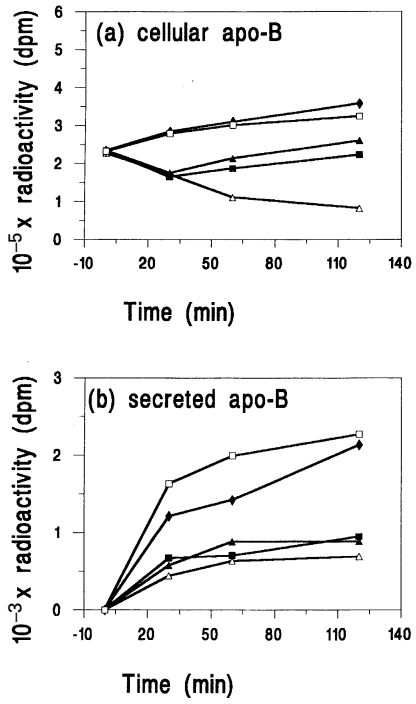

Figure 3 Effect of inhibitors of proteolysis on the degradation and secretion of newly synthesized apo-B by isolated rabbit hepatocytes

Isolated hepatocytes were pulse-labelled with [^{35}S]methionine for 30 min, isolated by centrifugation and reincubated with and without addition of protease inhibitors; at intervals the cells were pelleted by centrifugation and the radiolabel in cell and secreted apo-B determined as described in the Methods section. Results plotted are d.p.m. in apo-B per g of hepatocytes (**a**) and total d.p.m. secreted by 1 g of hepatocytes (**b**); 1 g of hepatocytes contains 40 mg of protein. Symbols: △, no addition; ▲, ALLN; ◆, leupeptin; ■, aprotinin; □, *o*-phenanthroline.

required centrifugation of a large number of samples, so it was not possible to perform sufficient repeated individual time points in a single experiment for statistical analysis. In repeated experiments the radiolabel added, but not the amount of methionine, varied owing to decay. Therefore the results illustrated in Figures 2 and 5 are from a single experiment. However, when the results from repeated experiments were normalized by calculating the percentage change in the radiolabel in apo-B in subcellular

Table 2 Effect of zinc on the inhibition of degradation of radiolabelled apo-B by *o*-phenanthroline

Isolated hepatocytes were pulse-labelled with [^{35}S]methionine for 30 min, isolated by centrifugation and reincubated with *o*-phenanthroline with a range of concentrations of zinc chloride for 120 min. The cells were pelleted by centrifugation and the radiolabel in cell and secreted apo-B determined as described in the Methods section. Results are means ± S.D. of the change in d.p.m. in apo-B relative to the beginning of the chase-incubation per mg of hepatocyte proteins, and total d.p.m. secreted by 1 g of hepatocytes, for three separate preparations of hepatocytes. The radiolabel in the hepatocyte apo-B at the start of the chase period was 13 465 ± 1152 d.p.m.

	Change in radiolabel in apo-B (d.p.m.)	
Treatment	Cellular apo-B	Secreted apo-B
No addition	−5925 ± 390	1094 ± 16
Plus *o*-phenanthroline	9999 ± 2046	2716 ± 103
Plus 10 μM zinc chloride	3393 ± 352	1828 ± 177
Plus 100 μM zinc chloride	413 ± 287	1423 ± 2.31
Plus 1 mM zinc chloride	−5923 ± 444	1211 ± 106

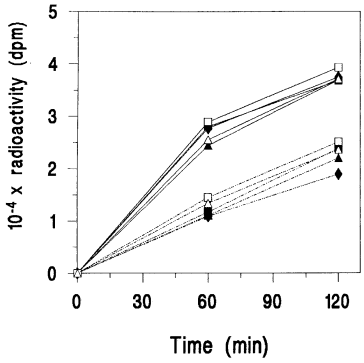

Figure 4 Effect of protease inhibitors on the incorporation of [^{35}S]methionine into total cellular and secreted proteins of isolated hepatocytes

Isolated hepatocytes were incubated with [^{35}S]methionine as described in the Methods section. At intervals the cells were isolated by centrifugation and the radiolabel in cellular and secreted proteins was determined [15]. Symbols: △, no addition; ▲, ALLN; ◆, leupeptin; ■, aprotinin; □, *o*-phenanthroline; solid lines, cellular protein; broken lines, secreted protein.

fractions during the 120 min chase period, similar effects of addition of protease inhibitors or oleate were observed (Table 3).

Effect of oleate on secretion of apo-B by hepatocytes treated with protease inhibitors

The above results indicate that newly synthesized apo-B does not mix with pre-existing apo-B in the membranes of the secretory compartment. Oleate seems to protect the total membrane apo-B from degradation and stimulates its transfer to the lumen for secretion, whereas *o*-phenanthroline and leupeptin protect cellular apo-B from degradation at specific sites and stimulate the secretion of newly synthesized apo-B. The effects of *o*-phenanthroline or leupeptin and oleate on secreted apo-B and cellular apo-B were additive (Table 4), suggesting that different mechanisms are involved. Oleate seems to inhibit degradation by diverting apo-B into a pool that is separated from the protease, whereas the inhibitors act directly on the protease. Sakata et al.

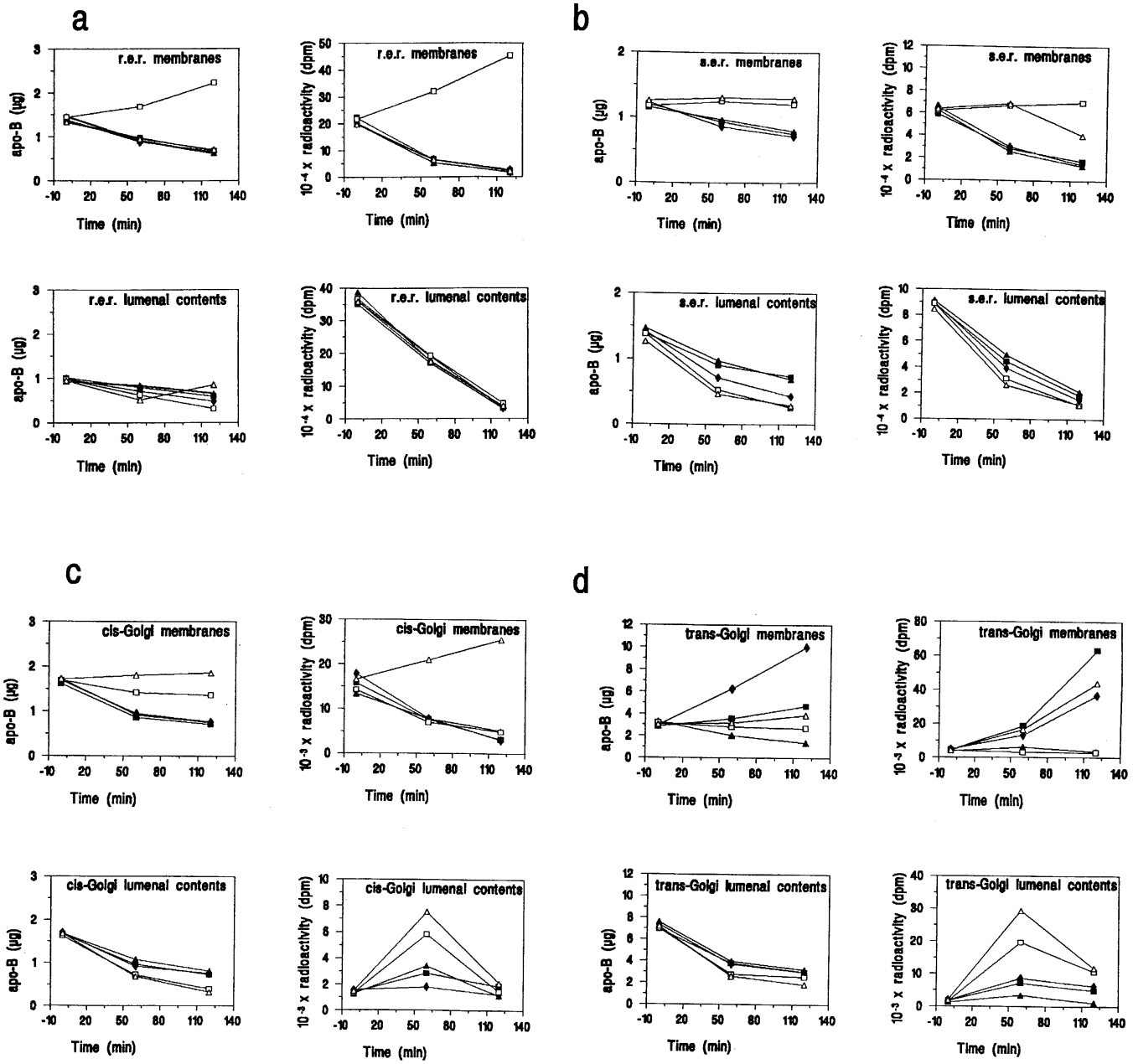

Figure 5 Effect of protease inhibitors on the intracellular degradation of apo-B in pulse-labelled hepatocytes

Isolated hepatocytes were pulse-labelled with [^{35}S]methionine for 30 min, isolated by centrifugation and reincubated with no addition or with aprotinin, ALLN, *o*-phenanthroline or leupeptin. At intervals the cells were pelleted by centrifugation, subcellular fractions prepared and radiolabel and mass of apo-B determined as described in the Methods section. The results plotted are d.p.m. in apo-B or μg of apo-B per mg of subcellular fraction protein. Data from a single experiment are shown. In repeated experiments the amounts of apo-B present in each subcellular fraction were within 10% (see Table 1). Symbols: ▲, no addition; ◆, ALLN; △, leupeptin; ■, aprotinin; □, *o*-phenanthroline. (**a**) RER fractions; (**b**) SER fractions; (**c**) *cis*-Golgi fractions; (**d**) *trans*-Golgi fractions. In each group the upper two panels are results from membranes and the lower two panels are from luminal contents.

[13] have also shown that in ALLN-treated Hep-G2 cells, secretion of apo-B in the ER membrane can be stimulated by the addition of oleate.

DISCUSSION

Our aim in these studies was to radiolabel apo-B in rabbit hepatocytes in a first incubation step and to follow its transit and fate in the different intracellular pools in a subsequent incubation in the presence of inhibitors of proteolysis. Ideally, in a pulse–chase experiment the radiolabel should be initially in a single pool. However, this is difficult to achieve in a complex cellular system. Under our experimental conditions [^{35}S]methionine is incorporated rapidly into all intracellular pools of apo-B. Therefore we selected an initial incubation of 30 min because this resulted in sufficient incorporation of radiolabel to allow determination of the specific activities of apo-B in the small amounts of subcellular fractions prepared. We then followed the fate of

Table 3 Percentage change in radiolabel in apo-B of subcellular fractions during the chase period

Isolated hepatocytes were pulse-labelled with [^{35}S]methionine for 30 min, isolated by centrifugation and reincubated for 120 min with no addition, or plus oleate, aprotinin, ALLN, *o*-phenanthroline or leupeptin as described in the Methods section. The cells were pelleted by centrifugation, subcellular fractions prepared and the radiolabel in apo-B was determined as described in the Methods section. Results are the percentage change in the radiolabel in apo-B in each subcellular fraction at the end of the reincubation relative to that at the begininning. Values given are the averages of up to three determinations. The range of values for different experiments was less than ±15% of the average.

Subcellular fraction	No addition	Oleate	Aprotinin	ALLN	*o*-Phenanthroline	Leupeptin
RER						
Membrane	−92.4	−87.7	−90.8	−87.4	+109.1	−89.2
Content	−90.1	−88.9	−80.8	−89.2	−87.1	−89.1
SER						
Membrane	−78.7	−61.8	−70.4	−86.2	+13.3	+36.3
Content	−76.9	−73.0	−79.4	−83.4	−87.9	−88.7
cis-Golgi						
Membrane	−62.1	−38.5	−80.3	−83.3	−66.0	+54.2
Content	−10.1	−47.9	+33.8	−22.0	+8.7	+41.4
trans-Golgi						
Membrane	+11.3	+299.8	+134.3	+648.8	−28.9	+937.0
Content	−10.1	+106.3	+177.6	+231.3	+563.2	+508.1

Table 4 Effect of oleate on the degradation and secretion of apo-B in the presence of *o*-phenanthroline or leupeptin

Isolated hepatocytes were labelled with [^{35}S]methionine for 30 min, isolated by centrifugation and reincubated with no addition, with leupeptin or with *o*-phenanthroline with and without addition of oleate. After 120 min of incubation the cells were pelleted by centrifugation and the radiolabel in cell (per mg of hepatocyte proteins) and secreted apo-B (total radiolabel secreted by 1 g of hepatocytes) were determined as described in the Methods section. Results given are the means ± S.D. of change in radiolabel in apo-B relative to the beginning of the chase-incubation for three separate preparations of hepatocytes. The radiolabel in the hepatocyte apo-B at the beginning of the chase period was 4931.7 ± 237.7 d.p.m.

	Change in radiolabel in apo-B (d.p.m.)	
Treatment	Cellular apo-B	Secreted apo-B
No addition	−2183.7 ± 69.2	595.3 ± 61.7
Plus oleate	2113.0 ± 423.2	1621.7 ± 108.5
Plus *o*-phenanthroline	2892.0 ± 223.1	1634.6 ± 128.6
Plus oleate and *o*-phenanthroline	4876.0 ± 441.5	2345.0 ± 25.5
Plus leupeptin	2708.7 ± 340.0	1379.3 ± 38.1
Plus leupeptin and *o*-phenanthroline	4122.7 ± 227.9	4266.7 ± 127.1

the radiolabelled apo-B in in the different subcellular pools. This protocol was used in previous investigations [20,21].

We have characterized our subcellular fractions from rabbit liver by using marker enzymes [20,22,24] and by using immunological markers for the *cis*-Golgi network (p58) and the *trans*-Golgi network (TGN38) [20]. These results have been discussed in detail elsewhere [20,22,24]; however, they show that RER has very low to undetectable contamination with Golgi elements, the SER contains some Golgi elements, the *cis*-Golgi-enriched fraction is significantly contaminated with ER and the *trans*-Golgi fraction does not contain ER or *cis*-Golgi network markers. However, it must be emphasized that the secretory compartment represents a functional continuum in which the ER is linked to the *cis*-Golgi through the *cis*-Golgi network and the *trans*-Golgi is linked to the plasma membrane through the *trans*-Golgi network [27–29] and that separation of this continuum is somewhat artificial. Our subcellular fractions thus sample each end of this continuum and the overlapping central part.

Investigations in which brefeldin A, monesin or nocodazole were used to perturb membrane traffic have suggested that the main site of apo-B degradation in Hep-G2 cells is a pre-Golgi compartment, probably the ER [10,12,14]. Consistent with this, isolated ER vesicles were found to contain a protease that acts on endogenous apo-B in the membranes; however, such activity could not be detected in Golgi fractions [12]. The results reported here also show that the major site of loss of radiolabelled apo-B is the RER membrane and lumen. However, the mass of apo-B does not behave in the same way as the radiolabel in apo-B, and our results indicate that degradation of total apo-B occurs in both the ER and the Golgi [20,21]. We have previously shown that feeding orotic acid to rats causes accumulation of apo-B in the *trans*-Golgi membranes, indicating that the membrane-bound form of apo-B does move to this site [21]. We have also shown that ER and Golgi membranes from rabbit liver, but not the luminal content fractions, contain fragments of apo-B [22]. As these are seen when a cocktail of protease inhibitors is included in all fractionation steps it is likely that the fragments were present in the membranes initially and are due to degradation *in vivo*. A similar observation was made by Davis et al. [8] in the ER, although fragments of apo-B were barely detectable in the Golgi fractions.

Degradation of apo-B in Hep-G2 cells has been shown to be dependent on ATP and pH and inhibited by ALLN [14]. Treatment of Hep-G2 cells with brefeldin A, which inhibits transfer of membrane and secretory proteins from the ER to the Golgi, has shown that degradation is ALLN-sensitive, indicating the involvement of a cysteine-ER protease [10,12,14]. In contrast, it has been shown that insulin-stimulated degradation of apo-B in rat hepatocytes is only partly inhibited by ALLN, is blocked by brefeldin A, and therefore occurs in a post-ER compartment, presumably the Golgi [9]. Our observations are consistent with this and indicate that ALLN inhibits degradation of apo-B but causes accumulation of apo-B in the *trans*-Golgi membranes. These apparently conflicting results may reflect differences between Hep-G2 cells and adult hepatocytes. In other systems either the Golgi or the ER, or both, have been implicated as sites of degradation of secretory and membrane proteins. ALLN-inhibited degradation of secretory IgM in B lymphocytes and degradation of apo-E in Hep-G2 cells both occur in a post-ER (Golgi) compartment [30–32]. Many proteins have also been shown to be degraded in the ER [33,34], including the T-cell antigen, 3-hydroxy-3-methylglutaryl-CoA reductase and the asialoglycoprotein receptor [35–37].

The observations reported here indicate that apo-B degradation is blocked by inhibitors of metalloproteases, serine proteases and cysteine proteases and that these cause accumulation of apo-B at different sites. Thus a metalloprotease could be involved in degradation of apo-B in the ER, serine protease in the SER/*cis*-Golgi and cysteine protease in the *trans*-Golgi. Interestingly, metalloproteases have also been implicated in the degradation of secretory IgM [30]. None of the inhibitors investigated affected degradation of apo-B in the lumen of the RER. One problem with our experimental approach, and indeed with all studies using inhibitors of proteases, metabolism or membrane traffic, is the complexity of the system studied. Thus the inhibition of transfer of apo-B from the RER to the *trans*-Golgi by *o*-phenanthroline might not be the direct effect of this inhibitor on a protease acting on apo-B, but could be due to inhibition of membrane traffic, thus resulting in accumulation of apo-B. However, in the presence of *o*-phenanthroline the synthesis and secretion of total proteins continues, the secretion of apo-B occurs and this is further stimulated by oleate. These observations suggest that *o*-phenanthroline does not perturb membrane traffic through the secretory compartment.

Our results indicate that apo-B in the membrane of the first part of the secretory pathway is secretion-competent, whereas once the protein has reached the *trans*-Golgi it is irretrievably committed to degradation. This suggests that fully translated apo-B in the ER membrane can be assembled with lipid post-translationally. Studies in which topography has been probed with monoclonal antibodies have shown that in the Golgi all of the membrane-bound apo-B is exposed at the cytosolic side, but that in RER and SER membranes apo-B exists in at least two pools, one exposed at the cytosolic surface and a second at the luminal surface [24]. The latter pool may be available for assembly into apo-B. This may also be the pool protected by *o*-phenanthroline, although this requires further investigation.

Studies of Hep-G2 cells suggest that oleate protects apo-B from degradation by diverting it into a compartment that is separated from the protease [11,13]. Our findings are consistent with this idea and demonstrate that oleate preferentially protects the mass of apo-B in membrane and luminal content fractions over the newly synthesized apo-B. In addition, degradation of endogenous apo-B in isolated ER vesicles from Hep-G2 only occurs if the membranes are disrupted with Triton X-100 [12]. This also suggests that the apo-B is topographically separated from the protease either in separate membrane vesicles or within the same vesicles. The mechanism of oleate action is not clear. However, as apo-B is hydrophobic with multiple lipid-binding sites [37] it can be postulated that oleate, or triacylglycerol, phospholipid or cholesterol ester derived from it, protects apo-B by binding to proteolytic sites or assisting in the proper folding of the protein. All of these lipids have been implicated in the translocation of apo-B into the lumen of the RER and the assembly of VLDL [39–48].

Degradation of apo-B, especially in the ER membranes, could play an important role in the regulation of secretion of VLDL. The finding that metalloproteases may be involved in degradation at this site is novel and could provide a therapeutic target for new methods of regulating VLDL secretion by the liver.

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REFERENCES

1 Pullinger, C. R., North, J. D., Teng, B.-B., Rifici, V. A., Ronhild de Brito, A. E. and Scott, J. (1989) J. Lipid Res. **30**, 1065–1077
2 Dashti, N., Williams, D. L. and Aulauovic, P. (1989) J. Lipid Res. **30**, 1365–1373
3 Kaptein, A., Roodenberg, L. and Princen, M. G. (1991) Biochem J. **278**, 557–564
4 Borchardt, R. A. and Davis, R. A. (1987) J. Biol. Chem. **262**, 16394–16402
5 Bostrom, K., Boren, J., Wettesten, M., Sjoberg, A., Bondjers, G., Wiklund, O., Carlsson, P. and Olofsson, S.-O. (1988) J. Biol. Chem. **263**, 4434–4442
6 Boren, J., Wettesten, M., Sjoberg, A., Thorlin, T., Bondjers, G., Wiklund, O. and Olofsson, S.-O. (1990) J. Biol. Chem. **265**, 10556–10564
7 Boren, J., Rustaeus, S., Wettesten, M., Andersson, M., Wiklund, A. and Olofsson, S.-O. (1993) Arteriosclerosis and Thrombosis **13**, 1743–1754
8 Davis, R. A., Thrift, R. N., Wu, C. C. and Howell, K. E. (1990) J. Biol. Chem. **265**, 10005–10011
9 Sparks, J. D. and Sparks, C. E. (1990) J. Biol. Chem. **265**, 8854–8862
10 Sato, R., Imanaka, T., Takasuki, A. and Tanako, T. (1990) J. Biol. Chem. **265**, 11880–11884
11 Dixon, J. L., Furukawa, S. and Ginsberg, H. N. (1991) J. Biol. Chem. **266**, 5080–5086
12 Furukawa, S., Sakata, N., Ginsberg, H. N. and Dixon, J. L. (1992) J. Biol. Chem. **267**, 22630–22638
13 Sakata, N., Wu, X., Dixon, J. L. and Ginsberg, H. N. (1993) J. Biol. Chem. **268**, 22967–22970
14 Adeli, K. (1994) J. Biol. Chem. **269**, 9166–9175
15 Dixon, J. L. and Ginsberg, H. N. (1993) J. Lipid Res. **34**, 167–179
16 Yao, Z. and McLeod, R. S. (1994) Biochim. Biophys. Acta **1212**, 152–166
17 Gibbons, G. F. (1994) Curr. Opin. Lipidol. **5**, 191–199
18 Gibbons, G. F., Khurawa, R., Odwell, A. and Seelaender, M. C. L. (1994) J. Lipid Res. **35**, 1801–1808
19 Spring, D. J., Chen-Lui, L. W., Chatterton, J. E., Elovson, J. and Schumaker, V. N. (1992) J. Biol. Chem. **267**, 14839–14845
20 Cartwright, I. J. and Higgins, J. A. (1995) Biochem J. **310**, 897–907
21 Cartwright, I. J., Hebbachi, A.-M. and Higgins, J. A. (1993) J. Biol. Chem. **268**, 20937–20952
22 Wilkinson, J., Higgins, J. A., Groot, P. H. E., Gherardi, E. and Bowyer, D. E. (1992) Biochem J. **288**, 413–419
23 Wilkinson, J., Higgins, J. A., Groot, P. H. E., Gherardi, E. and Bowyer, D. E. (1992) FEBS Lett. **304**, 24–26
24 Wilkinson, J., Higgins, J. A., Groot, P. H. E., Gherardi, E. and Bowyer, D. E. (1993) J. Lipid Res. **34**, 815–825
25 Cartwright, I. J. and Higgins, J. A. (1992) Biochem. J. **285**, 183–189
26 Higgins, J. A. and Hutson, J. L. (1984) J. Lipid Res. **25**, 1295–1305
27 Lahtinen, U., Dahllof, B. and Sareste, J. (1992) J. Cell Sci. **100**, 415–430
28 Sareste, J. and Svennsson, K. (1991) J. Cell Sci. **103**, 321–333
29 Luzio, J. P. and Banting, G. (1993) Trends Biochem. Sci. **18**, 395–398
30 Amitay, R., Shacher, I., Rabinovich, E., Haimovich, J. and Bar-Nun, S. (1992) J. Biol. Chem. **267**, 20694–20700
31 Oda, K. (1992) J. Biol. Chem. **267**, 17485–17471
32 Ye, S., Reardon, C. A. and Getz, G. S. (1993) J. Biol. Chem. **26**, 8497–8502
33 Klausner, R. D. and Sitia, R. (1990) Cell **62**, 611–614
34 Bonifacino, J. S. and Lippincott-Schwartz, J. (1991) Curr. Opin. Cell Biol. **3**, 592–600
35 Stafford, F. J. and Bonifacino, J. S. (1991) J. Cell Biol. **115**, 1225–1236
36 Urade, R. and Kito, M. (1992) FEBS Lett. **312**, 83–86
37 Wilkstom, L. and Lodish, H. F. (1992) J. Biol. Chem. **267**, 5–8
38 Chan, L. (1992) J. Biol. Chem. **267**, 25621–25624
39 Cianflone, K. M., Yasruel, Z., Rodriguez, M. A., Vas, D. and Sniderman, A. D. (1990) J. Lipid Res. **30**, 2045–2055
40 Craig, W. Y., Nutik, R. and Cooper, A. D. (1988) J. Biol. Chem. **263**, 13860–13890
41 Dashti, N. (1992) J. Biol. Chem. **267**, 7160–7169
42 Fungwe, T. V., Cagen, L., Wilcox, H. G. and Heimer, M. (1992) J. Lipid Res. **33**, 179–191
43 Khan, B. V., Fungwe, T. V., Wilcox, H. G. and Heimer, M. (1990) Biochim. Biophys. Acta **1044**, 297–304
44 Qin, W., Infante, J., Wang, S.-R. and Infante, R. (1992) Biochim. Biophys. Acta **1127**, 57–66
45 Riberia, A., Mangeney, M., Loriette, C., Thomas, G., Pepin, D., Janvier Chambez, J. and Bereziat, G. (1991) Biochim. Biophys. Acta **1086**, 279–286
46 Rusinol, A. E., Chan, E. Y. W. and Vance, J. E. (1993) J. Biol. Chem. **268**, 25168–25175
47 Tanaka, M., Jingami, H., Otani, H., Cho, M., Ueda, Y., Arai, H., Nagano, Y., Doi, T., Yokode, M. and Kita, T. (1993) J. Biol. Chem. **268**, 12713–12718
48 Verkade, H. J., Fast, D. G., Rusinol, A. E., Scraba, D. G. and Vance, D. E. (1993) J. Biol . Chem. **268**, 24990–24996

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