The presence and subcellular distribution of sterol carrier protein 2 in embryonic-chick tissues

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Transport of lipids from the yolk to the tissues of the chick embryo is slow during the first 2 weeks of development, but increases abruptly during the last week. Evidence suggests that the lipid traverses the cytoplasm of the yolk-sac membrane before secretion as lipoprotein into the fetal circulation. Little is known about the cytoplasmic transport of lipid in avian systems, but recently the presence of sterol carrier protein 2 (SCP₂) was reported in chicken liver. Here we examine the cells of yolk-sac membrane, liver and small intestine for the presence of this protein as a function of the time of embryonic development. The

quantity of SCP₂ present in the embryonic cells did not appear to correlate with the rate of lipid flux in these tissues. The abrupt appearance of a high-molecular-mass form of SCP₂ was detected in small intestine shortly before hatching, but the significance of this protein is not clear. The presence of SCP₂ in these tissues was also confirmed by immunocytochemical techniques. Similarly to SCP₂ of mammalian cells, avian SCP₂ is localized in both peroxisome-like structures and mitochondria. To a lesser extent it is associated with the endoplasmic reticulum.

INTRODUCTION

The developing chick embryo represents an excellent system for the study of lipid mobilization and transport. Throughout the 3-week developmental process, the yolk serves as the sole source of lipids, carbohydrate and also of protein. Although carbohydrate and protein are rapidly transported to the developing embryo during the first 2 weeks of development [1,2], only 350 mg of the 5–6 g of lipid in the yolk are mobilized in the same period. The rate of lipid mobilization increases, with an additional 230 mg being transported between day 13 and 15 and, during the last week of development, it reaches up to 1 g or more per day [3,4]. Evidence suggests that at least some lipid traverses the yolk-sac membrane cytoplasm before secretion as lipoproteins. For example, while yolk cholesterol is predominantly unesterified the cholesterol secreted by the yolk-sac membrane is mostly esterified (4–7). Since more than 70 % of the fatty acid moieties are oleate [3], acetyl-CoA:cholesterol *o*-acyltransferase (ACAT)-mediated esterification is suggested, and this implies that cholesterol travels through the cytoplasm of the yolk-sac-membrane cells. Although it is known that serum lipoproteins carry lipid from the yolk-sac membrane to the tissues [8], little is known about the machinery of intracellular lipid transport in chickens. In mammalian cells, the 12 kDa protein sterol carrier protein 2 (SCP₂) has been implicated in the transport of sterols through the cytoplasm [9] as well as in stimulating ACAT [10]. Recently, we demonstrated the presence of a protein in adult chicken liver which is similar to mammalian SCP₂ [11]. Here we report the presence of this SCP₂-like protein in embryonic chicken yolk-sac-membrane, liver and intestinal cells. The intracellular localization of this protein and its higher-molecular-mass forms was examined using immunocytochemical techniques.

METHODS AND MATERIALS

Growth of embryos

Fertile eggs of broiler-type chicken were obtained from Moyer's

Chicks, Quakertown, PA, U.S.A., and were incubated at 38 °C in a Lyon Electric Co. humidified incubator.

Tissue preparation

At various stages of development, eggs were selected at random, the embryos removed, and liver, intestine and yolk-sac membrane were sampled. Tissues used for Western-blot analysis were weighed, suspended in 3 vol. of homogenization buffer [1 mM Tris/HCl (pH 7.5)/250 mM sucrose/0.5 mM dithiothreitol (DTT)/20 µM leupeptin] and were homogenized by three strokes in a Potter–Elvehjem-type glass homogenizer. An aliquot was saved for protein determination and the remainder was immediately mixed with an equal volume of sample buffer [5 % (w/v) SDS/10 % (v/v) mercaptoethanol/0.01 % Bromophenol Blue/5 % glycerol] and boiled for 3 min.

Electron microscopy

Tissues to be used for electron microscopy were fixed in 1 % glutaraldehyde and embedded in Lowicryl K4M resin. Sections were cut and placed on Formvar-coated grids. The grids were inverted over droplets of 1 % gelatin in Tris-buffered saline [TBS; 20 mM Tris/HCl (pH 7.4)/500 mM NaCl] and were incubated for 10 min. After a brief rinse with TBS, the grids were inverted over 1 % gelatin in TBS containing 1:100 rabbit anti-(rat SCP₂) antiserum. This was incubated 3 h at room temperature, washed in 30 drops of TBS and incubated with 1:50 goat anti-rabbit IgG–10 nm immunogold label for 3 h at room temperature. Sections were washed and lightly counterstained with uranyl acetate and were examined in a Zeiss model 10B electron microscope.

Gel electrophoresis and Western blotting

Proteins (100 µg/sample as determined by the method of Bradford [12]) were analysed on duplicate SDS/12 % polyacrylamide gels. One finished gel was stained with Coomassie Brilliant Blue

Abbreviations used: SCP₂, sterol carrier protein 2; ACAT, acetyl-CoA:cholesterol *o*-acyltransferase; Tw, Tween 20.

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R250 stain, while the second was blotted to 0.2 μ m-pore-size nitrocellulose by the method of Burnette [13], using a Bio-Rad immunoblot chamber. The blot was washed, incubated in TBS containing 1% BSA (Sigma, St. Louis, MO, U.S.A.) for 15 min, and placed into TBS/Tween 20 (Tw; 0.05%)/1% BSA containing 1:2000 rabbit anti-(rat SCP₂) antiserum. After an overnight incubation at 4 °C and another 1 h at room temperature, the blot was washed with TBS/Tw and placed into TBS/Tw/1% BSA containing Goat anti-rabbit IgG (1:2000) conjugated with horseradish peroxidase (Bio-Rad). After a 2 h incubation at room temperature, blots were washed twice with TBS/Tw, three times with TBS and incubated in a 4-chloro-1-naphthol/H₂O₂ system. Development was terminated by rinsing with distilled water.

RESULTS

Tissues were obtained from chick embryos between 10 and 20 days of incubation. These were homogenized, treated with SDS, electrophoresed and either stained with Coomassie Blue stain or blotted for immunostaining. The results for the yolk-sac membrane are shown in Figure 1(a). The presence of SCP₂ in the yolk-sac membrane was confirmed throughout the developmental period. The molecular mass of the protein, however, shows heterogeneity, ranging between 12 and 16 kDa. This heterogeneity was observed even when tissues were homogenized directly in SDS buffer and heated promptly. Therefore it is not likely that this heterogeneity is due to proteolysis during sample preparation, but rather reflects actual multiple species of SCP₂ present in the tissue. The intensity of immunostaining of these 12–16 kDa proteins decreased steadily throughout development. The liver also contained SCP₂, as is indicated in the gels and blots shown in Figure 1(b). Heterogeneity of molecular mass also was observed in the 12–16 kDa region of the blot. The amount of immunoreactive material in the 12–16 kDa range increased dramatically between day 10 and 12, remained high through day 18 and then decreased at day 20. This pattern is also visible for another immunocross-reactive form at 44 kDa. This higher-molecular-mass form, which has been reported in adult chicken liver [11], is present as a doublet, but the nature of the doublet is not yet understood. A minor immunoreactive protein was observed at 64 kDa which exhibited the same time course of appearance/disappearance. The embryonic chick small intestine also contained the heterogeneous 12–16 kDa SCP₂. Figure 1(c) shows the gradual decline in the quantity of immunoreactive material in this range as embryonic development proceeds. Barely visible was the 44 kDa form which was more easily detectable in liver samples. The form also shows the quantitative decrease during later stages of development. Surprisingly, however, a 64 kDa form of the protein appears abruptly on the last day of development, before hatching. The reason for this is not apparent, but could be related to the impending need for the chick to begin digestive processes for extra-oval sources. To assure that this observation was significant, additional embryos were obtained and the intestinal tissues analysed. Five additional day-20 samples analysed showed the same abrupt appearance of this protein.

In order to localize SCP₂ using immuno-gold-labelling techniques, a protocol was developed using 14-day liver tissue samples as described in the Methods and materials section. When tissue was incubated first with rabbit anti-(rat SCP₂) antibodies, followed by colloidal gold-anti-rabbit IgG, labelling was observed predominantly in small spheroidal organelles resembling peroxisomes and mitochondria, as demonstrated in Figure 2. When tissue was first incubated with pre-immune serum rather than rabbit anti-(rat SCP₂) serum, the colloidal gold conjugate

failed to bind to sections other than (limited and non-specifically) to lipid droplets (results not shown).

Representative organelles which bound SCP₂ antibodies in the three tissue types at several stages of development are shown in Figure 3. The organelles which bound these antibodies ranged from 100 nm to 500 nm in diameter. While some appeared to contain internal structure, most resembled typical avian peroxisomes. In addition, in each tissue, moderate staining of mitochondria and light staining of endoplasmic reticulum was observed.

Intestinal cells consistently gave the most discrete labelling patterns for SCP₂, with gold particles seen mainly over peroxi-

Figure 1 Protein profile and SCP₂ content of chick tissues as a function of development

Protein content of chick yolk-sac membrane (a) as a function of the day of development was revealed by SDS/12%-PAGE (left). The SCP₂ content of these samples was determined by Western-blotting procedures (right). Only a barely detectable amount of immunoreactive material was observed in the 12–14 kDa range. The protein content (left) and SCP₂ content (right) of embryonic liver is shown in the centre panel (b). Here, 12–16 kDa SCP₂ is relatively abundant, and an additional major immunoreactive species was present at 44 kDa. Small amounts of a third protein were detectable at 64 kDa. The amount of immunoreactive protein was low on day 10, but increased between days 12 and 18, corresponding to the period of rapid lipid transport. The bottom panel (c), shows the protein content (left) and SCP₂ content (right) of embryonic intestine. While low amounts of 12–16 kDa SCP₂ were present throughout development, the major form present was the 64 kDa protein, which appeared abruptly on the day before hatching. Abbreviation: M, molecular mass.

Figure 2 Immunolocalization of SCP₂ in embryonic chicken liver

Tissue was fixed lightly in 1% glutaraldehyde and was embedded in Lowicryl. Thin sections were cut and incubated sequentially with: 1% BSA in TBS (30 min), rabbit anti-rat SCP₂ antiserum diluted 1:100 in TBS/1% gelatin, goat anti-rabbit IgG conjugated with 10 nm colloidal gold particles diluted 1:50 in TBS/1% gelatin. Sections were washed before each step with 30 drops of TBS. Gold conjugate was localized over small spheroidal membrane bound organelles resembling peroxisomes (arrows).

somes (Figure 4). At day 20, immediately before hatching, the predominant species of SCP₂ found is 64 kDa. Therefore it appears that this molecular-mass form is also confined to the peroxisomes.

DISCUSSION

Little is known about the mechanism(s) of intracellular lipid transport in avian systems, although lipid transfer activities have

Figure 3 SCP₂-positive organelles in embryonic tissues as a function of development

Yolk-sac membrane (top row, **a–c**), liver (centre, **d–f**) and intestine (bottom, **g–i**) are shown from left to right at day 10 (**a,d,g**), 14 (**b,e,h**) and 18 (**c,f,i**) [except intestine, which is day 14 (**g**), 18 (**h**) and 20 (**i**)], were treated as described in Figure 2. Structures representative of the typical organelle labelled for SCP₂ are shown here. While a few of the structures resemble multivesicular bodies (see **a** and **e**), most resemble peroxisomes. The bar represents 100 nm.

Figure 4 Localization of SCP₂ in intestinal epithelium of day-20 chick embryos

The predominant molecular-mass form of SCP₂ in day-20 intestinal epithelium was 64 kDa. Immunogold labelling revealed that this form of the protein is also localized almost exclusively to peroxisome-like organelles. Abbreviations: N, nucleus; M, mitochondria; PM, plasma membrane. The bar represents 100 nm.

been described [14]. Recently we demonstrated the presence of SCP₂ (a protein implicated in sterol biosynthesis, bile acid synthesis, cholesterol esterification and lipoprotein assembly in mammalian systems [9]) in homogenates of adult chicken livers [11]. Since the quantity of SCP₂ in specific mammalian tissues correlates well with the rate of lipid metabolism or flux in that tissue [9], we wanted to determine whether chicken SCP₂ is present in chicken tissues which are important in lipid transport and metabolism. Developing chick represents a useful model for these studies, since lipid transport undergoes an abrupt shift from a low to high rate during the last week of development. Tissues of particular interest include the yolk-sac membrane, which internalizes yolk lipids, assembles them into lipoproteins and secretes them to the embryo, the fetal liver, which does not synthesize cholesterol, but stores massive amounts of cholesterol ester from lipoprotein remnants [3], and small intestine. Our attention was therefore focused on these three tissues.

Western-blotting analysis of homogenates of these tissues taken from embryos at various stages of development revealed the presence of the 12 kDa form of SCP₂ and higher-molecular-mass immunocross-reactive proteins (Figure 1). The presence of these higher-molecular-mass species has been demonstrated previously in chicken tissues [11] and also in mammalian tissues [9,15,16]. Moreover, only the low-molecular-mass form was observed, although some microheterogeneity was evident (12–

16 kDa). The molecular basis for this heterogeneity is understood in the rat, where several mRNAs have been identified, each coding for a distinct molecular-mass species. Each species contains the full SCP₂ sequence at the C-terminus [17]. Whether this is true for the avian system remains to be established. Western blotting revealed that, while SCP₂ was present in the yolk-sac membrane, its abundance did not increase with the rise in lipid flux observed from day 13 to hatching.

The liver also contained the low-molecular-mass forms of SCP₂ at all stages of development. Additionally, a 44 kDa immunocross-reactive form was observed, and there is a correlation in the amount of this species with the stage of development. This is puzzling, however, since the liver of the developing embryo does not appear to be as highly active in lipid metabolism as it is in the adult. Instead the embryonic liver serves as a depot for cholesterol esters which remain after delivery of triacylglycerol to the tissues [3]. Another feature peculiar to embryonic chick liver is the secretion of cholesterol ester and triacylglycerols into the bile [18]. Adult bile of most species contains little or none of these lipid classes. While it remains to be determined whether this material is taken up and metabolized by the embryonic intestine, we have shown that both the 12 kDa and the 44 kDa forms of SCP₂ were present in the intestinal cells at all stages of development examined. Quantitatively, the amount of these forms of SCP₂ decreases as

the lipid flux in the embryo increases. At 1 day before hatching, however, a major immunocross-reactive 66 kDa protein appeared. The significance of this protein remains to be established, as does its actual relatedness to SCP₂.

In order to explore more fully the role of SCP₂ in lipid mobilization in the developing chick embryo, we examined its intracellular location using immunogold labelling. Studies with mammalian (rat) tissues have shown that the majority of SCP₂ is segregated into peroxisomes, with smaller amounts associated with mitochondria and endoplasmic reticulum [15,16,19]. Staining of yolk-sac membranes showed that most of the SCP₂ present is found in membrane-bounded organelles which resemble peroxisomes (see Figure 3). In addition, gold labelling was detected in mitochondria, but was largely absent from Golgi regions, even at peak times of lipoprotein secretion, indicating no role for any form of SCP₂ in the terminal stages of lipoprotein assembly and secretion. Some labelling was detectable near the periphery of large cytoplasmic lipid droplets, but this staining was also present when sections were not first exposed to anti-SCP₂ serum. Liver sections showed largely the same pattern of immunogold labelling (Figures 2 and 3). Most of the gold particles observed were associated with peroxisomes and mitochondria. In the vicinity of the lipid deposits, little or no specific staining was observed.

A highly specific pattern of immunogold labelling was observed in intestinal tissue, particularly on the last day (day 20) before hatching (Figures 3 and 4). Gold label was associated with peroxisome-like organelles and, to a lesser extent, with mitochondria. At day 20, when the 64 kDa form of SCP₂-like protein abruptly appears and is the major immunoreactive species, label was still predominantly localized over peroxisomes, indicating that all of the molecular-mass forms are similarly localized.

Previously it was hypothesized that SCP₂ might stimulate ACAT activity. The failure of SCP₂ levels to correlate with rates of cholesterol esterification in chick yolk-sac membrane, coupled with the low degree of immunogold labelling of endoplasmic reticulum (the site of ACAT), does not support this hypothesis. A role for SCP₂ in the terminal stages of lipoprotein secretion is also not supported. The failure of anti-SCP₂ to label endoplasmic reticulum to a greater degree in either avian or mammalian tissues [15,16,19] also fails to support a role in cholesterol synthesis, since that is the location of the enzymes of sterol biosynthesis [20,21]. Therefore the question of the true role(s) of SCP₂ in lipid metabolism remains unanswered. The specific localization of this protein to peroxisomes and to mitochondria suggests that it either plays a role within these organelles or in the delivery of lipids to them. Mammalian peroxisomes have been shown to contain all of the requisite enzymes of cholesterol biosynthesis [22–24]. They, as well as mitochondria, also carry

out some steps of bile acid production, and it is possible that SCP₂ in these organelles facilitates those processes [25–27]. Whether chicken peroxisomes carry out cholesterol biosynthesis is not known. It is possible that SCP₂ participates more generally in intracellular lipid transport, perhaps carrying triacylglycerols to sites of β -oxidation. This is currently under investigation in our laboratory.

Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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