Translational Control of the Circadian Rhythm of Liver Sterol Carrier Protein*

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Rat liver sterol carrier protein (SCP), a major regulator of lipid metabolism and transport, undergoes a rapid turnover and dramatic circadian variation in amount. The level of SCP was quantitated by a specific immunochemical assay using an antibody to homogenous liver SCP. During a 12-h dark, 12-h light cycle, liver exhibits a biphasic pattern in SCP level. A 7-fold increase in SCP (i.e. from 1 to 7 mg/g of liver) occurs in the dark period, peaking at the midpoint and returning to basal levels by the beginning of the light period. A similar but smaller pattern of variation in SCP amount occurs in the light cycle. To elucidate the basic mechanism responsible for these changes in SCP level, the relative synthetic rate of SCP and mRNA functional activity for SCP were measured during the dark-light cycle. Alterations in the rate of SCP synthesis can account for the variations in SCP concentration. Although large changes occur in relative synthetic rate, no significant changes were found in the level of mRNA for SCP. Therefore, the circadian rhythm in SCP synthesis and amount does not reflect variations in the concentration of mRNA for SCP, but instead is caused by some mechanism controlling the efficiency of translation of SCP mRNA.

SCP (also called fatty acid binding protein (1)) is considered to be a major regulator of lipid metabolism and transport (2). SCP is ubiquitous, abundant in rat liver, and required for activation of membrane-bound enzymes catalyzing cholesterol synthesis (2, 3). Since it is well known that the rate of liver cholesterol synthesis and the controlling enzyme in the pathway, 3-hydroxy-3-methylglutaryl-CoA reductase, undergo a circadian rhythm (e.g. Ref. 4), it was of interest to determine whether SCP also exhibits a rhythm. Preliminary findings showed that rat liver SCP undergoes a remarkable circadian variation in amount during a 12-h dark, 12-h light cycle (5). The amount increases 7–10-fold in the dark period and then returns to basal levels by the beginning of the light cycle (5). The present studies were designed to probe basic mechanisms controlling the level and circadian rhythm of SCP.

This report focuses, first, on the synthesis of liver SCP and describes experiments to determine if changes in the relative rate of formation of SCP are responsible for its circadian rhythm. Second, mRNA levels for liver SCP were measured throughout the dark-light cycle to gain insights regarding the role of mRNA in regulating SCP synthesis.

EXPERIMENTAL PROCEDURES

Materials—Radioactive L-[4,5-3H]-leucine (55 Ci/mmol) and L-[35S]methionine (1060-1455 Ci/mmol) were purchased from ICN and Amersham, respectively. [14C]Formaldehyde (57 mCi/mmol), En3-Hance, and Aqualose-II were obtained from New England Nuclear, and reagent grade guanidine HCl from Sigma. Heat-killed, formaldehyde-fixed SaC was purchased from Calbiochem-Behring and Protein A-Sepharose from Pharmacia.

Animals—Female Sprague-Dawley (150-200 g) rats were obtained from Bio-Labs, Inc., St. Paul, MN. The animals were housed two to a cage and allowed free access to Purina Rat Chow and water. The rats were adapted to a 12-h dark, 12-h light cycle for at least 2 weeks prior to use in experiments.

Assay of Liver SCP Amount by Immunoprecipitation—Rabbits were immunized with homogeneous rat liver SCP purified by the method of Dempsey et al. (2). The IgG fraction of the immune serum was purified using a Protein A-Sepharose affinity column (6). The monospecificity of the anti-SCP IgG was confirmed by immunoelectrophoresis and immunodiffusion (7) (see also Fig. 2). Liver homogenates were sonicated for 30 s, followed by complete solubilization with an equal volume of 1% Triton X-100 at 25 °C for 15 min. Immunoprecipitation of homogenates was carried out as follows (8): 100 μl of 1% (v/v) Triton X-100 was layered over 200 μl of anti-SCP IgG (4 mg of protein/ml). This amount of anti-SCP IgG was sufficient to precipitate 8 μg of homogeneous SCP. Then 100 μl of a solution containing an aliquot of the homogenate (5–200 μg of protein) was carefully layered on top of the 1% Triton solution. The assay tube was allowed to stand at 25 °C for 1 h and at 4 °C for 12 h. The immunoprecipitate was collected by centrifugation and washed 3 times with saline, and the total protein was measured. The amounts of immunoprecipitated protein from the various levels of homogenate assayed were plotted versus total protein. The amount of SCP was estimated by comparing the position of the initial linear slope of this plot to one for homogeneous SCP.

Relative Rate of SCP Synthesis—The relative rate of SCP synthesis was measured by immunoprecipitation of SCP from rat liver homogenates. Rats were given an intraperitoneal injection with 0.5 μCi of [3H]leucine or [35S]methionine at designated times in the dark-light cycle. Animals were killed by decapitation 30 min after injection. The livers were removed, and aliquots were frozen at −70 °C. For measurement of radioactivity incorporation into SCP and total protein, an aliquot of each liver was homogenized in 0.1 M phosphate buffer, pH 7.4 (2.5 ml/g). SCP was immunoprecipitated as described above and the immunoprecipitate was submitted to electrophoresis in a cylindrical SDS-polyacrylamide gel as described previously (2). Gels were fractionated into 2-mm portions, dissolved in 0.5 ml of 35% H2O2 at 60 °C and counted in Aqualose II with an efficiency of 50% for both isotopes (cf. Fig. 2). Radioactivity incorporation into total protein was determined by precipitation of an aliquot of each homogenate with cold 10% (w/v) trichloroacetic acid. Precipitates were dissolved in 100 μl of 88% formic acid and the [3H]- or [35S]-levels were measured by liquid scintillation spectrometry with an efficiency of 45 or 65%, respectively. The relative synthetic rate of SCP was estimated by correcting the net precipitable trichloroacetic acid-labeled radioactivity (i.e. in the SCP peak, cf. Fig. 2) as a per cent of total trichloroacetic acid-precipitable radioactivity. Values were corrected for relative counting efficiencies.

RNA Extraction—Total hepatic RNA was isolated by a modification of the method of Deeley et al. (9). One g of frozen liver tissue was homogenized in 20 ml of 8 M guanidine HCl containing 50 mm Tris-HCl, pH 7.6, 10 mM EDTA, and 10 mM diithiothreitol. RNA was
precipitated with 0.5 ml of 1 M acetic acid and 10.0 ml of ethanol for 6 h at -20 °C. The precipitate was dissolved in 10 ml of 6 M guanidine HCl containing 50 mM Tris-HCl, pH 7.6, 10 mM EDTA, and 10 mM dithiothreitol and reprecipitated with 0.25 ml of 1 M acetic acid and 5 ml of ethanol. The precipitations were repeated two additional times. The final RNA pellet was extracted three times with 1 ml of 6 M guanidine HCl, 0.5 ml of ethanol. The precipitate was dissolved in 10 ml of 6 M guanidine HCl and counted in Aquasol-2.

The precipitates, both before and after electrophoretic analysis (13), were treated with IgG obtained from a nonimmunized rabbit. The antigen/antibody mixture was precipitated with 0.3 ml of 2 M potassium acetate and 6 ml of ethanol. RNA was dissolved in 10 ml Tris-HCl, pH 7.6, containing 0.1 M EDTA and stored in liquid N2. The yield of total RNA was approximately 5 mg/g of liver.

**SCP Messenger RNA Translational Assay**—The micrococcal nuclease treated rabbit reticulocyte lysate system of Pelham and Jackson (10) was used for functional assay of SCP mRNA. Total cellular RNA was translated at a concentration of 500 µg/ml according to the procedure of Mikić and Towle (11). Twenty µl of anti-SCP IgG was added to an aliquot of the translation reaction containing 2.5 × 10⁶ cpm of labeled products. The antigen/antibody mixture was incubated at 4 °C for 30 min and adsorbed to 30 µl of a 10% suspension of Sac membranes. The complex was pelleted by centrifugation and the immunosorbed products were washed five times with 0.7 ml of 10 mM Tris-HCl, pH 7.6, 140 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, and 10 mM methionine. Antigen was released from the Sac membranes by incubation with 100 µl of 17 mM Tris-HCl, pH 6.8, containing 1% SDS, 2.5% 2-mercaptoethanol, and 5% glycerol. The Sac membranes were removed by centrifugation and the supernatant was submitted to electrophoresis on a 10-20% gradient SDS-polyacrylamide slab gel according to the method of Laemmli (12). Gels were fixed, impregnated with En'Harce, dried, and exposed for two days at -80 °C to Kodak X-Omat AR-5 film.

Radioactivity present in the translation products was excised (using an ultraviolet photodensiometer) and counted in Aquasol-2. Background radioactivity was measured in adjacent slices and subtracted from radioactivity in translation products. The level of SCP mRNA was estimated by expressing roacetic acid-precipitable radioactivity. All values were corrected for relative counting efficiencies.

**RESULTS AND DISCUSSION**

Circadian Rhythm of SCP Amount and Synthesis—The amount of liver SCP was measured at 3-h intervals in rats adapted to a 12-h dark, 12-h light cycle. SCP levels were quantitated by a specific immunoprecipitation assay using a purified antibody to homogeneous rat liver SCP (2). As shown in Fig. 1, SCP constitutes 1.3 mg/g of total liver protein at the beginning of the dark cycle. By 6 h into the dark cycle, SCP increases dramatically to 6.8 mg/g of total protein. A similar pattern occurs in the light cycle, but the change in SCP amount is only 2-fold.

In order to explore the basic mechanism(s) responsible for the rapid changes in amount of liver SCP, its relative synthetic rate was measured at 3-h intervals during the 24-h dark-light cycle (Fig. 1). At the beginning of the dark cycle, the synthetic rate of SCP is 5% of the incorporation of labeled leucine into total liver protein. Within 3 h, the synthetic rate of SCP surges to 20% of the incorporation into total protein and then falls to 3% by the beginning of the light cycle. Next, the relative synthetic rate increases 2-fold by 3 h into the light cycle and then declines to the level seen at the beginning of the dark period. In both the dark and light periods, the relative synthetic rate of SCP peaks prior to the peak in amount of SCP and otherwise parallels the pattern of SCP concentration. Thus, it appears that changes in the rate of synthesis of SCP are primarily responsible for the circadian rhythm in SCP amount and the degradative rate of SCP probably is constant during the dark-light period. The half-life of SCP was estimated from the decrease in amount during the 6-12-h dark period to be about 2 h. However, the true value for SCP half-life must be less than 2 h as some synthesis of SCP is occurring during this period. It is clear that SCP must have a rapid half-life to account for major changes in amount during, in particular, the dark cycle.

Several experiments were conducted to verify the specificity and accuracy of the relative synthetic rate measurements during the 24-h dark-light cycle. Analysis of immunoprecipitated SCP from a labeled tissue homogenate on a denaturing polyacrylamide gel system showed a single radioactive peak which co-migrated with homogeneous SCP (Fig. 2). No peak of radioactivity was seen when a labeled homogenate was precipitated with IgG obtained from a nonimmunized rabbit.

The quantitative nature of the immunoprecipitation reaction was verified by showing the absence of functional SCP activity in the supernatant after immunoprecipitation (2). Furthermore, addition of a second aliquot of antibody to the first supernatant did not precipitate additional radioactivity. Fatty acids and sterols are known to be associated with SCP (2). If [3H]leucine was converted into fatty acids or sterols in the 30-min labeling period, and these labeled lipids remained associated throughout the procedure, an artificially high relative synthetic rate value would be obtained. Thus, extraction with organic solvents was performed on labeled immunoprecipitates, both before and after electrophoretic analysis (13). No significant amount of radioactivity was removed from the immunoprecipitates by these extractions. To further confirm that the immunoprecipitated radioactive activity was not lipid associated, methionine, an amino acid which is not readily converted into lipids, was injected into rats. The per cent of radioactivity associated with [35S]methionine-labeled immunoprecipitates was approximately the same as SCP labeled with [3H]leucine at both high and low points in the dark-light cycle. Strong evidence that radioactive amino acids were associated throughout the procedure, an artificially high relative synthetic rate value would be obtained. Thus, extraction with organic solvents was performed on labeled immunoprecipitates, both before and after electrophoretic analysis (13). No significant amount of radioactivity was removed from the immunoprecipitates by these extractions. To further confirm that the immunoprecipitated radioactive activity was not lipid associated, methionine, an amino acid which is not readily converted into lipids, was injected into rats. The per cent of radioactivity associated with [35S]methionine-labeled immunoprecipitates was approximately the same as SCP labeled with [3H]leucine at both high and low points in the dark-light cycle. Strong evidence that radioactive amino acids were
Phoresis (2). Marker proteins are: thyroglobulin washed, and analyzed by SDS-urea-7.5% polyacrylamide gel electrophoresis as described under "Experimental Procedures." The liver was homogenized and incubated with anti-SCP IgG (○—○) or IgG from a nonimmunized rabbit (●—●), as also described in "Experimental Procedures." The immunoprecipitate was pelleted by centrifugation, and analyzed by SDS-urea-7.5% polyacrylamide gel electrophoresis (2). Marker proteins are: thyroglobulin (M, 330,000), ferritin (M, 220,000 and 18,500), albumin (M, 67,000), catalase (M, 60,000), lactate dehydrogenase (M, 36,000), and homogeneous SCP (M, 14,200). Actually incorporated into SCP was obtained by purification to homogeneity of labeled SCP from a liver soluble fraction (2). The specific activity of purified SCP was the same as that obtained by immunoprecipitation assay (cf. "Experimental Procedures" and Fig. 2). Following complete hydrolysis of purified SCP and extraction of the hydrolysate with organic solvents, radioactivity remained with the hydrolysate. Related experiments showed that incorporation of radioactive amino acids into total liver protein was linear with time (i.e. from 15-60 min), while the per cent incorporation of radioactivity into immunoprecipitable SCP remained the same. The latter findings indicate that degradation of SCP was not influencing estimation of relative synthetic rate. Also, incorporation of radioactive amino acids into total liver protein, e.g. following a 30-min pulse, was the same at each time point in the dark-light cycle (Fig. 1). Finally, values for relative synthetic rate of SCP were not influenced by lowering or raising the amount of radioactive amino acid injected, i.e. from 0.25 to 1 mCi of [3H]leucine or [35S]methionine. Thus, changes in labeled amino acid pool size were not occurring during these experiments.

Functional Activity of SCP Messenger RNA—The large fluctuations in the relative synthetic rate of SCP (cf. Fig. 1) could reflect alterations in the cellular concentration of SCP mRNA. Alternatively, translational events controlling the availability of template RNA, rate of initiation, or rates of elongation could affect the rate at which SCP is synthesized. To gain insights regarding these possibilities, the functional activity of liver SCP mRNA was measured at 3-h intervals during the 12-h dark, 12-h light cycle (Figs. 1 and 3). Total RNA was isolated from each of 3 rat livers at the specific time points, translated in a rabbit reticulocyte lysate system, and quantitatively immunoselected with a monospecific antibody to SCP. The immunoselected products were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 3). A major band of radioactivity migrated to the position of purified SCP. This band represents 7% of the total translational products; its M\textsubscript{s} was estimated to be 14,000, in close agreement with the M\textsubscript{s} = 14,900 determined from amino acid analysis of homogeneous SCP (2) and sequence analysis (M\textsubscript{s} = 14,184) (1). The minor band with an approximate M\textsubscript{s} = 20,000 (cf. Fig. 3) was not consistently observed and is considered to be an artifact.

However, total RNA yield and translational activity were consistent throughout the dark-light cycle. Although the SCP relative synthetic rate exhibits a large diurnal variation, no significant change was found in the mRNA levels for SCP (Figs. 1 and 3). Therefore, the variation in amount of liver SCP during the dark-light cycle is not due to changes in concentration of SCP mRNA, but instead, to some mechanism specifically altering the efficiency with which SCP mRNA is translated. Some variation was observed in functional SCP mRNA activity (cf. Fig. 1 legend); however, this variation was not methodological and was probably due to inter-animal differences.

In agreement with Gordon et al. (1), the results of Fig. 3 show that liver SCP is not synthesized in a larger precursor form. However, the per cent of SCP mRNA, determined by the translational assay (Fig. 1), is higher than that reported by these co-workers. The reason for this discrepancy is not known, but part of the difference could be due to the use of male rats by Gordon et al. (1). Male rats are known from our studies and those of others to have a lower amount of SCP protein than their female counterparts (e.g. Ref. 14).

The mechanisms controlling the periodicity of the few enzymes that have been studied are diverse. The circadian rhythm of tyrosine aminotransferase has been investigated using drugs to inhibit protein and RNA synthesis (15). It was concluded that the alterations of tyrosine aminotransferase were also due to regulation of the levels of the protein at a post-transcriptional step. However, studies of the mRNA levels for \(\beta\)-hydroxy-\(\beta\)-methyl glutaryl-CoA reductase indicate that the reductase mRNA levels do change during the course of its diurnal cycle (16). In this case, at least part of the diurnal rhythm is due to regulation at some pretranslational level. Therefore, although SCP and \(\beta\)-hydroxy-\(\beta\)-methyl glu-
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Taryl-CoA reductase are both involved in the biosynthesis of cholesterol, the basic mechanism controlling their circadian rhythm is not identical. A few examples of mechanisms of translational control have been investigated. Selective modification of RNA may alter its translation (17). Also, RNA can be compartmentalized and made specifically available for translation (18, 19) and rates of initiation and elongation can be altered in different physiological states (20). Further studies to define details of the structure of SCP mRNA and the components involved in its translation should explain how translational events regulate the circadian variation of SCP.

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