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Rat liver cytosol oxysterol-binding protein

Characterization and comparison with the HTC cell protein

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A cytosol protein that specifically binds cholesterol derivatives oxygenated on the side chain has been demonstrated in rat liver and transformed HTC cells. This protein, of which the sedimentation coefficient is about 8 S, was saturable and showed a high binding affinity (K_d about 5×10^{-9} M) for 25-hydroxycholesterol. Its molecular mass is about 160 kDa. The physicochemical characteristics of this protein were identical whether the model was normal or transformed. This oxysterol-binding protein differs from the well-known sterol carrier proteins.

Oxysterol; Oxysterol-binding protein; Cholesterol metabolism; Sterol carrier protein; (HTC cell, Rat liver)

1. INTRODUCTION

Proteins that specifically bind oxysterols (OSBPs) have been described in several cell models [1–5]. They are thought to play a regulatory role in either sterol synthesis [6], or cell proliferation [5], or both.

Purification of the OSBP is now an essential step for investigation of its biological role. This cannot be performed with cell cultures because of the great quantity of biological material needed.

Rat liver could be a good model for OSBP purification since (i) liver is a cholesterogenic organ in which OSBP might have a major role in the regulation of HMG-CoA reductase activity and (ii) preliminary work by Kandutsch and Thompson [2] seemed to indicate the presence of an OSBP in liver cell cultures.

The present work shows the presence of a high level of OSBP in rat liver cytosol and reports the physicochemical characters of this protein. Moreover, we compare the properties of normal rat liver and HTC cell OSBPs; HTC cells, derived from rat hepatoma, provide a model for studying the OSBP in cancerous cells and we demonstrate that cancerisation does not alter the properties of hepatic OSBP.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

2.1.1. Radioactive compounds

25-Hydroxy[26,27-^{3}H]cholesterol (87 Ci/mmol) (New England Nuclear) was checked for radioactive purity by thin-layer chromatography (two successive elutions in chloroform-methanol-ethanol, 80:2:1.5 then 80:1:0.5, v/v).

2.1.2. Unlabeled sterols and steroids

The following products were kindly donated: cholest-5-ene-3β,25-diol [25-hydroxycholesterol] from Roussel-Uclaf (Romainville, France); cholest-5,20(22)-diene-3β,25-diol [20(22)-dehydro-25-hydroxycholesterol], cholest-5,23-diene-3β,25-diol [23-dehydrocholesterol], cholest-5-ene-3β,7,25-triol and (22*R*)-2β,3β,14,20,22,25-hexahydroxycholest-7-ene-6-one [20-hydroxyecdysone]

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from G. Ourisson and Luu Bang (Strasbourg, France); cholest-5-ene-3β,23(*S*)-diol [23(*S*)-hydroxycholesterol], cholest-5-ene-3β,23(*R*)-diol [23-(*R*)-hydroxycholesterol], cholest-5-ene-3β,24(*R*)-diol [24(*R*)-hydroxycholesterol], cholest-5-ene-3β,24-(*S*)-diol [24(*S*)-hydroxycholesterol] and cholest-5-ene-3β,26(25*R*)-diol [26-hydroxycholesterol] from J.E. Van Lier (Sherbrooke, Canada). The following were of commercial origin: cholest-5-ene-3β-ol [cholesterol], 9α-fluoro-16β-methyl-11,17β,21-trihydroxypregna-1,4-diene-3,20-dione [dexamethasone], 1,3,5-estratriene-3,17β-diol [estradiol] and 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene [squalene] from Sigma; cholest-5-ene-3β,7α-diol [7α-hydroxycholesterol], cholest-5-ene-3β,7β-diol [7β-hydroxycholesterol], 3β-hydroxycholest-5-ene-7-one [7-ketocholesterol] and cholest-5-ene-3β,20α-diol [20α-hydroxycholesterol] from Steraloids; 1-{4-(2-dimethylaminoethoxyl)-phenyl}-1-(4-hydroxyphenyl)-*Z*-phenylbut-1(2)-ene [hydroxytamoxifen] from ICI. 24(*R,S*),25-Epoxycholesterol was synthesized in the laboratory by treating cholest-5,24-diene-3β-ol (desmosterol, Steraloids) with chloroperbenzoic acid according to [7]; 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene-2*S*,3*S*-epoxide [oxidosqualene] and 25-hydroxycholesterol-3β-acetate were synthesized in the laboratory.

2.1.3. Other materials

Aprotinin (5000 KIU/ml), PMSF, leupeptin and TPCK were from Sigma; Tris from Boehringer; EDTA, potassium mono- and diphosphate, saccharose and ethanol from Prolabo; Sephadex LH-20 from Pharmacia and Ultrogel AcA34 from IBF.

2.1.4. Buffers and solutions

Phosphate-buffered saline (PBS) consisted of 10 mM potassium phosphate buffer (pH 7.4) and 0.15 M NaCl supplemented or not with 50 mM NaF; Ringer's solution was from Flow; buffer A comprised 10 mM potassium phosphate buffer (pH 7.4), 1.5 mM EDTA and 50 mM NaF; buffer B consisted of 50 mM potassium phosphate buffer and 1.5 mM EDTA; buffer C was composed of 50 mM Tris (pH 7.4) and 1.5 mM EDTA; buffer D comprised 10 mM Tris (pH 7.4), 1 mM $CaCl_2$ and 1 mM $MgCl_2$.

2.2. *Methods*

2.2.1. HTC cell culture and cytosol preparation

HTC cells are a line of cells established from the ascites form of the rat hepatoma Morris 7288c. Cells were cultured in suspension (spinner) in Swim's medium (Gibco) buffered by 15 mM Tricine (Sigma) and supplemented with 10% newborn calf serum [8]. After seeding at 5×10^4 cells/ml and regular feeding when the cell concentration reached 5×10^5 cells/ml, the cells were collected at near confluency ($8-10 \times 10^5$ cells/ml). After centrifugation at $2000 \times g$ for 10 min, the cell pellet was washed twice with PBS and stored at $-80°C$. After cell thawing, Potter homogenization was performed in buffer D and cytosol was obtained by ultracentrifugation of the homogenate at $220000 \times g$ for 60 min.

2.2.2. Rat liver cytosol preparation

Sprague-Dawley male rats were killed by cervical dislocation. Livers were perfused in situ with Ringer's solution or PBS, to eliminate the maximum of hemoglobin, and stored at $-196°C$. After thawing, they were Potter-homogenized in buffer A containing 1 mM PMSF, 50 μM leupeptin, 50 μM TPCK and aprotinin (40 KIU/ml). The homogenate was centrifuged at $10000 \times g$ for 10 min, followed by centrifugation of the supernatant at $70000 \times g$ for 15 min. Cytosol was obtained by final ultracentrifugation at $220000 \times g$ for 60 min and stored in aliquots at $-80°C$.

2.2.3. 25-Hydroxycholesterol-binding assay

This was performed as described in [5]. Briefly, cytosol proteins were precipitated with $(NH_4)_2SO_4$ at between 25 and 50% saturation for liver and 25–40% for HTC cells. After dissolution of the protein precipitate in buffer B for liver and buffer C for HTC cells, proteins were incubated in the presence of a saturating concentration (20 nM) of 25-hydroxy[^{3}H]cholesterol with or without an excess of 10^{-6} M unlabeled sterol for 2 h at 4°C. Free sterol excess was cleared by Sephadex LH-20 gel filtration. The radioactivity of labeled proteins eluted in the void volume of the column was measured by liquid scintillation counting. Specific binding was calculated from the difference between total binding (radioactivity eluted after labelling in the presence of tritiated sterol alone) and nonspecific binding (radioactivity eluted after labeling in the presence of an excess of unlabeled sterol). In some experiments, an aliquot of the

eluate was subjected to ultracentrifugation on a linear sucrose density gradient (5–20%).

2.2.4. Molecular mass determination of the 25-hydroxy[^{3}H]cholesterol-protein complex

For HTC cells, a 25-hydroxy[^{3}H]cholesterol-protein complex was subjected to chromatography on a calibrated Ultrogel AcA34 column (elution buffer: buffer C + 0.1 M KCl). For rat liver, whole cytosol was layered on a Superose 12 column (FPLC system from Pharmacia) and eluted with buffer B. The collected fractions were precipitated with ammonium sulfate (0–50% saturation) and tested for 25-hydroxy[^{3}H]cholesterol binding. The molecular masses were calculated as a function of the Stokes radius and sedimentation coefficient of the protein as described in [5].

3. RESULTS

3.1. *Saturability and sedimentation coefficient of the OSBP*

Using experimental conditions reported in [5], a protein saturable by 25-hydroxy[^{3}H]cholesterol and with a sedimentation coefficient of 8 ± 0.2 S was demonstrated in the HTC cell model (fig.1). The binding site number was about 900 fmol/mg protein.

We had to modify the methodology for rat liver cytosol protein, particularly in the steps of liver perfusion and cytosol preparation. Fig.2 shows that the binding site number increased from 240 to 420 fmol/mg protein when the liver was perfused with PBS + NaF instead of Ringer's solution (fig.2B). The addition of protease inhibitors in buffer A (homogenization buffer) led to a sharp increase in binding sites (from 420 to 840 fmol/mg protein) (fig.2C). The nonspecific binding disappeared almost totally.

The sedimentation coefficient of the liver cytosol protein was about 7.7 ± 0.2 S.

3.2. *High affinity and stability of the OSBP*

Association of 25-hydroxy[^{3}H]cholesterol with the protein was rapid in the first 90 min of incubation and then increased slowly up to 180 min. The dissociation constant of the 25-hydroxycholesterol-protein complex was determined by Scatchard analysis (fig.3). There was only one population of binding sites. The K_d values of HTC

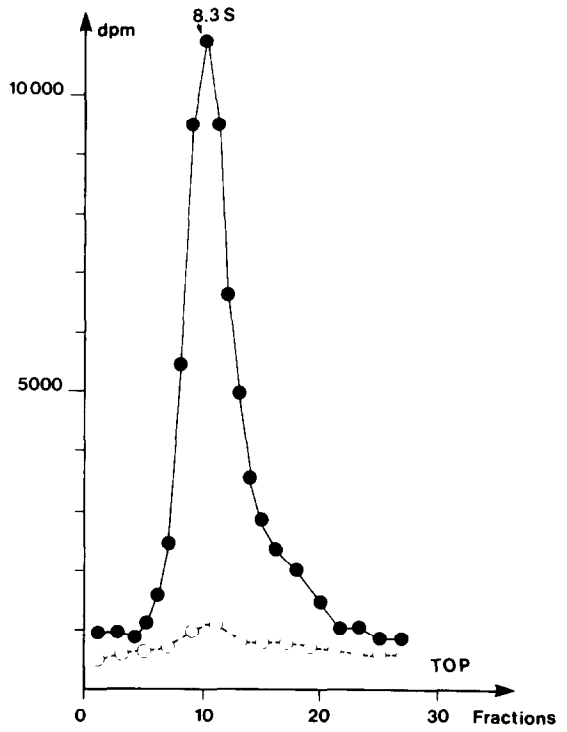

Fig.1. 25-Hydroxy[^{3}H]cholesterol binding to cytosol proteins of HTC cells. Binding assay was performed as described in section 2. After filtration on Sephadex LH-20, an aliquot of the proteins was subjected to ultracentrifugation on a linear (5–20%) sucrose gradient. (●——●) Labeling in the presence of 25-hydroxy[^{3}H]cholesterol (20 nM) alone. (○---○) Labeling in the presence of a 300 nM excess of unlabeled 25-hydroxycholesterol. Binding site number: 900 fmol/mg protein.

cell and rat liver OSBPs were very similar: 6.9×10^{-9} M (maximum binding site number: 960 fmol/mg protein, 25000 sites/cell) and 9.2×10^{-9} M (maximum binding site number: 655 ± 180 fmol/mg protein, mean of 3 experiments), respectively.

When the rats were killed at the maximum level of cholesterol biosynthetic activity (middle of the dark phase), no change in the K_d of the protein was observed, but the maximum binding site number increased to 1800 fmol/mg protein. The 25-hydroxy[^{3}H]cholesterol-protein complex was very stable after 24 h at 0°C. The unliganded protein was unstable after cytosol storage for 24 h at 0°C. By contrast, the 25–50% fraction retained its binding capacity when stored for 24 h at 0°C.

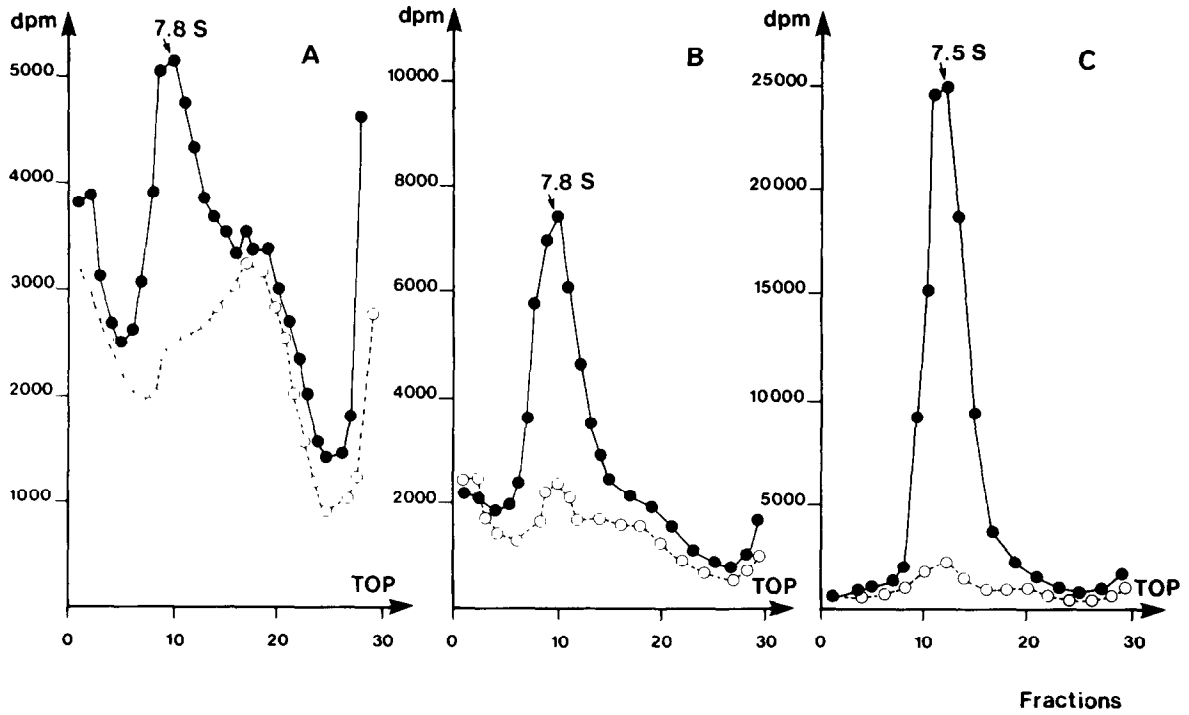

Fig.2. 25-Hydroxy[^{3}H]cholesterol binding to cytosol proteins of rat liver. Binding assay was performed as described in section 2 and the legend to fig.1. (A) Liver washing without PBS and NaF (240 fmol/mg protein); (B) cytosol preparation without protease inhibitors (420 fmol/mg protein); (C) combination of PBS + NaF washing and protease inhibitors during cytosol preparation (840 fmol/mg protein). Symbols as in fig.1.

3.3. *Molecular mass of the complex*

This differed very slightly between the models: 160600 Da for HTC cells (R_S = 47.5 Å) vs 162400 Da for rat liver OSBP (R_S = 48 Å).

3.4. *Specificity of cytosol OSBP*

We compared the relative binding affinities of several derivatives of cholesterol oxygenated either on the nucleus (C-7) or on the side chain. Fig.4 shows the competition curves obtained after incubation of the 25–50% fraction of rat liver cytosol proteins with 20 nM 25-hydroxy[^{3}H]cholesterol and increasing concentrations (10^{-8} to 10^{-5} M) of unlabeled sterol. No noticeable differences were found when the relative binding affinities of rat liver and HTC cell proteins were compared (table 1): both proteins preferentially bound the compounds oxygenated on the side chain, with a low affinity for the compounds oxygenated at C-7. The hepatic protein discriminated between the two (*R*) and (*S*) epimers of 23-hydroxycholesterol and, to a lesser extent, those of 24-hydroxycholesterol. 20α-Hydroxycholesterol was the best competitor in both models. 26-Hydroxycholesterol and 24(*R,S*),25-epoxycholesterol were less competitive than 25-hydroxycholesterol and 23(*S*)-hydroxycholesterol. The poor solubility of the Δ20(22) derivative of 25-hydroxycholesterol precluded testing its competition as for the other sterols. Nevertheless, it did not appear to be recognized by the liver OSBP, whereas the Δ23 derivative was an excellent competitor, as effective as 25-hydroxycholesterol itself.

The addition of a hydroxyl function at C-7 noticeably decreased the affinity of 25-hydroxycholesterol for the OSBP: 7,25-dihydroxycholesterol was a very poor competitor, as well as 7α- and 7β-hydroxycholesterol and 7-ketocholesterol (IC_{50} in the same range, 6×10^{-6} M). Neither oxidosqualene nor squalene was able to bind OSBP. Tested at 10^{-6} M, the following steroids were not recognized: cholesterol, 20-hydroxyecdysone, dexamethasone, estradiol, and hydroxytamoxifen, as well as the 25- and 1,25-hydroxylated derivatives of vitamin D_3 (only studied in HTC cells).

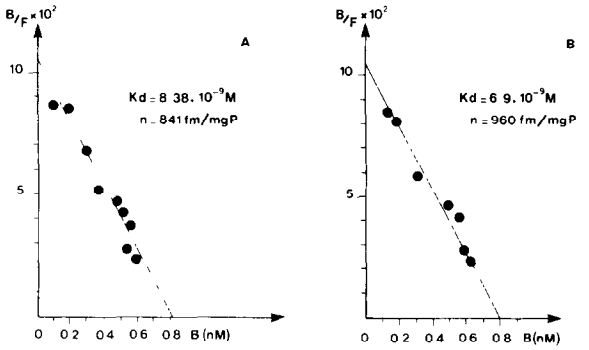

Fig.3. Scatchard plot of 25-hydroxy[^{3}H]cholesterol binding to the cytosol proteins of HTC cells and rat liver. Binding assays were performed, as described in section 2, in the presence of increasing concentrations (0 to 30 nM) of tritiated 25-hydroxycholesterol. Non-specific binding was determined with a 10^{-6} M (rat liver) or 3×10^{-7} M (HTC cells) excess of unlabeled 25-hydroxycholesterol. (A) Rat liver, (B) HTC cells.

4. DISCUSSION

The preliminary results obtained by Kandutsch and Thompson [2] on 25-hydroxycholesterol binding by fetal mouse liver cells were not convincing since, in the established line FL83B, two proteins of respectively 8 S and 5 S sedimentation coefficient were able to bind the tritiated 25-hydroxycholesterol but the characters of saturability and specificity of the binding were not firmly established. Moreover, in primary cultures of liver cells, the 5 S binding was largely predominant. Here we have demonstrated that the OSBPs of adult rat liver and HTC cell cytosol have physicochemical characteristics very similar to those of OSBPs demonstrated in L cells [1,2,9], normal human lymphocytes [4] and rat embryo fibroblasts [5]: sedimentation coefficient about 8 S, high affinity (K_d about 5×10^{-9} M), molecular mass of the complex (about 160 kDa).

The characterization of the hepatic protein has required some modification of the methodology described in [5]:

(i) The protein appears to be very sensitive to the presence of phosphate ions and NaF during cytosol preparation, indicating that it may have to be phosphorylated to bind its ligand. Nevertheless, when the 25-hydroxy[^{3}H]cholesterol-protein complex or the unliganded protein was treated with

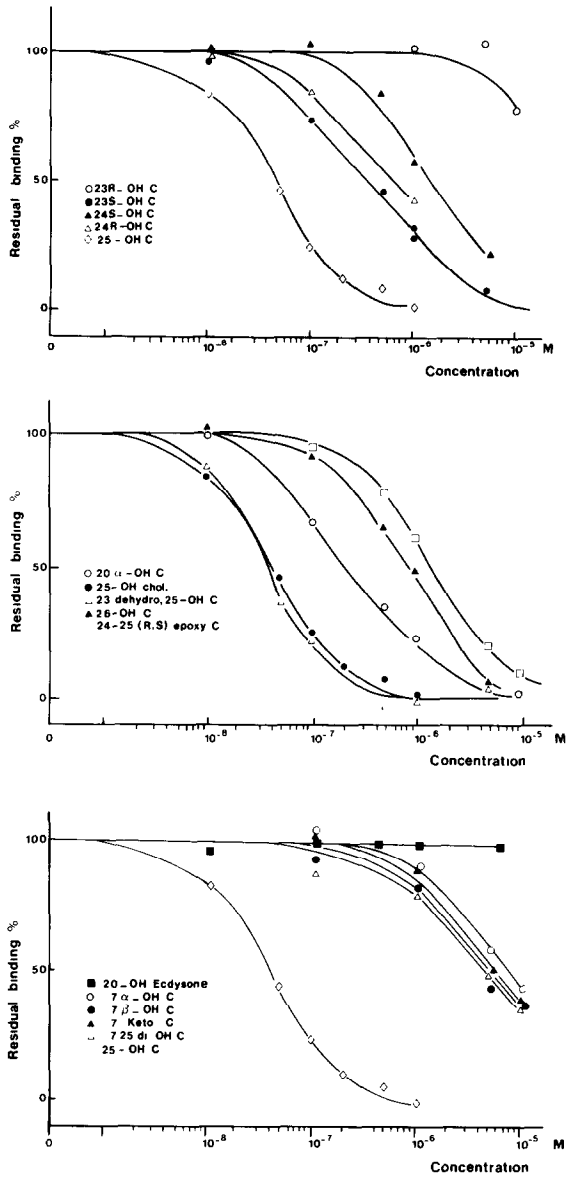

Fig.4. Competition of several oxygenated sterols with tritiated 25-hydroxy[^{3}H]cholesterol in rat liver cytosol.

alkaline phosphatases from *E. coli* or calf intestine, neither the quantity of 25-hydroxy[^{3}H]-cholesterol bound to the protein nor the sedimentation coefficient of the complex was modified.

(ii) The addition of protease inhibitors eliminated nonspecific binding and increased the specific 8 S labeling. These inhibitors were only necessary in the steps preceding cytosol protein

Table 1

Relative binding affinities of HTC cell and rat liver OSBPs for several sterols

	Rat liver		HTC cells	
	IC_{50} (M)	RBA	IC_{50}	RBA
25-OH-chol	5×10^{-8}	100	3×10^{-8}	100
23-Dehydro-25-OH-chol	4.5×10^{-8}	111		
20α-OH-chol	2.1×10^{-7}	23.8	8×10^{-8}	37.5
26-OH-chol	10^{-6}	5	3.4×10^{-7}	8.8
24,25(*R,S*)-epoxychol	1.7×10^{-6}	2.9		
23(*S*)-OH-chol	4×10^{-7}	12.5		
23(*R*)-OH-chol	–	0		
24(*R*)-OH-chol	8×10^{-7}	6.3		
24(*S*)-OH-chol	1.8×10^{-6}	2.8		
7-Keto-chol	6×10^{-6}	0.8	2×10^{-6}	1.5
7β-OH-chol	6×10^{-6}	0.8	7×10^{-6}	0.4
7,25-Dihydroxy-chol	6×10^{-6}	0.8		
7α-OH-chol	9×10^{-6}	0.5		

IC_{50}, sterol concentration yielding 50% competition with 20 nM 25-hydroxy[^{3}H]cholesterol; RBA, binding affinities relative to 25-hydroxycholesterol (100)

precipitation by ammonium sulfate. The 5 S binding protein in liver cell cultures [2] could be explained by the absence of protease inhibitors in the experiments.

Contrary to a classical steroid hormone receptor, the 8 S sedimentation coefficient of the complex did not change in a high-ionic-strength buffer or with a temperature increase (20°C for 120 min), indicating that there was no 'activation' of the protein.

The relative binding affinities were determined for 2 groups of oxysterols: 7-oxygenated sterols and side-chain-oxygenated sterols. With respect to the sterols tested in both models (rat liver and HTC cells), similar results were obtained: 7-oxygenated derivatives were weakly recognized contrary to the cholesterol derivatives hydroxylated on the side chain. Not only the presence of a hydroxyl function on the side chain was important for sterol affinity but also the localization and spatial configuration of the function:

(i) The most competitive sterols were, in decreasing order: 25-OH > 20α-OH > 23(*S*)-OH > 24(*R*)-OH > 26-OH.

(ii) Amongst the two (*R*) and (*S*) epimers of 23-hydroxycholesterol, only the (*S*) epimer was recognized by OSBP; 24(*R*)-hydroxycholesterol had a better affinity than its 24(*S*) epimer.

Desaturation of the side chain in position 23 did not change the affinity of 25-hydroxycholesterol. Desaturation in position 20(22) reduced the sterol solubility and did not allow extensive competition experiments.

Rat liver OSBP was also able to bind 24(*R,S*)-epoxycholesterol; the 24(*S*) epimer was recently described by Saucier et al. [10] as a possible endogenous sterol regulating cholesterol biosynthesis. Unfortunately, we were unable to use the pure 24(*S*) and 24(*R*) epimers, which could explain the relatively low relative binding affinity of the mixture.

The modification of the nucleus of 25-hydroxycholesterol (addition of a hydroxyl function on C-7, or acetylation of the 3β-hydroxyl function) prevented the binding of 25-hydroxycholesterol to OSBP. Nevertheless, Taylor et al. [6] report that the 3β,25-dihydroxycholest-5-en-7-one has a great affinity for the L cell OSBP.

Sterol carrier proteins (SCPs) are major regulators of lipid metabolism and transport, and have been described in liver and adrenals. SCP_1 [11] stimulates the conversion of squalene to lanosterol, whereas SCP_2 [12,13] (or FABP) activates the conversion of lanosterol into

cholesterol. In order to differentiate the hepatic OSBP from SCPs, we tested its affinity for squalene and oxidosqualene. Neither compound was bound by rat liver or HTC cell OSBP. Although SCP_2 appears to be able to bind 25-hydroxycholesterol [14], the differences between the molecular masses of SCPs (12–18 kDa) and OSBP clearly exclude a similarity between the proteins. Circadian variations of SCP_2 have been described [15], with an increase (× 7) in the dark period. Preliminary experiments with hepatic OSBP show similar results, i.e. an increase (× 3) of the binding site number without K_d modification in the dark period.

The biological role of the OSBP has not been firmly demonstrated. The main hypothesis is that the protein is a regulator of sterol biosynthesis, acting at the level of HMG-CoA reductase. Taylor et al. [6] have in fact demonstrated a correlation in L cells between the capacity of oxysterols to inhibit HMG-CoA reductase and their capacity to bind OSBP. This hypothesis cannot be excluded in a strong cholesterogenic organ such as the liver, and OSBP could be another regulatory protein in the sterol metabolism of liver. The low affinity of OSBP for cholesterol derivatives oxygenated on C-7 seems to exclude that 7α-hydroxycholesterol, classically described as the first intermediary compound in biliary acid synthesis, could be involved in liver HMG-CoA reductase regulation.

A loss of feedback control of HMG-CoA reductase by exogenous cholesterol has been described in hepatomas [16]. Nevertheless, recent works on sterol synthesis regulation in HTC cells demonstrate that the intracellular mechanism of cholesterol synthesis regulation by lipoproteins is intact in these cells [17] and that the phosphorylation-dephosphorylation mechanism of the reductase is functional [18]. It is thus not surprising that no difference was found between normal rat liver and cancerous liver OSBP.

In conclusion, rat liver seems to be a good model for OSBP purification since the physicochemical characters of the hepatic protein are comparable to those of OSBP found in cell cultures.

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