Sterol and fatty acid regulatory pathways in a *Giardia lamblia*-derived promoter: evidence for SREBP as an ancient transcription factor

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Abstract **The sterol regulatory element binding-proteins (SREBPs) are transcription factors that regulate the genes of lipid metabolism. Cholesterol and unsaturated fatty acids regulate SREBPs. *Giardia lamblia* (GL) is an intestinal parasite and one of the earliest derived members within the eukaryotic lineage. GLs exist as trophozoites and cysts. Growth in cholesterol depletion induces transcription of cyst-wall protein (CWP) genes that are upregulated during encystation. The hypothesis was investigated that SREBP-like pathways have a role in cwp gene transcription. Chinese hamster ovary cells were transfected with a cwp-2 promoter reporter construct. Incubation with cholesterol or oleate reduced cwp-2 mediated gene transcription to about half of the control. Incubation in sterol-depleted media, or in the presence of either an inhibitor of intracellular cholesterol movement or inhibitor of cholesterol synthesis, increased gene expression up to 3-fold. Overexpression of SREBPs increased reporter gene activity 2.5-fold. In the absence of functional SREBPs, cwp-2 was not regulated by cholesterol. Footprint analysis of cwp-2 reveals three novel binding sites for mammalian SREBPs with no homologies in other species or humans. The data show that SREBP binds to and can modulate transcription of a regulatory element from an ancient eukaryote and suggest the existence of an SREBP homolog in GL.**—Worgall, T. S., S. R. Davis-Hayman, M. M. Magana, P. M. Oelkers, F. Zapata, R. A. Juliano, T. F. Osborne, T. E. Nash, and R. J. Deckelbaum. **Sterol and fatty acid regulatory pathways in a *Giardia lamblia*-derived promoter: evidence for SREBP as an ancient transcription factor. *J. Lipid Res.* 2004.** 45: **981–988.**

Supplementary key words cyst-wall protein • encystations • sterol regulatory element binding protein

Giardia lamblia (GL) is a flagellated protozoan that resides in the intestines of different vertebrate hosts. GL is the most frequent cause of waterborne diarrhea worldwide (1). The disease can cause severe malnutrition (2), and infection has been correlated with impaired cognitive development in less-developed countries (3). GL, considered one of the most ancient eukaryotes (4–6), is a diplomonad with a simple life cycle. Infection occurs after the ingestion of cysts contaminating water or food or through person-to-person transmission. Excystation in the duodenum results in the development and release of trophozoites that proliferate in the small intestine. Cysts develop as the trophozoites are swept down the intestine. Importantly, in vitro cholesterol starvation induces trophozoites to differentiate into environmentally resistant cysts (7), a process that occurs in the lower small intestine after the absorption of bile salts and cholesterol. The precise mechanism of cyst wall formation is unknown, but its assembly is preceded by the synthesis, packing, and release of secretory components destined for the cyst wall, such as cyst-wall proteins 1 (CWP-1) and 2 (CWP-2). Upon formation, the cysts are passed in the feces, and the life cycle is completed.

The sterol regulatory element binding-proteins (SREBPs) were discovered in mammalian cells (8). SREBPs are pivotal transcription factors of the genes of lipid metabolism. Cholesterol starvation activates a proteolytic cascade that

Manuscript received 22 January 2004.

Published, JLR Papers in Press, February 16, 2004.
DOI 10.1194/jlr.M400024-JLR200

Abbreviations: CHO, Chinese hamster ovary; CWP, cyst wall protein; GL, *Giardia lamblia*; mSREBP, mature SREBP; RLU, relative light units; SRE, sterol regulatory element; SREBP, sterol regulatory element binding protein.

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results in the generation of the transcriptionally active mature SREBPs (mSREBPs) (9–11). Cholesterol and unsaturated fatty acids are known negative regulators of transcriptional and posttranscriptional regulation (12–16). SREBP is also present in *Drosophila melanogaster* (17), where regulation occurs through palmitic acid and phosphatidylethanolamine (18, 19). Of interest, neither GL nor *D. melanogaster* synthesize cholesterol.

Cholesterol starvation induces the transcription of SREBP-dependent genes important in lipid and membrane homeostasis in mammalian cells. We hypothesized that similar mechanisms exist in GL, resulting in the regulation of CWP. SREBP-dependent regulation of the upstream regulatory region of cwp-2 was investigated. As a model system, Chinese hamster ovary (CHO) cells were stably transfected with a cwp-2 promoter construct linked to the luciferase reporter gene. Our experiments indicate that the conditions known to increase or decrease transcriptionally active SREBPs similarly increase and decrease the expression of cwp-2 mediated gene transcription. In agreement with these findings, cwp-2 expression is not regulated in cells that do not process SREBP to the transcriptionally active mSREBP. Also, overexpression of mSREBP increases cwp-2 mediated gene transcription. Human SREBP-1 binds to three distinct sites in the cwp-2 promoter in a footprinting assay, and deletion of one of these sites abolishes sterol-mediated decrease in the cwp-2 transcription.

Our data provide evidence that gene regulatory sequences that originated from the earliest lineage of the eukaryotic tree can be regulated by the SREBP pathway, and that SREBP binds to unique binding sites within the cwp-2 promoter. The data suggest that SREBP-mediated transcriptional regulation is an ancient pathway.

MATERIALS AND METHODS

Materials

CHO cells were obtained from American Type Culture Collection (Rockville, MD). Ethanol, fatty acid BSA, cholesterol, 25-hydroxycholesterol (25-OH cholesterol), and fatty acids were obtained from Sigma (St. Louis, MI). All cell culture reagents and neomycin (G418) were obtained from Life Technologies, Inc. (Grand Island, NY). All organic solvents were purchased from Fisher Scientific Co. (Springfield, NJ).

Plasmids

The cwp-2 plasmids contain a 446, 300, 200, or 122 bp upstream regulatory sequence (to be called 'promoter' for the purpose of this paper) of the cwp-2 gene, which was amplified from plasmid pMM109 (20) by PCR using Precision Plus (Stratagene, La Jolla, CA) and cloned into pCR-Blunt (Invitrogen, Carlsbad, CA). To facilitate subcloning, all primers for upstream flanking sequences contain an ASP718 site at the 5′ end and an Nco1 site at the 3′ end. Placement of the Nco1 site required the addition of a CC dinucleotide prior to the ATG start codon (21). For mammalian transfection, the promoters were subcloned into the pGL2 basic vector (Promega, Madison, WI). Additional plasmids were constructed by subcloning upstream regulatory sequences that contained deletions in Sites I and II into the pGL2 basic vector. The deletions (D1, D2, D3, and D4) have been described (21). D1 comprises all of Site II, D2 comprises partially Site II, D3 comprises half of Site I, and D4 comprises the second half of Site I. None of the promoters contains a TATA-box or sterol regulatory elements (SREs). The pWLNeo and renilla luciferase plasmids were obtained from Stratagene, Inc. For activation studies with overexpressed SREBP-1a, a CMV promoter expression clone encoding amino acid 1-490 of SREBP-1a and a CMV β-galactosidase transfection control reporter gene were used as described (15, 22). The pSyn-SRE plasmid, used as a control to demonstrate the response of a mammalian SRE-promoter to cholesterol, contains a generic TATA-box and three SREs (15).

Cell culture and stable and transient transfections

CHO cells were grown in F12-nutrient mixture medium containing 10% FBS, 1% glutamine (v/v), 1% penicillin/streptomycin (v/v), and 10% FBS (v/v) at 37°C in humidified carbon dioxide (5%). To obtain stable transfectants, cells were plated in 12-well plates at 50% confluency and transfected for 5 h in the presence of serum-free DMEM, with pCWP-2 (1 μg/well) and pWLNeo (0.25 μg/well) using Lipofectamine 2000 (Gibco, Invitrogen) (1.5 μl/well). Cells were then incubated for 2 days in growth medium. On day 3, neomycin-containing medium (400 μg/ml) was added. Selection for neomycin-resistant colonies was continued for 3 weeks. Pooled clones were analyzed for luciferase expression. Experiments were performed with pooled clones as well as with cells derived from a single clone. Cells were grown in the presence of 400 μg/ml neomycin. For experimental use, cells were plated in the absence of neomycin at least 24 h ahead in regular growth medium. PCR was carried out to ensure that the upstream regulatory sequence of cwp-2 was stably transfected into cells. Primers were: 5′3′ (forward) TACCGAAGCAAAGCCATTTTCTTTTG (within cwp); 5′3′ (reverse) GGCGTATCTCT (within luciferase coding region). The PCR product of 519 bp was isolated and sequenced.

Transient transfections were carried out in 12-well plates at 90% confluency. Cells were transfected with plasmids D1–D4 (1 μg/well) [described in detail in ref. (21)] and a renilla luciferase control plasmid (Stratagene) (0.25 μg/well) or with CMV-SREBP-1 (amino acids 1-490) and a β-galactosidase (0.1 μg/well) transfection control reporter gene using Lipofectamine 2000 for 5 h in the presence of serum-free DMEM.

Enzyme assays

Cells to be analyzed for luciferase activity were lysed in lysis buffer A containing 0.1% Triton X-100, 50 mM Hepes, 10 mM $MgSO_4$, pH 7.7. Cells were scraped, collected, vortexed, and briefly centrifuged to pellet cell debris. An aliquot was used to measure luciferase activities in a luminometer (Berthold LB 9501, Wallace, Inc., Gaithersburg, MD) with a luciferin reagent from Promega. In experiments using stable transfections, luciferase activity in relative light units (RLUs) was divided by protein content (mg/ml) for each extract. In transient transfections, cells were lysed with dual luciferase assay buffer (Promega). Renilla luciferase activity and luciferase activity were determined separately. Luciferase activity, in RLUs, was divided by renilla luciferase activity for each extract.

Protein determination

The amount of cellular protein was determined by the Biorad method, and BSA was used as a standard.

DNA footprinting assay

DNase I footprinting was performed as described (23). SREBP-1 was incubated with 10 fmol of the ^{23}P-labeled DNA probe (nt −126 bp to +5 of CWP) (~2.5×10^4 cpm) on ice for 15 min in 50 μl of a buffer containing 25 mM Tris-HCl (pH 7.9), 6.25 mM $MgCl_2$, 50 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10% glycerol, and 2% polyvinyl alcohol. A solution containing 5 mM $CaCl_2$ and 10 mM $MgCl_2$ (50 μl) was added, followed by incubation at room temperature for 60 s. DNase I (25–375 ng in 1–15 μl) was added, and the samples were incubated for an additional 60 s, after which 50 μl of stop solution (1.0% SDS, 20 mM EDTA, 200 mM NaCl, and 100 μg/ml tRNA) was used to terminate the reaction. The samples were extracted with phenol/$CHCl_3$, precipitated with ethanol, and analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography.

Data analysis

Statistical significance was calculated by paired *t*-tests. Unless otherwise indicated, results are given as mean ± SD. All experiments were repeated on different days at least three times and each time in triplicates.

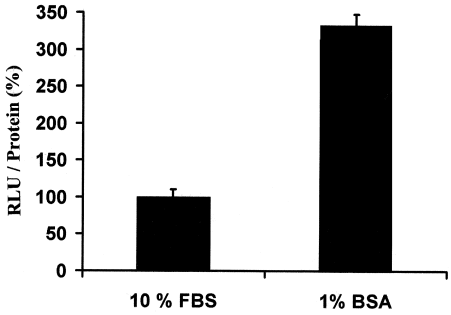

Fig. 1. Serum-free media induces cwp-2 mediated gene transcription in CHO cells. CHO cells stably transfected with the 466 bp cwp-2 promoter construct linked to the luciferase reporter gene were incubated for 8 h in the presence of control conditions (10% FBS) or serum-free experimental conditions (1% BSA). After cell lysis, luciferase activity was analyzed and expressed as a ratio of protein content. Data represent the average (±SD) of at least four different experiments, each performed in triplicate. Compared with controls, incubation with 1% BSA significantly increased luciferase expression ($P < 0.05$) measured as RLUs.

RESULTS

Serum-free medium induces cwp-2 mediated gene transcription in stably transfected mammalian cells (CHO)

In GL, cholesterol starvation induces the transcription of cwp-2, beginning 90 min after induction in the encystation medium, maximizing at 5–24 h, and greatly diminishing at 48 h (24, 25). It has been shown that 466 bp of the upstream regulatory region of the GL cwp-2 gene is sufficient to control regulated transcription (26). We chose to investigate the transcription of the cwp-2 promoter in a mammalian system because regulation of SREBP is well defined in mammalian cells. Stable transfectants for the 466 bp cwp-2 promoter were generated in CHO cells. Inasmuch as transcription in GL differs in fundamental ways compared with mammalian cells, and the requirements for regulated transcription are not fully defined (27, 28), it was unknown whether transcription would occur in CHO cells. Of note, in GL, cwp-2 is expressed only during encystation (encystation conditions in vitro). Cwp-2 is not expressed when cells are in the vegetative growth state, and it is not expressed when cells are mature cysts.In contrast, stable CHO transfectants for cwp-2 exhibit continuous cwp-2 mediated luciferase expression in the presence of cholesterol rich growth conditions. Therefore, it is possible to investigate conditions that increase and decrease cwp-2 mediated gene transcription. The first experiments demonstrated that the cwp-2 mediated luciferase reporter gene is transcribed and regulated by conditions of high and low nutrient content. CWP-CHO cells were incubated for 8 h in the presence of 10% FBS (control) or 1% BSA (experimental condition). Incubation with 1% BSA induces cwp-2 mediated gene transcription 3-fold (**Fig. 1**). In contrast, control cells expressing a β-galactosidase reporter gene are not regulated under the same conditions.

Cwp-2 mediated gene transcription is decreased by cholesterol and oleic acid and increased by inhibitors of intracellular cholesterol movement (U18666A) or inhibitors of cholesterol synthesis (compactin)

Next, we investigated if decreasing transcriptionally active mSREBP levels in mammalian cells would affect cwp-2 mediated gene transcription. In mammalian cells, cholesterol, and polyunsaturated fatty acids either together or alone are known to decrease mSREBP, leading to a decrease in SRE-mediated gene transcription. CWP-CHO cells were incubated for 5 h in the presence of cholesterol, together with 25-OH cholesterol (1 μg/ml/10 μg/ml). Compared to control conditions, cholesterol and 25-OH cholesterol significantly ($P < 0.05$) decreased cwp-2 mediated luciferase transcription to 66% (±6%), and oleic acid reduced cwp-2 mediated gene expression to 54% (±9%) ($P < 0.05$) (**Fig. 2**), similar to levels of inhibitions found for mammalian SRE-mediated gene expression (Fig. 2, inset) (15). Then, we investigated if conditions that increase cellular mSREBP levels would increase cwp-2 mediated gene transcription. Incubation for 5 h with U18666A, an inhibitor of intracellular cholesterol movement that induces intracellular cholesterol depletion (29), or compactin, an inhibitor of HMG-CoA reductase that decreases intracellular cholesterol synthesis, increases cwp-2 mediated gene transcription. The results of cholesterol-mediated inhibition of transcription and U18666A-mediated induction of transcription are similar to results obtained using the mammalian HMG-CoA synthase promoter, which is a classical SREBP responsive promoter (Fig. 2, inset) (30, 31).

Then the original 466 bp promoter was deleted to 300, 200, and 122 bp to determine the minimally necessary sequence that conveys cholesterol responsiveness. All four promoter constructs demonstrated equally decreased transcription in response to incubation with cholesterol (data not shown). Of note, in GL 64 bp upstream of cwp-2

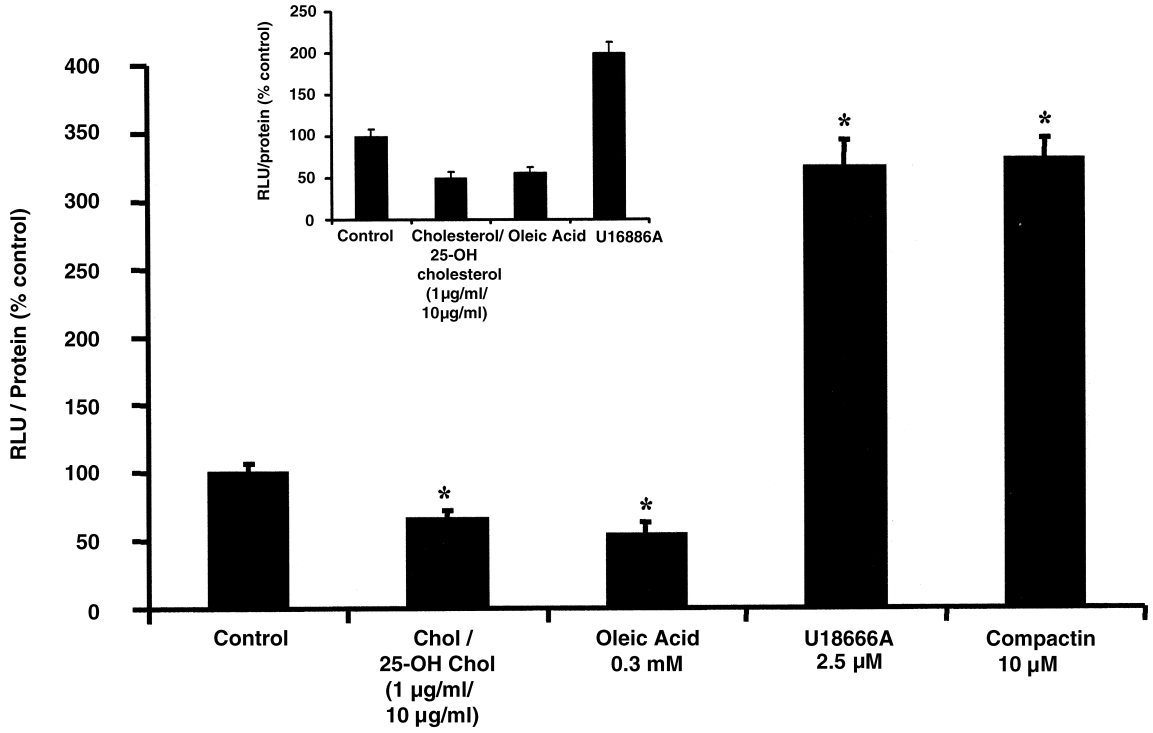

Fig. 2. Cwp-2 mediated gene transcription in CHO-cells is suppressed by cholesterol and hydroxycholesterol and oleic acid and is increased by inhibitors of intracellular cholesterol movement and HMG-CoA reductase. CHO cells stably transfected with the 466 bp cwp-2 promoter construct linked to the luciferase reporter gene were incubated for 8 h in the presence of 1% BSA (control condition), cholesterol (10 μg/ml)/25-OH cholesterol (1 μg/ml), oleic acid (0.3 mM), U18666A (2.5 μM), or compactin (HMG-CoA reductase inhibitor) (10 μM). After cell lysis, luciferase activity was analyzed and expressed as a ratio of protein content. Data represent the average (±SD) of three different experiments, each performed in triplicate. Compared with the control incubation with cholesterol, or oleic acid significantly decreased and U18666A and compactin significantly increased luciferase expression ($P < 0.05$) measured as RLUs. Inset: Comparison of sterol regulatory element binding protein (SREBP)-mediated regulation in a CHO cell line stably transfected with a mammalian HMG-CoA synthase reporter gene (15).

fully control regulated transcription of cwp-2 (21). These data are consistent with short 5′ untranslated regions described in GL mRNA sequences so far (32).

Cwp-2 mediated gene transcription not regulated by cholesterol in sterol regulation defective cells that do not process mSREBP

Sterol regulation defective cells, SRD-6 (33), do not process SREBP to its mature form due to a mutation in Site-2 protease. This mutation results in constitutively low mRNA levels of SREBP-regulated genes even under conditions of sterol deprivation (8, 34). Consequently, SRD-6 cells are cholesterol auxotrophs and a good tool to test the dependency of gene transcription on regulated levels of mSREBP. SRD-6 cells were transiently transfected with the 466 bp cwp-2 promoter construct and a β-galactosidase control plasmid before the medium was changed to experimental conditions. Reporter gene activity was not affected by the addition of cholesterol/25-OH cholesterol within 8 h (**Fig. 3**). These data are consistent with the hypothesis that suppression of cwp-2 mediated gene transcription by cholesterol is dependent on physiological processing of SREBP, resulting in the generation of mSREBP.

Overexpression of mSREBP increases cwp-2 mediated gene transcription

Next, we investigated if overexpression of mSREBP can increase cwp-2 mediated gene transcription (**Fig. 4**). Cells that stably express the 466 bp cwp-2 mediated luciferase were transiently transfected with 100–300 ng of plasmid encoding the transcriptionally active mSREBP-1 (amino acids 1–490) (22). Sixteen hours after transfection, cwp-2 mediated luciferase expression was measured. Compared with controls transfected with a β-galactosidase plasmid, cells transfected with mSREBP showed approximately a 2–2.5-fold increase of cwp-2 mediated luciferase expression (Fig. 4).

SREBP-1a binds to three distinct sites in the cwp-2 promoter

To investigate if SREBP can bind to the cwp-2 promoter, a DNA footprinting assay was carried out with recombinant SREBP-1a. End-labeled DNA probes were prepared from the 122 bp promoter that had been shown as equally regulated by sterols as the 466 bp promoter. The results of a representative experiment are shown in **Fig. 5**. There are three DNase I-protected regions that do not correspond to known SRE elements. They are located in reference to the ATG transcription site at nt (−20) to (−42) (Site I), nt (−52) to (−71) (Site II), and nt (−83) to (−110)

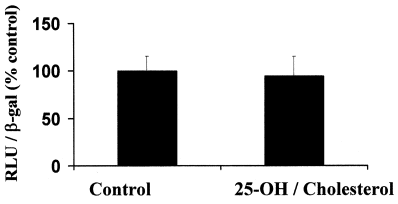

Fig. 3. Cholesterol fails to decrease cwp-2 mediated gene transcription in SRD-6 cells. SRD-6, sterol regulation defective CHO cells that do not generate mature SREBP (mSREBP), were transiently transfected with the 466 bp CWP-promoter construct linked to the luciferase reporter gene. Sixteen-hour posttransfection cells were incubated for up to 8 h in the presence of 1% BSA (control condition) or cholesterol (10 μg/ml)/25-OH cholesterol (1 μg/ml). After cell lysis, luciferase activity was analyzed and expressed as a ratio of protein content. Data represent the average (±SD) of three different experiments, each performed in triplicate. Compared with the control, incubation with cholesterol did not affect luciferase expression measured as RLUs.

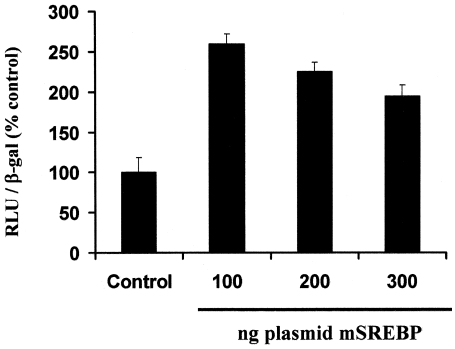

Fig. 4. Increased levels of mSREBP correlate with increased cwp-2 mediated gene transcription. On day 1, CHO cells that are stable transfectants for cwp-2 were transiently transfected for 5 h with 100, 200, or 300 ng of a plasmid for mSREBP-1 and a plasmid for β-galactosidase as a transfection control vector. Cells were incubated for 8 h in a medium containing 10% FBS. On day 2, cells were incubated for 8 h in the presence of 1% fatty acid free BSA. Cells were harvested, lysed, and analyzed for luciferase activity (measured in RLUs) and β-galactosidase expression. Data are expressed as percentage of control and represent the average (±SD) of three different experiments, each performed in triplicate.

(Site III) (Fig. 5). All three binding sites are not classical SRE sites and are located within the last 110 bp upstream of the translation of the CWP protein. Next, the role of Sites I and II in cholesterol-mediated regulation of transcription experiments were investigated. Four plasmids harboring deletions (21) comprising of Site I, Site II, or between both sites were generated (**Fig. 6**). Transient transfection experiments demonstrate the loss of sterol regulation in the deletion D1 (Site II) and the persistence of sterol regulation, although to a lower extent in plasmids D2, D3, and D4.

DISCUSSION

GL, an enteric parasite, represents the earliest branching lineage of descent among eukaryotes (4, 5). Infection begins when trophozoites colonize the small intestine of vertebrate hosts. When the nutritional environment changes, trophozoites differentiate into infective cysts,

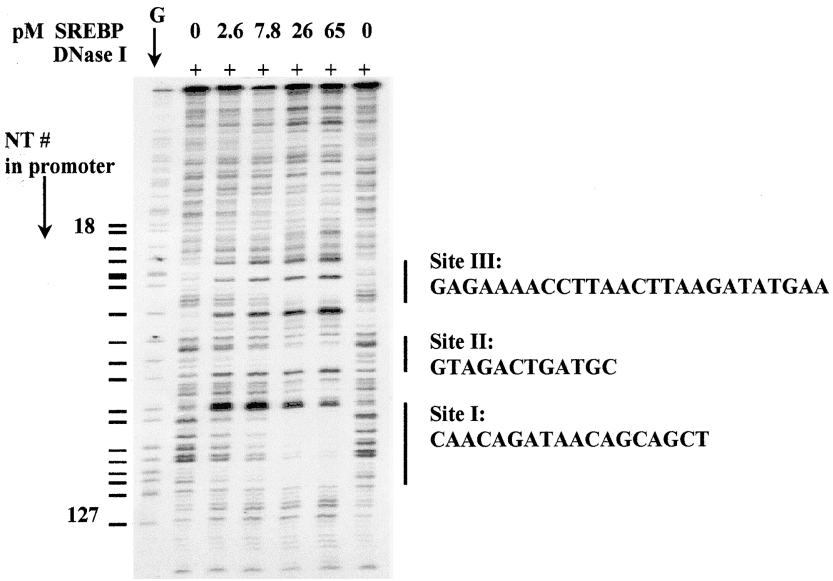

Fig. 5. SREBP-1a binds to three distinct sites within the upstream regulatory region of cwp-2. DNase I footprint analysis with SREBP-1a P^{32} end-labeled probes were prepared by digesting the plasmid encoding the 122 bp promoter with Sca I and Xba I. This fragment was labeled and subsequently cut with Pvu I to produce a 613 bp fragment labeled at the Xba I site. This probe was used for the G reactions and the footprinting reactions. Lane 1 corresponds to the G reactions, and the Gs are labeled corresponding to their position in the cwp-2 vector. The two lanes designated by a "0" (lanes 2 and 7) correspond to reaction conditions in which the probe was incubated in the presence of DNase I only. Lanes 3–6 correspond to reaction conditions in which the probe was incubated with increasing amounts (2.6, 7.8, 26, and 65 pM) of recombinant SREBP-1a prior to the DNase I incubation. The three DNase I-protected regions are designated as Sites I, II, or III.

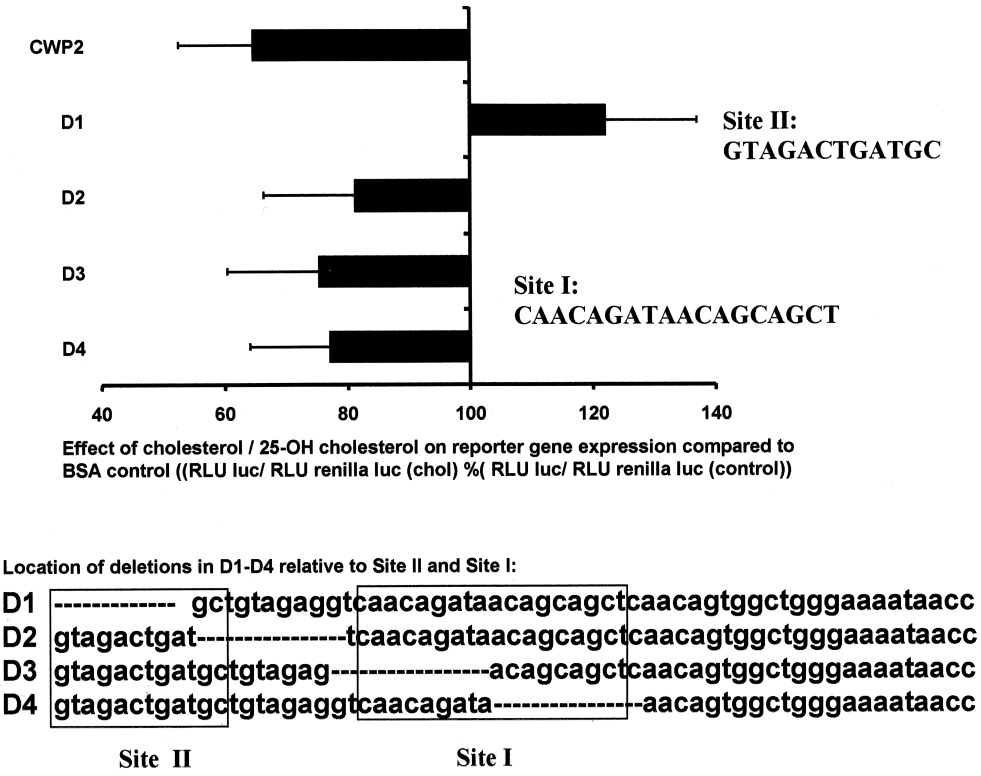

Fig. 6. Deletion of Site II results in the loss of sterol regulation. On day 1, Chinese hamster ovary (CHO) cells were transiently transfected for 5 h with plasmids cyst wall protein (CWP)-2 (full length promoter), D1 (deletion Site II), D2 (deletion between Site II and Site I), D3 (partial deletion Site I), and D4 (partial deletion Site I). Renilla luciferase (0.25 μg/ml) was cotransfected as a transfection control. After transfection, cells were incubated overnight in a growth medium containing 10% FBS. On day 2, cells were incubated for 6 h in the presence of 1% fatty acid free BSA (control) or in the presence of cholesterol (10 μg/ml)/25-OH cholesterol (1 μg/ml). Cells were harvested, lysed, and analyzed for luciferase and renilla activity [measured in relative light units (RLUs)]. Data demonstrate the effect of cholesterol/25-OH on luciferase-mediated gene transcription, expressed as a percentage of control (BSA) and representing the average (±SEM) of eight different experiments, each performed in triplicate.

which are excreted in the feces. Cholesterol deprivation induces encystation and initiates the transcription of CWPs (7, 20), including CWP-2, one of the three known CWPs (20, 25, 35). The precise mechanism of cholesterol-mediated encystation is not known.

In mammalian cells, SREBPs are key transcription factors that regulate genes of cholesterol, fatty acids, and carbohydrate metabolism, all essential components of membrane lipids (8). Deprivation of cholesterol and unsaturated fatty acid induces transcriptionally active mSREBP, which increases the synthesis of cholesterol and fatty acids. Cellular levels of mSREBP are decreased by sterols and unsaturated fatty acids (9, 12, 15), thereby providing a mechanism for the feedback regulation of lipid synthesis and membrane lipid composition (8).

The rationale to study the effect of SREBP on a GL promoter is that in mammals, as well as in GL, cholesterol deprivation induces the transcription of genes that contribute to membrane synthesis. Of note, GL is unable to synthesize cholesterol and other lipids and has a limited ability to alter host-derived lipids, and yet it senses and responds to cholesterol deprivation (36). Of interest, SREBP and its regulation have been well characterized in *D. melanogaster*, another organism that does not produce cholesterol (19). Although there are significant differences between transcription in GL and mammalian cells (21, 28), the cwp-2 mediated luciferase reporter gene was continuously transcribed, which allowed the assessment of induction as well as suppression of cwp-2 mediated transcription. Initial experiments demonstrated that cwp-2 mediated transcription is increased by nutrient deprivation (i.e., incubation with 1% BSA) when expressed in CHO cells (Fig. 1). This finding prompted further experiments that assessed conditions known to regulate the SREBP transcription factors in mammalian cells.

Our results strongly support SREBP regulation of this construct in CHO cells. As anticipated, transcription was induced by cholesterol deprivation and inhibited by conditions of cholesterol excess. In cells that cannot process SREBP, cholesterol did not alter transcription, whereas transfection of the transcriptionally active SREBP led to increased transcription of cwp-2 mediated gene transcription. In addition, there are other similarities in the regulation of cwp-2 mediated gene expressin in GL and CHO cells, such as the time it takes for peak expression and the sufficiency of short 5′ UTR. In GL, cwp-2 induction peaks around 5 h (25). When expressed in CHO cells, the cwp-2 mediated gene expression also occurs maximally at 5 h. In comparison, encystation induces expression of gmby2, a transcription factor recently implicated in the

transcriptional regulation of CWP-1, CWP-2, and CWP-3, maximally within 24 h (37). In our experiments, 122 bp are sufficient to regulate the cwp-2 mediated gene transcription. Gene regulation in GL has also been demonstrated to necessitate short sequences (21, 26, 27, 38).

In mammalian cells, SREBPs regulate gene transcription by binding to SRE found in the promoters of genes of cholesterol and fatty acid metabolism. Both cwp-2 and SRE promoters are similar with regard to substrate (cholesterol and unsaturated fatty acid) and magnitude of increase in reporter gene expression when incubated in a nutrient-deficient medium. GL does not synthesize cholesterol, but SREBP can induce a pathway that relates to "survival." Possibly, Giardia "lost" the pathway of sophisticated sterol synthesis, or maybe more likely the mammalian system adapted this more rudimentary pathway and evolved it to the synthesis of lipids. Of note, *D. melanogaster* also does not synthesize cholesterol, yet SREBP is present and regulated by palmitic acid and phosphatidylethanolamine (19).

Regulation of the cwp-2 promoter in CHO cells is distinct from mammalian SRE promoters with regard to timeframe of regulation and magnitude of repression. Induction and repression of cwp-2 mediated gene transcription occurs maximally within 5 h, whereas mammalian SRE-mediated gene transcription peaks at 16 h (31). Cholesterol or unsaturated fatty acids decrease the cwp-2 mediated gene transcription in CHO cells to 66% and 54% within 5 h compared with a decrease to 30% of control within 16 h in SRE-mediated gene transcription. Thus, inhibition of SRE-mediated gene transcription is more efficient than inhibition of cwp-2 transcription, possibly reflecting species differences (i.e., expression of a GL-derived promoter construct in mammalian cells) and ongoing unregulated transcription. Cwp-2 transcription is not decreased by cholesterol when expressed in SRD-6 cells that are defective in the generation of mSREBP (Fig. 3) (8, 34). The data demonstrate that physiological processing of SREBP is necessary in order for cholesterol to decrease cwp-2 mediated gene transcription in the mammalian model system. But mSREBP is not necessary to ensure baseline transcription of the cwp-2 mediated reporter gene.

Overexpression of mSREBP increases cwp-2 mediated gene transcription up to 2.5-fold 16 h after transfection (Fig. 4). The data suggest that high cellular levels of transcriptionally active SREBPs increase the transcription of cwp-2 mediated gene transcription. Of interest, in preliminary experiments, transfection and expression of SREBP-1a into GL led to an increase of cwp-2 transcription by 2–4-fold (data not shown).

Human recombinant SREBP-1 binds to three distinct sites within the last 110 bp of the cwp-2 promoter sequence, as shown by DNaseI footprinting analysis (Fig. 5). These sites are not classical SRE sites, and to our knowledge they are not present in humans or other species. Site I and II are located within the short 5′ region necessary for transcription in GL in general (32) and in cwp-2 specifically, where the last 64 bp upstream of ATG are sufficient for transcription (21). When Site II is deleted, transcription persists, but regulation secondary to sterols is abolished (Fig. 6). These data strongly imply Site II in sterol-mediated regulation of the cwp-2. Efforts are currently underway to identify and isolate a possible Giardia SREBP homolog. Of interest, the C(T/A)ACAG sequence that is found within Site I has been demonstrated to bind to gmyb2 (35, 37), a GL protein closely related to the Myb family of transcription factors that regulate developmental processes in fungi, plants, and mammals (39–41).

In this paper, we present evidence that SREBP can control transcription of a GL-derived promoter sequence that regulates the transcription of cwp-2, a gene important in encystation. The majority of experiments were carried out in CHO cells that were stably transfected with a cwp-2 reporter gene. CHO cells served as a model system in which the SREBP pathway has been well studied. Transcription of cwp-2 is increased in conditions of sterol depletion and decreased in the presence of cholesterol and oleic acid. In the absence of mSREBP, cholesterol does not suppress cwp-2 mediated gene expression. Furthermore, human SREBP binds to the cwp-2 promoter in a DNase footprinting analysis, and deletion of one binding site (Site II) abolishes cholesterol-mediated gene regulation. Together, the data demonstrate that SREBP can regulate a promoter derived from an ancient eukaryote and support the existence of an SREBP homolog in GL.

The authors gratefully acknowledge Fannie Keyserman for her excellent technical assistance. This work was supported by the National Institutes of Health Grant HL-40404 and the American Heart Association Grant-in-Aid 0255656 N (T.S.W.). The work was also supported in part by National Institutes of Health Grant T32DK-07715 (T.S.W.).

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