HLH106, a *Drosophila* Sterol Regulatory Element-binding Protein in a Natural Cholesterol Auxotroph*

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In mammalian cells, sterol regulatory element-binding proteins (SREBPs) coordinate metabolic flux through the cholesterol and fatty acid biosynthetic pathways in response to intracellular cholesterol levels. We describe experiments that evaluate the functional equivalence of mammalian SREBPs and the insect homologue of SREBP-1a, HLH106, in both mammalian and insect cell culture systems. HLH106 binds to both palindromic E-boxes and direct repeat sterol regulatory elements (SREs) efficiently, suggesting that it has a dual DNA binding specificity similar to the mammalian proteins. The amino-terminal “mature” protein activates transcription from mammalian SREs in both mammalian and *Drosophila* tissue culture cells. Additionally, HLH106 also requires a ubiquitous regulatory co-activator to efficiently activate transcription from mammalian SREs. These properties are shared with its mammalian counterparts. When expressed in mammalian cells, the carboxyl-terminal portion also localizes to perinuclear membranes similar to mammalian SREBPs. Furthermore, membrane-bound HLH106 is proteolytically processed in response to intracellular sterol levels in mammalian cells in an SREBP cleavage-activating protein-stimulated fashion. The presence of an SREBP homologue in *Drosophila* whose processing is regulated by intracellular sterol levels when expressed in mammalian cells suggests that related processing machinery exists in insect cells. This is notable, since insects are reportedly incapable of *de novo* sterol biosynthesis.

Mammalian sterol regulatory element-binding proteins (SREBPs),
activate transcription of key genes whose expression is required for cholesterol uptake and biosynthesis as well as fatty acid metabolism (1). Synthesized as ~125-kDa precursor proteins, SREBPs are anchored to intracellular membranes of the endoplasmic reticulum (ER) and nuclear envelope via two membrane-spanning domains. The mature, transcriptionally active portion of the molecules is released from the membrane through an elaborate mechanism that requires two sequential proteolytic events, the first of which is regulated by the sterol needs of the cell. This initial cleavage event is catalyzed by an as yet unidentified sterol-responsive protease that clips the SREBP molecule at a site within its ER luminal loop. Furthermore, this primary processing step is regulated at least partially by an ER membrane protein designated SREBP cleavage-activating protein (SCAP) (2). An interaction between the carboxyl-terminal domains of SREBPs with SCAP is required for sterol-regulated proteolysis (3).

The molecular mechanism by which SCAP regulates SREBP cleavage, however, is unknown. SCAP contains a putative sterol-sensing domain and has been proposed to stimulate the cleavage activity of the first SREBP protease only upon intracellular sterol deprivation. This initial ER luminal sterol-regulated cleavage divides the SREBP protein into two halves, both of which presumably remain anchored to the membrane by a single membrane span. The amino-terminal intermediate SREBP is then further processed by a large, hydrophobic, zinc metalloprotease designated the site 2 protease (4). Following this second cleavage, which occurs within transmembrane domain 1, the soluble mature transcription factor is released, translocates to the nucleus, and activates transcription of genes involved in cholesterol and fatty acid metabolism (1, 5).

This elegant mechanism for regulation allows the mammalian cell to tightly control production of cholesterol such that it occurs only when the cell absolutely requires new sterol accumulation. In this sense, processing of SREBPs in response to metabolic demand represents a novel signal transduction pathway, mediated by cholesterol or oxysterol derivatives and possibly other intermediates or products of the mevalonic acid pathway.

The recent identification of an apparent SREBP-1a homologue in *Drosophila*, termed HLH106 (6), is intriguing for several reasons. The *hlh106* gene encodes a protein of similar size and predicted domain structure to the mammalian SREBPs, including a putative acidic transcriptional activation domain, a basic helix-loop-helix leucine zipper (bHLHZip) DNA binding and dimerization domain, two central membrane-spanning domains, and an extensive carboxyl-terminal portion (see Fig. 1A).

HLH106 mRNA is expressed in a variety of tissues throughout *Drosophila* development, and initial experiments suggested that the protein may also be processed from a precursor form to a mature form in *Drosophila* (6). These properties are to be expected if it is a true functional homologue of the mammalian SREBPs.

Insects are thought to be incapable of *de novo* cholesterol biosynthesis, since they appear to lack key enzymes, such as...
squalene synthesis, which catalyzes the first committed step in the synthesis of cholesterol (7). Insects also do not have a well-documented pathway for receptor-mediated uptake of lipoprotein particles (8). In fact, the biological role of cholesterol in insect cells is further confused by observations that suggest certain insect cell lines can divide indefinitely in culture in the absence of exogenously supplied cholesterol and do not explicitly require cholesterol molecules in their membranes for cell function and division (9).

Cholesterol or closely related steroids, however, are required for steroid hormone biosynthesis (notably edysone) and are also added post-translationally to generate the mature form of the hedgehog protein (10). This sterol modification is absolutely required for proper hedgehog signaling in Drosophila during embryonic development.

Since they appear to be cholesterol auxotrophs, Drosophila must obtain cholesterol from dietary sterols, such as ergosterol from yeast and phytosterols from plants. Thus, a major question arises as to why an organism that is a cholesterol auxotroph contains a transcription factor (HLH106) that has a domain structure reminiscent of the mammalian proteins that regulate genes involved in cholesterol uptake and biosynthesis. As a first step toward addressing this question, we have initiated a study of the functional properties of HLH106.

In the present studies, we have compared its activity directly to the mammalian SREBPs in a variety of assays. The results demonstrate that the Drosophila protein has similar DNA binding and transcriptional activation properties as its mammalian counterparts. Additionally, we demonstrate that when HLH106 is expressed in mammalian cells, the precursor form is subject to regulated processing by cellular sterol levels. Thus, the signals required for sterol regulation are conserved in the HLH106 protein and can additionally be recognized by the mammalian regulatory processing machinery.

**EXPERIMENTAL PROCEDURES**

**Cells and Media**—Human embryonic kidney (HEK) 293 cells were obtained from Dr. Luis Villarreal (University of California, Irvine). Chinese hamster ovary CHO-7 cells were obtained from Peter Edwards (UCLA). The sources for other cell lines have been described previously (11). Cell culture materials were purchased from Life Technologies, Inc., except for Dulbecco’s modified Eagle’s medium and Ham’s F-12, which were obtained from Irvine Scientific, and Shields and Sang SL2 medium which was purchased from Sigma. Lipoprotein-deficient serum was prepared by ultracentrifugation as described (12).

**Vectors encoding fusion proteins between the green fluorescent protein (GFP) and the carboxyl-terminal membrane domains of the SREBPs were made as follows.** Oligonucleotides were synthesized and used in PCR reactions to amplify products using pSREBP-1a, pTK-HSV-SREBP-1a, and pBS-HLH106 as templates for pCMV-CSA10, pRC-CS1C, and pPAC-Sp1, respectively. This releases the COOH-terminal domain of SREBP2 from transmembrane domain 1 through the end of the open reading frames. Oligonucleotides contained SalI links, with the GFP-SREBP fusion to facilitate in frame cloning. The reverse oligonucleotide was the same utilized to generate pCMV-CSA10 for GFP-SREBP-1a and SREBP-2 GFP fusions were compared by direct fluorescence.

Vectors encoding fusion proteins between the green fluorescent protein (GFP) and the carboxyl-terminal membrane domains of the SREBPs were constructed as described below. The carboxyl-terminal portion of SREBP2 from transmembrane domain 1 through the end of the open reading frames. Oligonucleotides contained SalI links, with the GFP-SREBP fusion to facilitate in frame cloning. The reverse oligonucleotide was the same utilized to generate pCMV-CSA10 for GFP-SREBP-1a and SREBP-2 GFP fusions were compared by direct fluorescence.

**Cell Culture and Transfection Experiments**—HepG2 cells were cultured in modified essential medium containing 10% fetal bovine serum and supplements at 5% CO2 and transfected with DNA by the calcium phosphate co-precipitation method as described previously (11).
were harvested on day 3, 48 h post-transfection, by pooling dishes of scraped cells followed by three cycles of freeze-thawing.

*Drosophila* SL2 cells were cultured in Shields and Sang M3 media (Sigma) containing 10% heat-inactivated fetal bovine serum and transfected as described previously (17).

CHO-7 cells were maintained in a 1:1 mixture of Dulbecco’s modified Eagle’s medium:Ham’s F12 medium containing 5% delipidated serum at 8% CO₂. Cells were transfected by a standard calcium phosphate co-precipitation method as used for our other cell lines (17) with plasmids containing a neomycin resistance gene at 3 µg/dish. After transfection, colonies were allowed to grow out as stable transformants, which were doubly selected with 50 µg/ml G418 and 25 µg/ml 25-hydroxycholesterol, as described below. Selections for 25-hydroxycholesterol resistance were repeated three times with the identical cell populations.

CV-1 cells were cultured and transfected as described previously (11) with the following modifications. Following transfection with µEGFP vectors at 5 µg/dish on day 1, cells were refed with media containing 600 µg/ml G418 on day 3 and subsequently selected by feeding the dishes normal media containing 600 µg/ml G418 every 3 days until drug-resistant colonies were detected. G418-resistant colonies were subsequently pooled (roughly 10–30 colonies/100-mm dish), and cells were maintained in media containing 350 µg/ml G418 and processed for direct fluorescence confocal microscopy as described below.

HEK 293 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and transfected as described elsewhere (18) at 8% CO₂. Cells were transfected on day 0 by our standard calcium phosphate method as above with 5 µg/ml of pTK-HSV expression vectors, as indicated in the individual figure legends. On day 3, cells were refed identical media containing 700 µg/ml G418 and reseeded every 3 days until colonies were detected. Stably transfected cell populations were subsequently pooled (roughly 10–30 colonies/100-mm dish), and the cells were maintained in medium containing 350 µg/ml G418.

**Enzyme Assays—**Luciferase activities were measured in a luminometer with a luciferin reagent from Promega Biotech using equivalent volumes of cell extract. β-Galactosidase assays were performed by standard colorimetric assay using equivalent volumes of cell extract with 2-nitrophenyl-β-D-galactopyranoside as the substrate. The ratio of luciferase activity in relative light units was divided by the β-galactosidase activity (X₄₃₅ units) to obtain normalized relative light units/β-galactosidase units.

**Protein Purification—**Recombinant SREBP-1a and HLH106 proteins were purified from extracts of *E. coli* by metal chelation chromatography as described previously (17). The purity and yield of the proteins were assessed by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining. The concentration of active protein was determined by an electrophoretic mobility shift assay as described below with varying amounts of purified protein in the presence of a vast excess of [32P]-labeled oligonucleotide probe containing an E-box element (CAACGTG).

**Oligonucleotide Probes and Gel Mobility Shift Assay—**All oligonucleotides were double-stranded and contained GATC overhangs to facilitate labeling with polynucleotide kinase and γ-[32P]ATP. The E-box oligonucleotide used to determine protein activity and depicted in Fig. 2 was based upon the DNA binding site selection protocol of Kim et al. (19). The E-box, human LDL receptor, and hamster HMGC-CoA synthase oligonucleotides were described previously (20).

Gel mobility shift assays were performed as described (20) with the following modifications. Binding reactions were performed in the presence of 0.5 ng of labeled probes and equimolar quantities (0.07 pmol) of active recombinant human SREBP-1a (aa 1–490) or *Drosophila* HLH106 (aa 1–446). Samples were loaded onto a native polyacrylamide gel and analyzed by an electrophoretic mobility shift procedure as described previously (20).

**Cell Fractionation and Immunoblot Analysis—**Protein expression in transfected cell lines was analyzed as follows. For HepG2 cells, five 60-mm dishes of cells were transfected as indicated above with 5 µg/dish of the corresponding SREBP expression plasmid. Four hours prior to harvest, cells were refed with media with or without 25 µg/ml ALLN and were harvested 4 h later. Cells were fractionated (24), and membrane and nuclear extracts were subjected to immunoblot analysis as described above with the following modifications. Filters were blocked overnight with 10% nonfat dry milk plus 5% fetal calf serum in Tris-buffered saline (50 mM Tris, pH 7.4, 150 mM NaCl), and the HSV-Tag antibody (Novagen) was used as the primary antibody (0.4 µg/ml) in blocking buffer.

**Fluorescence Microscopy—**Stable cell lines expressing GFP-SREBP fusion proteins were generated in CV-1 cells as described above and plated at a density of 20,000 cells/dish on coverslips in media containing 10% fetal bovine serum and 300 µg/ml G418. GFP fusion proteins were excited according to the manufacturer’s instructions (CLONTECH), and direct fluorescence imaging was performed using a Bio-Rad MRC 1024 UV confocal laser microscope and a 60x oil immersion objective (UC Irvine Optical Biology Core).

**Protein Alignment and Sequences—**Protein sequences were aligned using Clustal X software (25) in combination with SeqVu software (The Garvin Institute of Medical Research, Sydney, Australia). The accession numbers are U00968, U09103, L16995, U02031, U12330, and U38238 for human SREBP-1, hamster SREBP-1, rat ADD1, human SREBP-2, and *Drosophila* HLH106, respectively.

**RESULTS**

HLH106 Can Recognize both E-boxes and Sterol Regulatory Elements (SREs) in Vitro—*The Drosophila* HLH106 and mammalian SREBP proteins appear to contain a similar organization of functional domains (Fig. 1A). This includes an acidic amino-terminal potential transactivation domain, a putative bHLHZip domain, and two likely transmembrane spans. The most similar domain is the bHLHZip domain, which exhibits 71% amino acid identity with human SREBP-1 (Fig. 1B). Interestingly, the fly protein has more residues in common with SREBP-1 than SREBP-2.

Additionally, residues at its extreme amino and carboxyl termini are more similar to the SREBP-1a isoform than to SREBP-1c. Of crucial importance, the HLH106 protein contains a unique tyrosine residue within its basic domain, which corresponds to an arginine in other related bHLHZip proteins. This tyrosine is a signature amino acid that identifies the SREBP subfamily of bHLH transcription factors. This residue is largely responsible for the ability of SREBP-1c to bind to both direct repeat SREs, as well as to the canonical palindromic E-box and equivalency of protein loading were ensured by staining the nitrocellulose membranes with Ponceau S (Sigma). Immunoblot analysis for SREBP-1 and HLH106 expression was performed using the IgG-2A4 monoclonal antibody that was raised against a peptide fragment encoding amino acids 301–407 of human SREBP-1a (22), anti-mouse horse-
sequence, which is the archetypal recognition site for the bHLHZip proteins (19). Thus, we predicted that HLH106 would bind both SRE and E-box elements efficiently, based solely upon amino acid conservation with the putative bHLHZip domain.

To test this hypothesis, we expressed a truncated version of the HLH106 cDNA that encodes amino acids 1–446, which, based on previous data for the mammalian SREBPs, represent the predicted mature transcriptionally active molecule (Fig. 2A). The recombinant protein was purified from E. coli and compared directly with similarly expressed human SREBP-1c for specificity in DNA site recognition using a standard gel shift DNA binding protocol (Fig. 2B). Both proteins bound similarly to a consensus E-box element (compare lanes 2 and 3) as well as to the direct repeat SRE elements from the promoters for both the human LDL receptor (lanes 5 and 6) and hamster HMG CoA synthase genes (lanes 8 and 9). These experiments demonstrate that HLH106 can recognize both an E-box and mammalian SRE elements and further suggests that the protein has binding site specificity similar to that of the human SREBPs.

**HLH106 Activates Transcription from Mammalian SREs in Human HepG2 Cells**—The in vitro DNA binding results indicate that the *Drosophila* protein has a similar DNA binding specificity as the mammalian SREBPs. To evaluate its potential transcriptional activation properties, we prepared a vector designed to express the putative “mature” amino-terminal portion in mammalian cells. Reporter constructs containing known SREBP regulated promoters were then transfected into human HepG2 cells in the presence and absence of either the HLH106-expressing plasmid or a similar plasmid that expresses the mature form of human SREBP-1a (14, 27).

The results demonstrate that the HLH106 protein activates both the human LDL receptor (Fig. 3A) and a hamster HMG CoA synthase (Fig. 3B) promoter-reporter plasmids in a dose-dependent fashion. At the 300-ng level of the HLH106 plasmid, maximal activation of 94- and 32-fold were observed for the LDL receptor and HMG CoA synthase promoters, respectively. It should be noted that the magnitude of activation was consistently 3–4-fold lower than that observed when equal amounts of an expression plasmid encoding a similar version of the human SREBP-1a protein was transfected.

To ensure that the difference in activation potential between the two proteins was not simply a result of differential levels of protein expression, we examined their expression levels directly by an immunoblotting procedure (Fig. 3C). Similar levels of immunoreactive material of the appropriate size were produced in cells transfected with either plasmid. ALLN treatment of untransfected cells was utilized to stabilize the endogenous mature SREBPs as reported previously (22). Although this treatment had no apparent stabilizing effect upon the overexpressed proteins, the artificially truncated HLH106 co-migrated with both transfected mature SREBP-1a and endogenous mature SREBPs (compare lanes 2, 4, and 6). The predicted molecular mass of the mature mammalian SREBPs is ~51 kDa, and they migrate aberrantly in SDS-polyacrylamide gel electrophoresis at ~68 kDa (29). This anomalous migration through SDS is also a characteristic of the mature HLH106, since it comigrates with the mammalian proteins despite a predicted M, of ~49,000.

**HLH106 Requires a Co-regulator to Stimulate LDL Receptor Transcription in Drosophila SL2 Cells**—Mammalian SREBPs require the assistance of a ubiquitous transcription factor in order to efficiently activate transcription (11). To test the possibility that HLH106 also requires a ubiquitous co-regulatory factor, we performed transient transfection assays in *Drosophila* Schneider SL2 cells, using an assay developed to study the co-regulatory requirement for the mammalian SREBPs (11).
Using this system, we examined the ability of HLH106 to activate transcription in the absence and presence of an exogenously supplied SREBP co-regulator.

Previous studies demonstrated that Sp1 was the required SREBP co-regulatory factor for the LDL receptor promoter (11). To evaluate the fly protein for a similar requirement, a vector designed to express amino acids 1–446 of the HLH106 protein in Drosophila SL2 cells was co-transfected with the human LDL receptor reporter plasmid described above in the presence and absence of a vector designed to express human Sp1 in insect cells. Neither SREBP-1a (aa 1–490) nor HLH106 (aa 1–446) was capable of activating transcription from the LDL receptor promoter in the absence of Sp1 (Fig. 4A, open squares and circles, respectively). However, when the Sp1 expression vector was also included, both SREBPs activated the LDL receptor, and the degree of activation was similar to that obtained from similar experiments performed in mammalian cells in Fig. 3 (maximal activation of 198-fold for SREBP-1a, 85-fold for HLH106). Consistent with our earlier studies, the Sp1 expression vector by itself was very inefficient in activating expression from the LDL receptor (data not shown).

Thus, transactivation of the LDL receptor promoter by HLH106 is dependent upon co-transfection of an Sp1-expressing plasmid, which indicates that, similar to the mammalian SREBPs, the HLH106 protein is an inefficient transcriptional activator without an additional ubiquitous co-regulatory factor. Once again, we performed an immunoblot analysis for SREBP expression in nuclear extracts of transfected cells to determine if the difference in activation by the two SREBPs was due to a difference in the levels of protein expressed from the different vectors (Fig. 4B). The level of expression of each of the transfected SREBP proteins was similar, but the mammalian protein was expressed at a slightly higher level (compare lanes 2 and 3 to lane 1). Thus, the lower activation level mediated by the HLH106 from the LDL receptor promoter is possibly due at least in part to the lower expression level. However, the parallel differences in activation between the mammalian (where protein expression was equivalent) and Drosophila transfection experiments suggest other fundamental differences in the ability of HLH106 to activate transcription from mammalian
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SRE-containing promoters. Interestingly, nuclear extracts from SL2 cells express an immunoreactive species that may represent a precursor length HLH106 species at 25-hydroxycholesterol. Spencer et al. showed that high levels of the corresponding proteins were expressed in transfected cells cultured in the absence of 25-hydroxycholesterol. This was not due to a failure of the G418-resistant cells to express the SREBP-1 or HLH106 proteins, since immunoblot analysis showed that high levels of the corresponding proteins were expressed in transfected cells cultured in the presence of 25-hydroxycholesterol. We were unable to directly compare the levels of overexpression of the SREBP-1 proteins to that of SREBP-2, since these proteins are immunologically distinct. However, we did perform similar experiments with both SREBP-1a:GFP- and SREBP-2:GFP-expressing cell lines, which showed identical growth patterns to those described above, and expression of both proteins was roughly equivalent as determined by direct GFP fluorescence (data not shown). This experiment suggests that neither SREBP-1a (aa 1–490), SREBP-1c (aa 1–466), nor HLH106 (aa 1–446) is capable of activating all of the genes required for CHO-7 cell growth in the presence of oxysterols.

**HLH106 Is Targeted to Intracellular Membranes in both Mammalian and Drosophila Cells**—The experiments presented thus far were designed to analyze the function of the amino-terminal portion of HLH106, which contains the bHLHZip and transcriptional activation domains. The following experiments were designed to examine the function of the carboxy-terminal portion of HLH106, which contains its putative transmembrane domains and signals for precursor processing. To determine if the COOH-terminal domain would function as a membrane anchor, the carboxyl domain of HLH106 was fused to the coding sequence of the GFP in a vector that also expresses the selectable G418 resistance marker. For mammalian SREBPs, this portion of the protein has previously been shown to be necessary and sufficient for membrane localization (24). For comparative purposes, similar vectors were prepared for human SREBP-1a, SREBP-1c, and SREBP-2. These GFP-SREBP expression plasmids were transfected into CV-1 cells, and G418-resistant colonies were selected in medium containing serum lipoproteins (Fig. 6, A–D). Under these conditions, properly targeted SREBP proteins should reside in the membranes of the ER and nuclear envelope. Analysis of the expression patterns by confocal fluorescent microscopy indicated that all of the GFP-SREBP proteins appeared to localize in a perinuclear reticular pattern, consistent with previous immunofluorescence localization experiments for mammalian SREBPs (22). Importantly, the GFP-HLH106 fusion protein was targeted to a similar pattern to that observed for the mammalian SREBPs, and little difference in subcellular distribution was observed for all four proteins (Fig. 6, A–D). These data support the hypothesis that HLH106, like mammalian SREBPs, is also synthesized as a membrane-anchored precursor protein.

In order to confirm the association of full-length HLH106 with intracellular membranes, Drosophila SL2 cells were fractionated into cytoplasmic, nuclear, and membrane fractions and subjected to immunoblot analysis. Fig. 7 demonstrates that endogenous HLH106 full-length protein is associated with cell membranes (lane 1, P), and a cross-reacting species similar in size to both artificially truncated HLH106 and mammalian mature SREBPs is observed in nuclear extracts (lane 2, M). The cytoplasmic fraction contains no detectable immunoreactive material (lane 3). This fractionation, as well as the localiza-
tion of the GFP-HLH106 protein, is consistent with the intracellular localization of mammalian SREBPs, suggesting that HLH106 is also anchored to intracellular membranes and processed to a mature length by a similar regulatory mechanism.
The fusion between SREBP-2 and HLH106 retains the HLH106 T445HSR sequence, which is located just prior to the first transmembrane domain. The analogous sequence in SREBP-2 is D478RSR, and previous studies have shown that this cleavage takes place, the mammalian SREBP is first cleaved at a site within its ER luminal loop (24). Mutagenesis studies have shown that the luminal peptide signal RXX is required for this first cleavage in SREBP-2 (2). In an earlier report, overexpression of SCAP resulted in sterol-independent processing of membrane-targeted SREBPs. To evaluate if the processing of the HLH106 protein was similarly affected by SCAP in mammalian cells, we devised a transient transfection assay where the activation of the HMG CoA synthase promoter was dependent on the expression, processing, and nuclear accumulation of SREBP-2 or the SREBP-2:HLH106 hybrid from a co-transfected vector (Fig. 9). When HepG2 cells were transfected with pCMV-SCAP in the absence of co-transfected SREBPs, a 17-fold induction in reporter activity was observed, presumably due to an increase in processing of endogenous SREBPs (compare samples 1 and 2). When the HSV-SREBP-2 or SREBP-2:HLH106 fusion plasmids were also co-transfected, there was a significant increase in HMG CoA synthase promoter activity that was dependent upon the inclusion of the SCAP-expressing plasmid (compare lanes 3 and 4 with lanes 5 and 6; compare lanes 7 and 8 with lanes 9 and 10).

Interestingly, there was a low level of HMG CoA synthase promoter activation by the SREBP-2:HLH106 fusion construct in the absence of SCAP (see Fig. 9, inset). This suggested that...
a basal level of sterol-independent processing of the SREBP-2:HLH106 protein occurred and is consistent with the low level of sterol-independent processing that was observed by the immunoblot analysis in Fig. 8B. Nonetheless, these experiments indicate that the *Drosophila* HLH106 is a substrate for the mammalian sterol-regulated SREBP processing pathway.

**DISCUSSION**

The experiments described in the current report were intended to compare the functional properties of a putative homologue of the mammalian sterol regulatory element-binding proteins in *Drosophila melanogaster* relative to its mammalian counterparts. When expressed in and purified from *E. coli*, the recombinant protein bound to DNA with the same dual specificity as the mammalian SREBPs (Fig. 1). Thus, the conserved tyrosine residue that allows the mammalian proteins to recognize both palindromic E-boxes and direct repeat SREs confers a similar dual binding property to the invertebrate SREBP.

We also showed that this putative "mature" form of HLH106 activated transcription from SREBP-regulated native mammalian promoters in a mammalian tissue culture transfection assay (Fig. 2). However, its expression resulted in consistently lower levels of transactivation relative to a similarly expressed mammalian SREBP-1a construct, despite similar levels of protein expression as determined by Western blot analysis. Thus, the "mature" HLH106 protein apparently has an inherently weaker potential to activate transcription when analyzed from mammalian promoters in mammalian cells. A more thorough understanding of the significance of this difference can only be addressed when natural target genes of HLH106 function are identified in *Drosophila*.

Mammalian SREBPs are inefficient transcriptional activators in isolation. However, they function efficiently in concert with ubiquitous transcription factors that bind to neighboring sites in target promoters. By using an SREBP trans-activation assay in *Drosophila* tissue culture cells that requires co-transfection of a plasmid that expresses the required co-regulatory factor, we showed that HLH106 also requires a co-factor to effectively activate transcription (Fig. 4). Consistent with the mammalian trans-activation experiments of Fig. 3, HLH106 reproducibly activated transcription to a lower level than a similarly expressed version of mammalian SREBP-1a in SL2 cells (Fig. 4).

Unregulated expression of SREBP-2 in the nucleus of CHO cells allows them to grow in the presence of toxic levels of oxysterols. This was first realized when a collection of independently selected oxysterol-resistant cell lines was shown to contain distinct gene rearrangements at the SREBP-2 locus (31). The mutations in all of these independently isolated cell lines resulted in the production of a truncated SREBP-2 protein that was constitutively targeted to the nucleus because it lacked the membrane-anchoring and COOH-terminal domains. Transfection of an artificially truncated version of the SREBP-2 cDNA resulted in a similar oxysterol-resistant phenotype (31).

No oxysterol-resistant cell line has been isolated that only expresses the nuclear form of SREBP-1a or SREBP-1c. Therefore, we wished to determine if expression of high levels of mature forms of either of the mammalian SREBP-1 isoforms or the *Drosophila* HLH106 protein would result in similar oxysterol resistance. Using expression of SREBP-2 as a positive control, we showed that high levels of mature SREBP-1a, SREBP-1c, or HLH106 were insufficient to confer oxysterol resistance. (Fig. 5). The molecular reason for this finding is presently unclear, but the results indicate that independent expression of nuclear SREBP-2 results in activation of a gene or set of genes that is essential for cells to overcome the cytotoxic effects of oxysterols and that other mature SREBPs cannot activate these essential genes.

These results are consistent with recent data on the effects of targeted knockouts of the SREBP-1 and -2 loci in mice. A targeted knockout of SREBP-2 resulted in embryonic lethality at day 8–9, whereas disruptions at the SREBP-1 locus reduced viability, but apparently homozygous survivors developed normally (26). Interestingly, the survivors also exhibited a slight up-regulation of nuclear SREBP-2 protein compared with control littermates. Thus, similar to our results in the oxysterol resistance assay in cultured cells, it appears that overexpression of SREBP-2 in animals can, under certain circumstances, overcome the growth restriction that results from the loss of SREBP-1, but the reverse is apparently not true.

The mammalian SREBPs can be loosely divided into three parts: the NH₂-terminal domain that contains all of the structural features required to activate gene transcription, a central portion that contains two transmembrane domains, and a
COOH-terminal region that is required for interaction with the sterol regulatory processing complex (1). In addition to the conservation in transcriptional activation and DNA binding properties, there is also putative structural similarity between the membrane and COOH-terminal domains of the mammalian and Drosophila proteins. To analyze this functionally, we prepared fusion proteins that contain bioluminescent GFP attached to the membrane anchoring and COOH-terminal domains of both the mammalian SREBPs and HLH106. When these fluorescently tagged proteins were expressed in cells, they all localized in similar perinuclear-reticular patterns (Fig. 6), indicating that the determinants within the coding sequence that are required for membrane localization are also conserved in HLH106. Similarly, Drosophila SL2 cells appear to express an endogenous precursor HLH106 that is targeted to intracellular membranes (Fig. 7). The significance of the mature sized species of HLH106 in SL2 nuclei (Fig. 7) is currently unknown, and we have thus far been unable to influence its nuclear accumulation by nutritional manipulation (data not shown). However, if the endogenous species is, in fact, bona fide HLH106 protein, then it is apparently not expressed at sufficient levels to activate SREBP requiring promoters in transfection assays. This observation is supported by the fact that our transient transfection assays require both exogenously supplied SREBP-expressing plasmids and the appropriate coregulatory expression plasmid to produce significant levels of promoter activation.

Probably the most intriguing observation from these studies is that the membrane-targeted HLH106 is processed in a sterol-regulated manner in mammalian cells, similar to vertebrate SREBPs (Fig. 8). This demonstrates that the Drosophila protein is not only similarly targeted to intracellular membranes but is also recognized by the complex sterol regulatory processing apparatus that involves at least two distinct proteases and a regulatory protein designated SCAP (4). Additionally, we also showed that co-expression of the mammalian SCAP protein stimulated the processing of the HLH106 precursor subunit (Fig. 9) as has been shown for the mammalian SREBPs (2).

Since the determinants for membrane insertion and sterol-regulated processing are conserved in the Drosophila protein, the next important question will be to determine why a protein in an organism that cannot synthesize cholesterol has retained, through evolution, the signals required to respond to cellular sterol levels. Recent reports suggest that mammalian SREBP-1 may be more critical for regulatory events involved with fatty acid metabolism, whereas SREBP-2 may be more critical for sterol metabolism (26). Since HLH106 is more similar in sequence to SREBP-1, HLH106 may be critically important in the regulation of fatty acid metabolism in insects. Furthermore, the sterol-regulated processing signals within the HLH106 protein are conserved, and it is therefore likely that a similar regulatory processing pathway functions within Drosophila.

Identification of both HLH106 target genes and regulatory metabolites that influence its processing will be fruitful areas of future research.

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