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The SREBP Pathway in *Drosophila*: Regulation by Palmitate, Not Sterols

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Summary

In mammals, synthesis of cholesterol and unsaturated fatty acids is controlled by SREBPs, a family of membrane-bound transcription factors. Here, we show that the *Drosophila* genome encodes all components of the SREBP pathway, including a single SREBP (dSREBP), SREBP cleavage-activating protein (dSCAP), and the two proteases that process SREBP at sites 1 and 2 to release the nuclear fragment. In cultured *Drosophila* S2 cells, dSREBP is processed at sites 1 and 2, and the liberated fragment increases mRNAs encoding enzymes of fatty acid biosynthesis, but not sterol or isoprenoid biosynthesis. Processing requires dSCAP, but is not inhibited by sterols as in mammals. Instead, dSREBP processing is blocked by palmitic acid. These findings suggest that the ancestral SREBP pathway functions to maintain membrane integrity rather than to control cholesterol homeostasis.

Introduction

Sterol regulatory element binding proteins (SREBPs) were discovered in mammalian cells, where they stimulate the synthesis of cholesterol (Wang et al., 1994; Yokoyama et al., 1993) and unsaturated fatty acids (Kim and Spiegelman, 1996; Shimano et al., 1996). The unusual feature of these transcription factors is their initial location on cell membranes, from which they are released by proteolysis in order to enter the nucleus. Proteolytic release of SREBPs is inhibited by sterols (Wang et al., 1994) and polyunsaturated fatty acids (Hannah et al., 2001; Thewke et al., 1998; Worgall et al., 1998), thereby providing a mechanism for the feedback regulation of lipid synthesis and membrane lipid composition (Brown and Goldstein, 1997).

Theopold et al. (1996) fortuitously isolated a *Drosophila melanogaster* cDNA encoding an apparent ortholog to mammalian SREBPs. Like the mammalian proteins, *Drosophila* SREBP contains an NH_2-terminal transcription factor domain of the basic-helix-loop-helix-leucine zipper (bHLH-zip) family, followed in order by a putative membrane attachment domain with two transmembrane helices and by a long COOH-terminal extension. Using antibodies raised against the bHLH-zip domain of human SREBP-1, these investigators showed that the full-length *Drosophila* SREBP is bound to cell membranes and that the cleaved bHLH-zip domain is present in nuclear extracts (Theopold et al., 1996). dSREBP was expressed in all body regions of the fly and at all stages of development. There was no information as to the mechanism of cleavage of dSREBP, nor was there information about its pattern of regulation. In the only other reported studies of dSREBP, Rosenfeld and Osborne (1998) transfected a dSREBP fusion construct into mammalian cells and showed that the cleavage of dSREBP was inhibited by sterols. Studies of the regulated processing of dSREBP in *Drosophila* cells have not been reported.

The finding of a dSREBP is somewhat surprising, since insects do not synthesize sterols (Clark and Bloch, 1959). Insects possess the early enzymes in the sterol biosynthetic pathway, which allows them to make a variety of isoprenoids, but they lack the enzymes necessary to convert farnesyl pyrophosphate to squalene and sterols. Insects require sterols for growth, but they obtain these sterols from their diet (Clark and Bloch, 1959). Since insects do not synthesize sterols, it seems unlikely that sterols are the end products that regulate dSREBP cleavage in *Drosophila* cells.

The present studies were designed to determine whether dSREBP cleavage in insect cells is carried out by the same type of proteolytic mechanism that is used by mammalian cells and to determine whether this cleavage is regulated by sterols or by some other lipid. We studied this process in Schneider S2 cells, a line of cultured cells derived from *Drosophila* embryos (Schneider, 1972). The results reveal that dSREBP processing is regulated not by sterols, but by palmitic acid or a lipid derived from palmitic acid. These findings suggest that SREBPs evolved to maintain the lipid composition of membranes and that their sterol regulatory function was acquired subsequent to a gene duplication event in the lineage leading from insects to vertebrates.

Results

We began our studies by searching *Drosophila* DNA sequence databases for genes encoding proteins related to mammalian SREBPs and to the three proteins known to be necessary for SREBP processing. Vertebrate organisms have genes encoding two isoforms of SREBP, designated SREBP-1 and SREBP-2. The *SREBP-1* gene produces two transcripts, designated SREBP-1a and SREBP-1c, that encode proteins with different NH_2-terminal sequences owing to the use of alternate promoters producing alternate first exons (Brown and Goldstein, 1997). Although the target genes for these transcription factors overlap, SREBP-2 is relatively more selective for genes in the pathway of sterol synthesis, whereas the SREBP-1 isoforms are more selective for production of unsaturated fatty acids (Horton et al., 1998).

Figure 1A shows a hydropathy plot of the single dSREBP, as predicted by the cDNA sequence of Theopold et al. (1996) and confirmed by us. The bHLH-zip

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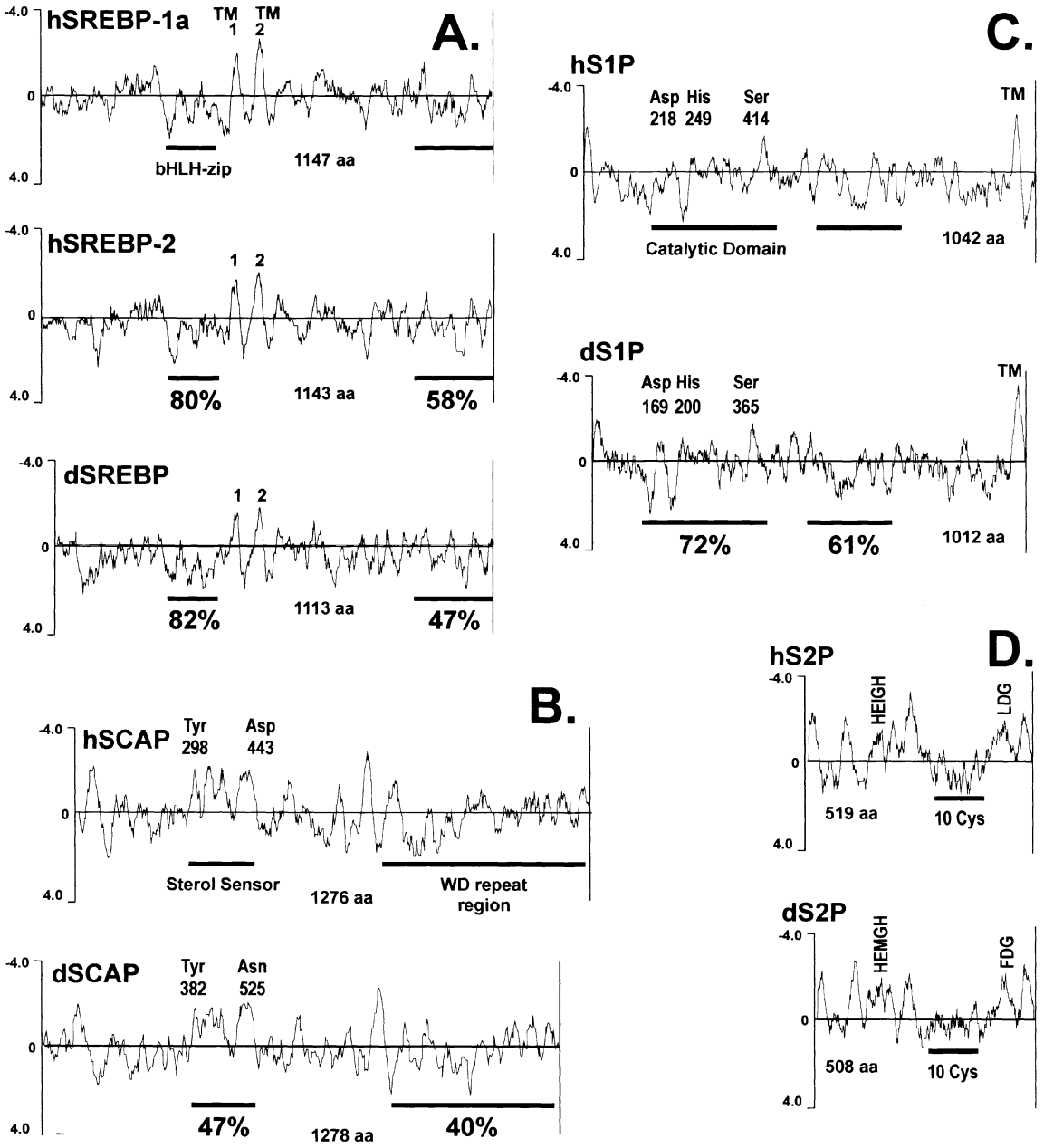

Figure 1. *Drosophila* Orthologs of SREBP Pathway Components

Hydropathy plots of the amino acid sequences of components of the human SREBP pathway (upper panels) and their *Drosophila* counterparts (lower panels) are shown, plotted by the method of Kyte and Doolittle (1982) over a window of 20 amino acids. The overall amino acid sequence identities of the *Drosophila* proteins to their mammalian orthologs are hSREBP-1a, 26%; hSREBP-2, 23%; S1P, 42%; S2P, 26%; and SCAP, 24%. These values are calculated from pairwise alignments using the Clustal algorithm in the DNAStar software package (LaserGene). The length of each sequence is indicated in amino acids (aa). Heavy bars denote regions of high sequence identity. For example, the percent identity between hSREBP-1 and dSREBP in the bHLH zipper domain is 82% as compared to 80% identity between hSREBP-1 and hSREBP-2. For S1P, the three residues comprising the catalytic triad are shown. For S2P, the sequences of the zinc binding motif and accessory ligand are shown. For SCAP, the location of two point mutations in the sterol-sensing domain that produce a dominant, constitutively active phenotype in CHO cells (Hua et al., 1996; Nohturfft et al., 1996) are shown. GenBank accession numbers are as follows: dSREBP (HLH106), U38238; dSCAP, AF441759; dS1P, AF441758; and dS2P, AF441757.

domain of dSREBP closely resembles that of human SREBP-1a (82% identity) and human SREBP-2 (66% identity). Importantly, dSREBP contains the unique tyrosine residue that was shown to permit SREBPs to bind to nonpalindromic target sequences (Kim et al., 1995). dSREBP has two hydrophobic sequences corresponding to the two transmembrane regions of the mammalian SREBPs, designated TM1 and TM2. The COOH-terminal sequences of the three proteins are also similar. Of note, dSREBP has a relatively long acidic NH_2-terminal

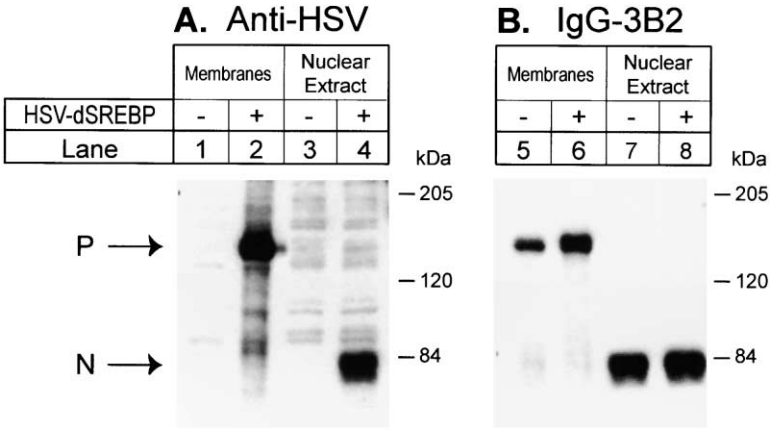

Figure 2. Detection of dSREBP Protein in S2 Cells by Immunoblotting

On day 0, S2 cells were set up in medium A as described in Experimental Procedures. On day 2, cells were transfected in medium A with 3 μg/dish of either empty vector (lanes 1, 3, 5, and 7) or pDS47-HSV-dSREBP (lanes 2, 4, 6, and 8). After incubation in medium A for 8 hr, the cells were washed and refed medium C. After an additional 16 hr, cells were harvested and fractionated into membrane and nuclear extract fractions as described in Experimental Procedures. Aliquots of protein (10 μg/lane) from transfected cells were subjected to SDS-PAGE and immunoblot analysis with either 50 ng/ml of monoclonal anti-HSV (A) or 2 μg/ml of monoclonal anti-dSREBP (IgG-3B2; [B]). The filters were exposed to film for 5 s. P and N denote the precursor and nuclear cleaved forms of dSREBP, respectively.

transcription-activating sequence, thereby resembling SREBP-2 and SREBP-1a in contrast to SREBP-1c, which has a shorter sequence and is less active (Brown and Goldstein, 1997). dSREBP also contains the crucial residues that are required for cleavage by the site 1 and site 2 proteases (S1P and S2P), which release the bHLH-zip domain from the membrane (Brown and Goldstein, 1999).

In mammalian cells, SREBPs form a complex with SCAP, a polytopic membrane protein that escorts SREBPs from the ER to the Golgi complex where SREBPs are cleaved (DeBose-Boyd et al., 1999; Sakai et al., 1998a). The *Drosophila* genome encodes a protein that we have named dSCAP (Figure 1B). Like human SCAP, the NH_2 terminus of dSCAP is predicted to contain eight membrane-spanning helices. Helices 2–6 in the mammalian protein have been called the sterol-sensing domain (Brown and Goldstein, 1999). Similar sequences are found in three other mammalian membrane proteins (HMG CoA reductase, NPC1, and Patched). In SCAP, this domain is postulated to sense the level of cholesterol in the ER membrane and thereby to regulate the movement of the SCAP/SREBP complex from ER to Golgi. When sterols accumulate, the SCAP/SREBP complex no longer leaves the ER, and SREBP processing is blocked (Nohturfft et al., 2000). Point mutations at either of two residues in this domain (Tyr-298 or Asp-443) render hamster SCAP insensitive to sterols (Nohturfft et al., 1998). As a result, SREBPs are processed constitutively, and the cells overaccumulate massive amounts of cholesterol. Tyr-382 in dSCAP corresponds to Tyr-298 in mammalian SCAP, but the position corresponding to Asp-443 is occupied by an asparagine in dSCAP (Asn-525). The COOH-terminal one-half of human and hamster SCAP contains five copies of a WD-40 repeat sequence. In other proteins, these sequences form propeller structures that engage in protein-protein interactions. The dSCAP sequence encodes multiple WD repeats, as in mammalian SCAP.

After SREBPs arrive at the Golgi complex, they are cleaved by S1P, a membrane-bound serine protease (Sakai et al., 1998b). As shown in Figure 1C, the *Drosophila* genome encodes a putative protease whose catalytic domain is 72% identical to that of human S1P, including a putative catalytic triad (Asp-169, His-200, and Ser-365 of dS1P). dS1P contains a second highly conserved region that is 61% identical to hS1P. The protein also shares a hydrophobic NH_2-terminal signal sequence and a COOH-terminal hydrophobic sequence that forms a membrane anchor. Thus, the active site of dS1P, like hS1P, should be located in the lumen of the ER and Golgi. The cytoplasmic COOH-terminal tail of dS1P, like that of hS1P, is notable for its high concentration of positively charged amino acids and the absence of negatively charged residues. The intraluminal location of the S1P active site is necessary because S1P cleaves SREBPs in the luminal loop between the two transmembrane helices, thereby separating the NH_2-terminal and COOH-terminal halves of the SREBP, both of which remain membrane bound (Duncan et al., 1997).

The NH_2-terminal product of S1P-mediated cleavage is designated the "intermediate" fragment of SREBP (Sakai et al., 1996). This intermediate is cleaved by S2P at a site that is three residues within the transmembrane helix (Duncan et al., 1998). This cleavage liberates the bHLH-zip domain, which enters the nucleus with three hydrophobic residues at its COOH-terminus. hS2P is an unusually hydrophobic zinc metalloprotease that contains two sequences necessary for zinc binding and catalysis. These are HEIGH (which fits the HEXXH consensus for other zinc metalloproteases) and a COOH-terminal LDG sequence (Brown et al., 2000). As shown in Figure 1D, dS2P has corresponding HEMGH and FDG sequences at the appropriate positions. dS2P also shares a cysteine-rich region (ten cysteines), which is luminally disposed in hS2P (Zelenski et al., 1999).

To study the proteolytic processing of dSREBP, we prepared a cDNA encoding the *Drosophila* protein with an NH_2-terminal epitope tag derived from a herpes simplex virus protein. After transfection into *Drosophila* S2 cells, immunoblotting with anti-HSV revealed the full-length dSREBP bound to cell membranes (designated P for precursor form; Figure 2A, lane 2) and a proteolyzed fragment corresponding to the mature form in nuclear extracts (designated N for nuclear form; Figure 2A, lane 4). To visualize endogenous dSREBP, we prepared a

monoclonal antibody against the NH_2-terminal domain of the protein and used it to blot membranes and nuclear extracts from S2 cells. The antibody revealed a membrane-bound protein (Figure 2B, lanes 5 and 6) and a smaller nuclear protein (Figure 2B, lanes 7 and 8) that corresponded in size to the precursor and nuclear forms of the HSV-tagged dSREBP, respectively. This protein was visualized in nontransfected cells (lanes 5 and 7) as well as in cells expressing HSV-tagged dSREBP (lanes 6 and 8).

To determine the role of dSREBP in gene regulation, we eliminated the dSREBP mRNA by treating the S2 cells with a double-stranded RNA fragment (dsRNA) corresponding to a region of the dSREBP transcript. Such treatment, called RNAi (Clemens et al., 2000), leads to the nucleolytic destruction of the endogenous mRNA. As shown in Figure 3A, treatment with dSREBP dsRNA caused the disappearance of the precursor and nuclear forms of dSREBP as determined by immunoblotting of the appropriate extracts. Treatment with a control dsRNA directed against a control mammalian mRNA (CYP7A1) had no effect on precursor or nuclear dSREBP.

We performed a series of Northern blots to measure changes in mRNA levels for genes that are potential targets of dSREBP in the cells treated with dSREBP dsRNA (Figure 3B). As expected, the mRNA for dSREBP was no longer detectable. We also observed marked reductions in mRNAs encoding acetyl CoA synthetase, acetyl CoA carboxylase (ACC), and fatty acid synthase (FAS). Levels of these three mRNAs, which encode enzymes of fatty acid biosynthesis, decline in mammalian cells when nuclear SREBPs decline (Luong et al., 2000; Matsuda et al., 2001). There was no change in two other mRNAs that decline in SREBP-deficient mammalian cells, namely, ATP-citrate lyase (an enzyme that generates acetyl CoA to be used for lipid synthesis) and farnesyl diphosphate synthase (an enzyme of isoprenoid biosynthesis). As a control, we found no change in the mRNA encoding ribosomal protein 49 (RP49).

To determine whether dSCAP is required for dSREBP processing, we incubated the cells with a dsRNA that targets dSCAP. This treatment led to a complete disappearance of the nuclear form of dSREBP and a marked reduction in the precursor form (Figure 3C). Similar results are observed in mutant hamster cells that lack SCAP (Rawson et al., 1999).

Table 1 shows measurements of several mRNAs, as determined by quantitative real-time PCR in S2 cells treated with dsRNA targeting either dSREBP or dSCAP. The level of each mRNA is expressed relative to the level found in cells treated with the control dsRNA directed against mammalian CYP7A1. As expected, we observed reductions in mRNAs encoding multiple enzymes of fatty acid biosynthesis, and these reductions were similar in the presence of the dSCAP or dSREBP dsRNAs. In contrast, no change was observed in the expression of mRNAs encoding multiple enzymes in the mevalonate pathway of isoprenoid biosynthesis, including 3-hydroxy-3-methylglutaryl CoA (HMG CoA) synthase, HMG CoA reductase, and farnesyl diphosphate synthase, all of which are prominent targets of SREBP in mammalian cells (Matsuda et al., 2001). Malic enzyme and 6-phosphogluconate dehydrogenase, which provide the reduced NADPH that is needed for lipid synthesis, are strongly regulated by SREBPs in mammalian liver (Shimomura et al., 1998), but they did not decline in the dSREBP-deficient S2 cells. In mammalian liver, stearoyl CoA desaturase is an important target of SREBPs, thereby providing a role for SREBPs in enhancing the synthesis of unsaturated fatty acids (Shimomura et al., 1998). However, in S2 cells, the stearoyl CoA desaturase ortholog did not change upon dSREBP deprivation.

Of note, we found a moderate decline of dSREBP mRNA in cells treated with dSCAP dsRNA (Table 1). This suggests that dSREBP transcription may be regulated by a positive feed-forward mechanism mediated by nuclear dSREBP. The same phenomenon has been observed in mammalian cells (Matsuda et al., 2001; Rawson et al., 1999). The modest increase in expression of

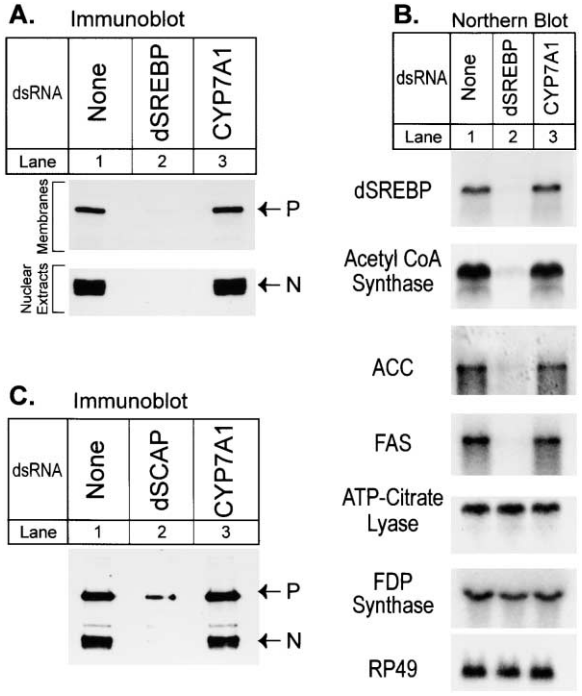

Figure 3. RNAi-Mediated Inhibition of dSREBP Expression and Its Effects on Target Gene Expression in S2 Cells

On day 0, S2 cells were suspended in medium B containing 50 μg of the indicated dsRNA per 10^6 cells and then plated as described in Experimental Procedures.

(A) Immunoblot analysis of dSREBP in membranes and nuclear extracts. On day 2, cells were harvested, and aliquots of protein (20 and 15 μg/lane, respectively) from the indicated cell fraction were analyzed by immunoblotting with IgG-3B2. Film was exposed for 30 s. P and N denote the precursor and nuclear cleaved forms of dSREBP, respectively.

(B) Northern blot analysis of target genes. Total RNA was isolated from the cells in the same experiment as shown in (A), and aliquots (20 μg) were analyzed by denaturing electrophoresis followed by Northern blotting with the indicated probes. Filters were exposed at −70°C for 2–48 hr.

(C) Immunoblot analysis of dSREBP in whole-cell extracts. On days 1, 2, and 3, fresh medium B containing 50 μg of the indicated dsRNA was added to each well. Cells were harvested on day 4 and lysed in RIPA buffer (Gielkens et al., 1976) supplemented with 50 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, and protease inhibitors (Hua et al., 1995). Aliquots of total cell protein (40 μg/lane) were analyzed by immunoblotting with monoclonal IgG-3B2 against dSREBP. Film was exposed for 40 s.

Table 1. Relative Amounts of mRNAs in *Drosophila* S2 Cells Treated with dsRNA Targeted against dSREBP or dSCAP

mRNA	Relative mRNA Expression Level	
	dsSREBP	dsSCAP
Reduced expression		
Acetyl CoA synthase	0.24	0.43
Acetyl CoA carboxylase	0.38	0.41
Fatty acid synthase	0.15	0.17
Fatty acyl CoA synthetase	0.39	0.62
dSREBP	*	0.66
No effect		
Actin	0.98	1.00
ATP citrate lyase	0.93	0.90
Farnesyl diphosphate synthase	1.03	1.02
Glycerol-3-phosphate acyltransferase	0.98	0.75
Glycerol-3-phosphate dehydrogenase	0.98	1.07
3-Hydroxy-3-methylglutaryl CoA synthase	1.00	1.02
3-Hydroxy-3-methylglutaryl CoA reductase	0.94	0.91
Malic enzyme	1.00	0.91
6-Phosphogluconate dehydrogenase	0.88	0.82
Pyruvate dehydrogenase	0.99	0.86
SCAP	1.40	*
Site 1 protease	0.99	1.21
Site 2 protease	1.04	1.06
Stearoyl CoA desaturase	0.98	1.10

On day 0, cells were treated with the indicated dsRNA as described in the legend to Figure 3A (dsSREBP) or Figure 3C (dsSCAP). Total RNA was extracted for analysis by real-time RT-PCR of the indicated mRNA. The values shown are relative to those in cells treated with control CYP7A1 dsRNA, which were arbitrarily assigned a value of 1.0. All measurements were normalized to acetaldehyde dehydrogenase in the same experiment. Each value shows the mean of triplicate measurements from the same pool of mRNA. Similar results were obtained in one other independent experiment.

*The level of mRNA was not determined.

SCAP mRNA (1.4-fold) might also reflect a feedback mechanism.

To confirm the functional effect of the reductions in the mRNAs encoding fatty acid-synthesizing enzymes, we treated S2 cells with dSREBP dsRNA, incubated them with [^{14}C]pyruvate, and measured the incorporation into [^{14}C]fatty acids. [^{14}C]Pyruvate was used instead of [^{14}C]acetate because pyruvate is converted into acetyl CoA directly by the enzyme pyruvate dehydrogenase, thereby avoiding the acetyl CoA synthetase step, which is not considered to be a major step in normal fatty acid biosynthesis (Luong et al., 2000). As shown in Table 2, we observed a 4-fold reduction in fatty acid synthesis in the cells treated with dSREBP dsRNA as compared with the control CYP7A1 dsRNA.

The above data indicate that dSREBP is required for normal rates of synthesis of fatty acids in *Drosophila* S2 cells. The major fatty acids in the lipids of *Drosophila melanogaster* are myristate (14 carbons, 0 double bonds), palmitate (16:0), palmitoleate (16:1), and oleate (18:1), each of which constitutes about 20% of the total fatty acid composition of the whole adult fly (De Renobales and Blomquist, 1984). To determine whether one of these fatty acids, palmitate, could regulate the processing of dSREBP, we performed immunoblotting on membranes and nuclear extracts of S2 cells using an antibody against the NH_2-terminal domain of dSREBP (Figure 4). When these cells were incubated with palmitate, the amount of nuclear dSREBP declined dramatically (Figure 4A). The effect of palmitate was detectable at 10 μM and maximal at 100 μM (Figure 4B). In contrast, a mixture of sterols (25-hydroxycholesterol plus cholesterol), which is a potent suppressor of SREBP cleavage in mammalian cells (Wang et al., 1994), failed to decrease the nuclear form of dSREBP in S2 cells (Figure 4A).

In mammalian cells, sterols block SREBP processing through specific inhibition of cleavage at site 1, which lies within the luminal loop (Sakai et al., 1996). The transfection experiments of Figure 5 were designed to determine whether palmitate blocks cleavage of dSREBP at

Table 2. Incorporation of [^{14}C]Pyruvate into Fatty Acids in S2 Cells Treated with dsRNA Targeted against dSREBP

Addition to medium	Protein content	[^{14}C]Pyruvate → [^{14}C]fatty acids
	μg/dish	pmol/hr per mg protein
None	213 ± 6	1035 ± 97
dsCYP7A1	206 ± 17	1320 ± 97
dsSREBP	203 ± 15	290 ± 49

On day 0, S2 cells were suspended in medium B containing 50 μg of the indicated dsRNA per 10^6 cells. Fresh medium B containing the appropriate dsRNA was added on days 1 and 2. On day 3, sodium [2-^{14}C]pyruvate was added directly to the culture medium at the final concentration of 1.5 mM (2.8 dpm/pmol). The cells were incubated for 1 hr and harvested for measurement of total protein and [^{14}C]fatty acid content as described in Experimental Procedures. Each value represents the mean ± SD of four incubations. Similar results were obtained in four independent experiments.

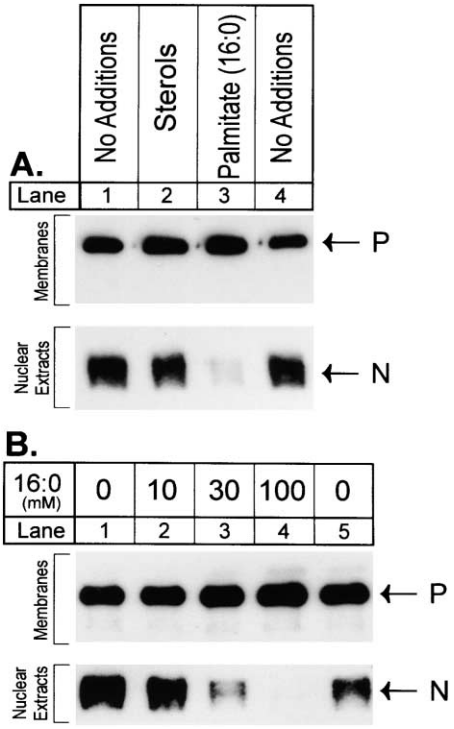

Figure 4. Proteolytic Processing of dSREBP in S2 Cells Is Regulated by Palmitate, but Not by Sterols

On day 0, S2 cells were set up in medium A as described in Experimental Procedures.

(A) On day 2, cells were refed medium C containing either no additions, sterols (10 μg/ml cholesterol plus 1 μg/ml 25-hydroxycholesterol), or 100 μM sodium palmitate. All culture medium was adjusted to contain 0.2% (w/v) BSA and 0.2% (v/v) ethanol.

(B) On day 2, cells were refed medium C containing the indicated concentration of sodium palmitate. All culture medium was adjusted to contain 0.2% BSA.

(A and B) After incubation for 4 hr, cells were harvested, and membrane and nuclear extract fractions were prepared as described in Experimental Procedures. Aliquots of protein (2.5 μg) were subjected to SDS-PAGE and immunoblot analysis with 2 μg/ml monoclonal anti-dSREBP (IgG-3B2). The filters were exposed to film for either 10 s (A) or 1 min (B).

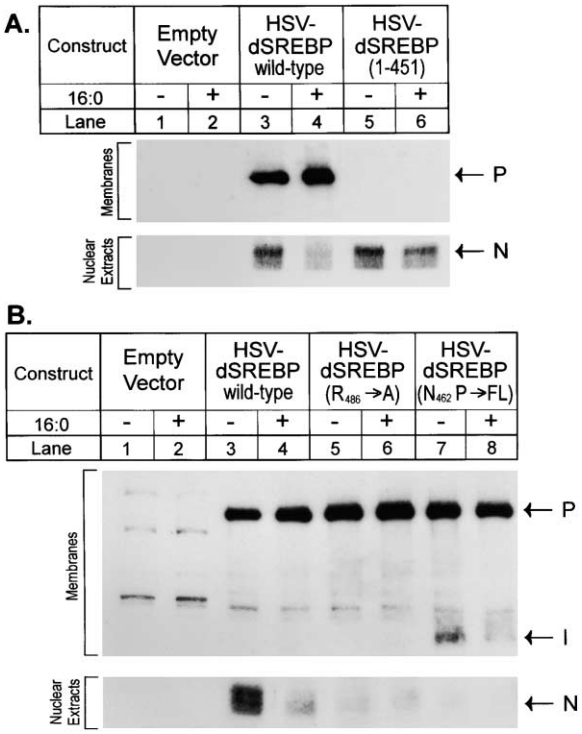

Figure 5. Palmitate Regulates Proteolytic Processing of dSREBP at Site 1

On day 0, S2 cells were set up in medium A as described in Experimental Procedures. On day 2, cells were transfected with 3 μg empty vector (lanes 1 and 2) or 100 ng of the indicated wild-type or mutant version of pAC-HSV-dSREBP. In each case, the total amount of transfected DNA in each dish was adjusted to 3 μg with empty vector. After 16 hr, the cells were washed and refed medium B supplemented with either 0.2% BSA or 100 μM sodium palmitate (16:0). After an additional incubation for 4 hr (A) or 6 hr (B), cells were harvested, and membrane and nuclear extract fractions were prepared as described in Experimental Procedures. Aliquots of protein (2.5 μg/lane of membrane fraction; 15 μg/lane of nuclear extract) were subjected to SDS-PAGE and immunoblot analysis with 50 ng/ml of monoclonal anti-HSV. The filters were exposed to film for either 30 s (A) or 1 min (B).

the same site. As shown in Figure 5A, when S2 cells were transfected with a cDNA encoding full-length dSREBP, palmitate reduced the nuclear form (lane 4). There was no reduction when the cells expressed a truncated form of dSREBP that terminates prior to the membrane attachment domain, and therefore does not require proteolytic cleavage in order to enter the nucleus (lanes 5 and 6). This experiment indicates that palmitate acts by blocking cleavage of dSREBP and not by accelerating degradation of the nuclear form.

To distinguish between cleavage at site 1 and site 2, we transfected the S2 cells with cDNAs encoding mutant forms of dSREBP with amino acid substitutions at sites corresponding to the recognition sites for mammalian S1P and S2P (Figure 5B). When the S1P recognition site was disrupted (R486A mutant; Duncan et al., 1997), the amount of nuclear dSREBP was markedly reduced in the absence or presence of palmitate (Figure 5B, lanes 5 and 6). This experiment indicates that dSREBP, like mammalian SREBP, must be cleaved at site 1 in order to enter the nucleus. Next, we substituted phenylalanine-leucine for an asparagine-proline sequence at position 462 in the transmembrane domain. The analogous substitution in mammalian SREBP prevents cleavage by S2P (Ye et al., 2000). When the cDNA encoding this mutant dSREBP was expressed in S2 cells, there was no detectable nuclear form (lanes 7 and 8). Instead, we observed a cleaved membrane-bound fragment that corresponds to the intermediate created by cleavage at site 1 (lane 7). The amount of this intermediate was markedly reduced in the presence of palmitate (lane 8). These results establish that palmitate in *Drosophila* cells blocks cleavage of dSREBP at site 1.

The effect of palmitate was remarkably specific (Figure 6). The only other fatty acid that completely blocked dSREBP cleavage in multiple experiments was the closely related (and unphysiological) 17-carbon saturated fatty acid. Partial inhibitory effects were observed for the 15-carbon and 18-carbon saturated fatty acids. The introduction of even a single unsaturated bond removed the ability of the 16-carbon fatty acid to suppress

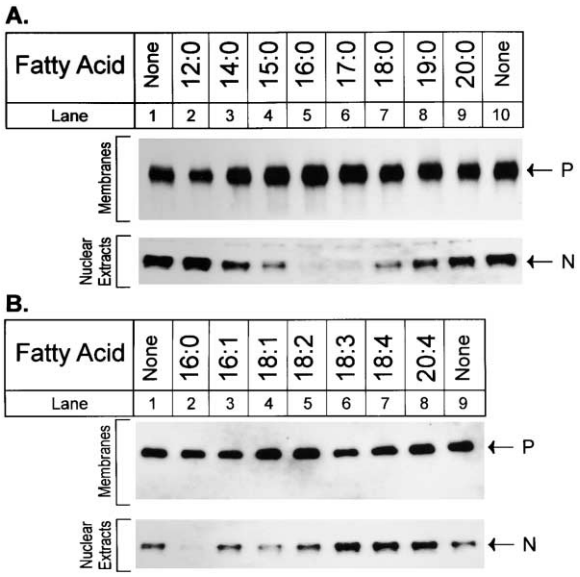

Figure 6. Palmitate, but Not Other Fatty Acids, Regulates Proteolytic Processing of dSREBP in S2 Cells

On day 0, S2 cells were set up in medium A as described in Experimental Procedures. On day 2, cells were refed medium C containing the indicated fatty acid at a final concentration of 100 μM. All culture medium was adjusted to contain 0.2% BSA. After incubation for 4 hr, cells were harvested, and membrane and nuclear extract fractions were prepared as described in Experimental Procedures. Aliquots of protein (2.5 μg) were analyzed as described in Figure 2B. The filters were exposed to film for 30 s ([A], membranes) or 2 min ([A and B], nuclear extracts; [B], membranes). P and N denote precursor and nuclear cleaved forms of dSREBP, respectively.

dSREBP cleavage (Figure 6B). We also tested a variety of other compounds for their ability to decrease nuclear dSREBP in *Drosophila* cells (see Supplemental Table S1 at http://www.developmentalcell.com/cgi/content/full/2/2/229/DC1). None of them had a significant effect.

Discussion

The current data establish the following points regarding the SREBP pathway in *Drosophila*: (1) the *Drosophila* genome contains one *SREBP* gene whose product resembles mammalian SREBP-1a and SREBP-2; (2) the *Drosophila* genome encodes orthologs of mammalian SCAP, S1P, and S2P; (3) dSREBP is processed at site 1 and site 2 in a process that requires dSCAP, thereby generating an NH_2-terminal nuclear fragment; (4) nuclear dSREBP enhances transcription of genes encoding enzymes of fatty acid biosynthesis, but not cholesterol or isoprenoid biosynthesis; and (5) processing of dSREBP at site 1 is decreased in the presence of palmitate, but not other fatty acids or sterols. It is not yet clear whether palmitate itself inhibits processing of dSREBP or whether palmitate must be incorporated into a more complex lipid, such as a sphingolipid or a glyceryl phospholipid, to exert its regulatory effect.

The studies in *Drosophila* cells provide interesting similarities and contrasts with the SREBP pathway in mammalian cells. In mammalian cells, SCAP transports SREBPs from the ER to the Golgi apparatus where S1P and S2P reside. This transport is inhibited by sterols, which thereby block SREBP processing (DeBose-Boyd et al., 1999; Nohturfft et al., 2000). Sterol inhibition is mediated through the membrane domain of SCAP, which contains a polytopic membrane-spanning segment of ~150 amino acids, termed the sterol-sensing domain. *Drosophila* SCAP contains a sequence that shares 47% identity with this sterol-sensing domain (Figure 1B). In mammalian SCAP, sensitivity to sterols is disrupted by one of two mutations; that is, when Tyr-298 is mutated to Cys or when Asp-443 is mutated to Asn (Nohturfft et al., 1998). In *Drosophila* SCAP, the amino acid corresponding to Tyr-298 is Tyr-382, as determined by sequence alignment. On the other hand, the *Drosophila* equivalent of mammalian Asp-443, rather than being an Asp, is Asn-525, which corresponds to one of the two mutant versions of mammalian SCAP that renders it sterol resistant. Whether this single amino acid difference is the basis for the sterol resistance of dSCAP remains to be determined.

Although the processing of dSREBP is not inhibited by sterols, it is effectively blocked by palmitate, which does not block processing in mammalian cells (Hannah et al., 2001). If palmitate acts through the sterol-sensing domain in dSCAP, this finding would suggest that this domain can respond to other lipids beside sterols. Perhaps this domain senses some physical property of the ER membrane, such as membrane thickness or fluidity, which in mammalian cells is altered by sterols and in *Drosophila* cells by palmitate itself or, more likely, a lipid derived from palmitate.

Although palmitate and other saturated fatty acids do not suppress SREBP cleavage in mammalian cells, we and others have provided evidence that polyunsaturated fatty acids, such as arachidonate, are capable of achieving such suppression (Hannah et al., 2001; Thewke et al., 1998; Worgall et al., 1998). Under certain conditions, these lipids act synergistically with sterols. In experiments not shown, we found no additive effect when palmitate or other fatty acids were added to *Drosophila* cells in combination with sterols. Whether arachidonate in mammalian cells acts by the same mechanism as palmitate in *Drosophila* cells remains to be determined.

In an evolutionary sense, the current data suggest that the original function of the SREBP pathway may have been to maintain the integrity of the cell membrane by adjusting fatty acid synthesis in response to an excess or deficiency of palmitic acid or a lipid derived from palmitic acid. During evolution, the single ancestral *SREBP* gene became duplicated in the lineage leading to vertebrates, and the resulting mammalian SREBP proteins acquired additional roles in regulating cholesterol homeostasis and in mediating increased lipid synthesis in liver in response to insulin (Matsuda et al., 2001). Further evolutionary insights will require the identification of the palmitate-derived lipid that regulates the processing of dSREBP.

Experimental Procedures

Materials

We obtained affinity-purified donkey anti-mouse IgG from Jackson Immunoresearch Laboratories; monoclonal antibody IgG-HSV-Tag™ from Novagen; free fatty acids from Sigma-Aldrich; N-acetyl-leucinal-leucinal-norleucinal (ALLN) from CalBiochem; defatted bovine serum albumin (BSA) from Boehringer Mannheim; vectors

pDS47/V5-HisA and pAc5.1/V5-HisB, *Drosophila* S2 cells, and a calcium phosphate transfection kit from Invitrogen; and [2-^{14}C]pyruvic acid, sodium salt (5.5 mCi/mmol) from American Radiolabeled Chemicals. Delipidated fetal calf serum and BSA-bound fatty acids were prepared as described previously (Hannah et al., 2001).

cDNA Cloning

Computer-based searches of the Berkeley *Drosophila* Genome Project (BDGP) databases using mammalian protein sequences identified partial genomic sequences for potential orthologs of SCAP and S1P. These sequences were used to design primers for PCR using fly genomic DNA as a template (dSCAP: 5′-GCTGAGCTGCACCCA AGGGGCTGG-3′, 5′-GTTCAAGGGTTGCCGTCATTGTGCC-3′; dS1P: 5′-AACATTTTTGAGCAGGGAGCTGG-3′, 5′-CTGACATCCAACCGG TCCACGGCC-3′). The amplified fragments were used to screen several *Drosophila* cDNA libraries (Tom Kornberg, University of California, San Francisco; LD cDNA library from BDGP). Inserts from purified clones were sequenced in their entirety from both strands. For SCAP, 5′ RACE with reverse transcription at 65°C was used to recover the complete sequence since none of the clones extended to the 5′ end of the transcript. Searches with human S2P identified several *Drosophila* EST clones encoding sequences that resembled different portions of the human cDNA. Two clones from the same cDNA library were obtained from the BDGP (LD14421 and LD11632). Sequencing of their inserts showed that each encoded the complete coding sequence of dS2P.

Expression Plasmids

pAc-HSV-dSREBP encodes full-length *Drosophila* SREBP (amino acids 2–1113) fused at its NH_2 terminus with two tandem copies of the HSV epitope tag (QPELAPEDPED; Isola et al., 1989) under the control of the *Drosophila* actin 5C promoter. This plasmid was constructed by simultaneous ligation of three DNA fragments: (1) a 5.3 kb fragment generated by digestion of the expression vector pAc5.1/V5-HisB with EcoRI and XbaI; (2) a 3.3 kb fragment including the entire dSREBP coding sequence (amino acids 2–1113) generated by PCR from a plasmid carrying dSREBP cDNA (obtained from D. Hultmark, Stockholm University, Stockholm, Sweden; previously referred to as HLH106; Theopold et al., 1996) using primers 5′-GAAGATATCGATGACACGACACTGATGAACTTAATAG-3′ and 5′-TTCATCTCTAGACTATCCTAAGCCTTCACGCTGCCAA-3′, followed by restriction digestion with BspDI and XbaI to cleave sites at the ends of each primer (underlined); and (3) a 179 bp fragment including a start codon and two copies of the HSV epitope tag generated by PCR from another HSV-tagged expression plasmid using primers 5′-GATGCAGAATTCAGCCATGCAGCCTGAACTCGCTCCA-3′ and 5′-CGTGTCATCGATATCTTCCGGATCCTCTGG-3′, followed by restriction digestion with EcoRI and BspDI to cleave sites at the ends of each primer (underlined).

pAc-HSV-dSREBP(1–451) encodes the NH_2-terminal cytosolic domain of dSREBP (amino acids 1–451) with an NH_2-terminal HSV epitope tag under control of the *Drosophila* actin 5C promoter. This plasmid was constructed in the same manner as pAc-HSV-dSREBP, except that the 3.3 kb dSREBP fragment was replaced with a 1.4 kb fragment including codons 2–451 of dSREBP followed by a stop codon. This fragment was generated by PCR from the dSREBP cDNA plasmid described above using primers 5′-GAAGATATCGAT GACACGACACTGATGAACTTAATAG-3′ and 5′-TTCTAGTCTAGAT CAGAGTCCGAGGCGAGAGTGGGTGG-3′. The PCR product was digested with BspDI and XbaI (underlined) and simultaneously ligated to the pAc5.1 vector and HSV tag fragments described above.

pDS47-HSV-dSREBP encodes full-length *Drosophila* SREBP (amino acids 2–1113) fused at its NH_2 terminus with two tandem copies of the HSV epitope tag under the control of the *Drosophila* DS47 promoter. This plasmid was constructed as follows: pAc-HSV-dSREBP described above was digested with EcoRI and XbaI to liberate the entire coding region of HSV-dSREBP. This fragment was ligated to EcoRI/XbaI-digested pDS47/V5-HisA empty expression vector.

Site-Directed Mutagenesis

Plasmids pAc-HSV-dSREBP(R^{486}→ A) and pAc-HSV-dSREBP(N^{462}P → FL) were created by site-directed mutagenesis of pAc-HSV-dSREBP using the QuickChange Site-Directed Mutagenesis kit (Stratagene). Plasmids were sequenced to confirm the mutations.

Antibodies

pGST-dSREBP(1–451) encodes the first 451 amino acids of dSREBP fused to glutathione-S-transferase in a pGEX-4T-1 bacterial expression vector (Novagen). The insert was generated by PCR using the following primers, which incorporate an EcoR1 site (underlined) and Xho1 site (underlined) preceded by an in-frame stop codon (bold) to facilitate directional cloning: 5′-GGAATTCGACACGACACTGAT GAACTTAATAGACGC-3′ and 5′-CTAGCTCGAG**CTA**GAGTCCGAG GCGAGAGTGGGTGGC-3′. Monoclonal antibody IgG-3B2 against dSREBP was produced by immunizing a mouse (Herz et al., 1990) with a fusion protein produced by expressing pGST-dSREBP(1–451) in *E. coli.* IgG1 fractions of antibody were purified by protein G-Sepharose.

S2 Cells

Stock cultures of *Drosophila* S2 cells were continuously grown in medium A (Schneider's *Drosophila* medium [Life Technologies] supplemented with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate) at 23°C. Cells were set up for experiments on day 0 in either medium A or medium B (Ultimate Insect Serum-Free medium [Invitrogen]) as described below.

Proteolytic Processing of dSREBP

Cells were set up for experiments in medium A at a density of 1 × 10^6 cells per 60 mm dish. On day 2, the cells were refed medium C (IPL-41 Insect medium [Life Technologies] supplemented with 10% heat-inactivated delipidated fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate) in the absence or presence of BSA-bound fatty acids (Hannah et al., 2001; at the indicated concentration) or sterols (10 μg/ml cholesterol plus 1 μg/ml 25-hydroxycholesterol added in a final concentration of 0.2% [v/v] ethanol). Cells were incubated for 3 hr followed by the direct addition of ALLN (3 μl into 3 ml of medium) to achieve a final concentration of 25 μg/ml. One hour later the cells were harvested by scraping into the medium, and the cell suspension from duplicate dishes was combined. Cells were homogenized and fractionated as described previously to yield membrane and nuclear extract fractions (Sakai et al., 1996).

Transfection of S2 Cells

Cells were set up on day 0 in medium A at a density of 1 × 10^6 cells per 60 mm dish. On day 1, the cells were transfected with a calcium phosphate transfection kit (Invitrogen) in medium A according to the manufacturer's instructions. Cells were incubated with the plasmid DNA/calcium phosphate precipitate for either 8 or 16 hr, after which the cells were washed twice with medium A without serum and refed with medium C as described in the figure legends.

SDS-PAGE and Immunoblot Analysis

Protein concentrations in membrane and nuclear extract fractions were measured using a BCA kit (Pierce). Prior to SDS-PAGE, the membrane and nuclear extract fractions were mixed with 0.25 volume of 5× SDS loading buffer (1× loading buffer contains 30 mM Tris-HCl [pH 7.4], 3% [w/v] SDS, 5% [v/v] glycerol, 0.004% [w/v] bromphenol blue, and 2.5% [v/v] β-mercaptoethanol). Aliquots of the fractions were boiled for 5 min, subjected to SDS-PAGE on 8% polyacrylamide gels, and transferred to Hybond-C extra nitrocellulose filters (Amersham Life Science). The filters were blocked with a solution of 5% (w/v) nonfat dry milk and 5% (v/v) newborn calf serum in PBS-Tween (Sigma-Aldrich), and then incubated with the antibodies listed in the figure legends. After washing, bound antibodies were visualized with peroxidase-conjugated donkey anti-mouse IgG using the SuperSignal CL-HRP substrate system (Pierce) as previously described (Sakai et al., 1996). Gels were calibrated with broad range prestained molecular weight markers (Bio-Rad). Filters were exposed to Kodak X-Omat Blue XB-1 film (NEN Life Science Products) at room temperature for the indicated time.

dsRNA Production and RNAi

DNA templates for the synthesis of double-stranded RNA (dsRNA) were generated by PCR and purified using the High Pure PCR Product Purification kit (Boehringer Mannheim). Primer sequences are shown in Supplemental Table S2A. dsRNA was synthesized using a MEGAscript T7 kit (Ambion) as described (Clemens et al., 2000; http://dixonlab.biochem.med.umich.edu/protocols/). The dsRNA fragments were analyzed by electrophoresis in 1% agarose gels, where they migrated predominantly as single 700 bp bands. The dsRNA was stored at −70°C.

On day 0, S2 cells were released from the stock flask by tapping and were then suspended in medium B containing 50 μg of the appropriate dsRNA per ml. One milliliter of cell suspension was plated at a density of 1×10^6 cells per 37 mm well. Six hours later, 2 ml of medium B containing 15% heat-inactivated fetal calf serum, 150 units/ml penicillin, and 150 μg/ml streptomycin sulfate was added to each well. When cells were retreated with dsRNA, the monolayers were washed once with phosphate-buffered saline, resuspended in medium B by gentle pipetting, and replated in the same well. Cells were harvested on day 2, 3, or 4 as described in the legends.

Northern Blot Analysis

Total RNA was prepared from S2 cells with the RNA STAT-60 reagent (TEL-TEST) according to the manufacturer's instructions. Total RNA (20 μg) was subjected to agarose gel electrophoresis and Northern blot analysis as previously described (Shimomura et al., 1999). A cDNA probe for dSREBP was generated with the same PCR fragment used for dsRNA synthesis. The probe for *Drosophila* RP49 was generated as previously described (O'Connell and Rosbash, 1984). The other cDNA probes were prepared by RT-PCR from first strand cDNA products generated from total S2 cell RNA with SuperScript First-Strand Synthesis system for RT PCR (Life Technologies). Primer sequences for the cDNA probes are shown in Supplemental Table S2B.

Real-Time PCR Quantification of mRNAs

Total RNA was prepared from S2 cells treated as described above. A 5 μg aliquot was used as a template for cDNA synthesis employing the SuperScript First-Strand Synthesis system and oligo dT primers. Specific primers for each gene were designed with Primer Express software (PE Applied Biosystems). The sequences of forward and reverse primers are shown in Supplemental Table S2C. Primers were verified by showing that the PCR reaction product produced a single band after agarose gel electrophoresis. Real-time quantitative TaqMan PCR analysis (Heid et al., 1996) was performed according to the manufacturer's instructions (PE Applied Biosystems). The reaction contained, in a final volume of 30 μl, 250 ng of reverse transcribed total RNA, 0.33–1.67 μM of the forward and reverse primers, and 2× SYBR Green PCR Master Mix. All reactions were performed in triplicate. The relative amounts of all mRNAs were calculated using the Comparative CT method as described in User Bulletin #2 (PE Applied Biosystems). *Drosophila* acetaldehyde dehydrogenase mRNA was used as an internal control for variations in amounts of mRNA. Levels of the different mRNAs were normalized to acetaldehyde dehydrogenase mRNA levels and presented as the difference between RNAi-treated and -untreated cells.

Fatty Acid Synthesis

Monolayers of S2 cells were incubated with 1.5 mM sodium [2-^{14}C]pyruvate (2.8 dpm/pmol), and pyruvate incorporation into fatty acids was determined as described previously (Brown et al., 1978). Before saponification, each sample received ~10^5 dpm [^{3}H]oleic acid as a recovery standard. Protein concentration was measured by the method of Lowry et al. (1951).

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