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Alternative Processing of Sterol Regulatory Element Binding Protein During Larval Development in *Drosophila melanogaster*

Krista A. Matthews, Amit S. Kunte,[1] Edward Tambe-Ebot and Robert B. Rawson[2]

Department of Molecular Genetics, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9046

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ABSTRACT

Sterol regulatory element binding protein (SREBP) is a major transcriptional regulator of lipid metabolism. Nuclear Drosophila SREBP (dSREBP) is essential for larval development in *Drosophila melanogaster* but dispensable in adults. d*SREBP*$^{-}$ larvae die at second instar owing to loss of dSREBP-mediated transcription but survive to adulthood when fed fatty acids. Activation of SREBP requires two separate cleavages. Site-1 protease (S1P) cleaves in the luminal loop of the membrane-bound SREBP precursor, cutting it in two. The NH_2- and COOH-terminal domains remain membrane bound owing to their single membrane-spanning helices. The NH_2-terminal cleavage product is the substrate for site-2 protease (S2P), which cleaves within its membrane-spanning helix to release the transcription factor. In mice, loss of S1P is lethal but the consequences of loss of S2P in animals remain undefined. All known functions of SREBP require its cleavage by S2P. We isolated Drosophila mutants that eliminate all dS2P function (*dS2P*$^{-}$). Unexpectedly, larvae lacking *dS2P* are viable. They are deficient in transcription of some dSREBP target genes but less so than larvae lacking *dSREBP*. Despite loss of dS2P, dSREBP is processed in mutant larvae. Therefore, larvae have an alternative cleavage mechanism for producing transcriptionally active dSREBP, and this permits survival of *dS2P* mutants.

INCREASED transcription of genes targeted by sterol regulatory element binding protein (SREBP) requires proteolytic release of the NH_2-terminal transcription factor domain from the membrane-bound precursor. This process, which involves two separate cleavages by two different proteases, is an example of regulated intramembrane proteolysis (Brown *et al.* 2000). Once SREBP is cleaved in the luminal loop of the precursor at site 1, the second cleavage occurs at site 2, which lies within the first membrane-spanning helix of SREBP (Duncan *et al.* 1998). This cleavage requires a highly hydrophobic integral membrane protein that contains a metalloprotease active site motif (Rawson *et al.* 1997). This protein is thus designated site-2 protease (S2P) and its homologs occur throughout all kingdoms of life. Recent structural analysis of an archaebacterial S2P shows that its active site is highly similar to that of thermolysin (Feng *et al.* 2007). Importantly, all currently known functions of SREBP require its cleavage by S2P (Bengoechea-Alonso and Ericsson 2007).

S2P is absolutely required for the survival of mammalian cells under standard culture conditions (Rawson *et al.* 1997). Cells lacking S2P cannot process SREBPs and are deficient in the transcription of many genes needed for synthesis and uptake of lipid (*e.g.*, genes of the biosynthetic pathways for cholesterol and unsaturated fatty acids and the low-density lipoprotein receptor gene). Mutant cells survive when the ultimate products of SREBP activation, cholesterol and unsaturated fatty acids, are added to the medium (Limanek *et al.* 1978; Goldstein *et al.* 2002), demonstrating that the essential role for S2P in cultured mammalian cells is to process SREBPs and thereby enable them to mediate the transcriptional upregulation of the genes of lipid metabolism.

The SREBP pathway is also found in insects (Seegmiller *et al.* 2002), even though they cannot make cholesterol from acetyl–coenzyme A and must get sterols from their diet (Clark and Bloch 1959). Accordingly, cleavage of their single isoform of SREBP (*dSREBP*, also called *HLH106*; Theopold *et al.* 1996) is regulated by phospholipids rather than by sterols (Dobrosotskaya *et al.* 2002). We have shown that dS2P is required for release of dSREBP from the membranes in Drosophila S2 cells (Seegmiller *et al.* 2002). An asparagine–proline (NP) motif found in the first membrane-spanning helix of all SREBP homologs is necessary for cleavage by S2P (Ye *et al.* 2000a). When $N_{462}P$ is mutated to phenylalanine–leucine (FL), dSREBP is still correctly inserted into the membrane but no longer serves as a substrate for dS2P (Seegmiller *et al.* 2002).

In Drosophila larvae, *dSREBP* itself is an essential gene. Without it, larvae raised on standard cornmeal–molasses–agar culture medium die at second instar (Kunte *et al.* 2006). Supplementing the culture medium

[1]*Present address:* Department of Internal Medicine, Residency Training Programs, Yale School of Medicine, P.O. Box 208030, New Haven, CT 06520-8030.

[2]*Corresponding author:* Department of Molecular Genetics, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-9046. E-mail: rob.rawson@utsouthwestern.edu

with fatty acids affords substantial rescue of *dSREBP* mutant flies. Expressing a truncated form of dSREBP that ends before the first membrane-spanning helix (and therefore bypasses proteolytic regulation) also rescues mutant flies. Rescued larvae show restored transcription of dSREBP target genes. The remaining portions of dSREBP neither rescue mutants nor are required for their rescue (Kunte *et al.* 2006). Once *dSREBP* mutants reach adulthood, dSREBP is dispensable (Cherry *et al.* 2006). These data indicate that the essential role for dSREBP in larvae requires it to reach the nucleus and mediate the increased transcription of target genes required for fatty acid synthesis and uptake.

There are currently no animal models lacking S2P and the consequences of its loss in whole animals are unknown. To address the role of S2P in the SREBP pathway *in vivo*, we isolated mutant Drosophila lacking *dS2P* owing to deletion of the locus. We also obtained mutants harboring a transposon insertion in exon 3. These mutations eliminate dS2P function. Unexpectedly, we found that flies lacking dS2P function are viable.

dS2P mutant larvae show modest transcriptional deficits in some dSREBP target genes but the deficits are less severe than those observed in *dSREBP* mutants. The present data indicate that some protease(s) other than dS2P can release the transcriptionally active NH_2-terminal domain of dSREBP from the membrane, freeing it to go to the nucleus. This alternative cleavage thus supports larval development in the absence of dS2P.

MATERIALS AND METHODS

Plasmids: pUAS-dSREBP is described elsewhere (Kunte *et al.* 2006). pUAS-dSREBP(NP–FL) was constructed by subjecting pUAS-dSREBP to *in vitro* mutagenesis using the Quickchange-XL kit (Stratagene). The primers used for mutagenesis were 5′-GCCATCCTGGCCGTCTTTCTCTTCAAGACCTTTCTCC-3′ and 5′-GGAGAAAGGTCTTGAAGAGAAAGACGGCCAGGAT GGC-3′. The mutant dSREBP cDNA fragment was then excised and recloned into the original pUAST vector and the open reading frame was completely sequenced. P{*GAL4-dSREBPg*} and P{UAS-GFP} are described in Kunte *et al.* (2006).

Genetic strains: All marker mutations and balancer chromosomes are described in and referenced by FlyBase Consortium (2003). Crosses were maintained at 25° in vials containing freshly yeasted cornmeal–molasses–agar (Kunte *et al.* 2006) except where noted. Oregon-R flies served as wild type. *P*-element transposon insertion lines EP(2)2245 (~1 kb upstream of *dS2P*) and KG08356 (in exon 3 of *dS2P*) were obtained from the Bloomington Drosophila Stock Center. Transposon alleles were allowed to recombine freely with wild type for three generations prior to being formally isogenized and tested for lethal and sterile phenotypes. Deletion mutants were obtained as described (Kunte *et al.* 2006). *dSREBP*^{189} is a deletion extending into the open reading frame of *dSREBP* isolated in a screen for imprecise excisants of a nearby *P* element (Kunte *et al.* 2006). The UAS-dSREBP and UAS-dSREBP(NP → FL) transgenes used are inserted on the second chromosome. These stocks were created as described (Kunte *et al.* 2006). The 6487 GAL4 driver line is a P{GAWB} enhancer trap insertion (P{*w*[+*mW.hs*]=GawB}OK376) obtained from the Bloomington Stock Center. The P{*GAL4-dSREBPg*} and P{UAS-GFP} transgenes were recombined onto a single third chromosome.

Characterization of alleles: The following primers were used in PCR analysis and sequencing of mutant alleles (F, forward; R, reverse; number indicates the nucleotide position relative to the predicted start site of transcription): 5′-GGA ATTCCATGGATCCCTTCGTGTTCTTCATA-3′ (F, 285), 3′-GTG TAAACACCTACTTAAATTTGGC-3′ (F, −2381), 5′-CTAGTCT AGATTCTTAAAGCAGGGGTCGCAG-3′ (R, 1915), 5′-CTCAG TTAAGGTGAACTTGGTGGTGG-3′ (F, −1041), 5′-CATATAAG ACTTTTGCCGACTTGC-3′ (R, −256), 5′-GTATTTTAAGTCAC TTAACACAATGG-3′ (F, −202), 5′-GGTGAGGTCTCAAGATG TCATTGG-3′ (R, 258), 5′-CGACGACTCAGGGTCAAGAGCG AGG-3′ (F, −3977), 5′-GTGCATAGGTTTAACCAGCGTTGGG-3′ (R, −3338), 5′-CCCAACGCTGGTTAAACCTATGCAC-3′ (F, −3338, 5′-GTTGGCAATTCTATCAAGAAACCCGG-3′ (R, 3441).

Immunoblot analysis of dSREBP cleavage: On day 0, 24–48 hr old male flies were collected and fed for 1 day in freshly yeasted vials. On day 1, flies were distributed 60–70 flies/vial. Flies were harvested on day 4. After dead flies were discarded, the remaining flies were anesthetized under CO_2 and the sample was placed on ice. Flies were homogenized in buffer A (Kunte *et al.* 2006) supplemented with a mixture of protease inhibitors in 1.5 ml microcentrifuge tubes using pellet pestles (Kontes) for 15 strokes by hand followed by 30 sec with a motorized pestle (Fisher). Homogenates were filtered through 100 μm^2 Nitex mesh by centrifuging twice at 1000 × *g* for 1 min. The filtrates were passed through a $22\frac{1}{2}$ gauge needle 20 times and centrifuged at 1000 × *g* for 7 min. The resulting pellets, designated as the nuclear fraction, were resuspended in an equal volume of buffer C (Hua *et al.* 1996) supplemented with protease inhibitors and agitated for 1 hr at 4°. Nuclear fractions were then centrifuged at 100,000 × *g* for 30 min at 4°. The supernatant from this spin was designated the nuclear extract. The supernatant from the 1000 × *g* spin was further centrifuged at 100,000 × *g* for 30 min. The resultant pellets, designated membrane fraction, were then resuspended in SDS lysis buffer (1% SDS) and boiled in 1× SDS sample buffer immediately after resuspension. Nuclear extract (35 μg) and 50 μg of solubilized membrane were then subjected to electrophoresis on 8% SDS-polyacrylamide gels and transferred to nitrocellulose membranes for immunodetection with the IgG 3B2.

cDNA rescue experiments: The 6487 GAL4 driver was first crossed into a *dSREBP*^{189} background to generate *w*$^{-}$;P[*w*$^{+}$, *GAL4*]/P[*w*$^{+}$, *GAL4*]; *dSREBP*^{189}/*TM6B, Tb Hu e* flies. Similarly, the responder transgenes were crossed into the *dSREBP*^{189} background to generate *w*$^{-}$;P[*w*$^{+}$, *UAS-dSREBP*]/P[*w*$^{+}$; *UAS-dSREBP*]; *dSREBP*^{189}/*TM6B, Tb Hu e*. This was also done for P[*w*$^{+}$, *UAS-dSREBP(NP-FL)*] stocks. For rescue experiments, the driver and responder lines described above were crossed and the emergence of various classes of adults was scored using the *Hu* and *Cy* markers. The genotype of sampled individuals was verified by PCR analysis.

Quantitative analysis of transcripts: Transcript abundance was determined by real-time PCR as described (Kunte 2006, no. 3121). Briefly, total RNA was prepared from ~100 larvae for each genotype and time point examined using the RNA-Stat 60 reagent (Tel-Test). Real-time PCR was performed on an ABI 7900HT instrument, using SYBR green fluorescent probe and the primers described (Dobrosotskaya *et al.* 2002; Kunte *et al.* 2006). The relative abundance of all mRNAs was calculated using the comparative CT method as described in User Bulletin No. 2 (PE Applied Biosystems).

Viability: Standard cornmeal–molasses–agar supplemented with 0.075% (w/v) Na myristate and 0.15% (w/v) Na oleate was prepared as described (Kunte *et al.* 2006). Embryos from

the indicated crosses were collected overnight at 25°. Embryos (2 mg) were added to vials containing 9 ml supplemented medium. Adults were scored as they emerged and scoring was repeated multiple times daily through day 21 after egg laying (AEL) so that no mature adults remained in the cultures to produce F_1 offspring. Percentage rescue was calculated by dividing the observed ratio of homozygotes to heterozygotes by the expected ratio (the expected ratio is 1 for crosses of heterozygotes with homozygotes and 0.5 for crosses of heterozygotes with heterozygotes owing to embryonic lethality of balancer chromosome homozygotes). The day of median eclosion is that day at which ≥50% of adults had emerged from each culture.

Mass of flies: Mass was determined by placing 3–10 flies/tube into 8–10 preweighed tubes for each sex and genotype. These were then reweighed on a Mettler/Toledo XS105 dual range balance and the initial mass was subtracted from the subsequent mass to determine the mass of flies in each tube. This value was divided by the number of flies to determine mass/fly.

RESULTS

Flies lacking *dS2P* are viable: We used a *P*-element excision approach (Robertson *et al.* 1988) to isolate events that removed transposon EP(2)2245 and extended into the *dS2P* locus. The extent of each candidate deletion was determined using Southern blotting, PCR, and sequencing. Excision line 74 harbors a deletion that removes all *P*-element sequences and encompasses the entire *dS2P* locus (Figure 1A). We designate this allele $dS2P^{1}$. We also obtained a *P*-element insertion in the *dS2P* locus from the Bloomington Stock Center (KG08356). We designate this allele $dS2P^{2}$.

We determined the site of transposon insertion in $dS2P^{2}$ to be 255 bp into exon 3 (Figure 1A) by sequencing multiple PCR products generated from mutant genomic DNA using primers specific for *dS2P* and for *P*-element sequences. This insertion disrupts the open reading frame of the transcript at codon 261 of the 508-amino-acid coding sequence (Figure 1B). We detected no dS2P transcripts from $dS2P^{1}$ homozygous larvae by Northern blot analysis and only a truncated, ~1.4-kb transcript from $dS2P^{2}$ homozygous larvae (not shown).

Sequencing of PCR-amplified cDNAs from $dS2P^{2}$ mutants revealed an in-frame stop codon arising from *P*-element sequences four codons after the insertion site and no additional *dS2P*-derived sequence thereafter. A putative protein produced from this transcript could comprise only the first half of dS2P, plus three amino acids encoded by *P*-element sequences. The final 247 amino acids of dS2P include an aspartate residue at position 453 that is the third coordinating ligand for the active site metal atom (Kinch *et al.* 2006; Feng *et al.* 2007) and is essential for S2P function. In all S2P homologs tested, alteration of this aspartate renders S2P inactive (Rudner *et al.* 1999; Zelenski *et al.* 1999). Thus, any protein product of the $dS2P^{2}$ allele cannot be proteolytically active.

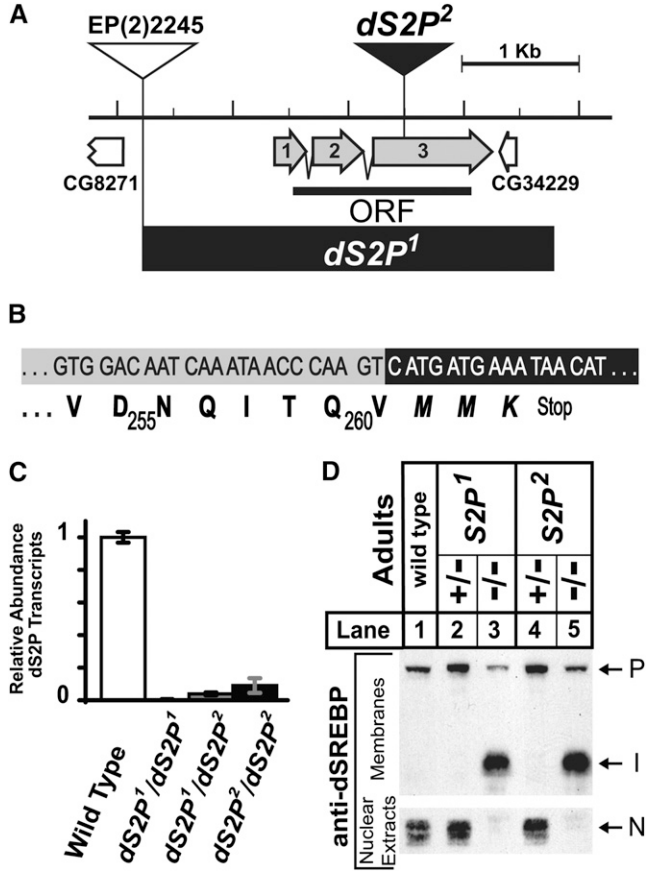

Figure 1.—$dS2P^{1}$ and $dS2P^{2}$ alleles. (A) Map of the dS2P locus. Shaded arrows represent the position of exons. The extent of the open reading frame (ORF) is shown by the thick solid line. The location of the *P* element that was mobilized to yield the $dS2P^{1}$ is indicated by the open triangle. The extent of the deficiency is shown by the solid box, which includes the entire dS2P gene. The solid triangle represents the location of the $dS2P^{2}$ *P*-element insertion (KG08356) at bp 255 of exon 3. CG34229 is a predicted gene encoding a putative component of the higher eukaryotic NADH complex. (B) Sequence of the dS2P transcript from $dS2P^{2}$ flies at the junction with *P*-element sequences. Sequences from the dS2P gene are indicated by the shaded box. Sequences from *P*-element KG08356 are indicated by the solid box. The encoded protein sequence is shown below, numbered as the wild-type sequence. (C) $dS2P^{1}$ and $dS2P^{2}$ are null alleles. Quantitative real-time PCR measurements of dS2P transcripts in wild type (open), $dS2P^{1}$ (shaded), $dS2P^{1}/dS2P^{2}$ mutants (dark shading), and $dS2P^{2}$ (solid) homozygous first instar larvae, using probe against exon 1, which is present in both wild-type and truncated, chimeric dS2P transcripts. (D) Immunoblot analysis of dSREBP cleavage in *dS2P* mutant adults shows that $dS2P^{1}$ and $dS2P^{2}$ are null alleles. Samples were prepared as described in materials and methods and subjected to immunoblot analysis using lgG 3B2 against dSREBP.

The $dS2P^{1}$ deletion also removes a recently predicted gene (CG34229, annotation of release 5.2 of the *Drosophila melanogaster* genome). Two independent transposon insertions within CG34229 exhibit no associated phenotypes (FlyBase Consortium 2003). To eliminate possible phenotypic effects due to loss of this putative gene in $dS2P^{1}$ mutants, we performed experiments with mutants *trans*-heterozygous for $dS2P^{1}$ and $dS2P^{2}$.

Figure 1C shows the results of a real-time PCR analysis of dS2P transcript abundance in first instar mutant larvae relative to wild-type larvae. The primers used here are specific for exon 1, which is present in the aberrant *dS2P*^{2} transcript. No transcript is detectable from *dS2P*^{1} mutants; only low levels of the aberrant transcript in *dS2P*^{2} mutants and levels intermediate to these are apparent in *trans*-heterozygotes.

We also examined cleavage of dSREBP in adult flies. dSREBP is the only confirmed substrate for dS2P in flies (Seegmiller *et al.* 2002). Both in wild-type and in heterozygous adult flies, cleaved dSREBP is clearly detected in nuclear extracts (Figure 1D). No nuclear dSREBP is seen in homozygotes (Figure 1D, lanes 3 and 5). Instead, the intermediate form, which is the product of the cleavage of dSREBP at site 1 (and which is the substrate for dS2P), accumulates in membranes (Figure 1D, lanes 3 and 5, top). Thus, in adults, both alleles of *dS2P* are profoundly deficient for cleavage of dSREBP.

Mammalian cells lacking S2P die unless grown in medium supplemented with cholesterol and unsaturated fatty acids (Rawson *et al.* 1997). This is owing to their inability to cleave SREBPs at site 2 and the consequent loss of transcriptional upregulation of target genes. *dSREBP* is itself essential in flies (Kunte *et al.* 2006). We expected that loss of *dS2P* would phenocopy loss of *dSREBP* due to inability of d*S2P* mutants to cleave dSREBP. It was therefore surprising that Drosophila mutants lacking *dS2P* survive well enough to be easily maintained as homozygous stocks.

We have maintained both homozygous and balanced heterozygous stocks of d*S2P*^{1} for >200 generations (and of *dS2P*^{2} for >100 generations) without intentional selection. Maintenance of homozygous stocks demonstrates that, in flies, *dS2P* is not essential for viability. By contrast, maintenance of the lethal-allele-carrying balancer chromosome at high frequency in the heterozygous stocks for so many generations indicates that loss of *dS2P* puts homozygotes at a substantial competitive disadvantage relative to their heterozygous culture mates (see below).

***dS2P* mutants grow more slowly than heterozygotes:** We compared the growth of *dS2P*^{1}/*dS2P*^{2} mutants (from crosses of *dS2P*^{2}/*dS2P*^{2} virgin females with *dS2P*^{1}/*CyO* males) to wild-type larvae raised in parallel cultures. Up to 48 hr AEL, there is no difference in size between *dS2P*^{1}/*dS2P*^{2} mutants and wild-type larvae (Figure 2A). By 60 hr AEL, *dS2P*^{1}/*dS2P*^{2} mutants are distinctly smaller than wild type. Disparity in size persists through 84 hr. By the time that mutants reach adulthood, they display a greater variability of body size than do their heterozygous siblings (males shown; Figure 2B) and are somewhat less massive, on average (Figure 2C). Morphologically, mutants are normal. These data show that the homozygotes grow more slowly than wild type or heterozygotes, taking longer to approach normal size.

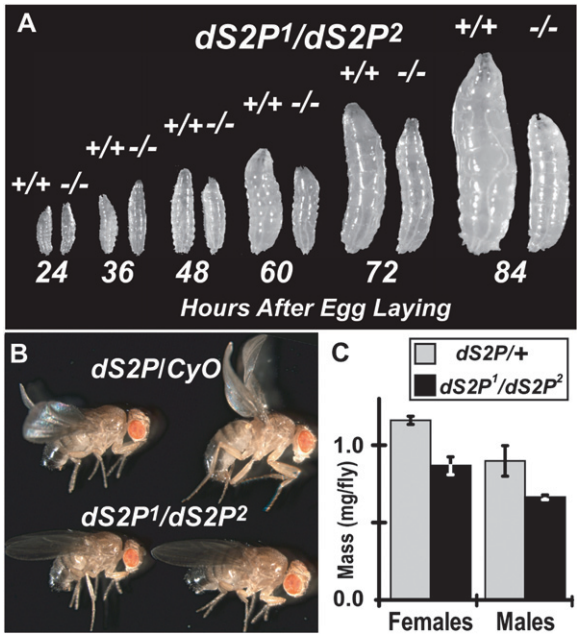

Figure 2.—*dS2P*^{1}/*dS2P*^{2} mutants grow more slowly than wild type. (A) Representative larvae of each genotype were photographed at the indicated time after egg laying (24–84 hr). By 84 hr, wild type have reached the third instar on the basis of mouth hook and anterior spiracle morphology. (B) Male offspring of mothers homozygous for dS2P. Heterozygotes above, *trans*-heterozygotes below. (C) Mass of offspring of mothers homozygous for dS2P. Shaded bars indicate heterozygotes, solid represent *trans*-heterozygotes. Male and female *trans*-heterozygotes show a similar reduction in average mass compared with heterozygotes. Error bars represent the SEM.

Figure 3A shows typical emergence data from a heterozygous cross of *dS2P*^{1}/*dS2P*^{1} virgin females with *dS2P*^{2}/*CyO* males. While the majority of heterozygotes emerge by day 11 AEL, the bulk of their *dS2P*^{1}/*dS2P*^{2} siblings emerge 2 days later. In multiple experiments, we consistently observe this ~2-day delay irrespective of the alleles used or the direction of the *trans*-heterozygous cross. This delay becomes more pronounced with crowding (Figure 3B). We set up cultures with the indicated masses of embryos on standard medium and scored adults as they emerged. The delay is shown as the day AEL of median eclosion for homozygotes minus the day of median eclosion for heterozygotes. At 10 mg of embryos per culture, the delay for *dS2P*^{1}/*dS2P*^{1} flies was 2 days. Doubling the mass of embryos in the culture increased the delay to 5 days. At 40 or 70 mg of embryos, the delay extends to ~2 weeks. Results from flies lacking dSREBP (*dSREBP*^{189}; Kunte *et al.* 2006) are shown for comparison.

Maternally supplied dS2P functions in *dS2P* mutant larvae: We conducted extensive fertility, fecundity, and viability studies on *dS2P* mutant stocks. In the course of these studies, we noted that the frequency of emergence of homozygotes was strongly affected by the maternal genotype. In experimental cultures, the homozygous offspring of heterozygous mothers emerged at about

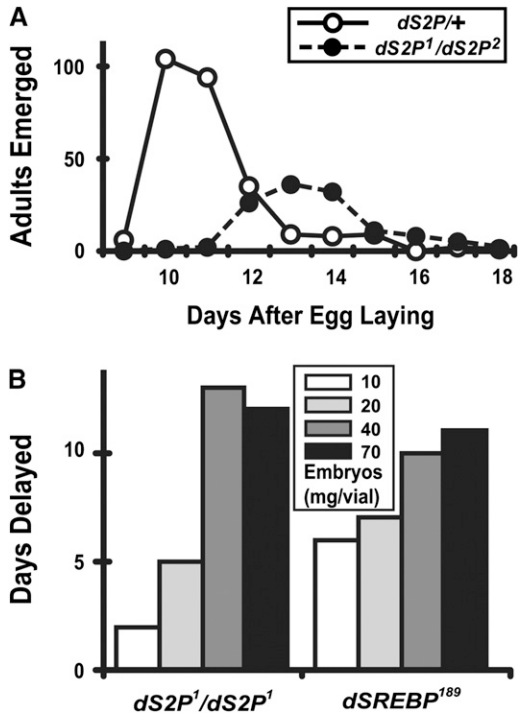

FIGURE 3.—*dS2P* mutants develop more slowly than wild type. (A) Plot of the number of adults emerging *vs.* days after egg laying. On day 0, 3 mg of embryos was introduced into vials of standard cornmeal–molasses–agar medium. Beginning on day 9, and each day thereafter, adults were cleared from the culture and counted. (B) Crowding substantially exacerbates the developmental delay. The indicated mass of embryos was introduced into flasks of standard cornmeal–molasses–agar medium (~80 ml/flask) on day 0. Beginning on day 9, and each day thereafter, adults were cleared from the culture, scored, and counted. "Days delayed" was calculated as the day of median eclosion for homozygotes minus that of heterozygotes.

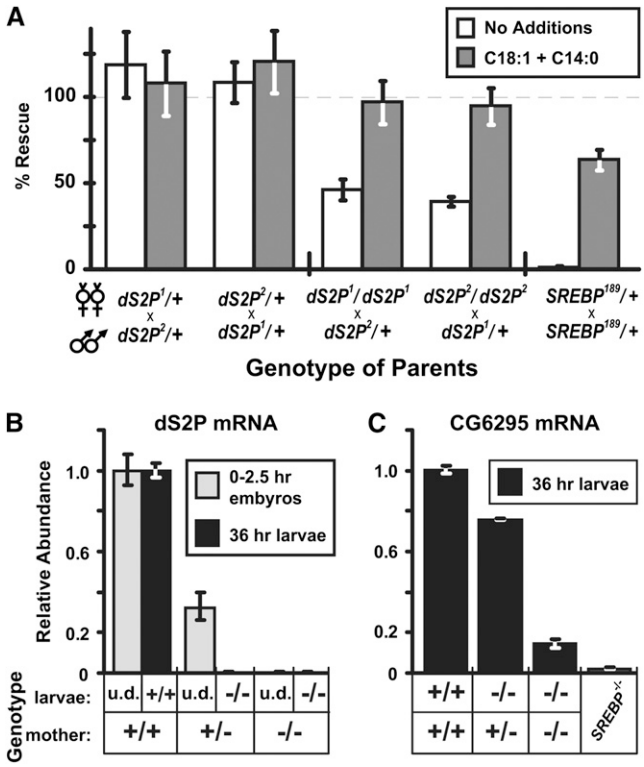

FIGURE 4.—Effect of maternal *dS2P* genotype. (A) Survival of homozygous offspring. Virgin females and males of the indicated genotypes were crossed. Embryos from these crosses were collected and cultures were set up on standard cornmeal–molasses–agar medium or on medium supplemented with fatty acids as described in MATERIALS AND METHODS. On day 10 AEL, each vial was cleared and the newly emerged adults were scored for *dS2P* genotype. Standard culture medium, open bars; culture medium supplemented with fatty acids, shaded bars. Error bars represent SEM. (B and C) Virgin females homozygous for $dS2P^2$ were crossed to males heterozygous for $dS2P^1$. A minus sign ("−") indicates the *dS2P* allele. Embryos from these crosses were collected and divided into two groups that were allowed to develop for the indicated times after which total RNA was isolated and subjected to real-time PCR analysis with primers for the indicated transcripts. Larvae were genotyped on the basis of expression of a GFP transgene on the balancer chromosome. The 0- to 2.5-hr embryos were not genotyped owing to the lack of zygotic transcription at this early time in development (u.d., undetermined). Transcript abundance is plotted relative to wild-type controls. Error bars represent the SEM.

the expected frequencies (Figure 4A, left, open bars). By contrast, the homozygous offspring of homozygous mothers survived markedly less well on unsupplemented medium, emerging at less than half the expected frequency (Figure 4A, middle, open bars). To determine if reduced viability resulted from disruption of fatty acid metabolism subsequent to deficient processing of dSREBP, we tested sibling cultures on medium supplemented with fatty acids (KUNTE *et al.* 2006). Supplementation with fatty acids permitted near-expected survival of the homozygous offspring of homozygous mothers (Figure 4A, middle, shaded bars). $dSREBP^{189}$ flies served as a control for rescue by fatty acid supplementation (Figure 4A, right).

Differential survival of homozygotes depending on the maternal genotype indicates that maternally supplied *dS2P* ameliorates the effects of the lack of *dS2P* in the zygotic genome. We tested the hypothesis that at least some maternal dS2P activity is supplied via mRNA. Figure 4B shows real-time PCR analysis of transcript abundance in 0- to 2.5-hr AEL embryos and 36-hr AEL larvae. At 0–2.5 hr AEL, before the onset of most zygotic transcription, the offspring of heterozygous mothers show significant levels of dS2P transcript, about one-third of wild-type levels, while no dS2P transcript is detectable in the offspring of homozygous mothers. By 36 hr AEL, no dS2P transcript is detectable in *dS2P* mutant larvae irrespective of the maternal genotype.

Transcript abundance of CG6295, a highly transcriptionally responsive target of dSREBP (KUNTE *et al.* 2006), is shown as an indicator of dSREBP activity in these larvae. We found reduced transcript abundance in the homozygous offspring of heterozygous mothers and a much more substantial deficit in offspring of homozygous mothers (Figure 4C). Interestingly, these later

TABLE 1

Rescue of *dSREBP* lethality by wild-type and mutant *dSREBP* cDNA

Transgene construct lines		Either transgene (GAL4 or UAS-dSREBP)		Both transgenes (GAL4 and UAS-dSREBP)		
		dSREBP^{189}		*dSREBP*^{189}		
GAL4 driver	UAS-dSREBP	+	−	+	−	% rescue
6487	NP → FL (A)	140	0	658	176	63.3
	NP → FL (B)	325	0	658	114	44.3
	NP → FL (C)	419	0	574	155	63.8
	Totals	884	0	1890	445	
6487	Wild type (A)	282	2	473	219	94.9
	Wild type (B)	348	1	601	189	71.8
	Wild type (C)	28	0	739	288	84.1
	Wild type (D)	291	2	502	213	89.4
	Totals	949	5	2315	909	

We prepared *P*-element-based germline transformation constructs that encode either wild-type *dSREBP* cDNA or cDNA carrying the mutation $N_{462}P \rightarrow FL$, which abolishes cleavage by dS2P. Independent second chromosome insertions of each transgene were isolated (designated A, B, C, and D) and used to generate stocks of the genotypes *w*$^{-}$;P{*w*$^{+}$, *UAS-dSREBP*}/P{*w*$^{+}$,*UAS-dSREBP*}; *dSREBP* ^{189}/ *TM6B, Tb Hu e* (for homozygous viable transgene insertions) and *w*$^{-}$; *UAS-dSREBP/CyO; dSREBP* ^{189}/ *TM6B, Tb Hu e* (for homozygous lethal transgene insertions). These were crossed to flies carrying the 6487 GAL4 driver (expressed predominantly in anterior gut, fat body, and oenocytes) of the genotype *w*$^{-}$;P{*w*$^{+}$, *GAL4*}/P{*w*$^{+}$, *GAL4*}; *dSREBP*^{189}/ *TM6B, Tb Hu e*. Adult progeny were scored for homozygosity at the *dSREBP* locus and for the presence of the responder transgene. Wild-type and mutant *dSREBP* cDNA transgenes rescue *dSREBP* mutants (range 44.3–63.8% for NP → FL and 71.8–94.9% for wild type).

animals show greater abundance of CG6295 transcript than do *dSREBP*^{189} larvae, even in the complete absence of detectable dS2P transcripts (see below).

dSREBP mutated at site 2 rescues dSREBP null flies: The NH_2-terminal transcription factor domain of dSREBP, which is the product of cleavage by dS2P, is needed to rescue dSREBP mutants (Kunte *et al.* 2006). Cleavage of dSREBP by dS2P requires an $asp_{462}pro$ motif in the first membrane-spanning helix of dSREBP (Ye *et al.* 2000a). When $N_{462}P$ is mutated to phenylalanine–leucine, dSREBP cleavage is abolished (Seegmiller *et al.* 2002). Since flies entirely lacking *dS2P* can survive, cleavage of dSREBP by dS2P is not essential for survival. Accordingly, an $N_{462}P \rightarrow FL$ mutant dSREBP that cannot be cleaved by dS2P should be able to rescue flies otherwise lacking dSREBP.

To test this hypothesis, we prepared transgenic flies expressing either wild-type or $N_{462}P \rightarrow FL$ mutant dSREBP cDNAs under the control of the yeast GAL4 upstream activating sequence. Expression was driven by the 4687 GAL4 enhancer trap line, which we have previously show is able to rescue *dSREBP* null mutant animals to adulthood when driving expression of dSREBP (Kunte *et al.* 2006). These transgenes were tested in a *dSREBP* null background. Samples of emerging flies were analyzed by sequencing PCR products to confirm the presence of the indicated *dSREBP* transgenes. Table 1 shows that both wild-type and mutant SREBPs can substantially rescue *dSREBP* null flies to adulthood, although the NP → FL mutant does so less efficiently than wild-type dSREBP.

Alternative cleavage of dSREBP in flies: Nuclear dSREBP is essential for larval survival but cleavage of dSREBP by dS2P is not. This implies that transcriptionally active dSREBP must be present in the nuclei of *dS2P*^{1}/*dS2P*^{2} larvae owing to a mechanism that does not require dS2P. To test this hypothesis, we used the previously described P{*GAL4-dSREBPg*} and P{UAS-GFP} binary reporter system (Kunte *et al.* 2006) to assess dSREBP processing in *dS2P* mutants (Figure 5A). Virgin females homozygous for either *dS2P*^{1} or *dS2P*^{2} on the second chromosome and homozygous for both the P{*GAL4-dSREBPg*} and P{UAS-GFP} transgenes on the third chromosome were crossed to males of the same genotype heterozygous on the second chromosome. Embryos were raised on standard medium until third instar when they were examined by fluorescence microscopy. Fluorescence owing to GFP expression is readily detectable in *dS2P* mutants (Figure 5B, top and middle), although at levels lower than seen in heterozygous siblings (Figure 5B, bottom). Thus, release of the amino-terminal transcription factor domain from dSREBP occurs even in the absence of dS2P.

We noted above (*cf.* Figure 4) that the *dS2P*^{1}/*dS2P*^{2} homozygous offspring of homozygous mothers showed transcription of CG6295 that was greater than in *dSREBP* mutants. This is consistent with the presence of the dSREBP transcription factor domain in the nuclei of

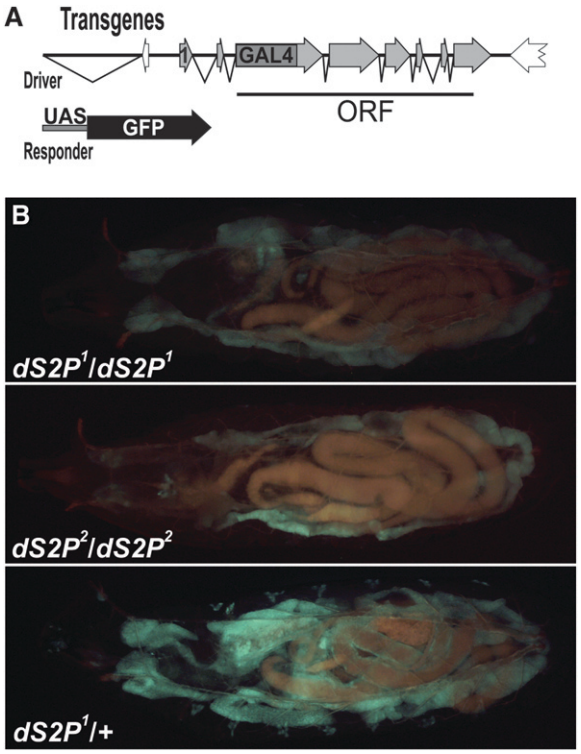

Figure 5.—Larvae lacking *dS2P* nevertheless process dSREBP. (A) A binary reporter system for dSREBP activity (Kunte *et al.* 2006). The transcription factor domain of pP{*dSREBPg*} was replaced by a GAL4-VP16 transcription factor to generate pP{*GAL4-dSREBPg*}. (B) Animals homozygous for both P{*GAL4-dSREBPg*} and P{UAS-GFP} transgenes in the indicated *dS2P* background were examined for spatial localization of GFP fluorescence. In larvae homozygous for either *dS2P* allele, fluorescence is detectable in fat body but levels are decreased relative to heterozygous siblings. No fluorescence is detectable in the midgut of *dS2P* homozygotes, in contrast to heterozygotes. Although not clearly visible in photographs, we detect a faint fluorescence in the oenocytes of many *dS2P* homozygotes. All larvae are the offspring of mothers homozygous for the indicated dS2P allele. Images are 1-sec exposures taken using a Leica MZ16FA fluorescence microscope equipped with an Evolution MP digital camera (Media Cybernetics) and In Focus software (Meyer Instruments, Houston). GFP fluorescence was visualized using a GFP2(+) filter set for MZ16 FA, 480/40, 510 nm, and images were captured using ImagePro software.

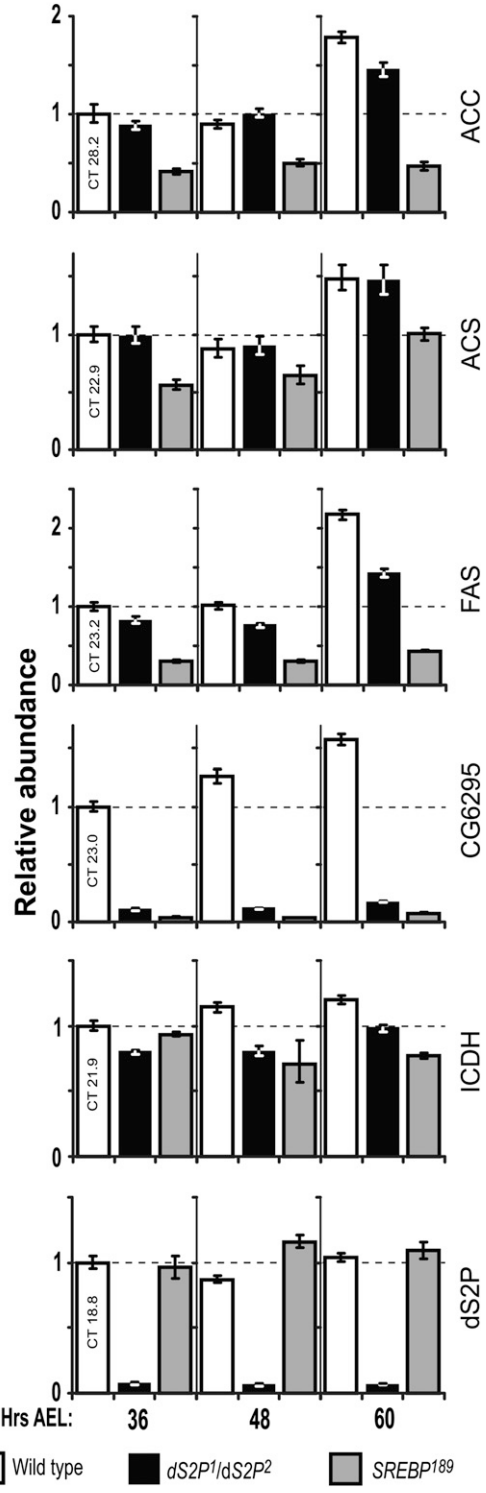

Figure 6.—Larvae null for *dS2P* show a less severe transcriptional deficit in genes of fatty acid synthesis than *dSREBP* null larvae. Total RNA was extracted from larvae at the indicated times, and transcript abundance was determined by real-time PCR as described in materials and methods. Relative abundance was normalized to wild type at 36 hr AEL.

dS2P larvae. To determine if this pattern held true for other target genes, we performed real-time PCR analysis. Figure 6 shows mRNA abundance at 36, 48, and 60 hr AEL for the indicated dSREBP target genes (Dobrosotskaya *et al.* 2002; Kunte *et al.* 2006). At 36 hr, $dS2P^{1}/dS2P^{2}$ and wild-type larvae show similar abundance of transcripts for acetyl–coenzyme A carboxylase, synthase, and fatty acid synthase. These transcripts are less abundant in $dSREBP^{189}$ larvae. This pattern continues through 60 hr. By contrast, transcripts for CG6295 are much less abundant in $dS2P^{1}/dS2P^{2}$ than in wild type, more closely matching their abundance in $dSREBP^{189}$ larvae. We consistently observe the small increase in abundance in $dS2P^{1}/dS2P^{2}$ larvae *vs.* $dSREBP^{189}$ larvae. Thus, $dS2P^{1}/dS2P^{2}$ larvae have less severe transcriptional deficits than do $dSREBP^{189}$ larvae.

DISCUSSION

We isolated mutant *D. melanogaster* harboring a deficiency that removes the entire *dS2P* transcription unit (Figure 1A). No dS2P mRNA is detectable in these animals and no dSREBP processing is observed in mutant adults under conditions where it is readily observed in wild-type flies. Instead, the substrate for dS2P cleavage, the intermediate form of dSREBP, accumulates in membranes (Figure 1D). Therefore, the $dS2P^{1}$ deletion is a null allele of *dS2P*.

Phenotypes of the *P*-element insertion allele, $dS2P^{2}$, are indistinguishable from $dS2P^{1}$ and are no more severe in *trans* to the deletion allele. Transcripts from $dS2P^{2}$ cannot yield catalytically active dS2P (Figure 1B). Thus, $dS2P^{2}$ is a null allele by genetic and molecular criteria. Surprisingly, animals harboring either allele are viable and can be readily maintained as homozygous stocks. Reciprocally, $dSREBP^{189}$ flies can be rescued by expressing a dSREBP cDNA harboring an $N_{462}P \rightarrow FL$ mutation that renders dSREBP refractory to cleavage by dS2P (Table 1). Thus, the site-2 protease is not essential for the development and growth of *D. melanogaster*.

The $dS2P^{1}$ allele must also be null for the predicted gene CG34229 (Figure 1A) that encodes a putative component of the higher eukaryotic NADH complex. The predicted sequence of the encoded polypeptide is highly conserved, supporting the case for this gene.

Are there consequences of the loss of CG34229 that influence the phenotypes that we report? We cannot absolutely exclude the possibility that some phenotypes could result, in part, from haplo-insufficiency for CG34229 in *dS2P trans*-heterozygotes. However, CG34229 cannot be an essential gene; $dS2P^{1}$ homozygotes are viable. We performed most of the experiments presented here with mutants *trans*-heterozygous for $dS2P^{1}$ and $dS2P^{2}$. In parallel experiments, we found indistinguishable results with flies homozygous for either $dS2P^{1}$ or d$S2P^{2}$ (not shown), which indicates that the phenotypes that we observe are not the result of the loss of CG34229. Further, the reduced survival of *dS2P* mutants is rescued by feeding fatty acids, a treatment that also rescues lethality in animals lacking *dSREBP*. This indicates that reduced survival is a consequence of reduced dSREBP activity.

The phenotype informative for the most important finding described here is cleavage of dSREBP in the absence of dS2P (Figure 5B). Whether or not insufficiency for CG34229 (or any gene yet to be identified in this region) contributes in some way to reduced viability, smaller-average-size, or delayed development in *dS2P* homozygotes, dS2P is absent and dSREBP does reach the nucleus without cleavage by dS2P (Figures 5 and 6).

In mammals, S2P is needed to process other membrane-bound transcription factors, ATF-6α and -β, that play a crucial role in the endoplasmic reticulum (ER)-stress response [also known as the unfolded protein response or UPR (Ye *et al.* 2000b)]. The Drosophila genome encodes a protein highly similar to mammalian ATF-6, CG3136. In mammals, ATF6 is required to transcribe XBP1 mRNA, and mutant cells lacking S2P are deficient in the induction of the spliced form of XBP1 mRNA (Yoshida *et al.* 2006). When $dS2P^{-}$ larvae are challenged with dithiothreitol or tunicamycin, treatments that elicit the UPR, we see no difference in XBP1 splicing compared to wild-type larvae (supplemental Figure 1). If the Drosophila UPR is closely similar to the mammalian UPR, these data suggest that ATF6 processing is relatively unimpaired in $dS2P^{-}$ larvae. It might be that the Drosophila homolog of ATF6 is not required for the fly UPR or that its activity does not require cleavage by dS2P. If dS2P *is* required to activate this homolog in flies, the observed developmental delay of $dS2P^{-}$ larvae may result from defects in ATF6 activation. Nevertheless, while these putative additional functions of dS2P may be important, the crucial function of dS2P in flies is to process dSREBP.

In striking contrast to $dS2P^{-}$ adults, which lack nuclear dSREBP under conditions where it is readily detected in wild type, dSREBP can reach the nucleus and activate transcription of target genes in *dS2P* mutant larvae (Figure 5B). Thus, Drosophila larvae lacking *dS2P* have an alternative means of releasing the nuclear transcription factor domain of dSREBP from the membrane-bound precursor. This explains the greater abundance of dSREBP target transcripts in $dS2P^{1}/dS2P^{2}$ mutants compared with $dSREBP^{189}$ mutants (Figures 4 and 6).

What is the role of this alternative mechanism for producing nuclear dSREBP? The current data show only that it occurs in the absence of dS2P. We do not yet know if it is a normal, physiologically relevant mechanism or whether it happens fortuitously in the absence of normal dSREBP processing. It is, however, sufficient to afford the survival, over many generations, of flies completely lacking dS2P.

How is the transcription factor domain of dSREBP produced in *dS2P* mutants? A possible mechanism is production of alternative transcripts that encode only the dSREBP transcription factor domain without the membrane-spanning helices. These might arise from different promoter usage or from differential splicing. Arguing against these possibilities is the fact that only a single transcript is detected for dSREBP in flies from embryogenesis through adulthood and in various tissues examined (Theopold *et al.* 1996). We likewise observe a single band on Northern blots for dSREBP (not shown). Any putative alternative transcripts or splice forms would have to be present at levels too low to be detected in these assays, while the activity of nuclear dSREBP in $dS2P^{1}/dS2P^{2}$ larvae is readily detected (Figures 5 and 6). Moreover, a cDNA construct harboring the $N_{462}P \rightarrow FL$ mutation and under control of a single, heterologous promoter rescues *dSREBP* mutants (Table 1). This construct has no exons; it is not subject to alternative splicing nor is it cleaved by dS2P (Seegmiller *et al.* 2002).

We favor the hypothesis that in larvae lacking dS2P, dSREBP is released from the membrane by some other protease(s). This posited protease is unlikely to cleave within the first membrane-spanning helix of dSREBP: flies have no other S2P homologs, and other intramembrane-cleaving proteases display different substrate preferences (*cf.* Hooper and Lendeckel 2007). The signal peptide peptidase (SPP) is an intramembrane protease of the ER (Weihofen *et al.* 2002). SPPs are unlikely candidates for cleavage of SREBPs, however. Like S2P, the SPPs require prior cleavage of the substrate by a separate protease. Chinese hamster ovary (CHO) cells express active SPP (Dev *et al.* 2006), but multiple, independently isolated lines of CHO cells lacking S2P show no processing of SREBPs (Sakai *et al.* 1996). If SPPs could cleave SREBPs, one would expect some evidence of SREBP processing in $S2P^-$ cells. Cleavage of dSREBP *following* its first membrane-spanning helix cannot release the NH_2-terminal domain. It is most probable that the alternative cleavage occurs in the cytoplasm, between the transcription factor domain and the first membrane-spanning helix of dSREBP. We term this portion of dSREBP the "stalk."

Cleavage of SREBPs within the stalk has been reported previously. Wang *et al.* showed that caspases 3 and 7 could each cleave mammalian SREBPs (Wang *et al.* 1995; Pai *et al.* 1996) and that this cleavage was detectable during apoptosis (Wang *et al.* 1996). The physiological significance of this cleavage is presently unclear. The caspase cleavage sites identified by Wang *et al.* (1995) are highly conserved among vertebrate SREBP isoforms, however, and all metazoan SREBPs (except those from Nematoda) contain potential caspase cleavage sites within their stalk regions (R. B. Rawson, unpublished observations). Using reporter constructs, Higgins and Ioannou showed that SREBP cleaved during apoptosis by caspases can be transcriptionally active (Higgins and Ioannou 2001). There is precedent for caspase cleavage of SREBPs releasing the functional transcription factor.

Current data do not suggest that the production of nuclear dSREBP in dS2P mutants has any involvement with apoptosis. However, nonapoptotic roles of caspases have been found in Drosophila (Huh *et al.* 2004) and other systems (*e.g.*, reviewed in Algeciras-Schimnich *et al.* 2002). Cleavage of dSREBP in the absence of dS2P may be an example of a nonapoptotic caspase function. We are currently testing the hypothesis that dSREBP is cleaved by caspases to produce transcriptionally active dSREBP in *dS2P*$^-$ larvae.

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