Drosophila syntaxin Is Required for Cell Viability and May Function in Membrane Formation and Stabilization

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ABSTRACT

The role of the Drosophila homologue of syntaxin-1A (syx) in neurotransmission has been extensively studied. However, developmental Northern analyses and in situ hybridization experiments show that SYX mRNA is expressed during all stages and in many tissues. We have isolated new mutations in syx that reveal roles for syx outside the nervous system. In the ovary, SYX is present in the germarium, but it is predominantly localized to nurse cell membranes. Mitotic recombination experiments in the germ-line show SYX is essential for oogenesis and may participate in membrane biogenesis in the nurse cells. In the early embryo, a large contribution of maternally deposited RNA is present, and the protein is localized at cell membranes during cellularization. After the maternal contribution is depleted, zygotically produced SYX assists secretion events occurring late in embryogenesis, such as cuticle deposition and neurotransmitter release. However, SYX is also required in larval imaginal discs, as certain hypomorphic mutant combinations exhibit rough eyes and wing notch defects indicative of cell death. Furthermore, recombinant clones that lack syx cause cell lethality in the developing eye. We propose that, similar to its roles in cuticle secretion and neurotransmitter release, SYX may mediate membrane assembly events throughout Drosophila development.

The study of vesicle-mediated transport in a number of systems has greatly enhanced our understanding of the process by which membranes and their protein constituents are delivered to the proper target site during secretion and membrane addition. The observation that the molecular mechanisms that underlie vesicle trafficking in two distinct systems, constitutive secretion in yeast and regulated neurotransmitter release in higher eukaryotes, are conserved has led to the hypothesis that an ubiquitous machinery exists to execute the fusion event (reviewed in Bennett and Scheller 1993; Ferro-Novick and Jahn 1994). Extensive studies of the molecules that are proposed to mediate vesicle docking and fusion events in both systems have generated a number of theories regarding how these events occur. The model that has enjoyed the most popularity is the "SNARE hypothesis." The basic premise relies upon the interaction of putative membrane-anchored receptors, termed v- (vesicle) and t- (target) SNAREs, dependent on the membrane within which they reside. The SNAREs (SNAP receptors) are so named for their ability to attract the soluble NSF attachment proteins (SNAPS) and the ATPase NSF (N-ethylmaleimide-sensitive fusion protein) to the appropriate position to effect vesicle fusion with its target membrane (Sollner et al. 1993a,b). In brain, the SNAREs have been identified as synaptobrevin on the synaptic vesicle and syntaxin and SNAP-25 within the presynaptic membrane. These proteins have homologues in yeast that have independently been identified as essential genes that lie along the secretory pathway. It has therefore been speculated that the association of tissue- or cell-specific vesicle and target receptors with each other and/or with the NSF-SNAP complex may mediate the docking and fusion event (Sollner et al. 1993b; Pevsner et al. 1994; Schiaovo et al. 1995).

The phenotypes of yeast SNARE mutants (secretion blockade and post-Golgi vesicle accumulation) confirm the absolute necessity for these proteins in exocytosis (Novick et al. 1980). Furthermore, the brain SNARE homologues have also been shown to be essential to neurotransmitter release, as they are the targets of proteolytic cleavage by the clostridial and botulinum neurotoxins that block neurotransmission (Schiaovo et al. 1992; Hayashi et al. 1994; reviewed in Jahn and Niemann 1994). Though these data certainly do not contradict the premises of the theory, recent evidence has challenged the fundamental tenets of the SNARE hypothesis. Mutational analyses of two proteins, the putative v-SNARE n-synaptobrevin and the potential t-SNARE syntaxin, have been performed in Drosophila (Sweeney et al. 1994; Brodie et al. 1995; Schulze et al. 1995). These experiments have established that synaptobrevin and syntaxin both function downstream...
of the vesicle docking event, in contrast to what was originally proposed in the SNARE hypothesis. More importantly, genetic manipulations such as those that removed N-SYB or SYX function revealed that although syntaxobrevin may assist in Ca\(^{2+}\)-regulated neurotransmitter release, its function is not essential to the fusion event per se as spontaneous fusions still occur (Sweeney et al. 1994; Broadie et al. 1995). On the other hand, the role of syntaxin was shown to be absolutely essential to vesicle fusion in neurotransmitter release, as all forms of exocytosis, both spontaneous and evoked, are absent in syx null mutants (Broadie et al. 1995; Schulze et al. 1995). The precise function of syntaxin, whether in a prefusion or “priming” step, or as a catalyst of the fusion event itself, has yet to be elucidated.

Clues to syntaxin’s function have been revealed by rigorous analysis of its protein structure and its interactions with other proteins. Syntaxin has been shown to bind in vitro to all the components that may form the SNARE complex (syntaxobrevin, SNAP-25, NSF and α-SNAP), and amino acid domains within the protein that are crucial for these interactions have been delineated (Calakos et al. 1994; Hayashi et al. 1994; Peusner et al. 1994; Hanson et al. 1995; Kee et al. 1995). Syntaxin was originally isolated due to its interaction with the synaptic vesicle membrane protein synaptotagmin, and this interaction has been shown to occur in a Ca\(^{2+}\)-dependent manner (Bennett et al. 1992; Chapman et al. 1995). Finally, syntaxin also interacts with presynaptic N-type calcium channels (Sheng et al. 1994) in a manner that may effect their function (Bezprozvanny et al. 1995). The association of syntaxin with synaptotagmin and Ca\(^{2+}\) channels in the neuron strongly supports the placement of syntaxin’s function late in the cascade of events that culminate in vesicle fusion upon Ca\(^{2+}\) influx.

We initially reported that the expression of the Drosophila syntaxin-IA homologue is not restricted to the nervous system as is thought to be the case for its vertebrate counterpart. syx functions in cuticle secretion and possibly in the garland cells where it may assist in the clearance of waste from the hemolymph via endo- and exocytosis (Schulze et al. 1995). To gain further insight into SYNTAXIN’s (SYX) potential role in vesicle fusion, we have examined its function in a variety of secretory processes occurring throughout Drosophila development. Herein we present preliminary evidence that SYX may be required for many exocytotic events during fusion or prefusion steps that may occur in a similar manner as has been suggested for neurotransmission. Cumulatively, these data point to a critical and essential role for SYX in membrane fusion and biogenesis.

**MATERIALS AND METHODS**

**Drosophila stocks and mutagenesis:** All Drosophila strains were maintained on standard cornmeal-molasses medium at 25°C unless otherwise noted. Canton-S was used as the wild-type strain. A deficiency that uncovers all syx alleles (Df(3R)cyb\(^{56-5}\), breakpoints 95D7–11; 95F15) was provided by Dr. Elizabeth Knust (Tepass et al. 1990). Some syx alleles were recombined over TM6B and placed in a y w background using either the balancer strain y w/y w; D\(^{2+}\)TM6B, Tb P[w\(^{1}\)$]Abd-a-lacZ or y w/y w; l/Cyo; P[w\(^{1}\), lacZ]sp; D\(^{2+}\)TM6B, Tb P[w\(^{1}\),$]Abd-a-lacZ.

The P-element insertion strain P[w\(^{1}\), syx\(^{1}\)]syx\(^{3}\)/TM3, Sb sy (abbreviated syx\(^{4}\)) was obtained from the Drosophila Stock Center in Bloomington, Indiana (P[syx], Schulze et al. 1995). Excision of the syx\(^{4}\) insertion was achieved by mating individual P[w\(^{1}\), syx\(^{1}\)]syx\(^{3}\)/TM3 males with virgin females of the genotype y w/y w; K\(^{1}\)$\Delta25; K\(^{1}\)$\Delta25-3. Individual y w; P[w\(^{1}\), syx\(^{1}\)]syx\(^{3}\)/K\(^{1}\)$\Delta25; K\(^{1}\)$\Delta25-3 males (or females) were backcrossed to females (males) of the original insertion strain to balance the excision chromosome. The progeny of these crosses were scored for individuals with rosy eye color, indicative of an excision event, from each of which \(\Delta syx\(^{4}\)y w/TM3, Sb sy balanced stocks were established. Complementation tests with the original insertion strain were subsequently performed to define the nature of the excision events as precise or imprecise.

To isolate point mutations, isogenized y w males were fed 25 mM EMS suspended in 1% sucrose using a standard protocol (Lewis and Bacher 1968). The mutagenized chromosomes (10,500) were balanced over TM6B, Tb and assessed for failure to complement the original P-element insertion syx\(^{4}\) in vivo at 28°C.

**Induction of mosaic animals and germline clones:** Genetic mosaics were generated using the FLP/FRT recombinase system (Chao and Perrimon 1992) essentially as outlined by Xu and Rubin (1993). The alleles syx\(^{255}\), syx\(^{5}\) (Df(3R)\Delta229 and Df(3R)\Delta6, Schulze et al. 1995) and syx\(^{5}\) (each in a y w background) were recombined onto a chromosome containing P[w\(^{1}\)$ hs-neo FRT/S2B using selection for resistance to G418 (Genetec, Gibco) and verification of failure to complement the original syx allele. Males containing the heat-inducible FLP recombinase (y w P[w\(^{1}\) hsFLP]/N) and the chromosomes P[w\(^{1}\)$ hs-neo FRT/S2B P[w\(^{1}\)$ hs-neo FRT/S2B (for assaying germline clone development) were constructed and crossed to y w; P[w\(^{1}\)$ hs-neo FRT/S2B syx females. First instar larvae (24–48 hr AEL) were incubated in a 38°C water bath (in the vials in which they were laid) for 1 hr to induce mitotic recombination. Adult females of the appropriate genotype were examined for clones (w\(^{5}\)) or assayed for egg-laying ability after several days of mating to y w; syx\(^{255}$ TM6b, Tb males. In some cases, the ovaries of females that failed to lay eggs were dissected (see below). P[FRT] and P[marker] strains were obtained from the Bloomington, Indiana Stock Center.

**Construction of genomic rescue transgenes:** An 11.0 kb XbaI fragment from genomic phage A10 and a 6.0-kb Not fragment from genomic phage AB was subcloned into the vector pcSAp63, which contains the 5′ and 3′ P-element integration sequences flanking the white gene (Pirrotta 1988). The constructs were injected into y w; K\(^{1}\)$\Delta25;+/ embryos (Bellen et al. 1992), and surviving adults were backcrossed to y w. Progeny bearing w\(^{5}\) eyes (indicating stable integration of the construct at a chromosomal locus) were selected and served as founders of stocks. Seven second chromosome insertion strains were generated that contained the A10 XbaI construct, and six second chromosome insertion strains were created that bore the A6 Not construct.

To determine if either of the two P[w\(^{1}\)$ syx\(^{1}\)] constructs could rescue the lethality of syx\(^{255}\) and syx\(^{255}$, y w; P[w\(^{1}\)$ syx\(^{1}\)]/CyO; D\(^{2+}\)TM6B, Tb males were crossed to y w; syx\(^{255}$ TM6B, Tb females. O: the progeny of this cross, brothers and sisters of the genotype y w; P[w\(^{1}\)$ syx\(^{1}\)]+/; syx\(^{255}$ TM6B, Tb were mated. Recovery of non-Tubby progeny was indicative of rescue.

**In situ hybridization:** To determine the distribution of SYX mRNA in wild-type embryos, antisense RNA probes were synthesized from the full-length 1.4 kb cDNA subcloned into Bluescript II SK+. RNA digoxigenin-labeled probes were pre-
pared as described in the Genius RNA nonradioactive labeling detection kit (Boehringer Mannheim). In situ hybridization to whole mount embryos was performed as previously described by Ingham et al. (1991) and Tautz and Pfeifle (1989).

**Immunohistochemistry:** Appropriately staged wild-type and syx mutant embryos were collected and processed for immunocytochemical staining according to standard techniques outlined in Salzberg et al. (1994). MAb 8C3, which recognizes Drosophila Syntaxin-1A and was given by Dr. Sev- 

Mour Benzer, was used at a final dilution of 1:10 (Fujita et al. 1982). MAb 1D4, which recognizes the cytoplasmic domain of Drosophila Fasciclin II (Van Factor et al. 1993), was obtained from Dr. Corey S. Goodman and was used at a final dilution of 1:50 to detect if motoneuron growth cone guidance and fasciculation occurred normally. MAb 22C10, a neuronal marker that recognizes a membrane antigen on all cells of the Drosophila peripheral nervous system (Fujita et al. 1982; Goodman et al. 1984) was utilized at a 1:200 dilution to analyze overall neuronal morphology. Nuclear staining of embryos was achieved by treating fixed embryos with 125 units ribonuclease A for 30 min at 37° C. Propidium iodide (50 

pg/mL) was applied concurrently with the primary antibody.

Ovaries were dissected and fixed as described in Xue and Cooley (1993). Fixed ovaries were processed for immunohistochemistry using the procedure for embryos outlined in Salzberg et al. (1994). To visualize SYX protein distribution, MAb 8C3 was utilized at a dilution of 1:10. Biotinylated goat anti-mouse secondary antibody (Vector) was used at a 1:200 dilution. The Vecta-Stain ABC-HRP kit (Vector) was used to augment the DAB-peroxidase signal. Fluorescin isothiocya-

nate-conjugated anti-mouse IgG was utilized at a 1:200 dilution.

Paraffin sections of adult Canton-S heads were prepared for mass histochemistry using a modification of the protocol of Jäger and Fischbach (1987). The primary antibody (anti-rat Syntaxin polyclonal I378, Hata et al. 1995) was diluted 1:200.

**Molecular techniques:** Total RNA for Northern analysis was isolated by LiCl precipitation of phenol-chloroform extracted Drosophila homogenates, and poly-A+ RNA was purified with oligo-dT cellulose (Sambrook et al. 1989). Approximately 5 

pg poly-A+ RNA was loaded per lane. The probe was generated by PCR of the 3' untranslated region of the syx cDNA, including 430 bp between the stop codon and the polyadenylation signal. A 3.0-kb EcoRI genomic fragment served as the template for the reaction. The primers used were 5'-CGACCACACGTTATGTTTCCATCACTG-3' as well as two primers designed from syx sequence (5'-GTTATTGTTGACTACACTGTTACC-3' and 5'-GTTTACACTGAAAAATTGTGTTGCC-3') to determine if the P element had been completely removed or internally deleted (see Schulze et al. 1995). The conditions for the PCR were as follows: 1 cycle at 95° (5 min); 35 cycles at 94° (1.5 min), 51–53° (2 min) and 72° (3 min); 1 cycle at 72° (5 min).

**RESULTS**

**syx expression is widespread throughout Drosophila development:** We initially reported the expression and distribution of SYX transcripts and SYX protein during the latter half of embryogenesis (Schulze et al. 1995). The extent of expression of SYX during Drosophila development was further investigated by Northern blotting of RNA representing all stages of embryogenesis, larval life and adulthood. We tested three probes: the 3' end of the cDNA containing coding region that would be most conserved (G-terminal portion of the protein), a probe containing 5' untranslated sequence and sequence coding for the first 44 amino acids (not well conserved among syntaxins), and a probe containing only 3' untranslated sequence. All three probes recognized the same complex pattern composed of up to six transcripts, ranging in size from 3.5 to 12 kb (see Figure 1). SYX messages are present during every stage of Drosophila development. Although the pattern of transcripts is very complex considering that the small (1.4 kb) cDNA we and others (Cerezzo et al. 1995) have recovered is contained within a single exon, it is likely that alternative splicing, various promoters, and different polyadenylation signals are used to generate this heterogeneity. We feel confident that each of these transcripts represent SYX messages and not transcripts from other related and possibly highly homologous Drosophila syntaxins that may exist, since three different probes generated identical patterns. Further, these transcripts are likely to encode a single protein as developmental Westerns display only a single band (Schulze et al. 1995; data not shown). In addition, low stringency hybridization of genomic Southern blots failed to reveal any other bands than those corresponding to SYX (data not shown).

In young embryos [0–3 hr after egg lay (AEL)], the 3.5-, 4.2- and 8.0-kb transcripts are abundant. At 3–9 hr AEL, the 3.5-kb transcript slowly disappears while the 4.2-, 8.0- and 9.0-kb messages predominate until the end of embryogenesis. Between 9–15 AEL, two additional transcripts (7.0 and 12 kb) appear. During first instar larval life, five messages are present, whereas second and third instars utilize three and two messages,
To determine the distribution of SYX protein during oogenesis and embryogenesis, we immunocytochemically stained ovaries and embryos with either a polyclonal antibody raised against rat syntaxin-1A (Hata et al. 1993) or monoclonal antibody 8C3 (a gift from Konrad Zinsmaier and Seymour Benzer). The former antibody specifically recognizes a protein of the appropriate molecular weight in extracts prepared from Drosophila heads and identifies the protein encoded by the syx gene (Schulze et al. 1995), whereas the latter was prepared against the native Drosophila SYX protein (Fujita et al. 1982). In wild-type ovaries stained with MAb 8C3, we detected SYX protein in regions 2 and 3 of the germarium, outlining the membranes of germline cyst cells. SYX continues to be abundantly expressed in the nurse cell membranes of egg chambers during stages 1–8, and SYX levels fade during stages 8 and 9 (Figure 2A).

In young embryos during mitotic cycles 9–13, the plasma membrane partially envelopes each dividing nucleus, forming "cytoplasmic buds". Beginning in mitotic cycle 9, "pole buds" form at the posterior tip of the embryo, marking the formation and segregation of the germ cells (Foe et al. 1993). As is the case for syx mRNA, SYX is present ubiquitously in precellularized embryos but is concentrated at the posterior tip, and accumulates beneath these pole buds as they undergo cytokinesis (Figure 2C and D). Similarly during cycle 11 "somatic buds" form around dividing somatic nuclei but undergo several rounds of formation and collapse as the nuclei beneath the buds divide. At metaphase of each mitotic division, the membrane of the bud invaginates markedly, and "pseudocleavage furrows" form between the buds (Foe et al. 1993; Schejter and Wieschaus 1993). SYX expression appears to follow the outline of the buds as they partially enwrap the dividing nuclei during mitotic cycles 10–14 (Figure 2C). Finally, during cycle 14, the somatic nuclei become completely surrounded by the elongating membranes of the cytoplasmic buds, and cellularization is completed. SYX localization outlines these membranes as they form (Figure 2D). SYX continues to be expressed in the membranes of each cell of the fully cellularized embryo (Figure 2E).

Syntaxin protein expression does not become restricted to any tissue or cell type until stage 9 of embryogenesis. During germ band extension, the protein is present in the mesoderm, ectoderm and invaginating neurectoderm but is excluded from the amnioserosa (data not shown). During later stages of embryogenesis, the protein is most prominent in the CNS and the garland cells and is weakly expressed in the midgut and ectoderm (Schulze et al. 1995).

SYX protein is also present in the adult brain and the synaptic subations of the visual system (Figure 5A). Staining of paraffin sections through adult wild-type heads with an anti-rat syntaxin polyclonal antibody reveals SYX is concentrated in the neuropil regions of the brain and is somewhat enriched at synaptic regions.
Role of syx in Membrane Biogenesis

FIGURE 2.—Distribution of SYX mRNA and SYX protein in ovarioles and young embryos. Wild-type ovariole and embryos stained for SYX with MAb 8C3 or hybridized with an antisense SYX RNA probe. Embryos are oriented anterior to the left, dorsal up. Staging is according to CAMPOS-ORTEGA and HAETENSTEIN (1985). (A) Syntaxin is present in the nurse cell membranes of the ovary. The protein is abundant in the nurse cell membranes of the germarium (g) and the early stages of oocyte development but dissipates beginning at stage 8 (S8). (B) SYX RNA is present ubiquitously in embryos before cellularization but is concentrated at the posterior pole. (C) SYX distribution in an embryo that has not yet completed mitotic cycle 13 is observed in large, roughly hexagonal-shaped outlines that correspond to the pseudocleavage furrows of the cytoplasmic buds. (D) An embryo that nears the completion of somatic cellularization (cycle 14) demonstrates SYX staining along the membranes of each cell (green). SYX is also concentrated beneath the pole cells. Nuclei are labeled with propidium iodide (red). (E) A completely cellularized embryo is beginning gastrulation (stage 6), as the cephalic and ventral furrows are forming. SYX is present in all the membranes of all cells at this stage. (F) A stage 9 embryo exhibits SYX RNA localization in the anterior (am) and posterior (pm) midgut invaginations, the ventral neuroblasts (n) and the ectoderm.

such as in the lamina and medulla of the optic lobes where photoreceptor axons form synaptic contacts with second order neurons. However, SYX distribution in the brain differs from that of synaptic vesicle-specific proteins such as synaptotagmin (LITTLETON et al. 1993), as SYX is also present in axons and cell bodies whereas synaptotagmin is restricted to synaptic terminals.

Generation and molecular characterization of mutants in syx: To gain a better insight into syx's function, and to identify possible new phenotypes associated with partial loss of function alleles of syx, we performed two mutagenesis screens. In the first, the P element in syx'/ was imprecisely excised (SCHULZE et al. 1995). This P element created an hypomorphic syx mutation that compromised neurotransmission at the embryonic neuromuscular junction. Mature syx'/ embryos failed to exhibit
muscle contraction waves and emerge from the egg case. In addition, two of the embryonic lethal mutations recovered in this screen were phenotypically characterized in the previous work. syx\textsuperscript{229}, a null allele, exhibits complete blockade of all forms of neurotransmitter release. In addition, nonneuronal forms of secretion are impaired, as yolk digestion in the gut is abnormal and several layers of the cuticle are missing (SCHULZE et al. 1995; A. PROKOP, personal communication). syx\textsuperscript{6} also exhibits secretory defects that are intermediate in severity between syx\textsuperscript{229} and syx\textsuperscript{6}.

In the second mutagenesis, ~10,500 chromosomes were screened for EMS-induced mutations. The molecular defects borne by the excision mutations were analyzed by genomic Southern and PCR analyses, and the EMS-induced mutations were sequenced. The data is described below and pictorially in Figure 3.

syx\textsuperscript{229} is a null allele that almost entirely deletes the ORF and flanking regulatory regions (1.7 kb), whereas all other excision events produced alleles of varying severity. syx\textsuperscript{96} and syx\textsuperscript{284} appear to be severe hypomorphs or null alleles on the basis of their molecular deficiencies and the fact that, like syx\textsuperscript{229}, they fail to secrete most cuticle and are embryonic lethal (Table 1). We have previously shown that syx\textsuperscript{6} is a severe hypomorph but is not a null allele, as some residual protein is present (SCHULZE et al. 1995). syx\textsuperscript{2}, syx\textsuperscript{4}, syx\textsuperscript{17}, syx\textsuperscript{118}, syx\textsuperscript{177}, syx\textsuperscript{204}, syx\textsuperscript{294} and syx\textsuperscript{494} bear incomplete internal deletions of the P element (Figure 3). syx\textsuperscript{4}, syx\textsuperscript{17}, syx\textsuperscript{118} and syx\textsuperscript{294} have an intermediate phenotype similar to that caused by the original P element insertion syx\textsuperscript{6}; these alleles are embryonic lethal, display wild-type cuticular structures, but fail to exhibit muscle contraction waves. Finally, on the basis of their viability, syx\textsuperscript{2}, syx\textsuperscript{177}, syx\textsuperscript{204} and syx\textsuperscript{494} are weak hypomorphs.

Only two point mutations were recovered from the EMS mutagenesis (1/5250 chromosomes screened). syx\textsuperscript{r} carries a premature termination signal (Q101 to stop) and behaves as a null allele (see Table 1). syx\textsuperscript{175a} contains no detectable aberrations in the coding region and probably carries a temperature-sensitive mutation in a regulatory domain (see below and discussion). Table 1 summarizes the complementation data among all the syx alleles recovered, as well as the lethality of each individual allele when tested in trans to itself and the two null alleles, syx\textsuperscript{229} and syx\textsuperscript{6}. Almost all the alleles are embryonic lethal as homozygotes and as transheterozygotes with the null alleles. Their phenotypes range in severity from that of the null allele itself, which lacks cuticle and bears other secretory defects, to that of the original P element allele, which exhibits a normal cuticle but no spontaneous muscle contractions. These two phenotypic categories seem to correspond well to the molecular defects, with the null-like phenotype restricted to alleles with an imprecise deletion of the P element that removed surrounding genomic DNA (syx\textsuperscript{6}, syx\textsuperscript{17}, syx\textsuperscript{26} and syx\textsuperscript{284}). The hypomorphic phenotype is displayed by alleles bearing small internal deletions within the P element (syx\textsuperscript{2}, syx\textsuperscript{17}, syx\textsuperscript{118} and syx\textsuperscript{294}). However, a third group, represented by alleles with <1 kb of the P element insertion remaining (syx\textsuperscript{6}, syx\textsuperscript{177}, syx\textsuperscript{204} and syx\textsuperscript{294}), are very weak hypomorphs and often produce viable progeny in trans to other syx alleles. Furthermore, syx\textsuperscript{6} homozygotes are completely normal and fertile adults, whereas males homozygous for syx\textsuperscript{177} (an allele with a molecular defect indistinguishable from syx\textsuperscript{6}) are sterile (data not shown).

**Rescue of the lethality associated with syx\textsuperscript{6} and syx\textsuperscript{229}**

To determine if syx\textsuperscript{6} and the deletions generated from its excision were bona fide mutations in syx, we rescued the lethality associated with the original P element insertion allele and the most severe loss of function (null) allele, syx\textsuperscript{229}. Two rescue constructs (shown in Figure 3) were comprised of the genomic region encompassing the ORF and up- and downstream sequences. This genomic DNA was engineered into the pCaSpeR3 vector and injected into embryos, and from those that had been stably integrated, 12 second chromosome transformant lines were generated. The second chromosomes bearing these constructs were crossed into the syx\textsuperscript{6} and syx\textsuperscript{229} genetic backgrounds and assessed for their ability to rescue the lethality associated with these syx alleles. Two of the 12 transformants completely rescued the phenotypes associated with both syx\textsuperscript{6} and syx\textsuperscript{229}, as homozygotes carrying the rescue transgene were viable and fertile and overtly morphologically normal (data not shown). These results demonstrate that the P element and the deletion associated with syx\textsuperscript{229} both disrupt the syx gene and further, that a number of the transcripts may not be essential for viability and fertility, as their length exceeds that of the rescue construct.

**Syntaxin is required in the female germline for oocyte development**

Since Northern analysis and in situ hybridizations to very young embryos (0–3 hr AEL) indicated an abundance of SYX transcripts, we wished to determine the effect on early embryonic development of the removal of all maternally derived SYX activity. Mitotic recombination was induced by activating FLP recombinase expression in first instar larvae bearing the syx null allele, syx\textsuperscript{229} (located at 95E1-2), distal to a flippase recognition target site (FRT) on one chromosome arm X and two copies of P[w\textsuperscript{+} ovo\textsuperscript{P1}] distal to the FRT on the homologous chromosome arm. These animals were analyzed for egg-laying ability upon eclosion. ovo\textsuperscript{P1} on the third chromosome causes dominant female sterility leading to rudimentary ovaries that fail to develop beyond stage 8 or 9. Hence, females heterozygous for this P element insertion carrying ovo\textsuperscript{P1} cannot lay eggs. Nevertheless, individual ovarioles that have developed from a progenitor germline cell in which a mitotic recombination event has taken place, exchanging ovo\textsuperscript{P1} for syx, produce viable oocytes. In control experiments of this type, 60% of heat-induced females examined exhibited restoration of fertility (see Table 2). However, none of the heat-shocked FRT syx\textsuperscript{229}/ FRT w\textsuperscript{+} ovo\textsuperscript{P1} females were able to produce eggs. Of
the 918 FRT syx<sup>229</sup>/FRT w<sup>+</sup> ovo<sup>01</sup> females tested for egg-laying ability, 212 were dissected and their ovaries examined. All were rudimentary in their appearance and indistinguishable from that of ovo<sup>01</sup> heterozygotes.

We repeated these experiments using two weaker syx alleles, syx<sup>a</sup> and syx<sup>s</sup>. We have previously shown by Western blotting that some residual SYX activity is present in syx<sup>s</sup> mutants, whereas syx<sup>a</sup> homozygotes display even more reduced SYX levels than syx<sup>s</sup> mutants (Schulze et al. 1995). Although heat-shocked FRT syx<sup>a</sup>/FRT w<sup>+</sup> ovo<sup>01</sup> females are sterile, as shown in Table 2, 84% of the heat-shocked FRT syx<sup>a</sup>/FRT w<sup>+</sup> ovo<sup>01</sup> females tested demonstrated egg-laying ability. When these females were mated to y w syx<sup>a</sup>/TM6B, Tb males, 54% of their progeny survived because the zygotic syx<sup>a</sup> provided by the balancer chromosome of the male was sufficient to rescue the maternal syx<sup>a</sup> effect. The remainder (zygotic genotype syx<sup>a</sup>/syx<sup>229</sup>, maternal syx<sup>s</sup>/syx<sup>s</sup>) displayed a phenotype similar to that of syx zygotic null embryos (lack of cuticle structures and accumulation of yolk in the gut). Therefore, although SYX is essential for oogenesis to proceed to completion, the requirements for SYX in the development of the germline and for maternally derived SYX during zygotic development can be provided sufficiently by the weak syx<sup>a</sup> allele. Furthermore, any maternal effects of the syx<sup>a</sup> allele upon early embryonic development can be fully rescued by a wild-type zygotic contribution from the male.

**A temperature-sensitive mutation reveals syx's role in eye and wing development:** The isolation of a series of partial loss of function alleles of syx immediately revealed that syx plays a role in imaginal tissues. The most intriguing adult phenotype was observed among allelic combinations including the EMS-induced syx<sup>192</sup>. In every combination (except in trans to syx<sup>s</sup>, see discussion), decreased viability, roughened eyes and notched wings were observed in varying percentages of the transheterozygous viable progeny when the cross was performed at 25°C. The ommatidial array of these flies is irregular compared to wild type and resembles a weak Bar phenotype. The most severely affected eyes appear to have fewer ommatidia than wild type. The ommatidia
are sometimes fused and often are improperly rotated and misaligned with respect to one another (Figure 4B). These defects are often observed near an invagination of the photoreceptor array that occurs at the edge of the cuticle near the antenna. At times, this region of cuticle is enlarged and protrudes into the eye proper (compare arrows in wild type and mutant, Figure 4C). This rough eye phenotype was exacerbated by raising these progeny at 28°, as roughened eyes were observed in 100% of the transheterozygotes. However, when the cross is performed at 18° the effect is abolished, and all transheterozygotes expected emerge without defects (data not shown).

Another defect present in many of the \(~yx'~''\) transheterozygous combinations is notching along the posterior margin of the wing (Figure 4D). This effect is observed more prevalently in the transheterozygous \(syx\) progeny of a cross performed at 25° than in a similar cross raised at 28°. Interestingly, the number of viable progeny that emerge from crosses at 28° is reduced compared to the

### Table 1

**syx complementation and lethality**

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Complementation of given \(syx\) allele (name of allele indicated in left column) with other \(syx\) allele (name of allele indicated in top row), performed at 25°C and scored for viability. Lethality expressed as percentage of total fertile progeny that fail to emerge. - , no adults survive; -/+ , ≤10% of adults are viable (fair number of escapes); +/− , 11–22% transheterozygous viable (slightly less than 30% expected); +, ≥26% transheterozygous viable progeny (30% expected if complements); +*, transheterozygous viable progeny with eye and wing phenotypes; V, viable; 1L-2L, first or second instar lethal; 3L-P, third instar or pupal lethal; n.d., not determined.

### Table 2

**syx** clone frequency

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<th>P ([w^d])</th>
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<tr>
<td>Allele</td>
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<tr>
<td>P</td>
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<th>Eye color</th>
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<td>29</td>
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<td>31</td>
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<td>36</td>
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Frequency of recombination between an FRT \(syx\) chromosome (\(syx\) allele tested shown in left column) with an FRT \(P\) marker chromosome (\(P\) marker tested shown in top row) is expressed as a percentage. In the case of eye color, the percentage of FRT \(syx/FRT\, P[w^d]\) female progeny that exhibited one or more mosaic patches (either red or white) is listed. The total number of females of this genotype scored is given (n). Egg-laying ability was determined as described in MATERIALS AND METHODS and is expressed as the percent of the total number (n) of FRT \(syx/FRT\, P[w^d\, ov8]\) females examined for this phenotype. ND, not determined.
same cross at 25°C (e.g., syx14, syx284, syx17, syx287; data not shown), suggesting that a more severe wing phenotype or a complete penetrance of the wing phenotype among the progeny cannot be observed due to the concurrent effect on viability. Both the notched wing and reduced viability phenotypes are completely nullified by raising syx150 transheterozygotes at 18°C. The roughened eye and notched wing defects exhibited by these flies suggest that SYX functions during eye and wing development.

**Syntaxin is required for cell viability in the eye:** Although the defects in syx150 transheterozygotes are mild, we proceeded to further investigate the role SYX plays during eye development. Since the complete absence of syx results in embryonic lethality, mitotic recombination experiments were again performed to analyze the effects of lack of SYX function in individual cells of the developing eye. Recombination experiments were performed using the FRT/FLP recombination system as described above for the construction of w- ovoD1 mosaic animals. In this case, a second P[marker] chromosome was utilized that carried a P-element insertion at 90E containing only the eye color marker w+. As shown in Table 2, 18% of the adult FRT syx+/FRT w- females examined displayed w- clones. Similar results were obtained when FRT syx+/FRT w- ovoD1 females were scored for eye clones (20%). However, none of these females exhibited the concomitant syx150 and thus w- clone, indicating that the syx- sister cell clone born of the recombination event failed to survive in the absence of SYX. In control experiments using an FRT on a wild-type syx chromosome, 56% of the female FRT syx+/FRT w- progeny displayed both w+ and w- sister clones. Hence, lack of SYX activity in cells of the developing eye causes lethality.

The weaker syx alleles syx6 and syx7 both produce a cell lethal effect in the eye. As shown in Table 2, of the heat shocked FRT syx+/FRT w- or FRT syx+/FRT w+ females examined, none displayed w- clones. Hence, when SYX is either abolished (syx+1229) or reduced (syx+ or syx-), the w- clone bearing two copies of either of these alleles fails to develop alongside its sister w+/w- clone. No trace of the syx- clone is visible, such as a scar or a change in the overall size or pattern of the eye, suggesting that the clone dies soon after the recombination event. These results show that the requisites for SYX in the germline and the eye are different, with the eye requiring more SYX to complete its development.

**Syntaxin plays a role in membranes of the nervous system:** As we have previously shown, SYX’s principal role in the nervous system of Drosophila is its essential function in neurotransmitter release (SCHULZE et al. 1995). However, in syx1229 homozygotes, incomplete compaction of the ventral nerve cord is observed (SCHULZE et al. 1995). The effects of zygotic loss of SYX were closely examined by immunohistochemistry to syx1229 and syx6 embryos using MAbs 1D4 and 22C10. These antibodies respectively recognize fasciclin II and a neuronal membrane glycoprotein, and are excellent markers to observe defects in axonal structure. Late-stage homozygous syx1229 or syx6 embryos (stage 16 and 17) display incomplete fasciculation of the intersegmental (ISN) and segmental (SN) nerve bundles, which contain motor and sensory nerve fibers exiting and entering the CNS to and from the periphery (Figure 5C). A wild-type embryo stained with MAb 22C10 (Figure 5B) displays properly fasciculated axons in its ISN and SN bundles; the axons are tightly apposed and no gaps can be distinguished. The staining of the ISN and SN of a syx6 homozygote is quite diffuse, suggesting that the individual axons cannot adhere to one another perhaps due to inconsistencies in their membrane constituents (Figure 5C). Similar defects are observed in the longitudinal tracts of the CNS when observed with anti-fasciclin II antibody (Figure 5E). In the wild-type CNS, three longitudinal tracts are observed on either side of the midline, and each set of axon fibers exhibits a tight arrangement (Figure 5D). In the CNS of a syx1229 homozygote, the axon bundles are irregular and slightly defasciculated (Figure 5E). These data indicate that SYX affects properties of neuronal membranes. In addition, these defects worsen in aging embryos, suggesting that SYX plays a critical role in maintaining membrane composition and integrity.

**DISCUSSION**

Here we have utilized a variety of syx mutations to identify processes in Drosophila development that require syx. We have shown that the activity of SYX is
Thus rely upon syntaxin’s essential function in the fusion of vesicles with target membrane.

Developmental Northern analysis and in situ hybridization to wild-type embryos clearly indicate a large amount of maternally derived RNA is deposited into the oocyte. Furthermore, the localization of syntaxin protein during the early stages of embryogenesis suggests that SYX’s function may contribute to the tremendous amount of membrane biogenesis that occurs at this time. It is estimated that 23 times the amount of membrane overlying the syncytium of nuclei at mitotic cycle 14 is needed to completely cellularize the 6000 blastoderm cells of the Drosophila embryo (FOE ET AL. 1993; SCHEJTER AND WIESCHAUS 1993). Although the mechanism by which membrane addition occurs during cellularization is unknown, investigators have divided the process into two phases: a slow phase involving saltatory transport along astral microtubules and a fast phase in which the apical microvilli disappear. The contribution to the membranes of the developing blastoderm cells during the fast phase is believed to involve a flattening of the microvilli and hence may not require the synthesis and addition of new membrane (FULLILOVE AND JACOBSON 1971). However, during the earlier slow phase, particles have been observed to move along the microtubules with anterograde (toward the yolk) and retrograde (toward the nucleus at the apex) speeds resembling that observed in extruded squid axoplasm (FOE AND ALBERTS 1983; VALE 1987). Hence, in a similar way that vesicles are transported to the presynaptic terminals of neurons, phospholipid vesicles may be transported further away from the periphery of the cellularizing embryo to sites of membrane addition. SYX may play a role in the fusion of the vesicles at these sites, as it is enriched within the cytoplasmic buds that envelop the dividing blastoderm nuclei. This localization corresponds to that of the cytoskeletal elements and products of such genes as nullo and serendipity-A, which are believed to form the structural foundation for somatic membrane invagination (SCHEJTER AND WIESCHAUS 1993). Its localization to the apical and lateral walls at cycle 14 appropriately places SYX where it may perform this putative function in the fusion of membranous vesicles to achieve cellularization.

To determine if most of the RNA and hence protein requirements for syx during cellularization are supplied by the mother and to analyze the effects upon embryonic development when the maternal contribution is removed, we constructed females either totally lacking or bearing deficits in germline-derived syx. Females in which syx was completely absent in their germline primordia failed to complete oogenesis and produced no oocytes. On the basis of detection of SYX protein in the nurse cell membranes in the germarium and during the first eight stages of oocyte development, we propose SYX’s role in oogenesis may involve the production of additional membrane by the nurse cells, as they grow considerably during these stages to prepare for transfer...
of their cytoplasmic contents into the oocyte. However, a weak syx allele estimated to produce only 30% of wild-type levels of SYX was sufficient to rescue the defect in oogenesis such that viable eggs could be produced. In fact, when these eggs are fertilized by sperm carrying a syx’ chromosome, the zygotes are capable of developing normally. Hence, these data suggest the maternal and zygotic SYX components overlap in embryonic development.

A role for SYX in eye development or function was suggested by the observation that a temperature-sensitive allele, syx<sup>150</sup>, produces roughened eye phenotypes in trans to other syx alleles. The rough eye phenotype could be the result of improper secretion of factors that influence cell-cell communication between developing ommatidia or direct decision-making events that normally guarantee the precision and regularity of the ommatidial array. The defect could also arise from anomalies in membrane biogenesis within cells comprising each ommatidium. Further, the presence of nodules of cuticle at the edge of the ommatidial array indicates that secretion of cuticle is aberrant in these mutants. However, no molecular defects were detected in the ORF of the syx<sup>150</sup> chromosome. The observation that the phenotype is observed only in trans to alleles exhibiting a loss of part of the syx regulatory region suggests that the defect of this allele lies within the syx promoter. In addition, syx<sup>150</sup> / Df(3R)ob<sup>199A</sup> progeny are inviable, confirming that the mutation is cytologically near the syx locus. The fact that syx<sup>150</sup> / syx<sup>1</sup> embryos are viable and exhibit no defects suggests that, in the presence of at least one functional syx promoter, transcription is directed via transfection (Lewis 1954; Müller and Schaffner 1990). Although the precise functional anomaly caused by the syx<sup>150</sup> mutation cannot be predicted from these data, the phenotype does suggest a role for SYX in eye development and prompted further investigation.

When SYX production is abolished or diminished by the presence of either homozygous null or hypomorphic syx alleles in single cells of the developing eye imaginal disc, these cells or the clonal descendants derived from them are never observed in the adult. If a cell were to lose SYX function due to a mitotic recombination event, it is conceivable that it or its descendants may not be able to complete its division due to an inability to fuse and pinch off the membranes of the resultant sister cells. Such a cytokinesis defect has been suggested to underlie the phenotype of knolle mutants. The KNOLLE gene encodes an Archidopsis thaliana syx homologue, and in these mutants the concentric pattern of tissue primordia is absent due to a failure of the highly ordered cell divisions that produce this array to progress to completion (Lukowitz et al. 1996). The authors suggest that the Knolle protein may function in the addition of membrane at the cell plate during cytokinesis. Similarly, in dividing cells of the Drosophila eye imaginal disc as well as at cellular blastoderm and during pole cell formation, SYX may be required to assist in cytokinesis and membrane biogenesis by directly affecting the fusion of vesicles containing newly synthesized membranes. It is interesting to note that mutations in rop, a nSec1/Munc18/UNC-18 homologue (Salzberg et al. 1994), a gene encoding a protein thought to interact with SYX in vivo (Schulze et al. 1994), cause cell lethality in the eye (Harrison et al. 1994). In addition, mutations in rop cause very similar defects (Harrison et al. 1994) to those observed in syx mutants throughout development (this work), suggesting that both genes may function in general secretion and membrane biogenesis.

In conclusion these lines of evidence suggest but do not demonstrate that SYX may function in membrane assembly events throughout Drosophila development. All of the tissues in which SYX is highly expressed or in which mutations in syx have a dramatic effect on development or function share the common characteristic that they have a high requirement for fusion of vesicles to form membranes or secrete or release vesicle contents. We feel this evidence is highly suggestive of a role for SYX in membrane biogenesis in these tissues.

To this end, additional experiments to define SYX function in these tissues can now be performed using the panel of mutations described in this study. Electron microscopic analysis can be utilized to ascertain the severity of membrane instability in the nervous system of syx null and hypomorphic alleles. Immunolocalization of SYX in the cellularizing embryo can also be ascertained with electron microscopy. Since it was not possible to obtain embryos lacking the maternal syx contribution, the function of SYX during cellularization could be disrupted by the application of anti-syntaxin antibodies or the production of antisense SYX RNA. syx clones derived from mitotic recombination in the eye imaginal disc could be followed microscopically to determine if and when division by cells in the clone goes awry. These experiments should provide more conclusive answers as to the role of SYX in the development of these tissues.

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LITERATURE CITED


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