# Probable Mechanisms Underlying Interallelic Complementation and Temperature-Sensitivity of Mutations at the *shibire* Locus of *Drosophila melanogaster*

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ABSTRACT

The *shibire* locus of *Drosophila melanogaster* encodes dynamin, a GTPase required for the fission of endocytic vesicles from plasma membrane. Biochemical studies indicate that mammalian dynamin is part of a complex containing multiple dynamin subunits and other polypeptides. To gain insight into sequences of dynamin critical for its function, we have characterized in detail a collection of conditional and lethal *shi* alleles. We describe a probable null allele of *shi* and show that its properties are distinct from those of two classes of lethal alleles (termed I and II) that show intergroup, interallelic complementation. Sequenced class I alleles, which display dominant properties, carry missense mutations in conserved residues in the GTPase domain of dynamin. In contrast, the sequenced class II alleles, which appear completely recessive, carry missense mutations in conserved residues of a previously uncharacterized "middle domain" that lies adjacent to the GTPase region. These data suggest that critical interactions mediated by this middle domain are severely affected by the class II lethal mutations; thus, the mutant sequences should be very useful for confirming the *in vivo* relevance of interactions observed *in vitro*. Viable heteroallelic combinations of *shi* lethals show rapid and reversible temperature-sensitive paralytic phenotypes hitherto only described for the ts alleles of *shi*. When taken together with the molecular analysis of *shi* mutations, these observations suggest that the GTPase domain of dynamin carries an intrinsically temperature-sensitive activity: hypomorphic mutations that reduce this activity at low temperatures result in conditional temperature-sensitive phenotype. These observations explain why screens for conditional paralytic mutants in Drosophila inevitably recover ts alleles of *shi* at high frequencies.

DROSOPHILA carrying ts alleles of *shibire* display rapid and reversible temperature-dependent paralysis (Grigliatti *et al.* 1973). The paralysis of *shi*$^{ts}$ flies is due to a conditional block in synaptic vesicle recycling that results in vesicle depletion at nerve terminals and impaired synaptic transmission (Poodry and Edgar 1979; Kosaka and Ikeda 1983). In *shi*$^{ts}$ mutants, vesicle recycling is arrested at a very specific stage of membrane retrieval from presynaptic plasma membrane (Kosaka and Ikeda 1983). At this "collared pit" stage, nascent endocytic vesicles are attached to plasma membrane via a narrow neck bearing an electron-dense collar. Similar conditional blocks in endocytosis occur in all other cellular contexts that have been examined in *shi*$^{ts}$ mutants (Kosaka and Ikeda 1983; Masur *et al.* 1990; Tsuruhara *et al.* 1990; Kramer *et al.* 1991; Tabata and Kornberg 1994). Thus, the *shi* gene product is required for the formation of endocytotic vesicles. Molecular-genetic analyses revealed that the *shibire* gene encodes the Drosophila homolog of dynamin, a GTPase previously identified in mammals as a microtubule-binding protein (Shpetner and Vallee 1989; Obar *et al.* 1990; Chen *et al.* 1991; van der Bliek and Meyerowitz 1991). Detailed analyses of dynamin function have not revealed any microtubule-associated functions *in vivo*. Rather, dynamin appears exclusively involved in vesicle formation at the plasma membrane (Vallee 1992).

The initial conclusion from these analyses, that dynamin is essential for a late stage in the formation of endocytotic vesicles, has been borne out and extended by a large number of experiments in mammalian preparations (Warnock and Schmid 1996). Most significantly, in the presence of a nonhydrolyzable GTP analog, mammalian dynamin has been localized by immunoelectron microscopy to the electron-dense collars seen on the neck of endocytic pits on plasma membrane (Takei *et al.* 1995). This critical observation suggests that dynamin is an integral part of a protein complex, localized at the neck of nascent endocytotic vesicles, that functions to drive membrane fission during vesicle budding. As the first member of this fission complex to be identified, dynamin has received considerable interest not only from the context of its own structure, func-

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tion, and regulation, but also as a tool in affinity chromatography designed to identify other proteins that participate in this basic cellular process (De Camilli and Takei 1996).

Dynamin is a member of a superfamily of structurally related GTP binding proteins that may play roles in many types of membrane budding events. Dynamin-related proteins include the mammalian Mx-1 proteins, molecules that are induced by interferon and confer viral resistance (Arnheiter and Meier 1992); Mgm1p, a protein required for yeast mitochondrial DNA maintenance (Jones and Fangman 1992); Vps1p, a protein involved in exocytic trafficking of proteins from the Golgi apparatus to the yeast vacuole (Vater *et al.* 1992); and Dnm1p, a protein involved in a late stage of endocytotic traffic (Gammie *et al.* 1995). Several structural domains described in dynamins are also found in dynamin-related proteins. These domains include a GTPase domain comprising ~300 residues at the N terminus, a pleckstrin homology domain (PHD) that could participate in protein-protein interactions or membrane localization, a coiled-coil domain that could mediate dimerization, and a proline-rich domain (PRD) that probably mediates critical protein-protein interactions and serves as a site for regulatory kinases (Warnock and Schmid 1996). In addition, a region linking the GTPase domain to the PH domain, which we here term the "middle domain," is conserved across dynamin-related proteins. Pleckstrin homology domains and proline rich domains are absent in the yeast and virus-induced dynamin-related proteins; thus, dynamins may have unique features to their functions.

Biochemical experiments have made some progress toward identifying the distinct roles of dynamin domains. *In vitro*, dynamin can spontaneously assemble into open rings similar in size and morphology to the collars of collared pits, a process sensitive to the state of the GTPase domain (Hinshaw and Schmid 1995). Thus, GTP-dynamin can assemble into rings, but subsequent GTP hydrolysis causes disassembly into dynamin units. These data suggest that *in vivo*, GTP-bound dynamin may assemble at the neck of the endocytotic vesicle and subsequent GTP-hydrolysis would initiate a disassembly of dynamin collars following membrane fission. Modulation of dynamin's GTPase activity could be important for controlling the timing of dynamin collar assembly or disassembly. The PRD of dynamin appears to participate in such modulatory interactions (Warnock *et al.* 1997). Although a variety of potential PRD-binding molecules has been identified *in vitro* (microtubules, phospholipids, Grb2, c-src, Fyn, Lyn, PLCg, p85 subunit of $PI_3K$, and a novel family of SH3-domain-containing proteins), there is little evidence that these molecules modulate dynamin function *in vivo* (Gout *et al.* 1994; Ringstad *et al.* 1997). The only PRD-mediated interactions known to be relevant to vesicle budding are homotypic interactions between dynamin polypeptides and interactions with an SH3-domain-containing protein known as amphiphysin (Shupliakov *et al.* 1997). Interactions mediated by the other domains of dynamin, or the functions served by these other domains, remain unknown.

To correlate dynamin sequences with *in vivo* activity, we have characterized a collection of *shi* lethal alleles that should produce nonfunctional mutant dynamins. We describe for the first time a putative null allele of *shibire*, as well as the two intragenic lethal complementation groups at the Drosophila *shibire* locus. Phenotypic and molecular analysis of these lethal alleles offer interesting insights into the functions and structure of dynamin *in vivo*, as well as new information regarding critical functional domains and residues in dynamin. Results described in this article validate existing biochemical data that suggest that dynamin functions as a multimer composed of several functional domains. Further, our genetic analysis shows that the unheralded middle domain in dynamin is likely to be involved in molecular interactions vital for dynamin function. Single amino acid substitutions in this domain result in nonfunctional dynamins. The identification of these mutations should be valuable for future biochemical and cell biological studies.

Since the original isolation of $shi^{ts}$ mutants in 1970, several screens have been performed for temperature-sensitive paralytic mutants in Drosophila. These screens have recovered dozens of conditional alleles of *shi* and several of *para* (a gene that encodes the major Drosophila voltage-gated sodium channel), but very few other genes have been identified. Observations described in this article suggest, in the context of dynamin functional domains, a molecular explanation for why conditional *shibire* alleles are particularly easily obtained.

## MATERIALS AND METHODS

**Cultures and stocks:** Drosophila cultures were maintained between 22° and 25° in a medium consisting of Carolina Biological formula 4-24 Instant Drosophila medium (63 g/liter; Carolina Biologicals Supply Company, Burlington, NC), Quaker Quick Oatmeal (45 g/liter), agar (0.5% w/v), and Nipagin (p-hydroxybenzoic acid methyl ester, 0.1% w/v; NIPA Labs, Wilmington, DE) (Condie and Brower 1989). Oregon-R and $shi^{ts}$ mutants were from the Krishnan and Ramaswami laboratory stock collections (Ramaswami *et al.* 1993; Estes *et al.* 1996); $w^{1118}$ flies were obtained from D. Brower at the University of Arizona. Transgenic P($w^+$, $shi^+$) flies expressing the entire *shibire* genomic region, including the transcriptional initiation site and 3′ untranslated sequence, were obtained from A. van der Bliek (van der Bliek and Meyerowitz 1991). Duplications of *shibire* (Df(1) $sd^{72b}$/C(1)DX/$y^+$Y$shi^+$) and deficiencies spanning the *shibire* locus (Df(1)$sd^{72b}$) were obtained from V. Rodrigues at the TATA Institute of Fundamental Research. $shi^{12.12B}$ and $shi^{12.18H}$ alleles were generated in a gamma-ray mutagenesis screen and kindly given to us by Clifton Poodry (National Institutes of Health, Bethesda, MD). Lethal alleles of *shibire* were isolated in an EMS screen

for lethals uncovered by the Df(1)$sd^{72b}$ chromosome and were generated as described previously (Katzen and Bishop 1996).

**Behavioral testing:** Temperatures for paralysis of heterozygous lethal alleles were measured in an apparatus as described in Ramaswami *et al.* (1993). A smooth glass chamber was enclosed in a sealed water jacket connected by a tube through which water was passed by means of a circulating water bath (Haake, Paramus, NJ). Temperature was controlled accurately with a precision of at least 0.5°. Paralysis was defined as the condition in which flies were on their backs with little or no movement of wings and legs. The temperature of paralysis was determined by the occurrence of paralysis in 100% of flies within 2 min at a given temperature. All flies tested were 2–4 days old, and ~20 flies were tested at each temperature.

**Western blotting and *shibire* antibodies:** Rabbit polyclonal antibodies against fly dynamin, Ab2073, and shi-3 were raised as described previously (Estes *et al.* 1996): 2073 serum was raised against a GST fusion to a truncated dynamin entirely lacking the PRD; shi-3 to a C-terminal peptide (CRPGGS LPPPMLPSRR).

Drosophila head homogenates were prepared by homogenization in 2× sodium dodecyl sulfate (SDS) buffer (0.125 Tris pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 8 m Urea) and centrifuged to remove debris. Protein concentration was determined by the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Twenty-five micrograms of protein samples were mixed with gel loading buffer, boiled for 5 min, electrophoresed, and blotted onto polyvinylidene difluoride (PVDF) membrane. Following transfer, the membrane was blocked in 5% powdered milk, 0.1% Tween in phosphate buffered saline (PBS) at room temperature for 30 min, then incubated with a 1:2500 dilution of shi-3 serum or a 1:1000 dilution of Ab2073 overnight at 4°. Membranes were then incubated for 2 hr at room temperature in goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (1:1000). After three washes in PBS, pH 7.5, at room temperature for 10 min each, signals were detected using enzyme catalyzed luminescence (ECL) Western blotting detection reagents (Amersham, Buckinghamshire, UK) according to manufacturer's directions.

**Oligonucleotides:** Oligonucleotides were used for PCR amplification of *shibire* genomic sequences or for sequencing amplified DNA. Oligonucleotides used for PCR were as follows (in 5′ to 3′ orientation): D6, TGC AGT GAA GTA AAG CCA GC; 67, GTC TCC GAG TTA CGA TAC; D11, GCC CAA CAA CAC TCT TGG TGT CC; D12, GCA TCC GAA TTG GCC AGA TCC GT; 64, GGC GAG TTC CTT CAC ATT; 12, GGC CAA TGC CTG GTG GAT AT; 9, GGT GTG CGA ACC ATT GGC GTT ATA; D13, CGG TCT CCT CAC GCA GAC GTG G; D7, GCC TCT TCA CAC CCG ACA TGG; D10, ATC GCC GTT TTC CTG AGT CTC C; D4, ATC TTA TAG TCG GAC TCT CG; D1, AGG TTG GGT ACT CAC GAT GG. Oligonucleotides used for sequencing included 67, 64, 12, D11, D12, 9, D7, and D4 as well as the following (in 5′ to 3′ orientation): for the region amplified between D6 and 67, 31, CGG ACC TCG CCG CAA TGG ATA G; for the region amplified between 9 and D13, J20, CAC ATG GCC GAC CGT; and J28, GTG CAC ATG CGC ACG AC; for the region amplified between D7 and D10, J21, ACC CAC GTC TGC GTG AG; and MS02, CTT ATA AAC ATT ACG TCC ATC G; and for the region amplified between D4 and D1, J23, GTA CCG TGC TTG CAA GG. The oligonucleotides used for the rapid amplification of genomic ends (RAGE) protocol include the AP1 adaptor supplied in Marathon kit (Clontech, Palo Alto, CA) and RAGE2: CAG CTC GAT GCT TTA AAT GTG CAT T.

**Sequencing lethal *shi* alleles:** The sequencing of the lethal alleles was performed by direct sequencing of overlapping PCR products. Sequencing reactions were performed using a PCR sequencing kit (U.S. Biochemical Corp., Cleveland) and the manufacturer's directions, except that dimethyl sulfoxide (DMSO) was added at the primer annealing, labeling reaction, and termination reaction steps. PCR fragments varied in length from 500 to 2000 bp and spanned most of the *shibire* genomic region and all of the coding region. Four of the six PCR fragments (D6-67, D11-D12, 64-12, and 9-D13) were amplified from genomic DNA isolated from hemizygous embryonic lethals that allowed for easier detection of mutations, and two (D7-D10 and D4-D1) were amplified from heterozygous animals. Embryos were laid at 25° on grape juice agar plates. Hemizygous lethal embryos were selected by allowing eggs to mature in a humidified chamber for 2 days, at which time any remaining embryos were either unfertilized or dead. The dead embryos could be differentiated by their amber hue. Three to five embryos were homogenized in 30 ml of Gloor and Engel's buffer (DIS 71: 148-149, 1992), incubated at 37° for 15 min, heat denatured at 95° for 10 min, and 5 ml were used for each PCR reaction.

**Genomic Southern analysis:** Genomic DNA was extracted from flies homogenized in 20% SDS and incubated at 65° for 10 min. Proteins were extracted using an equal volume of chloroform and half-volume of 5 m NaCl and removed by centrifugation. DNA was precipitated from the aqueous layer using 100% ethanol, and the resultant pellet was washed with 70% ethanol. Ten micrograms of DNA was digested with *Eco*RI / *Bam*HI, *Cla*I, and *Cla*I / *Pst*I restriction enzyme combinations and analyzed by standard Southern hybridization procedures (Sambrook *et al.* 1989). Southern blots were incubated with various *shibire* cDNA and genomic DNA probes in order to define a 1.35-kb region in which the chromosomal rearrangement originates. DNA used for probes was PCR amplified, subsequently digested with enzymes, if appropriate, and $^{32}$P-labeled using the random priming method (Boehringer Mannheim, Indianapolis).

**Breakpoint sequencing:** Identification of the breakpoint at the nucleotide level was achieved using a variation of the Marathon cDNA amplification or RACE kit (Clontech) that we call RAGE for rapid amplification of *genomic* ends. Genomic DNA from $shi^{12.18H}$ flies was isolated as described in methods for Southern analysis and digested with *Cla*I for 2 hr at 37°. The 5′ *Cla*I site was restored by filling in nucleotides under the 5′ overhangs using 1 mm dNTPs and Klenow enzyme for 15 min at 37°. Adaptor supplied in the Marathon kit was ligated to the treated DNA for 7 hr at 16°. An adaptor-specfic primer (AP1) and a gene-specific primer (RAGE2) were used to amplify a 4.3-kb fragment from wild-type sequence and a 3.3-kb fragment from sequence containing the breakpoint. Each fragment was cloned using the restored *Cla*I site and a *Not*I located within the adaptor sequence. Automated sequencing of wild-type and mutant clones was performed by the Laboratory of Molecular Systematics and Evolution (LMSE) at the University of Arizona.

## RESULTS

**Lethal *shi* alleles fall into three groups based on interallelic complementation analysis:** In a screen for EMS-induced lethals in the 13F region of the *X* chromosome, a collection of mutations defining 16 different complementation groups, one of which did not complement *shi*, were generated (Katzen and Bishop 1996). We confirmed that this collection of *shi* lethal chromosomes could be rescued by a *shibire* P($w^+$, $shi^+$) genomic transgene inserted on the second chromosome. Of all the *shi* lethals we characterized, only $shi^{EM53}$ is not res-

cued by one or two copies of the *shi* transgene, although it is rescued by a duplication Dp(1,Y) $sd^{72b}$ that includes several other genes in the 13F cytogenetic region. Thus, the EM53 chromosome probably carries an independent lethal mutation closely linked to *shi*. Two alleles, $shi^{12.18H}$ and $shi^{12.12B}$, were isolated by Clifton Poodry in a screen for gamma-ray-induced lethal alleles of *shi* that typically results in chromosome rearrangements. As for the EMS-induced alleles, $shi^{12.18H}$ and $shi^{12.12B}$ lethality was completely rescued by the P($w^+$, $shi^+$) transgene; thus, the lethality caused by these mutations is solely due to their effect on the *shibire* locus.

On further characterization of this collection of *shi* lethals, we discovered that three classes (I, II, and III) could be defined on the basis of intragenic complementation analysis (Table 1). Class I alleles ($shi^{EM14}$, $shi^{EM38}$, $shi^{EM53}$, $shi^{EM33}$, $shi^{EM55}$, $shi^{EM65}$, and $shi^{EM66}$) and class II alleles ($shi^{EM18}$, $shi^{EM27}$, $shi^{EM35}$, $shi^{EM42}$, $shi^{EM44}$, $shi^{EM56}$, and $shi^{EM59}$) were lethal when homozygous. However, the two groups showed interallelic complementation such that classI / classII heterozygotes were viable (Table 1). A subset of mutants in class I ($shi^{EM14}$, $shi^{EM38}$, and $shi^{EM53}$) did not complement any of the *shibire* alleles, but we categorized them as class I alleles for reasons explained below (Table 1). Class III mutants $shi^{12.18H}$ and $shi^{12.12B}$ did not complement any of the *shi* lethals.

**Null, dominant-negative, and recessive properties of *shi* lethal alleles:** The observation of intragenic complementation groups in *shibire* led us to a more detailed phenotypic characterization of these lethal alleles. It has previously been observed that $shi^{ts1}$/+ and $shi^{ts2}$/+ heterozygous flies are paralyzed at 37°, whereas wild-type animals and flies heterozygous for a *shibire* deficiency are not affected at temperatures below 42°.

To test if any of the lethal alleles showed dominant-negative effects similar to $shi^{ts1}$ and $shi^{ts2}$, we examined the temperature of paralysis for the lethal alleles when heterozygous with balancer or wild-type chromosomes (Table 2). We also tested for dominance by examining the paralytic behavior of *shi* lethals rescued by a wild-type *shibire* transgene. On the basis of their ts paralytic behavior in these tests, the lethal alleles fell into two groups: class I alleles had dominant effects and caused temperature-sensitive paralysis in heterozygous flies; in contrast, class II and class III alleles heterozygous with a wild-type allele of *shibire* did not affect the temperature of paralysis. The class I alleles $shi^{EM14}$/+ and $shi^{EM33}$/+ paralyzed at 36°, $shi^{EM38}$/+ and $shi^{EM55}$/+ paralyzed at 37°, and $shi^{EM65}$/+ and $shi^{EM66}$/+ paralyzed at 40° and 41°, respectively. In addition, class I mutants rescued by a *shibire* transgene displayed even lower temperatures of paralysis, ranging from 33° to 41° (Table 2), probably due to lower levels of dynamin expressed from the *shibire* transgene. The allele $shi^{EM38}$ required two copies of the *P* element for rescue of lethality, suggesting strong dominant-negative properties. A very strong dominant-negative effect could explain why $shi^{EM38}$, and the two other

**TABLE 1**

**Interallelic complementation among *shibire* lethal alleles**

| | $shi^{EM14}$ | $shi^{EM38}$ | $shi^{EM53}$ | $shi^{EM33}$ | $shi^{EM55}$ | $shi^{EM65}$ | $shi^{EM66}$ | $shi^{EM18}$ | $shi^{EM27}$ | $shi^{EM35}$ | $shi^{EM42}$ | $shi^{EM44}$ | $shi^{EM56}$ | $shi^{EM59}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $shi^{EM14}$ | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| $shi^{EM38}$ | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| $shi^{EM53}$ | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| $shi^{EM33}$ | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| $shi^{EM55}$ | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| $shi^{EM65}$ | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| $shi^{EM66}$ | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| $shi^{EM18}$ | − | − | − | − | − | − | − | + | + | + | + | + | + | − |
| $shi^{EM27}$ | − | − | − | − | − | − | − | + | + | + | + | + | + | − |
| $shi^{EM35}$ | − | − | − | − | − | − | − | + | + | + | + | + | + | − |
| $shi^{EM42}$ | − | − | − | − | − | − | − | + | + | + | + | + | + | − |
| $shi^{EM44}$ | − | − | − | − | − | − | − | + | + | + | + | + | + | − |
| $shi^{EM56}$ | − | − | − | − | − | − | − | + | + | + | + | + | + | − |
| $shi^{EM59}$ | − | − | − | − | − | − | − | − | − | − | − | − | − | − |

Results of heteroallelic combinations are indicated by a (−) for lethality and (+) for viability.

**TABLE 2**

**Temperature of paralysis for heterozygous or transgene-rescued *shi* lethals**

| Class | Line | $\frac{shi}{FM7c}$ | $\frac{shi}{Y}; \frac{P(w^+ shi^+)}{+}$ | $\frac{shi}{shi^{ts1}}$ | Phenotype |
|---|---|---|---|---|---|
| I | $shi^{EM14}$ | 36–37° | 34–35° | no survivors | dom.-neg. |
| I | $shi^{EM38}$ | 37° | no survivors | no survivors | dom.-neg. |
| I | $shi^{EM53}$ | wild type | no survivors | 33° | ? |
| I | $shi^{EM33}$ | 36° | 33° | no survivors | dom.-neg. |
| I | $shi^{EM55}$ | 37–38° | 36° | <28° | dom.-neg. |
| I | $shi^{EM65}$ | 40° | 39° | 30° | dom.-neg. |
| I | $shi^{EM66}$ | 37° | 33° | ND | dom.-neg. |
| II | $shi^{EM18}$ | wild type | wild type | 34° | recessive |
| II | $shi^{EM27}$ | wild type | wild type | 36° | recessive |
| II | $shi^{EM35}$ | wild type | wild type | 35.5° | recessive |
| II | $shi^{EM42}$ | wild type | wild type | 34° | recessive |
| II | $shi^{EM44}$ | wild type | wild type | 34° | recessive |
| II | $shi^{EM56}$ | wild type | wild type | 35.5° | recessive |
| II | $shi^{EM59}$ | wild type | wild type | 36° | recessive |
| III | $shi^{12.18H}$ | wild type | wild type | 26° (as $shi^{ts1}$) | recessive |

Paralysis is defined as the condition in which the animal lies on its back with little movement of the wings and legs. The temperature of paralysis is assessed by the occurrence of paralysis within 2 min at a specific temperature. Wild-type flies (ORR) are incapacitated at 42°; however, this behavior cannot be confused with the typical features of *shi* paralysis, which is preceded by characteristic behavioral seizures and uncontrolled wing-beating.

class I alleles $shi^{EM14}$ and $shi^{EM53}$, did not complement any of the other *shi* lethal alleles. Thus, although they do not strictly belong to the same complementation group as the other dominant-negative *shi* lethals, we include them in the group of class I *shi* alleles (Table 2).

Class II and Class III alleles showed no effect when heterozygous with a wild-type allele of *shibire*. Thus, the heterozygotes $shi^{EM18}/+$, $shi^{EM27}/+$, $shi^{EM35}/+$ $shi^{EM42}/+$, $shi^{EM44}/+$, $shi^{EM56}/+$, $shi^{EM59}/+$, $shi^{12.12B}/+$, and $shi^{12.18H}/+$ were not affected in any noticeable way at temperatures <42°. A unique feature of class III alleles was their behavior when heterozygous with conditional alleles of *shi*, where they behaved in the same way as *shi* deficiencies. $shi^{ts1}/shi^{12.18H}$ and $shi^{ts2}/shi^{12.18H}$ heterozygotes are paralyzed at 26° and 26.5°, respectively, which is identical to the temperature of paralysis of $shi^{ts1}/Df(1)sd^{72b}$ or $shi^{ts2}/Df(1)sd^{72b}$ flies. However, class II alleles heterozygous with ts alleles generally had greatly elevated restrictive temperatures compared to homozygous ts mutants for example, $shi^{ts2}/shi^{ts2}$ homozygotes are paralyzed at 27.5°, but $shi^{ts2}/shi^{EM44}$ flies are not affected until 36° (Table 2).

In summary, class I alleles showed dominant-negative properties, as previously observed for conditional *shi* alleles; class II alleles complemented the lethality of class I alleles but otherwise had no dominant properties; and class III alleles behaved in all tests in a manner indistinguishable from a deficiency for *shi*. To account for these data, we reached a model with three main points. First, the dominant-negative phenotype of class I alleles suggests that they are affected in the domain of dynamin mutated in $shi^{ts1}$ and $shi^{ts2}$, namely, the GTPase domain (van der Bliek and Meyerowitz 1991). Second, class II mutations affect a different functionally separable dynamin domain. Third, class III alleles $shi^{12.18H}$ and $shi^{12.12B}$ are probably null for dynamin function. To test this working model and to gain insights into the molecular details of dynamin function *in vivo*, we performed a molecular analysis of the *shi* lethals.

***shi*$^{12.18H}$ is potentially a null allele of *shibire*:** Identical large-scale rearrangements were observed at the *shi* locus of $shi^{12.18H}$ and $shi^{12.12B}$ mutants, and so data is presented here only for $shi^{12.18H}$. Southern analysis of $shi^{12.18H}/+$ DNA using *shibire* probes revealed that an *Eco*RI, *Bam*HI fragment was altered in the $shi^{12.18H}$ mutant as compared to wild-type animals (Figure 1A). To test whether this reflected an alteration in a single restriction site or a more large-scale genomic rearrangement, genomic DNA was treated with two independent enzymes (*Cla*I and *Pst*I) and hybridized to various *shibire* probes (Figure 1B). When *Cla*I, *Pst*I-digested DNA was probed with a 1.35-kb region between a *Cla*I and *Pst*I site near the 3′ end of the *shibire* coding sequence (probe 6), an altered band was visible. Probes specific to regions 5′ and 3′ of this region did not reveal any fragment different from wild type. These results demonstrate that the lesion in $shi^{12.18H}$ involves a chromosome rearrangement in this 1.35-kb region of genomic DNA (Figure 1C).

In order to define the exact position at which the breakpoint occurs, we used PCR and chromosomal "walking" or RAGE (see materials and methods) to amplify and sequence the region, including the breakpoint. A 4.3-kb piece was amplified from the wild-type genomic DNA, and a smaller 3.3-kb piece was amplified

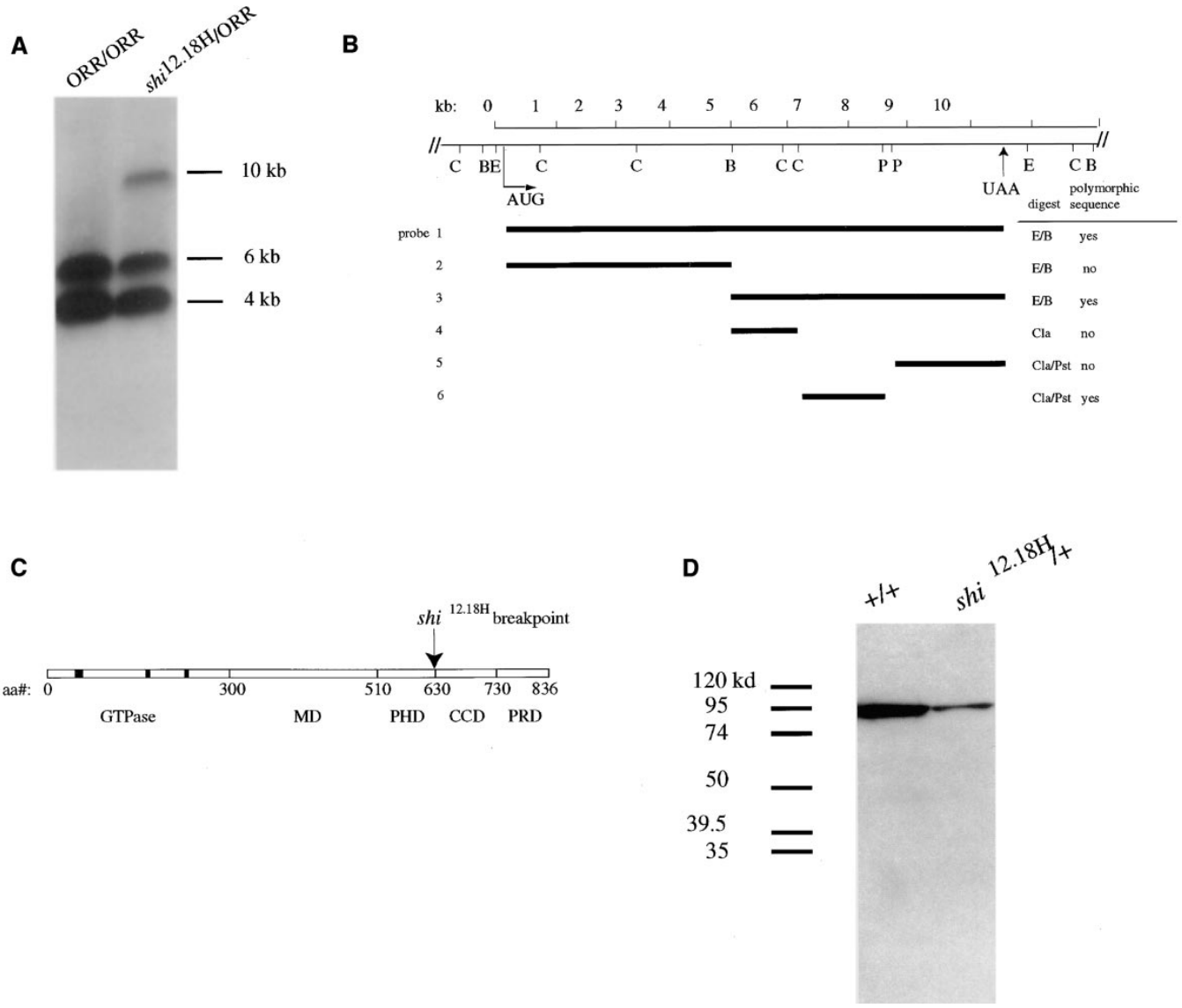
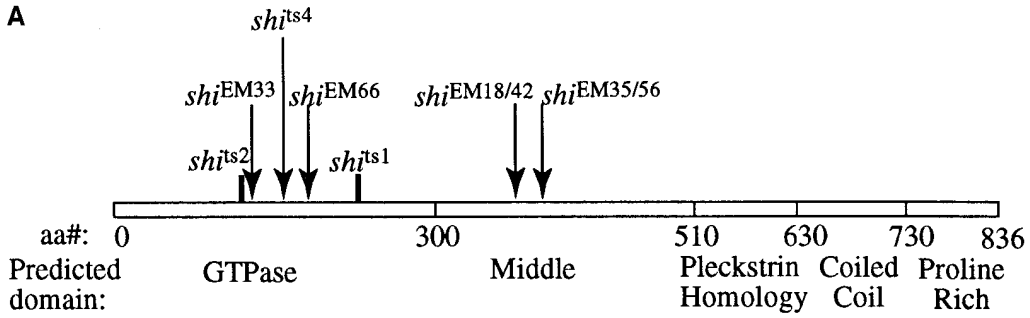



Figure 1.—The *shi*$^{12.18H}$ allele is associated with a breakpoint within the COOH-terminal portion of *shibire*. (A) Genomic Southern blot analysis indicates an altered fragment that is present in heterozygous *shi*$^{12.18H}$/+. Ten micrograms of genomic DNAs were digested with *Eco*RI and *Bam*HI and probed with *shibire* cDNA. Oregon R (ORR) flies were used as a homozygous wild-type strain for a control, and heterozygous *shi*$^{12.18H}$/+ flies were balanced with an FM7c balancer chromosome that was shown to have the same fragment pattern as wild type (data not shown). (B) A restriction map of the *shibire* genomic region is shown. Restriction sites are indicated by B (*Bam*HI), C (*Cla*I), E (*Eco*RI) and P (*Pst*I). Probes specific to various portions of *shibire* genomic DNA reveal that the breakpoint lies within a 1.35-kb region flanked by *Cla*I and *Pst*I restriction sites. (C) The *shi*$^{12.18H}$ allele contains a breakpoint that originates in an intron between the coding sequence that encodes the predicted coiled-coil domain. Abbreviations are as follows: middle domain (MD), pleckstrin homology domain (PHD), coiled-coil domain (CCD), proline-rich domain (PRD). (D) An immunoblot of *shi*$^{12.18H}$/+ heterozygotes, stained with an antibody raised against the middle portion of fly dynamin, reveals decreased levels of full-length dynamin and absence of a 70-kD fragment that would represent a truncated protein.

from *shi*$^{12.18H}$/+ animals. This 3.3-kb fragment included 5′ *shi* sequences, a translocation breakpoint, and a new *Cla*I site within the rearranged sequence. Genomic sequence from the 3.3-kb fragment is identical to the wild-type *shi* genomic sequence until the ninth *shibire* intron, which occurs after nucleotides encoding amino acid residue 629 of *shibire* product. At this point, the sequence diverges completely and extends the remainder of the decipherable sequence reaction (~200 bp). Thus, the *shi*$^{12.18H}$ mutant shows a major chromosomal rearrangement within the *shibire* gene.

To test whether a truncated 629-residue protein is made, we stained an immunoblot containing identically loaded homogenates of heads from wild-type (Oregon R) and *shi*$^{12.18H}$/+ heterozygotes using an antibody against a Drosophila dynamin fragment encompassing amino acids 331 to 651, which would encode most of the middle domain, the PHD, and some of the coiled-coil do-

A




B

| | Phenotype | cDNA bp | nucleotide transversion | amino acid position | amino acid change | domain |
|---|---|---|---|---|---|---|
| *shi*EM33 | dom. neg. | 493 | G--> A | 148 | Glu--> Lys | GTPase |
| *shi*EM66 | dom. neg. | 616 | G--> A | 189 | Asp--> Asn | GTPase |
| *shi*ts4 | dom. neg. | 563 | C--> T | 171 | Pro--> Ser | GTPase |
| *shi*EM18 | recessive. | 1192 | G--> A | 381 | Arg--> Gln | MD |
| *shi*EM42 | recessive | 1192 | G--> A | 381 | Arg--> Gln | MD |
| *shi*EM35 | recessive | 1251 | C--> T | 401 | Pro--> Ser | MD |
| *shi*EM56 | recessive | 1251 | C--> T | 401 | Pro--> Ser | MD |

Figure 2.—(A) Schematic diagram of mutant *shibire* proteins. The location of amino acid changes within *shibire* product are indicated by arrows. *shi*$^{ts1}$ and *shi*$^{ts2}$ (van der Bliek *et al.* 1991) are placed for reference. (B) The table shows the exact nucleotide and predicted amino acid substitutions for the *shi* alleles sequenced in this study. All the substitutions appear to be nucleotide transversions that occur most frequently with EMS mutagenesis.

main (CCD) of dynamin (Figure 1D). Although the predicted size of the truncated protein is 70 kD, no such band was visible, suggesting that a functional protein is either not translated (perhaps due to instability of the mutant mRNA) or rapidly degraded (Figure 1D). The decreased intensity of the 95-kD band in *shi*$^{12.18H}$/+ head extracts is consistent with functional dynamin deriving exclusively from the chromosome wild type for *shibire*. The molecular-genetic and biochemical data indicate that no functional dynamin is generated from the *shi*$^{12.18H}$ allele. Thus, *shi*$^{12.18H}$ mutants, which in genetic tests are indistinguishable from a deficiency for *shi*, are probably null for zygotic *shi* function.

**Missense lethal mutations in two dynamin domains:** The simplest explanation for our observation of intragenic complementation among *shi* lethals is that class I and class II lethals affect two separable domains of Drosophila dynamin. Previous observations that two temperature-sensitive alleles with dominant-negative properties (*shi*$^{ts1}$ and *shi*$^{ts2}$) mapped to the GTPase domain suggested that class I mutants could represent more extreme alleles that also map to this domain. To identify these domains as well as critical residues within these regions, we sequenced the *shibire* coding regions in a selection of class I and class II lethal mutants. We identified mutations associated with two class I alleles and four different class II alleles that clustered in distinct domains.

To identify the lesions contained in the lethal alleles, fragments of the *shibire* genomic sequence were PCR amplified from genomic DNA isolated from each of the lethal alleles, and the entire coding sequence was directly sequenced. The results are shown in Figure 2. Interestingly, of four recessive class II alleles we sequenced, *shi*$^{EM18}$ and *shi*$^{EM42}$ had identical mutations, and similarly, *shi*$^{EM35}$ and *shi*$^{EM56}$ were identical. All these mutations were point substitutions that mapped to a region between the GTPase and PHDs at residues conserved among dynamins (Figure 2). We refer to the region defined by the class II mutations, between the GTPase and the PHD, as the "middle domain." This middle domain is generally conserved in a yeast dynamin homolog, *VPS1*, although the specific residues Arg$^{381}$ and Pro$^{401}$ are not conserved (Vater *et al.* 1992).

**Temperature-sensitive phenotypes in allelic combinations of *shi* lethals:** We observed that class I *shi* alleles heterozygous over wild-type alleles or viable heteroallelic class I/class II flies showed tight and sharply temperature-sensitive paralytic phenotypes previously described for *bona fide* conditional alleles of *shi*. For instance, *shi*$^{EM33}$/FM7c flies were paralyzed within 2 min at 34.5°, and *shi*$^{EM33}$/*shi*$^{EM42}$ flies are similarly paralyzed at 36° (Figure 3). The features of *shi*$^{ts}$ alleles, including rapid onset, fast reversal, tight temperature dependence, as well as typical behavioral seizures prior to onset of paralysis, are all seen in these animals. The observation is remarkable because the alleles were selected solely for their lethal phenotype and not for any

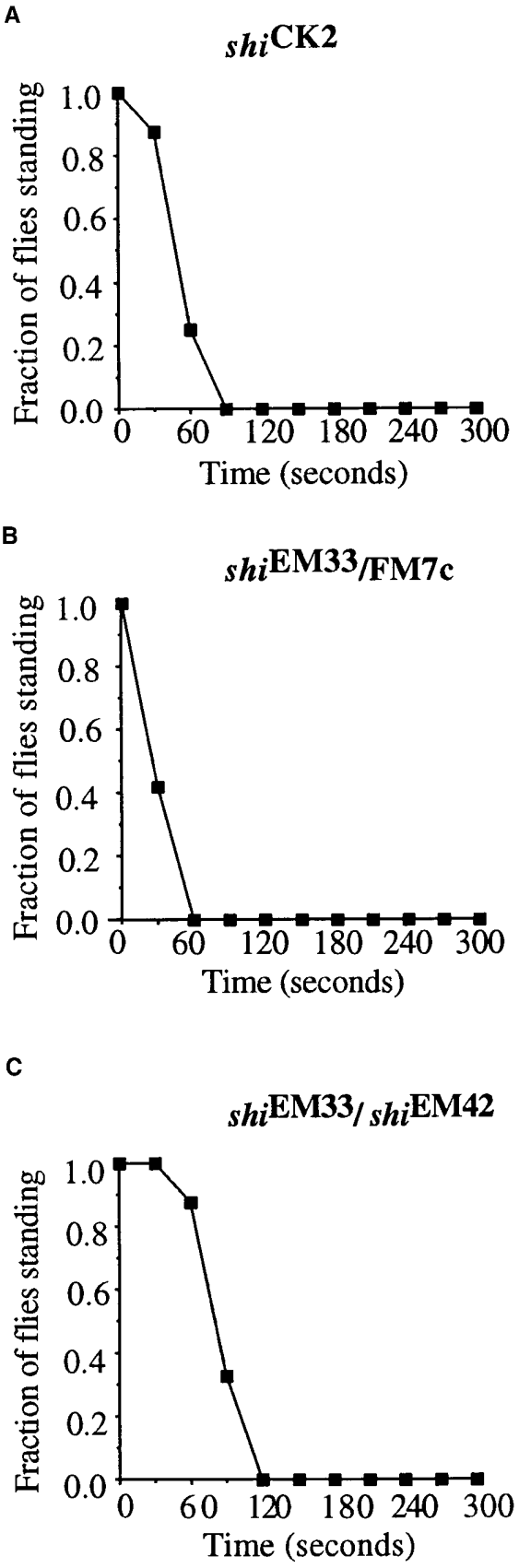
temperature sensitivity. The implication of these studies for the molecular basis of the *shibire* temperature-sensitive phenotype is considered in the discussion.

## DISCUSSION

Studies of Drosophila *shibire* mutants have led to major advances in understanding of vesicle budding mechanisms (Kosaka and Ikeda 1983; Chen *et al.* 1991; van der Bliek and Meyerowitz 1991). Stimulated by the original discovery that dynamin is a critical regulator of endocytosis, cell biologists working with mammalian tissue have accumulated a vast amount of biochemical information on the properties of purified dynamin (Warnock and Schmid 1996). To correlate these *in vitro* studies with *in vivo* functions, a genetic analysis of *shibire* mutants in Drosophila is extremely valuable. In this article we have described the first set of lethal *shibire* alleles using genetic, phenotypic, and molecular methods. Our phenotypic studies confirm that dynamin functions *in vivo* as a multimeric protein with independent functional domains. Molecular analysis of lethal mutations suggests that a previously ignored region of dynamin, which we term the middle domain, has critical functions distinct from those of the GTPase domain. Our identification of loss-of-function point mutations in this domain should stimulate biochemical and genetic experiments to identify middle-domain binding proteins involved in endocytosis. Finally, we provide some insight into molecular transitions that could underlie temperature sensitivity caused by a large collection of conditional *shibire* alleles. This insight allows us to rationalize the high susceptibility of the *shi* locus to conditional mutations.

**Lethal alleles of *shibire*:** Of roughly 14 alleles described in this study, only one appears to be null in our genetic tests. The others fall into two intragenic complementation groups, whose properties clearly indicate that they are not null alleles. We consider the implications of these findings below.

*A null allele of shibire:* A null allele of dynamin would be useful to examine the *in vivo* function and regulation of Drosophila dynamin, as well as to provide a genetic background for transgenic studies. We have identified an allele of *shibire*, $shi^{12.18H}$, that is likely to be null for dynamin function. Genetic arguments, by their nature, cannot prove that a mutation is a true null unless the entire coding sequence for a gene has been deleted. Because this is not the case for $shi^{12.18H}$, our arguments in favor of its being a null allele are based on several lines of compelling circumstantial data. Genetic and phenotypic experiments show that $shi^{12.18H}$ (and the identical $shi^{12.12B}$) is the only *shi* allele indistinguishable from a *shibire* deficiency when heterozygous over wild-type, *shi* lethal, or $shi^{ts}$ chromosomes. Genomic analyses demonstrate a large chromosomal rearrangement at the *shi* locus in $shi^{12.18H}$ mutants: this rearrangement begins in



the ninth intron of *shi*, and it is likely to result in a severely truncated dynamin lacking the entire C-terminal PRD. Biochemically, we find reduced levels of full-length dynamin; the data are consistent with a 50% reduction in $shi^{12.18H}$/+ heterozygotes compared to wild-type flies. There is no evidence of a stable truncated protein present in these heterozygotes; this is not unexpected because truncated proteins, as well as transcripts encoding truncated polypeptides, are often rapidly degraded. Thus, several lines of genetic and molecular data are consistent with our working conclusion that $shi^{12.18H}$ is a null allele of *shibire*.

*Intragenic complementation between two classes of shi lethals:* A collection of EMS-induced lethal *shi* alleles was recovered from a genetic screen designed to isolate lethal mutations in the 13F region of the *X* chromosome (Katzen and Bishop 1996). These lethal alleles fell into three groups: one group that did not complement any other lethal allele and two intragenic complementation groups. Intragenic complementation is most often observed in genes whose products have two (or more) independent functional domains. In these cases, the gene product is inactive as a monomer and self-associates to form an active functional multimer (Raz *et al.* 1991; Gepner *et al.* 1996). In some unusual cases, however, interallelic complementation has been observed in a monomeric, multifunctional protein (Ohya and Botstein 1994). Taken together with the genetic dominance of several *shi* alleles, the observed interallelic complementation in *shi* strongly suggests that dynamin functions as a multimer *in vivo*. This conclusion, previously suggested by weak but distinct interallelic complementation between two different conditional alleles of *shi* (Kim and Wu 1990), is consistent with *in vitro* studies on purified dynamin (Hinshaw and Schmid 1995).

A dominant ts phenotype shown by alleles in the first complementation group is also shown by the three alleles that do not complement any other *shi* lethals. For this reason, we have tentatively classified these three alleles, along with the first intragenic complementation group, as class I lethal alleles. The rest of the lethals fell cleanly into the second complementation group that we refer to as class II. It is likely that noncomplementation of the class II lethals by specific class I alleles reflects the strong dominant-negative properties of class I mutants. For several reasons, we predicted that class I alleles would map to sequences encoding the GTPase domain of dynamin. First, similar dominant ts paralytic phenotypes have been previously observed with $shi^{ts1}$ and $shi^{ts2}$ alleles that have mutations in the GTPase domain (Kim and Wu 1990; Ramaswami *et al.* 1993). Second, studies on *VPS1* in yeast, a gene encoding a homolog of dynamin, have shown that a subset of *VPS1* mutations with dominant-negative properties affects the GTPase domain of Vps1p (Vater *et al.* 1992). Finally, the induction of mutant dynamin with specific mutations in the GTPase domain has dominant-negative effects on endocytosis in mammalian cells (Herskovits *et al.* 1993; van der Bliek *et al.* 1993).

Because class I and class II alleles show intragenic complementation, it is likely that all mutants in our collection are capable of multimer formation. However, the specific dynamin domain affected by class II lethals was hard to predict. Multiple functional domains exist in dynamin that could associate with different substrates during different stages of the dynamin GTPase cycle (Figure 2). Several lines of evidence have implicated the PRD of dynamin in protein-protein interactions essential for dynamin function (Shupliakov *et al.* 1997). Studies on *VPS1*, which lacks C-terminal coiled-coil and PRD domains of dynamin, have shown that the middle domain of Vps1p is essential for function, although mutations in this domain do not have measurable dominant phenotypes (Vater *et al.* 1992). To identify mutations in class I and class II *shi* lethals, we sequenced *shi* coding sequences from a selection of the mutant alleles.

**Distinct functional domains in dynamin:** There are several reasons to identify sequence alterations in *shi* lethal alleles. First, it is interesting to know which dynamin domains are most easily inactivated by mutations. Second, because of their subtlety when compared with large deletions, point mutations that inactivate dynamin are very useful to evaluate the significance of *in vitro* binding observations. This is a particularly significant reason because residues critical for protein function cannot be identified by phylogenetic conservation alone: conserved residues, like conserved genes, are often not essential for protein activity.

We sequenced the entire coding region of dynamin in a selection of class I and class II *shi* lethals and in $shi^{ts4}$. The conditional allele $shi^{ts4}$, as well as both class I alleles we identified ($shi^{EM33}$ and $shi^{EM66}$), have single nucleotide transversions within the GTPase domain, similar to $shi^{ts1}$ and $shi^{ts2}$ (van der Bliek and Meyerowitz 1991). These missense mutations result in single amino acid substitutions at residues conserved among dynamins (Figure 2). It is interesting that the mutation in $shi^{EM33}$ that results in a lysine substitution for a conserved glutamate is identical to that reported for a dominant-negative allele of *VPS1* (Vater *et al.* 1992). These observations suggest that the GTPase domain plays a similar role during function of dynamin and Vps1p, an interesting observation because interactions between

Figure 3.—Rapid, reversible paralysis of *shi* allelic combinations. (A) A conditional *shi* allele, $shi^{CK2}$ is paralyzed within 2 min at 36° (Ramaswami *et al.* 1993). This presumably includes the time required for synaptic vesicle utilization and depletion at critical synapses in the fly nervous system; thus, the onset of the ts block in vesicle recycling is very rapid. (B) Flies heterozygous for a class I *shi* lethal allele ($shi^{EM33}$/ FM7c) show similar rapid onset temperature-sensitive paralysis at 36.5°. (C) Heteroallelic *shi* class I/class II flies ($shi^{EM33}/shi^{42}$) also show rapid, reversible conditional paralysis at 34.5°.

the GTPase domain and proline-rich C-terminal extensions, lacking in Vps1p, are believed essential for dynamin assembly and function (Hinshaw and Schmid 1995; Warnock and Schmid 1996). Our interest in *shi*$^{ts4}$ stems from a behavioral study of *shibire*$^{ts}$ alleles in which different heteroallelic combinations were analyzed for the degree of paralytic severity at a variety of temperatures. Heterozygous *shi*$^{ts2}$/*shi*$^{ts4}$ flies paralyze at a slower rate than either homozygote and so exhibit partial interallelic complementation (Kim and Wu 1990). This complementation between conditional alleles is very subtle compared with the robust intragenic complementation observed between class I and class II lethals. Our finding that the *shi*$^{ts4}$ mutations also map within the GTPase domain (proline$^{171}$-serine), 30 residues downstream of the *shi*$^{ts2}$ lesion, suggests that, in the functional oligomer, GTPase domains show interdomain associations and do not operate as isolated subunits. Finally, the interallelic complementation between conditional *shi* alleles may indicate functionally distinct regions within the so-called GTPase domain, analogous to subdomains at the C-terminal, PRD of dynamin (Okamoto *et al.* 1997).

We did not discover any sequence alterations in regions encoding the PHD, CCD, or PRD dynamin domains that have received most attention from the field. Rather, the four class II alleles that we sequenced altered a 20-residue segment of the middle domain, a region of dynamin adjacent to the GTPase domain. Potential interactions mediated by this middle domain are unknown. The mutants *shi*$^{EM18}$ and *shi*$^{EM42}$ were found to have identical sequence alterations, as did the alleles *shi*$^{EM35}$ and *shi*$^{EM56}$; this could reflect the very special nature of the residues altered or, more likely, that the two sets of mutant alleles, isolated in the same genetic screen, derived from the same premeiotic mutational events. The middle domain is ~64% identical among Drosophila and mammalian dynamins and significantly conserved in Vps1p and dnm1. The residues that are mutated in *shi*$^{EM18}$/*shi*$^{EM42}$ and *shi*$^{EM35}$/*shi*$^{EM56}$ are residues conserved among dynamins. In addition, the proline residue altered in *shi*$^{EM35}$/*shi*$^{EM56}$ is conserved in dnm1 as well, and Vps1p contains a glycine at this position. Because proline is a helix breaker and glycine is often found in turns, it is possible that a proline-serine substitution alters local secondary structure. Along with the GTPase domain, the middle domain appears to be the region most easily inactivated by point mutations. Although there are alternative possibilities, it is most likely that the middle domain mediates yet unknown protein-protein interactions required for dynamin activity. The conservation of this domain across dynamin-related proteins suggests that these middle domain interactions are required for the functions of Vps1p, Dnm1p, and Mx proteins, as well as of dynamins.

**The origin of ts paralysis caused by conditional alleles of *shibire*:** Temperature-sensitive alleles of *shi* probably have conditional effects on an activity of dynamin's GTPase domain. This is most directly indicated by the observation that ts mutations in *shibire* alter the GTPase domain. It is strengthened by the finding that a precisely assayed conditional phenotype of *shi*$^{ts}$ mutants (accumulation at elevated temperatures of a collared-pit intermediate in vesicle budding) is remarkably similar to structures observed when GTP hydrolysis of mammalian dynamin is inhibited by GTP-γ-S (Warnock and Schmid 1996). Because the molecular function of dynamin can be assayed to a high degree of precision *in vivo*, the data indicate that conditional phenotypes of *shi*$^{ts}$ mutants result directly from a temperature-sensitive effect on an activity of the GTPase domain. We report here that, in the appropriate genetic background, lethal mutations in the GTPase domain cause temperature-dependent phenotypes indistinguishable from those caused by previously characterized ts alleles. Because the lethal alleles were selected solely for their lethal phenotype, it is likely that the vast majority of them are simple hypomorphic mutations. The most parsimonious explanation for conditional phenotypes arising from hypomorphic mutations is that an activity of the dynamin GTPase domain is intrinsically temperature-sensitive and that hypomorphic mutations that reduce this activity reveal the intrinsic temperature sensitivity at lower temperatures.

Temperature sensitivity has traditionally been believed to result from specific mutations that affect the stability of a normally folded protein (Varadarajan *et al.* 1996). In this model, a restricted set of amino acid substitutions in a given protein would be expected to cause tight temperature-dependent phenotypes. However, it has long been known that only a small subset of genes can be mutated to yield ts phenotypes at any reasonable frequency (Harris and Pringle 1991). The concept that certain proteins are more easily identified by ts mutagenesis is supported by genetic screens for ts paralytic mutants on the Drosophila *X* chromosome. These screens require not only that the mutants show a temperature-sensitive phenotype but also demand a rapid onset of the ts defect and rapid reversibility. The vast majority of mutations isolated in these screens are in the *shibire* and *para* genes (Grigliatti *et al.* 1973; M. Ramaswami and K. S. Krishnan, unpublished results). Previous studies on the molecular basis for temperature sensitivity in *para*$^{ts}$ mutants showed that conditional *para* alleles carry insertions in intronic sequences (Loughney *et al.* 1989). Several lines of evidence argued that reduced expression of functional voltage-gated sodium channels caused by such insertions result in ts phenotypes by revealing the intrinsic temperature-dependent lability of sodium channels or an increased requirement for sodium channels at elevated temperatures (Stern *et al.* 1990). Direct measurements of the temperature sensitivity of macroscopic sodium currents in Drosophila have never been performed, and so the two alternatives, that is, increased lability of channels or an

increased requirement for them, have never been distinguished for *para*. These alternatives have been distinguished for *shibire* at identified synapses where synaptic vesicle fusion and endocytosis may be monitored. In *shi*$^{ts}$ synapses, elevated temperatures do not result in increased vesicle fusion and hence an increased demand for vesicle recycling; rather, a conditional block in endocytosis is seen.

We propose that an activity of the dynamin GTPase domain is intrinsically thermolabile and that this intrinsic temperature sensitivity is revealed by relatively nonspecific hypomorphic mutations. This sensitivity of dynamin to temperature appears phylogenetically conserved; homologous substitutions in mammalian dynamins also result in ts phenotypes (Damke *et al.* 1995). It is possible that the temperature-induced lability of dynamin results from particular functional requirements for the GTPase domain, such as rapid and large-scale conformational transitions. A similar sharp temperature sensitivity has been observed for a mutant GTPase in mammals. The mutation, in the alpha subunit of a trimeric G protein, causes Gs misregulation in testes that are about 2° cooler than in the rest of the body where the same mutation causes complete loss of Gs activity (Iiri *et al.* 1994). It is interesting that a testes-specific isoform exists for mammalian dynamin: it is possible that testes-specific isoforms of dynamin are adapted for function at lower temperatures.

In the annals of genetics, there is very sparse information available on molecular and biophysical bases for temperature-sensitive phenotypes. It is possible that most temperature-sensitive mutations affect proteins that are intrinsically thermolabile or that are required at higher concentrations at elevated temperatures.

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