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 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 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 mutants (Kosaka and Ikeda 1983; Masur et al. 1990; Tsuruhara et al. 1990; Kramer et al. 1991; Tabata and Korner 1994). Thus, the shi gene product is required for the formation of endocytic 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 Vale 1989; Obar et al. 1990; Chén 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 (Vale 1992).

The initial conclusion from these analyses, that dynamin is essential for a late stage in the formation of endocytic 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 endocytic 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-
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 endocytic 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 virusinduced 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 endocytic 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-K, 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 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 dynamin, as a site for regulatory kinases (Gammie et al. 1995). In vitro cell biological studies. Since the original isolation of shi 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 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 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 mutants were from the Krishnan and Masamwami laboratory stock collections (Masamwami et al. 1993; Est et al. 1996); w1118 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)sd129/C(1)DX/y+Yhi+) and deficiencies spanning the shibire locus (Df(1)sd270) were obtained from V. Rodrigues at the Tata Institute of Fundamental Research. sh112 and sh118b alleles were generated in a gamma-ray mutagenesis screen and kindly given to us by Cliffo 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 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 (Haahe, Paramus, NJ). Temperatures were controlled accurately with a precision of at least 0.5°C. 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 (Est 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). 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, D4-12, and D9-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 hemizygous animals. Embryos were laid at 25°C 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 (DS 71: 148-149, 1992), incubated at 37°C for 15 min, heat denatured at 95°C 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°C 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 EcoRI/BamHI, Clal, and Clal PstI restriction enzyme combinations and analyzed by standard Southern hybridization procedures (Sambrück 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 32P-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 shi121Lethal Alleles of shibire

**L o g i n u c l o n e t e l o s i d e s :** 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, TGG CAG GAA GTA AAG CCA GC; 67, GTC TCC GAG TTA CGA TAC D; 112, GCC CAA CAA CAC TCT TGG TCT CC; D12, GAC TCA AAG TGG GCC AGA TCA CT; 64, GGA GAG TTC CTT ATT ACC; 12, GCCCAA TCG CGT CGT GAT AT; 9, GGT GAT CCA ACC ATT GCC GTT ATT A; D13, CGG TCT CCT ACC GCA GAC GTG G; D7, GCC TCT TCA CAC CGC ACA TGG; D10, ATC GCC GTC TCT CTC ATC C; D4, ATC TTA TAG TCG GAC TCT CG; D11, AGG TGG GTG ACT CAC GAT GG. Oligonucleotides were used for sequencing including 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 TGT AGA T; for the region amplified between D7 and D10, J21, ACC CAC GTC TGC GAT AG; and MS02, CTT ATA AAC ATT AGC TCT ATC G; and for the region amplified between D4 and D1,J23, GTA CGG TCG 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

**RESULTS**

**Lethal shi allelles 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 12 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 shiEMS is not res-
duced by one or two copies of the shi transgene, although it is rescued by a duplication Dp(1, Y) 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, shi12.18h and shi12.12b, 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, shi12.18h and shi12.12b 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 (shiEM14, shiEM38, shiEM33, shiEM35, shiEM55, shiEM65, and shiEM66) and class II alleles (shiEM12, shiEM27, shiEM35, shiEM42, shiEM44, shiEM56, and shiEM59) were lethal when homozygous. However, the two groups showed interallelic complementation such that class I/class II heterozygotes were viable (Table 1). A subset of mutants in class I (shiEM14, shiEM38, and shiEM53) 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 shi12.18h and shi12.12b 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 shi102/ + and shi102/+ 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 shi102 and shi108, 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 shiEM14/+ and shiEM33/+ paralyzed at 36°, shiEM38/+ and shiEM55/+ paralyzed at 37°, and shiEM65/+ and shiEM66/+ 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 shiEM38 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 shiEM38, and the two other
class I alleles \( \text{shi}^{EM14} \) and \( \text{shi}^{EM53} \), did not complement any of the other \( \text{shi} \) lethal alleles. Thus, although they do not strictly belong to the same complementation group as the other dominant-negative \( \text{shi} \) lethals, we include them in the group of class I \( \text{shi} \) alleles (Table 2).

Class II and Class III alleles showed no effect when heterozygous with a wild-type allele of \( \text{shi} \). Thus, the heterozygotes \( \text{shi}^{EM11}/ + \), \( \text{shi}^{EM27}/ + \), \( \text{shi}^{EM35}/ + \), \( \text{shi}^{EM42}/ + \), \( \text{shi}^{EM44}/ + \), \( \text{shi}^{EM59}/ + \), and \( \text{shi}^{12.18H}/ + \) were not affected in any noticeable way at temperatures \(<42^\circ\text{C}\). A unique feature of Class III alleles was their behavior when heterozygous with conditional alleles of \( \text{shi} \), where they behaved in the same way as \( \text{shi} \) deficiencies. \( \text{shi}^{12.18H}/ + \) and \( \text{shi}^{12.18H}/ + \) heterozygotes are paralyzed at 26° and 26.5°, respectively, which is identical to the temperature of paralysis of \( \text{shi}^{12.18H}/ \text{Df}(1)\text{sd}^{72b} \) or \( \text{shi}^{12.18H}/ \text{Df}(1)\text{sd}^{72b} \) flies. However, class II alleles heterozygous with ts alleles generally had greatly elevated restrictive temperatures compared to homozygous ts mutants for example, \( \text{shi}^{12.18H}/ \text{shi}^{12.18H} \) homozygotes are paralyzed at 27.5°, but \( \text{shi}^{12.18H}/ \text{shi}^{EM44} \) flies are not affected until 36° (Table 2).

In summary, class I alleles showed dominant-negative properties, as previously observed for conditional \( \text{shi} \) 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 \( \text{shi} \). 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 \( \text{shi}^{12.18L} \) and \( \text{shi}^{12.18H} \), 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 \( \text{shi}^{12.18H} \) and \( \text{shi}^{12.12B} \) 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 \( \text{shi} \) lethals.

\textbf{Table 2}

**Temperature of paralysis for heterozygous or transgene-rescued \( \text{shi} \) lethals**

<table>
<thead>
<tr>
<th>Class</th>
<th>Line</th>
<th>( \text{shi} )</th>
<th>( \text{shi} ) ( P(w^+ \text{shi}^+) )</th>
<th>( \text{shi} )</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>( \text{shi}^{EM14} )</td>
<td>36-37°</td>
<td>34-35°</td>
<td>no survivors</td>
<td>dom.-neg.</td>
</tr>
<tr>
<td>I</td>
<td>( \text{shi}^{EM38} )</td>
<td>37°</td>
<td>no survivors</td>
<td>no survivors</td>
<td>dom.-neg.</td>
</tr>
<tr>
<td>I</td>
<td>( \text{shi}^{EM53} )</td>
<td>wild type</td>
<td>no survivors</td>
<td>33°</td>
<td>?</td>
</tr>
<tr>
<td>I</td>
<td>( \text{shi}^{EM33} )</td>
<td>36°</td>
<td>33°</td>
<td>no survivors</td>
<td>dom.-neg.</td>
</tr>
<tr>
<td>I</td>
<td>( \text{shi}^{EM55} )</td>
<td>37-38°</td>
<td>36°</td>
<td>&lt;28°</td>
<td>dom.-neg.</td>
</tr>
<tr>
<td>I</td>
<td>( \text{shi}^{EM65} )</td>
<td>40°</td>
<td>39°</td>
<td>30°</td>
<td>dom.-neg.</td>
</tr>
<tr>
<td>I</td>
<td>( \text{shi}^{EM66} )</td>
<td>37°</td>
<td>33°</td>
<td>ND</td>
<td>dom.-neg.</td>
</tr>
<tr>
<td>II</td>
<td>( \text{shi}^{EM18} )</td>
<td>wild type</td>
<td>wild type</td>
<td>34°</td>
<td>recessive</td>
</tr>
<tr>
<td>II</td>
<td>( \text{shi}^{EM27} )</td>
<td>wild type</td>
<td>wild type</td>
<td>36°</td>
<td>recessive</td>
</tr>
<tr>
<td>II</td>
<td>( \text{shi}^{EM35} )</td>
<td>wild type</td>
<td>wild type</td>
<td>35.5°</td>
<td>recessive</td>
</tr>
<tr>
<td>II</td>
<td>( \text{shi}^{EM42} )</td>
<td>wild type</td>
<td>wild type</td>
<td>34°</td>
<td>recessive</td>
</tr>
<tr>
<td>II</td>
<td>( \text{shi}^{EM44} )</td>
<td>wild type</td>
<td>wild type</td>
<td>35.5°</td>
<td>recessive</td>
</tr>
<tr>
<td>II</td>
<td>( \text{shi}^{EM56} )</td>
<td>wild type</td>
<td>wild type</td>
<td>35°</td>
<td>recessive</td>
</tr>
<tr>
<td>II</td>
<td>( \text{shi}^{EM59} )</td>
<td>wild type</td>
<td>wild type</td>
<td>36°</td>
<td>recessive</td>
</tr>
<tr>
<td>III</td>
<td>( \text{shi}^{12.18H} )</td>
<td>wild type</td>
<td>wild type</td>
<td>26° (as ( \text{shi}^{12.18L} ))</td>
<td>recessive</td>
</tr>
</tbody>
</table>

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 \( \text{shi} \) paralysis, which is preceded by characteristic behavioral seizures and uncontrolled wing-beating.

\( \text{shi}^{12.18H} \) is potentially a null allele of \( \text{shi} \): Identical large-scale rearrangements were observed at the \( \text{shi} \) locus of \( \text{shi}^{12.18H} \) and \( \text{shi}^{12.12B} \) mutants, and so data is presented here only for \( \text{shi}^{12.18H} \). Southern analysis of \( \text{shi}^{12.18H}/+ \) DNA using \( \text{shi} \) probes revealed that an EcoRI, BamHI fragment was altered in the \( \text{shi}^{12.18H} \) 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 (Clal and PstI) and hybridized to various \( \text{shi} \) probes (Figure 1B). When Clal, PstI-digested DNA was probed with a 1.35-kb region between a Clal and PstI site near the 3' end of the \( \text{shi} \) 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 \( \text{shi}^{12.18H} \) 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 shi12.18H 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 shi12.18H/+. Ten micrograms of genomic DNAs were digested with EcoRI and BamHI and probed with shibire cDNA. Oregon R (ORR) flies were used as a homozygous wild-type strain for a control, and heterozygous shi12.18H/+ 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 (BamHI), C (ClaI), E (EcoRI) and P (PstI). Probes specific to various portions of shibire genomic DNA reveal that the breakpoint lies within a 1.35-kb region flanked by ClaI and PstI restriction sites. (C) The shi12.18H 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 shi12.18H/+ heterozygotes, stained with an antibody 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 shi12.18H/+ animals. This 3.3-kb fragment included 5′ shi sequences, a translocation breakpoint, and a new ClaI 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 shi12.18H 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 shi12.18H/+ 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-
Lethal Alleles of *shibire*

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 defined by the class II mutations, 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 Arg181 and Pro401 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°C, and *shi*EM33/FM7c flies are similarly paralyzed at 36°C (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, shi12.18H, 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 shi12.18H, 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 shi12.18H (and the identical shi12.12B) is the only shi allele indistinguishable from a shibire deficiency when heterozygous over wild-type, shi lethal, or shi15 chromosomes. Genomic analyses demonstrate a large chromosomal rearrangement at the shi locus in shi12.18H 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 shi12.18/+/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 shi12.18 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 shi134 and shi152 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 (Her skovits 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 I 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 shi134. The conditional allele shi134, as well as both class I alleles we identified (shiEM33 and shiEM66), have single nucleotide transversions within the GTPase domain, similar to shi134 and shi152 (van der Bliek and Meyeroowitz 1991). These missense mutations result in single amino acid substitutions at residues conserved among dynamins (Figure 2). It is interesting that the mutation in shiEM33 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...
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 alleles stems from a behavioral study of shibire alleles in which different heteroallelic combinations were analyzed for the degree of paralytic severity at a variety of temperatures. Heterozygous shi/shi 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 alleles also map within the GTPase domain (proline-serine), 30 residues downstream of the shi lesion, suggest, 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 and shi were found to have identical sequence alterations, as did the alleles shi and shi; 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 and shi and shi are residues conserved among dynamins. In addition, the proline residue altered in shi and shi 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 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 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 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 the 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 and dnm1 mutants showed that conditional para alleles carry insertions in intronic sequences (Loughney et al. 1989). Several lines of evidence argue 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 (Stevenson 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 tests that are about 2°C 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. We thank Clifton Poodry for his gamma-ray-induced lethal alleles of shi, Alexander van der Bliek for providing us with unpublished genomic sequence from the shi locus, Veronica Rodrigues and Danny Brower for frequent advice. The manuscript was improved by comments from Alex van der Bliek, Chun-Fang Wu, Jane Robinson, Dave Sandstrom, and Robin Stappes and by discussions with members of the Ramaswami lab. The work was funded by a National Science Foundation grant to M.R. and a grant from the Department of Science and Technology of the Government of India to K.S.K. and M.R. M.R. is a Sloan Research Fellow and a McKnight Neuroscience Scholar.

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