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Synaptic Clustering of Fasciclin II and Shaker: Essential Targeting Sequences and Role of Dlg

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Summary

Previous studies have shown that both the Fasciclin II (Fas II) cell adhesion molecule and the Shaker potassium channel are localized at the Drosophila neuromuscular junction, where they function in the growth and plasticity of the synapse. Here, we use the GAL4-UAS system to drive expression of the chimeric proteins CD8–Fas II and CD8–Shaker and show that the C-terminal sequences of both Fas II and Shaker are necessary and sufficient to drive the synaptic localization of a heterologous protein. Moreover, we show that the PDZ-containing protein Discs-Large (Dlg) controls the localization of these proteins, most likely through a direct interaction with their C-terminal amino acids. Finally, transient expression studies show that the pathway these proteins take to the synapse involves either an active clustering or a selective stabilization in the synaptic membrane.

Introduction

The precise localization of proteins at the pre- and postsynaptic membranes is required for the fidelity and plasticity of synaptic transmission. Transmitter release machinery must be positioned at the presynaptic active zones, poised to release neurotransmitter in response to changes of membrane potential. Rapid response to chemical transmitter requires spatial localization and clustering of postsynaptic neurotransmitter receptors. In addition, ion channels, modulatory receptors, and signal transduction machinery all must be precisely positioned to modulate and integrate synaptic signals reliably and accurately. It is likely that the ability of proteins to cluster at the synapse is required for other aspects of synapse formation, stabilization, and growth. Until recently, the mechanisms by which proteins become localized at the synapse were largely unknown.

A major insight came with the identification of the interaction of PSD-95, a member of the MAGUK family of membrane-associated guanylate kinases, with the C terminus of NMDA receptors (Kornau et al., 1995) and potassium channels (Kim et al., 1995). MAGUK proteins contain three PDZ domains at their N termini, an SH3 domain, and a C-terminal guanylate kinase domain. The PDZ domains are modular protein–protein interaction domains of ~90 amino acids each. The presence of multiple PDZ domains in a MAGUK protein enables it to cross-link membrane proteins and to link them to the cytoskeleton or to signal-transducing enzymes (reviewed by Gomperts, 1996; Sheng, 1996). Initially, putative PDZ-interacting proteins were identified on the basis of a C-terminal consensus motif, -S/T-X-V (Kornau et al., 1995); however, it has become clear that different PDZ domains have preferences for different C-terminal binding motifs (Songyang et al., 1997; Stricker et al., 1997). The diversity of PDZ-containing proteins and their diverse specificities of interaction have been proposed to underlie the differential distribution of different proteins at the same synapse, to allow differential regulation of protein density, and to couple different proteins physically to distinct downstream signaling pathways (Sheng, 1997).

Evidence for the functional roles of PDZ-containing proteins has been obtained through in vitro cell culture assays and in vivo loss-of-function genetic studies. Coexpression studies in heterologous cells have shown that members of the MAGUK family, PSD-95 and chapsyn-110, can cause the clustering of potassium channels and NMDA receptors (Kim et al., 1995, 1996). These studies indicate a role for MAGUK proteins in cross-linking proteins at the synapse but leave unclear a possible role in targeting these proteins to their specific synaptic locations. Genetic loss-of-function studies show that PDZ-interacting proteins are often mislocalized in the absence of their PDZ-containing partners (Simske et al., 1996; Chevesich et al., 1997; Tejedor et al., 1997; Tsunoda et al., 1997), implicating PDZ proteins as mediators of subcellular localization.

The homophilic cell adhesion molecule Fasciclin II (Fas II) and the potassium channel Shaker both contain -S/T-X-V sequences at their C termini (Kornau et al., 1995), identifying them as proteins that could interact with PDZ domains. Interestingly, both proteins are known to localize at the synapse of the Drosophila neuromuscular junction (NMJ; Schuster et al., 1996a; Tejedor et al., 1997). Although there is evidence for the interaction of Shaker with a PDZ-containing protein (Tejedor et al., 1997), there is no such evidence for Fas II. The localization of Fas II to the synapse is essential for synaptic stabilization and growth, and the regulation of Fas II levels at the synapse has been shown to control structural plasticity (Schuster et al., 1996a, 1996b). Likewise, the level of Shaker expression at the same synapse has been implicated in the growth and plasticity of this synapse (Budnik et al., 1990; Zhong et al., 1992). The presence of PDZ-interaction sequences at the C termini of Fas II and Shaker suggests a possible PDZ-dependent mechanism for the precise regulation of their localization and expression levels at the synapse.

The MAGUK protein Discs-Large (Dlg; Woods and Bryant, 1991), a Drosophila homolog of PSD-95, has been implicated in the clustering of Shaker at the NMJ (Tejedor et al., 1997). These results suggest that the C-terminal PDZ-interaction sequences of Shaker may be involved in its synaptic localization and that, by analogy, similar sequences in Fas II might also control synaptic localization. In order to determine whether these C-terminal sequences are responsible for synaptic targeting,

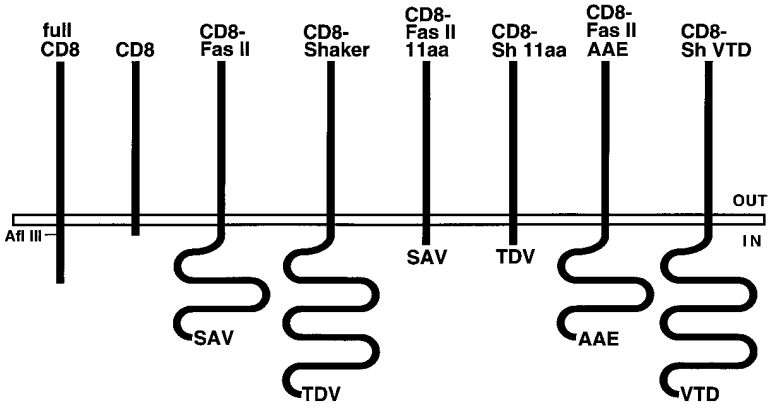

Figure 1. CD8–Shaker and CD8–Fas II Chimeric Proteins

The extracellular and transmembrane domains of CD8 were used to make the chimeras. For the control CD8 (CD8), a stop codon was inserted directly following the AflIII site, creating a truncated CD8 protein that contains only the CD8 sequences present in the chimeras, terminating six amino acids into the cytoplasmic domain. CD8–Fas II and CD8–Shaker are fusions of CD8 to the full C-terminal sequences of the $PEST^{+}$ isoform of Fas II or to the cytoplasmic C terminus of Shaker. CD8–Fas II 11aa and CD8–Shaker 11aa are fusions to the final 11 amino acids of Fas II (-SGEIIGKNSAV) or of Shaker (-NALAVSIETDV). CD8–Fas II AAE and CD8–Shaker VTD are almost identical to the full C-terminal CD8 chimeras, except that the final three amino acids are altered from SAV to AAE for CD8–Fas II AAE or from TDV to VTD for CD8–Shaker VTD.

we asked whether they are capable of targeting a heterologous membrane protein to the synapse. We find that the C-terminal sequences of Fas II and Shaker are both necessary and sufficient for targeting to the subsynaptic muscle membrane at the larval NMJ of Drosophila. This localization depends on Dlg and can be accounted for by an active clustering or selective retention of the protein at the synapse.

Results

C Termini of Fas II and Shaker Direct Synaptic Localization

In order to test whether the C-terminal sequences of Fas II and Shaker are sufficient to direct synaptic localization, we constructed a series of chimeric molecules with the human T lymphocyte membrane protein, CD8 (Littman et al., 1985), which is not normally expressed in nerve or muscle (Figure 1). The extracellular and transmembrane domains of CD8 were fused to the C-terminal cytoplasmic domains of Shaker (CD8–Shaker) or Fas II (CD8–Fas II). A control CD8 construct (CD8) was engineered to include solely the CD8 sequences present in the chimeric molecules by introducing a stop codon at the site of fusion, creating a truncated CD8 protein that terminates six amino acids into the cytoplasmic domain. These molecules were placed under the control of the UAS promoter (Brand and Perrimon, 1993) and transformed into flies.

We examined synaptic localization of the chimeric CD8 constructs at the postsynaptic membrane of the Drosophila NMJ, referred to as the subsynaptic reticulum (SSR), which is composed of multiple layers of elaborately folded muscle membrane surrounding the presynaptic terminal. The use of chimeric proteins eliminated two potential problems in interpreting results on the postsynaptic localization of Fas II and Shaker: (1) their simultaneous expression at the presynaptic terminal and (2) the homophilic binding function of Fas II. Expression of the CD8 chimeric constructs was driven using the *MHC^{82}-GAL4* line that promotes GAL4 expression in all muscles. Wandering third instar larvae were dissected and stained with a monoclonal antibody to CD8. We observed a strong concentration of staining at the synaptic boutons for CD8–Shaker and CD8–Fas II but not for CD8 (Figures 2A–2C). Electron micrographs using anti-CD8 antibodies and horseradish peroxidase (HRP) immunocytochemistry confirmed this result and showed that CD8–Fas II and CD8–Shaker are concentrated in the SSR (Figures 2D–2F). Thus, the C-terminal sequences of Fas II and Shaker are sufficient to direct synaptic localization.

Although much lower than the synaptic levels of CD8–Fas II and CD8–Shaker, a small amount of bouton staining was apparent for CD8. We suspect that this is due to the concentration of postsynaptic membrane in the SSR. If a protein is uniformly distributed throughout the muscle membrane, including in the SSR layers, then one would expect to see a concentration of staining at the boutons. Consistent with this idea, in first instar larvae, where the SSR is composed of one or only a few layers of membrane, there was no concentration of the control CD8 protein at the boutons (Figure 3B). In contrast, there was a strong concentration of CD8–Shaker and CD8–Fas II at the boutons of first instar larvae (Figures 3A, 6A, and 6C).

The muscles of third instar Drosophila larvae are innervated by synaptic terminals of two major types (Johansen et al., 1989). Type I synapses have larger boutons containing glutamate-filled vesicles, while type II synapses have small boutons containing a variety of vesicles, including those with dense cores (Atwood et al., 1993). We observe localization of our CD8 chimeras at type I boutons and not at type II boutons. This is consistent with the observed localization for the endogenous Shaker protein (Tejedor et al., 1997); however, endogenous Fas II is localized at both type I and type II boutons (data not shown). In light of this, Fas II must not be directed to type II boutons by its C-terminal sequences. Therefore, it is likely either that the observed Fas II staining at type II boutons is entirely presynaptic or that the extracellular domain of Fas II is required for its postsynaptic concentration at type II boutons,

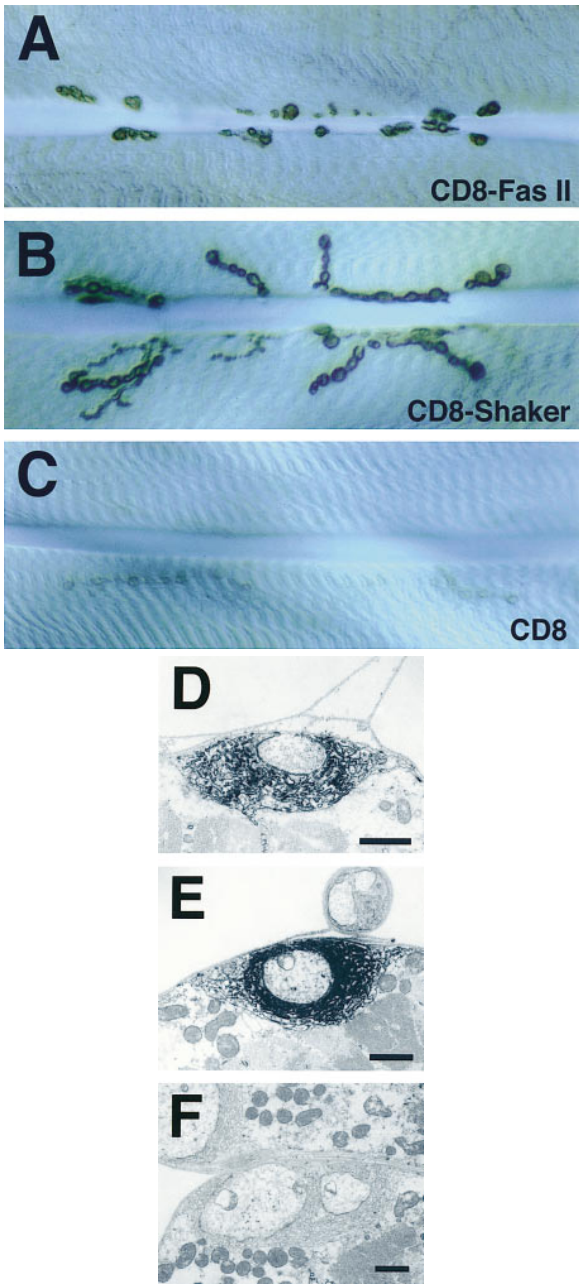

Figure 2. C-Terminal Sequences of Fas II and Shaker Direct Synaptic Localization

Muscle expression of CD8 chimeras is driven in third instar larvae by *MHC-GAL4*, which expresses in all muscles.
(A–C) Anti-CD8 immunocytochemistry at 1:500 dilution of the CD8 antibody. CD8–Fas II (A) and CD8–Shaker (B) are concentrated at the synaptic boutons, whereas CD8 (C) is not.
(D–F) Electron micrographs using anti-CD8 antibodies and HRP immunocytochemistry of type Ib boutons of third instar larvae. CD8–Fas II (D) and CD8–Shaker (E) are concentrated in the subsynaptic reticulum, whereas CD8 (F) is not.
Scale bar, 1 μm.

perhaps via homophilic interactions with presynaptic Fas II.

The relative efficiency of localization of the two chimeric proteins to the synapse is not the same. We observe

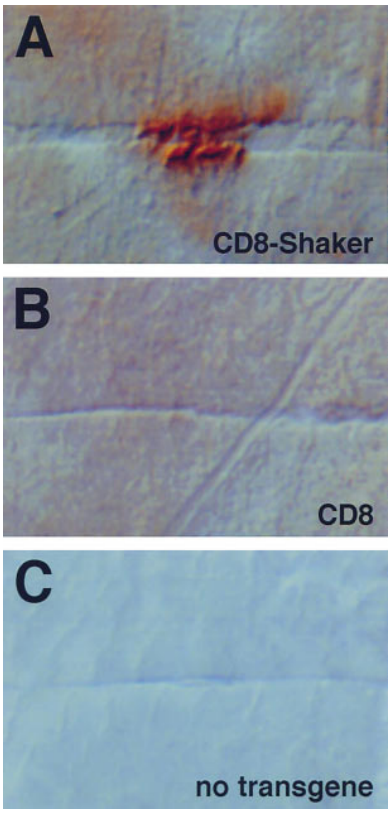

Figure 3. Synaptic Localization of CD8 Chimeras in First Instar Larvae

Anti-CD8 immunocytochemistry at 1:500 dilution of the CD8 antibody. Muscle expression of CD8 chimeras is driven in first instar larvae by *24B-GAL4*.
(A) CD8–Shaker is concentrated at the synaptic boutons.
(B) CD8 is not concentrated at the synaptic boutons. The slight background bouton staining seen in third instar larvae is not observed here, consistent with the undeveloped subsynaptic reticulum in first instar larvae.
(C) Animals that do not express a transgene show no muscle-membrane staining with the CD8 antibody.

that CD8–Shaker is more concentrated at the boutons than is CD8–Fas II. This was true in third instar larvae, where smaller type Is boutons were difficult to detect with staining for CD8–Fas II (Figure 2A) but not for CD8–Shaker (Figure 2B), as well as in first instar larvae (Figures 6A and 6C). We quantitated the differential localization of these two constructs in third instar larvae using fluorescent secondary antibodies and confocal microscopy (see Experimental Procedures). The targeting efficiency for CD8–Shaker was 7.6 $\pm$ 0.2, significantly higher than the value of 2.2 $\pm$ 0.2 found for CD8–Fas II ($p < 0.00001$, Student's t test; values expressed as mean $\pm$ SEM). Thus, despite their similar PDZ-interaction consensus sequences, the C-terminal sequences of Fas II and Shaker have different abilities to direct synaptic localization.

C-Terminal 11 Amino Acids of Fas II and Shaker Are Sufficient to Direct Synaptic Localization

There is considerable evidence that PDZ interactions are directed by a relatively small number of protein–protein

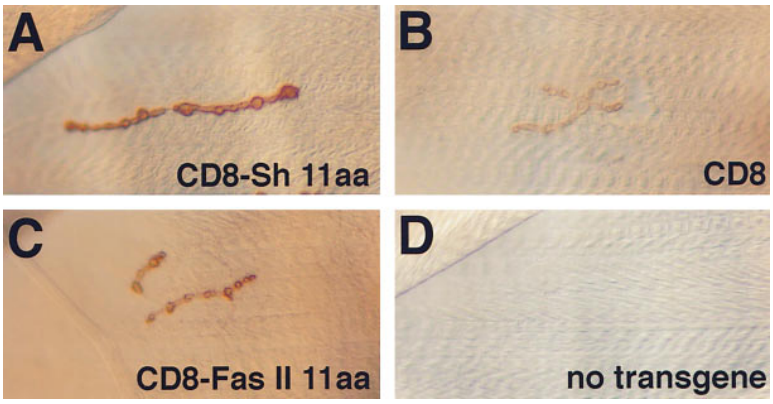

Figure 4. C-Terminal 11 Amino Acids of Shaker and Fas II Are Sufficient to Direct Synaptic Localization

Anti-CD8 immunocytochemistry at 1:500 dilution of the CD8 antibody. Muscle expression of the CD8 chimeras is driven in third instar larvae by *MHC-GAL4*. CD8–Sh 11aa (A) is concentrated at the synaptic boutons, whereas CD8 (B) is not. CD8–Fas II 11aa (C) is also concentrated at the synaptic boutons, though at a lower level than CD8–Shaker. Animals that do not express a transgene show no muscle-membrane staining with the CD8 antibody (D).

contacts (Doyle et al., 1996). Therefore, we tested whether these final amino acids are sufficient to direct synaptic localization of the heterologous CD8 protein to the NMJ in vivo.

Chimeras were constructed between CD8 and the final 11 amino acids of Shaker (CD8–Sh 11aa) or of Fas II (CD8–Fas II 11aa). As for the entire C-terminal fusions, CD8–Sh 11aa and CD8–Fas II 11aa were localized to the synaptic boutons of third instar larvae, and the synaptic concentration of CD8–Sh 11aa was stronger than that for CD8–Fas II 11aa (Figures 4A and 4C). Although statistically significant above background levels for CD8 alone ($p < 0.0001$; Figure 4B), the concentration of the 11aa chimeras at the boutons was not as strong as that observed for the chimeras containing the entire C-terminal sequences (Figure 2). The targeting efficiency of CD8–Sh 11aa was 1.69 ± 0.07, and that for CD8–Fas II 11aa was 1.44 ± 0.04 (as compared to 7.6 ± 0.2 for CD8–Shaker and 2.2 ± 0.2 for CD8–Fas II, reported above). Therefore, we conclude that although the final 11 amino acids of Shaker and Fas II are sufficient to direct synaptic localization, they are not nearly as effective as the entire C termini at concentrating CD8 at the synaptic boutons.

C-Terminal Three Amino Acids of Fas II and Shaker Are Necessary for Synaptic Localization

Since the entire C termini of Fas II and Shaker are more effective at directing synaptic localization than the last 11 amino acids, which contain the PDZ-interaction motif, it is possible that other portions of the C termini are involved in synaptic targeting. We tested whether the terminal consensus PDZ-interaction sequence (-S/T-X-V) is necessary for synaptic localization. Two new chimeras were constructed that are essentially identical to the CD8–Shaker and the CD8–Fas II chimeras, except that the last three amino acids are altered. To insure disruption of PDZ interactions, we mutated both the S/T at position -2 and the C-terminal valine. The final three amino acids of CD8–Shaker were scrambled from T-D-V to V-T-D (CD8–Sh VTD), and the final three amino acids of CD8–Fas II were altered from S-A-V to A-A-E (CD8–Fas II AAE).

Expression of CD8–Sh VTD and CD8–Fas II AAE was driven in third instar larvae. Double staining with the CD8 antibody and synaptotagmin revealed that the strong concentration of CD8–Shaker and CD8–Fas II at the synaptic boutons is abolished when the final three amino acids are altered to destroy interaction with PDZ domains (Figure 5). Instead, CD8–Fas II AAE and CD8–Sh VTD are uniformly expressed on the muscle membrane (Figures 5C and 5G). Animals that do not express a transgene show no muscle-membrane staining with the CD8 antibody (data not shown). Similar results were obtained in first instar larvae (data not shown). Thus, the PDZ-interaction domains of Fas II and Shaker are necessary for their localization to the synapse.

Dlg Is Required for Synaptic Localization of CD8–Fas II and CD8–Shaker

The dependence of CD8–Shaker and CD8–Fas II localization on the C-terminal -S/T-X-V sequences suggests that the synaptic localization process requires an interaction with a PDZ-containing protein. In Drosophila, a good candidate for such a protein is Dlg. Using the yeast two-hybrid assay, we observed a direct interaction between the C-terminal sequences of both Fas II and Shaker and the PDZ domains of Dlg (data not shown). These interactions were disrupted upon alteration of the final three amino acids from TDV to VTD for Shaker or from SAV to AAE for Fas II. In addition, an earlier study showed that the clustering and localization of endogenous Shaker protein are disrupted in third instar *dlg* mutant larvae (Tejedor et al., 1997). However, this result on its own is difficult to interpret, since Dlg is required for normal synaptic bouton structure in third instar larvae (Lahey et al., 1994). Is the absence of Shaker localization due to a requirement for an interaction with Dlg, or is it secondary to the disruption of normal bouton structure in third instar *dlg* mutant larvae? To get around this problem, we examined the localization of CD8–Shaker and CD8–Fas II in first instar *dlg* mutant larvae, where we found that the synapse looks normal.

CD8–Shaker and CD8–Fas II were concentrated at the synaptic boutons of first instar *dlg*/+ heterozygous larvae (Figures 6A and 6C), as expected. In *dlg* mutant first instar larvae, the localization of CD8–Shaker and CD8–Fas II was lost (Figures 6B and 6D). Instead, the protein was diffusely and uniformly distributed across the muscle membrane. The neuromuscular synapses of the *dlg* mutant first instar larvae were found to be intact, as evidenced by normal synaptotagmin staining (Figure

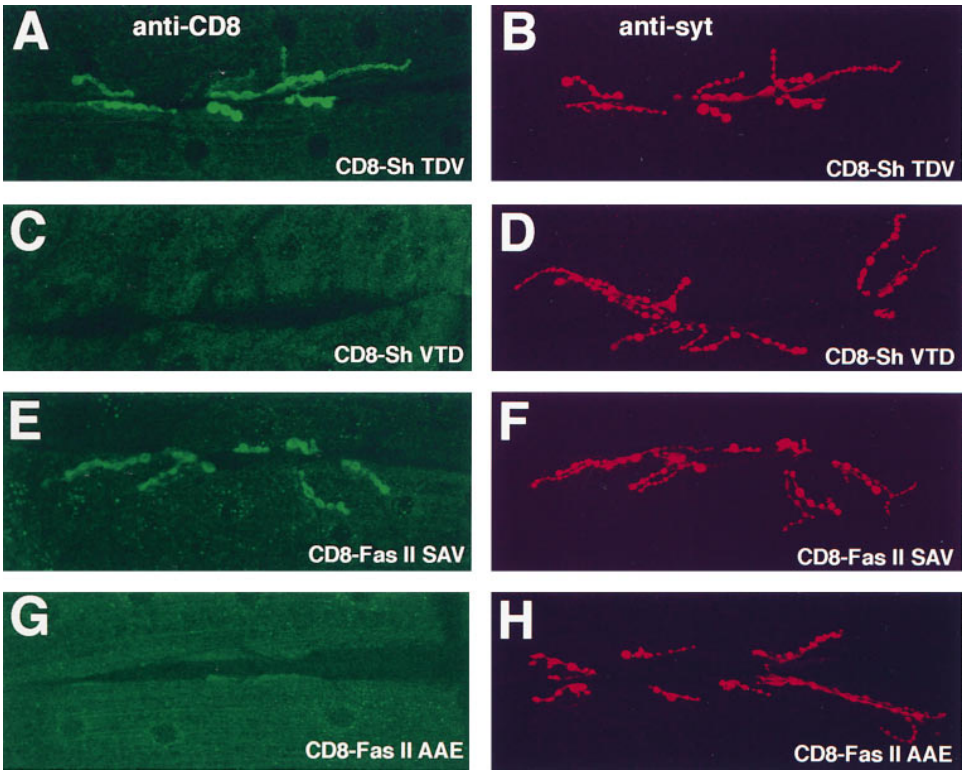

Figure 5. C-Terminal Three Amino Acids of Fas II and Shaker Are Necessary for Synaptic Localization

Muscle expression of the CD8 chimeras is driven in third instar larvae by *MHC-GAL4*.

(A, C, E, and G) Anti-CD8 immunocytochemistry at 1:500 dilution of the CD8 antibody.

(B, D, F, and H) Synaptic boutons of the same neuromuscular junctions as in (A), (C), (E), and (G) are visualized by immunostaining with anti-synaptotagmin.

(A and B) CD8–Shaker with a wild-type C-terminal PDZ-interaction domain (TDV) is localized at the synaptic boutons.

(C and D) CD8–Shaker with a disrupted PDZ-interaction domain (VTD) is not localized to the synaptic boutons; instead, it is distributed uniformly on the muscle membrane.

(E and F) CD8–Fas II with a wild-type C-terminal PDZ-interaction domain (SAV) is localized to the synaptic boutons.

(G and H) CD8–Fas II with a disrupted PDZ-interaction domain (AAE) is no longer localized to the synaptic boutons; instead, it is uniformly distributed on the muscle membrane.

6F). Thus, the uniform expression observed for CD8–Shaker and CD8–Fas II in the *dlg* mutants indicates a loss of localization, despite the presence of normal synapses in the mutant larvae. These results demonstrate that Dlg is required for synaptic localization of CD8–Shaker and CD8–Fas II. This result is supported by the observed localization of the CD8 chimeras to type I and not to type II boutons. Because endogenous Dlg is localized exclusively to type I boutons (Lahey et al., 1994), it follows that any protein that is localized exclusively in a Dlg-dependent manner must also be localized only to type I boutons.

In *dlg* mutants, we observe that the localization of endogenous Fas II to the synapse is not lost, although the levels appear lower than at wild-type synapses (data not shown). It is not surprising that Fas II persists at the synapse for two reasons. First, there is presynaptic Fas II expression, and at the light level it is not possible to distinguish pre- from postsynaptic expression. Second, the Fas II molecule self-clusters in a homophilic fashion if it is expressed on opposing cell surfaces (Grenningloh et al., 1991). Therefore, even without Dlg, some Fas II may cluster at the synapse simply due to homophilic binding between pre- and postsynaptic Fas II.

CD8–Shaker Uniformly Distributes on the Muscle Membrane Prior to Concentrating at the Synapse

Models for the mechanism by which synaptic localization is accomplished can be divided into two major categories: (1) direct synaptic targeting or (2) uniform membrane targeting followed by active clustering or selective retention at the synapse. A direct synaptic-targeting model involves a vesicular-mediated synaptic-targeting event that carries the proteins on a path directly from the ER/Golgi to the subsynaptic reticulum. The second model involves a uniform targeting pathway to the muscle membrane, followed either by an active recruitment to the synapse or by a selective retention at the synapse through stabilizing interactions with synapse-specific proteins. In an attempt to distinguish between these two possibilities, we performed a "pulse-chase" experiment to examine the pathway that CD8–Shaker follows to the synapse.

A heat shock promoter driving GAL4 expression was used to promote a pulse of CD8–Shaker expression during different stages of larval development. The expression pattern was monitored in third instar larvae at specific time points after the heat shock by staining with

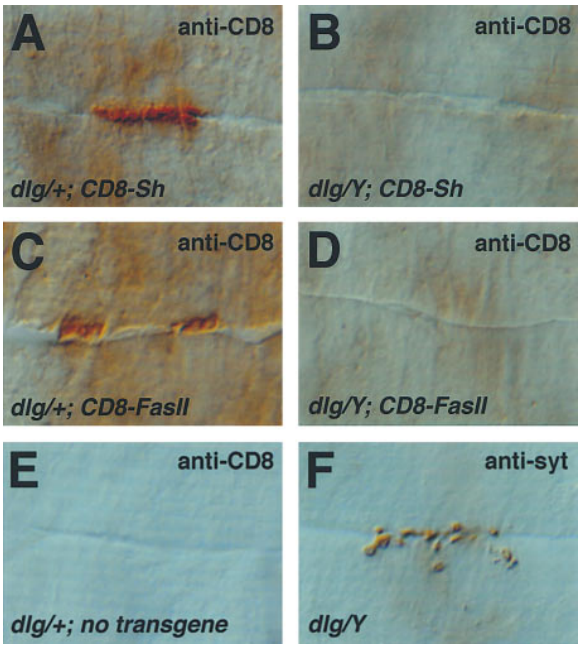

Figure 6. Dlg Is Required for Synaptic Localization of CD8–Fas II and CD8–Shaker

(A–E) Anti-CD8 immunocytochemistry at 1:100 antibody dilution. Muscle expression of CD8–Shaker and CD8–Fas II is driven in first instar larvae by *24B-GAL4*, which expresses in all muscles. CD8–Shaker (A) and CD8–Fas II (C) are localized to the synaptic boutons of first instar *dlg/+* heterozygotes. CD8–Shaker (B) and CD8–Fas II (D) are not localized to the synaptic boutons of first instar *dlg/Y* mutants; instead, they are uniformly expressed on the muscle membrane. Staining of the CD8 antibody in the absence of transgene expression (E) shows that the expression on the membrane in (B) and (D) is CD8 immunoreactivity and not background. Anti-synaptotagmin staining of synaptic boutons (F) shows that synapses appear normal in mutant *dlg/Y* first instar larvae, despite the absence of localization for CD8–Shaker and CD8–Fas II.

the CD8 antibody. Seven hours following heat shock, expression of the CD8–Shaker protein was low and was uniformly distributed over the muscle membrane (Figure 7B). Three days following heat shock, the protein expression was high both on the membrane and at the synaptic boutons (Figure 7C). After six days, membrane staining was again low, whereas bouton staining remained high (Figure 7D). Thus, initially the CD8–Shaker protein was uniformly distributed on the muscle membrane, and this distribution was followed by concentration at the synapse. These results do not support a direct synaptic-targeting model, but they are consistent with a model of uniform membrane targeting followed by active clustering or selective retention at the synapse.

To control for possible abnormalities due to heat shock, we performed a similar experiment using two muscle-specific promoters that promote GAL4 expression at different times during development. Expression of CD8–Shaker by *24B-GAL4*, which drives expression very early in myoblasts, even before muscle fusion occurs (Luo et al., 1994), resulted in dark bouton staining in early first instar larvae (Figure 6A). However, when CD8–Shaker expression was driven by *MHC-GAL4*, which promotes expression beginning in early first instars, a uniform light muscle staining was observed in first instar larvae, with no concentration at the synaptic boutons (data not shown). These results further support a model whereby synaptic membrane proteins are initially uniformly distributed throughout the muscle membrane, followed by concentration at the synapse.

Discussion

In this paper, we demonstrate that the C-terminal sequences of Fas II and Shaker are sufficient to direct synaptic localization of a heterologous protein, the lymphocyte membrane protein CD8. Precise alteration of two amino acids in the PDZ-interaction motif of the Fas II and Shaker C termini completely eliminated targeting of the CD8–Fas II and CD8–Shaker chimeras, suggesting a role for a PDZ-containing protein, such as Dlg, in localizing these proteins to the synapse. We provide genetic and biochemical support for such a role by demonstrating that localization of the CD8 chimeras is lost in a *dlg* mutant background and that the C-terminal sequences of Fas II and Shaker directly interact with the PDZ domains of Dlg. Finally, we use transient expression

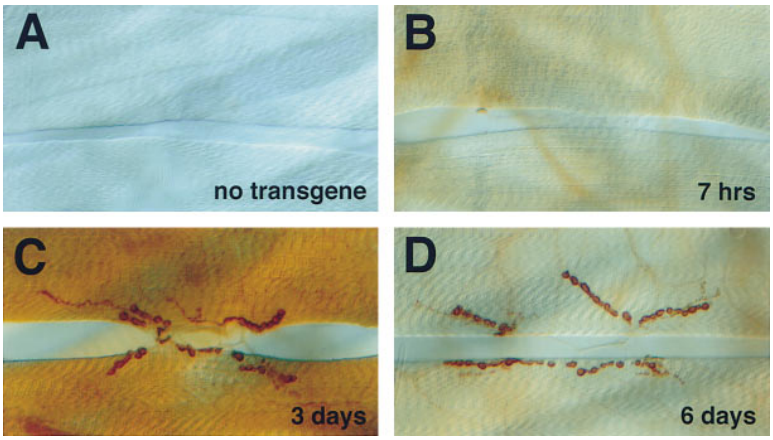

Figure 7. Uniform Muscle Membrane Expression Is Followed by Concentration at the Synapse

A heat shock promoter driving GAL4 expression was used to promote a pulse of CD8–Shaker expression during different stages of larval development. Animals were heat shocked for a total of 4 hr at 37°C and then were allowed to develop at 18°C. The expression pattern in third instar larvae was monitored at specific time points after heat shock by CD8 immunocytochemistry at a 1:100 antibody dilution.

(A) Control animals 7 hr after heat shock show no CD8 immunoreactivity.

(B) Seven hours after heat shock, CD8–Shaker is uniformly distributed over the muscle membrane, and no bouton staining is observed.

(C) Three days after heat shock, CD8–Shaker is present at high levels on the muscle membrane and at the synaptic boutons.

(D) Six days after heat shock, membrane staining is much lower, whereas staining at the synaptic boutons remains at high levels.

studies to show that these proteins first distribute uniformly on the muscle membrane and then concentrate at the synapse over time.

PDZ Interactions Direct Synaptic Localization

The ability to direct an unlocalized protein to the synapse positively identifies the C termini of Fas II and Shaker as synaptic-targeting signals. Aside from the terminal PDZ-interaction motif (-S/T-X-V), the C-terminal sequences of Fas II and Shaker are not related. We therefore propose that the C-terminal PDZ-interaction domain directs synaptic localization of Fas II and Shaker. Indeed, we have shown that alteration of two amino acids in the PDZ-interaction motif completely eliminated targeting in both cases, demonstrating that the PDZ-interaction domain is necessary for synaptic localization. Previous studies have shown that the absence of the C-terminal sequences of Shaker in Sh^{102} mutants results in no detectable Shaker protein at the NMJ (Tejedor et al., 1997). However, in addition to the absence of the entire C-terminal region, these mutants are missing the pore region and the final transmembrane domain. Here, we demonstrate that the three amino acid PDZ-interaction consensus motif is absolutely required for the synaptic localization of these proteins.

We then show that the final 11 amino acids of Shaker and Fas II are sufficient to direct synaptic localization in vivo in third instar larvae. Thus, we provide further evidence that the C-terminal PDZ-interaction domain directs synaptic localization of Fas II and Shaker. However, the synaptic localization of the 11aa chimeras is not as efficient as that directed by the entire C-terminal sequences. We conclude that the C-terminal 11 amino acids of Fas II and Shaker contain information sufficient to direct synaptic localization but that the efficiency of this process depends upon other factors. Perhaps other proteins interacting with Dlg hold it distant from the membrane and unable to reach the short, 11 amino acid tail of the CD8 chimeras. Alternatively, it is possible that additional protein interactions strengthen the synaptic localization process and that these interactions are directed by sequences outside the PDZ-interaction motif. In fact, in vitro clustering in COS7 cells of the Shaker channel by Dlg is extremely weak, observed in only 5%–10% of cotransfected cells, indicating a possible requirement for an additional factor (Tejedor et al., 1997). Whatever the role of these other portions of the targeted proteins, or of additional factors, they only function when the PDZ-interaction sequence is intact.

We have provided genetic support for a role of PDZ interactions in directing synaptic localization of Fas II and Shaker by demonstrating that localization of CD8–Fas II and CD8–Shaker is lost in dlg^{m52} mutant larvae. Instead, the proteins are diffusely and uniformly distributed across the muscle membrane. Thus, Dlg either plays an active role in recruiting Fas II and Shaker to the synapse or a passive role in stabilizing these proteins once they arrive at the subsynaptic membrane. Using the yeast two-hybrid assay, we observed that the C-terminal sequences of Fas II and Shaker directly interact with the PDZ domains 1–2 of Dlg. Therefore, CD8–Fas II and CD8–Shaker should still interact with the truncated dlg^{m52} allele, which contains the N-terminal sequences and the PDZ 1–2 domains (Woods et al., 1996). However, despite this interaction, the truncated protein is unable to direct or stabilize synaptic localization of Fas II and Shaker. This failure may be due to an inability of the truncated Dlg protein to localize to the synapse.

Synaptic Targeting by Active Clustering or Selective Retention

We considered two general models for the mechanism of synaptic localization: (1) direct synaptic targeting or (2) uniform membrane targeting followed by active clustering or selective retention at the synapse. We have shown that following a pulse of protein expression, the CD8–Shaker protein is initially distributed uniformly on the muscle membrane, followed by concentration at the synapse. These results do not support a direct synaptic-targeting model in which a vesicular-mediated synaptic-targeting event carries the proteins directly from the ER/Golgi to the subsynaptic reticulum. If this were the case, one would expect first to see dark staining at the boutons, possibly followed by a spillover onto the muscle membrane due to overexpression. We cannot rule out the possibility that a direct targeting to the synapse is more time consuming than a general targeting to the muscle membrane, and therefore if the heat shock promoter drives protein expression at much higher levels than endogenous promoters for synaptic proteins, we would observe muscle membrane distribution before bouton concentration, despite an intact direct-targeting mechanism. However, because the results obtained using the endogenous MHC promoter are consistent with the results obtained using the heat shock promoter, we feel that this is unlikely.

Instead, our results are consistent with the second model of uniform membrane targeting followed by active clustering or selective retention at the synapse. An active-clustering model would involve a mechanism to recruit proteins already positioned on the muscle membrane to the synapse. A selective-retention model would involve a process whereby proteins improperly positioned in the muscle membrane are degraded, whereas proteins at the synapse are stabilized through protein–protein interactions with synapse-specific proteins. Because Dlg is localized to the Drosophila NMJ and is required for the synaptic localization of Shaker and Fas II, we propose that interactions with Dlg either actively cluster or selectively stabilize Fas II and Shaker at the synapse.

A Role for PDZ Interactions in Synaptic Plasticity and Synapse Formation

Activity-dependent down-regulation of Fas II levels at the synapse has been shown to control structural plasticity (Schuster et al., 1996a, 1996b). In addition, the level of Shaker expression has been implicated in plasticity of the synapse (Budnik et al., 1990; Zhong et al., 1992). The presence of PDZ-interaction sequences at the C termini of Fas II and Shaker suggests a possible PDZ-dependent mechanism for regulation of their localization and expression levels. If interactions with Dlg stabilize

Fas II and Shaker at the synapse, as proposed above, one mechanism to regulate the levels of Fas II and Shaker could be through regulation of this interaction. Such a regulation has been demonstrated for the inwardly rectifying potassium channel Kir 2.3, in which phosphorylation of the −2 serine residue of its PDZ-interaction motif by cAMP-dependent protein kinase (PKA) inhibits its binding to PSD-95 (Cohen et al., 1996). A similar mechanism to regulate interactions between Dlg and Fas II or Shaker could provide an activity-dependent mechanism for regulation of the levels of these proteins at the synapse.

In what way might the localization of these proteins to the synapse be critical for their functions? For a membrane that is essentially isopotential (Jan and Jan, 1976), the positioning of a voltage-gated potassium channel might seem irrelevant. However, the colocalization of a modulatory enzyme, such as PKA, and the Shaker channel to the subsynaptic membrane could provide a rapid activity-dependent mechanism for the regulation of the channel kinetics and thus of postsynaptic excitability. In addition, due to the close apposition of the pre- and postsynaptic membranes, the potassium concentration is likely to rise substantially in the small extracellular volume during muscle activity, resulting in depolarization of the presynaptic bouton. Thus, it is possible that the potassium efflux through the densely packed postsynaptic potassium channels directly feeds back to the presynaptic membrane, retrogradely influencing presynaptic transmitter release. Finally, the synaptic localization of Shaker is critical for its proposed structural role in cross-linking large rafts of synaptic proteins via PDZ-containing proteins like Dlg (Hsueh et al., 1997).

It has been demonstrated that during the period of growth-cone exploration and synapse formation, increased levels of Fas II on the muscle can lead to the inappropriate stabilization of growth-cone contacts and the formation of ectopic synapses (Davis et al., 1997). The authors propose that localization of Fas II during normal synapse formation serves two functions. First, it increases the concentration of Fas II under certain growth cones, thereby stabilizing those contacts as they transform into presynaptic terminals. At the same time, the decrease in the concentration of Fas II over the rest of the muscle makes the muscle refractory to synapse formation by other growth cones. Thus, the Dlg-dependent localization of Fas II may play an important role in the selective stabilization of synaptic contacts and thus in the patterning of synapse formation.

The interaction of Dlg with both Fas II and Shaker implies that these proteins are linked together in a synaptic complex. The cross-linking of diverse transmembrane signaling molecules via a PDZ protein suggests a model in which the PDZ complex regulates the synapse by integrating information about synaptic structure and chemical transmission via the connection of adhesion molecules and transmitter-activated receptors or voltage-activated channels. Such a model is particularly intriguing in view of the fact that PDZ proteins have also been shown to be concentrated in the presynaptic nerve terminal (Kistner et al., 1993), where they would likely interact with presynaptic Fas II. This could provide a physical transsynaptic link between PDZ-associated proteins, permitting their interaction and coordination in the control of synaptic transmission and plasticity.

Experimental Procedures

UAS Constructs

A three-way ligation was performed with an EcoRI-AflIII fragment of the CD8 gene (amino acids 1–214; Littman et al., 1985), an EcoRI-XbaI fragment of the *UASt* vector (Brand and Perrimon, 1993), and an AflIII-XbaI fragment of the appropriate C-terminal sequences. For the control CD8 construct, two complementary oligos were synthesized to insert a stop codon directly following the AflIII site, creating a truncated CD8 protein. For the fusions with the full-length C termini of Fas II (amino acids 770–873, $PEST^+$, TM isoform; Grenningloh et al., 1991) and Shaker B (amino acids 479–656; Schwarz et al., 1988), PCR primers were designed to insert an AflIII site in frame directly preceding the intracellular C-terminal sequences and an XbaI site following the stop codon. For the C-terminal constructs that alter the final three amino acids of Fas II (SAV to AAE) and of Shaker (TDV to VTD), the XbaI primer was modified to insert the altered sequences followed by a stop codon. The CD8–Shaker VTD construct was amplified using the Shaker H4 cDNA (Kamb et al., 1987), which contains three additional changes that are naturally occurring polymorphisms (insertion of a valine and deletion of one or addition of two glutamines in the polyglutamine regions). However, these amino acids are not the cause of the disrupted localization in the VTD construct because CD8–Shaker constructs containing the ShH4 C terminus are fully targeted to the synapse (data not shown). To construct the 11aa CD8 chimeras, complementary oligos were designed to form an AflIII-XbaI fragment containing the final 11 amino acids of Shaker or Fas II followed by a stop codon.

Genetic Stocks

At least two independent transformants were tested for all experiments reported. The *UAS* insert lines that were used for the majority of experiments are as follows: CD8 (*33E*), CD8–Shaker (*42D*), CD8–Fas II (*C8A*), CD8–Shaker VTD (*8Z*), CD8–Fas II AAE (*59B*), CD8–Shaker 11aa (*12G*), and CD8–Fas II 11aa (*26–2A*). The dlg^{m52} stock was obtained from Dan Woods and Peter Bryant (University of California, Irvine). MHC^{82}-*GAL4* is a homozygous viable line containing a myosin heavy-chain promoter fused to the open reading frame of *GAL4* (provided by Margaret Winberg, University of California, Berkeley), which drives GAL4 expression in all muscles beginning in the first larval instar. *24B-GAL4* is a GAL4 enhancer-trap line expressing GAL4 in all embryonic and somatic muscles (Luo et al., 1994). The heat shock *GAL4* line is a homozygous lethal second chromosome insert containing the *hsp70* promoter fused to *GAL4* (provided by Andrea Brand).

Larval Culture, Dissection, and Immunocytochemistry

Larval dissections and immunostainings were performed as described previously (Lin et al., 1994). The monoclonal antibody against CD8 (OKT8) was generously provided by Tom Livelli and used at the indicated dilution. The serum antibody against CD2 was obtained from Harlan Bioproducts for Science, Incorporated. The serum antibody directed against synaptotagmin (Syt, provided by Troy Littleton and Hugo Bellen) was used at a final dilution of 1:2000. The monoclonal antibody against Fas II (MAb 1D4; Gregg Helt and C. S. G., unpublished data) was used at a dilution of 1:5. HRP-conjugated secondary antibodies were used at 1:400. FITC-conjugated goat anti-mouse and rhodamine-conjugated donkey anti-rabbit antibodies were used at a dilution of 1:1000.

Confocal Quantitation

Experimental and control animals were stained under identical conditions with the OKT8 monoclonal against CD8 (1:1000) and rabbit anti-synaptotagmin at 1:2000. Fluorescent secondary antibodies were used as described above. All images were collected on a Leica TCS NT confocal microscope at identical settings for laser power, photomultiplier gain, and offset. Settings were chosen for these parameters so that the pixel densities for the brightest samples were just below saturation levels. For CD8–Shaker and CD8–Fas II,

a single image of each synapse was taken. For the CD8–Sh and CD8–Fas II 11aa chimeras and for CD8 alone, a z series (eight sections each averaged eight times) that included the brightest bouton and muscle-membrane staining was projected to a single image using the maximum-intensity method. Using the NIH Image software, boutons were outlined, and mean pixel density was measured for the included area as well as for a similar area on the muscle surface. Background pixel density was obtained by measuring animals stained in identical conditions that were not expressing the CD8 transgene. Background pixel density was subtracted from the pixel density values obtained for bouton and muscle. The bouton/muscle ratio was then determined by dividing the mean pixel density at the boutons by the mean pixel density at the muscle surface. The bouton/muscle ratio for each chimera was then divided by the bouton/muscle ratio for CD8 alone to obtain the targeting efficiency. A minimum of eight hemisegments was analyzed for each chimera, corresponding to no less than 400 boutons.

Immunoelectron Microscopy

Larvae were prepared for electron microscopy according to procedures previously described (Lin et al., 1994), with the following modifications. Hydrogen peroxide was used instead of glucose oxidase for the HRP reaction. Larvae were incubated sequentially with MAb OKT8 against CD8 (1:10) for 2 hr, biotinylated goat anti-mouse secondary antibody (1:100) for 1–2 hr, and then streptavidin-conjugated HRP (1:100) for 1–2 hr. The HRP development time was 2 min instead of the standard 10 min. This HRP reaction time was chosen to be within the linear range for reaction product generation based upon a time course of membrane staining for the CD8 control larvae.

Yeast Two-Hybrid Protein Interaction Assay

The sequences for the C-terminal 11 amino acids of Shaker and Fasciclin II, with or without the disrupted PDZ-interaction domain, were synthesized as complementary oligonucleotides and fused to the GAL4 DNA binding domain in the *pAS2–1* plasmid (Clontech). To disrupt the PDZ-interaction domain, the final three amino acids were altered from TDV to VTD for Shaker and from SAV to AAE for Fas II. The sequences for two or all three PDZ domains of Dlg (amino acids 27–277 for PDZ 1–2, amino acids 27–593 for PDZ 1–3; Woods and Bryant, 1991) were PCR-amplified and directly fused with the GAL4 activation domain in the *pACT2* plasmid (Clontech). Combinations of experimental and control plasmids containing the GAL4 DNA binding and activation domains were transformed into the yeast strain *Y187* (Clontech). β-galactosidase activity was determined by an X-Gal filter lift assay at 30°C.

Heat Shock Experiments

The CD8–Shaker line was crossed to *hsGAL4/CyO* and flipped daily to generate a set of vials with larvae at different developmental stages. The vials were then exposed to 37°C for a period of 1 hr followed by incubation at 18°C for 30 min or 1 hr. This heat shock cycle was repeated three times, for a total exposure of 3–4 hr. The larvae were then allowed to develop at 18°C. Third instar larvae were dissected from the appropriately staged vial 7 hr after the final heat shock, after 3 days, and after 6 days and stained for CD8 immunoreactivity.

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