Erratum

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The present address of **Masaki Sone** is Kyoto University, Japan, and **Chihiro Hama** is the corresponding author (hama@ncnp.go.jp)

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Synaptic development is controlled in the periactive zones of *Drosophila* synapses

Masaki Sone[1,*], Emiko Suzuki[2,3], Mikio Hoshino[4], Dongmei Hou[5], Hiroshi Kuromi[5], Masaki Fukata[6,7], Shinya Kuroda[6], Kozo Kaibuchi[6], Yo-ichi Nabeshima[4] and Chihiro Hama[1,2,‡]

[1]Department of Molecular Genetics, National Institute of Neuroscience, NCNP, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan
[2]Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, Japan
[3]Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan
[4]Kyoto University School of Medicine, Kyoto 606-8501, Japan
[5]Gunma University School of Medicine, Maebashi 371-8511, Japan
[6]Nara Institute of Science and Technology, Ikoma 630-0101, Japan
[7]Hiroshima University School of Medicine, Hiroshima 734-8551, Japan
*Present address: Kyoto University School of Medicine, Kyoto 606-8501, Japan
‡Author for correspondence (e-mail: hama@ncnp.go.jp)

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SUMMARY

A cell-adhesion molecule fasciclin 2 (FAS2), which is required for synaptic growth and still life (SIF), an activator of RAC, were found to localize in the surrounding region of the active zone, defining the periactive zone in *Drosophila* neuromuscular synapses. βPS integrin and discs large (DLG), both involved in synaptic development, also decorated the zone. However, shibire (SHI), the *Drosophila* dynamin that regulates endocytosis, was found in the distinct region. Mutant analyses showed that *sif* genetically interacted with *Fas2* in synaptic growth and that the proper localization of SIF required FAS2, suggesting that they are components in related signaling pathways that locally function in the periactive zones. We propose that neurotransmission and synaptic growth are primarily regulated in segregated subcellular spaces, active zones and periactive zones, respectively.

Key words: Periactive zone, Synapse, SIF, RAC, Cell adhesion molecule, *Drosophila*

INTRODUCTION

Increasing evidence suggests that the cellular plasma membrane is organized into multiple domains responsible for distinct biological functions (Fanning and Anderson, 1999). These domains are characterized by the presence of specific proteins that are, in many cases, assembled to form protein complexes (Tsunoda et al., 1997; Xu et al., 1998; Nagano et al., 1998). These complexes efficiently mediate a signal transduction to locally control particular cellular functions. Therefore, extensive analyses of the molecular anatomy on the plasma membrane would provide clues to reveal the presence or organization of functional domains.

Among a variety of differentiated cells, neurons are highly polarized and their membrane surfaces comprise several morphologically, biochemically and functionally different domains. It has been suggested that the somatodendritic and axonal surfaces of neurons correspond to the basolateral and apical domains of epithelial cells, respectively (Dotti and Simons, 1990). However, the neuronal plasma membrane appears to be further specialized into a complex of functional domains, because the behavior of some neuronal proteins do not reflect this analogy (Winckler and Mellman, 1999).

One of the most functionally and structurally complex apparatus in the neurons is possibly the synaptic structure. The presynaptic terminal membrane contains the active zone for neurotransmission, which is identified as an electron-dense region where a number of proteins involved in the exocytosis of synaptic vesicles are assembled (Bajjalieh, 1999). Following exocytosis in the active zone, the fused vesicle membranes are recycled through endocytosis, which is regulated by dynamin (Betz and Angleson, 1998; Cremona and DeCamilli, 1997). The membrane domain abundant in SHI, *Drosophila* dynamin, has been identified as a possible region for endocytosis in the *Drosophila* neuromuscular junctions (NMJs) (Estes et al., 1996; Roos and Kelly, 1999). Thus, the presynaptic terminals are organized into at least two functional domains for exocytosis and endocytosis. While these domains execute vesicle cycling for neurotransmission, the shape of the synaptic terminals dramatically changes during development and possibly even at the mature stages (Sanes and Lichtman, 1999; Keshishian et al., 1996). Therefore, the synaptic membrane should be also involved in the structural control. However,

several fundamental questions remain to be answered. Is there any specific membrane domain for synaptogenesis in the synaptic terminal? Where are the signaling molecules involved in synaptogenesis localized? Are they localized in or outside the active zone?

In *Drosophila* NMJs, several cell-surface or intracellular signaling molecules have been identified as factors regulating synaptic development. For example, FAS2, an NCAM-like neural cell-adhesion molecule with immunoglobulin domains (Grenningloh et al., 1991), is localized both presynaptically and postsynaptically, and plays an important role in synaptic growth or maintenance. In *Fas2* null mutants, synapses form and mature at the first stage, but then fail to grow and eventually retract (Schuster et al., 1996a). FAS2 is also implicated in synaptic structural plasticity (Schuster et al., 1996b). Another class of cell-adhesion molecules involved in synaptic development are the PS integrins. In the loss-of-function and gain-of-function mutants of βPS integrin, the growth of larval NMJ is altered (Beumer et al., 1999). Furthermore, the localization of FAS2 to the synaptic structures is regulated by a multiple PDZ-domain containing protein, DLG. This protein is closely related to the mammalian PSD-95 (Dlgh4 – Mouse Genome Informatics) and serves as a scaffold protein that tethers FAS2 and Shaker K^+ channel to the synaptic site by directly binding to the cytoplasmic tails of these proteins (Thomas et al., 1997; Zito et al., 1997; Tejedor et al., 1997). DLG is involved in the structural development of both presynapses and postsynapses (Thomas et al., 1997; Lahey et al., 1994).

We previously identified the *sif* gene, which was suggested to control synaptic development. SIF is a putative guanine-nucleotide exchange factor (GEF) for RHO family small G proteins. *sif* mutants exhibit behavioral phenotypes that include sluggishness, which can be rescued by expression of *sif* in neurons. The neuronal localization of SIF is confined to the synaptic terminals. In addition, an activated form of SIF can induce alterations in cellular morphology (Sone et al., 1997; unpublished). These data suggest that SIF functions in the process of formation or maintenance of synapses by activating RHO family G proteins that regulate structural changes in nerve terminals (Luo et al., 1994, 1996; Threadgill et al., 1997; Kozma et al, 1997; Lamoureux et al., 1997; Gallo and Letourneau, 1998). Moreover, immunoelectron microscopic observations revealed that the SIF localization in the presynaptic terminals of the NMJs and neuron-neuron synapses in the brain is specifically detected at the lateral regions of the active zones (Sone et al., 1997). This finding tempted us to hypothesize that the lateral region of the active zone may be a specific region that controls synaptic development.

In this paper, we have attempted to identify the synaptic domain responsible for the control of synaptic structures in the *Drosophila* glutamatergic NMJs. Our data indicate that the molecules involved in synaptic development are localized at a specific domain, periactive zone, which surrounds the active zone on the synaptic plasma membrane. This domain was characterized by the presence of SIF and FAS2, and was also found to be decorated with βPS integrin and DLG. The dynamin-rich domain was distinct from the periactive zone. Mutant phenotypes of all these genes exhibit alterations in the structural properties of the synaptic terminals (Schuster et al., 1996a; Beumer et al., 1999; Thomas et al., 1997; Lahey et al., 1994; this study). Our genetic data show that *sif* interacted with *Fas2* in the control of synaptic growth, and that the proper localization of SIF requires FAS2. Finally, SIF acts as a GEF for RAC, a member of RHO family G protein. These data indicate that FAS2, SIF and RAC are components in related signaling pathways that control synaptic development in the periactive zones. We propose that the active and periactive zones together constitute a major part of a structural unit of the presynaptic terminal and that neurotransmission and synaptic development are basically segregated into the two distinct zones, respectively.

MATERIALS AND METHODS

Immunohistochemistry

Canton-S was used as a wild type. Wandering third instar larvae were dissected in PBS and fixed in 4% paraformaldehyde, 0.5 mM EGTA in PBS for 20 minutes. The fixed larvae were washed several times with PBS, blocked in 5% skimmed milk in PBT (PBS + 0.2% Triton X-100) for 30 minutes and followed by overnight incubation at 4°C with antibodies in the blocking buffer. Antibodies used in this study were rat AbI3 against SIF (diluted 1:2; Sone et al., 1997), rabbit antiserum against DPAK (1:500), mouse MAb1D4 against FAS2 (1:2), mouse MAb6G11 against βPS integrin (1:10), guinea pig antiserum against DLG (1:1000; Hough et al., 1997) and rabbit antiserum against SHI (1:200; Estes et al., 1996). They were visualized with FITC- or Texas Red-conjugated secondary antibodies (Jackson, USA), mounted in Vectashield (Vector Labs) and observed with Leica TCS NT or TCS SP laser-scanning microscopes. We always used boutons on muscle 6 and 7 for the observation.

Immunoelectron microscopy

For immunoelectron microscopy, larvae were dissected in PBS and fixed in PLP fixative (4% paraformaldehyde, 0.075 M lysine, 0.01 M $NaIO_4$, 0.0375 M Na phosphate buffer, pH 6.2) for 1.5 hours. The fixed larvae were washed several times with PBS, treated with 50 mM glycine in PBS for 5 minutes, blocked in 5% skim milk in PBSS (PBS + 0.1% saponin) and followed by overnight incubation at 4°C with antibodies in the blocking buffer. After the larvae were washed three times for 20 minutes each in PBS, they were incubated with secondary antibody (nanogold anti IgG Fab', Nanoprobe, USA) for 1 hour. For anti-SIF antibody, biotin-conjugated anti-rat IgG antibody (Vector Labs, USA) and nanogold-conjugated streptavidin (Nanoprobe, USA) were used instead of nanogold-conjugated secondary antibody. After three 20-minute washes, larvae were fixed in 1% glutaraldehyde in PBS for 10 minutes. They were washed three times with water and treated with HQ silver (Nanoprobe, USA). The specimens were processed for electron microscopy using a standard technique as previously described (Suzuki and Hirosawa, 1994).

Statistical analyses of SIF distribution in NMJ were performed as follows. The medial portion of the electron-dense region was defined as an area farther than 100 nm from both edges of the region in a section of a bouton. Only sections that had more than 15 grains of SIF signal were analyzed. SIF signals were counted in a blind manner. The total number of larvae examined was two or three for each genotype.

Mutagenesis

Multi marker chromosomes (ru^1 h^1 th^1 st^1 cu^1 sr^1 e^s ca^1) were mutagenized with ethyl methanesulfonic acid (Sigma) as previously described (Grigliatti, 1986). 8921 mutagenized chromosomes were screened for sluggishness in the next generation after being crossed to $sif^{98.1}$. 13 new *sif* alleles were isolated.

Western blot analysis

Western blot analysis was performed essentially as previously described (Harden et al., 1996). Five adult heads were homogenized in SDS sample buffer (3% SDS, 100 mM Tris (pH8.0), 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 100 mM dithiothreitol, 1% Triton X-100, 10 mM $MgCl_2$, 100 mM NaCl, 50 μg/ml aprotinin). The samples were boiled for 5 minutes, centrifuged to remove cell debris, and electrophoresed on a SDS 5-10% gradient polyacrylamide gel. The gel was electroblotted onto a Hybond ECL membrane (Amersham). The membrane was blocked in PBTw (PBS + 0.1% Tween 20) containing 1.5% skimmed milk and 1.5% bovine serum albumin for 1 hour, and incubated with mouse monoclonal anti-SIF antibody, MAbH24.8.2 (diluted 1:250), which was raised to the fusion protein consisting of a portion of SIF protein (amino acids 1080 to 1269) and metal-binding domain derived from pTrcHisA (Invitrogen). After three 15-minute washes with PBTw, the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (Jackson) for 1 hour. After three washes, the signal was detected with the ECL system (Amersham).

Counting the bouton number

First instar larvae were collected and reared in a plastic vial. To avoid crowded conditions, care was taken that each vial contained less than 100 larvae. Wandering third instar larvae were dissected and synaptic boutons were visualized by anti-HRP antibody (Cappel, USA). The synaptic bouton number on muscles 6 and 7 in one of the A4 hemisegments was counted in a blind manner for the genotype. Only muscles with no ectopic innervation were used for counting.

Overexpression of *sif*

UAS-SIF transgene was constructed by introducing the full-length type 1 *sif* cDNA into the downstream of the UAS promoter of pUAST (Brand and Perrimon, 1993). The transgenic strain *P[UAS-SIF]F11-1* was used in combination with the *P[elav-Gal4]* strain (Luo et al., 1994).

Electrophysiology

Membrane potentials were recorded from muscle 6 in abdominal segment A3 in HL3 medium (70 mM NaCl, 115 mM sucrose, 5 mM KCl, 20 mM $MgCl_2$, 0.7 mM $CaCl_2$, 10 mM $NaHCO_3$, 5 mM trehalose, 5 mM HEPES (pH 7.3)), using glass microelectrodes filled with 4 M potassium acetate. To elicit evoked junctional potentials (EJPs), pulses (1 ms in duration and 2× threshold voltage intensity) were delivered to an appropriate segmental nerve via a glass suction electrode at a frequency of 0.1 Hz. Miniature junctional potentials (MJPs) were recorded for 1 minute, and the mean frequency and amplitude were calculated. The ratio of mean EJP amplitude to mean MJP amplitude (corrected for nonlinear summation using a reversal potential of 0 mV; Martin, 1955) was used as an estimate of the quantal content.

GEF assay

The 1.2 kb DNA fragment encoding the DH-PHc domain of SIF (amino acids 1404-1812) was subcloned into pGEX-4T-1 vector (Amersham). Expression and purification of recombinant proteins and GEF assays were performed as previously described (Hoshino et al., 1999).

RESULTS

Identification of anti-DPAK antibody as a dense-region marker

The *Drosophila* NMJs on the body-wall muscles of the third instar larvae have been used as a good model system to study synapse formation (Keshishian et al., 1996). The motor nerves innervate the muscles in highly stereotyped patterns, and their endings form synaptic contacts with muscles in the swelling structures called synaptic boutons or varicosities, each containing multiple active zones for neurotransmission (Atwood et al., 1993; Jia et al., 1993). To investigate whether synaptic terminals can be further dissected into functional domains, we started to screen antibodies that label specific regions along the plasma membrane in a single bouton.

The antibodies for the synaptic vesicle proteins like synaptotagmin and cysteine string protein have been used to stain vesicle-clustering regions (Estes et al., 1996), but their staining signals are diffused throughout the boutons and not largely associated with the plasma membrane, possibly because the nerve terminals are filled with synaptic vesicles. We then searched for other synaptic markers and found that the antibody raised against the *Drosophila* serine/threonine kinase, DPAK protein (Harden et al., 1996), stained synaptic boutons of NMJs in a 'dotted' manner (Fig. 1A). Using an electron microscope, we found that anti-DPAK antibody clearly labeled the postsynaptic side of the electron-dense regions that contain the active zones for neurotransmission (Atwood et al., 1993; Jia et al., 1993; Fig. 1B). From now on, we shall refer to the electron-dense region as the active zone, although it is not known whether exocytosis for neurotransmission occurs in the whole dense region. We then took advantage of the anti-DPAK antibody as a marker of the active zone to investigate molecular anatomy of the synaptic terminals further.

SIF and FAS2 are localized at the periactive zone

The SIF protein is a putative GEF that converts RHO-like small G proteins from the GDP-bound inactive state to the GTP-bound active state. Given that the small G proteins are widely distributed in a variety of tissues and regulate multiple cellular functions, one can hypothesize that their activation is spatiotemporally controlled by their regulatory factors, such as

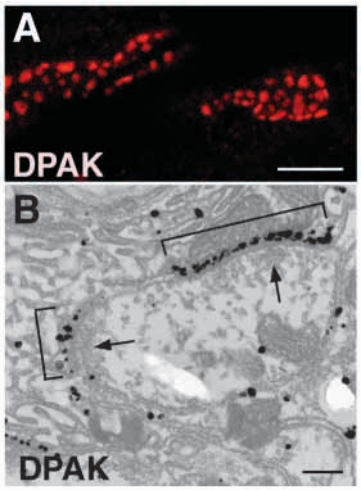

Fig. 1. Anti-DPAK antibody labels the dense regions of the neuromuscular synapses. (A) Anti-DPAK antibody stained synaptic boutons of larval neuromuscular synapses in a dotted pattern. (B) Immunoelectron micrograph of the neuromuscular synapse stained by anti-DPAK antibody. Postsynaptic sides of the electron-dense regions (brackets) are marked. The T-bar structures (arrows) that mark the position of active zones are observed in these dense regions. Scale bars: 1 μm in A; 200 nm in B.

GEFs. It is therefore important to examine the regions where the GEFs are localized at the subcellular level. Our previous study has shown that SIF is confined to the synaptic terminals in the neurons. Immunoelectron microscopic analyses have further revealed that SIF is localized in the restricted regions of presynapses near the plasma membrane. The localization was often observed on the lateral sides of the active zones for neurotransmission (Sone et al., 1997).

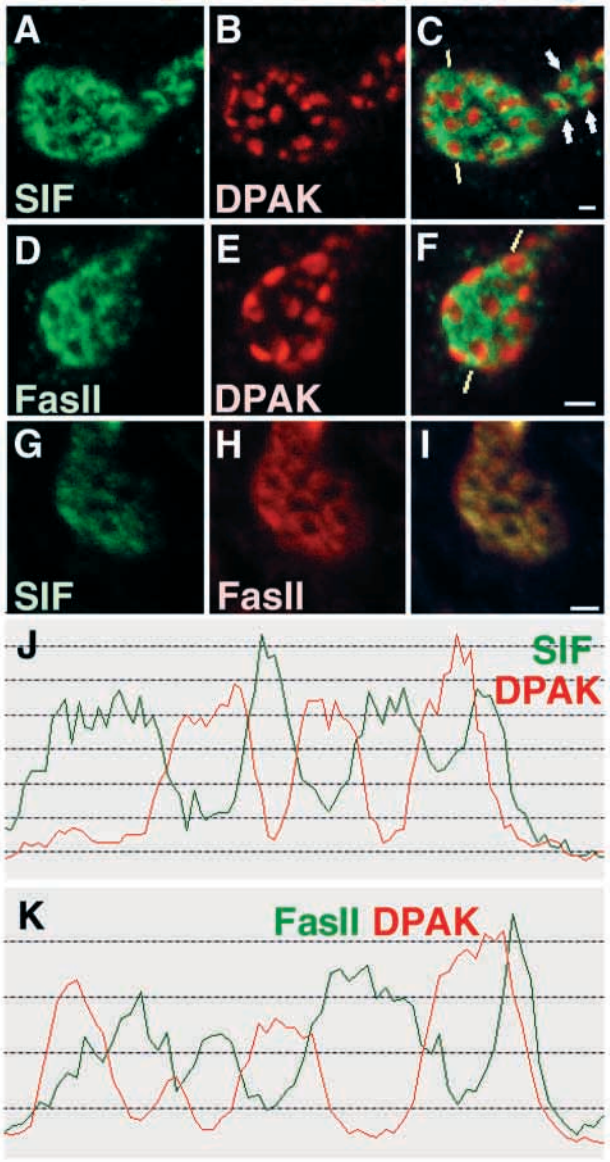

Fig. 2. The localization of SIF and FAS2 (FasII) defines the periactive zone. (A-C) Double staining of NMJs with anti-SIF (green) and anti-DPAK (red) antibodies. These staining patterns are mostly complementary to each other and are locally arranged in concentric figures (arrows). (D-F) Anti-FAS2 (green) and anti-DPAK (red) antibodies produced complementary patterns. (G-I) Anti-SIF (green) and anti-FAS2 (red) antibodies exhibit overlapping network-like patterns. (J) Fluorescent intensity profile of the SIF-DPAK co-staining pattern on the cross section indicated by the yellow line in (C). SIF (green) and DPAK (red) were found to label the synaptic bouton in a mostly complementary manner. (K) Fluorescent intensity profile of the FAS2-DPAK co-staining pattern on the cross section indicated by the yellow line in (F). FAS2 (green) and DPAK (red) were again found to stain in a mostly complementary pattern. Scale bars: 1 μm.

To characterize this SIF localization pattern better, particularly in reference to the functional domains, we further examined the subcellular distribution of SIF in the boutons of larval neuromuscular junctions by concomitant staining with anti-DPAK antibody using laser-scanning confocal microscopy. We found that anti-SIF antibody labeled the synaptic boutons in a network-like pattern (Fig. 2A), which was strikingly complementary with DPAK staining in the boutons (Fig. 2A-C). The cross-section profile of the fluorescent intensity (Fig. 2C, yellow lines; Fig. 2J) also showed that the staining patterns of SIF and DPAK are mostly complementary to each other. These staining patterns demonstrated that the areas stained for SIF surround the active zones. Close examinations further revealed that anti-SIF and anti-DPAK antibodies produced a number of concentric figures that were occasionally separated from each other (Fig. 2C, arrows). Fig. 2C is an accumulated image of several focal planes panning the surface of the bouton, and therefore the concentric figures appear to be continually separated at some depth from the surface. These data suggest that the active zone and the outer ring together form a structural unit that constitutes a synapse. We hereafter refer to the SIF-positive regions around the active zones as periactive zones.

To characterize the periactive zone, especially in identifying its functional significance, we investigated the distribution patterns of other molecules with the aid of DPAK staining. Monoclonal antibody, MAb1D4, against FAS2 (Van Vactor et al., 1993) labeled the boutons in a complementary pattern with DPAK staining. FAS2 staining surrounded the DPAK-positive regions and formed concentric patterns as observed for SIF staining (Fig. 2D-F). The cross-section profile also showed similar patterns as SIF and DPAK double staining (Fig. 2F, yellow lines; Fig. 2K). SIF and FAS2 were indeed co-localized in overlapping network-like patterns (Fig. 2G-I). FAS2 is involved in synaptic growth, stabilization and structural plasticity, possibly through its homophilic adhesion (Schuster et al., 1996a,b). These data suggest that FAS2 controls these synaptic events locally in the periactive zones. Thus, the periactive zone was characterized by the specific localization of two distinct types of molecules: a cell adhesion molecule (FAS2) that controls synaptic development and an intracellular molecule (SIF) that is a GEF to RAC (see later).

PS integrin and DLG also decorate the periactive zone

In an attempt to understand the function of the periactive zone further, we continued the search for molecular markers that stained the zone. The monoclonal antibody, MAb6G11, against βPS integrin that is structurally similar to vertebrate integrin β1 subunit (Brower et al., 1984; Gotwals et al., 1994) also showed staining that was complementary to DPAK staining (Fig. 3A-C). Unlike SIF and FAS2, however, MAb6G11 staining was observed much more diffusely on the muscle surfaces surrounding the outside of the bouton, suggesting the staining in the postsynaptic specialization, subsynaptic reticulum. In the mutants of the *mys* gene that encodes βPS integrin, the extent of the cell contact between nerve terminals and muscles is altered by either a primary or secondary effect

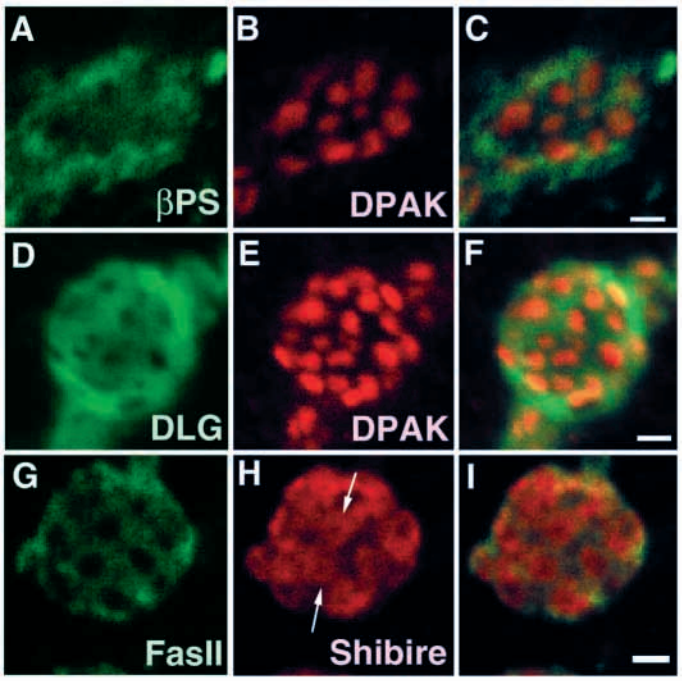

Fig. 3. Further characterization of the periactive zone. (A-C) Anti βPS integrin (green) and anti-DPAK (red) antibodies decorated a bouton in a complementary manner. The distribution of βPS integrin appears to largely extend to the postsynapses since the region stained with βPS integrin is much wider than the bouton size demarcated by the distribution of DPAK staining. This suggests the presence of βPS integrin in the subsynaptic reticulum. (D-F) Anti DLG (green) and anti-DPAK (red) antibodies also stained a synaptic bouton in a complementary manner. DLG moderately diffuses in the postsynapse. Compare this with other staining patterns for βPS integrin(A-C), SIF (Fig. 2A-C) and FAS2 (Figs 2D-F) relative to the DPAK patterns. (G-I) Double staining of the synaptic bouton with anti-FAS2 (green) and anti-shibire (red) antibodies. shibire staining, which indicates the active site for the vesicular endocytosis, showed donut-like patterns (H, arrows). But the shibire donuts were located mostly within the holes of the FAS2 rings. Scale bars: 1 μm.

of the mutation (Prokop et al., 1998), and the growth of larval neuromuscular synapses is affected (Beumer et al., 1999). These synaptic defects observed in the βPS integrin mutants may represent its function in the periactive zones.

The polyclonal antibody against DLG protein stained synaptic boutons in a similar way to MAb6G11. The DLG staining also appeared to be moderately diffused on the muscle surfaces surrounding the bouton. This pattern was again found to be complementary with the anti-DPAK staining when we scanned the bouton at the surface level (Fig. 3D-F). In *dlg* mutants, the structural properties of synapses, including the formation of subsynaptic reticulum at the postsynapses and the number of active zones at the presynapses, are altered (Lahey et al., 1994; Thomas et al., 1997). Furthermore DLG regulates the synaptic localization of FAS2 by binding directly to the cytoplasmic tail of FAS2 (Thomas et al., 1997; Zito et al., 1997). Therefore, one of the roles for DLG in synaptic development is probably the localization of FAS2 to the periactive zone.

These observations indicate that two additional molecules, βPS integrin and DLG, are present in synaptic areas including the peri-active zones. They are both involved in the structural development of the neuromuscular synapses, and therefore appear to participate in the control of synaptic development in the periactive zones.

The periactive zone is distinct from the dynamin-rich domain

It has been suggested that a dynamin-rich domain in the presynaptic terminal functions as a site for the vesicular endocytosis (Estes et al., 1996). A recent study has also indicated that this domain is distinct from the active zone for exocytosis and instead surrounds the active zone (Roos and Kelly, 1999). We were interested in whether the periactive zone is involved in endocytosis and therefore examined the spatial relationships between the dynamin-rich domain and the periactive zone. Synaptic boutons were co-stained with anti-FAS2 antibody and a polyclonal antibody against the SHI protein that is the *Drosophila* homolog of dynamin (Chen et al., 1991; van der Bliek and Meyerowitz, 1991). SHI is an essential factor for endocytosis, as endocytosis is completely blocked in the *shi* mutant (Kosaka and Ikeda, 1983; Estes et al., 1996). Anti-SHI antibody stained synaptic boutons in donut-like patterns but these patterns were found almost within the holes of the FAS2 rings (Fig. 3G-I). This SHI staining may partly overlap with FAS2 staining, but these staining patterns were distinct from each other. Thus, we conclude that the periactive zone does not coincide with the dynamin-rich zone and therefore does not likely represent a functional domain for endocytosis.

Fas2 and *sif* genetically interact in synapse formation

Our data show that the molecules involved in the synaptic development are present in the periactive zone. This implies that the periactive zone is the membrane domain that is specialized for the control of synaptic development. To test this possibility, we analyzed the NMJ phenotype of the *sif* mutants. As the originally identified *sif* mutation was a hypomorphic allele (data not shown), we further tried to isolate stronger *sif* mutant alleles induced with a chemical mutagen, ethyl methanesulfonic acid. Among the alleles newly isolated in this study, one allele, sif^{ES11}, was possibly functionally null, because the wild-type protein of more than 200 kDa disappeared in the mutant flies, as revealed by a western blot (Fig. 4A). Sequence analyses of the cDNAs indicated that the allele produced a truncated protein as a result of a frameshift mutation (Fig. 4B). The truncated protein lacks the Dbl-homology (DH) domain that catalyzes the GEF activity (Whitehead et al., 1997). The mutant flies exhibited reduced locomotor activity as observed for the original *sif* allele. In the mutant larvae, the synaptic bouton number of NMJ was slightly but significantly reduced when compared with the control larvae (Fig. 4C, left panel; $P<0.05$). In these experiments, the data of the bouton number were normalized by the muscle area as the synaptic growth often correlates with the muscle surface area (Schuster et al., 1996a). Moreover, the reduced bouton number found in the mutant was rescued by expression of a *sif* minigene in neurons under the control of the *elav* promoter (Sone et al., 1997). These results suggest that *sif* functions in the regulation of

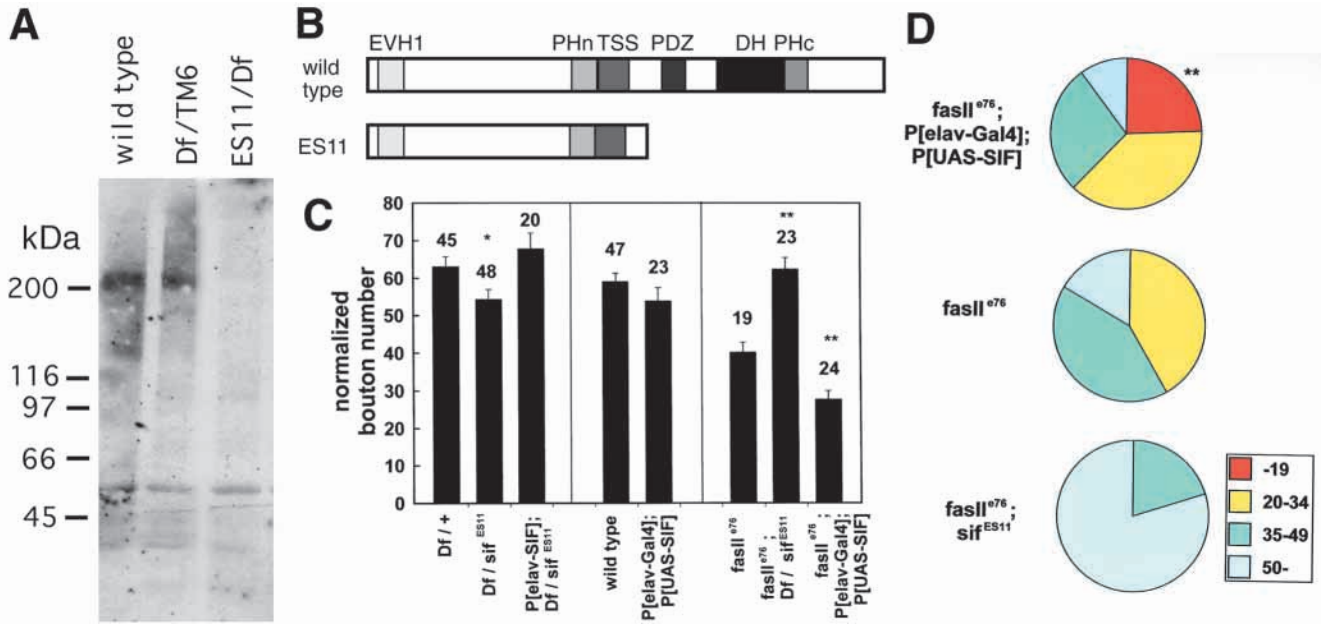

Fig. 4. Characterization of the *sif* null mutant and the genetic interaction between *sif* and *Fas2*. (A) Western blot analysis of sif^{ES11} mutant probed with anti-SIF antibody. SIF protein detected at over 200 kDa disappeared in sif^{ES11}*/Df(3L)64DF*. *Df(3L)64DF* completely lacks the *sif* locus. TM6 is a balancer chromosome that carries the wild-type *sif* allele. (B) Predicted structure of the truncated SIF protein produced by sif^{ES11} allele. The wild-type SIF protein contains an Ena/Vasp-homology domain 1 (EVH1; Callebaut et al., 1998), an N-terminal PH domain (PHn), a TSS domain (Hoshino et al., 1999), a PDZ domain, a DH domain and a C-terminal PH domain (PHc). In sif^{ES11}, an 11 bp sequence (CCTTTTTCCAG) was inserted after nucleotide 3502, which caused a frameshift mutation. The truncated gene product contains amino acids 1-1112 and four additional amino acids, and only retains EVH1, PHn and TSS domains. (C) Average bouton numbers of NMJs on the muscle 6 and 7 of the larval A4 segment. The bouton number was normalized by the muscle surface area. The average muscle area of the wild type was used as a standard. (left) The bouton number was slightly but significantly reduced in sif^{ES11} */ Df(3L)64DF* when compared with the control larvae, *Df(3L)64DF/+* (P<0.05). + represents the original genotype of the chromosome used to generate the *sif* alleles. The reduced bouton number was rescued by expression of a *sif* minigene in neurons (*P[elav-sif]; Df(3L)64DF /* sif^{ES11}; P<0.01, compared with *Df(3L)64DF/*sif^{ES11}). (middle) No significant difference was observed between the wild-type and the transgenic animal overexpressing *sif* in neurons (*P[elav-Gal4]; P[UAS-SIF]*; P=0.23). (right) In $Fas2^{e76}$ larvae, the bouton number decreased by 34% compared with the wild type (P<0.001). But in the $Fas2^{e76}sif^{ES11}$ double mutants, the bouton number was restored to the wild-type level (P<0.001, compared with $Fas2^{e76}$). When SIF was overexpressed in $Fas2^{e76}$ (*$Fas2^{e76}$;P[elav-Gal4]; P[UAS-SIF]*), the bouton number decreased (P<0.001; compared with $Fas2^{e76}$) and exhibited extremely reduced bouton numbers (see D). Error bars indicate s.e.m. The numbers of larvae examined are indicated above the bars. *, P<0.05; **, P<0.01, student's *t*-test, compared with the control larvae shown at the left of each panel. (D) Comparison of the distribution of the bouton number data among $Fas2^{e76}$ and *$Fas2^{e76}$;P[elav-Gal4]; P[UAS-SIF]*, and $Fas2^{e76}$; sif^{ES11}*/ Df(3L)64DF* larvae. The measured bouton numbers were sorted into four groups for simplicity, and the proportion of each group is indicated in a circle. Note that 24% of the *$Fas2^{e76}$; P[elav-Gal4]; P[UAS-SIF]* larvae showed a extremely small number (<20) of boutons (marked with **, P=0.01, chi-square test, compared with $Fas2^{e76}$).

synaptic growth. However, properties of basic synaptic transmission at the NMJs, including the frequency and amplitude of spontaneous MJPs, the amplitude of EJPs and the quantal content, were not significantly altered in the sif^{ES11} mutants (Fig. 5A-C). In addition, repetitive stimulation at 0.35 mM Ca^{2+} caused the decrease of EJPs in the mutants similarly as observed in the wild-type NMJs, and vesicle endocytosis was apparently unchanged, as estimated by the uptake of FM1-43 (data not shown). These data indicate that the lack of GEF activity of SIF results in no apparent effects on basic electrophysiological functions, which is consistent with the separate location of the active and periactive zones in the synaptic terminals.

As SIF and FAS2 are co-localized in the periactive zone, we next tested whether there is a genetic interaction between *sif* and *Fas2* loci. The hypomorphic allele of *Fas2*, $Fas2^{e76}$, shows a reduced number of boutons (Schuster et al., 1996a; Stewart et al., 1996), and we examined whether changing the dose of sif^{+} affected this bouton number phenotype. We found that the double mutant of $Fas2^{e76}$ and sif^{ES11} strikingly recovered the bouton number phenotype to the wild-type level (Fig. 4C, right panel; P<0.001), which suggests the presence of a suppressive genetic interaction between the two loci. To assess this genetic interaction further, we examined the effect of SIF overexpressed in the $Fas2^{e76}$ background. SIF overexpression did not clearly affect the synaptic bouton number in the wild-type background (Fig. 4C, middle panel; P=0.23), but caused a significant reduction in $Fas2^{e76}$ (Fig. 4C, right panel; P<0.001). Moreover, the NMJs with extremely few synaptic boutons (less than 20) were significantly observed in the $Fas2^{e76}$ larvae overexpressing SIF when compared with the NMJs in $Fas2^{e76}$ (P=0.01; Fig. 4D). Taken together, our data suggest that SIF and FAS2 are the components of related signaling pathways that control synaptic development, and SIF may, in an inhibitory manner, modulate the effect of FAS2 that regulates synaptic growth.

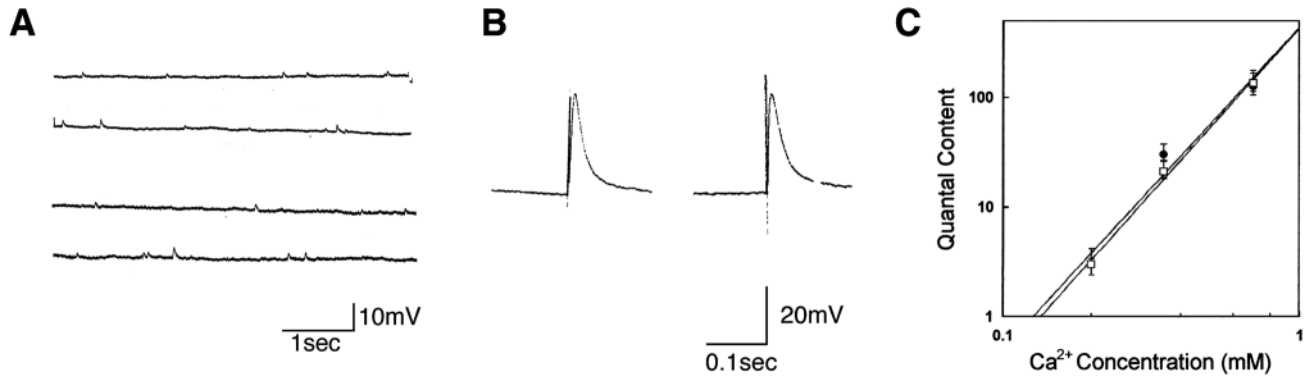

Fig. 5. Basic electrophysiological properties of *sif* mutant NMJs. (A) Spontaneous miniature potentials of control (top; *Df(3L)64DF*/+; amplitude and frequency are 0.9±0.1 mV and 3.4±1.6 /s; mean±s.e.m., *n*=5) and *sif* mutant (bottom; *Df(3L)64DF / sif*^{ES11}; 1.0±0.1 mV and 2.1±0.7 /s; mean±s.e.m., *n*=5) at NMJs. (B) Evoked potential of control (left; *Df(3L)64DF*/+; 34.4±2.8 mV, *n*=5) and *sif* mutant (right; *Df(3L)64DF/sif*^{ES11}; 36.8±3.9 mV; mean±s.e.m., *n*=5). The Ca^{2+} concentration is 0.7 mM. (C) Quantal contents of control (*Df(3L)64DF*/+; black circle) and *sif* mutants (*Df(3L)64DF / sif*^{ES11}; open square) measured at 0.2, 0.35 and 0.7 mM Ca^{2+}. These basic electrophysiological properties of the neuromuscular synapse were not significantly altered in the *sif* mutants.

FAS2 affects the proper localization of SIF to the periactive zone

We next assessed the possibility that the molecules in the periactive zone may affect each other in establishing their zonal localization. Because SIF and FAS2 co-localize typically in the periactive zones and interact genetically, we focused on these proteins and investigated if any perturbation occurs in the distribution of several molecular markers in the mutant background of *Fas2* or *sif*. In the *sif* mutants, the localization of FAS2 was indistinguishable from the wild type. Conversely the SIF localization was normal in a hypomorphic allele of *Fas2*, *Fas2*^{e86}. However, in the boutons of a more severe hypomorphic allele of *Fas2*, *Fas2*^{e76}, the SIF localization to the periactive zones was perturbed. The *Fas2*^{e76} mutation reduces *Fas2* expression to 10% of the wild-type level (Grenningloh et al., 1991). In most *Fas2*^{e76} boutons, the network pattern of SIF staining was still observed, but frequently in an irregular or diffused fashion (Fig. 6A-F; compare D-F with Fig. 2A-C). In more extreme cases, SIF was distributed almost evenly throughout the boutons (Fig. 6A, arrow) or was largely concentrated on one side of the boutons (Fig. 6D, arrow) so that SIF staining considerably overlapped DPAK staining. We could distinguish the mutant larvae from the wild type by the blind test for the SIF and DPAK co-staining patterns. Similar results were obtained in the heterozygotes with *Fas2*^{e76} and *Fas2*^{eB112} (*Fas2* null allele), suggesting that the alteration of SIF localization is not due to a second-site mutation on *Fas2*^{e76} chromosome (data not shown). To investigate the altered distribution of SIF further, the mutant boutons were examined under the electron microscope. We found that a large number of SIF signals were occasionally present in the medial portions of the electron-dense regions in the *Fas2*^{e76} boutons (Fig. 6G,H; this altered distribution was statistically significant; $P<0.05$; see Fig. 6 legend) and those

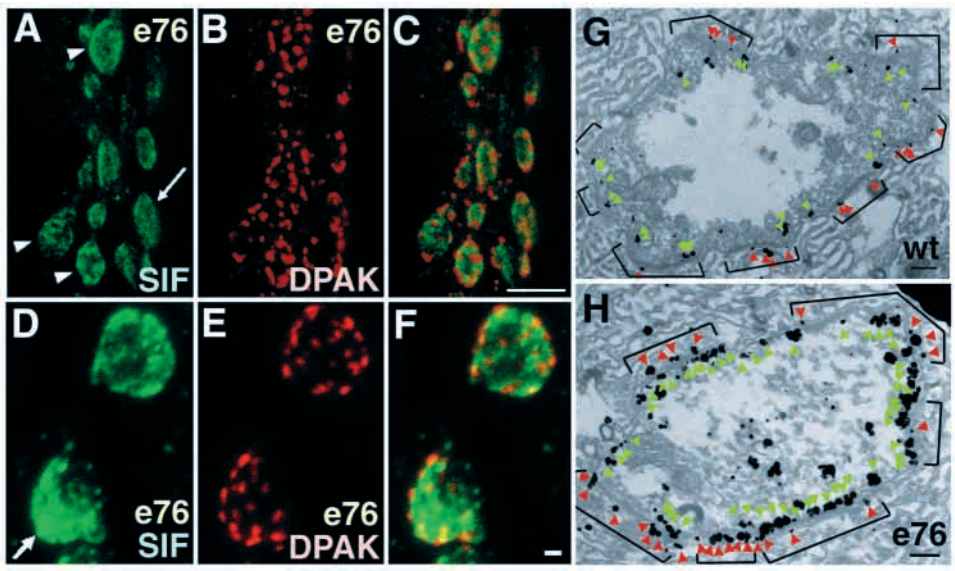

Fig. 6. Altered localization of SIF in *Fas2*^{e76} boutons. (A-F) Altered staining patterns in *Fas2*^{e76} NMJs labeled with anti-SIF (green) and anti-DPAK (red) antibodies. The distribution of SIF was frequently irregular (A, arrowheads), diffuse (A, arrows) or concentrated on one side (D, arrow), while some boutons still show a network pattern (A, open arrowhead). (G,H) Immunoelectron micrograph of the wild-type (G) and *Fas2*^{e76} (H) NMJs doubly stained with anti-SIF (green arrowheads at the presynaptic side) and anti-DPAK (red arrowheads at the postsynaptic side) antibodies. In *Fas2*^{e76} NMJ, SIF signals were distributed even in the medial portions of the electron-dense regions that contain the active zones (brackets); in *Fas2*^{e76}, 23% of the sections (*n*=56) exhibited more than 25% of the SIF signals in the medial portions, whereas 9% of the sections (*n*=65) showed such a distribution pattern in the wild type. The difference was significant ($P<0.05$, chi-square test). NMJs were co-stained with anti-DPAK antibody to assist in the identification of the electron-dense regions. Scale bars: 5 μm in A; 1 μm in D; 200 nm in G,H.

signals were still associated with the plasma membrane (Fig. 6H), as were the signals observed in the wild type. We therefore concluded that the reduction of FAS2 in the periactive zones resulted in the improper localization of SIF along the plasma membrane.

Previous study showed that the synaptic localization of FAS2 requires DLG (Thomas et al., 1997). Therefore, we examined the localization of SIF in the $dlg^{X1\text{-}2}$ mutant background, but no apparent alteration was found in the network pattern (data not shown). In our observation, a considerable amount of FAS2 was still present in the periactive zones of $dlg^{X1\text{-}2}$ boutons (see also Thomas et al., 1997), while faint or no staining was detected in the $Fas2^{e76}$ boutons. This residual FAS2 seems to be sufficient to sustain the proper localization of SIF in the $dlg^{X1\text{-}2}$ mutants.

SIF acts as a GEF for RAC

The structure and localization of SIF suggest that SIF activates RHO-family G proteins in the periactive zones. However, the family comprises several members including RAC, CDC42 and RHO, each playing a distinct role in a variety of cellular events (Van Aelst and D'Souza-Schorey, 1997; Hall, 1998). It is therefore important to estimate which member of the family is activated by SIF in the periactive zone. We examined the GEF activity of SIF by assaying its catalytic ability to dissociate [^{3}H]GDP from mammalian RAC1, CDC42 and RHOA using bacterially expressed a glutathione-S-transferase (GST)-fusion protein carrying the DH domain and adjacent Pleckstrin-homology (PH) domain of SIF. We found that the GST-SIF stimulated the dissociation of GDP from RAC1 both in a time-dependent (Fig. 7A) and dose-dependent manner (Fig. 7B), but did not show the activity for CDC42 and RHOA. GST-Dbl, used as a positive control (Yaku et al., 1994), clearly increased the release of GDP from all the three G proteins (Fig. 7B). Thus, these data demonstrate that SIF specifically activates RAC1 in vitro, suggesting that SIF activates RAC in the periactive zones.

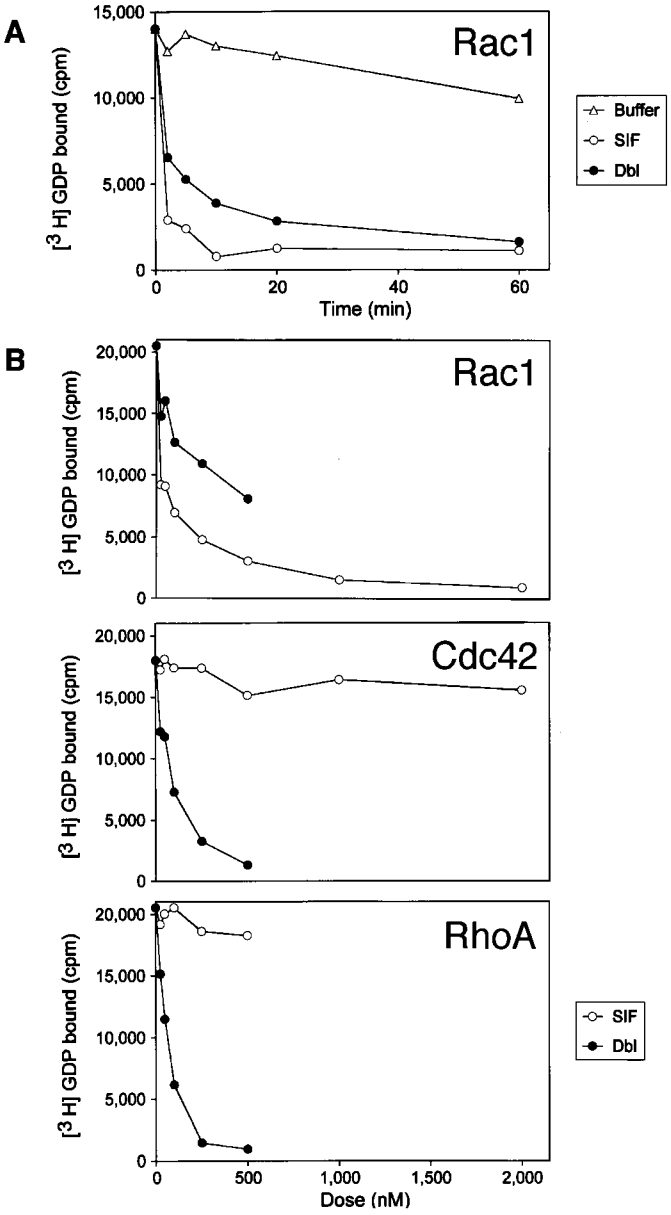

Fig. 7. SIF acts as a GEF for RAC1 *in vitro.* GEF activity of SIF for RHO family G proteins. (A) SIF dissociates [^{3}H]GDP from RAC1 in a time-dependent manner, as does a control molecule, Dbl. (B) SIF specifically acts on RAC1 in a dose-dependent manner, but not on CDC42 and RHOA. Dbl showed a GEF activity for all the three G proteins.

DISCUSSION

Identification of the periactive zone as a region regulating synaptic development

Active zones are well-characterized structures specialized for exocytosis of synaptic vesicles and a number of molecules participating in the process have been identified. However, the molecular characteristics of other synaptic regions on the plasma membrane are largely unknown. In this report, our data suggest the presence of another functional domain, the periactive zone, which serves in the control of changing synaptic structures.

We have characterized the periactive zone in three respects. Initially, the periactive zone was defined on the basis of the distribution patterns of SIF and FAS2, both clearly surrounding the electron-dense region marked with anti-DPAK antibody. The concentric staining patterns that represent pairs of the periactive zone and electron-dense region were often separated from the adjacent pairs and therefore suggested that these two regions constitute a structural unit in the NMJ. Secondly, the biochemical properties of the molecules found in the periactive zone characterize this zone. We found cell adhesion molecules, FAS2 and integrin, on the plasma membrane, and DLG and SIF inside the membrane of the zone, although βPS integrin and DLG were more widely distributed, at least on the postsynaptic side. These findings suggest that the periactive zone may link the information from cell adhesion molecules to the intracellular signaling pathways. Finally, the role for the periactive zone was suggested by the genetic evidence for the molecules localized in the zone. The mutant analyses in this and the previous studies have shown that all these molecules are involved in synaptic development. In *Fas2* null mutants, neuromuscular synapses fail to grow and eventually retract (Schuster et al., 1996a). In the mutants affecting βPS integrin,

the growth of larval NMJ is increased or decreased dependent on the alleles (Beumer et al., 1999). The mutation in *dlg* caused the reduction in size of the subsynaptic reticulum in postsynapses and the increase in the number of active zones in presynapses (Lahey et al., 1994; Thomas et al., 1997). Also, the *sif* mutation exhibited a modulatory effect on synaptic growth when combined with the *Fas2* mutation as shown in this report. Taken together, all gene products localized in the peri-active zone participate in the synaptic development. These findings provide genetic evidence suggesting that the periactive zone serves as a membrane domain for the structural control of synaptic terminals.

In addition, our data indicate that the periactive zone is distinct from the zone for endocytosis recently reported (Roos and Kelly, 1999). Staining for SHI, which is in a donut-like pattern, does not overlap with most of the FAS2-positive region, and is nearly within the hole of the FAS2 rings. This observation suggests that endocytosis actively occurs near or within the electron-dense region, which is consistent with the previous finding that one type of endocytosis occurs near the active zone in the *Drosophila* optic lobe synapses (Koenig and Ikeda, 1996). Although it is possible that endocytosis occurs in the periactive zone at some frequency, these data suggest that the major role for the zone is directed to events other than *shi*-dependent endocytosis.

Our genetic data suggested that FAS2 and SIF are components in the related signaling pathways that locally function in the periactive zone. The altered localization of SIF in the *Fas2* mutants suggests that a certain level of FAS2 expression is required to maintain the configuration of the intracellular molecules including SIF in the periactive zone. Further evidence that shows the functional interaction between SIF and FAS2 was also obtained by mutant analyses. While each of the *sif* and *Fas2* mutations causes the reduction in bouton number, the *sif* and *Fas2* double mutations exhibited a suppressive genetic interaction in synaptic growth. This suppression suggests that the two pathways for FAS2 and SIF signals are both involved in synaptic development and the balance between the pathways is important for the regulation of synaptic growth. These notions were supported by the exacerbated reduction of the bouton number when SIF was overexpressed in the $Fas2^{e76}$ background. It has been shown that FAS2 mediates synaptic stabilization, the varying extent of which seems to cause the increase or decrease in synaptic growth (Schuster et al, 1996a,b). Our data may be therefore consistent with the idea that SIF acts to regulate synaptic stabilization that is mediated by FAS2 or other adhesion molecules. Moreover, SIF may modulate synaptic growth in an inhibitory manner when the FAS2-mediated synapse stabilization is reduced. It may be further noteworthy that the $Fas2^{e76}$ sif^{ES11} double mutants exhibit low viability and are difficult to maintain as a stock even as heterozygotes (data not shown). This observation may be also interpreted as indicating that eliminating the doses of both *sif* and *Fas2* impairs a regulatory cascade that is established by the balance of the two signaling pathways.

In summary, the cell adhesion molecule FAS2 and intracellular signaling molecule SIF interact with each other, both controlling synaptic development in the periactive zone. This finding enforces the idea that the periactive zone is the functional membrane domain where various types of proteins constitute signaling networks or protein complexes that control synapse formation. This notion is further supported by the fact that DLG regulates the localization of FAS2 in the periactive zone (Thomas et al., 1997; Zito et al., 1997). In addition, FAS2, in turn, may function to organize the arrangement of the molecules including SIF in the periactive zones.

SIF may activate RAC in the periactive zones

Our in vitro assay shows that SIF catalyzes the guanine-nucleotide exchange reaction for mammalian RAC1. This is consistent with the previous observation that SIF induces ruffling membranes in human KB cells, as does the constitutive active form of RAC1 (Sone et al., 1997). In addition, *Drosophila* RHO family G proteins are more than 85% identical in amino acid sequences to the corresponding mammalian G proteins (Luo et al., 1994; Hariharan et al., 1995; Harden et al., 1995). These lines of evidence suggest that SIF activates *Drosophila* RAC in the periactive zones. As SIF contains multiple domains that potentially mediate interaction with other molecules, RAC and several other molecules may be recruited to make a protein complex in the zone.

In mice, TIAM1 and STEF have been identified as GEFs that specifically activate RAC1 (Michiels et al., 1995; Hoshino et al., 1999), and both are highly related to SIF in domain organization and amino acid sequence in several domains, indicating that these two proteins are likely to be the mouse orthologs of SIF. Interestingly, both *Tiam1* and *Stef* are expressed in the brain (Habets et al., 1994; Hoshino et al., 1999), and *Tiam1* expression is observed in the adult hippocampus (Ehler et al., 1997). It would be important therefore to examine whether TIAM1 and STEF are localized in the synaptic terminals.

RAC is well known as a regulator of the actin-based cytoskeleton and cell adhesion in various cells (Van Aelst and D'Souza-Shorey, 1997; Hall, 1998; Kuroda et al, 1997; Takaishi et al., 1997; Hordiik et al., 1997). RAC is also implicated in the structural changes of nerve terminals including growth cones and dendrites (Luo et al., 1994, 1996; Kozma et al., 1997; Lamoureux et al., 1997; Threadgill et al., 1997; Gallo and Letourneau, 1998). Therefore, in the periactive zones, activated RAC may locally regulate the processes of the structural change in the synaptic terminals, which include reorganization of the actin-based cytoskeleton and cell adhesion.

Previous studies have shown that RAC acts in the neurite outgrowth of neuroblastoma cells that depends on the signal from integrin on the cell surface (Sarner et al., 2000). The mammalian SIF homolog TIAM1, which functions as a RAC GEF, recruits integrin to specific adhesive contacts at the cell periphery (van Leeuwen et al., 1997). Moreover, expression of TIAM1 increases cadherin-mediated cell adhesion in epithelial MDCK cells (Hordijk et al., 1997). Therefore, there appear to be signaling links between the RAC and cell-adhesion molecules. Our study shows that SIF activates RAC, *sif* genetically interacts with *Fas2* in synaptic growth and the SIF localization was perturbed in the *Fas2* mutants. Taken together, these data suggest that the SIF-RAC pathway is linked to the cell-adhesion molecule FAS2 in close vicinity in the periactive zone.

Spatial segregation of two synaptic functions: neurotransmission and synaptic development

We have indicated the periactive zone as a region for the

control of synaptic development. The periactive zone surrounds the active zone, which is the site for vesicle exocytosis or neurotransmission. This concentric organization suggests that the two zones specialized for the different cellular functions constitute an elemental unit for the presynaptic structure. Investigation of how these zones are incorporated into the synaptic bouton during development will be interesting but remains to be carried out.

The segregated distribution of the two zones suggests that the mechanisms controlling synaptic development and neurotransmission may be separable. This view was supported by the mutant analyses for FAS2 and SIF; both mutations affect structural properties of synapses without changing basic electrophysiological functions. In the NMJs of *Fas2* mutants, the bouton number is decreased or increased depending on the alleles but the total synaptic strength is maintained at the normal level (Stewart et al., 1996; Schuster et al., 1996b). Functional strength of the synapse is regulated only through the activity of a transcription factor, cAMP-response-element-binding protein (CREB), which functions independently of FAS2 (Davis et al., 1996). Also in *sif* mutants, basic electrophysiological properties of NMJs are normal. These observations clearly contrast with the mutant phenotypes for the proteins controlling vesicle exocytosis: synaptotagmin, cysteine string protein, n-synaptobrevin and syntaxin 1A. All these mutants show the impaired EJPs (Littleton et al., 1993; Umbach et al., 1994; Sweeney et al., 1995; Schulze et al., 1995; Deitcher et al., 1998). Taken together, these results indicate that synaptic development and neurotransmission are genetically separable phenomena and are regulated by independent pathways. We propose that these genetically separable phenomena are spatially segregated into the two zones on the presynaptic plasma membrane (Fig. 8), although we do not exclude the possibility that the two zones interact with each other.

Recently, new members were identified to be added to the periactive zone proteins (Wan et al., 2000; Zhen et al., 2000; Schaefer et al., 2000). In these studies, *Drosophila* highwire protein and its *C. elegans* homolog, RPM-1, were demonstrated to function in the growth or structural development of synapses, and highwire was found to localize in the periactive zone. Discovery of these proteins further enforces our current view and highlights the importance of periactive zones for synaptic growth and stability (see also Chang and Balice-Gordon, 2000).

In the nervous system, various cell-adhesion molecules and signaling molecules may provide characteristic attributes for each synapse. In addition to the molecules described in this article, cadherins and catenins are likely to play such a role in synapses (Uemura, 1998). Notably, N-cadherin and SIF are localized laterally to the active zones in the neuron-neuronal synapses of the mammalian and *Drosophila* brain, respectively (Fannon and Colman, 1996; Uchida et al., 1996; Sone et al., 1997). This suggests that the presence of the specialized area surrounding the active zone is a general feature among synapses. Further examination of signaling pathways that function locally in the periactive zone would provide insights as to how synapses grow or retract during development and plasticity.

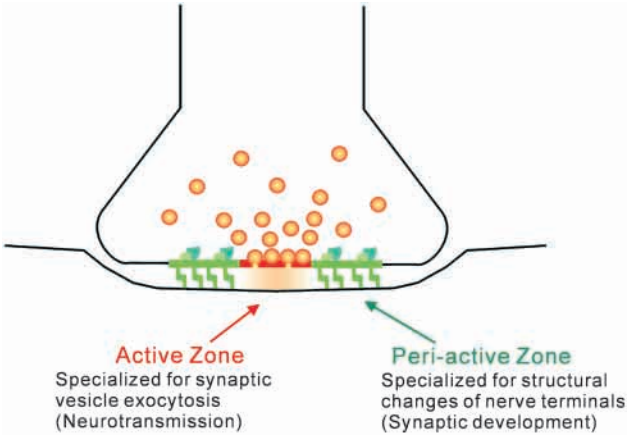

Fig. 8. Model of synaptic structure with two distinct zones. The periactive zone hypothesis proposes that the synaptic structure is organized into at least two functionally specialized domains. The active zone is specialized for neurotransmission on the presynaptic membrane. The periactive zone surrounds the active zone and is specialized for the regulation of structural changes in the synapse.

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