Interactions between Piccolo and the Actin/Dynamin-binding Protein Abp1 Link Vesicle Endocytosis to Presynaptic Active Zones*

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Piccolo is a high molecular weight multi-domain protein shown to be a structural component of the presynaptic CAZ (cytoskeletal matrix assembled at active zones). These features indicate that Piccolo may act to scaffold proteins involved in synaptic vesicle endo- and exocytosis near their site of action. To test this hypothesis, we have utilized a functional cell-based endocytosis assay and identified the N-terminal proline-rich Q domain in Piccolo as a region that interferes with clathrin-mediated endocytosis. Utilizing the Piccolo Q domain as bait in a yeast two-hybrid screen, we have identified the F-actin-binding protein Abp1 (also called SH3P7 or HIP-55) as a potential binding partner for this domain. The physiological relevance of this interaction is supported in vivo binding studies, colocalization in nerve terminals, in vivo recruitment studies, and immunoprecipitation experiments. Intriguingly, Abp1 binds to both F-actin and the GTPase dynamin and has been implicated in linking the actin cytoskeleton to clathrin-mediated endocytosis. Our results suggest that Piccolo, as a structural protein of the CAZ, may serve to localize Abp1 at active zones where it can actively participate in creating a functional connection between the dynamic actin cytoskeleton and synaptic vesicle recycling.

Presynaptic boutons are highly specialized cellular compartments designed for the rapid, efficient, and regulated release of neurotransmitter. Morphologically, the most prominent feature of these boutons is a region of the presynaptic plasticity, called the active zone. Here synaptic vesicles (SVs) dock, fuse, and release their neurotransmitter contents into the synaptic cleft. Much progress has been made in understanding the molecular machinery involved in the regulated fusion of SVs (1), as well as the endocytic events that lead to the recycling of SV membranes (2). However, our knowledge is more limited regarding how active zones function to spatially restrict SV endo- and exocytosis and dynamically regulate these activities.

The fine filamentous structure and intimate association of the CAZ (cytoskeletal matrix assembled at active zones) with sites of SV exo- and endocytosis suggests that the CAZ may serve as a platform to coordinate and spatially organize the machineries regulating SV recycling in nerve terminals (3–6). This hypothesis is supported by studies aimed at defining the molecular composition of the CAZ. For example, in the last several years, a number of structural multi-domain proteins of the CAZ have been characterized including Piccolo (7, 8) (also referred to as Aczonin; see Ref. 9), Bassoon (10, 11), the Rab3-interacting molecule RIM1α and RIM2 (12, 13), Munc13s (14, 15), and CAST (15). Of these, we understand the RIMs and Munc13–1 the best. Functionally, RIM1α appears to be involved in SV priming (16) and to be required for presynaptic long-term potentiation (17); however, its ability to directly interact with Rab3A/C (12), Munc13–1–18, α-liprins (16), RIM-BPs (13, 19), and CAST (15) indicates that it also performs a scaffold function networking together different CAZ proteins. Munc13s have also been found to be important for vesicle priming and are phorbol-ester-binding proteins (20, 21). RIM-BPs may link RIMs to voltage gate Ca2+ channels (19). CAST is a novel 120-kDa CAZ protein that contains a putative C-terminal consensus motif for binding to PDZ (PSD-95/Disc large/ZO-1 homology) domains that allows it to interact with the PDZ domain of RIM1α (15).

The largest CAZ proteins are a pair of structurally related high molecular weight proteins, Piccolo and Bassoon (3). They have molecular masses of 550 and 420 kDa, respectively, and are comprised of numerous modular subdomains that include ten Piccolo/Bassoon homology domains and a pair of double zinc finger domains (8, 10, 22). In addition, Piccolo contains a PDZ domain and two Ca2+/phospholipid binding (C2) domains (8, 9). The C2A domain has been suggested to sense changes in Ca2+ in nerve terminals similar to Synaptotagmin (23). Searches for Piccolo and Bassoon binding partners have identified proteins involved in both SV trafficking including the prenylated Rab3A-associated protein (PRA1) (8) and actin assembly based on the ability of Piccolo to bind Profilin, an actin monomer-binding protein (9), and GIT1 (24), an Arf GTPase main; PBS, phosphate-buffered saline; MAP, microtubule-associated protein.
activating protein (GAP) implicated in the regulation of membrane traffic and the actin cytoskeleton (25). These data suggest a role for these proteins in regulating actin dynamics and SV recycling, features fundamental to nerve terminals (4, 26). Studies on a mouse mutant of Bassoon support this conclusion, as we have found that the absence of wild-type Bassoon disrupts SV recycling at a fraction of excitatory synapses (27); however, the molecular details of how it regulates specific steps in this cycle remain unclear.

Clearly, one aspect of SV recycling in nerve terminals involves a functional cross-talk between the cytoskeleton in general and membrane trafficking machinery, which is thought to be important in ensuring high efficiency and accuracy of vesicle formation and recycling (5, 6, 28). In nerve terminals, the primary mode of SV endocytosis, subsequent to neurotransmitter release, is compensatory endocytosis. Here a specialized form of clathrin-mediated endocytosis similar to that found in non-neuronal cells has been described (2, 29). Intriguingly, many of the proteins associated with clathrin-mediated endocytosis, including Clathrin, AP-2, AP180, Epsin, Eps15, Intercstin, Dynamin, and Amphiphysin (29), are enriched in nerve terminals and can be found associated with the CAZ or presynaptic web (30, 31). In addition, nerve terminals contain a number of actin-associated adapter proteins, including HIP1R (32), Profilin II (33), Syndapin I (34), and Abp1 (35), that appear to link dynamic aspects of the actin cytoskeleton and the interlinked membrane endocytotic trafficking machinery (36).

To gain direct clues as to whether Piccolo is involved in SV endocytosis, we have examined whether Piccolo is functionally associated with components of the SV endocytotic machinery. Specifically, we have examined whether specific subdomains in Piccolo can interfere with clathrin-mediated endocytosis. Here, we find that the Q domain in Piccolo has such an activity and has the capacity to interact in vivo with the actin/dynamin-binding protein Abp1. Our results suggest Piccolo may work with Abp1 at or near the active zone to regulate actin rearrangement and membrane dynamics events that are spatially restricted within nerve terminals.

EXPERIMENTAL PROCEDURES

Eukaryotic Expression Vectors—The following FLAG-tagged vectors were subcloned as restriction digestion fragments from an original rat cDNA fragment-7If (8) into the pCMV-Tag2 vector system (Stratagene) including pFLAG-Piccolo-N-Zn2a (amino acid (aa) 1–1382), pFLAG-Piccolo-N (aa 1–373), pFLAG-Piccolo-N-Q (aa 1–526), pFLAG-Piccolo-Q-Zn1 (aa 369–588), and pFLAG-Piccolo-Q (aa 369–494). pEGFP-Q-Zn1 (aa 369–588) and pEGFP-Q (aa 369–494) were constructed using the pEGFP-C1 vector (Clontech). N-terminally Myc-tagged full-length Abp1 and Abp1-SH3 constructs for overexpression in mammalian cells were described previously (37).

Transferrin Uptake Assays—For receptor-mediated endocytosis assay, COS-7 cells were plated onto poly-lysine-coated glass coverslips and grown overnight. Cells were then either subjected to protein import experiments or transfected with different FLAG-tagged Piccolo constructs using the LipofectAMINE PLUS transfection reagent method. For protein import into COS-7 cells, the BioPorter® (Gene Therapy Systems, Inc.) method was used. The conditions were analogous to a procedure described previously (38). Cells were incubated with complexes containing the His6-Q protein used for the blot overlay analyses and BioPorter® or with BioPorter® reagent and PBS for 4 h.

Transferrin uptake assays were performed 48 h after transfection and after a 1-h recovery period following BioPorter® treatment, respectively, as described previously (37–39). The expression of Piccolo constructs was visualized with the monoclonal anti-FLAG antibody M2 (Sigma) and the anti-thio mouse monoclonal antibody (Invitrogen), respectively. The percentages of cells showing no detectable uptake of transferrin, significantly reduced transferrin signals, and normal levels of internalized transferrin were calculated by scoring the transferrin signals and counting cells in several independent experiments.

Expression of tagged Piccolo fragments was confirmed by SDS-PAGE and Western blotting. Homogenates of COS-7 cells overexpressing FLAG-Piccolo fusion proteins were prepared from high density cultures in 6-well plates 36 h after transfection. Cells were harvested and resuspended in 30 μl of lysis buffer (1% Triton X-100 in PBS supplemented with protease inhibitors (complete protease inhibitor tablet, EDTA-free; Roche Molecular Biochemicals)) and incubated for 20 min on ice. The samples were then spun for 20 min at 14000 × g at 4 °C. Supernatants were separated on 10% SDS-PAGE and immunoblotted with anti-FLAG antibody. The intensity of the His6-Q protein was analyzed by SDS-PAGE and Coomassie staining.

Yeast Two-hybrid Assays—For the yeast two-hybrid screen, the Q-Zn1 region of Piccolo (aa 369–629) was subcloned into the EcoRI/SalI site of pLexA (Clontech). Yeast two-hybrid assay was performed as described previously (8) using pLexA-Q-Zn1 as bait to screen a mouse cDNA library (Clontech). Peptide overlapping analysis of the interaction between Abp1 and the Q-Zn1 region of Piccolo, the prey construct pB42AD-Abp1.9.2 and bait constructs containing pC-Q-Zn1 (aa 369–629), Pic-C (aa 369–494), and Pic-Zn1 (aa 485–629) were cotransformed into EGY48(4ploplac23) yeast cells. Yeast clones cotransformed with bait and prey constructs were selected on plates lacking uracil and tryptophan. Positive clones were selected and assayed for His growth and β-galactosidase activity as described in MATCHMAKER protocols.

Fusion Protein Overlay Assays—The vector pGEX2T.1 was modified from pGEX2A (Amersham Biosciences) as described previously (8). GST-Abp1.9.2 (aa 194–433 of Abp1) was generated by restriction digestion of the cDNA fragment from pB42AD bait vector as an EcoRI/SalI fragment and ligated into pGEX2T.1. Additional GST-Abp1 fusion protein constructs were generated as described previously (35). His6-Q was generated by PCR amplification of the Q domain of Piccolo (aa 369–494) and subcloning into pET32α (Novagen) vector. For the overlay assay, 100 pmol of GST fusion protein was separated on a 10% SDS-PAGE gel and transferred to nitrocellulose membrane. The membrane was blocked for nonspecific binding in blocking solution (1× TBS, pH 7.4, 5% skim milk, and 0.1% Tween 20) for 30 min, followed by incubation with 0.1 μg His6-Q in blocking solution for 1 h at room temperature. The membrane was further incubated with anti-thio mouse monoclonal antibody (Invitrogen) followed by a goat anti-mouse antibody conjugated to alkaline phosphatase. Horseradish peroxidase fusion proteins was visualized by colorimetric alkaline phosphatase reaction with nitro blue tetrazolium and 5-bromo-4-chloro-3-indoyl-phosphate.

Antibodies—Piccolo mouse and rabbit polyclonal antibodies were generated against sap44a and 44a2 sequences fused to GST, respectively, and affinity purified as described previously (7). The polyclonal anti-Abp1 antibody, anti-Abp1.SF1, was raised in rabbit against a purified His6-tagged fusion protein of Abp1 (aa 194–433) as immunizing antigen. Antibodies were affinity purified on an analogous GST fusion protein of Abp1 bound to Actigel beads (Sterogene) as described previously (35). Affinity purified guinea pig anti-Abp1 antibodies were affinity purified previously (35). The polyclonal anti-Abp1 antibody, anti-Abp1.MK1, was raised in rabbit against a purified GST fusion protein of Abp1 lacking its SH3 domain. Antibodies were depleted against GST and subsequently affinity purified using the respective maltose-binding protein fusion protein blotted to nitrocellulose membranes, analogous to a method described previously (34). Monoclonal anti-MAP2 antibody (clone HM-2) was from Sigma.

Coimmunoprecipitation Assays—pFGLQ-Q-Zn1 was constructed by subcloning the Q-Zn1 sequence of Piccolo into the EcoRI/SalI site of pCMV-Tag2B (Stratagene). 3T3 cells were transiently transfected with pFLAG-Q-Zn1 using FuGENE reagent (Roche Molecular Biochemicals), harvested, and lysed in radioimmune precipitation assay buffer with a mixture of protease inhibitors (Roche Molecular Biochemicals). Following sonication and clarification in bovine serum albumin, extracts were incubated with either anti-Abp1.SF1 or anti-FLAG antibodies coupled to Actigel beads at 4 °C for 6 h, followed by immunoblot analysis of the immunoprecipitates with FLAG and Abp1 antibodies. In vivo coimmunoprecipitation was performed using soluble rat brain lysates as described previously (9). Briefly, the brain membranes were solubilized in homogenization buffer (150 mM NaCl, 1 mM EDTA, 10 mM Tris, 100 mM Na2CO3, adjusted to pH 11.5) with a mixture of protease inhibitor (Roche Molecular Biochemicals), centrifuged at 100,000 × g for 1 h and the supernatant adjusted to pH 7.6. The supernatant was incubated with Piccolo antibody, Abp1 antibody (anti-Abp1.MK1), or rabbit IgG coupled to Actigel beads. After washing 6 h at 4 °C, binding of beads in homogenization buffer (pH 7.6 lacking 100 mM Na2CO3), immunoprecipitates were subject to immunoblot analysis with affinity purified anti-Piccolo and anti-Abp1.MK1 antibody.

Lamellipodial Recruitment and Cell Perforation Experiments—Rest-
incubated at 37 °C with FITC-transferrin (green) for 30 min, fixed, and analyzed by confocal microscopy. Cells were viewed using a Leica TCS NT laser confocal microscope with a Leica TCS software package (Leica) or a Nikon Diaphot 300 microscope equipped with a Photometrics CH250 CCD camera. Images were recorded digitally and processed using IP Lab Spectrum (Signal Analytics) and Adobe Photoshop software (Adobe).

RESULTS

Overexpression of N-terminal Segments of Piccolo Blocks Receptor-mediated Endocytosis—Previous studies indicate that Piccolo may function to scaffold proteins critically involved in the dynamic assembly of actin and recycling of SVs in nerve terminals (8, 9, 24). However, to date, functional evidence for a specific role of Piccolo in SV endocytosis has not been obtained.

Processing of N-terminal Segments of Piccolo Blocks Receptor-mediated Endocytosis—Previous studies indicate that Piccolo may function to scaffold proteins critically involved in the dynamic assembly of actin and recycling of SVs in nerve terminals (8, 9, 24). However, to date, functional evidence for a specific role of Piccolo in SV endocytosis has not been obtained.

37°C fibroblasts transfected with GFP-Piccolo-Q-Zn1, GFP-Piccolo-Q-Zn1, and Myc-tagged Abp1 or GFP alone were activated by replating the cells onto fibronectin-coated coverslips and additional stimulation with 300 ng/ml phorbol 12-myristate 13-acetate (Sigma) and 5 ng/ml of human recombinant platelet-derived growth factor (Sigma) as described previously (35). The cells were then washed with warm PBS and subsequently either briefly perfused with 0.02% saponin (Sigma) for 5 s or directly, PBS, pH 7.4, paraformaldehyde in PBS, containing 0.5 mM MgCl₂, and 0.9 mM CaCl₂, for 20 min. The cells were then processed for immunofluorescence microscopy using a monoclonal anti-Myc antibody (37°C fibroblasts transfected with GFP-Piccolo-Q-Zn1, GFP-Piccolo-Q-Zn1, and Myc-tagged Abp1 or GFP alone were activated by replating the cells onto fibronectin-coated coverslips and additional stimulation with 300 ng/ml phorbol 12-myristate 13-acetate (Sigma) and 5 ng/ml of human recombinant platelet-derived growth factor (Sigma) as described previously (35). The cells were then washed with warm PBS and subsequently either briefly perfused with 0.02% saponin (Sigma) for 5 s or directly, PBS, pH 7.4, paraformaldehyde in PBS, containing 0.9 mM CaCl₂ and 0.5 mM MgCl₂, for 20 min. The cells were then processed for immunofluorescence microscopy using a monoclonal anti-Myc antibody (9E10, BabCO) and Alexa Fluor™ 568 goat anti-mouse antibody conjugates (Molecular Probes). The samples were examined using a DMRD fluorescence microscope (Leica) and a confocal microscope.

For quantitative analyses, the number of GFP-Piccolo-Q-Zn1-expressing cells per coverslip was quantitated live 45 min after replating using an inverted Eclipse TE300 fluorescence microscope (Nikon). Cells were then perfused, fixed, and processed for fluorescence microscopy. Cells with remaining GFP-Piccolo-Q-Zn1 signal were counted again. Several independent experiments were performed and the extent of GFP-Piccolo-Q-Zn1 retention was expressed as percentage of the number of observed GFP-Piccolo-Q-Zn1-positive cells per number of GFP-Piccolo-Q-Zn1-expressing cells. Standard deviations were calculated from independent experiments.

Cell Culture and Immunofluorescence Microscopy—Primary hippocampal cultures were prepared and grown on poly-L-lysine-coated glass coverslips according to Ref. 41 and transfected at day 4 in culture employing the calcium phosphate method according to Ref. 42. Transfected neurons were fixed and processed for immunofluorescence at day 13 in culture as described below. COS-7 and 3T3 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Primary hippocampal neurons cultured at low density for 20 days were fixed in 4% paraformaldehyde for 5 min at room temperature. After incubation with 25 mM glycine in PBS for 15 min, cells were permeabilized and blocked for 1 h in 2% bovine serum albumin, 10% horse serum, and 0.02% saponin in PBS. Primary hippocampal neurons kept in culture for 3 days were processed for immunofluorescent microscopy as described previously (43).

Incubations with antibodies were performed according to Ref. 35. As primary antibodies, affinity purified polyclonal anti-Abp1 guinea pig antibody GP5 (35) and rabbit anti-Piccolo serum were used. Secondary antibodies used in this study include FITC goat anti-guinea pig (ICN and Alexa Fluor™ 568 goat anti-rabbit (Molecular Probes).

Cells were viewed using a Leica TCS NT laser confocal microscope with a Leica TCS software package (Leica) or a Nikon Diaphot 300 microscope equipped with a Photometrics CH250 CCD camera. Images were recorded digitally and processed using IP Lab Spectrum (Signal Analytics) and Adobe Photoshop software (Adobe).

RESULTS

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We, therefore, designed a strategy to assess possible effects of subdomains of Piccolo on the endocytic uptake of a fluorescent-labeled tracer, *i.e.* FITC-transferrin, undergoing fast recycling. For this analysis, we created several FLAG-epitope-tagged constructs from the N-terminal region of Piccolo (Fig. 1a). Each was successfully expressed in COS-7 cells, as shown by immunoblotting with anti-FLAG antibodies (Fig. 1b). In untransfected cells, FITC-transferrin is rapidly internalized after 30 min of incubation, and readily detectable accumulations are seen in the perinuclear region of most cells (Fig. 1c). In contrast, when constructs encompassing the N terminus of Piccolo up to the second zinc finger (N-Zn2) were overexpressed, the uptake of FITC-labeled transferrin into transfected cells was inhibited significantly (Fig. 1d). Quantitative analyses revealed that only about 30% of transfected cells displayed a transferrin uptake that could be considered as wild-type. More than a third of the cells displayed a total block of uptake, and another third showed significantly reduced levels of endocytosis of transferrin, as seen by the lack or the only faint traces of yellow in the merged images (Fig. 1d), respectively. Quantification of the transferrin signals within transfected cells confirms these qualitative observations (Fig. 1h) indicating the presence of a region in the N-terminal third of Piccolo that negatively influences clathrin-mediated endocytosis.

In a previous study (8), we found that the double zinc finger domains in the N terminus of Piccolo interact with the Rab3A-associated protein PRA1. We therefore evaluated whether a construct including only the first of these double zinc fingers (Q-Zn1) or one that lacked the zinc fingers entirely (N-Q) influenced transferrin uptake. Both constructs exhibited an impaired endocytosis similar to the larger N-Zn2 construct (Fig. 1, e and f). A FLAG construct containing N-terminal sequences preceding the Q domain did not lead to a significant impairment of transferrin uptake (Fig. 1, g and h). These data suggest that perhaps the Q domain, a proline-rich decapeptide repeat region (see below), in Piccolo, rather than the zinc fingers, is responsible for impairing endocytosis. Attempts to confirm this conclusion by overexpressing constructs comprising the Q domain alone fused to FLAG or GFP were unsuccessful, as FLAG-tagged Q domain fusion proteins did not express in COS-7 cells, and the GFP-Q appeared to form intracellular aggregates. To overcome these complications, we utilized the BioPorter® (38) to import a bacterially expressed His6-Q fusion protein, found to be functional in several biochemical assays (see below), into COS-7 cells. With this system, the His6-Q fusion protein was imported successfully into a small subset of cells and caused an endocytosis phenotype similar to that observed upon overexpression of FLAG-Piccolo-N-Zn2, FLAG-Piccolo-Q-Zn1, and FLAG-Piccolo-N-Q. Quantifying data from eight independent assays revealed that more than a third of the His6-Q-containing cells were blocked for endocytosis, whereas another third showed severely impaired uptake of the fluorescent tracer (Fig. 1h). No effect on transferrin uptake was detected when cells were treated solely with BioPorter®, Taken together, these data suggest that it is the Q domain that is responsible for the observed block of endocytosis and that flanking regions are not required for the observed phenotype.

The Q Domain of Piccolo Interacts In Vitro with the SH3 Domain of A bp1—In an attempt to unravel the molecular mechanism underlying how the Q domain in Piccolo could participate in membrane trafficking, we used the short rat Piccolo cDNA fragment Q-Zn1 as bait in a yeast-two hybrid screen to identify interacting proteins. Screening 1 × 10⁶ transformants yielded several independent clones that interacted with Q-Zn1. Two of these encoded the C-terminal half of the actin-binding protein Abp1 (aa 194–433; named Abp1.9.2). Abp1 is a multi-domain protein containing an N-terminal actin-depolymerizing factor homology domain (ADF-H; see Ref.
44) and a central helical region both shown to mediate F-actin binding (35) (Fig. 2c). At the C terminus, Abp1 proteins contain an SH3 domain. Sequences separating the ADF-II and SH3 domains have been identified as ligands for SH3, WW, and related domains (48). SH3 domains, in particular, recognize proline-rich sequences in numerous proteins, proline-rich ligands (37, 51) were mutated, the Abp1 SH3 domain containing a proline-rich sequence interaction. Furthermore, we found that when residues P (49, 50) and a PEST sequence predicted to trigger proteolytic degradation (35, 44) and a central helical region both shown to mediate F-actin binding (35) (Fig. 2c). At the C terminus, Abp1 proteins contain an SH3 domain. Sequences separating the ADF-II and SH3 domains contain two Src family kinase phosphorylation sites and a PEST sequence predicted to trigger proteolytic degradation (35, 45). Intriguingly, Abp1 was initially identified, along with other SH3 domain-containing proteins such as Intersectin, Endophilin, and Amphiphysin, in a screen for SH3-containing proteins (46). The latter three proteins are enriched in nerve terminals and have been implicated as mediators of SV recycling, Dynamin1, Synapsin, and Synaptojanin. It has therefore been suggested to functionally link and coordinate the actin cytoskeleton and SV recycling in nerve terminals (35, 37).

Analysis of the deduced amino acid sequence of the cDNA clone Q-Zn1 revealed that the Q domain is proline-rich and contains twelve copies of a degenerated decapeptide repeat (35). At the N terminus, Abp1 is predicted to contain an SH3 domain, sequences separating the ADF-II and SH3 domains contain two Src family kinase phosphorylation sites and a PEST sequence predicted to trigger proteolytic degradation (35, 45). In numerous proteins, proline-rich regions have been identified as ligands for SH3, WW, and related domains (48). SH3 domains, in particular, recognize proline-rich sequences containing the core peptide sequence, PXXP (49, 50). The presence of an SH3 domain in our prey construct (Abp1.9.2), as well as a number of PXXP sequences in the Q domain, suggests that Q-Zn1 and Abp1 may interact via these two regions. We initially tested this hypothesis in our yeast-two hybrid assay using the Q or Zn1 domain alone as bait constructs. The Q domain (aa 369–494) but not the Zn1 domain (aa 485–629) was found to interact with the C-terminal half prey construct (Fig. 2a). To further evaluate these observations, we used a yeast-two hybrid assay using the Q or Zn1 domain alone as bait constructs. The Q domain (aa 369–494) but not the Zn1 domain (aa 485–629) was found to interact with the C-terminal half prey construct (Fig. 2a). To further evaluate these observations, we examined whether the Q domain from Piccolo alone (fused to His6-Q) could interact with various Abp1 fragments presented as a series of GST fusion proteins in a filter overlay assay (Fig. 2, c and d). His6-Q interacted robustly with two fusion proteins containing the SH3 domain of Abp1 (lanes 2 and 3) and with the Abp1 SH3 domain alone (lane 4) but not with fusion proteins lacking the SH3 domain (lanes 5 and 6) or GST alone (lane 1). Furthermore, we found that when residues within the SH3 domain known to be essential for binding to proline-rich ligands (37, 51) were mutated, the Abp1 SH3 domain failed to bind the Q domain of Piccolo in protein overlay assay (Fig. 2, lane 7). These data indicate that the Abp1-Piccolo interaction is not only direct but is mediated by a classical SH3 domain proline-rich sequence interaction.

Cellular Interactions between the Piccolo Q Domain and Abp1—To confirm our in vitro binding data, we also examined whether Abp1 and Piccolo could interact in a cellular environment. To detect and follow the spatial distribution of endogenous Abp1, rabbit polyclonal antisera were generated against a His6 fusion protein containing the C-terminal half of Abp1 (His6-Abp1.9–2). The anti-Abp1 antisera (anti-Abp1.SF1) detected endogenous Abp1 as a 55-kDa band in both 3T3 cells and brain extracts, as reported previously (Fig. 3a) (35, 45). To test whether Piccolo could interact with Abp1 in cells, a FLAG-tagged Q-Zn1 construct (FLAG.Q-Zn1) was transfected into 3T3 cells. An interaction between endogenous Abp1 and transfected FLAG.Q-Zn1 was assessed by immunoprecipitation with anti-Abp1 and anti-FLAG antibodies, respectively. Both proteins were found to be soluble under mild detergent conditions (radioimmune precipitation assay buffer) (Fig. 3b). Antibodies against Abp1 were able to immunoprecipitate endogenous Abp1 (Fig. 3b, upper panel), as well as coprecipitate transiently expressed FLAG.Q-Zn1 (Fig. 3b, lower panel). In a parallel experiment, antibodies against the FLAG antigen were found to immunoprecipitate FLAG.Q-Zn1 (Fig. 3b, lower panel) and to coinmunoprecipitate endogenous Abp1 (Fig. 3b, upper panel). Control IgG neither precipitated FLAG.Q-Zn1 nor Abp1 indicating that the communoprecipitation of FLAG.Q-Zn1 with Abp1 is specific.

Association of Abp1, the Piccolo Q Domain, and the Actin Cytoskeleton—In 3T3 cells, signaling cascades leading to the activation of Rac1 cause a dramatic redistribution of Abp1 to the actin-rich cell periphery within minutes (35). This visualization of the association of Abp1 with the actin cytoskeleton can be used to assess whether specific proteins can interact with Abp1 in association with F-actin in vivo. We therefore transfected 3T3 cells with different Abp1 constructs and with a GFP-tagged Q-Zn1 construct (GFP.Q-Zn1). The cells were then re-plated and activated with platelet-derived growth factor and phorbol 12-myristate 13-acetate, as described (35). In nonperforated resting cells, both endogenous and epitope-tagged Abp1 are found diffusely distributed throughout the cytosol of the cells and exhibiting mainly in the perinuclear pattern (Fig.
Abp1 can recruit Piccolo fragments to the leading edge of spreading, growth factor-treated ST3 fibroblasts via its association with F-actin. a–c, confocal microscopy of resting cells double transfected with Myc-Abp1 (red) and GFP-Piccolo-Q-Zn1 (green). c shows an overlapping cytosolic staining of both proteins (b), d–f, cells activated with growth factors extend actin-rich lamellipodia, which contain F-actin-bound Abp1 at the leading edge (d), also show Piccolo-Q-Zn1 accumulations resistant to cell perforation (f) at these Abp1-rich sites (yellow) (e). Lamellipodial accumulations of both Abp1 and Piccolo-Q-Zn1 are marked by arrows. Insets represent high magnifications of the boxed areas in d–f; g–i, control cells double transfected with Myc-Abp1 and GFP alone and treated in parallel lost almost entirely their cytosolic GFP staining upon cell perforation. Bar for a–c in e, 20 μm. Bar for d–i in i, 20 μm. j, the in vivo association of Piccolo-Q-Zn1 is dependent on the SH3 domain of either endogenous Abp1 or overexpressed Abp1. Quantification of GFP-positive cells transfected with either GFP-Piccolo-Q-Zn1 alone or double transfected with Myc-Abp1 and GFP-Piccolo-Q-Zn1 or with Myc-Abp1-SH3 domain and GFP-Piccolo-Q-Zn1 after cell perforation is shown. The frequencies of GFP-positive cells on coverslips (dark gray columns) were compared with those of GFP-positive cells prior to perforation (100%). Error bars represent standard deviations of two independent assays. Conditions were as follows: GFP-Piccolo-Q-Zn1-transfected cells, 79.5 ± 12.1% post-perforation, 1050 cells scored; Myc-Abp1/GFP-Piccolo-Q-Zn1-double transfected cells, 88.0 ± 5.1% post-perforation, 1866 cells scored, cotransfection rate, 99.5 ± 0.5% of GFP-Piccolo-Q-Zn1-positive cells; Myc-Abp1-SH3 domain/GFP-Piccolo-Q-Zn1-double transfected cells, 43.3 ± 2.3% post-perforation, 1182 cells scored, cotransfection rate, 37.4 ± 3.4% of GFP-Piccolo-Q-Zn1-positive cells.

A similar localization pattern was observed for GFP.Q-Zn1 (Fig. 4c). In activated cells, endogenous and recombinant Abp1 was observed to accumulate at the very leading edge of the cells, whereas staining within the cell bodies decreased (Fig. 4, d and g). The same held true for GFP.Q-Zn1 (Fig. 4f), which colocalized with cotransfected Myc-Abp1 at the leading edge of lamellipodia formed upon cell activation (Fig. 4e). The fact that this accumulation of Myc-Abp1 and GFP.Q-Zn1 was observed by confocal microscopy rules out any volume effects within membrane ruffles protruding upward. Importantly, Abp1 and GFP.Q-Zn1 localization at the cell periphery was maintained even after a brief perforation of the cells with 0.02% saponin prior to fixation (Fig. 4, d–f). These latter data strongly indicate an association of both proteins with the actin cytoskeleton. Under identical conditions, GFP cotransfected with Abp1 was almost completely removed from the cytosol of these cells. Only the GFP present in the nucleus was retained after cell perforation (Fig. 4i).

The colocalization of Abp1 and GFP.Q-Zn1 in the lamellipodia of platelet-derived growth factor- and phorbol 12-myristate 13-acetate-stimulated 13-activated cells, together with our in vitro binding and coimmunoprecipitation data, strongly suggest that Abp1 mediates the attachment of the GFP.Q-Zn1 with the actin cytoskeleton in these activated cells. This scenario was further evaluated by assessing the effect of an Abp1 dominant-negative construct on the recruitment of GFP.Q-Zn1 to the actin-rich lamellipodia. Here, cells were transfected with GFP.Q-Zn1 alone or doubly transfected with either a Myc-tagged SH3 domain of Abp1 or Myc-tagged full-length Abp1. Twenty-four h after transfection, living cells were counted for GFP expression, detergent-extracted, fixed, and recounted for retained GFP fluorescence. In cells expressing GFP.Q-Zn1 alone, 79.5 ± 12.1% of the cells were still positive for some GFP signal after perforation (Fig. 4j) indicating that endogenous Abp1 is able to retain GFP.Q-Zn1 expressed in the cells. In cells doubly transfected with Myc-Abp1 and GFP.Q-Zn1, almost 90% of the GFP.Q-Zn1 transfected cells were still identifiable as GFP-positive after perforation (Fig. 4j), indicating that the additional exogenous Abp1 is able to more efficiently recruit GFP.Q-Zn1 to the actin-rich lamellipodia. In contrast, when cells were doubly transfected with GFP.Q-Zn1 and the Myc-tagged Abp1 SH3 domain, we observed a significant reduction in the percentage of cells retaining GFP fluorescence (42.5 ± 12%) after perforation and fixation (Fig. 4j). Importantly, this percentage appears to be an underestimation of the true effectiveness of Myc-tagged SH3 domain as only about 40% of the cells transfected with GFP.Q-Zn1 were also determined to be positive for Myc-SH3. These data, taken together, reveal that the recruitment of GFP.Q-Zn1 to the actin cytoskeleton involves its association with the SH3 domain in Abp1. The data also indicate that Abp1 can function as a bifunctional molecule able to simultaneously interact with F-actin and its SH3 binding partner. This creates the possibility that in vivo Abp1 might form a physical link between Piccolo and the actin cytoskeleton.

Overlapping Distribution of Piccolo and Abp1 in Cultured Hippocampal Neurons—Mammalian Abp1 is expressed in nearly all tissues examined where it is often found associated with actin-rich structures (35, 37). In contrast, Piccolo is expressed almost exclusively in the central nervous system, where it is specifically localized at the presynaptic active zone (7) in mature neurons. In immature neurons, Piccolo is expressed almost exclusively in the central nervous system, where it is often found associated with actin-rich structures (35, 37). In contrast, Piccolo is expressed almost exclusively in the central nervous system, where it is specifically localized at the presynaptic active zone (7) in mature neurons. In immature neurons, Piccolo is concentrated in axonal growth cones (40, 43) where it is found primarily associated with 80-nm dense core vesicles along F-actin filaments. These Piccolo transport vesicles have been shown to carry numerous active zone proteins and thus appear to be active zone precursor vesicles (40). The patterns of Piccolo subcellular distribution and Abp1 association with actin structures provide several distinct situations where a Piccolo and Abp1 interaction may come into play. For example, as an actin-binding protein, Abp1 may tether Piccolo transport vesicles to the actin cytoskeleton in growth cones regulating when and where they are permitted to fuse with the plasma membrane to establish active zones. Alternatively, Piccolo could use its association with Abp1 to either link the actin cytoskeleton to the...
active zone in nerve terminal, thus facilitating the mobilization of SVs from the reserve to the readily releasable pool, and/or coordinate endo- and exocytosis. It was therefore of some importance to examine when and where the Abp1-Piccolo interaction occurs in nerve cells.

This was initially accomplished by double-labeled immunofluorescence microscopy of primary hippocampal cultures stained with antibodies against Abp1 and Piccolo. In our initial experiments we stained cultures grown for 3 days in vitro, a stage prior to synaptogenesis in these cultures. Here, Abp1 immunoreactivity was observed throughout the axonal shaft and growth cone. Piccolo staining is less intense with distinct puncta throughout the shaft and growth cone; these are presumably Piccolo transport vesicles. Overlay of the two images indicates little to no colocalization of the Piccolo and Abp1 puncta along the growing process or growth cone. Bar, 10 μm.

Although Piccolo has been shown to be strictly presynaptic (6), Abp1 has been reported to be postsynaptic (52). To assess whether Abp1 is also axonal and presynaptic, we compared the spatial distribution of recombinant Myc-Abp1 (Fig. 7, a–c) and endogenous Abp1 (Fig. 7, d–f) in primary hippocampal neurons with the dendritic microtubule-associated protein, MAP2. In both cases Abp1-positive processes, not stained with antibodies against MAP2, were readily observable (Fig. 7, examples marked by arrows). Double labeling these processes with Piccolo antibodies to label presynaptic boutons revealed that a subset of Piccolo-positive punctae (Fig. 6, e and f) colocalize with Abp1 clusters situated along dendritic profiles (Fig. 6, c and d, arrows; also see high magnification insets). These data indicate that Piccolo and Abp1 can be found together at synapses.

Fig. 5. Spatial distribution of Piccolo and Abp1 in hippocampal growth cones. Cultured rat hippocampal neurons were fixed at 3 days in vitro and double stained with anti-Abp1 (red) (a) and anti-Piccolo antibodies (green) (c). Abp1 is broadly expressed throughout the axonal shaft and growth cone. Piccolo staining is less intense with distinct puncta throughout the shaft and growth cone; these are presumably Piccolo transport vesicles. Overlay of the two images (b) indicates little to no colocalization of the Piccolo and Abp1 puncta along the growing process or growth cone. Bar, 10 μm.

Fig. 6. Spatial distribution of Piccolo and Abp1 in mature hippocampal neurons. Colocalization of Abp1 and Piccolo by confocal immunofluorescence microscopy in primary hippocampal neurons kept in culture for 20 days, as revealed by confocal immunofluorescence microscopy, is shown. a and b, anti-Abp1 staining outlines neurites (arrowheads) and displays punctate accumulations adjacent to neurites (a few examples are marked by arrows). c and d, the immunostaining for Abp1 (green) and Piccolo (red) overlaps at synapses marked by anti-Piccolo antibodies (e and f, a few examples are marked by arrows). Insets in b, d, and f show a clear overlap of the synaptic Abp1 and Piccolo staining (yellow) at high resolution (2.5-fold enlargements of arrow-marked areas adjacent to insets). Bar, 10 μm.
Abp1-positive neurites (some examples are marked by arrows) (marker MAP2 (red) and green) with guinea pig anti-Abp1 antibodies (Fig. 7). Primary hippocampal neurons 20 days in culture. Immunolabeling for Abp1 using the dendritic marker MAP2 (red) (a and d) and for the dendritic compartment in vitro. Abp1 and immunolabeled after 13 days in culture. d-f, primary hippocampal neurons 20 days in culture. Immunolabeling for Abp1 using guinea pig anti-Abp1 antibodies (green) (a and d) and for the dendritic marker MAP2 (red) (c and e) shows the presence of MAP2-negative but Abp1-positive neurites (some examples are marked by arrows in a, c, d, and f). Bars, 30 μm in c and 15 μm in f, respectively.

Fig. 7. The subcellular localization of Abp1 is not restricted to dendrites and cell bodies but also includes the axonal compartment. a-c, primary hippocampal neurons were transfected with Myc-Abp1 and immunolabeled after 13 days in culture. d-f, primary hippocampal neurons 20 days in culture. Immunolabeling for Abp1 using guinea pig anti-Abp1 antibodies (green) (a and d) and for the dendritic marker MAP2 (red) (c and e) shows the presence of MAP2-negative but Abp1-positive neurites (some examples are marked by arrows in a, c, d, and f). Bars, 30 μm in c and 15 μm in f, respectively.

Fig. 8. Piccolo forms a complex with Abp1 in brain. Solubilized rat brain membranes were immunoprecipitated with anti-Piccolo, anti-Abp1.MK1 antibodies, or rabbit IgG and analyzed by immunoblotting with anti-Piccolo and anti-Abp1.MK1. Piccolo antibodies selectively coimmunoprecipitate Abp1. Abp1 antibodies also coimmunoprecipitated Piccolo although immunostaining was weaker reflecting the broader distribution of Abp1 and its ability to associate with proteins other than Piccolo. Control IgG conjugated beads did not pull down Piccolo or Abp1. The high frequency of repeats may hereby assure high binding affinity.

DISCUSSION

Initial structural studies on the active zone proteins Piccolo (7–9) and Bassoon (5, 10) suggested that they might play specific roles in the formation, maintenance, and/or functional interconnection of the different molecular machineries within the presynapse. The interaction of Piccolo with PRA-1 (8), as well as with the GTPase-activating protein GIT1 (24), also suggested some involvement in membrane trafficking. For example, PRA-1 has been shown to be an integral Golgi membrane and presynaptic protein, which is thought to mediate the membrane targeting and intracellular trafficking of small, prenylated GTPases (such as ras, rhoA, and rabs) (54, 55). GIT proteins are ubiquitously expressed, occur both pre- and postsynaptically in neurons, localize to focal adhesions in Chinese hamster ovary cells, and associate with focal adhesion molecules such as β-PIX (56), FAK (57), and Paxillin (58).

In line with an association of the Piccolo double zinc fingers with the GTPase-interacting protein PRA1, we observed that constructs encoding for the N-terminal region of Piccolo interfere with membrane trafficking events in heterologous cells. Surprisingly, an N-terminal region that does not contain the double zinc finger domain in Piccolo was sufficient to inhibit receptor-mediated endocytosis. Clues to a possible molecular explanation for this effect were obtained by searching for Q domain binding partners. One such candidate, Abp1, is an F-actin-binding protein (34) that is thought to provide a molecular link between the actin cytoskeleton and membrane trafficking (27, 36). Our in vitro analysis revealed that the SH3 domain of Abp1 is responsible for its association with Piccolo. Because purified components were used to reconstitute the interaction in vitro, we conclude that the interaction is direct and that no additional bridging proteins are required. Consistently, all fusion proteins lacking the SH3 domain or containing two point mutations, which abolish SH3 domain interactions, failed to bind to the Piccolo Q domain, whereas constructs containing the functional Abp1 SH3 domain bound. This is compatible with our sequence analysis of the Q domain, which revealed the presence of a degenerated decapptide repeat, most of which contain two PXXP motifs (P(A/T)KP and PQ/Q/P) that could function as SH3 domain recognition sites. The high frequency of repeats may hereby assure high binding affinity.

Our immunoprecipitation analyses demonstrated that endogenous Abp1 can associate with epitope-tagged Piccolo constructs comprising the Q domain in cell extracts. Moreover, we find that Abp1 is able to recruit Q domain-containing Piccolo constructs to the actin-rich leading edge when cells are activated with growth factor, a treatment that leads to the relocation of Abp1 to lamellipodia (35). This distribution was maintained even in the presence of detergents indicating that Abp1...
is recruiting the Piccolo Q domain to the actin cytoskeleton. Consistently, this recruitment to sites of high actin turnover was suppressed in cells transfected with the Abp1 SH3 domain. These data are consistent with our previous in vitro analysis assigning the F-actin binding capabilities of mAbp1 to the N-terminal domains (35). Our new studies also demonstrate that Abp1 has the capacity to simultaneously interact with the actin cytoskeleton and its SH3 domain binding partners such as Piccolo.

Piccolo, a Potential Organizer of the Presynaptic Actin Cytoskeleton—Actin cytoskeletal structures have long been thought to be restricted to the postsynaptic side (59, 60). During recent years, however, various studies demonstrated a role for actin in the presynapse. Actin filaments have been shown to form and disassemble in dependence of neurotransmitter release and refil cycles (61). Recently, Morales et al. (60) reported that the major GFP-actin pool in the presynaptic terminal of cultured hippocampal neurons colocaled with Bassoon, which, like Piccolo, is exclusively localized to the active zone. It is likely that at least a large portion of this pool represents G-actin, because F-actin labeling by phalloidin displayed only partial spatial overlap (60). Upon tetanic stimulation GFP-actin was observed to shift toward the synaptic junction. The yet unknown mechanism underlying this response seemed dependent on glutamate receptor activation and calcium and cAMP signaling (62). An important role for actin in the presynapse is also consistent with the identification of actin as part of the presynaptic particle web by biochemical purification and the analysis of these preparations by mass spectroscopy (30). However, no molecular links between the actin cytoskeleton and the vesicle fusion and recycling machinery were identified in these preparations. With the interaction of Abp1 and Piccolo, we have identified a possible molecular connection between F-actin structures and the active zone cytoskeletal matrix.

Piccolo has also been reported to interact with Profilin1 presumably via the capability of Profilin to bind to poly-proline regions such as those occurring in the central area of Piccolo/ Aczonin (9). Profilin1 binds to actin monomers and catalyzes the nucleotide exchange on actin (63). Because Profilin does not bind to F-actin, its interaction with Piccolo is unlikely to contribute to an association of Piccolo to the actin cytoskeleton. A more likely scenario is that Piccolo acts to dynamically retain Profilin1 near active zones where, once activated, perhaps in a synaptic activity-dependent manner, it could rapidly generate ATP-G-actin and promote F-actin assembly. Interestingly, a number of studies suggest that Abp1 may also actively participate in actin dynamics. For example, the yeast ortholog of Abp1 has been shown recently (64) to be an activator of the Arp2/3 complex promoting actin polymerization in vitro. Mammalian Abp1 shows a strong colocalization with the Arp2/3 complex at the leading edge of lamellipodia of 3T3 cells treated with growth factors (35). Although this has not been investigated in nerve terminals, the association of Abp1 with Piccolo may function in dynamic and rather stable actin structures.

Functional Implication of the Piccolo-Abp1 Interaction—Although the functional importance of a Piccolo-Abp1 interaction is still unclear, our data suggest that an association between the two molecules may occur in mature synapses but not in axonal growth cones. The most remarkable colocalization between Piccolo and Abp1 was seen in the presynaptic boutons of mature neurons. These data include double-labeled immunofluorescent microscopy and commounoprecipitation studies. In the former, we have observed, as reported previously, that Abp1 is a broadly expressed protein, present in dendrites, axons, and synapses. Although not examined here, recent immunoelectron micrograph studies, demonstrating the presence of Abp1 in both postsynaptic and presynaptic compartments (52), are consistent with an in vivo association of Piccolo and Abp1 in nerve terminals. What then could be the functional significance of the association of Abp1 with Piccolo in nerve terminals? Based on the known functions and characteristics of Piccolo and Abp1, we envision both static and possibly dynamic roles for these interactions.

Structurally, the cytoskeletal matrix associated with active zones is marked by high structural order (65). For example at the active zone, regular patches of electron dense material of about 50 nm in diameter are arranged in a web-like superstructure that is connected by fine filaments. Fine filaments were also observed to extend from the grid into the presynaptic cytosol (65). Immunogold electron micrograph studies on Piccolo have revealed that it is a structural component of the active zone cytoskeletal matrix extending some 200 nm into the presynaptic bouton (6). As such, an association of Abp1 with Piccolo may act to link either the web-like structure together or actin filaments in the bouton with the active zone. Functionally, the latter may facilitate the translocation of SVs in the reserve pool, through the presynaptic web, to the active zone plasma membrane (3–6). Clearly, careful double-labeled immunogold electron micrograph and functional studies will help to clarify these possibilities.

Alternatively, the Piccolo-Abp1 interaction may support dynamic aspects of presynaptic function, in particular SV endocytosis that is thought to depend on the precise recruitment and tethering of participating molecules. In this regard, it has been suggested that the active zone cytoskeletal matrix may help organize the endocytotic machinery and thus the site of endocytosis adjacent to active zones (3, 5, 6, 19, 29, 30). In the present study, we show that the proline-rich Q domain of Piccolo serves as molecular recognition site for the SH3 domain of Abp1 and can interfere with receptor-mediated endocytosis. Our previous studies on Abp1 are consistent with it being a cytoskeletal protein, which is also linked to membrane trafficking events in the presynapse. Of particular note, we have found that Abp1 interacts with a set of proteins involved in presynaptic membrane trafficking (Synaptojanin, Synapsins, and Dynamin1) in vitro (37). For Dynamin1, a key component of clathrin-mediated SV endocytosis, we have also demonstrated an Abp1 interaction in vitro. Dominant-negative constructs interfering with the Abp1-dynamin interaction blocked receptor-mediated uptake of transferrin in vivo (37). Together these findings lead to the view that Abp1 may interconnect membrane trafficking and the cytoskeleton in the presynapse, as well as other subdomains such as dendrites, axons, and the postsynapse. With regard to its presynaptic function, a role in SV endocytosis is the most attractive. As a structural protein of the cytoskeletal matrix at active zones, we hypothesize that Piccolo may perform a scaffold function by tethering Abp1 dynamically near sites of SV endocytosis.

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