Multiple RIBEYE–RIBEYE Interactions Create a Dynamic Scaffold for the Formation of Synaptic Ribbons

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Synaptic ribbons are large, dynamic structures in the active zone complex of ribbon synapses and important for the physiological properties of these tonically active synapses. RIBEYE is a unique and major protein component of synaptic ribbons. The aim of the present study was to understand how the synaptic ribbon is built and how the construction of the ribbon could contribute to its ultrastructural plasticity. In the present study, we demonstrate that RIBEYE self-associates using different independent approaches (yeast two-hybrid analyses, protein pull downs, synaptic ribbon–RIBEYE interaction assays, coaggregation experiments, transmission electron microscopy and immunogold electron microscopy). The A-domain [RIBEYE(A)] and B-domain [RIBEYE(B)] of RIBEYE contain five distinct sites for RIBEYE–RIBEYE interactions. Three interaction sites are present in the A-domain of RIBEYE and mediate RIBEYE(A)–RIBEYE(A) homodimerization and heterodimerization with the B-domain. The docking site for RIBEYE(A) on RIBEYE(B) is topographically and functionally different from the RIBEYE(B) homodimerization interface and is negatively regulated by nicotinamide adenine dinucleotide. The identified multiple RIBEYE–RIBEYE interactions have the potential to build the synaptic ribbon: heterologously expressed RIBEYE forms large electron-dense aggregates that are in part physically associated with surrounding vesicles and membrane compartments. These structures resemble spherical synaptic ribbons. These ribbon-like structures coassemble with the active zone protein bassoon, an interaction partner of RIBEYE at the active zone of ribbon synapses, emphasizing the physiological relevance of these RIBEYE-containing aggregates. Based on the identified multiple RIBEYE–RIBEYE interactions, we provide a molecular mechanism for the dynamic assembly of synaptic ribbons from individual RIBEYE subunits.

Key words: synaptic ribbon; ribbon synapse; RIBEYE; retina; active zones; exocytosis

Introduction

Ribbon synapses are specialized chemical synapses, e.g., in the retina and inner ear, capable to maintain rapid exocytosis of synaptic vesicles for prolonged periods of time (for review, see Fuchs, 2005; Heidelberger et al., 2005; Prescott and Zenisek, 2005; Sterling and Matthews, 2005; Nouvian et al., 2006; Singer, 2007). For this purpose, ribbon synapses are equipped with presynaptic specializations, the synaptic ribbons, which are considered to speed vesicle trafficking (for review, see tom Dieck and Brandstätter, 2006; Nouvian et al., 2006; Sterling and Matthews, 2005). Synaptic ribbons are large presynaptic structures associated with the active zone complex of ribbon synapses (for review, see Wagner, 1997). Synaptic ribbons of photoreceptor synapses are plate-like structures in three-dimensional representations that can be >500

nm in length and depth. In EM sections, retinal synaptic ribbons usually appear bar-shaped, and inner ear synaptic ribbons are usually spherical structures (for review, see Nouvian et al., 2006). Also in the retina, the assembly of the bar-shaped ribbon is believed to go through spherical ribbon intermediates, the so called synaptic spheres (for review, see Vollrath and Spiwoks-Becker, 1996; Spiwoks-Becker et al., 2004). The dimensions of synaptic ribbons in the retina can vary and are subject to changes, e.g., in response to different stimuli (lighting conditions/circadian rhythm), probably reflecting structural adaptations to different degrees of synaptic activity (for review, see Vollrath and Spiwoks-Becker, 1996; Wagner, 1997). Regardless of their shape, synaptic ribbons are associated with large amounts of synaptic vesicles and other membrane compartments (for review, see Sterling and Matthews, 2005).

We have previously identified a novel protein, “RIBEYE,” as a unique and specific component of synaptic ribbons (Schmitz et al., 2000). RIBEYE is present in synaptic ribbons of all vertebrate ribbon synapses (Schmitz et al., 2000, 2006; Zenisek et al., 2004; Khimich et al., 2005; tom Dieck et al., 2005; Wan et al., 2005) (for review, see tom Dieck and Brandstätter, 2006). RIBEYE consists of a unique A-domain [RIBEYE(A)] and B-domain [RIBEYE(B)] that is identical to CtBP2 except for the first 20 aa (Schmitz et al., 2000). The B-domain of RIBEYE binds nicotinamide adenine dinucleotide (NAD⁺ or NADH [NAD(H)]) with high affinity

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and belongs to a family of D-isomer-specific 2-hydroxy acid dehydrogenases (Schmitz et al., 2000). The structural analysis of these proteins, e.g., of CtBP1, revealed the presence of two globular subdomains, namely an NAD(H)-binding subdomain (NBD) and the substrate-binding subdomain (SBD) (for a review, see Chinnadurai, 2002; Kumar et al., 2002; Nardini et al., 2003).

Previous data indicated that RIBEYE is the major component of synaptic ribbons (Schmitz et al., 2000; Zenisek et al., 2004; Wan et al., 2005). In the present study, we analyzed functional properties of RIBEYE and demonstrate that RIBEYE is capable to interact with itself. RIBEYE–RIBEYE interactions are mediated through three binding sites in the A-domain and two binding sites in the B-domain enabling multiple RIBEYE–RIBEYE interactions. RIBEYE–RIBEYE interactions can generate the three-dimensional scaffold of the synaptic ribbon and provide a molecular mechanism for the ultrastructural plasticity of these presynaptic structures.

Materials and Methods

Plasmids. Details on all plasmids and antibodies used in this study are posted in the supplemental Methods (available at www.jneurosci.org as supplemental material).

Yeast two-hybrid methods. We used the galactosidase-4 (Gal4)-based Matchmaker Yeast Two-Hybrid System (Clontech) according to the manufacturer's instructions. The cDNA of the respective bait proteins were cloned in frame with the Gal4-DNA-binding domain of pGBKT7. The cDNA of the indicated prey proteins were cloned in frame with the Gal4-activation domain of pACT2 or pGADT7. The bait and prey plasmids confer tryptophan and leucine prototrophy to the respective auxotrophic yeast strains. Yeast strains Y187 and AH109 were used that contain distinct auxotrophic marker genes: AH109 contained *MATa*, *trp1-901*, *leu2-3,112*, *ura3-52*, *his3-200*, *gal4Δ*, *gal80Δ*, *LYS2::GAL1_{UAS}-GAL1_{TATA}-HIS3*, *GAL2_{UAS}-GAL2_{TATA}-ADE2*, and *URA3::MEL1_{UAS}-MEL1_{TATA}-lacZ* (James et al., 1996); Y187 contained *MATa*, *ura3-52*, *his3-200*, *ade2-101*, *trp1-901*, *leu2-3,112*, *gal4Δ*, *met*, *gal80Δ*, and *URA3::GAL1_{UAS}-GAL1_{TATA}-lacZ* (Harper et al., 1993). Bait plasmids were always electroporated into AH109 yeast, whereas all prey plasmids were transformed into Y187. Preparation of electrocompetent yeasts and electroporation of yeasts were done as described previously (Helmuth et al., 2001). For identifying transformants, yeasts were plated on the respective selective plates to identify the resulting revertants to the respective prototrophy (drop out media Clontech/QBiogene). For interaction analyses, AH109 yeasts containing the respective bait plasmid were mated with Y187 yeasts containing the respective prey plasmid. Mating was performed for 5 h at 30°C in 1 ml of YPD medium (yeast extract, peptone, and dextrose) with heavy vortexing. For assessing mating efficiency, half of the mated sample was streaked on –LW plates [containing synthetic complete yeast medium without leucine (L) and without tryptophan (W)], and the other half was plated on –ALWH selective plate [containing synthetic complete yeast medium without adenine (A), L, W, and histidine (H)] with 10 mM 3-amino-1,2,4-triazole added. For the matings, pSE111 and pSE112 (Bai and Elledge, 1996) as well as the empty bait and prey vectors were used as negative controls. Expression of β-galactosidase (β-gal) marker gene expression were qualitatively analyzed by filter assays and quantitatively with liquid assays as described previously (Wang et al., 1997; Stahl et al., 1999).

Expression of RIBEYE(A)-domain. RIBEYE(A)-glutathione S-transferase (GST) is difficult to express in conventional prokaryotic expression systems because of its high contents of proline, serine, glycine, and arginine residues (Schmitz et al., 2000) (our unpublished observations). We identified two expression systems to express full-length RIBEYE(A)-GST fusion protein. One source were LPAAT (lysophosphatidic acid acyltransferase)-deficient JC201 bacteria (Coleman, 1990), which express full-length RIBEYE(A)-GST fusion protein although part of it is processed to smaller fragments (see Figs. 1B, 4B, 6A, B, 10B; supplemental Fig. 1B, available at www.jneurosci.org as supplemental material). The second source

were methylotropic yeast *Pichia pastoris* (Cereghino and Cregg, 2000). Electroporation of JC201 and expression and purification of RIBEYE(A)-GST fusion protein was performed according to standard procedures (Schmitz et al., 2000). A certain degree of proteolytic processing of RIBEYE(A) is present in both of these systems. The proteolytic processing cannot be prevented even under optimized fermenting conditions using the BioFlo 110 fermenter (New Brunswick Scientific) with constant oxygenation of the medium, pH control, different induction times, and different induction temperatures (data not shown).

Intracellular expression of untagged RIBEYE(A) in *Pichia pastoris*. *Pichia pastoris* yeast strain GS115 (*his4*) (Invitrogen) was used for heterologous protein expression (Lin-Cereghino et al., 2005). Yeast cultures were grown at 30°C on synthetic minimal medium containing 0.67% yeast nitrogen base (without amino acids supplemented with ammonium sulfate and appropriate amino acid-base; Formedium). RE(A)pPIC3.5K was electroporated into freshly made electrocompetent yeasts GS115 (*his4*) as described (Lin-Cereghino et al., 2005). For electroporation, 10 μg of purified and *SalI*-linearized plasmid DNA was used. Electroporation was performed at 1500 V (BTX ECM 399 electroporator; Biogenetrix) with 2 mm gapped prechilled cuvette (Pqclab). Recombinant His⁺ clones were selected on MD (minimal dextrose) plates (0.67% yeast nitrogen base without amino acids, 0.077% CSM-His, 2% dextrose, 0.0004% biotin, 1 M sorbitol, 1.5% agar-agar). Genomic integration of the electroporated construct was confirmed through genomic PCR (5'-AOX1-primer: GACTGGTTCGAATTGCAAGC; 3'-AOX1-primer: GCAAATGGCATTCTGACATCC). For induction of fusion protein, yeasts were first cultured in BMGY medium (1% yeast extract, 2% peptone, 0.67% yeast nitrogen base without amino acids, 0.0004% biotin, 1% glycerol, 0.1 M potassium-phosphate buffer, pH 6) at 30°C to an optical density at 600 nm (OD₆₀₀) of 2–6. Induction was achieved in BMMY medium (same as BMGY with 0.5% methanol instead of glycerol) at 30°C, starting the culture at an OD₆₀₀ of 1. After every 24 h, methanol was replenished to the final volume of 0.5%. After 36 h of induction, the cells were pelleted (1500 rpm, 5 min, 4°C) and processed for extraction of fusion protein. Induced *Pichia pastoris* yeasts were mechanically cracked with 0.5 mm glass beads (Biospec/Roth) in breaking buffer (50 mM sodium phosphate, pH 7.4, 100 mM NaCl, 1 mM EDTA, 5% glycerol, 1 mM PMSF). For this purpose, 100 μl cell pellet were resuspended in 890 μl of ice-cold breaking buffer. To this mixture, ~500 μl of glass beads were added. The cracking was performed at +4°C using high-speed vortex (25× vortexing for 30 s; between vortexing, samples were chilled 30 s on ice). The lysate was centrifuged twice (13,000 rpm, 1 h at 4°C). Subsequently, the supernatant was precleared with 20 μl empty glutathione-agarose beads (Fluka) for 1 h at 4°C on a rotary wheel.

Protein pull-down assays using bacterial fusion protein. For pull-down experiments using pairs of GST- and maltose-binding protein (MBP)-tagged fusion proteins, the GST-tagged fusion proteins were usually kept immobilized on glutathione beads whereas MBP fusion proteins were used as solubilized prey proteins if not denoted otherwise. Bait and prey proteins were used in equimolar amounts along with the respective control proteins. Protein concentrations were determined using the Bradford method (Bradford, 1976). For pull-down experiments, fusion protein eluates were precleared with 10 μl of empty glutathione Sepharose beads (per 1 ml of eluate) for 1 h at 4°C. Binding was performed in PBS that contained 0.5% Triton X-100 at 4°C for 12 h on a rotary wheel (500 μl incubation volume) if not denoted otherwise. Pellets were washed five times by adding an excess of PBS/Triton X-100 and subsequent spinning (13,000 rpm, 1 min, 4°C). Pellets were boiled in SDS-sample buffer and subsequently subjected to Western blot analyses with the indicated antibodies.

Miscellaneous methods. For the preparation of synaptic ribbons, synaptic ribbons were purified as described previously (Schmitz et al., 1996, 2000). Standard protein techniques were performed as described previously (Schmitz et al., 2000). For reprobing of Western blots, nitrocellulose sheets were treated with prewarmed (90°C) stripping buffer (1% SDS, 10 mM β-mercaptoethanol in PBS) and incubated at room temperature for 1 h. Immunofluorescence microscopy was performed as described previously (Schmitz et al., 2000, 2006) using a Zeiss Axiovert 200M microscope (Carl Zeiss) equipped for conventional epifluorescence microscopy with the respective filter sets for enhanced green fluorescent protein (EGFP) and monomeric red fluorescent protein (mRFP)

and equipped with an Apotome (Zeiss) to make optical sections. Transfection of COS cells was done as described previously with the DEAE-dextran method (Schmitz et al., 2000). R28 cells were transfected by lipofection using perfectin (PepLab) according to the manufacturer's instructions. Transfected cells were usually analyzed by fluorescence microscopy 48 h after transfection if not denoted otherwise. Conventional transmission, immunogold electron microscopy, and quantification of radioactive NAD^+ -binding to RIBEYE fusion proteins were performed as described previously (Schmitz et al., 1996, 2000). Thrombin cleavage of GST-tagged fusion protein was performed mostly as described previously (Chadli et al., 2000).

Results

Homodimerization of RIBEYE(A)

We used the yeast two-hybrid (YTH) system to determine whether the A-domain of RIBEYE can homodimerize. In YTH, we observed a strong self-interaction between the A-domains of RIBEYE as judged by growth on $-\text{ALWH}$ -selective plates and expression of the β -galactosidase marker gene activity compared with the respective control matings (Fig. 1A, mating 1; supplemental Fig. 1A, available at www.jneurosci.org as supplemental material). RIBEYE(A) also interacted with full-length RIBEYE [RIBEYE(AB)] (Fig. 1A, mating 6). The RIBEYE-expressing yeasts were not autoactivating in YTH, as demonstrated by the lack of growth on $-\text{ALWH}$ plates and analysis of β -galactosidase expression of the respective control matings (Fig. 1A, matings 2–5, 7, 8). Quantitative β -galactosidase activities determined in liquid assays are shown in supplemental Figure 1A (available at www.jneurosci.org as supplemental material). The homodimerization of RIBEYE(A) observed in the YTH system was also confirmed at the protein level using two different pull-down assays (Fig. 1B; supplemental Fig. 1B, available at www.jneurosci.org as supplemental material). Immobilized RIBEYE(A)-MBP fusion protein (but not immobilized MBP alone) bound soluble RIBEYE(A)-GST fusion protein (but not GST alone) (Fig. 1B). Similarly, immobilized RIBEYE(A)-GST specifically bound RIBEYE(A) from crude protein extracts of RIBEYE(A)-transgenic *Pichia pastoris* (supplemental Fig. 1B, available at www.jneurosci.org as supplemental material). GST control protein alone did not bind RIBEYE(A) from the *Pichia pastoris* extract. Thus, both YTH and protein pull-down data independently demonstrated that RIBEYE(A) interacts with RIBEYE(A).

Mapping of RIBEYE(A)–RIBEYE(A) interaction

In the rat, RIBEYE(A) consists of the N-terminal 563 aa. To map the interaction sites important for RIBEYE(A)–RIBEYE(A)-interaction, we generated C- and N-terminal deletion constructs of RIBEYE(A) and tested them for their capability to interact with

A) RIBEYE(A) interacts with RIBEYE(A) in YTH

B) RIBEYE(A) interacts with RIBEYE(A) in pull-down assays

Figure 1. RIBEYE(A) interacts with RIBEYE(A). **A**, RIBEYE(A) interacts with RIBEYE(A) in YTH. Summary plates of YTH analyses obtained with the indicated bait and prey plasmids. The indicated yeast clones growing on selective medium either for the presence of bait and prey plasmids ($-\text{LW}$ dropout medium) or selective for protein–protein interaction ($-\text{ALWH}$ dropout medium). For convenience, experimental bait–prey pairs are underlayered in color (green in case of interacting bait–prey pairs; control matings are not colored). RIBEYE(A) interacts with RIBEYE(A) and RIBEYE(AB) as judged by growth on selective plates ($-\text{ALWH}$) and expression of β -galactosidase expression (yeast matings 1, 6; **Ab, Ac**). The respective control matings (autoactivation controls; yeast matings 2–5, 7–8) did not show growth on $-\text{ALWH}$ plates and expression of β -galactosidase activity. For quantification of the β -gal activities, see also supplemental Figure 1A (available at www.jneurosci.org as supplemental material). Growth on $-\text{LW}$ plates (**Aa**) demonstrates the presence of the bait and prey plasmids in the mated yeasts. **Ba, Bb**, RIBEYE(A) interacts with RIBEYE(A) in protein pull-down experiments (Western blot analyses). RIBEYE(A)-MBP and MBP alone (control) were used as immobilized bait proteins and RIBEYE(A)-GST and GST alone (control) as soluble prey proteins. RIBEYE(A)-GST specifically binds to RIBEYE(A)-MBP (**Ba**, lane 5, arrowhead). RIBEYE(A)-GST does not bind to MBP alone (**Ba**, lane 6). GST alone also does not bind to RIBEYE(A)-MBP (**Ba**, lane 7). **Bb** shows the same blot as in **Ba** after stripping and reprobing of the nitrocellulose with anti-MBP antibodies to show equal loading of bait proteins. RIBEYE(A)-GST also specifically binds intracellularly expressed RIBEYE(A) from a crude extract of RIBEYE(A)-transgenic *Pichia pastoris* (supplemental Fig. 1B, available at www.jneurosci.org as supplemental material). RE(AB), full-length RIBEYE; RE(A), RIBEYE(A).

full-length RIBEYE(A) in YTH (Fig. 2A,B; supplemental Fig. 2, available at www.jneurosci.org as supplemental material). Most of the C-terminal region of the A-domain could be removed without abolishing the interaction with RIBEYE(A). RIBEYE(A)1–105 was the shortest N-terminal construct that could interact with full-length RIBEYE(A)-domain (Fig. 2B, prey 7). Therefore, the first 105 N-terminal amino acids contain a binding site for RIBEYE(A), which is subsequently denoted as the “A1” interaction site. We also analyzed N-terminal deletions of RIBEYE(A) for their interaction with full-length RIBEYE(A) (Fig. 2B; supplemental Fig. 2, available at www.jneurosci.org as supplemental material). Interestingly, N-terminal deletion constructs of RIBEYE that did not contain the previously identified RIBEYE(A1)-binding site also interacted with RIBEYE(A) (Fig.

Mapping of RIBEYE(A)–RIBEYE(A) interactions

Figure 2. Mapping of RIBEYE(A)–RIBEYE(A) interactions. **A**, Schematic domain structure of RIBEYE showing the N-terminal A-domain and the C-terminal B-domain. **B**, **C**, Summary of mapping analyses. The indicated bait and prey plasmids were used to test for the interaction of the respective proteins in the YTH system. YTH analyses of the C-terminal deletion constructs of RIBEYE(A) reveal an N-terminal site (within the first 105 aa) that interacts with RIBEYE(A). This interaction site is denoted as A1 (**C**). YTH analyses of N-terminal deletion constructs of RIBEYE(A) reveal a second interaction site in the C-terminal region of RIBEYE(A) that interacts with RIBEYE(A). This C-terminal interaction site is denoted as A2 (**C**) and covers amino acids 438–563. The A3 region (**C**) in the middle of RIBEYE(A) (amino acids 106–363) that does not contain A1 and A2 is also able to interact with full-length RIBEYE(A). **C**, Schematic representation of the identified RIBEYE–RIBEYE interaction modules (A1, A2, A3) in the A-domain of RIBEYE. **D–G**, The indicated minimal interaction modules (A1, A2, and A3) were tested in the YTH system for interaction with each other. For convenience, experimental bait–prey pairs are underlined in color (green for interacting bait–prey pairs; yellow for noninteracting bait–prey pairs); control matings are not colored. **D**, RIBEYE(A1) interacts with RIBEYE(A1) (mating 1). Matings 2–5 show the respective indicated control matings. **E**, RIBEYE(A2) interacts with RIBEYE(A2) (mating 6). Matings 7–10 show the respective indicated control matings. **F**, RIBEYE(A1) interacts with RIBEYE(A2) (mating 11). Matings 12–15 show the respective indicated control matings. **G**, RIBEYE(A1) and RIBEYE(A3) interact with RIBEYE(A3) (matings 21, 16). RIBEYE(A3) does not interact with RIBEYE(B) (mating 27). Matings 22, 23, 17–20, 28, and 29 show the respective indicated control matings. No used RIBEYE constructs were autoactivating. RIBEYE(A2) does not interact with RIBEYE(A3) (mating 24). Matings 25 and 26 show the respective control matings. RE(A), RIBEYE(A); RE(B), RIBEYE(B).

2B, preys 9, 10; supplemental Fig. 2, available at www.jneurosci.org as supplemental material), pointing to a second homodimerization site in the C-terminal region of RIBEYE(A). We identified RIBEYE(A)438–563 as the smallest C-terminal portion of RIBEYE(A) that interacts with RIBEYE(A) (Fig. 2B; supplemental Fig. 2, available at www.jneurosci.org as supplemental material). This C-terminal RIBEYE(A) interaction site is denoted as “A2” in the following text. We further tested whether the midregion of RIBEYE(A), which does neither contain the N-terminal A1 interaction site nor the C-terminal A2 interaction site for its capability to interact with RIBEYE(A). This region in the midportion of RIBEYE(A), denoted as “A3,” also interacted with RIBEYE(A) (Fig. 2B, prey 11). Thus, the A-domain of RIBEYE has three independent sites which are able to interact with full-length RIBEYE(A) (summarized in Fig. 2C). Supplemental Figure 2 (available at www.jneurosci.org as supplemental material) demonstrates that all of the tested RIBEYE constructs were not autoactivating as judged by the absence of growth on –ALWH and lack of expression of β -galactosidase activity.

The A1, A2, and A3 interaction modules in the A-domain of RIBEYE allow multiple RIBEYE–RIBEYE interactions

Next, we tested whether the identified RIBEYE(A1), RIBEYE(A2), and RIBEYE(A3) interaction modules in the A-domain of RIBEYE could interact with each other. We tested all possible interaction combinations between RIBEYE(A1), RIBEYE(A2) and RIBEYE(A3) in the YTH system and found that multiple interactions could take place between them. RIBEYE(A1) interacts with RIBEYE(A1), RIBEYE(A2), and RIBEYE(A3) (Fig. 2D,F,G). Similarly, RIBEYE(A2) interacted with RIBEYE(A2) and RIBEYE(A1) but not RIBEYE(A3) (Fig. 2E–G). RIBEYE(A3) interacted with RIBEYE(A1) and RIBEYE(A3) but not RIBEYE(A2) (Fig. 2G). All of these interactions between RIBEYE(A) subdomains characterized in the YTH system were confirmed by protein pull-down analyses using the respective fusion proteins (Fig. 3A–D,F).

Homodimerization of RIBEYE(B)

With the YTH system, we confirmed homodimerization of the B-domain of RIBEYE (supplemental Fig. 3A,B, available at www.jneurosci.org as supplemental material). The homodimerization of RIBEYE(B) is not very surprising: CtBP2,

Figure 3. Multiple RIBEYE–RIBEYE interactions identified by YTH are confirmed by fusion protein pull-down experiments. Interaction analyses of the indicated RIBEYE subdomains in fusion protein pull-down analyses. GST-tagged RIBEYE proteins were used as immobilized bait proteins and eluted MBP-tagged RIBEYE proteins as soluble prey proteins. Binding of the prey proteins was analyzed by Western blotting with antibodies against MBP. Equal loading was verified by reprobing the respective blot (after stripping) with antibodies against GST. **Aa, Ab**, RIBEYE(A1) interacts with RIBEYE(A1). RIBEYE(A1)-GST (lanes 5, 6) and GST alone (lanes 7, 8; control protein) were tested for their ability to pull down RIBEYE(A1)-MBP. MBP alone served as control prey protein. Only RIBEYE(A1)-GST pulled down RIBEYE(A1)-MBP (lane 5) but not the control bait protein GST (lane 7). RIBEYE(A1)-GST does not pull down MBP alone (lane 6). **Ab** shows the same blot as in **Aa** after stripping and reprobing of the nitrocellulose with anti-GST antibodies to show equal loading of bait proteins. Lanes 1–4 show the indicated input proteins. **Ba, Bb**, RIBEYE(A2) interacts with RIBEYE(A2). RIBEYE(A2)-GST (lanes 5, 6) and GST alone (lanes 7, 8; control protein) were tested for their ability to pull down RIBEYE(A2)-MBP. MBP alone served as control prey protein. Only RIBEYE(A2)-GST pulled down RIBEYE(A2)-MBP (lane 5) but not the control bait protein GST (lane 7). RIBEYE(A2)-GST does not pull down MBP alone (lane 6). **Bb** shows the same blot as in **Ba** after stripping and reprobing of the nitrocellulose with anti-GST antibodies to show equal loading of bait proteins. Lanes 1–4 show the indicated input proteins. **Ca, Cb**, RIBEYE(A3) interacts with RIBEYE(A3). RIBEYE(A3)-GST (lanes 5, 6) and GST alone (lanes 7, 8; control protein) were tested for their ability to pull down RIBEYE(A3)-MBP. MBP alone served as control prey protein. Only RIBEYE(A3)-GST pulled down RIBEYE(A3)-MBP (lane 5) but not the control bait protein GST (lane 7). RIBEYE(A3)-GST does not pull down MBP alone (lane 6). **Cb** shows the same blot as in **Ca** after stripping and reprobing of the nitrocellulose with anti-GST antibodies to show equal loading of bait proteins. Lanes 1–4 show the indicated input proteins. **Da, Db**, RIBEYE(A2) interacts with RIBEYE(A1). RIBEYE(A2)-GST (lanes 5, 6) and GST alone (lanes 7, 8; control protein) were tested for their ability to pull down RIBEYE(A1)-MBP. MBP alone served as control prey protein. Only RIBEYE(A2)-GST pulled down RIBEYE(A1)-MBP (lane 5) but not the control bait protein GST (lane 7). RIBEYE(A2)-GST does not pull down MBP alone (lane 6). **Db** shows the same blot as in **Da** after stripping and reprobing of the nitrocellulose with anti-GST antibodies to show equal loading of bait proteins. Lanes 1–4 show the

which is identical to RIBEYE(B) (except for the first 20 aa) has been shown previously to homodimerize (Thio et al., 2004). CtBP1 also homodimerizes (Sewalt et al., 1999; Balasubramanian et al., 2003), and the structure of the CtBP1 dimer (tCtBP1) has been resolved (Kumar et al., 2002; Nardini et al., 2003). NAD(H) was found to stimulate the homodimerization of both CtBP1 and CtBP2 (Balasubramanian et al., 2003; Thio et al., 2004).

RIBEYE(B) also interacted with RIBEYE full-length protein indicating that the A-domain of RIBEYE does not prevent homodimerization of RIBEYE(B)-domains (supplemental Fig. 3A,B, available at www.jneurosci.org as supplemental material). The homodimerization of RIBEYE(B) is dependent on amino acids 689–716, which form the α B-loop- α C motif [homodimerization loop (HDL)] of RIBEYE(B) as judged by homology modeling (supplemental Fig. 3D, available at www.jneurosci.org as supplemental material). The α B-loop- α C motif in CtBP1 is important for homodimerization of CtBP1 (Nardini et al., 2003). In agreement with this prediction, homodimerization of RIBEYE(B) is completely abolished if the HDL is deleted (supplemental Fig. 3C, matings 1, 2, available at www.jneurosci.org as supplemental material). RIBEYE(B) Δ HDL no longer interacted with RIBEYE(B) (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). The RIBEYE(B) homodimerization interface is denoted as “B1” in the following text.

Heterodimerization of RIBEYE(B) and RIBEYE(A)

We used the YTH system to test whether RIBEYE(B) can also interact with

indicated input proteins. **Ea, Eb**, RIBEYE(B) interacts with RIBEYE(A2). RIBEYE(B)-GST (lanes 5, 6) and GST alone (lanes 7, 8; control protein) were tested for their ability to pull down RIBEYE(A2)-MBP. MBP alone served as control prey protein. Only RIBEYE(B)-GST pulled down RIBEYE(A2)-MBP (lane 5) but not the control bait protein GST (lane 7). RIBEYE(B)-GST does not pull down MBP alone (lane 6). **Eb** shows the same blot as in **Ea** after stripping and reprobing of the nitrocellulose with anti-GST antibodies to show equal loading of bait proteins. Lanes 1–4 show the indicated input proteins. **Fa, Fb**, RIBEYE(A3) interacts with RIBEYE(A1). RIBEYE(A3)-GST (lanes 5, 6) and GST alone (lanes 7, 8; control protein) were tested for their ability to pull down RIBEYE(A1)-MBP. MBP alone served as control prey protein. Only RIBEYE(A3)-GST pulled down RIBEYE(A1)-MBP (lane 5) but not the control bait protein GST (lane 7). RIBEYE(A3)-GST does not pull down MBP alone (lane 6). **Fb** shows the same blot as in **Fa** after stripping and reprobing of the nitrocellulose with anti-GST antibodies to show equal loading of bait proteins. Lanes 1–4 show the indicated input proteins. RE(A), RIBEYE(A); RE(B), RIBEYE(B).

A) RIBEYE(A) interacts with RIBEYE(B) in YTH

B) RIBEYE(A) interacts with RIBEYE(B) in pull-down assays

Figure 4. RIBEYE(A) interacts with RIBEYE(B). **A**, Analyses of RIBEYE(A)–RIBEYE(B) interactions using the YTH system. Summary plates of YTH analyses obtained with the indicated bait and prey plasmids. For convenience, experimental bait–prey pairs are underlayered in color (green for interacting bait–prey pairs; control matings are not colored). RIBEYE(A) interacts with RIBEYE(B) as judged by growth on –ALWH plates and expression of β -galactosidase activity (**Ab, Ac**, mating 1). **Ba, Bb**, RIBEYE(A) interacts with RIBEYE(B) in protein pull-down experiments. RIBEYE(A)-GST and GST alone (control protein) were used as immobilized bait proteins and RIBEYE(B)-MBP and MBP alone (control protein) as soluble prey proteins. After incubation, binding of the soluble prey proteins to the immobilized bait proteins was tested by Western blotting with the indicated antibodies. **Ba**, RIBEYE(B)-MBP binds to RIBEYE(A)-GST (lane 5, arrowhead) but not to GST alone (lane 6). MBP alone binds neither to RIBEYE(A)-GST (lane 7) nor to GST alone (lane 8). **Bb**, The same blot as in **Ba** after stripping and reprobing of the nitrocellulose with anti-GST antibodies to show equal loading of bait proteins. RE(A), RIBEYE(A); RE(B), RIBEYE(B).

RIBEYE(A). RIBEYE(B) showed a robust interaction with RIBEYE(A) in the YTH system as judged by growth on –ALWH selective plates and β -galactosidase marker gene expression (Fig. 4, mating 1; supplemental Figs. 4, 5, mating 1, available at www.jneurosci.org as supplemental material). This interaction between RIBEYE(A) and RIBEYE(B) was verified at the protein level using protein pull-down analyses (Fig. 4B). RIBEYE(A)-GST fusion protein, but not GST alone, specifically interacted with RIBEYE(B)-MBP fusion protein (but not with MBP alone) as judged by protein pull-down analyses (Fig. 4B). We used the YTH system to map the respective interaction sites for RIBEYE(B)–RIBEYE(A) interaction. Mapping analyses revealed that the A2 interaction site in the C-terminal portion of the RIBEYE(A) is the binding site for RIBEYE(B) (Fig. 5; supplemental Fig. 4B, available at www.jneurosci.org as supplemental ma-

terial). On RIBEYE(B), the NBD is responsible for the interaction with RIBEYE(A) (Fig. 5C; supplemental Fig. 4C, available at www.jneurosci.org as supplemental material). These mapping data obtained by YTH analyses were confirmed by protein–protein pull-down analyses that showed interaction between RIBEYE(A2) and RIBEYE(B) (Fig. 3E). We used deletion and point mutants of RIBEYE(B) to further analyze the binding site of RIBEYE(A) on the NBD of RIBEYE(B) in detail. First, we tested whether the RIBEYE(B) Δ HDL deletion mutant that is no longer able to homodimerize with RIBEYE(B) (supplemental Fig. 3C,D, available at www.jneurosci.org as supplemental material) is still able to interact with RIBEYE(A). Indeed, RIBEYE(B) Δ HDL is able to interact with RIBEYE(A) as well as with full-length RIBEYE [RIBEYE(AB)] (Fig. 5C; supplemental Figs. 4C, 7, available at www.jneurosci.org as supplemental material). Next, we

Mapping of RIBEYE(A)–RIBEYE(B) interaction

Figure 5. Mapping of RIBEYE(A)–RIBEYE(B) interaction in YTH. **A**, Schematic domain structure of RIBEYE. The A-domain of RIBEYE is depicted in blue, the SBD of RIBEYE(B), which consists of the N- and C-terminal portions of the B-domain, is depicted in red. The HDL is depicted in green within the yellow-labeled NBD of RIBEYE(B). **B**, **C**, The indicated bait and prey plasmids were used to test for the interaction of the respective proteins in the YTH system. **B**, YTH analyses of C- and N-terminal deletion constructs of RIBEYE(A) reveal that the RIBEYE(A2) site is responsible for interaction with RIBEYE(B). **C**, YTH analyses of the indicated constructs of RIBEYE(B) reveal that the NBD (prey 2), but not the SBD (prey 3), is responsible for interaction with RIBEYE(A). The RIBEYE(B) HDL is not essential for the interaction between RIBEYE(A) and RIBEYE(B). RIBEYE(B)ΔHDL (prey 1) still interacts with RIBEYE(A). **Da**, **Db**, Summary of the location of distinct amino acids on the NBD that are essential for binding of RIBEYE(A). If these amino acids on the NBD of RIBEYE(B) are point mutated, RIBEYE(A) can no longer bind to RIBEYE(B) (supplemental Fig. 5, available at www.jneurosci.org as supplemental material). The lack of binding of the RIBEYE(B) point mutants to RIBEYE(A) is not caused by misfolding of the respective point mutants because all of these RIBEYE(B) point mutants homodimerized with RIBEYE(B) (supplemental Fig. 6, available at www.jneurosci.org as supplemental material). **Da**, A lateral view of the NBD of RIBEYE(B). **Db**, A top view on the NBD from the position of the bound NADH to the "bottom" of the molecule as seen in **Da**. RE(A), RIBEYE(A); RE(B), RIBEYE(B).

tested different point mutants of the NBD of RIBEYE(B) for their interaction with RIBEYE(A) and RIBEYE(B). We analyzed RIBEYE(B) point mutants RIBEYE(B)G730A, D758N, I796A, E844Q, F848W, and K854Q, which are located at the outer face of the NBD (Fig. 5D). All of these point mutations did not prevent homodimerization with RIBEYE(B) (supplemental Figs. 5C, 6, available at www.jneurosci.org as supplemental material). Furthermore, RIBEYE(B)D758N, I796A, E844Q, F848W, and K854Q bound NADH as judged by NADH-dependent energy transfer from tryptophan W867 to bound NADH [performed as described by Fjeld et al. (2003)] (data not shown), demonstrating the proper folding of these point mutants. Although these point

mutants did not prevent homodimerization of RIBEYE(B), all of these point mutations [except for RIBEYE(B)K854Q] completely abolished interaction with RIBEYE(A) (supplemental Fig. 5A–C, available at www.jneurosci.org as supplemental material). This shows that the two binding interfaces on RIBEYE(B) available for interaction with RIBEYE(A) and RIBEYE(B) are distinct from each other, albeit spatially closely related. The binding site for RIBEYE(A) covers a large portion of the NBD (Fig. 5D; supplemental Fig. 5, available at www.jneurosci.org as supplemental material). Interestingly, RIBEYE(B)G730, which is an essential component of the conserved NAD(H)-binding motif of RIBEYE (Schmitz et al., 2000), appears to be part of the interaction interface for RIBEYE(A): the point mutant RIBEYE(B)G730A (Fig. 5D) that does not bind NAD(H) (supplemental Fig. 5D, available at www.jneurosci.org as supplemental material) can no longer interact with RIBEYE(A) (supplemental Fig. 5C, available at www.jneurosci.org as supplemental material). We interpret the latter result to mean that the binding sites for NAD(H) and for RIBEYE(A) are overlapping to a certain extent (see below and Discussion). The docking site on RIBEYE(B) for RIBEYE(A) is denoted as "B2" in the following text.

NADH and NAD⁺ inhibit RIBEYE(A)–RIBEYE(B) interaction

Because RIBEYE(A) docks to a broad interface of the NAD(H)-binding subdomain of RIBEYE(B), we analyzed whether this interaction is dependent on NAD(H). To analyze this question, we applied the pull-down assay described in Materials and Methods. We used RIBEYE(A)-GST as immobilized bait and eluted RIBEYE(B)-MBP as soluble prey protein and checked for interaction of these proteins in the presence of increasing concentrations of NADH/NAD⁺ (Fig. 6). Increasing concentrations of NADH/NAD⁺ strongly inhibited RIBEYE(A)–RIBEYE(B) interaction. Both NAD⁺ as well as NADH strongly inhibited RIBEYE(A)–RIBEYE(B) interaction already at low physiological concentrations. The tested concentrations of NAD(H) are within the cellular concentration range of NAD(H) known from other studies (Zhang et al., 2002; Fjeld et al., 2003). The NAD(H) concentrations did not have any influence on the control pull downs. At all NADH/NAD⁺ concentrations used, there was no unspecific binding of RIBEYE(B)-MBP to GST alone (supplemental Fig. 8, available at www.jneurosci.org as supplemental material). The identified interactions between the different subdomains of RIBEYE and their regulation via NAD(H) are summarized in Figure 11B.

RIBEYE coaggregates with other RIBEYE molecules in transfected R28 and COS cells

To determine whether the identified RIBEYE–RIBEYE interactions can also occur within the cellular context, we performed cell transfections with the indicated RIBEYE expression constructs that were differentially tagged either with EGFP or with mRFP. For transfection, we used COS7 cells and the R28 retinal progenitor cell line (Seigel, 1996; Seigel et al., 2004). R28 cells express retinal and neuronal marker proteins (e.g., opsins, β-2 arrestin, recoverin, neurotransmitter receptors, and various presynaptic and postsynaptic proteins) in addition to stem cell/precursor cell markers (e.g., nestin) (Seigel et al., 2004). If transfected alone, both RIBEYE(A) as well as RIBEYE(B) displayed a discrete, spot-like distribution, whereas RIBEYE(B) is diffusely distributed (Fig. 7; supplemental Figs. 9–11, available at www.jneurosci.org as supplemental material), as also described previously (Schmitz et al., 2000). If RIBEYE(A)-EGFP was cotrans-

NAD⁺ and NADH inhibit RIBEYE(A)–RIBEYE(B) interaction

Figure 6. NADH and NAD⁺ inhibit RIBEYE(A)–RIBEYE(B) interaction. **Aa–Bb**, Immobilized RIBEYE(A)–GST fusion protein (0.3 μ M) was incubated with 0.3 μ M RIBEYE(B)–MBP in the presence of the indicated concentrations of NAD⁺ (**Aa, Ab**) or NADH (**Ba, Bb**) for 3 h at 4°C in binding buffer (100 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100). After several washes with binding buffer, the pellets were boiled with SDS-sample buffer and analyzed for binding of RIBEYE(B)–MBP by Western blot analyses and probing of the Western blots with anti-MBP antibodies. Both NAD⁺ and NADH strongly inhibited binding of RIBEYE(B) to RIBEYE(A). **Ab, Bb**, Respective loading controls in which the same blots as shown in **Aa** and **Ba** were incubated with GST antibodies after stripping of the respective blots. RE(A), RIBEYE(A); RE(B), RIBEYE(B).

fectured with RIBEYE(A)-mRFP both coaggregated to the same protein clusters as judged by the large extent of colocalization of the EGFP and mRFP signals (Fig. 7C, arrows; supplemental Figs. 10C, 11A, available at www.jneurosci.org as supplemental material). Identical results were obtained when full-length RIBEYE(AB)-EGFP was cotransfected with RIBEYE(A)-mRFP (Fig. 7B, arrows). If RIBEYE(B)-EGFP was cotransfected with RIBEYE(B)-mRFP, both signals remained diffusely distributed (Fig. 7E). Interestingly, whenever RIBEYE(B)-mRFP was cotransfected with RIBEYE(A)-EGFP, RIBEYE(B)-mRFP redistributed from a diffuse distribution [as typical for single transfected RIBEYE(B)] to a patchy, spot-like distribution that is typical for RIBEYE(A) (Fig. 7D, arrow). Part of RIBEYE(B) remained diffusely distributed (Fig. 7D, arrowhead; supplemental Fig. 10D,E, available at www.jneurosci.org as supplemental material) probably because of the NAD(H) sensitivity of the RIBEYE(B)–RIBEYE(A) interaction (see above). NAD(H) is ubiquitously present in the cytoplasm and expected to partly dissociate RIBEYE(A)–RIBEYE(B) complexes. Interestingly, in cells double-transfected with full-length RIBEYE(AB) and RIBEYE(B), RIBEYE(B) virtually completely redistributed from the diffuse distribution to the spot-like distribution typical for RIBEYE(AB) and perfectly colocalized with RIBEYE(AB) (Fig. 7A, arrows; supplemental Figs. 9, 10B, 11B, available at www.jneurosci.org as supplemental material). From these latter experiments, we conclude that both homotypic domain interactions [RIBEYE(B)–RIBEYE(B) interactions] as well as heterotypic domain interactions [RIBEYE(A)–RIBEYE(B) interactions] support the interaction between RIBEYE(AB) and RIBEYE(B). As judged by the nearly complete colocalization of RIBEYE(AB) and RIBEYE(B) compared with cells double-transfected with RIBEYE(A) and RIBEYE(B), we assume that a combination of homotypic and heterotypic domain interactions is probably stronger than a single type of homotypic interactions. Qualitatively identical results were obtained for R28 cells (Fig. 7; supplemental Fig. 9, available at www.jneurosci.org as supplemental material) and COS cells (supplemental Figs. 10, 11, available at

www.jneurosci.org as supplemental material). Also in COS cells, RIBEYE(A) coaggregated with RIBEYE(AB) (supplemental Fig. 10A, available at www.jneurosci.org as supplemental material) and RIBEYE(A) (supplemental Figs. 10C, 11A, available at www.jneurosci.org as supplemental material). Similarly, RIBEYE(B) translocated from a completely diffuse distribution to a spot-like distribution if cotransfected with RIBEYE(A) or RIBEYE(AB) [in 98 and 99%, respectively, of double-transfected cells in 100 randomly picked double-transfected cells vs 3% spot-like distribution in cells transfected with RIBEYE(B) only].

Interestingly, if RIBEYE(AB)-EGFP-transfected cells were analyzed already a few hours after transfection, the RIBEYE(AB)-containing aggregates appeared smaller and more numerous than at later time points suggesting that the smaller protein clusters could mature/coalesce to the bigger protein aggregates that are predominant at later time points (supplemental Fig. 9, available at www.jneurosci.org as supplemental material).

www.jneurosci.org as supplemental material).

In conclusion, the coaggregation and colocalization data in the transfected COS and R28 cells indicate that the interaction sites between RIBEYE(A) and RIBEYE(B), either between the same type of domains (A–A, B–B) or between different domains (A–B), are also available within a cellular context.

Electron microscopy of RIBEYE-containing aggregates in transfected R28 cells

RIBEYE is the major component of synaptic ribbons and RIBEYE forms large protein aggregates in transfected cells (Fig. 7). We analyzed the ultrastructural appearance of the RIBEYE-containing aggregates by electron microscopy to find out whether these structures have similarities with synaptic ribbons (Fig. 8). Using conventional transmission electron microscopy, we observed large electron-dense aggregates in RIBEYE-EGFP-transfected R28 cells (Fig. 8A–J), which were absent in control cells (K). Similar, large electron-dense protein aggregates were also present in RIBEYE(AB)-EGFP-transfected COS cells but not in EGFP-transfected COS cells (data not shown). The large aggregates typically displayed a spherical shape with a diameter between of 200–500 nm. These electron-dense structures were often surrounded by vesicles which in part were physically attached to the electron-dense aggregates via thin electron-dense stalks (Fig. 8A–J, arrowheads). These large spherical structures were strongly positive for RIBEYE by immunogold labeling with antibodies against RIBEYE (Fig. 8L–N) but not reactive with antibodies against tubulin (O) or RIBEYE preimmune serum (P) (control incubations). These spherical structures have similarities to spherical synaptic ribbons of inner hair cells (for review, see Novian et al., 2006). Beside the large electron-dense particles we also found smaller aggregates which showed physical contacts between each other and which sometimes appeared to coalesce into larger, electron-dense structures (Fig. 8E,F). These structures were also partly physically linked to surrounding vesicles and show some resemblance to synaptic spheres, intermediate structures in the assembly and disassembly of synaptic ribbons

Co-clustering of different RIBEYE proteins in co-transfected R28 cells

Figure 7. Codistribution of different RIBEYE-proteins in cotransfected R28 cells. **A–H**, R28 cells were transfected with the indicated mRFP- or EGFP-tagged RIBEYE constructs. Transfected cells were analyzed for the intracellular distribution of the respective proteins via direct epifluorescence microscopy. RIBEYE(AB) (**H**) and RIBEYE(A) (**I**) show a discrete spot-like distribution, as already shown previously (Schmitz et al., 2000). In contrast, RIBEYE(B) is diffusely distributed in single-transfected cells (Schmitz et al., 2000) (**F, G**). **A**, If RIBEYE(B) is cotransfected with RIBEYE(AB) (supplemental Fig. 9, available at www.jneurosci.org as supplemental material), RIBEYE(B) virtually completely redistributed from a diffuse distribution into a spot-like, RIBEYE(AB)-typical distribution and colocalized with RIBEYE(AB) (arrows) (supplemental Fig. 9A, B, available at www.jneurosci.org as supplemental material). **D**, RIBEYE(B) also redistributed from a diffuse to spot-like distribution if cotransfected with RIBEYE(A) (supplemental Fig. 9C, available at www.jneurosci.org as supplemental material); part of RIBEYE(B) remained diffusely distributed (arrowhead). The higher degree of codistribution of RIBEYE(B) with RIBEYE(AB) compared with RIBEYE(A) probably

(for review, see Vollrath and Spiwok-Becker, 1996) (see Discussion).

RIBEYE coaggregates at bassoon-containing sites in retinal R28 progenitor cells

To further address the physiological relevance of the RIBEYE aggregates, we tested whether these structures are related to bassoon, a physiological interaction partner of RIBEYE at the active zone of ribbon synapses (tom Dieck et al., 2005). Bassoon is endogenously expressed in R28 retinal precursor cells as judged by immunocytochemistry (Fig. 9), Western blotting (supplemental Fig. 9D, available at www.jneurosci.org as supplemental material) and reverse transcription-PCR (data not shown). Bassoon is distributed in R28 in a spot-like manner (Fig. 9D, arrowheads). The RIBEYE clusters in RIBEYE(AB)-EGFP-transfected R28 cells primarily formed around this bassoon-containing clusters and colocalized with bassoon (Fig. 9A–C, arrows). The preferential colocalization between RIBEYE and its physiological interaction partner bassoon emphasizes the physiological relevance and ribbon-like partial function of the RIBEYE-containing protein aggregates.

Purified synaptic ribbons recruit externally added RIBEYE(A) and RIBEYE(B)

Next, we tested whether isolated, purified synaptic ribbons can recruit externally added RIBEYE(B)-GST and RIBEYE(A)-GST fusion proteins (Fig. 10). GST alone was used as control protein. Purified synaptic ribbons bound soluble RIBEYE(A)-GST and RIBEYE(B)-GST fusion proteins (Fig. 10A, B). GST control protein did not bind to synaptic ribbons (Fig. 10Aa, lane 6, Ba, lane 6) demonstrating the specificity of binding. Thus, the RIBEYE–RIBEYE in-

← represents the fact that more types of interactions can be formed between RIBEYE(B) and RIBEYE(AB) than between RIBEYE(B) and RIBEYE(A) alone (for a summary, see Fig. 11). RIBEYE(A) also coaggregated and colocalized with RIBEYE(A) (**C**, arrows). **E**, If RIBEYE(B)-EGFP was cotransfected with RIBEYE(B)-mRFP, both proteins remained diffusely distributed and did not generate a spot-like distribution. For additional examples of transfected R28 cells, see supplemental Figure 9 (available at www.jneurosci.org as supplemental material). The arrows in **A–D** point to intracellular RIBEYE aggregates that contain both types of the indicated differentially tagged RIBEYE proteins. The arrow in **I** points to an intracellular RIBEYE(A)-containing aggregate. COS cells transfected with the respective plasmids produced qualitatively identical results (supplemental Figs. 10, 11, available at www.jneurosci.org as supplemental material). n, Nucleus. Scale bars, 10 μ m.

RIBEYE forms spherical, synaptic ribbon-like structures in transfected R28 cells

Figure 8. Electron microscopy of RIBEYE-containing aggregates in transfected R28 cells. **A–K**, Conventional transmission microscopy of RE(AB)-EGFP- (**A–J**) and EGFP-transfected cells (**K**). **L–P**, Immunogold electron microscopy of RE(AB)-EGFP-transfected cells immunolabeled with antibodies against RIBEYE (**L–N**), tubulin (**O**), and control immunoglobulins (RIBEYE preimmune; **P**). **A**, Low magnification of RE(AB)-EGFP-transfected cells. Note the presence of large electron-dense material (200–500 nm in diameter) in RIBEYE-transfected cells (**A–J**, asterisks and black arrows). These electron-dense structures are mostly spherical in shape (**A–G**, asterisks), although more irregular profiles are also present (**H**, asterisks). These electron-dense structures (**A–J**) were often surrounded by vesicles, which in part were physically attached to the electron-dense aggregates via thin electron-dense stalks (**A–J**, arrowheads). In addition to the large electron-dense spheres, smaller electron-dense structures could be observed (**E**, white arrows). Neighboring small electron-dense aggregates (**E**, white asterisk) appear at least partly physically connected to each other (**F**, black arrow) and sometimes appeared to coalesce into larger, electron-dense structures (**E**, white asterisk). **K**, Ultrastructure of a control-transfected cell. **L–N**, Both the large (**L**, **N**) as well as the small (**M**) electron-dense aggregates were strongly immunolabeled by RIBEYE antibodies. The aggregates were densely decorated by immunogold particles. **O**, **P**, RE(AB)-EGFP-transfected cell immunolabeled with antibodies against tubulin (**O**) and RIBEYE-preimmune serum (**P**). In no case was a specific labeling of the electron-dense aggregates (asterisks) observed. n, Nucleus; m, mitochondria; G, Golgi apparatus; v, vesicles; tub, membrane tubule; pm, plasma membrane. Scale bars: **A**, 500 nm; **B**, 250 nm; **C**, 400 nm; **D–F**, 250 nm; **G–I**, 300 nm; **J**, 250 nm; **K**, 400 nm; **L–P**, 200 nm.

teraction sites are accessible on synaptic ribbons and available to recruit externally added, additional RIBEYE proteins. We verified that the binding of RIBEYE(A) to purified synaptic ribbons is independent of the attached GST tag by removing the GST tag by thrombin cleavage (Fig. 10C). Untagged RIBEYE(A) cosedimented with purified synaptic ribbons but not without synaptic ribbons, further confirming the specific binding of RIBEYE(A) to purified synaptic ribbons. To further evaluate the binding of RIBEYE(A) to synaptic ribbons, we also tested whether RIBEYE(A1), RIBEYE(A2), and RIBEYE(A3) (used as purified MBP-tagged fusion proteins) were able to bind to purified synaptic ribbons. RIBEYE(A1)-MBP and RIBEYE(A3)-MBP bound to synaptic ribbons whereas MBP alone did not demonstrating

the specificity of the interaction. Interestingly, RIBEYE(A2)-MBP did not bind to purified synaptic ribbons although it efficiently interacted with RIBEYE(A) subunits [RIBEYE(A1), RIBEYE(A2)] in protein pull-down assays (Fig. 3). We interpret these findings that the RIBEYE(A2)-binding sites/options are probably unavailable or blocked by other proteins on purified synaptic ribbons (see Discussion). The recruitment of additional RIBEYE subunits to preexisting ribbons could explain the known dynamic growth and ultrastructural plasticity of synaptic ribbons (see Discussion). Figure 11 depicts a simplified model that schematically shows how synaptic ribbons could be built from individual RIBEYE subunits via the identified RIBEYE–RIBEYE interactions.

Discussion

Synaptic ribbons are large and dynamic macromolecular constructions in the active zone of ribbon synapses. At present, it is not clearly understood how the synaptic ribbon is made and how it functions in the synapse. In the present study, we demonstrated that RIBEYE is a scaffold protein that contains multiple interaction sites for other RIBEYE molecules. Noteworthy, the RIBEYE–RIBEYE interactions involve sites in the A-domain as well as in the B-domain of RIBEYE, i.e., three distinct interaction sites in the A-domain (A1, A2, A3) and two in the B-domain (B1, B2). We have shown that these five interaction sites allow either homotypic domain interactions [interactions between same type of domains: RIBEYE(A)–RIBEYE(A), RIBEYE(B)–RIBEYE(B)] or heterotypic domain interactions [RIBEYE(A)–RIBEYE(B)]. Homotypic domain interactions can be either homotypic or heterotypic concerning the subdomain involved. A homotypic domain interaction, e.g., RIBEYE(A)–RIBEYE(A), can be mediated either by homotypic subdomain interactions, e.g., RIBEYE(A1)–RIBEYE(A1), or by heterotypic subdomain interactions, e.g., RIBEYE(A1)–RIBEYE(A2). The co-

transfection experiments demonstrated that RIBEYE proteins interact with each other and coaggregate into the same protein clusters. Given the fact that RIBEYE is the major component of synaptic ribbons (Schmitz et al., 2000; Zenisek et al., 2004; Wan et al., 2005), the multiple protein interactions of RIBEYE provide a molecular mechanism how the scaffold of the synaptic ribbon can be created. RIBEYE–RIBEYE interactions could directly link the individual RIBEYE units to each other. Because RIBEYE is present throughout the entire synaptic ribbon, RIBEYE–RIBEYE interactions could thus generate and stabilize the macromolecular structure of the synaptic ribbon. The proposed modular model of synaptic ribbons could explain how the scaffold of the

synaptic ribbon is formed mostly from a single protein component (RIBEYE). In agreement with this hypothesis, the RIBEYE aggregates in transfected R28 cells possess structural and functional similarities with synaptic ribbons. RIBEYE(AB)-transfected R28 cells formed electron-dense large protein aggregates that were partly associated with surrounding vesicles and membrane compartments. The electron-dense aggregates were usually round in shape and resembled spherical synaptic ribbons of inner hair cells (Nouvian et al., 2006). Bar-shaped/plate-shaped ribbons were not observed in the RIBEYE-transfected cells. Thus, the spherical synaptic ribbon appears to be the “basal” type of synaptic ribbon structure that is built from RIBEYE and most likely additional factors are needed to build plate-shaped ribbons from spherical ribbons. The colocalization of RIBEYE with its physiological interaction partner bassoon in R28 cells emphasizes the physiological relevance of the RIBEYE-containing protein aggregates and suggest that the RIBEYE-containing aggregates fulfill partial ribbon-like functions. Because RIBEYE is not the only component of synaptic ribbons (Schmitz et al., 2000; Wan et al., 2005), it cannot be expected that RIBEYE alone makes fully mature ribbons, e.g., with a dense and regular association of synaptic vesicles. Very likely, additional ribbon components are necessary to provide full-ribbon function and structure.

In principle, the multiple interaction sites present on RIBEYE can be important for both intramolecular and intermolecular RIBEYE–RIBEYE interactions. Intermolecular RIBEYE–RIBEYE interactions could provide the three-dimensional scaffold of the synaptic ribbon as discussed above. Intramolecular RIBEYE–RIBEYE interactions could shield the interaction sites from unwanted intermolecular interactions to keep the protein soluble. Such a shielding of binding sites could be particularly important during development and to prevent the assembly of synaptic ribbons at unwanted, unphysiological subcellular sites (e.g., outside of the presynaptic terminal).

It is likely that the interaction between different RIBEYE domains and RIBEYE molecules is regulated. In the present study, we found that NAD(H) is an important regulator of RIBEYE interactions. RIBEYE(A)–RIBEYE(B) interactions are efficiently inhibited by low, physiological concentrations of NAD(H). Both NADH and NAD^+ are very efficient in disrupting RIBEYE(A)–RIBEYE(B) complexes. Thus, NAD(H) appears to act as a molecular switch that distinguishes between two different types of RIBEYE–RIBEYE interactions: in the presence of NAD(H), RIBEYE(A)–RIBEYE(B) interactions are disassembled (this study), whereas RIBEYE(B)–RIBEYE(B) interactions are favored as judged by the NAD(H)-induced dimerization of CtBP2 (Thio

RIBEYE co-assembles with the active zone protein bassoon in R28 cells

Figure 9. The RIBEYE-induced protein aggregates recruit endogenous bassoon. R28 retinal precursor cells were transfected with plasmids encoding for the indicated EGFP-tagged proteins. **A–E**, The distribution of the endogenously present active-zone protein bassoon (**A–D**) or tubulin (**E**) was visualized by indirect immunofluorescence microscopy. In R28 cells, bassoon is endogenously present as discrete protein clusters (**A–C**, middle, arrows). Heterologously expressed RIBEYE–EGFP coaggregates with these preexisting bassoon clusters (**A–C**, arrows) but not EGFP alone (**D**). **D**, Endogenous bassoon (arrowheads) did not recruit EGFP alone. The arrowheads in **A** and **B** show RIBEYE clusters that aggregated independent of the endogenous bassoon. **E**, The RIBEYE(AB)–EGFP clusters (arrowhead) do not colocalize with microtubules, which were visualized by immunostaining with antibodies against tubulin. n, Nucleus. Scale bars, 10 μm .

et al., 2004). The binding interface on RIBEYE(B) for RIBEYE(B) interaction is spatially closely related but distinct from the binding interface on RIBEYE(B) for RIBEYE(A). This was shown by the analyses of point and deletion mutants of RIBEYE(B) that affect one type of interaction [RIBEYE(A)–RIBEYE(B) interaction] but not the other [RIBEYE(B)–RIBEYE(B) interaction] (Fig. 5C,D; supplemental Figs. 5, 6, available at www.jneurosci.org as supplemental material). The binding of NAD(H) could induce a conformation of RIBEYE(B) that favors homodimerization of RIBEYE(B) and that is incompatible with the formation of RIBEYE(B)–RIBEYE(A) heterodimers. RIBEYE(B)G730 is an essential part of the NADH-binding motif and the RIBEYE(B)G730A point mutant no longer interacts with RIBEYE(A). Therefore, one possible mechanism for the NADH-induced dissociation of the RIBEYE(A2)–RIBEYE(B) interaction

Purified synaptic ribbons recruit externally added RIBEYE subunits

Figure 10. Synaptic ribbons recruit externally added RIBEYE subunits. Purified synaptic ribbons (180 μ g) were incubated with the indicated RIBEYE fusion proteins ($\sim 3.5 \mu$ M) and then sedimented by a 1 min spin at 3,500 rpm. Fusion proteins that cosedimented with synaptic ribbons were detected by Western blotting with the indicated antibodies. **Aa–Bb**, Lanes 1 and 2 show the respective input fractions, and lanes 3 and 4 the respective autoaggregation controls of the soluble fusion proteins to test whether ribbon-independent sedimentation of fusion proteins occurs. The autoaggregation controls show that in the absence of synaptic ribbons, no fusion proteins are found in the pellet. In contrast, if synaptic ribbons were incubated with the fusion proteins, both RIBEYE(A)-GST (**B**) as well as RIBEYE(B)-GST (**A**) sedimented with purified synaptic ribbons indicating binding to synaptic ribbons. In contrast, GST alone did not cosediment with synaptic ribbons (lane 6), demonstrating the specificity of the binding of RIBEYE fusion proteins to synaptic ribbons. As outlined in Materials and Methods, RIBEYE(A)-GST cannot be expressed exclusively as an unprocessed protein even under fermenter conditions, probably because of its high contents of proline residues and the extended shape of the molecule. In addition to full-length RIBEYE(A), degradation bands of RIBEYE(A)-GST are also visible. But full-length RIBEYE(A) is clearly expressed and binds to purified synaptic ribbons (**Ba**, lane 7), whereas GST alone does not (**Ba**, lane 6). The indicated lower band in lane 7 at ~ 25 kDa is probably GST split-off from RE(A)-GST that piggybacks on RE(A)-GST bound to synaptic ribbons because GST is known to dimerize (Connell et al., 2008). In **Ab** and **Bb**, the same blot as in **Aa** and **Ba** was stripped and reprobed with antibodies against RIBEYE (U2656) to show that equal amounts of purified synaptic ribbons were used as bait for the protein pull downs. RIBEYE signals of isolated bait synaptic ribbons are denoted by arrowheads (**Aa–Bb**, lanes 5–7). Recruitment of RIBEYE fusion protein is independent of the tag. **Ca**, A further control to show that the recruitment of RE(A) to purified ribbons is mediated by RIBEYE(A) and not the GST-tag. Purified synaptic ribbons recruited RIBEYE(A), from which the GST-tag was removed by thrombin cleavage [RIBEYE(A)-TC, lane 4]. The autoaggregation control (lane 2) demonstrates that the coaggregation is dependent on the presence of synaptic ribbons and does not occur without ribbons. Binding of thrombin-cleaved rat RE(A) was detected by an antibody against RIBEYE(A) from the rat [anti-RE(A)] (tom Dieck et al., 2005). **Cb**, Equal loadings of purified ribbons in lanes 3 and 4 was verified by Western blotting with a monoclonal antibody against RIBEYE(B)/CtBP2 (BD Transduction Laboratories). Lane 1 shows the input protein, RIBEYE(A) without GST-tag. **Da**, RIBEYE(A) subdomains expressed as MBP fusion proteins are recruited to synaptic ribbons in the same manner as GST fusion proteins. RIBEYE(A1)-MBP and RIBEYE(A3)-MBP bound to synaptic ribbons (lanes 7, 8), whereas MBP alone did not (lane 6), demonstrating the specificity of the interaction. Interestingly, RIBEYE(A2)-MBP did not bind to purified synaptic ribbons (lane 9) although it efficiently interacted with RIBEYE(A) subunits [i.e., RIBEYE(A1), RIBEYE(A2)] in protein pull-down assays (Fig. 3). **Db**, The same blot as in **Da** was stripped and reprobed with antibodies against RIBEYE (U2656) to show that equal amounts of purified synaptic ribbons were used as bait for the protein pull downs. RIBEYE signals of isolated bait synaptic ribbons are denoted by arrowheads (lanes 3–4 in **Ca**, **Cb**; lanes 5–9 in **Da**, **Db**). Lane 1–4 shows the input proteins (**Da**, **Db**). RE, RIBEYE; RIBEYE(A)-TC, RIBEYE(A) generated from RIBEYE(A)-GST by a thrombin-mediated cleavage of the GST tag.

could be that the NAD(H)-binding region of RIBEYE(B) is also part of the binding interface with RIBEYE(A). If NADH binds to RIBEYE it could displace RIBEYE(A) from RIBEYE(B) and stimulate homodimerization of RIBEYE(B). By this way of thinking,

NAD(H) would favor RIBEYE complexes that contain a homodimerized B-domain, which is likely important for RIBEYE function. Additionally, RIBEYE(B) displaced from RIBEYE(A2) would make the A2-binding module available for RIBEYE(A)–RIBEYE(A) interactions. By this mechanism, binding of NAD(H) could potentially initiate the assembly of synaptic ribbons. The NADH concentrations used in the present study are well in the range of the known cellular concentrations of NADH (Zhang et al., 2002; Fjeld et al., 2003) and thus very likely capable in regulating RIBEYE–RIBEYE interactions *in situ*.

The suggested modular assembly of the synaptic ribbon from individual RIBEYE units also provides a molecular explanation for the ultrastructural dynamics of synaptic ribbons by the addition or removal of RIBEYE subunits or rearrangements of RIBEYE–RIBEYE complexes. The ribbon recruitment experiments showed that binding sites for additional RIBEYE subunits are accessible and available on synaptic ribbons at a molecular level. Isolated synaptic ribbons (Schmitz et al., 1996, 2000) are able to bind externally added RIBEYE(B) and also RIBEYE(A). The multiple RIBEYE–RIBEYE interaction sites in the A-domain suggest a predominantly structural role of the A-domain as previously suggested (Schmitz et al., 2000). Probably large portions of RIBEYE(A) are likely “buried” in the core of the synaptic ribbons. Still, part of the A-domain is accessible in isolated synaptic ribbons and therefore partly exposed. In ribbon pull-down experiments (Fig. 10), RIBEYE(A1) and RIBEYE(A3) but not RIBEYE(A2) did bind to purified synaptic ribbons. Because RIBEYE(A2) can bind to both A1 and A2 interaction sites but not to the A3 interaction site, we suggest that A1 and A2 are located in the core of the ribbon, where these sites are not available for interaction with RIBEYE(A2). In contrast, the A3 region appears at least partly exposed on purified synaptic ribbons where it is free to interact with other protein, i.e., externally added RIBEYE(A1) and RIBEYE(A3) (Fig. 10D). Binding of RIBEYE(B) probably occurs via homodimerization of RIBEYE(B)-domains based on homologous findings with CtBP2 (Balasubramanian et al., 2003; Thio et al., 2004). This homodimerization is favored by the presence of NADH. Interestingly,

RIBEYE(B) of synaptic ribbons does not bind RIBEYE(A2), although the respective fusion proteins can interact in an NAD(H)-dependent manner. Therefore, the RIBEYE(B)-binding site for RIBEYE(A2) might be blocked or the binding disfavored, e.g., by

Figure 11. Schematic RIBEYE-RIBEYE interaction model. **A**, Summary of the identified RIBEYE-RIBEYE interaction modules. In the A-domain of RIBEYE, three interaction modules are present, which are denoted as A1, A2, A3. In the B-domain of RIBEYE, two interaction modules, denoted as B1 and B2, are present. **B**, Interaction combinations between the identified interaction modules are summarized. Only for the top RIBEYE molecule are all possible intermolecular homotypic domain interactions shown. Homotypic domain interactions, e.g., RIBEYE(A)-RIBEYE(A) interactions, can be mediated by homotypic subdomain interactions, e.g., RIBEYE(A1)-RIBEYE(A1), or by heterotypic subdomain interactions, e.g., RIBEYE(A1)-RIBEYE(A2). **C**, A simplified, schematic working model shows how RIBEYE-RIBEYE interactions could build the scaffold of the synaptic ribbon from individual RIBEYE subunits. In this model, RIBEYE is depicted as a "linear" protein. For simplicity, a nonstaggered association of RIBEYE units is depicted based on homotypic RIBEYE-RIBEYE interactions. A possible, staggered interaction based on heterotypic RIBEYE-RIBEYE interactions is not included. *x*, *y*, and *z* represent the three-dimensional axis.

RIBEYE(B) homodimerization, or inhibited by NAD(H) bound at synaptic ribbons via the NBD of RIBEYE. Clearly, these working hypotheses have to be analyzed by future investigations and testing these assumptions will shed further light on the understanding of the construction and assembly of synaptic ribbons and how they work in the synapse.

In conclusion, our data show that RIBEYE is a scaffold protein with ideal properties to explain the assembly of synaptic ribbons as well as its ultrastructural dynamics via the modular assembly mechanism. The capability to interact with other RIBEYE proteins in multiple ways could explain how a single protein, RIBEYE, builds the scaffold for the entire ribbon (Fig. 11). Our transfection experiments actually show that RIBEYE can form aggregates that resemble spherical synaptic ribbons. The proposed modular assembly of the synaptic ribbon from individual RIBEYE subunits provides a molecular basis for the ultrastruc-

tural plasticity of synaptic ribbons (e.g., changes in size and shape of the ribbon). The binding of externally added RIBEYE to purified synaptic ribbons mimics the growth of synaptic ribbons that occurs *in situ*, e.g., under darkness in the mouse retina (Balkema et al., 2001; Spiwoeks-Becker et al., 2004; Hull et al., 2006). Similarly, RIBEYE-aggregates increased in size over time in light microscopy (supplemental Fig. 9, available at www.jneurosci.org as supplemental material) and RIBEYE aggregates appeared to be able to coalesce into larger structures at the ultrastructural level. The regulation of RIBEYE-RIBEYE interactions, e.g., by NAD(H), could contribute to the regulation of structural plasticity of synaptic ribbons.

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