BRAG2a, a Guanine Nucleotide Exchange Factor for Arf6, Is a Component of the Dystrophin-Associated Glycoprotein Complex at the Photoreceptor Terminal

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Purpose. Mutations in genes encoding the dystrophin-associated glycoprotein complex (DGC) can cause muscular dystrophy and disturb synaptic transmission in the photoreceptor ribbon synapse. However, the molecular composition and specific functions of the photoreceptor DGC remain unknown. Brefeldin A–resistant Arf-GEF 2 (BRAG2), also known as IQSEC1, is a guanine nucleotide exchange factor for ADP-ribosylation factor 6 (Arf6), a critical GTPase that regulates endosomal trafficking and actin cytoskeleton remodeling. In the present study, we characterized the expression of BRAG2a, an alternative splicing isoform of BRAG2, in the adult mouse photoreceptor.

Methods. Immunofluorescence and immunoelectron microscopic analyses of adult mouse retinas were performed using a novel anti-BRAG2a antibody. Pull-down, immunoprecipitation, and in situ proximity ligation assays were performed to examine the interaction between BRAG2a and the DGC in vivo.

Results. Immunofluorescence demonstrated punctate colocalization of BRAG2a with β-dystroglycan in the outer plexiform layer. Immunoelectron microscopy revealed the localization of BRAG2a at the plasma membrane of lateral walls and processes of photoreceptor terminals within the synaptic cavity. Pull-down and immunoprecipitation assays using retinal lysates demonstrated the protein complex formation between BRAG2a and β-dystroglycan in the outer plexiform layer.

Conclusions. The present study provided evidence that BRAG2a is a novel component of the photoreceptor DGC, suggesting functional involvement of the BRAG2a–Arf6 pathway downstream of the DGC.

Keywords: dystrophin, dystroglycan, ribbon synapse, small GTPase

Mutations in genes encoding components of the dystrophin-associated glycoprotein complex (DGC) can cause various forms of muscular dystrophy such as Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD). In addition to muscle-related symptoms, DMD and BMD patients show altered retinal responses to light/dark stimuli without an apparent loss of visual acuity.1–4 The DGC in skeletal muscles, which is composed of dystrophin, α- and β-dystroglycans, sarcoglycans, sarcospan, syntrophins, and dystrobrevins, is proposed to function not only as a molecular bridge between the intracellular cytoskeleton and the extracellular matrix but also as a molecular scaffold that recruits signaling molecules downstream of mechanical stimuli.5,6 Dystroglycan and dystrophin constitute the core components of the DGC. Dystroglycan itself is composed of α and β subunits cleaved from a single precursor protein: α-dystroglycan is an extensively glycosylated extracellular protein that functions as a receptor for extracellular matrix components, whereas β-dystroglycan is a transmembrane protein that is associated with α-dystroglycan extracellularly and with dystrophin intracellularly. Dystrophin exists in various isoforms (Dp427, Dp260, Dp140, Dp116, and Dp71) with tissue-specific expression patterns. In photoreceptors, dystrophin Dp427 and Dp260, dystroglycans, and β-dystrobrevin were immunohistochemically shown to localize in a specific microdomain of the plasma membrane of photoreceptor presynaptic terminals.7–10 Recently, pikachurin was identified as a novel physiologic ligand for α-dystroglycan and shown to regulate photoreceptor synaptic transmission and structure with the photoreceptor DGC.11,12 However, the exact molecular composition of the DGC differs depending on tissue and cell types,13 and we still have a poor understanding of the molecular components and functions of the DGC in nonmuscle tissues.

The ADP-ribosylation factors (Arfs) are critical small GTPases that regulate vesicle biogenesis and transport between various subcellular compartments.14,15 Arfs are divided into three classes based on structural similarity: class I (Arf1, Arf2, and Arf3), class II (Arf4 and Arf5), and class III (Arf6). Compared with the other Arfs, Arf6 is the most divergent in terms of its molecular structure and subcellular localization. Furthermore, in contrast to the primary function of other Arfs in the Golgi complex, Arf6 regulates endosomal trafficking between endo-
somes and the plasma membrane. Like other small GTPases, the interconversion of Arfs between GTP-bound active and GDP-bound inactive states is under tight control by two types of regulatory proteins: guanine nucleotide exchange factors (GEFs) that activate Arfs by catalyzing the exchange of GDP for GTP and GTPase-activating proteins (GAPs) that inactivate Arfs by triggering the hydrolysis of bound GTP to GDP.

Brefeldin A–resistant Arf-GEF 2 (BRAG2), originally named Arf-guanine exchange protein 100 (Arf-GEP100) and also known as IQSEC1, was the first identified member of the BRAG/IQSEC family of Arf-GEFs, and it functions to activate Arf6 in most cellular contexts.17,18 There are at least two alternative splicing isoforms, BRAG2a and BRAG2b, which share a conserved domain structure among the BRAG/IQSEC family, consisting of an N-terminal calmodulin-binding IQ-like motif, a central catalytic Sec7 domain, and a pleckstrin homology (PH) domain, but differ by the alternative use of N-terminal and C-terminal regions (Fig. 1A). Particularly, BRAG2a is unique in terms of the presence of a long C-terminal region containing a proline-rich domain and a type I postsynaptic density protein-95 (PDS-95)/Disc large/Zonula occludens 1 (PDZ)-binding motif.

In the retina, we recently demonstrated using immunohistochemical analyses with an anti-panBRAG2 antibody that recognizes all BRAG2 isoforms that BRAG2 colocalizes with the DGC at photoreceptor presynaptic terminals in contrast to its postsynaptic localization in retinal bipolar cells and hippocampal neurons.19,20 In the present study, we provide several lines of evidence for BRAG2a as a novel DGC component in photoreceptor presynapses with the use of independent assays including immunohistochemistry, pull-down, immunoprecipitation, and in situ proximity ligation assay (PLA). Furthermore, we show the effect of dystrophin (Dp427, Dp260, and Dp140) loss on the photoreceptor presynaptic localization of BRAG2a using dystrophin exon 52 knockout mice lacking dystrophin isoforms, Dp427, Dp260, and Dp140, which are expressed in photoreceptors.

MATERIALS AND METHODS

Animals

We purchased male C57BL/6N mice at postnatal week 10 from CLEA Japan (Tokyo, Japan). Dystrophin exon 52 knockout mice were kindly provided to us by Motoya Katsuki.10,21 Animals were kept on a 12-hour day/night cycle with free access to food and water. All experimental procedures were in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the Animal Experimentation and Ethics Committee of the Kitasato University School of Medicine.

Plasmids

For bacterial expression vectors, the C-terminal region (amino acids 1014–1089) of mouse BRAG2a (BRAG2a [1014–1089]) and the 120-amino acid C-terminal intracellular region of β-dystroglycan (β-dystroglycan [C120aa]) were amplified by PCR and subcloned into pGEX4T-2 (GE Healthcare, Piscataway, NJ).
USA). For mammalian expression vectors, the coding regions of BRAG2a and BRAG2b lacking the N-terminal 121 amino acids (BRAG2b-N121/GEP100), which corresponded to the human counterpart of GEP100,16 were amplified by PCR and subcloned into pCAGGS-FLAG.22 A mammalian expression vector for a BRAG2a mutant (P-PRD-A), in which proline residues that conform to the consensus WW domain-binding motif were replaced by alanine residues in the C-terminal proline-rich domain, was prepared as described elsewhere (Fukaya M, unpublished observations, 2017).

Antibodies

A glutathione S-transferase (GST) fusion protein of BRAG2a (1014–1089) was used as an antigen to immunize a rabbit. The serum was affinity-purified with the antigen. A guinea pig polyclonal anti-panBRAG2 antibody was characterized previously.20 Antibodies used in the present study were as follows: mouse anti-β-dystroglycan IgG2a (clone 43DA1/G8D5; Novocastra Laboratories, Newcastle upon Tyne, UK); mouse anti-PSD-95 IgG1 (clone 16; BD Transduction Laboratories, San Jose, CA, USA); rabbit anti-dystrophin IgG (ab15277; Abcam, Cambridge, MA, USA); mouse anti-Bassoon IgG2a (clone SAP7F407; Stressgen, Victoria, BC, Canada); rabbit anti-RIBEYE IgG2a; and mouse anti-FLAG IgG1 (clone M2; Sigma-Aldrich, St. Louis, MO, USA).

Immunohistochemistry

Immunohistochemical procedures were described previously.20,24,25 Briefly, eyeballs, from which the cornea and lens had been removed, were immersed in 4% paraformaldehyde for 10 minutes and cryoprotected in 30% (wt/vol) sucrose. Cryostat sections (20 µm) were treated with pepsin (1 g/L; DAKO, Carpinteria, CA, USA) in 0.2 N HCl for 5 minutes at 37°C. For immunofluorescence staining, sections were incubated with anti-BRAG2a IgG and antibodies against panBRAG2 or β-dystroglycan overnight at room temperature. Immunoreactions were visualized with species-specific secondary antibodies conjugated with Alexa Fluor 488 or 594 (Invitrogen, Carlsbad, CA, USA). Sections were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) and analyzed using a confocal laser scanning microscope (LSM 710; Carl Zeiss, Jena, Germany). For immunoelectron microscopy, after incubation with primary antibodies, sections were incubated with nanogold-conjugated secondary antibodies (1:100; Nanoprobes, Yaphank, NY, USA) and subjected to silver enhancement of gold particles (HQ kit; Nanoprobes, Yaphank, NY, USA) and subjected to silver incubation with primary antibodies, sections were incubated with 4% paraformaldehyde for 10 minutes and cryoprotected in 30% (wt/vol) sucrose. Cryostat sections (20 µm) were treated with pepsin (1 g/L; DAKO, Carpinteria, CA, USA) in 0.2 N HCl for 5 minutes at 37°C. For immunofluorescence staining, sections were incubated with anti-BRAG2a IgG and antibodies against panBRAG2 or β-dystroglycan overnight at room temperature. Immunoreactions were visualized with species-specific secondary antibodies conjugated with Alexa Fluor 488 or 594 (Invitrogen, Carlsbad, CA, USA). Sections were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) and analyzed using a confocal laser scanning microscope (LSM 710; Carl Zeiss, Jena, Germany). For immunoelectron microscopy, after incubation with primary antibodies, sections were incubated with nanogold-conjugated secondary antibodies (1:100; Nanoprobes, Yaphank, NY, USA) and subjected to silver enhancement of gold particles (HQ kit; Nanoprobes). Ultrathin sections (70 nm) were analyzed using a transmission electron microscope (Zeiss, Jena, Germany). For immunoelectron microscopy, after incubation with primary antibodies, sections were incubated with nanogold-conjugated secondary antibodies (1:100; Nanoprobes, Yaphank, NY, USA) and subjected to silver enhancement of gold particles (HQ kit; Nanoprobes). Ultrathin sections (70 nm) were analyzed using a transmission electron microscope (Zeiss, Jena, Germany). For immunoelectron microscopy, after incubation with primary antibodies, sections were incubated with nanogold-conjugated secondary antibodies (1:100; Nanoprobes, Yaphank, NY, USA) and subjected to silver enhancement of gold particles (HQ kit; Nanoprobes). Ultrathin sections (70 nm) were analyzed using a transmission electron microscope (Zeiss, Jena, Germany). For immunoelectron microscopy, after incubation with primary antibodies, sections were incubated with nanogold-conjugated secondary antibodies (1:100; Nanoprobes, Yaphank, NY, USA) and subjected to silver enhancement of gold particles (HQ kit; Nanoprobes).

For quantification of immunofluorescent signals, coronal sections of the retinas from three mice for each genotype were cut using a cryostat at the distance between 400 and 1000 µm from the posterior pole of the retina and mounted onto the same one glass slide. In our preliminary experiments, we confirmed that the immunoreactive intensities of puncta for BRAG2a in the outer plexiform layer (OPL) on retinal sections taken within this range were constant without significant regional differences. Immunofluorescent images of retinas immunostained with anti-BRAG2a or RIBEYE antibodies were acquired from five areas randomly selected in the OPL from each mouse using a confocal laser scanning microscope (LSM 710; Carl Zeiss) using a 63× oil-immersion objective lens and a digital zoom of 3.5 within the linear range below the saturation of detection signals. The intensities of immunoreactive puncta (100–200 puncta per mice) were measured using ZEN imaging software (Carl Zeiss), and statistical analyses were performed using Student’s t-test.

Immunoblot Analysis

Total lysates from the adult mouse brain and retina were prepared as described previously.20 HeLa cells were transfected with pCAGGS-FLAG-BRAG2a or pCAGGS-FLAG-BRAG2b-N121/GEP100 using Lipofectamine 2000 (Invitrogen) and harvested with loading buffer. The lysates (10 µg) were subjected to immunoblot analyses with anti-BRAG2a, anti-panBRAG2, or anti-FLAG antibodies as described previously.20 Experiments were repeated at least three times.

Pull-Down

HeLa cells were transfected with pCAGGS-FLAG vectors encoding BRAG2a, BRAG2b-N121/GEP100, or BRAG2a (P-PRD-A) using Lipofectamine 2000. Twenty-four hours after transfection, the cells were solubilized with lysis buffer consisting of 0.05 M Tris-HCl (pH 7.5), 0.15 M NaCl, 1% Triton X-100, and a cocktail of protease inhibitors (Complete Mini; Roche, Mannheim, Germany). The retinal lysate was obtained by the solubilization of retinas from six to eight adult C57BL/6N mice in the lysis buffer by brief sonication and incubation for 30 minutes at 4°C. The lysates (500 µg) were incubated with 20 µg GST-dystrophin (2937–3685),26 GST-β-dystroglycan (C120aa), or GST alone, which had been immobilized on glutathione-Sepharose 4B, for 1 hour at 4°C. After the beads were washed with the lysis buffer, proteins bound to the beads were dissociated by boiling with loading buffer. Samples were subjected to immunoblotting with anti-FLAG or anti-panBRAG2 antibodies. Experiments were repeated at least three times.

Immunoprecipitation

The rabbit anti-dystrophin IgG and normal rabbit IgG were conjugated with protein G-coupled magnetic beads (Dynabeads Protein G, Life Technologies AS, Oslo, Norway) in phosphate-buffered saline (PBS; pH 7.4) containing 0.1% Triton X-100 overnight at 4°C. The complexes were cross-linked by incubation with 1 mM dithiobis(succinimidyl propionate) (DSP; ThermoFisher Scientific, San Jose, CA, USA) in PBS for 2 hours at 4°C. After the cross-linking reaction was quenched by the addition of Tris-HCl (pH 7.5) for 15 minutes at room temperature, beads were washed with PBS containing 0.1% bovine serum albumin and stored at 4°C.

Retinas from six to eight adult C57BL/6N mice were solubilized in buffer consisting of 0.05 M Tris-HCl (pH 7.5), 0.15 M NaCl, 0.5% digitonin, 0.05% Nonidet P-40, and a cocktail of protease inhibitors (Complete Mini; Roche) by brief sonication and subsequent incubation for 30 minutes at 4°C. The retinal lysates (500 µg of 1 mg/mL) were incubated with the cross-linked beads (5 µg IgG) overnight at 4°C. The beads were then washed three times with a buffer consisting of 0.05 M Tris-HCl (pH 7.5), 0.15 M NaCl, 0.1% digitonin, and 0.05% Nonidet P-40 and boiled with loading buffer. Samples were subjected to immunoblotting with antibodies against panBRAG2 and β-dystroglycan. Experiments were repeated three times.

In Situ PLA

In situ PLA was carried out in accordance with the manufacturer’s instructions (Olink Bioscience, Uppsala, Sweden). Briefly, cryostat sections of retinas from three mice were treated with pepsin for 5 minutes at 37°C, blocked with 5% normal donkey serum, and incubated overnight at 4°C with...
anti-BRAG2a IgG or β-dystroglycan IgG alone or with the combination of anti-BRAG2a IgG and anti-β-dystroglycan IgG or anti-Bassoon IgG. Sections were then incubated with species-specific secondary antibodies conjugated with oligonucleotides, anti-rabbit PLUS, and anti-mouse MINUS PLA probes (Sigma-Aldrich) for 1 hour at 37°C and subjected to a rolling circle amplification reaction with the amplification-polymerase solution (Duolink In situ Detection Reagent Red; Sigma-Aldrich) for 100 minutes at 37°C. Fluorescent signals were analyzed using a confocal laser scanning microscope (LSM 710; Carl Zeiss). Experiments were repeated twice.

**RESULTS**

**Expression of BRAG2a in the Mouse Retina**

BRAG2 exists in at least two alternative splicing isoforms: BRAG2a and BRAG2b (Fig. 1A). In the present study, we produced an antibody specific for BRAG2a using a BRAG2a-specific C-terminal region (amino acids 1014–1089) as an antigen (Fig. 1A). In the immunoblot analysis, the anti-BRAG2a antibody recognized FLAG-BRAG2a but not FLAG-BRAG2bΔN121/GEP100 in the lysates of transfected HeLa cells and detected two major immunoreactive bands of 160 and 140 kDa in the lysates of mouse brain and retina (Fig. 1B). Meanwhile, an anti-panBRAG2 antibody, which had previously been raised against the common amino-terminal region,29 recognized both FLAG-BRAG2a and FLAG-BRAG2bΔN121/GEP100 and detected three immunoreactive bands of 160, 140, and 110 kDa in the mouse brain and retina lysates, as described previously (Fig. 1B). The sizes of the upper two panBRAG2-immunoreactive bands were consistent with those detected by the anti-BRAG2a antibody. Because the 140-kDa band detected by both anti-BRAG2a and panBRAG2 antibodies was consistent with FLAG-BRAG2a, it is likely that the 140- and 110-kDa bands correspond to BRAG2a and BRAG2b, respectively. The nature of the remaining 160-kDa species detected by the anti-BRAG2a remains unknown at present, but it could be explained by post-translational modification of BRAG2a such as phosphorylation or the presence of additional BRAG2 isoforms. Indeed, a previous proteomic analysis isolated BRAG2 as a phosphoprotein in the postsynaptic density,30 and the National Center for Biotechnology Information (NCBI) database predicted the presence of at least 12 possible BRAG2 isoforms, 8 of which possessed the same C-terminal region as BRAG2a and could be recognized by the anti-BRAG2a antibody (Supplementary Fig. S1). To further examine the specificity of the anti-BRAG2a antibody, HeLa cells were transiently transfected with FLAG-BRAG1a, BRAG2a, BRAG2bΔN121/GEP100, or BRAG3a and subjected to double immunofluorescence with the anti-BRAG2a and anti-FLAG antibodies (Fig. 1C). The anti-BRAG2a antibody only labeled HeLa cells expressing BRAG2a but not other BRAG isoforms, whereas the anti-FLAG antibody labeled HeLa cells expressing all FLAG-tagged isoforms (Fig. 1C). These findings suggested the specificity of the anti-BRAG2a antibody without any cross-reactivity to other BRAG family members.

**Localization of BRAG2a at Photoreceptor Ribbon Synapses**

Our previous immunohistochemical analysis using the anti-panBRAG2 antibody demonstrated that BRAG2 colocalizes with the DGC at photoreceptor presynaptic terminals.20 To examine the expression of BRAG2a in the photoreceptor, we performed double immunofluorescence staining of the mouse retina with the anti-BRAG2a antibody and either an anti-BRAG2 or anti-β-dystroglycan antibody. In the retina, the anti-BRAG2a antibody strongly labeled the OPL and slightly labeled the inner plexiform layer (Fig. 2A). In the OPL, the anti-BRAG2a antibody perfectly traced puncta labeled by anti-BRAG2 IgG (Figs. 2A–2C) or anti-β-dystroglycan IgG (Figs. 2E–2G). The detection of BRAG2a-immunoreactive puncta in the OPL depended on the pepsin pretreatment of retinal sections with the optimal incubation time set at 5 minutes (Supplementary Fig. S2), suggesting that the BRAG2a epitopes may be masked in the protein complex, as suggested by the requirement for pepsin treatment in the immunodetection of various postsynaptic density proteins such as PSD-95.26 In the forebrain, BRAG2 was shown to form a protein complex with PSD-95 in the postsynaptic density fraction.27 Thus, we also examined the colocalization of BRAG2a with PSD-95. In the OPL, the anti-PSD-95 antibody labeled the plasma membrane of the photoreceptor terminals as described previously, whereas BRAG2a-immunoreactive puncta were positioned within the PSD-95-labeled contour of photoreceptor terminals without any apparent overlapping (Figs. 2H–2J). In the control experiment, pre-adsorption of the anti-BRAG2a antibody with the antigen completely abolished the immunolabeling described above (Fig. 2D).

To further examine the ultrastructural localization of BRAG2a at photoreceptor ribbon synapses, we performed pre-embedding immunoelectron microscopy using the silver enhancement method. With a subcellular distribution similar to those of panBRAG2 and β-dystroglycan, BRAG2a-immunoreactive metal particles accumulated along the plasma membrane of the lateral walls and processes of photoreceptor terminals within the synaptic cavity (Fig. 3). Taken together, these findings suggest that BRAG2, especially BRAG2a, is localized at the specific plasma membrane domain of photoreceptor presynaptic terminals with the DGC.

**Interaction of BRAG2a with Dystrophin and β-Dystroglycan**

BRAG3, another member of the BRAG/IQSEC family, was previously shown to interact with a WW domain of dystrophin and utrophin through a proline-rich domain.26 Because BRAG2a also contains a similar proline-rich domain in the C-terminal region (Fig. 1A), we examined the ability of BRAG2a to interact with dystrophin. HeLa cells were transiently transfected with BRAG2a, BRAG2bΔN121/GEP100, or a BRAG2a mutant (P-PRD-A) in which proline residues that conform to the consensus WW domain-binding motif were mutated to alanine residues and subjected to pull-down assays with the GST fusion protein of the dystrophin C-terminal 7-49 amino acids containing a WW domain (GST-dystrophin [2937–3685]). GST-dystrophin (2937–3685) pulled down wild-type FLAG-BRAG2a but not mutant BRAG2aΔN121/GEP100 or BRAG2a (P-PRD-A) (Fig. 4A), suggesting a specific interaction of BRAG2a with dystrophin through the proline-rich domain. We further examined the possibility of an interaction between BRAG2 and β-dystroglycan using pull-down assays with GST fusion proteins of the β-dystroglycan C-terminal intracellular domain (GST-β-dystroglycan [C120]). GST-β-dystroglycan (C120) pulled down BRAG2a and BRAG2a (P-PRD-A) (Fig. 4B). In addition, a small amount of FLAG-BRAG2aΔN121/GEP100 was also detectable in the precipitates pulled down with GST-β-dystroglycan (C120). On the other hand, in the control experiment, GST alone did not pull down any BRAG2 isoforms.

We also performed a pull-down assay using retinal lysates (Fig. 4C). GST-dystrophin (2937–3685) efficiently pulled down two protein species detected by the anti-BRAG2a antibody, with the lower band corresponding to BRAG2a being much more intense than the upper band.
more efficiently pulled down than the upper band. On the other hand, GST–β-dystroglycan (C120) pulled down two species equally, although the efficiency of pull-down with GST–β-dystroglycan (C120) was much lower than that with GST-dystrophin (2937–3685). In the control, GST alone did not pull down any BRAG2 species from the retinal lysate.

To further confirm the in vivo interaction between BRAG2 and the DGC in the retina, we performed immunoprecipitation from the retina with anti-dystrophin IgG (Fig. 4D). Anti-dystrophin IgG precipitated β-dystroglycan and three BRAG2 species detected by the anti-panBRAG2 antibody, with the upper band being most densely labeled. On the other hand, normal rabbit IgG immunoprecipitated only a small amount of the lower BRAG2 species but not the two upper species. The reversed immunoprecipitation with the anti-panBRAG2 or anti-BRAG2a antibodies did not yield detectable immunoprecipitates for dystrophin or β-dystroglycan, because these antibodies for BRAG2 did not work well for the immunoprecipitation from the retinal lysate under the present condition (data not shown).

Finally, to verify the in situ interaction in photoreceptor synapses, we performed in situ PLA of the mouse retina. This is a sensitive method to visualize pairs of endogenous proteins that are localized in close proximity to form a protein complex in vivo.31 When retinal sections were subjected to in situ PLA with antibodies against BRAG2a and β-dystroglycan, discrete punctate fluorescent signals were detected in the OPL (Fig. 5A). In the control experiment, in situ PLA performed with the anti-BRAG2a or β-dystroglycan antibody alone, or the combination of antibodies against BRAG2a and Bassoon, an irrelevant presynaptic active zone protein, did not produce any fluorescent signals in the OPL (Figs. 5B–5D), thus confirming the specificity of the present in situ PLA.

**Synaptic Localization of BRAG2 in Photoreceptors of Dystrophin Exon 52 Knockout Mice**

The interaction of BRAG2a with the DGC led us to examine whether the presynaptic localization of BRAG2a in the OPL depends on the expression of dystrophin. Photoreceptors were shown to express three dystrophin isoforms, Dp426,
Dp260, and Dp140, at the mRNA level. We examined the expression of BRAG2a in the OPL of dystrophin exon 52 knockout mice, in which the expression of these three isoforms were abolished. Although the density of immunofluorescent puncta was indistinguishable between wild and mutant retinas, the intensity of BRAG2a-immunoreactive puncta in the OPL was drastically decreased in mutant retinas by approximately 50% compared with wild-type retinas (Fig. 6; control, 100 ± 10.2%; mutant, 48.9 ± 6.2%; P = 0.00428). On the other hand, we also examined the expression of RIBEYE, a component of synaptic ribbon in photoreceptor terminals, and found that there were no significant differences in the pattern of distribution and the immunofluorescent intensity of puncta between wild-type and mutant retinas (Supplementary Fig. S3; control, 100 ± 22.3%; mutant, 107.0 ± 15.9%; P = 0.68), excluding the possibility that the loss of dystrophin isoforms could generally affect the expression of presynaptic components in photoreceptor terminals. Therefore, our results suggested that synaptic localization of BRAG2a in photoreceptors was partially dependent on dystrophin isoforms Dp426, Dp260, and Dp140.

**DISCUSSION**

Recent evidence of the existence of multiple Arf regulators in the photoreceptor terminal, including BRAG1, BRAG2, EFA6A, and ArfGAP3, suggests the functional importance of Arfs in the photoreceptor ribbon synapse. We previously demonstrated by immunohistochemistry using the anti-panBRAG2 antibody that BRAG2, a GEF specific for Arf6, colocalizes with the DGC at the photoreceptor presynaptic terminal. In the present study, we produced a novel antibody that recognized the C-terminal region of BRAG2a and extended our previous finding by showing that BRAG2a is a novel component of the DGC at the photoreceptor terminal using independent assays. First, both immunofluorescence and immunoelectron microscopy demonstrated that BRAG2a exhibited subcellular localization in the same subcompartment of the photoreceptor terminal as β-dystroglycan. Second, pull-down assays showed both GST fusion proteins of dystrophin and β-dystroglycan could interact with BRAG2a that was artificially overexpressed in HeLa cell and endogenously expressed in the mouse retina. Third, immunoprecipitation
from retinal lysates with the anti-dystrophin antibody demonstrated the formation of an immunoprecipitable protein complex between BRAG2a and the DGC. Finally, in situ PLA showed a close spatial relationship between BRAG2a and β-dystroglycan in the OPL. Taken together, these findings strongly supported the in vivo complex formation of BRAG2a and the DGC in the photoreceptor terminal.

Concerning the targeting mechanism for BRAG2a to the photoreceptor terminal, accumulating evidence for the existence of multiple BRAG2-interacting proteins suggested that BRAG2a can be recruited to various subcellular locations by partner proteins, thereby regulating the precise localization of BRAG2a in the photoreceptor terminal.44 Among the DGC components in the skeletal muscle, syntrophin and neuronal nitric synthase are known to be PDZ domain-containing proteins. Particularly, the syntrophin family consists of α1-, α2-, β1-, β2-, γ1-, and γ2 isoforms and functions as a scaffold for signaling proteins.5,45 Although α1 syntrophin exhibits a distinct punctate distribution from the DGC in the OPL, information on the subcellular localization of other syntrophin isoforms in the photoreceptor is not available at present. Therefore, further studies are necessary to elucidate the targeting mechanisms for the synaptic localization of BRAG2 in photoreceptors and to investigate the potential interaction between BRAG2a and syntrophins.

What is the functional role of BRAG2a in the DGC at the photoreceptor terminal? Although the functions of the DGC in the photoreceptor are not yet fully understood, the recent discovery of pikachurin as a ligand for α-dystroglycan has advanced our understanding of the functions of the DGC in the photoreceptor terminal.11 Pikachurin is a 1017-amino-acid extracellular matrix protein that is synthesized by the β-dystroglycan intracellular domain. The dystrophin WW motif can interact specifically with dystrophin and BRAG2a. Thus, multiple interactions of BRAG2a with dystrophin and β-dystroglycan enable BRAG2a to build a more specific and stable complex with the DGC than BRAG2b. However, the present immunofluorescence showed that BRAG2a was significantly decreased but still present in the OPL of dystrophin exon 52 knockout mice lacking photoreceptor dystrophin isoforms, although this finding remains to be further verified by independent quantitative assays such as immunoblot and Northern blot analyses. Because this mutant retina was previously shown to contain very little β-dystroglycan in the OPL,10 it was suggested that dystrophin isoforms (Dp426, Dp260, and Dp140) and β-dystroglycan are not essential for the presynaptic localization of BRAG2a in the photoreceptor. Therefore, additional mechanisms are required to anchor BRAG2a to photoreceptor terminals. In the brain, BRAG2a is enriched at the postsynaptic density of excitatory synapses probably through the direct interaction of the C-terminal PDZ-binding motif of BRAG2a with PSD-95. However, the PSD-95 family members are unlikely to be responsible for the synaptic targeting of BRAG2a in the photoreceptor terminal, because PSD-95 and PSD-93 exhibit a distinct subcellular localization from BRAG2a and the DGC in the photoreceptor terminal.

In situ PLA. Cryosections of adult mouse retinas were subjected to in situ PLA using the following combination of primary antibodies: (A) anti-BRAG2a and anti-β-dystroglycan antibodies; (B) anti-BRAG2a antibody alone; (C) anti-β-dystroglycan antibody alone; and (D) anti-BRAG2a and anti-Bassoon antibodies. Note the occurrence of fluorescent signals in the OPL only with the combination of anti-BRAG2a and anti-β-dystroglycan antibodies. Scale bar denotes 5 μm.

### Table. Summary of BRAG2-Interacting Proteins

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Photoreceptors and present in the synaptic cleft of the photoreceptor ribbon synapse. These findings suggested that the pikachurin–DGC pathway plays an essential role in the synaptic transmission and structure of the photoreceptor ribbon synapse. As summarized in the Table, one of the consequences of the association of BRAG2 with its interacting proteins is the enhancement of its GEF activity, thereby initiating the Arf6-dependent internalization of AMPARs during synaptic long-term depression. Thus, it is attractive to speculate that BRAG2 regulates the formation and maintenance of synaptic connections and transmission in the photoreceptor ribbon synapse through Arf6-dependent membrane traffic and actin cytoskeleton remodeling downstream of the pikachurin–DGC.

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