Localization of Caveolin-1 in Photoreceptor Synaptic Ribbons

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Purpose. The purpose of this study is to determine whether caveolin-1 is a constituent of photoreceptor synaptic ribbons.

Methods. Immunoblot assay and electron microscopic immunocytochemistry were used to localize caveolin-1 in synaptic ribbons.

Results. Synaptic ribbons were localized close to the active site of presynaptic membranes and surrounded by a halo of synaptic vesicles. Immunosignals of caveolin-1 were clearly detected on the synaptic ribbons in rod and cone photoreceptors. However, the signal was seen neither on synaptic vesicles nor on presynaptic plasma membranes.

Conclusions. Caveolin-1 is a component protein of synaptic ribbons and may be involved in the regulation of transmitter release. (*Invest Ophthalmol Vis Sci.* 2001;42:850–852)

The photoreceptor synaptic-terminal is characterized by synaptic ribbons, which are located at fusion sites of the photoreceptor synapse and surrounded by halos of synaptic vesicles. The ribbon is a platelike structure that consists of a bilayer of highly ordered elementary particles and tethers vesicles with fine filaments.[1] The ribbon seems to be changed based on illumination conditions.[2,3] These morphologic observations suggest that synaptic ribbons may be involved in transmitter release. However, mechanisms of transmitter release, ribbon morphologic changes, and formation are unknown. One reason is that information about the composition of synaptic ribbons has been very limited.

Caveolin is an intrinsic membrane protein with a molecular weight of approximately 25 kDa.[4] Three types of the protein, caveolin-1, caveolin-2 and caveolin-3, have been identified, and caveolin-1 and -2 are abundantly expressed in many tissues.[5] Caveolin is involved in many signal transduction mechanisms, including protein phosphorylation by protein kinases and G-protein–mediated signal transduction.[6] Caveolin also controls cholesterol transport.[7] In addition, caveolin seems to be related to caveolae formation,[8] indicating that caveolin plays important roles in the specific localization of proteins and other components involved in signal transduction. In this report, we show that caveolin-1 is localized in synaptic ribbons. We suggest that caveolin may be a protein crucial for regulating transmitter release and formation of synaptic ribbons.

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Materials and Methods

Animals

Bovine and mouse retinas were used. Bovine eyes were obtained in a local slaughterhouse, and retinas were used for detection of caveolin-1 by immunoblot assay and immunocytochemistry. Mouse (strain BALB/c) retinas were isolated and used for immunocytochemistry. All animal procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Antibodies

An affinity-purified rabbit polyclonal antibody raised against an N-terminal recombinant fragment of caveolin-1 was used for immunocytochemistry and immunoblot analysis. This antibody is specific to caveolin-1 and reacts with the protein in mouse, rat, bovine, and human tissues. In some experiments, an antibody against caveolin-2 was also used. The antibody was prepared against a C-terminal fragment of caveolin-2. These antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Transduction Laboratories (Lexington, KY), respectively.

Preparation of OPL and ROS Fractions

The outer plexiform layer (OPL) fraction was obtained according to the methods described previously.[9] Briefly, bovine retinas suspended in 35 ml of buffer A (15 mM Na_2HPO_4, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride [PMSF], pH 7.4) were homogenized by a polytron homogenizer (Iuchiseieido, Co., Osaka, Japan) on ice. The homogenate (20 ml) was overlaid on 10 ml of buffer A containing 50% sucrose and centrifuged (50 minutes, 4°C, 15,000 rpm) using a rotor (RPR-20; Hitachi Co., Ltd., Mito, Japan). The layer between the sucrose solution and the overlaid solution was collected and suspended in the same volume of buffer A. This mixture was overlaid again onto a linear sucrose gradient (35%–50%) in buffer A and centrifuged (75 minutes, 4°C, 13,000 rpm) using a second rotor (SW28). The OPL fraction was collected at approximately 40% sucrose. The rod outer segment (ROS) fraction was obtained by a floatation method, as described previously.[10]

Immunoblot Assay

Proteins of OPL and ROS fractions (protein 10 μg) were separated by sodium dodecyl sulfate–polyacryamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. The nitrocellulose membranes were incubated with an anti-caveolin antibody solution (1 hour, room temperature) and visualized with alkaline phosphatase-conjugated anti-rabbit IgG.

Electron Microscopic Immunocytochemistry

Bovine and mouse retinas were fixed with 4% paraformaldehyde and 1% glutaraldehyde in phosphate buffer adjusted to pH 7.4 for 2 hours and dehydrated with an ascending series of ethanols (up to 95%). Dehydrated samples were embedded in Lowicryl K4M (Polysciences, Warrington, PA) or LR White embedding medium (Electron Microscopy Sciences, Fort Washington, PA). Ultrathin sections (65 nm) were incubated with an anti-caveolin antibody solution (0.2 μg/ml) for 2 hours (room temperature). After they were washed several times, the sections were incubated with 10 nm gold-conjugated goat anti-rabbit

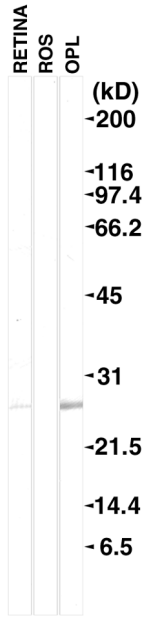

FIGURE 1. Specificity of anti-caveolin-1 antibody used in this study and presence of caveolin-1 in bovine retina. Anti-caveolin antibody recognized a protein of approximately 25 kDa in a whole retinal homogenate (retina) and OPL fraction. No band was found in the ROS fraction.

IgG (1:20 dilution: Amersham, Amersham, UK). For control experiments, an anti-caveolin antibody preabsorbed with a caveolin peptide was used as the primary antibody.

RESULTS

Immunoblot Assay

The anti-caveolin-1 antibody recognized only one protein in a retinal homogenate and in the OPL fraction (Fig. 1). Its molecular weight was slightly higher than 25 kDa. The immunologic signal in the OPL fraction was stronger than that of the retinal homogenate and was not detected in the ROS fraction. These results indicate that caveolin-1 is present in retinas, especially in the OPL fraction. Caveolin-2, another isoform, was not detected in the retina (data not shown). We note that only a monomeric form of caveolin-1 was recognized in these retinal fractions, although caveolin-1 isolated from other tissues and cells tends to aggregate or to form oligomers in the process of SDS-PAGE.[8,11,12]

Electron Microscopic Localization of Caveolin-1

Subcellular localization of caveolin-1 in photoreceptor synaptic terminals of bovine and mice retinas was examined by electron microscopic immunocytochemistry. As shown in Figure 2, in synaptic terminals of rod and cone photoreceptors, synaptic ribbons were localized close to the active sites of presynaptic membranes and surrounded by halos of synaptic vesicles. The immunolabeling was mainly found on the synaptic ribbons. The outer segments were not labeled. Although some labeling was scattered in the synaptic cytoplasm, it was barely above background level. No labeling was found in the controls (Fig. 2C). These observations indicate that caveolin-1 is a constituent of synaptic ribbons in both rod and cone synaptic terminals.

DISCUSSION

Generally, synapsin tethers synaptic vesicles,[13] although it is not found in photoreceptor synapses.[13] Instead, synaptic ribbons tether vesicles with fine filaments in photoreceptor synaptic terminals.[1] It is believed that synaptic ribbons are involved in the transmitter release by tethering synaptic vesicles. The present study indicates that caveolin-1 is localized in synaptic ribbons. This finding is crucial for studying functions of synaptic ribbons, because caveolin specifically binds to various proteins involved in signal transduction such as G-proteins, protein kinases,[5,6] and a calcium pump.[14,15] It is conceivable that caveolin-1 in synaptic ribbons is involved in transmitter release by forming complexes with proteins related to signal transduction.

Our results indicate that caveolin-1 was located in synaptic ribbons. However, the caveolin-1 detected may be slightly different from the protein reported previously[9] because oligomerization of the protein was not detected by SDS-PAGE, and its molecular weight was slightly higher than 25 kDa. It is

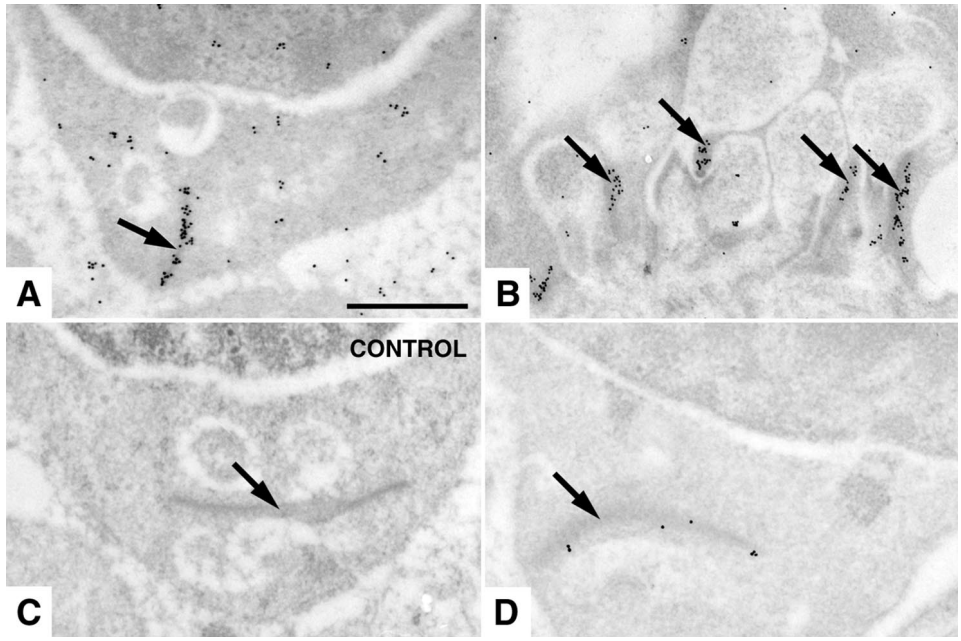

FIGURE 2. Immunocytochemical electron micrograph of synaptic terminals in bovine (**A**, **B**, and **C**) and mouse (**D**) photoreceptor cells. Anti-caveolin-1 antibody binding sites were found exclusively on synaptic ribbons (*arrows*) in both rod and cone synaptic terminals. (**A**) Bovine rod, (**B**) bovine cone, and (**C**) control (bovine rod) photoreceptor synaptic terminals. (**D**) Mouse rod photoreceptor synaptic terminal. Scale bar, 500 nm.

possible that caveolin-1 found in synaptic ribbons has a slightly different amino acid sequence (a caveolin-1 homologue), and/or the protein in synaptic ribbons is modified.

It is not known how synaptic ribbons are formed. One reason is that constituents of the synaptic ribbon have not been completely identified. Schmitz et al.[9] showed that a synaptic ribbon fraction contains several proteins. Nguyen and Balkema[16] demonstrated that antigenic epitope against the amino acid sequence DTYQHPPKD colocalized with α-actinin at the synaptic ribbon. Muresan et al.[17] have indicated that KIF3A, a member of the heteromeric family of kinesins, is a filament that tethers vesicles, suggesting that tubulin may be involved in synaptic ribbon formation. In this study, we found that caveolin-1 is localized in synaptic ribbons. A 30-kDa protein found by Schmitz et al.[9] in synaptic ribbons may be the caveolin-1 described in the present study. Caveolin-1 was originally identified as an intrinsic membrane protein,[4,5] suggesting that the synaptic ribbon could form from a membrane. However, immunocytochemical data (Fig. 2) suggest that vesicles and vacuoles do not contain caveolin-1. Information about incorporation of caveolin-1 into synaptic ribbons may be crucial for revealing the mechanism of synaptic ribbon formation.

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