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MCN Molecular and Cellular Neuroscience

www.elsevier.com/locate/ymcne
Mol. Cell. Neurosci. 35 (2007) 311–319

Localization and differential interaction of R7 RGS proteins with their membrane anchors R7BP and R9AP in neurons of vertebrate retina

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Received 22 December 2006; revised 6 March 2007; accepted 9 March 2007
Available online 15 March 2007

G protein signaling in the retina is crucially regulated by the R7 family of regulators of G protein signaling (RGS) proteins, which act to stimulate the rate of G protein inactivation. Recent findings indicate that R7 RGS proteins form complexes with two newly identified membrane anchors: RGS9 Anchor Protein (R9AP) and R7 Binding Protein (R7BP), which play essential roles in modulating the expression and localization of R7 RGS proteins. Here we demonstrate that the four R7 RGS proteins: RGS6, RGS7, RGS9 and RGS11 differentially associate with two membrane anchors. R9AP was found to form complexes with RGS9 and RGS11 which were substantially enriched in the photoreceptors. In contrast, complexes of R7BP with R7 RGS proteins were predominantly localized to the synaptic projections of retina neurons, suggesting their involvement in regulation of synaptic transmission between retina neurons. Furthermore, studies of knockout mice revealed that R9AP is necessary for the expression of only RGS9 but not for RGS6, 7 or 11. Together these data suggest that R7 RGS proteins in the retina are present as macromolecular complexes with their membrane anchors that could differentially regulate their function in various retina neurons.

Keywords: Retina; G protein signaling; RGS proteins

Introduction

The reception and processing of light stimuli by neurons in the vertebrate retina crucially depend on signaling through G-protein-coupled receptors (GPCR). The contributions of several GPCR pathways operating in individual neurons are required to accurately encode information about image brightness, contrast and motion (Rodieck, 1998).

The phototransduction cascade in rod and cone photoreceptors is a prototypic GPCR pathway that illustrates the organizational principles of G protein signaling in the retina (see Arshavsky et al., 2002; Burns and Arshavsky, 2005 for recent reviews). The key event in this pathway is the activation/deactivation cycle of the G protein, transducin. Transducin is activated upon GTP binding to its α subunit, a process triggered by the photoexcited receptor, rhodopsin. Activated transducin stimulates the activity of an effector enzyme, cGMP phosphodiesterase (PDE), which reduces the level of intracellular cGMP leading to the closure of cGMP gated ion channels. The resulting membrane hyperpolarization inhibits glutamate release from photoreceptor terminals, which is sensed by bipolar neurons located downstream from the photoreceptors. Termination of signaling requires the deactivation of transducin, which occurs upon GTP hydrolysis. The slow GTPase activity of transducin is potentiated by the short splice isoform of the Regulator of G Protein Signaling 9–1 (RGS9-1) in complex with its constitutive binding partner, G protein β subunit type 5 (Gβ5), ensuring rapid termination of signaling (He et al., 1998; Makino et al., 1999; Chen et al., 2000; Krispel et al., 2003). Recent evidence indicates that deactivation of transducin determines the overall duration of the photoreceptor response to light, making RGS9-1 the rate-limiting enzyme in the termination of signaling in the phototransduction cascade (Krispel et al., 2006). Indeed, studies with knockout mice indicate that genetic ablation of RGS9-1 (Chen et al., 2000) or Gβ5 (Chen et al., 2003; Krispel et al., 2003) results in vastly prolonged durations of photoresponses, whereas overexpression of the RGS9-1/Gβ5 complex (Krispel et al., 2006) shortens the response time. In accordance with these observations, mutations in the *RGS9-1* gene have been shown to result in a human visual disease, bradyopsia, which

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Available online on ScienceDirect (www.sciencedirect.com).

1044-7431/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.mcn.2007.03.006

symptomatically presents itself as difficulty adjusting to changes in luminance and an inability to detect moving objects (Nishiguchi et al., 2004).

The key role of RGS9-1/Gβ5 in phototransduction is underlied by several distinct features in its organization. In addition to its catalytic RGS domain, RGS9-1/Gβ5 contains several auxiliary modules that determine its catalytic activity (He et al., 2000; Skiba et al., 2001; Hu et al., 2003; Martemyanov et al., 2003), intracellular localization (Martemyanov et al., 2003), proteolytic stability (Chen et al., 2003; Keresztes et al., 2004) and ability to cooperate with PDE in the deactivation of transducin (He et al., 2000; Skiba et al., 2000). Recent findings have revealed that most of these functions critically depend on the association of RGS9-1/Gβ5 with its membrane anchor, RGS9 Anchor Protein (R9AP), adding another component to the GTPase activating complex of the photoreceptors (Hu and Wensel, 2002; Hu et al., 2003; Keresztes et al., 2003, 2004; Nishiguchi et al., 2004; Baker et al., 2006). More recently a new homolog of R9AP called R7 family binding protein (R7BP) was discovered (Drenan et al., 2005; Martemyanov et al., 2005). R7BP forms tight complexes with the brain-specific splice isoform of RGS9, RGS9-2 (Martemyanov et al., 2005). In addition to RGS9-2, R7BP was shown to form complexes with three other RGS proteins, RGS6, RGS7 and RGS11, which are closely related to RGS9 and together constitute the R7 subfamily of RGS proteins (Drenan et al., 2005; Martemyanov et al., 2005). R7BP is post-translationally modified by palmitoylation, and its association with R7 RGS proteins serves to anchor RGS proteins to the plasma membrane (Drenan et al., 2005; Song et al., 2006). Intriguingly, both R7BP and all of the R7 RGS proteins were reported to be expressed in the retina (Cabrera et al., 1998; Snow et al., 1998; Chen et al., 2003). The retina therefore represents a unique structure where related GTPase activating complexes composed of R7 RGS protein, Gβ5 and membrane anchors are uniquely positioned in the neuronal circuitry to regulate G protein pathways with demanding timing characteristics. However, the molecular composition and operational principles of the signaling pathways involving R7 RGS proteins are entirely unknown.

In this study we began a systematic analysis of the functional roles that R7 RGS protein complexes play in the vertebrate retina. We report our findings on the composition of the individual GTPase activating complexes involving R7 RGS proteins and their spatial distribution across retina neurons.

Results

Since R7 RGS proteins share significant homology (Ross and Wilkie, 2000) we began our studies by characterizing the cross-reactivity of the antibodies in our possession. In the experiment illustrated in Fig. 1A we determined the ability of antibodies against individual R7 RGS proteins to recognize recombinant R7 RGS/Gβ5 complexes by Western blotting. The results show that our antibodies demonstrate a high degree of specificity. Antibodies against the highly homologous RGS6 and RGS7 were capable of approximately 10-fold discrimination between those proteins as judged by densitometry analysis of Western blot data. Antibodies against the more diverse RGS9-1 and RGS11 were even more selective and exhibited no detectable cross-reactivity among the R7 RGS proteins. Anchor proteins R7BP and R9AP share much less similarity than the R7 RGS proteins (Drenan et al., 2005; Martemyanov et al., 2005), allowing the corresponding antibodies to be raised against unique non-overlapping epitopes and yielded predictably non-cross-reacting antibodies (data not shown).

We next analyzed the expression of individual R7 RGS proteins and their binding partners, Gβ5, R7BP and R9AP in the retina. In agreement with previous studies our Western blot analysis of retina extracts showed the presence of all four R7 RGS proteins: RGS6, RGS7, RGS9-1 and RGS11 (Fig. 1B) (He et al., 1998; Witherow et al., 2000; Chen et al., 2003), and their constitutive subunit, Gβ5 (Watson et al., 1996). Also in agreement with published studies (Rahman et al., 1999), the long splice isoform of RGS9, RGS9-2, was not detected in the retina when RGS9-2-specific antibodies were used for the analysis. At the same time, both splice isoforms of Gβ5 (Gβ5S and Gβ5L), which are known to be expressed in the retina (Watson et al., 1996), were detected with our specific

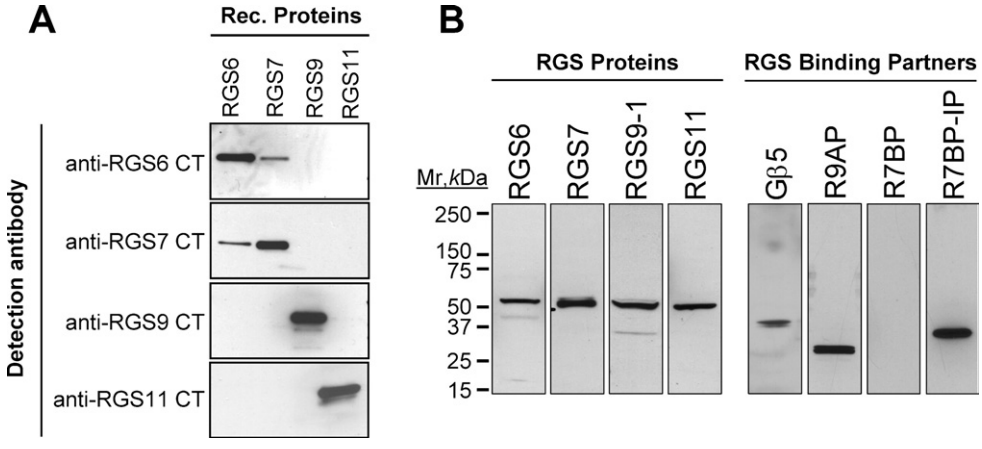

Fig. 1. Expression of R7 RGS proteins and their binding partners in the retina. (A) Characterization of the cross-reactivity of antibodies raised against individual R7 RGS proteins. Purified recombinant complexes of RGS6, RGS7, RGS9-1 and RGS11 with Gβ5 were loaded on the gel in the amount of 1 pmol per lane, separated by SDS PAGE and transferred to a PVDF membrane. Proteins were detected by Western blotting with affinity-purified antibodies as described in the Experimental methods. Antibodies were diluted to 1 μg/ml in 1% non-fat milk. (B) Detection of proteins in retina extracts. Retinas removed from mice were lysed in PBS containing 1% Triton X-100. Lysates were clarified by centrifugation, mixed with SDS sample buffer and analyzed by Western blotting. 25 μg of total protein was loaded in each lane. R7BP IP was performed as described in the Experimental methods section, and precipitate from lysate containing 200 μg total protein was loaded in one lane.