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Ras-associating domain proteins: A new class of cyclic nucleotide gated channel modulators

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Abstract

The Ras is a protein subfamily of small GTPases that are involved in cellular signal transduction. Members of Ras family are all related in structure and regulate diverse cell behaviors. Ras-associating/binding (RA/RBD) domain containing proteins perform several different functions ranging from tumor suppression to being oncoproteins. Their role in different biological processes may be unclear and highly divergent but what is clear is that they convergently function by interacting with Ras proteins through their RA/RBD subdomains directly or indirectly. Apart from interacting with Ras proteins, there is no perceptible relationship between these proteins or their highly unrelated protein bodies. The heterogeneity among these RA domains allows them to interact with Ras proteins of different types as well as several other proteins which contain similar motifs. Very recently we have demonstrated that growth factor receptor bound protein 14 (Grb14) RA-domain binds to photoreceptor cyclic nucleotide-gated channel (CNG) and inhibits its activity *in vivo*. In this study we have examined two other RA domain containing protein phosphates expressed in retina, PHLPP1 and PHLPP2 on CNG channel activity. Our data indicate that not all RA domain proteins are modulators of CNG channel, suggesting the existence of heterogeneity among several RA domain proteins.

XX.1 Introduction

Like almost all major biological processes, phototransduction is achieved by the interaction of proteins, leading to manifold functional outcomes. Cyclic nucleotide gated (CNG) channels comprise an indispensable element in phototransduction and as such are apt and inclined to play a regulatory role. CNG channels play important roles in visual and olfactory transduction (Yau and Baylor, 1989; Biel et al, 1999; Kaupp and Seifert, 2002; Yau and Hardie, 2009; Gerstner et al, 2000). In the visual system, a cyclic guanosine monophosphate (cGMP)-gated channel is found in the outer membrane of retinal photoreceptor cells. The cGMP-sensitive cation channel is the sensor of the changes in cytoplasmic cGMP concentration brought about by light (Yau and Baylor, 1989). CNG channels comprise two structurally related subunit types: A and B (Kaupp and Seifert, 2002). The rod channel consists of CNGB1 and CNGB1 subunits, whereas the cone channel contains CNGB3 and CNGB3 subunits. Heterologous expression studies have shown that the A subunits are responsible for the ion-conducting activity of the channel, whereas the B subunits function as modulators (Gerstner et al, 2000). The CNG channel sensitivity towards cGMP has been

studied extensively and has been shown to depend on and regulated by several other factors prominent of which are $\text{Ca}^{2+}/\text{CaM}$, divalent ions, diacylglycerol, phospholipids and phosphorylation state (reviewed in Kaupp and Seifert, 2002). These channels have an N-terminal and a C-terminal cytoplasmic region which are freely available in the cytoplasm for a multitude of factors to interact with. We recently reported that Ras-associating domain of growth factor receptor-bound protein 14 (Grb14) binds to the C-terminal region of rod CNGA1 subunit and modulate its activity *in vivo* (Gupta et al., 2010). Studies from several laboratories have shown the expression RA/RBD domain containing proteins in the retina which include c-Raf (Pimentel et al., 2000), Pi3k (Rajala et al., 2002), AF-6 (Kanai-Azuma et al., 2000), Tiam1 (Tanaka et al., 2004), PHLPP1 and 2 (Kanan et al., 2010), Grb14 (Rajala et al., 2005) and EPAC (Ivins et al., 2004), however, no information is available on their effect on channel. PHLPP1 and PHLPP2 (PH domain and Leucine rich repeat Protein Phosphatases) (Gao et al., 2005) are the two putative RA domain containing proteins which play an important role in IR signaling (Kanan et al., 2010). In this study we have investigated the effect of RA-domain containing proteins, PHLPP1 and PHLPP2 on rod CNG channel sensitivity.

XX.2 Materials and Methods

XX.2.1 Cell Culture studies and vectors

HEK293T cells (2.5×10^5) were seeded in each dish 12–18 h before transfection and transfected with the cDNA constructs (Wigler et al., 1978). The generation of CNGA1, Grb14 (Myc), PHLPP1 and PHLPP2 (FLAG) vectors has been described previously (Gupta et al., 2010, Kanan et al., 2010). The fluorescent indicator Indo-1/AM was used to quantify Ca^{2+} influx through CNGA1 channels in HEK293T cell suspensions (Gupta et al., 2010).

XX.2.2 Sequence alignment and domain assessment

The RA domain of Grb14 (106–192; Uniprot id. Q6ZVD8) was used to align with the human PHLPP2 (Uniprot id. Q14449) sequence using multiple sequence alignment portals (Corpet, 1988). Having ascertained the span of RA domain in PHLPP2, this sequence was further aligned with the human PHLPP1 (β) (Uniprot id. Q14449) and the exact position of PHLPP1(β) RA domain determined.

XX.2.3 Statistical Methods

Data were analyzed using Graphpad Prism software (GraphPad Software, San Diego, CA), expressed as the mean $\pm$ S.D. and compared the differences by Student's *t* test. The critical level of significance was set at $p < 0.05$.

XX.3 Results

XX.3.1 Assessment of RA domains in PHLPP1 and PHLPP2

We have reported recently that Grb14 inhibits CNGA1 channel activity through its RA domain (Gupta et al., 2010). Both PHLPP1 (β) and 2 have been speculated to possess a putative RA domain (Gao et al., 2009). Primary structural analysis indicates that both PHLPP1 (β) and PHLPP2 possess a discernible RA domain followed by a PH domain like the Grb14 family members (Fig. XX.1). The domain organization of both PHLPP1 (β) and PHLPP2 are shown in Fig. XX.1. The RA domain like sequence was assigned to regions 430–515 and 44–129 in human PHLPP1 and PHLPP2 isoforms based on the primary structure comparison with human Grb14 RA domain (106–192). No such sequence was observed in PHLPP1 (α) isoform. A similarity of 20–25% was observed at the primary structural level (with what). Both of these isoforms were observed to be present in retina, however, PHLPP2 is highly expressed in rod photoreceptors (Kanan et al., 2010). The

sequence comparison indicates that RA domain in the two families of proteins is not highly homologous further highlighting the structural and functional heterogeneity amongst the RA domains, although a phylogenetic convergence cannot be ruled out. A similar structural organization of the PHLPP (1, 2) isoforms with Grb14 protein, suggest that PHLPP1 and PHLPP2 isoforms may also modulate the CNG channel activity.

XX.3.2 PHLPP1 and 2 do not have channel modulatory properties

PHLPP1 and 2 are present in the retina and their activity is known to be regulated by the Akt which in turn gets activated upon activation of IR pathway (Kanan et al., 2010). The effect of each of the PHLPP1 (β) and PHLPP2 was investigated on CNGA1 channel function upon overexpression in HEK293 cells and it was observed that none of these proteins is capable of modulating CNG channel function (Fig. XX.2A). Grb14 co-expression with CNGA1 was used as a control (Gupta et al., 2010). CNGA1 co-expression with empty plasmid was used as a positive control. The lack of modulation was not due to lack of the protein expression as each of these proteins was expressed to comparable levels (Fig XX.2B). The individual protein expression of Grb14, PHLPP1 (β) and PHLPP2 was used to subtract the non-specific effect of autofluorescence and Indo-1AM/ Ca^{2+} bleeding through membranes (Fig. XX.2A).

XX.4 Discussion

Cyclic guanosine monophosphate (cGMP) gated channels play a pivotal role in phototransduction and light adaptation through maintenance of photoreceptor homeostasis. Mutations in the rod CNG channel are found in patients with *retinitis pigmentosa* (Driyja et al., 1995) whereas mutations in the cone CNG channel are associated with achromatopsia, progressive cone dystrophy and early-onset macular degeneration (Kohl et al., 2005; Kohl et al., 2000; Nishiguchi et al., 2005; Wissinger et al., 2001). This necessitates a thorough understanding of the physiological role and regulation of these channels for eventual therapeutic intervention. We recently reported that the RA domain of Grb14 is self sufficient to promote the channel to be in an *off* state (Gupta et al., 2010). This opens up myriad possibilities that the other RA domain containing proteins may have analogous role in modulating CNG channel function. Even though, PHLPP1 and 2 are RA-domain proteins, but they failed to inhibit the channel activity. Our data suggest that not all RA domain proteins are modulators of CNG channel, suggesting the existence of heterogeneity among several RA domains. Interestingly, several RA-domain containing proteins also contain PH domains (Raaijmakers and Bos, 2009) which help these proteins to localize to the plasma membrane through their interaction with phosphoinositides (PIs). Grb14 also contains a PH domain, however, we did not observe any channel modulatory property associated with this domain (Gupta et al., 2010). Proper localization of RA domain proteins through PH-PI interaction on CNG channel modulation cannot be ruled out. This study also opens up numerous possibilities for looking up other RA-domain proteins as modulators of CNG channel in photoreceptors and perhaps these domains may be used as therapeutic agents to treat retinal degenerations.

One of the biggest future challenges is to understand the RA/RBD containing protein interactions with Ras or Ras like domain containing proteins in a quantitative manner in their three dimensional orientations and study the underlying energetics. This study however makes it clear that effect of RA domain containing proteins is discriminatory and that RA domains cannot be considered as rigid bodies.

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Fig. XX.1.

Comparative domain analysis of the Grb14 with PHLPP1 (β) and PHLPP2. A. The primary structure analysis of the PHLPP1 (β) and PHLPP2 shows that these proteins contain RA and PH domain in a pattern similar to the Grb14 family. Various domains of the proteins are shown diagrammatically. B. The primary structural homology between RA domains of these proteins was established by multiple sequence alignment. The similarity consensus is 90% (red: Grb14 and PHLPP1 (β) and 2; Blue: Grb14 and either PHLPP1 (β) or PHLPP2). Pro, proline rich region; RA, Ras-associating; PH, pleckstrin homology; BPS, between PH and SH2; SH2, Src-homology-2 region; LRR, leucine-rich repeat; PP2C, Protein Phosphatase 2C; PDZ, post synaptic density protein (PSD95), *drosophila* disc large tumor suppressor (DlgA), and zonula occludens-1 protein (zo-1).

Fig. XX.2.

Effect of PHLPP1 and PHLPP2 on CNG channel activity. A. Myc- CNGA1 was transiently co-expressed with myc-Grb14, FLAG-PHLPP1 or FLAG-PHLPP2 and examined for their effect on channel activity. Cells transfected with Grb14, PHLPP1 (β) or PHLPP2 alone were used to measure the non-channel mediated Ca^{2+} permeability. CNGA1 co-transfected with empty plasmid was used as control. B. Lysates were subjected to immunoblot analysis with anti-Myc antibodies for CNGA1 and anti-FLAG antibodies for PHLPP1 and PHLPP2 respectively. Data mean $\pm$ SD, $n=3$; $p < 0.05$.