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A novel Gαs-binding protein, Gas-2 like 2, facilitates the signaling of the A_{2A} adenosine receptor

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ABSTRACT

The A_{2A} adenosine receptor ($A_{2A}R$) is a G-protein-coupled receptor that contains a long cytoplasmic carboxyl terminus ($A_{2A}R$-C). We report here that Gas-2 like 2 (G2L2) is a new interacting partner of $A_{2A}R$-C. The interaction between $A_{2A}R$ and G2L2 was verified by GST pull-down, co-immunoprecipitation, immunocytochemical staining, and fluorescence resonance energy transfer. Expression of G2L2 increased the intracellular cAMP content evoked by $A_{2A}R$ in an $A_{2A}R$-C-dependent manner. Immunoprecipitation and pull-down assays demonstrated that G2L2 selectively bound to $A_{2A}R$-C and the inactive form of Gαs to facilitate the recruitment of the trimeric G protein complex to the proximal position of $A_{2A}R$ for efficient activation. Collectively, G2L2 is a new effector that controls the action of $A_{2A}R$ by modulating its ability to regulate the Gαs-mediated cAMP contents.

1. Introduction

Adenosine was shown to play an essential role in modulating neuronal functions *via* four adenosine receptors (*i.e.*, A_1, A_{2A}, A_{2B}, and A_3), which belong to the seven-transmembrane G-protein-coupled receptor superfamily [1,2]. Previous studies suggest that the A_{2A} adenosine receptor ($A_{2A}R$) is coupled to Gαs and Gαolf in the peripheral tissues and the striatum, respectively [3,4]. Because $A_{2A}R$ is colocalized with dopamine D2 receptors (D2R) in enkapherine-containing GABAergic striatopallidal neurons (*i.e.*, the indirect pathway) at post-synaptic sites [5], it is a therapeutic target in Parkinson's disease [6] and Huntington's disease [7]. Ample evidence implicates $A_{2A}R$ in the regulation of important neuronal functions, including synaptic transmission and neuronal protection [8–10].

$A_{2A}R$ transmits signals through both G protein-dependent and -independent pathways. We and others have demonstrated that $A_{2A}R$ stimulation activates at least two major G protein-dependent signaling cascades, including adenylyl cyclase (AC)/cAMP/protein kinase A (PKA)- and PKC-mediated signaling [11,12]. In addition to conventional G protein-mediated signaling pathways, growing evidence has demonstrated that the carboxyl terminus of $A_{2A}R$ ($A_{2A}R$-C) renders it able to function independently of G proteins [13–15]. Compared to other adenosine receptors, $A_{2A}R$ contains a relatively long cytoplasmic carboxyl terminus (C-terminus, Fig. 1A) that is highly conserved among species. Recently, seven interacting proteins were identified as follows: (i) α-actinin, which tethers $A_{2A}R$ to the actin cytoskeleton [16]; (ii) a guanine nucleotide exchange factor of small monomeric G proteins of the ADP-ribosylation factor (ARF) family (ARNO/cytohesin-2), which is required for mitogen-activated protein kinase (MAPK) activation [15]; (iii) calmodulin (CaM), which modulates the function of $A_{2A}R$–D2R complexes [17]; (iv) neuronal Ca^{2+}-binding protein 2 (NECAB2), which retains $A_{2A}R$ in the intracellular compartment and enhances MAPK signaling [18]; (v) a synapse-associated protein of 102 kDa (SAP102), which reduces the mobility of $A_{2A}R$ [13]; (vi) translin-X-associated protein (TRAX), which regulates neuritogenesis [14,19]; and (vii) ubiquitin-specific protease 4 (USP4), which deubiquitinates $A_{2A}R$ and thus increases the number of receptors on the plasma membrane [20]. Together with the homo- or hetero-oligomerization of the receptors, these C-terminus-interacting proteins form a protein complex named $A_{2A}R$ signalosome. The mechanism depicting how the components within the $A_{2A}R$ signalosome interact has yet to be elucidated.

Growth-arrest-specific 2-like protein 2 (G2L2) is a protein of 860 amino acids (aa). Its amino acid sequence is conserved among species, suggesting that its function is evolutionally important. The N terminus (aa 1–282) of G2L2 shares 60% amino acid similarity with the growth-arrest-specific 2 [GAS2, [21]]. Although GAS2 was implicated in apoptosis, membrane ruffling, and embryogenesis [22–24], the function of G2L2 remains unclear. Similar to GAS2 [25], G2L2 contains a calponin homology (CH) domain (aa 1–159) and a GAS2-related (GAR) domain (aa 201–280), which might mediate the binding of G2L2 to the actin cytoskeleton

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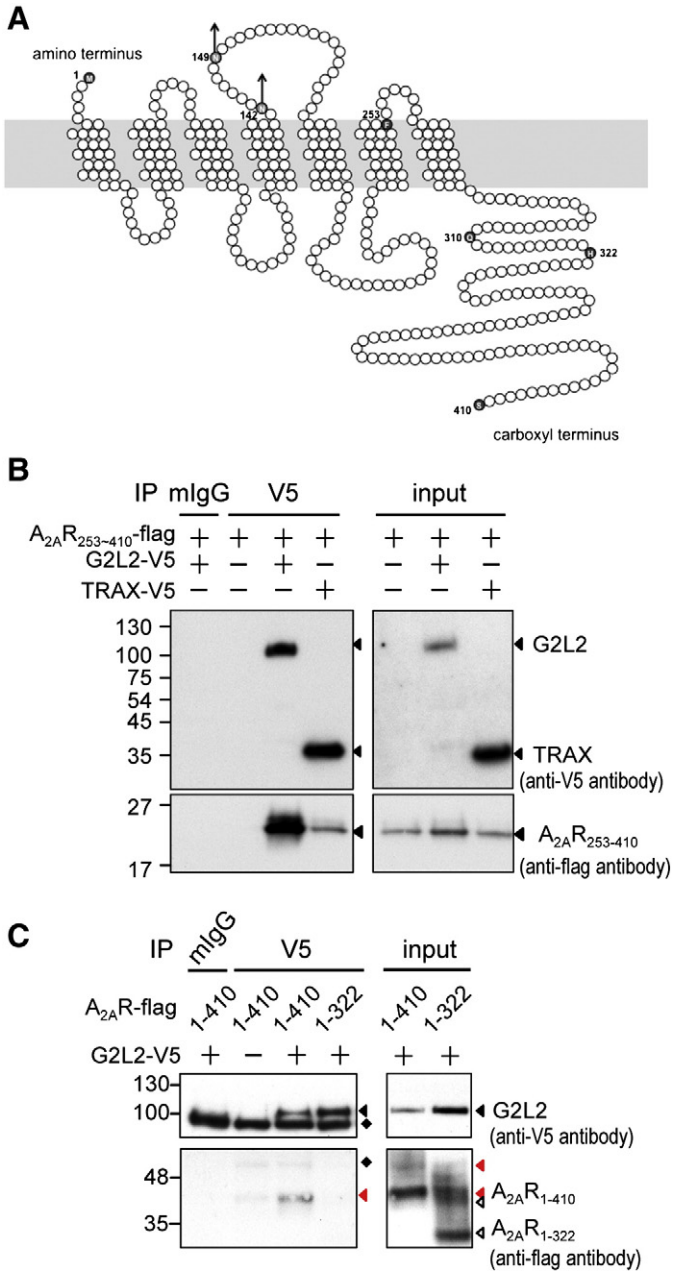

Fig. 1. G2L2 interacts with A^{2A}R-C. (A) Schematic structure of rat A^{2A}R. (B) HEK293T cells were transfected with $A^{2A}R^{253–410}$-flag and G2L2-V5 or TRAX-V5 for 48 h. (C) HEK293T cells were transfected with G2L2-V5 and the indicated A^{2A}R variant for 48 h. The cells were collected for immunoprecipitation.↑, N-glycosylation site; ♦, non-specific bands appeared in the immunoprecipitated protein complexes. Red arrowheads mark the $A^{2A}R^{1–410}$ proteins, while open arrowheads mark those of $A^{2A}R^{1–322}$.

and microtubules, respectively. Except for those two domains, no other defined domain exists in G2L2. There are three paralogs of the GAS2-related proteins: G2L1, G2L2, and G2L3. All of these proteins contain the CH and GAR domains and are considered "mini-versions of spectraplakins" [26]. These GAS2-related proteins therefore might associate with and/or coordinate various cytoskeletal proteins, including microtubules, actin filaments, and intermediate filaments [25–28]. In the present study, we report that G2L2 is a new binding protein of A_{2A}R-C. In addition, G2L2 regulates the cAMP-elevating ability of A_{2A}R by recruiting Gsα protein to its proximal location, for G2L2 is also a novel interacting protein of Gsα. Our study suggests that the A_{2A}R-C is crucial for effective stimulation of Gsα-mediated cAMP signaling by A_{2A}R.

2. Materials and methods

2.1. Reagents and antibodies

Adenosine deaminase (ADA), 50× protease cocktail, and 10× phosphostop were purchased from Roche (Basel, Switzerland). The mouse anti-flag monoclonal antibody (mAb) (M2), rabbit anti-actin polyclonal antibody (pAb), and CGS21680 (CGS) were obtained from Sigma (St. Louis, MO, USA). The mouse anti-V5 mAb was purchased from Invitrogen (Carlsbad, CA, USA). The mouse anti-A_{2A}R mAb was either from Millipore (Bedford, MA, USA) or Santa Cruz Biotechnology (Santa Cruz, CA, USA). The rabbit anti-A_{2A}R pAb was generated against a peptide (NH2-VQARVGASSWSSEFAPSC-COOH; Yao-Hong Biotechnology, Taipei, Taiwan) comprising aa 394–410 of A_{2A}R plus an additional cysteine residue at its C terminus to facilitate the purification of the resultant antibody. The anti-G2L2-C pAb was generated against a peptide (NH2-CQEPLKLGGTPLSPEEESWV-COOH) (Yao-Hong Biotechnology, Taipei, Taiwan) comprising the last C-terminal 20 amino acids of G2L2 plus an additional cysteine at the N terminus. The anti-G2L2-N pAb was generated against a peptide (NH2-VRSIRPFKSSEQYLEC-COOH) (Angene, Taipei, Taiwan) comprising aa 18–32 of G2L2 plus an additional cysteine at the C terminus. The mouse anti-myc mAb was obtained from LTK BioLaboratories (Taipei, Taiwan). The anti-G2L2-D pAb was generated against the purified recombinant GST–$G2L2_{579–860}$ protein (Yao-Hong Biotechnology). The resultant antiserum (20 ml) was first incubated with GST protein (1 mg) conjugated with glutathione beads (1 ml) to remove those antibodies that recognized GST. The absorbed anti-G2L2-D antibody was prepared by incubation with GST–$G2L2_{579–860}$ coupled to glutathione beads for 16 h at 4 °C. Alexa Flour 488, 568, and 647 and rhodamine–phalloidin were purchased from Molecular Probes (Eugene, OR, USA).

2.2. Plasmid constructions

All primers employed in the preparation of our constructs are listed in Supplementary Table S1. The DNA fragments of $A_{2A}R_{1–410}$, $A_{2A}R_{1–322}$ were amplified from rat complementary (c)DNA encoding A_{2A}R [1] by the polymerase chain reaction (PCR) using specific primers, and it was subcloned into the pcDNA3.1/V5-His-TOPO vector (Invitrogen). The full-length and C-terminus-deleted A_{2A}R-3 × flag constructs were amplified by PCR from pcDNA3.1-$A_{2A}R_{1–410}$ using specific primers, and they were subcloned into the p3X FLAG-CMV14 vector (Sigma). To generate the $A_{2A}R_{253–410}$-flag construct, DNA fragments encoding $A_{2A}R_{253–410}$ [14] were cleaved by *Kpn* I and *Xho* I and subcloned into the p3X FLAG-CMV14 vector. Mouse Gas2-related protein 2 (NM_001013759) was amplified by PCR from mouse brain cDNA and subcloned into pcDNA3.1-V5-His-TOPO. Truncated mutants of G2L2 were amplified from the full-length G2L2-V5 fragment (flG2L2-V5) by PCR using specific primers, and they were subcloned into pcDNA3.1-V5-His-TOPO. G2L2–CFP was generated from flG2L2-V5 by a PCR using specific primers, digested by *Bgl* II and *Sma* I, and then subcloned into the eCFP vector (Clontech, Palo Alto, CA). The YFP fragment was amplified by PCR using specific primers, digested with *Sac* II and *Xho* I, and subcloned into the pcDNA3-$A_{2A}R_{1–410}$-V5 to encode the fusion protein (A_{2A}R-YFP). Expression constructs of the Gα proteins were purchased from Missouri S&T cDNA Resource Center (Rolla, MO, USA). The DNA fragments encoding Gαs variants were amplified by PCR using specific primers, cleaved at the *Kpn* I and *Xba* I sites, and subcloned into pcDNA3.1-myc-His (Invitrogen). DNA fragments encoding Gαolf, Gαi, GαoA, Gαq, or Gα13 were amplified by PCR using specific primers, cleaved at the *Kpn* I and *EcoR*V sites, and subcloned into pcDNA3.1-myc-His (Invitrogen). The AMPK-V5 construct was prepared as previously described [10]. To generate prokaryotic expression constructs of