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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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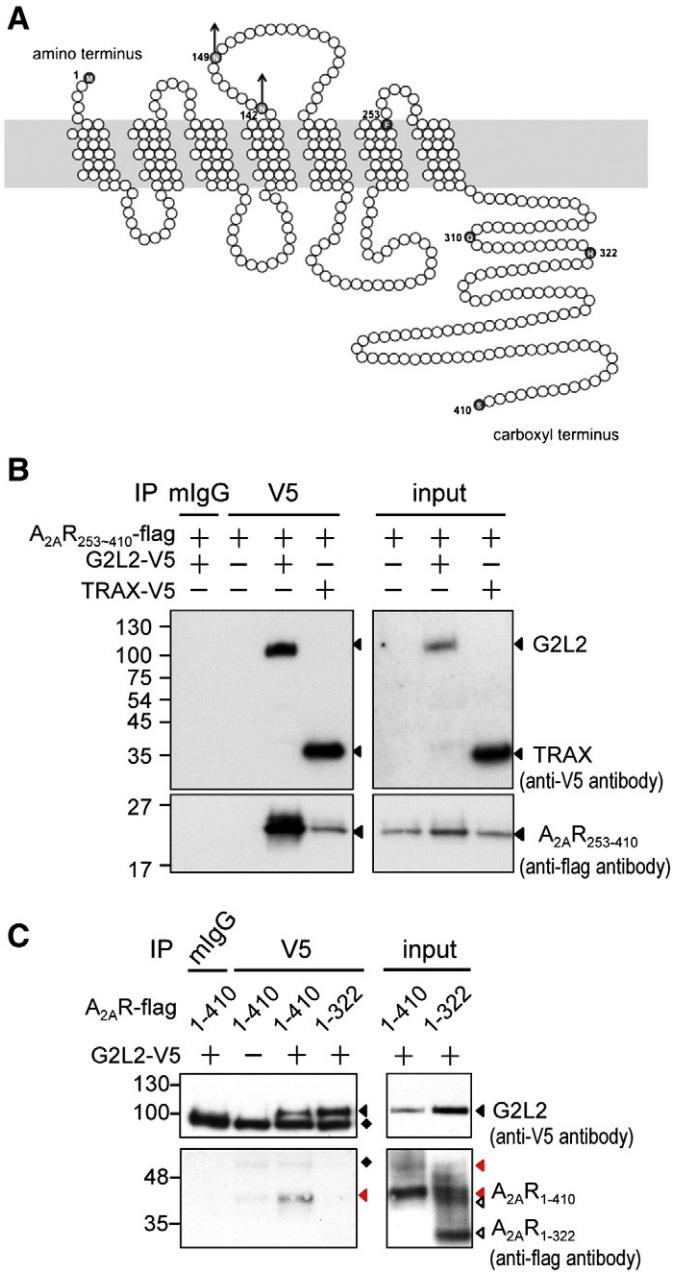

Fig. 1. G2L2 interacts with A^{2A}R-C. (A) Schematic structure of rat A^{2A}R. (B) HEK293T cells were transfected with $A^{2A}R^{253–410}$-flag and G2L2-V5 or TRAX-V5 for 48 h. (C) HEK293T cells were transfected with G2L2-V5 and the indicated A^{2A}R variant for 48 h. The cells were collected for immunoprecipitation.↑, 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

GST fusion proteins, $A_{2A}R_{310–410}$ and $A_{2A}R_{322–410}$ were first amplified from pcDNA3.1-$A_{2A}R_{1–410}$ by PCR using specific primers and subcloned into pcDNA3.1-V5-His-TOPO (Invitrogen). The resultant constructs were then cleaved at the *Bgl* II and *Eco* RI sites and subcloned into the *Bam* HI and *Eco* RI sites of pGEX-2T (GE Healthcare, Piscataway, NJ, USA) to produce the recombinant glutathione S-transferase (GST)-A_{2A}R-C protein. DNA fragments encoding aa 579–860 of mouse G2L2 were amplified by PCR using specific primers, and subcloned into pET-42a(+) as previously described [29] to produce recombinant GST–$G2L2_{579–860}$ proteins. GST–Gαs and -Gαi were also amplified from GNA0SL0000 and GNAI100000 by PCR using specific primers, and they were subcloned into pET-42a(+). All resultant plasmids were analyzed by nucleotide sequencing to verify the inserts.

2.3. Cell culture and transfection

HEK-293T cells [human embryonic kidney 293 cells expressing the large T-antigen of simian virus 40 (SV40)] were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1500 mg/l sodium bicarbonate, and 1% penicillin/streptomycin (Invitrogen GibcoBRL) with 5% CO_2 at 37 °C. Primary striatal neurons were prepared from Sprague–Dawley rat brains on embryonic day 18 as described previously [30] and grown in Neurobasal medium supplemented with B27 (Invitrogen). Transfection was carried out using Lipofectamine™ 2000 (Invitrogen) following the manufacturer's protocol.

2.4. In vitro binding

Recombinant GST-fusion proteins were expressed in JM109 (DE3) and purified using glutathione-sepharose beads (Promega) following the manufacturer's protocol. The GST pull-down assay was conducted by incubating purified GST fusion proteins with ^{35}S-G2L2 at 4 °C for 2 h in binding buffer (10 mM HEPES at pH 8, 150 mM NaCl, 1 mM benzamidine, 1% bovine serum albumin (BSA), 0.1% Triton X-100, and a protease inhibitor cocktail) on a rolling wheel. The bound protein complexes were harvested using glutathione-agarose beads, extensively washed with wash buffer (10 mM HEPES at pH 8, 150 mM NaCl, and 1 mM benzamidine) six times, and separated by a 12% SDS-PAGE, followed by autoradiography to detect the amount of ^{35}S-labeled G2L2 variant bound by the GST fusion protein (upper panels), and Coomassie blue staining of SDS-PAGE (bottom panels) to verify the amount of GST fusion protein loaded.

2.5. Immunoprecipitation

Protein lysates (3 mg) in lysis buffer (20 mM HEPES at pH 8, 2 mM $MgCl_2$, 1 mM EDTA, 0.5% $C_{12}E_9$, 1 μM MG132, 150 mM NaCl, and a protease/phosphatase inhibitor cocktail) were incubated with 1 μg of the indicated antibody coupled to 40 μl of protein A beads (Sigma-Aldrich) at 4 °C for 2 h on a rolling wheel. After extensive washing using ice-cold washing buffer (50 mM HEPES, 1 mM $MgCl_2$, 1 mM EDTA, 0.05% $C_{12}E_9$, 150 mM NaCl, and a protease inhibitor cocktail), the resultant immunoprecipitation complexes were resolved in 40 μl of 2× sampling buffer and analyzed by SDS-PAGE and western blotting.

2.6. FRET assay

HEK293T cells were seeded onto glass-bottom cell culture dishes (NEST Biotech, Wuxi, China; 1.6×10^4 cells per 20-mm well), transfected for 48 h, and monitored under a confocal microscope (LSM510, Carl Zeiss, Oberkochen, Germany). FRET images were collected following the sensitizing emission protocol per the manufacturer's manual. FRET values were calculated and analyzed by the "FRET plus analysis" software in Aim Image Browser (Carl Zeiss).

2.7. Adenylyl cyclase (AC) assay

HEK293T cells were seeded at a density of 6×10^6 cells per 100-mm plate, transfected with the indicated DNAs for 24 h, re-plated into two 150-mm plates, and cultured for another 24 h. To remove interference from endogenous adenosine in the medium, the cells were treated with adenosine deaminase (ADA, 1 U/ml) in regular culture medium for 24 h before being harvested. The transfected HEK293T cells were homogenized using a Sonicator (MISONIX, Farmingdale, New York, USA) at a setting of output 2 for 30 s. Cell debris and nuclear fragments were removed by low-speed spinning (1500 ×*g* for 5 min). For the AC assay, the collected total lysates (100 μg per reaction unless stated otherwise) were treated with the indicated reagent for 10 min at 37 °C in a 400-μl reaction mixture containing 1 mM ATP, 100 mM NaCl, 50 mM HEPES, 21 μM vinpocetine, 20 μM EHNA, 0.7 μM cilostamide, 20 μM rolipram, 10 μM papaverine, 6 mM $MgCl_2$, 1 μM GTP, and 0.2 mM EGTA. The reactions were terminated by the addition of 600 μl of 10% trichloroacetic acid. The cAMP formed was isolated by Dowex chromatography (Sigma) and assayed by a radioimmunoassay as described previously [31].

2.8. Immunocytochemistry

The cells were plated onto poly-L-lysine-coated (Sigma) coverslips and then transfected with the desired construct(s) for 48–72 h. The cells were fixed with 4% paraformaldehyde in PBS at room temperature for 30 min, permeabilized with 0.05% NP40/PBS for 10 min, blocked with PBS/2% BSA/2% goat serum, stained with the desired primary antibody reconstituted in PBS/2% BSA at 4 °C for 14–16 h, followed by the corresponding secondary antibody conjugated with Alexa Fluor-488 and Alexa Fluor-568 at room temperature for 2 h, and analyzed by laser confocal microscopy (MRC-1000; Bio-Rad, Hercules, CA, USA; LSM510, Carl Zeiss).

3. Results

3.1. G2L2 interacts with the A_{2A}R-C

To search for novel interacting partners of the A_{2A}R-C (aa 310–410, Fig. 1A), we generated and purified a recombinant protein (GST–A_{2A}R-C) composed of GST fused to the N terminus of A_{2A}R-C. The purified GST–A_{2A}R-C was incubated with rat cortical lysates for a GST pull-down assay as described in "Supplementary methods". The identities of those proteins bound to the GST–A_{2A}R-C were determined by mass spectrometry. Among them, we were particularly interested in G2L2 (Supplementary material Fig. S1A, C) because functional annotation indicated that this protein might bind to actin cytoskeleton, which plays an important role in the organization of signaling microdomains. We noticed that the G2L2 fragments identified in GST pull-down assays were much smaller than the predicted size of G2L2 (~95 kDa) and were likely to be the degradation products of G2L2. Analyses of the peptide hits revealed the existence of both N- and C- terminal degradation products of G2L2, suggesting that A_{2A}R-C might associate with large multiprotein complexes. In addition, G2L2 appeared to be unstable in the experimental conditions tested. To further investigate the role of G2L2, we generated an expression construct of mouse G2L2 (which shares 90% similarity of amino acids with rat G2L2) fused to a V5 tag at its C terminus. Immunoprecipitation of exogenously expressed G2L2-V5 in HEK293T cells using an anti-V5-antibody or an anti-G2L2 antibody pulled down both the full-length G2L2 and a small degradation product of G2L2 (~30 kDa, a C terminal fragment harboring aa 561–572; Fig. S1B, C), supporting the hypothesis that G2L2 is prone to degradation.

To verify the interaction between A_{2A}R-C and G2L2, an A_{2A}R mutant ($A_{2A}R_{253–410}$) that contained only the 7th transmembrane span and the entire A_{2A}R-C [14] was co-transfected into HEK293T cells with G2L2-V5 or another interacting protein of A_{2A}R-C, TRAX-V5 [14] as indicated. Similar to TRAX-V5, immunoprecipitation of G2L2-V5 brought down $A_{2A}R_{253–410}$ (Fig. 1B), supporting the hypothesis that A_{2A}R-C interacted with G2L2. To identify the domain located on A_{2A}R-C that interacted with G2L2, we produced a flag-tagged A_{2A}R variant ($A_{2A}R_{1–322}$-flag) with a C-terminal truncation. The major immunoreactive bands of these A_{2A}R variants are consistent with the predicted sizes for $A_{2A}R_{1–410}$-flag (WT) and $A_{2A}R_{1–322}$-flag (46 kDa and 35 kDa, respectively, Fig. 1C). Each A_{2A}R variant also had a slightly larger species, which was assumed

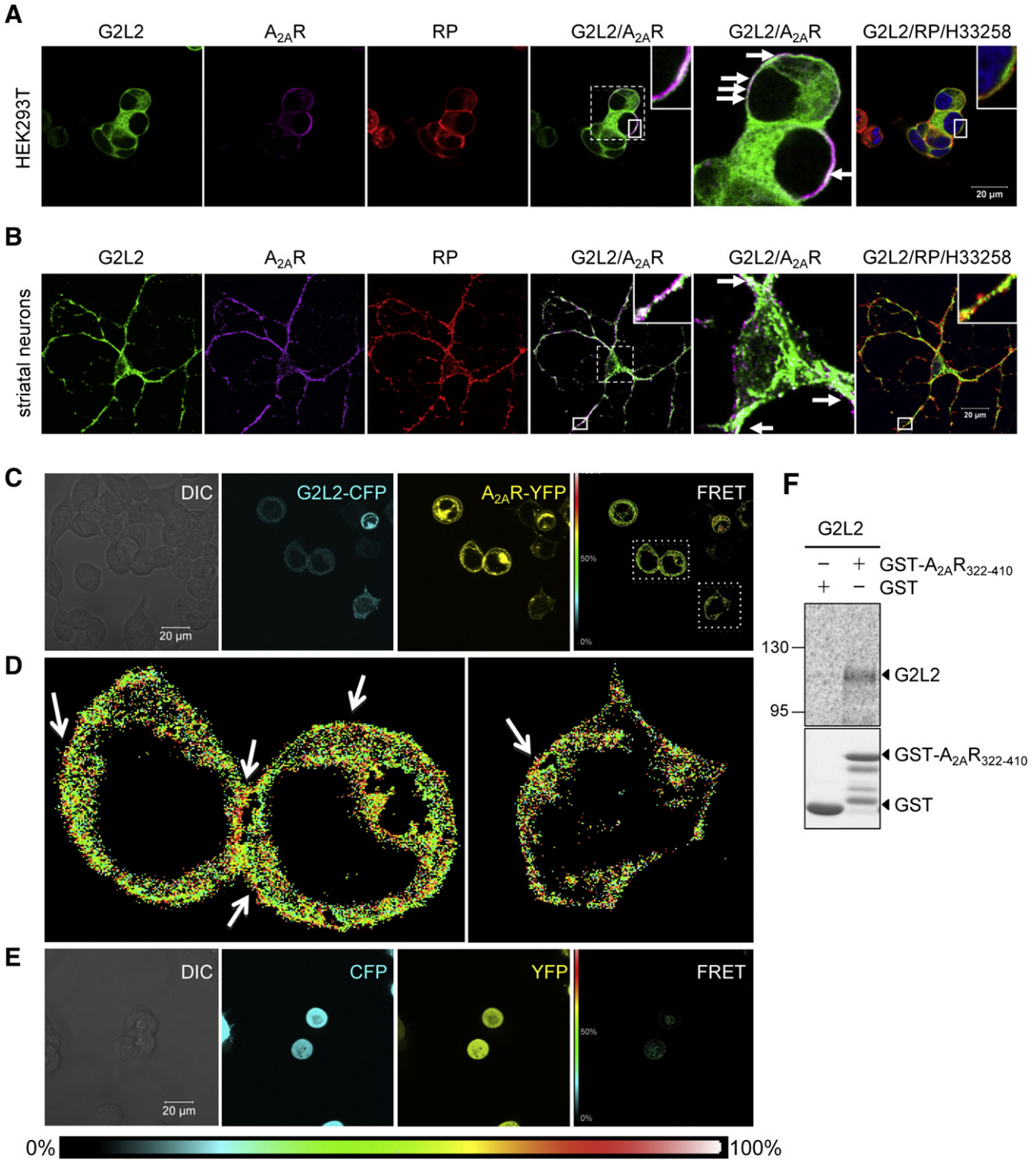

Fig. 2. G2L2 directly interacts with A_{2A}R. (A) HEK293T cells transfected with G2L2-V5 and A_{2A}R-flag were fixed and immunostained with the anti-V5 antibody (green), anti-flag antibody (purple), and rhodamine–phalloidin (RF, red) to detect F-actin. (B) Primary striatal neurons (days *in vitro* 18) were fixed and immunostained with the anti-G2L2-D antibody (green), anti-A_{2A}R antibody (purple), and RF (red). Nuclei were stained with Hoechst 33259 (H33258, blue). Arrows indicate colocalization signals. Higher-magnification images of the region of interest, indicated by squares, are shown in the upper-right corner of the same panel or in a separate panel. (C) HEK293T cells were co-transfected with G2L2–CFP and A_{2A}R-YFP. (D) Higher magnifications of the indicated regions shown in the FRET images of (C). Arrows indicate regions that showed positive FRET values. (E) The FRET values of cells co-transfected with CFP and YFP were measured. (F) GST–$A_{2A}R_{322–410}$, but not by GST, pulled down G2L2 variants (upper panel). Coomassie blue staining of the recombinant proteins used in the binding experiments is shown in the lower panel.

to be its glycosylated form. Immunoprecipitation of G2L2-V5 pulled down the full length $A_{2A}R$ ($A_{2A}R_{1-410}$). Removal of the C terminal 88 aa of $A_{2A}R$ (*i.e.*, $A_{2A}R_{1-322}$) greatly reduced its ability to interact with G2L2 (Fig. 1C). The domain comprising aa 322–410 of $A_{2A}R$ therefore might be the major binding site for G2L2.

Immunohistochemical staining showed that when expressed exogenously in HEK293T cells, G2L2-V5 was widely distributed throughout the cell (Fig. 2A). Consistent with the above finding that $A_{2A}R$ interacted with G2L2, the membrane-associated G2L2-V5 was colocalized with $A_{2A}R$ (Fig. 2A). Overexpression of $A_{2A}R$, but not G2L2, alone was sufficient to alter the morphology of HEK293T cells to a round shape. We next generated an anti-G2L2 antibody (designated anti-G2L2-D) using a recombinant protein comprising aa 579–860 of G2L2 as the antigen. The resultant anti-G2L2-D antibody recognized endogenous G2L2 in the primary striatal neurons, while the anti-G2L2-D antibody absorbed with the recombinant antigen ($G2L2_{579-860}$) detected no signal (Fig. S2). Colocalization of endogenous $A_{2A}R$ and G2L2 was observed mostly in the neuronal processes. Only very few colocalization signals were found on the plasma membrane of the soma (Fig. 2B). Because $A_{2A}R$ is known to interact with α-actinin (Fig. S3) [16] and G2L2 contains a CH domain that binds to the actin cytoskeleton (Fig. S4) [25], we found that the $A_{2A}R$/G2L2 complex was associated with cytoskeletal F-actin, which was stained using rhodamine–phalloidin (Fig. 2A, B). This finding is consistent with the observation that a peptide hit (aa 124–130) located in the N terminus of G2L2 was found in the GST–$A_{2A}R$-C pulldown complex (Fig. S1A, C).

To further assess whether the interaction between $A_{2A}R$-C and G2L2 was direct, we generated expression constructs of $A_{2A}R$-YFP and G2L2–CFP and transfected these constructs into HEK293T cells for fluorescence resonance energy transfer (FRET) analyses. If the distance between $A_{2A}R$–YFP and G2L2–CFP was less than 100 Å, energy transfer from the donor (CFP) to the acceptor (YFP) would occur. The images were pseudocolored with the calculated FRET values accordingly. Warmer colors represented higher FRET values and shorter distances between the tested protein pairs. As shown in Fig. 2C, expression of $A_{2A}R$–YFP and G2L2–CFP, but not YFP plus CFP (Fig. 2E), together resulted in positive FRET signals predominantly on cell membranes (Fig. 2D), which is consistent with the cellular location of $A_{2A}R$. This

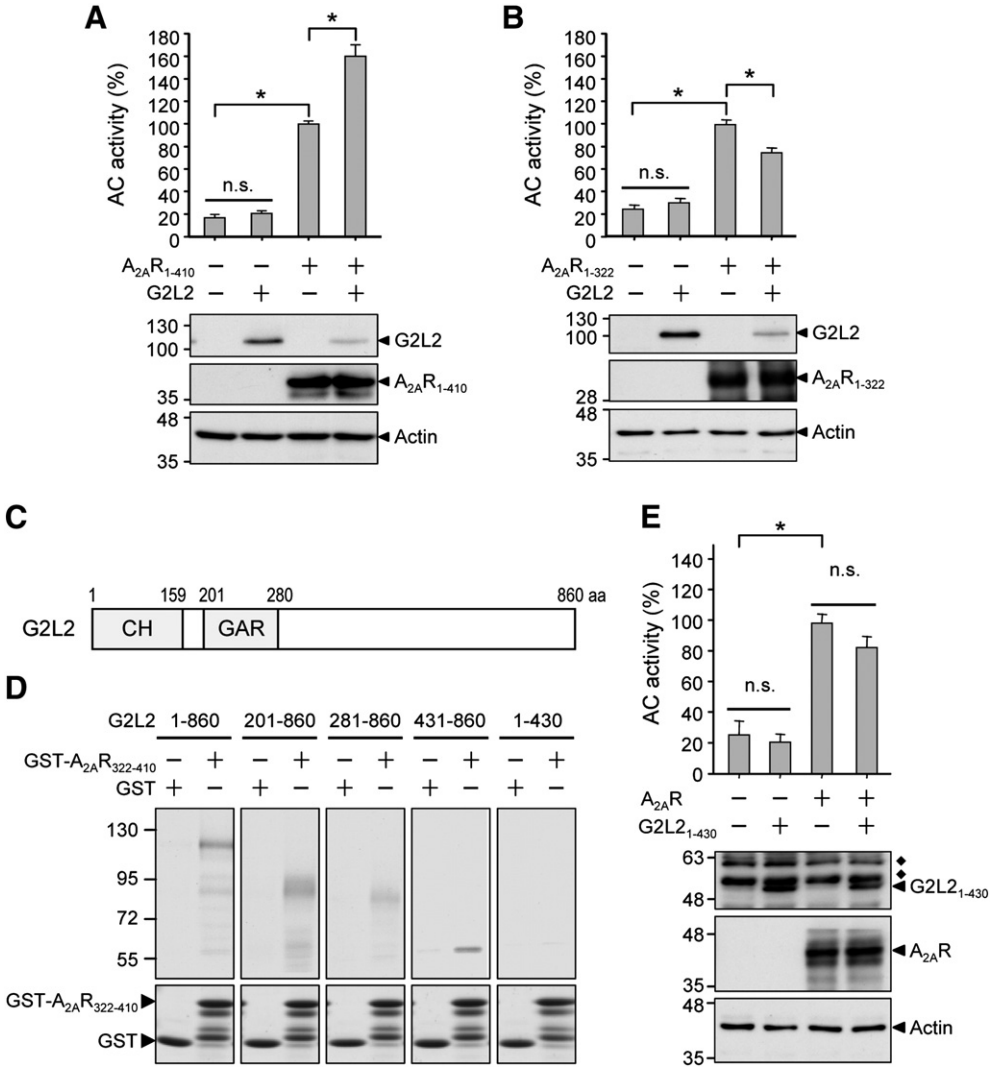

Fig. 3. Direct interaction is required for G2L2 to enhance the $A_{2A}R$-mediated adenylyl cyclase (AC) activation. (A) HEK293T cells were transfected with an empty vector, G2L2-V5, $A_{2A}R_{1-410}$-V5, or G2L2-V5 plus $A_{2A}R_{1-410}$-V5. (B) The cells were transfected with the vector, G2L2-V5, $A_{2A}R_{1-322}$-V5, or G2L2-V5 plus $A_{2A}R_{1-322}$-V5. (E) The cells were transfected with the vector, $G2L2_{1-430}$-V5, $A_{2A}R_{1-410}$-V5, or $G2L2_{1-430}$-V5 plus $A_{2A}R_{1-410}$-V5. Expression levels of G2L2 and the $A_{2A}R$ variant were analyzed using western blot analyses (30 μg per lane). The data are presented as the mean ± S.E. from three or four independent experiments. (C) The schematic structure of G2L2 depicts an N-terminal Pfam:CH domain of amino acids (aa) 1–159, a GAR domain of aa 201–280 [25], and a long C-terminal undefined region. (D) Purified GST and GST–$A_{2A}R$ proteins were incubated with the indicated ^{35}S-G2L2 variant (15 fmol) at 4 °C for 1 h. Coomassie blue staining of recombinant proteins used in the binding experiments is shown in the lower panel. * $p < 0.001$, by one-way ANOVA. The levels of the indicated proteins were analyzed by western blotting using the anti-G2L2-D (A,B), -N (E), anti-$A_{2A}R$, and an anti-actin antibody as indicated. ◆, non-specific bands.

finding supports that the distance between $A_{2A}R$–YFP and G2L2–CFP is sufficiently close for direct interactions. In addition, the purified recombinant GST–$A_{2A}R_{322–410}$ protein, which contains the $A_{2A}R$-C (aa 322–410) fused to GST, but not GST alone, successfully pulled down *in vitro*-translated G2L2 (Fig. 2F), further demonstrating that $A_{2A}R$-C directly interacts with G2L2.

3.2. G2L2 enhanced $A_{2A}R$-mediated cAMP signaling via direct binding

Many interacting proteins have been reported to bind $A_{2A}R$, yet only Usp4 could indirectly control the signaling by preventing membrane $A_{2A}R$ from recycling into the cytoplasm [20]. As a new binding partner of $A_{2A}R$, we overexpressed G2L2 together with $A_{2A}R$ in HEK293T cells to test whether G2L2 would affect $A_{2A}R$ signaling. This cell line was chosen because it did not contain a detectable level of endogenous G2L2 protein using the anti-G2L2-D antibody (Fig. 3). Expression of G2L2 together with the WT $A_{2A}R$ (*i.e.*, $A_{2A}R_{1–410}$) enhanced AC activity triggered by an $A_{2A}R$-selective agonist (CGS21680, CGS; 10 μM; Fig. 3A). In contrast, G2L2 did not enhance activation of AC triggered by stimulation of an $A_{2A}R$ variant that lacked the major binding domain (aa 322–410) of G2L2 (Figs. 1C, 3B). Therefore, binding with $A_{2A}R$-C is required to enhance $A_{2A}R$-mediated AC activation by G2L2. Note that the $A_{2A}R_{1–322}$ preserved most of the functional properties (such as ligand binding and the ability to stimulate AC) of the WT $A_{2A}R$, as previously reported [32]. Immunocytochemical staining also demonstrated that $A_{2A}R_{1–322}$ was located properly in the plasma membrane, as did WT $A_{2A}R$ (Fig. 2A, Supplementary material Fig. S3). To our surprise, expression of G2L2 caused a slight but statistically significant reduction of AC activity triggered by $A_{2A}R_{1–322}$ (Fig. 3B), suggesting that an additional factor might be involved in the regulation of $A_{2A}R$'s activity by G2L2. Another interesting observation was that the level of exogenously expressed G2L2 was consistently lower in the presence of $A_{2A}R$ variants (including $A_{2A}R_{1–410}$ and $A_{2A}R_{1–322}$), which activated the Gαs/AC/cAMP pathways upon stimulation (Fig. 3). The protein level of G2L2 thus might be regulated by a cAMP-dependent pathway.

G2L2 contains two cytoskeleton-associated domains (*i.e.*, CH and GAR) at its N terminus and a highly variable C terminal domain with no similarity to other known protein domains (Fig. 3C). To search for the $A_{2A}R$-interacting domain of G2L2, we created a series of G2L2 mutants as follows: $G2L2_{201–860}$, which had no CH domain, $G2L2_{281–860}$, which had neither the CH nor the GAR domain, $G2L2_{1–430}$, and $G2L2_{431–860}$. These G2L2 variants were *in vitro* translated and labeled with ^{35}S-methionine (Fig. S5). In the GST pull-down assays, the recombinant GST–$A_{2A}R$-C protein pulled down all G2L2 variants except $G2L2_{1–430}$. The $A_{2A}R$ binding domain of G2L2 therefore was located at its C terminus (aa 431–860, Fig. 3D). Consistently, a peptide hit

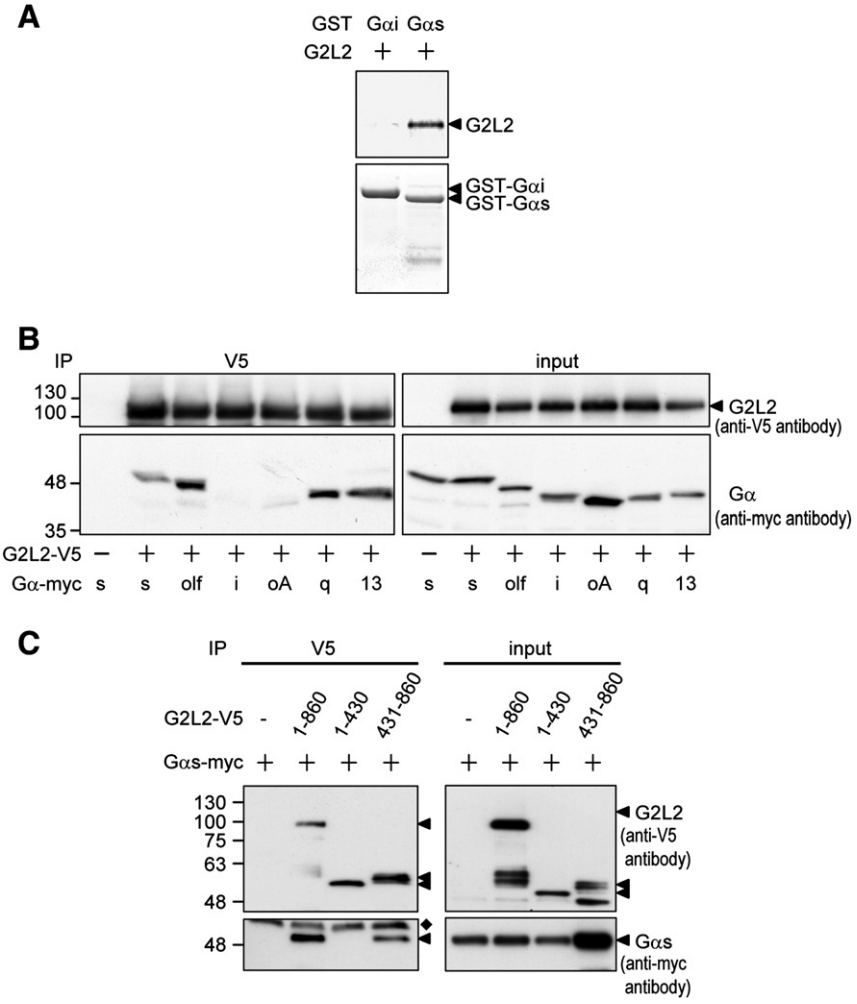

Fig. 4. Gαs-2 like 2 (G2L2) selectively bound to the Gαs protein. (A) Purified GST-Gαi or GST–Gαs proteins (15 μg) were incubated with ^{35}S-labeled G2L2 (30 fmol) at 4 °C for 1 h. Coomassie blue staining of the recombinant proteins employed in binding experiments is shown in the lower panel. (B) HEK293T cells were transfected with G2L2-V5 and the indicated Gα protein for 48 h. The cells were treated with ADA (1 U/ml) to remove endogenous adenosine for 24 h before being harvested. The cell lysates (3 mg) were incubated with the anti-V5 antibody (1 μg) for immunoprecipitation. Equal volumes of total lysate (1: 100; Input) were loaded as a control. (C) HEK293T cells were transfected with the indicated constructs for 48 h and treated with adenosine deaminase (ADA, 1 U/ml) and MG132 (10 μM) overnight to prevent interference by endogenous adenosine receptors and degradation of G2L2, respectively. ♦, a non-specific band.

harboring aa 578–600 of G2L2 was identified in the complex pulled down from brain lysate using GST–A_{2A}R-C (Fig. S1A, C). In line with this conclusion, expression of a G2L2 variant ($G2L2_{1-430}$), which did not bind A_{2A}R, did not affect A_{2A}R-mediated activation of AC (Fig. 3E). This result further strengthened our hypothesis that direct binding between G2L2 and A_{2A}R is critical for the enhancement of A_{2A}R's signal by G2L2.

3.3. G2L2 is a novel interacting protein of Gαs

Although G2L2 enhanced A_{2A}R-mediated AC activation as described above (Fig. 3A), forskolin (10 μM)-evoked AC activity did not significantly differ in the absence or presence of G2L2 (Fig. S6, 100.0 ± 1.4 and 102.02 ± 5.7, respectively, mean ± SEM, $n = 11$; $p = 0.401$, Student's *t*-test). Because forskolin directly activates ACs [33] and G2L2 did not alter the cAMP response triggered by forskolin, we reasoned that G2L2 might mediate its enhancing effect on A_{2A}R's cAMP signaling by altering the coupling between A_{2A}R and Gαs. Given the importance of the local microdomain concentration of the targeted protein(s) in the cAMP signaling pathway [34,35], we tested whether G2L2 also interacted with Gαs. As shown in Fig. 4A, the interaction between G2L2 and Gαs appeared to be direct because purified GST–Gαs (but not GST-Gαi) successfully pulled down the *in vitro*-translated ^{35}S-labeled G2L2.

We next examined whether G2L2 selectively bound to Gαs by co-expression of different Gα isoforms and G2L2 in HEK293T cells, followed by immunoprecipitation and western blot analyses as shown in Fig. 4B. Among the six Gα proteins tested, G2L2 selectively bound to Gαs, Gαolf, Gαq, and Gα13. No binding of G2L2 with Gαi or GαoA was detected. Since A_{2A}R may also be coupled to G proteins other than Gαs [36,37], the potential binding to Gαq/Gα13 raised the possibility that G2L2 might also facilitate other Gα protein-mediated signaling pathways triggered by A_{2A}R.

3.4. The inactive form of Gαs competed with the A_{2A}R and bound to the C-terminus of G2L2

Immunoprecipitation of Gαs coexpressed with the indicated G2L2 variant in HEK293T cells indicated that Gαs bound the C terminus of

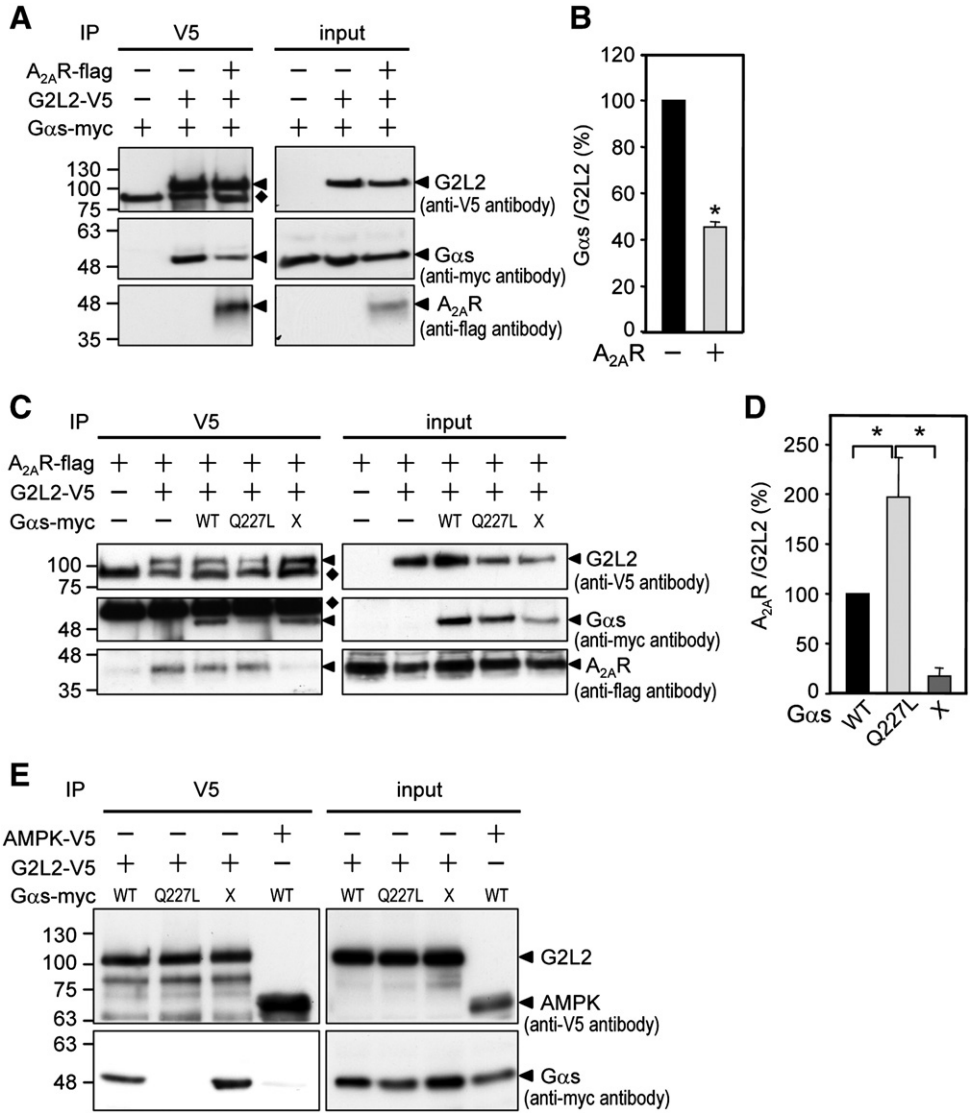

Fig. 5. Inactive form of Gαs competed with A_{2A}R to bind to the C-terminus of G2L2. (A, C, E) HEK293T cells were transfected with the indicated constructs for 48 h and incubated with ADA (1 U/ml) overnight before being harvested. The immunocomplexes were analyzed by western blotting. ♦, a non-specific band. (B) Quantification of the level of Gαs bound to G2L2. The amount of Gαs was normalized to that of immunoprecipitated G2L2 in each condition and was compared with those in the absence of A_{2A}R. The data are presented as the mean ± S.E. from three independent experiments. * $p < 0.001$, by Student's *t*-test. (D) Quantification of the level of A_{2A}R bound to G2L2. The amount of A_{2A}R was normalized to that of immunoprecipitated G2L2 in each condition and was compared to those in the presence of Gαs-WT. The data are presented as the mean ± S.E. from 3 independent experiments. * $p < 0.05$, by one-way ANOVA.

G2L2 (aa 431–860; Fig. 4C). Because $A_{2A}R$ also bound to the C terminus of G2L2 (Fig. 3D), we tested whether $A_{2A}R$ and Gαs could bind to G2L2 in a competitive manner. Elevated expression of $A_{2A}R$ in HEK293T cells significantly reduced the amount of Gαs bound by G2L2 (Fig. 5A, B). Most importantly, we found that expression of the dominant negative Gsα mutant, which binds xanthine nucleotides instead of guanine nucleotides (Q227L/D295N, Gαs-X; [38]), but not the dominant positive Gsα mutant with reduced GTPase activity (Gsα-Q227L, [39]), of Gαs reduced the interaction between G2L2 and $A_{2A}R$ (Fig. 5C, D) due to the preferential binding of G2L2 to the inactive Gαs (Fig. 5E). Collectively, these data showed that binding sites of $A_{2A}R$-C and Gαs both existed in the C terminus of G2L2 and likely overlapped. G2L2 might facilitate the recruitment of trimeric G protein complexes to the proximal position of $A_{2A}R$ for efficient assembly of the G protein with $A_{2A}R$ upon stimulation (Fig. 6). Once stimulated by activated $A_{2A}R$, the GTP-bound Gαs protein would be released from the C terminus of G2L2 to activate its downstream effectors, while the empty C terminus of G2L2 would then be recruited by $A_{2A}R$-C until its next encounter with inactive Gαs proteins.

4. Discussion

In the present study, we identified G2L2 as a novel scaffold protein that directly binds to $A_{2A}R$-C and the inactive form of Gαs. Because G2L2 has a CH domain at its N terminus as does GAS2 (Fig. 3C, [25]), it is colocalized with F-actin, as revealed by immunocytochemical analysis (Figs. 2A, B, S4). Similarly, $A_{2A}R$ also colocalizes with F-actin (Fig. S3) because it tethers to the cytoskeleton *via* direct binding to an F-actin cross-linking protein (α-actinin, [16]) at its C terminus (aa 291–322 of $A_{2A}R$). Even without the direct interaction between $A_{2A}R$-C and G2L2 as shown in the present study (Figs. 1–3), $A_{2A}R$ would have the opportunity to indirectly co-localize with G2L2 through binding to α-actinin/F-actin (Figs. 2A, B, S3, 4). Nonetheless, G2L2 did not enhance the activation of AC evoked by $A_{2A}R_{1-322}$, the $A_{2A}R$ variant that lacked the major G2L2-binding site but contained an intact α-actinin-binding site and therefore was tethered to F-actin (Figs. 3B, S3). The G2L2 variant ($G2L2_{1-430}$), which contained the CH domain and thus bound to F-actin (Fig. S4), did not increase the activation of AC evoked by $A_{2A}R$ either (Fig. 3E). G2L2 therefore is unlikely to modulate those G-protein-coupled receptors (GPCRs) that are bound to the cytoskeleton [40–43] unless they also directly bind to G2L2, as was observed for $A_{2A}R$. Our data showed that the C terminus of G2L2 preferentially bound to the inactive form of Gαs (Fig. 5C, E) and facilitated the efficient assembly of trimeric G protein complexes with $A_{2A}R$ upon stimulation (Fig. 6). *Via* binding to G2L2, $A_{2A}R$-C controls an appropriate and effective microdomain environment and thus enhances stimulation of Gαs and activation of its downstream effectors (such as AC, Fig. 3A). Our data collectively suggest that the synergistic interplay among $A_{2A}R$, Gsα, and G2L2 is critical for enhancement of $A_{2A}R$'s signal. In addition, such facilitation of $A_{2A}R$'s signal highly depends on the physical interaction between $A_{2A}R$ and G2L2. The Gsα-binding site of the $A_{2A}R$ variant ($A_{2A}R_{1-322}$) that did not bind to G2L2 remained intact and could activate Gsα and AC upon stimulation (Fig. 3B). Expression of G2L2, another Gsα-binding protein, is likely to compete for available Gsα proteins with $A_{2A}R_{1-322}$, and thus suppressed AC activity evoked by $A_{2A}R_{1-322}$ (Fig. 3B). Likewise, removal of the $A_{2A}R$-interacting domain (aa 431–860) of G2L2 also eliminated the enhancing effect of G2L2 on AC activation triggered by $A_{2A}R$ (Fig. 3E). We noted that expression of $G2L2_{1-430}$ also slightly reduced $A_{2A}R$'s signal, although the difference was too small to be statistically significant (Fig. 3E). While $G2L2_{1-430}$ lacks the $A_{2A}R$-binding site, its CH domain is intact and therefore is associated with the F-actin cytoskeleton (Figs. 3C, S4). It is possible that $G2L2_{1-430}$ might function as a dominant negative mutant to compete with the endogenous wildtype G2L2 for an association with the cytoskeleton, and therefore might compromise the enhancing function of endogenous G2L2. Nonetheless, because we did not detect endogenous G2L2 in HEK293T cells, the level of endogenous G2L2 in HEK293T cells might have been too low to produce any impact on our experimental observations. This would also explain why the effect of $G2L2_{1-430}$ on $A_{2A}R$'s signal was not significant (Fig. 3E). Alternatively, the slight decrease in $A_{2A}R$'s signal by $G2L2_{1-430}$ might have been due to nonspecific alteration of the cytoskeletal structure due to overexpression of a cytoskeleton-binding protein ($G2L2_{1-430}$). Taken together, these results suggest that the direct interaction between $A_{2A}R$ and G2L2 is essential for constructing a microdomain that favors activation of Gαs by $A_{2A}R$.

In addition to being coupled to Gαs and Gαolf, $A_{2A}R$ was reported to trigger signaling pathways *via* other G proteins [37,44]. Because G2L2 also bound to Gαq and Gα12/13 (Fig. 4B), G2L2 might also modulate $A_{2A}R$-triggered signals other than cAMP. It is unlikely that other members of the GAS2 family also possess similar functions to G2L2 because their homologous regions are mostly in the N-terminus [21,25,26,45], not in their C-terminal domains, which usually exert distinct functions [45] (Fig. S7). It should be noted that human G2L2 was previously reported to exist in HEK293T cells [25]. However, our in-house anti-G2L2-D antibody, which was raised against a recombinant protein containing the most variable C terminus of mouse G2L2 (aa 579–860),

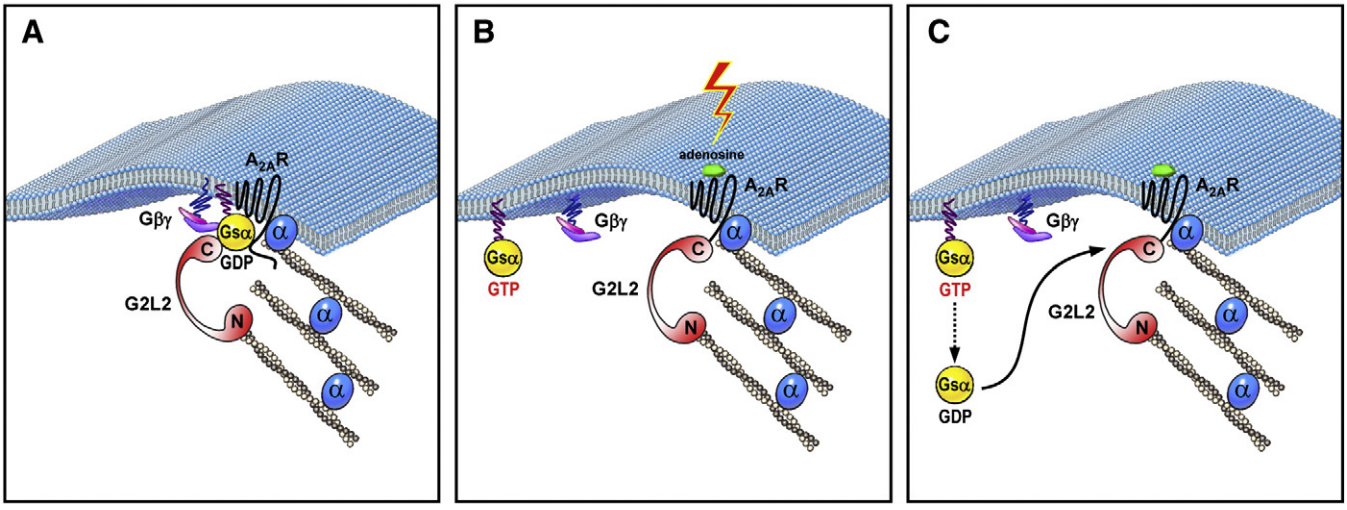

Fig. 6. A schematic representation showing G2L2 as a novel scaffolding protein that recruits components of $A_{2A}R$ signalosome to the microenvironment. The N terminus of G2L2 colocalized with F-actin, whereas the $A_{2A}R$ also colocalized with F-actin *via* α-actinin (α). The C terminus of G2L2 bound to 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 (A). Once activated by adenosine, the GTP-bound Gαs protein was released from the C terminus of G2L2 to activate its downstream effectors (B), while the empty C terminus of G2L2 was then recruited by $A_{2A}R$-C until its next encounter with inactive Gαs proteins (C).

failed to detect the presence of endogenous human G2L2 in HEK293T cells (Fig. 3). This discrepancy most likely occurred because of the highly variable C-terminus of mouse G2L2, which shares only 66% amino acid similarity (Fig. S8) with that of human G2L2.

To date, at least 8 proteins (including G2L2) that interact with the C terminus of $A_{2A}R$ have been reported and characterized [13–16,20]. Among them, G2L2 is the first $A_{2A}R$-interacting partner that directly contributes to the $A_{2A}R$-mediated cAMP signaling pathway. Such an enhancing effect of G2L2 on $A_{2A}R$ relies on the direct binding between $A_{2A}R$ and G2L2, which contributes to the formation of a microdomain that favors the activation of Gαs by $A_{2A}R$. Because Gαs-activated cAMP signaling is the major pathway that mediates the functions of $A_{2A}R$ [10,46], G2L2 might serve as a key determinant that controls the action of $A_{2A}R$ by modulating its ability to regulate the cellular cAMP contents. Because G2L2 binds to the same domain of $A_{2A}R$ as does D2R [47], the potential role of G2L2 in $A_{2A}R$–D2R heteromerization is also of great interest. In addition to direct intra-membrane interactions, an important intracellular electrostatic interaction occurred between the N-terminus of the 3rd intracellular loop of D2R (*i.e.*, the Arg-rich epitope) and the C-terminal segment of $A_{2A}R$ (aa 370–378 in humans; aa 365–373 in the rat) that overlaps with the G2L2-binding domain (aa 322–410, Fig. 1C). Moreover, two Arg-rich regions exist in the $A_{2A}R$-binding domain of G2L2 ($_{576}$ALRRREQAL$_{585}$ and $_{759}$LRPRIRPRRD$_{768}$; the Arg-rich regions are underlined), supporting G2L2 possibly competing with D2R for $A_{2A}R$. This is particularly important because our data showed that G2L2 bound to Gαolf (Fig. 4B), which is the major Gαs-like protein in the striatum where $A_{2A}R$ and D2R colocalize [5]. It is possible that G2L2 might interfere with $A_{2A}R$–D2R heteromerization and facilitate the $A_{2A}R$/Gαolf-mediated cAMP signal in the striatum. In addition to potentially interfering with the physical interaction between $A_{2A}R$ and D2R, the "biochemical fingerprint" of the $A_{2A}R$–D2R heteromer including ligand binding affinity, G protein coupling switch, and receptor internalization [48], might also be affected by G2L2 and is worth further evaluation in the future. Given that $A_{2A}R$ drugs were implicated in neuronal neurodegenerative diseases [9,46,49–52], further characterization of the expression and subcellular localization of G2L2 during development and degenerative disorders would greatly advance our current understanding of $A_{2A}R$ as a drug target for neuronal diseases and traumas.

Author contributions

YCW designed and conducted the experiments and wrote the manuscript. HLL and WCC performed the cAMP assay and the transfection experiments, respectively. JTL carried out the immunocytochemical staining. YJL performed the FRET analysis. YC designed the experiments and wrote the manuscript.

Conflict of interest

The authors have declared that they have no conflict of interests.

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Appendix A. Supplementary data

(Supplementary information is available at *BBA*: *Molecular Cell Research* Online). Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2013.08.009.

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