The Adenosine A_1 and A_{2A} Receptor C-termini are Necessary for Activation but not the Specificity of Downstream Signaling

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Abstract

Recent efforts to determine the high-resolution crystal structures for the adenosine receptors (A1R and A2AR) have utilized modifications to the native receptors in order to facilitate receptor crystallization and structure determination. One common modification is a truncation of the unstructured C-terminus, which has been utilized for all the adenosine crystal structures obtained to date. However, the C-terminus has been identified as a location for protein-protein interactions that may be critical for physiological function of these important drug targets. Here, we determine whether the presence of the full-length C-terminus affected downstream signaling using a yeast MAPK response-based fluorescence assay. Upon ligand binding, the A1Δ291R or A2AΔ316R variants were unable to couple to human-yeast chimeric G-protein chimeras to generate a downstream signal in yeast, though full-length receptors showed native-like G-protein coupling. Further, constructs transfected into mammalian cells (HEK-293) showed similar behavior – i.e. the variants with C-terminal truncations lacked cAMP-linked signaling compared to the full-length receptors. Although the C-terminus was essential for Gα protein- associated signaling, chimeras of A1R with a C-terminus of A2AR coupled to the A1R-specific Gα (i.e. Gαi1 versus Gαs). This surprising result suggests that the C-terminus is important in signaling, but not specificity, for the interaction with Gα protein. This result has further implications in drug discovery both in enabling the experimental use of chimeras for ligand design, and in cautious interpretation of structure-based drug design based on truncated receptors.

Receptor	Expression host	Ligand	Modification	Modification	Modification
			Chimera	**Stabilization**	**Thermo-stabilizatio**
$A_{2A}R$	*S. frugiperda*	ZM241385	**X**		
	S. frugiperda	*UK-432097*	**X**		
	Trichoplusia ni	*Adenosine*; Synthetic ligand			**X**
	S. frugiperda	Caffeine; ZM241385; XAC			**X**
	P. pastoris	ZM241385		**X**	
	S. frugiperda	Novel compound			**X**
	S. frugiperda	ZM241385	**X**		
	Trichoplusia ni	*CGS21680*			**X**
	Trichoplusia ni	ZM241385 and novel compounds	**X**		**X**
	Trichoplusia ni	*NECA*	**X**	**X**	
	S. frugiperda	ZM241385	**X**		
	S. frugiperda	Novel compound	**X**		
	S. frugiperda	ZM241385	**X**		
	S. frugiperda	ZM241385	**X**		
	Trichoplusia ni	Theophylline; caffeine; PSB36	**X**		**X**
	Trichoplusia ni	ZM241385	**X**		**X**
	S. frugiperda	ZM241385	**X**		**X**

Receptor	Expression host	Ligand	Modification	Modification	Modification
	P. pastoris	ZM241385	**X**		
	Trichoplusia ni	Theophylline and novel compounds	**X**		**X**
	P. pastoris	*UK-432097*	**X**		
	Trichoplusia ni	*NECA*	**X**	**X**	
	S. frugiperda	ZM241385	**X**		
A_1R	*S. frugiperda*	DU172	**X**		
	Trichoplusia ni	PSB36	**X**		**X**
	Trichoplusia ni	*Adenosine* and DU172	**X**		

Receptor chimeras have traditionally been used to understand the role of the receptor domains in ligand recognition, G-protein coupling and specificity, and the ability to produce downstream signaling. In our previous study (Jain et al., 2018), we created an adenosine A_1/A_{2A} receptor chimera to improve membrane localization and expression in yeast for A_1receptor (A_1R) variants and reported exceptional yields of the active receptor compared to parental A_1R expressed in any host system to date.

Adenosine receptors are a GPCR subfamily of four receptors (A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R) that recognize the natural ligand adenosine, an important energy metabolite (Fredholm et al., 2001; Fredholm et al., 2011). Adenosine is produced in tissues under stressful conditions like ischemia or hypoxia or energy "demand-supply" imbalance (Fredholm et al., 2005; McIntosh et al., 2012). All four adenosine receptor subtypes provide critical protection under stressful conditions and therefore, are therapeutic targets for Parkinson's disease, Alzheimer's disease, cardiovascular diseases, and many others (Chen et al., 2013). Multiple crystal structures of $A_{2A}R$ have been resolved with bound agonists or antagonists (Table 1). Recently, three crystal structures have been reported for A_1R (Cheng et al., 2017; Draper-Joyce et al., 2018; Glukhova et al., 2017). All structures reported for the adenosine receptors contain a C-terminal truncation, except a recently published cryo-EM structure of A_1R (Draper-Joyce et al., 2018). The C-terminus of A_1R is 34 amino acids long, whereas the $A_{2A}R$ C-terminus is relatively long with 122 amino acids. The two crystal structures of A_1R contain a truncation from residues 311 and 316. Most crystal structures of $A_{2A}R$ contain a truncation from residue 316 ($A_{2A}\Delta316R$), corresponding to only 26 out of the 120 aminos acids or approximately 20% of the total $A_{2A}R$ C-terminus. The long C-terminus of $A_{2A}R$ has been hypothesized to be involved in receptor expression (Britton, 2012; Jain et al., 2018; Moriyama et al., 2010), interactions with other signaling partners (Gsandtner et al., 2006; Zezula et al., 2008), oligomerization (Navarro et al., 2018) and receptor turnover (Singh et al., 2010; Weiss et al., 2002). However, previous studies have suggested that the $A_{2A}\Delta316R$ has native-like signaling (Bennett et al., 2013; Klinger et al., 2002; Palmer et al., 1997).

Yeast share many functionally exchangeable proteins involved in the GPCR signaling pathway with higher eukaryotes (Dohlman et al., 1991; Elion, 2000), and have served as a useful microbial platform for rapid ligand screening and lead development for orphan GPCRs (Huang et al., 2015). The GPCR-mediated pathway in yeast is responsive to the presence of peptide mating pheromones that regulate metabolism related to mating. Activated receptors catalyze dissociation of Gpa1, the yeast G protein, activating a mitogen-activated protein kinase (MAPK) cascade, which has been used as a unique platform to study human GPCR signaling (King et al., 1990). In contrast, the presence of multiple GPCRs and G_α proteins in native mammalian systems can confound the results from downstream signaling assays =. Yeast provides a relatively simple and inexpensive platform without the complexities of multiple GPCRs, receptor promiscuity, and crosstalk that occurs in native mammalian hosts (Chen et al., 2007; Saito, 2010).

Engineered yeast strains with modification to the native MAPK-based signaling pathway to report on ligand-mediated downstream signaling from human GPCRs (Figure 1A) were obtained both from the Broach laboratory (Fowlkes et al., 1997) and the Dowell laboratory at GlaxoSmithKline (GSK, Brentford, UK) (Brown et al., 2000). In these yeast strains, the last five amino acids of native yeast G_α (Gpa1) were replaced with the last five amino acids residues from a human G_α to yield native-like GPCR-$G_\alpha$interactions. This replacement has been shown to be sufficient for coupling with many human GPCRs, including human

$A_{2A}R$, resulting in a native-like dose reponse and ligand binding order preference (Brown et al., 2000). Because of structure-based drug discovery efforts that rely on truncated receptors for in silico screening, we investgated one of the key protein-protein interactions of the C-terminus, coupling to G-protein to activate downstream signaling, by utilizing this engineered yeast pheromone response pathway. In addition, the results were validated in transiently transfected mammalian cells to provide further evidence of the value of screening these signaling pathways in yeast.

Materials and Methods

2.1 Materials

Adenosine receptor ligands NECA (5'-N-ethylcarboxamidoadenosine), CPA (N^6-cyclopentyladenosine) and CGS21680 were purchased from Tocris (Minneapolis, MN). Forskolin was obtained from Sigma-Aldrich (St. Louis, MO). Precision Plus Protein Western C Standards was purchased from Biorad (Hercules, CA). Human embryonic kidney cells (HEK-293; ATCC), Dulbecco's modified eagle medium (DMEM, 11995-065), Opti-MEM I reduced serum media (31985-070), fetal bovine serum (FBS, 16000-044), Lipofectamine 2000 transfection reagent (11668-019), RIPA buffer, Halt Protease and Phosphatase Inhibitor Cocktail, mammalian expression vectors (pCEP4) and Alexa 568- donkey anti-rabbit antibody (A10042) were obtained from Invitrogen Life Technologies (Carlsbad, CA). The cAMP dynamic 2 kit was purchased from Cisbio US Inc (Bedford, MA). The mouse monoclonal $A_{2A}R$ antibody was obtained from Santa Cruz Biotechnology (sc-32261, Dallas, TX). The rabbit anti-GFP antibody (ab6556) and goat pAb to Mouse IgG HRP antibody (ab97265) were obtained from Abcam (Cambridge, MA).

2.2 Strains and culture conditions

E. coli strain DH5α was used for amplifying yeast expression plasmids and mammalian expression vectors. *E. coli* was grown in Luria-Bertani media supplemented with 100 μg/mL ampicillin at 37°C at 250 rpm.

Table 2. List of yeast strains used.

Yeast Strain	G protein	Last 5 amino acids at C-terminal	Εχυιαλεντ ηυμαν Γ_α
MMY12, BY4741	Gpa1	KIGII^{COOH}	GPA1 (yeast)
MMY14	Gpa1-G_αq(5)	EYNLV^{COOH}	GNAQ, GNA11
MMY16	Gpa1-G_α16(5)	EINLL^{COOH}	GNA15, GNA16
MMY19	Gpa1-G_α12(5)	DIMLQ^{COOH}	GNA12
MMY20	Gpa1-G_α13(5)	QLMLQ^{COOH}	GNA13
MMY21	Gpa1-G_α14(5)	EFNLV^{COOH}	GNA14
MMY22	Gpa1-G_αo(5)	GCGLY^{COOH}	GNAO
MMY23, CY13393	Gpa1-G_αi1(5)	DCGLF^{COOH}	GNAI1, GNAI2, GNAT1, GNAT2, GNAT3
MMY24	Gpa1-G_αi3(5)	ECGLY^{COOH}	GNAI3
MMY25	Gpa1-G_αz(5)	YIGLC^{COOH}	GNAZ
MMY28, CY13399	Gpa1-G_αs(5)	QYELL^{COOH}	GNAS, GNAL

All yeast strains used in this study are summarized in Table 2. Yeast strains with modified pheromone response pathway and human-yeast chimeric G_α proteins (Fig 1A) were obtained from the Broach laboratory (Fowlkes et al., 1997) and Glaxo-Smith-Kline (GSK) (Brown et al., 2000). These parental yeast strains were grown in YPD media (2% bacto peptone, 2% glucose, 1% yeast extract) and depending on the fus1 transformation, supplemented with 300 μg/mL hygromycin B or 200 μg/mL G418. Yeast expression plasmids were constructed using homologous recombination in *S. cerevisiae* strain BY4741 (*MATa ησ3Δ1 λευ2Δ0 μετ15Δ0 υρα3Δ0*) and were grown in synthetic media. The synthetic media (SD or SG) was comprised of 2% dextrose or galactose, respectively, 0.67% yeast nitrogen base, citrate buffer at pH 5.4 (4.2 g/L citric acid and 14.7 g/L sodium citrate) and supplemented with amino acids and essential nutrients per Burke et al. (2000) (Burke et al., 2000). Uracil was omitted from this media (SD-ura or SG-ura) to select for

plasmid-containing cells. Yeast was grown in culture tubes and multiwell plates at 30°C at 275 rpm.

Human embryonic kidney (HEK-293) cells were maintained in growth media containing DMEM with 10% FBS at 37 °C in a 5% CO_2incubator. Transient transfections were performed by seeding cells on day 0 to be approximately 70% confluent on day 1. On day 1, cells were transfected using 10 µL Lipofectamine 2000 reagent, and 1 µg DNA in two mL Opti-MEM reduced serum media (per 25 cm^2 flask). On day 2 cells were placed back in growth media, and used for experimentation on day 3, approximately 36 hours post-transfection. The cAMP accumulation assay described below (Section 2.6) was performed on cells with passage number less than 25.

2.3 Yeast genomic transformation

To develop a fluorescence-based assay to measure the downstream signaling response in yeast following ligand binding, monomeric Cherry fluorescent protein (mCherry) (Shaner et al., 2004) was introduced into the FUS1 locus under control of the FUS1 promoter. To this end, overlapping fragments were first assembled in yeast using homologous recombination as described below. The fragment consisted of the mCherry fluorescent protein and hygromycin resistance gene hphMx6 or kanamycin resistance gene KanR2 with the translation elongation factor 1 promoter and terminator (pTEF and TEFt). The fragment was flanked with approximately 300 base pairs of the Fus1 promoter and Fus1 terminator to aid in genomic recombination. The Fus1 promoter and Fus1 terminator sequences were amplified from BY4741 using colony PCR. The mCherry protein and pTEF-hphMx6-TEFt fragments were amplified from the pBS35 plasmid, while the pTEF-KanR2-TEFt fragment was amplified from the pBS7 plasmid (Figure 1B). Both pBS7 and pBS35 were received from the Yeast Resource Center at the University of Washington. The fragments were assembled in BY4741 using homologous recombination using pRS316 as a template. Fragment assembly was verified using Sanger sequencing (Operon, Louisville, KY). The resulting fragment was then amplified using PCR and transformed into yeast using the protocol from Gietz and Woods (Gietz et al., 2002). Colony PCR was used to confirm successful genomic integration. Partial sequencing confirmation of final clones was obtained for some of the transformants.

2.4 Subcloning and plasmid construction

A set of yeast expression plasmids (Table 3) containing a GPCR and C-terminal protein tags, necessary for adenosine receptor detection and quantification, was constructed using homologous recombination in BY4741 as described previously (Jain et al., 2018). The plasmid contains a galactose ($pGAL_{1\text{-}10}$) promoter, a pre-pro leader sequence (PP) (Arnold et al., 1998) for targeting to the secretory pathway and the CYC1 terminator ($CYC1_t$) (Jain et al., 2018). For fluorescence microscopy, the GPCRs were C-terminally tagged for easier detection of protein expression with monomeric Citrine fluorescent protein (mCitrine) (Young et al., 2012). Single-point $A_{2A}R$ mutants were created using the QuikChange II Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA). Sequencing was used to confirm the correct gene sequence for the constructs (Operon, Louisville, KY).

Table 3. List of plasmids used for receptor expression in yeast and mammalian cells. Yeast expression plasmids contain an N-terminal leader sequence (PP) to improve receptor expression and trafficking to the plasma membrane (Arnold et al., 1998).

Name	**Plasmid**
ARJ001	pRS316 $pGal_{1\text{-}10}$ PP A_1R mCit cyc_t
ARJ089	pRS316 $pGal_{1\text{-}10}$ PP $A_1\Delta291R$ mCit cyc_t
ARJ002	pRS316 $pGal_{1\text{-}10}$ PP $A_1/A_{2A}R$ mCit cyc_t
ARJ051	pRS316 $pGal_{1\text{-}10}$ PP $A_1/A_{2A}\Delta316R$ mCit cyc_t
ARJ030	pRS316 $pGal_{1\text{-}10}$ PP $A_{2A}R$ mCit cyc_t
ARJ057	pRS316 $pGal_{1\text{-}10}$ PP $A_{2A}\Delta316R$ mCit cyc_t
ARJ194	pCEP4 A_1R
ARJ195	pCEP4 $A_1/A_{2A}R$

Name	Plasmid
ARJ196	pCEP4 $A_1/A_{2A}\Delta316R$
ARJ320	pCEP4 $A_1\Delta291R$
CM001	pCEP4 $A_{2A}R$
CM002	pCEP4 $A_{2A}\Delta316R$
ARJ073	pRS316 $pGal_{1\text{-}10}$ PP pFus1 mcherry pTEF-kanR2-tTEF $Fus1_t$ cyc_t
ARJ172	pRS316 $pGal_{1\text{-}10}$ PP pFus1 mcherry pTEF-hphMx6-tTEF $Fus1_t$ cyc_t

Mammalian expression vector pCEP4 was used for expressing receptors in transiently transfected HEK-293 cells. Untagged A_1R, $A_1/A_{2A}R$, and A_1[?]291R were inserted into the pCEP4 multiple cloning site between HindIII and NotI restriction enzyme sites, whereas A_1/A_{2A}[?]316R, $A_{2A}R$, and A_{2A}[?]316R were inserted between KpnI and XhoI restriction enzyme sites. Transformations of *E. coli* were performed by the heat shock method. Sequencing was used to confirm the correct gene sequences for the plasmids (Operon, Louisville, KY).

2.5 MAPK response signal determination

All ligand stock solutions were prepared to the highest soluble concentration (typically around 40-100mM) in dimethyl sulfoxide (DMSO), according to the recommendations of the manufacturer. Working concentrations of 5 mM ligand (50x) in DMSO were used for all yeast signaling experiments. Yeast cultures were grown overnight in SD-ura selection in 400µL or 1mL media in 48-well or 24-well plates (Falcon 353047 and 353078, Corning, NY), respectively, at 30°C at 275 rpm. Recombinant GPCR expression was induced by transferring 12.5 µL of overnight culture into 400 µL SG-ura. For some strains 0.125% glucose was used to supplement the SG-ura media to improve cell growth of the engineered yeast strains. This level of glucose supplementation has been shown to result in minimal glucose-based suppression of the galactose promoter, as described previously (Bitter et al., 1988). After 24 hours of GPCR expression, twelve µL of the overnight culture was added to 380 µL fresh SG-ura media per well of a 48-well plate. Eight µL of ligand or DMSO was added to each well (final DMSO concentration at 2% (v/v) per well). A high ligand concentration has been shown previously to be needed for effective downstream signaling in yeast (Hara et al., 2012; Niebauer et al., 2005; Price et al., 1995; Price et al., 1996). After ligand addition, the 48-well plate was incubated at 30 °C at 275 rpm for 24 hours. Adenosine deaminase treatment was not required for working with the yeast-based assay as previous studies show this treatment does not impact downstream signaling measurements (Bertheleme et al., 2013; Peeters et al., 2012). Similar results were obtained from 4-hour incubations, but the signal:noise ratio was not as pronounced. Fluorescence intensities of 100 µL of resulting liquid culture were measured in triplicate in a 96-well plate (Costar 3915, Corning, NY) using a BioTek Synergy H1 microplate reader (Winooski, VT) maintained at 30 °C. Experiments were performed for six independent transformants.

2.6 Cyclic adenosine monophosphate accumulation assay

The cyclic adenosine monophosphate (cAMP) accumulation assay was performed as previously described in McGraw et al. (2019) (McGraw et al., 2019). Briefly, transiently transfected HEK-293 and control cells were incubated for 30 minutes in the presence or absence of ligand at a cell density of 1,000 cells/well in a white 384 well plate (Grenier Bio-One #784075, Monroe, NC). Excess cells were pelleted and stored at -80 °C for subsequent Western blotting. The concentration of cAMP per well was determined using the cAMP dynamic 2 kit using a BioTek Synergy H1 Plate Reader according to the manufacturer's protocol. Our previous study (McGraw et al., 2019) has shown that adenosine deaminase (ADA) pre-treatment of cells did not alter the ligand binding or downstream signaling, and therefore the cells were not treated with ADA prior to ligand treatment while utilizing the CisBio HTRF kits (McGraw et al., 2019). Experiments were performed in triplicates for three independent transfections. Data was analyzed as per manufacturer's recommendation and mean and standard error were plotted using Prism (GraphPad, La Jolla, CA). Student's t-test was performed using Prism to obtain the significance of the data.

2.7 Western blotting

Yeast cell pellets (10 OD_{600}) were resuspended in 250 µL lysis buffer (10% glycerol, 50 mM sodium phosphate, 300 mM sodium chloride, pH 8) supplemented with cOmplete EDTA-free protease inhibitor cocktail (Roche, Indianapolis, IN). An equal volume of 0.5 mm zirconia/silica beads (BioSpec, Bartlesville, OK) was added to the cells and lysis was performed using a vortexer or a BeadBug homogenizer (Benchmark Scientific, Edison, NJ). Cell lysates were combined with 4X Laemmli sample loading buffer supplemented with β-mercaptoethanol (Bio-Rad, Hercules, CA). One OD_{600} equivalent of cell lysate was loaded per well for Western blotting. Precision Plus Protein WesternC Standard (BioRad)was used as a standard to enable molecular weight estimation. Rabbit anti-GFP antibody (1:5000 dilution) and Alexa 568-donkey anti-rabbit (1:2500) was used to detect mCitrine protein-tagged receptors.

Transiently transfected HEK-293 cells were scraped, pelleted, and resuspended in ice-cold 1X TE buffer (1% 1M Tris-Cl pH 7.5, 0.2% 500mM EDTA pH 8) with protease inhibitors. Cells were sonicated with a Branson Sonifier 450 at 50% power for 30 pulses and then centrifuged at 2,000 xg for 5 min at 4°C to remove cell debris and unlysed cells. The supernatant was then centrifuged at 100,000 x g for 1 hr at 4°C to pellet cell membranes. Membranes were solubilized in 1X RIPA buffer (10 mM Tris-Cl (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl) with protease inhibitors; if necessary, membranes were sonicated again for 5 pulses at 50% power to break up any visible pieces of membrane. BCA assay (Pierce; Rockford, IL) was performed to determine the total protein concentration of isolated membrane, using bovine serum albumin (BSA; Thermo Fisher, Waltham, MA) as a standard.

Isolated HEK cell membranes were utilized for $A_{2A}R$ and $A_{2A}\Delta316R$ protein quantification via Western immunoassay. Western blotting analysis could not be performed for A_1R and its variant due to the lack of an effective antibody against the receptor. 10 µg of total protein per sample was loaded onto a 12% Tris-Glycine gel and electrophoresed in SDS buffer at 125V for 65 minutes. Western immunoassay was performed using adenosine $A_{2A}R$ mouse monoclonal IgG antibody (sc-32261, Santa Cruz Biotechnology, Dallas, TX) at 1:5000 dilution, and Goat pAb to Mouse IgG HRP antibody at 1:5000 dilution. Membranes were imaged with the UVP BioSpectrum imaging system.

3. Results

3.1 Ωηολε ςελλ φλυορεσςενςε το μονιτορ λιγανδ-μεδιατεδ ΓΠΡ- $Γ_α$ ινταραςτιον

Because the yeast G protein-couled signaling pathway contains homologues to proteins in the human signaling pathway, engineered yeast have been used to successfully recapitulate native ligand binding preferences and G protein coupling for human GPCRs (Bertheleme et al., 2013; Brown et al., 2000; Fowlkes et al., 1997; Peeters et al., 2012). To further the utility of these previously engineered yeast strains, we replaced the original Fus1 modification that relied on growth-dependent signaling (via His3 expression) in the Broach lab strain (Fowlkes et al., 1997) or β-galactosidase reporter activity in the GSK strains (Brown et al., 2000) with an easily detectable fluorescence signal, monomeric Cherry fluorescent protein (mCherry). mCherry is produced in the cells upon ligand-mediated downstream signal activation via the human GPCR-G_α protein coupling (Figure 1A). The signal/noise ratio of mCherry fluorescence in these strains can be easily compared by addition of agonist relative to a control. Here, each yeast strain (Table 2) acts as an independent downstream signaling reporter for a GPCR-G_α interaction.

Twelve strains containing different yeast-human $G_\alpha$chimeras reproduce downstream signaling responses of human G_α proteins (Brown et al., 2000). These strains can be classified into five G_α families: G_αi/o, G_αs, G_αq, G_α12 and native G_α. With the modified G_α chimera strains, we successfully mapped the interaction between the A_1 adenosine receptors and the appropriate G_α using the non-selective high-affinity adenosine receptor family agonist, NECA (100 µM, Figure 1C). A_1R showed a signaling response upon agonist binding with the inhibitory G_α family (G_αi1, G_αi3, G_αo and G_αz) and the promiscuous G_α16. The highest signal was observed for the yeast strain expressing the Gpa1-G_αo chimera. Note that ligand levels are well above expected $K_D$values; however, this behavior is consistent with earlier studies (Bertheleme et al., 2013; Brown et al., 2000; Peeters et al., 2012; Stewart et al., 2009), and perhaps reflects ligand incapable of penetrating the chitosan-rich yeast cell wall to reach the plasma membrane, resulting in an apparent reduced effective

ligand concentration at the membrane.

To ensure our results were not strain-dependent, we compared the signaling response obtained from engineered yeast strains modified from those of the Broach laboratory. A_1R and $A_{2A}R$ were expressed in yeast strains expressing Gpa1-G_αi1 and Gpa1-G_αs. Both receptors maintained their native G_α coupling-specificity, as observed in Figure 1C for A_1R signaling in the GSK strains. The Broach laboratory strains showed a higher signal/noise ratio compared to the GSK strains for both receptors (Figure 2 A & B). Because of the higher signal/noise ratio compared to the GSK strains, the Broach strains were utilized for subsequent studies investigating the role of the C-terminus in downstream signaling.

3.2 Loss of the cytoplasmic C-terminus results in loss of downstream signaling

To investigate the role of the C-terminus on downstream signaling, truncated A_1R and $A_{2A}R$ were constructed. A_1R was truncated at residue 291($A_1\Delta291R$), corresponding with the end of transmembrane 7 and the start of the cytoplasmic tail; this A_1R truncation lacks helix 8. The $A_{2A}R$ truncation at residue 316 ($A_{2A}\Delta316R$) was constructed based on the agonist-bound crystal structure of the receptor obtained by Lebon et al. (Lebon et al., 2011). This $A_{2A}\Delta316R$ contains the helix loop 8 and some residues of the cytoplasmic tail, and has been reported previously to have native-like affinity for the agonist NECA and antagonist ZM 241385 by Magnani and colleages (Magnani et al., 2008). Expression of the truncated receptors with C-terminal tagged mCitrine fluorescent protein fusions was confirmed using Western blot analysis (Figure 3A). Previous studies from our laboratory show that the C-terminal fluorescent protein fusion does not impact trafficking or activity of $A_{2A}R$ (Niebauer et al., 2006; Niebauer et al., 2004; O'Malley, 2009; Wedekind et al., 2006).To evaluate the localization of these receptors inside the yeast, confocal microscopy was performed (Figure 3B ii and iv). Both full-length and truncated $A_{2A}R$ showed efficient localization of the receptor to the plasma membrane, whereas both A_1R constructs showed puncta inside the cell with minimal receptor located at the cell periphery (Figure 3B i and iii).

Upon agonist binding, there was no downstream signaling observed in the truncated $A_1\Delta291R$ and $A_{2A}\Delta316R$ as compared to their full-length receptors in the inhibitory or stimulatory yeast strains (Figure 3C and 3D). Similarly, a previous study performed on rat A_1R had identified that the loss of the C-terminus resulted in a loss of downstream signaling (Pankevych et al., 2003). But, the loss of signaling for the truncated $A_{2A}\Delta316R$ was surprising, as the agonist-bound crystal structures have been reported to be in an active state (Carpenter et al., 2016; Lebon et al., 2011), and the truncation localizes well to the cell surface (as shown in Fig 3B and reported by our lab previously in (Jain et al., 2018)) and binds ligand in mammalian cells (Magnani et al., 2008).

To validate that our observations in yeast reflect native-like behavior, we assayed cyclic adenosine monophosphate (cAMP) accumulation following ligand addition in mammalian cells, as described in the Materials and Methods. HEK-293 cells were transiently transfected with pCEP4 encoding full-length or truncated receptor using lipofectamine. $A_{2A}R$ couples to G_αs, and thus agonist binding activates adenylyl cyclase, resulting in cAMP synthesis. As expected, cells transfected with empty plasmid showed negligible cAMP synthesis in the absence of ligand and remained unchanged following the addition of a selective $A_{2A}R$ agonist, CGS21680 (1 μM) (Figure 4A). The presence of the full-length $A_{2A}R$ led to constitutive activation in the absence of ligand as well as a significant increase in cAMP levels following agonist treatment, consistent with previous studies (McGraw et al., 2019). As expected, based on the yeast results, the $A_{2A}\Delta316R$ showed no increase in cAMP levels upon agonist addition. These data show that $A_{2A}\Delta316R$ does not activate G_αs, suggesting the C-terminus is necessary for downstream signaling of the receptor.

Western blot analysis of membrane preparations was utilized to verify that the absence of $A_{2A}\Delta316R$ activity was not due to reduced protein expression. Similar levels of $A_{2A}R$ and $A_{2A}\Delta316R$ expression were detected in HEK-293 cells; therefore, the lack of a C-terminus had no effect on protein expression levels in cell lysates or extracted total membrane fractions (Figure 4B). Non-transfected cells did not show any receptor expression via western blot analysis (data not shown).

A_1R couples to G_αi/o, which inhibits activation of adenylyl cyclase, so in the absence of ligand there should

be minimal changes to cAMP levels, consistent with our results (Fig. 5). Forskolin directly activates adenylyl cyclase, which leads to stimulation of the production of cAMP even in cells not expressing A_1R, so treatment with 10 μM forskolin was used to elevate the basal level of cAMP. Cells expressing A_1R showed a reduction in cAMP following treatment with an A_1R-selective agonist (1 μM CPA in the presence of 10 μM forskolin). In cells expressing $A_1\Delta 291R$, activation of adenylyl cyclase via forskolin treatment led to the synthesis of cAMP, while agonist treatment showed a negligible change in cAMP levels, in contrast to full-length A_1R. The $A_1\Delta 291R$ behaved similarly to the empty plasmid control, as well as non-transfected cells (not shown). This data along with data obtained from engineered yeast strains show that $A_1\Delta 291R$ does not activate $G_\alpha i/o$ following ligand treatment, suggesting the C-terminus is necessary for downstream signaling of the A_1R.

The observations for both adenosine receptors are consistent with our results from the yeast pheromone response. Our observation is important as the yeast system can be utilized to screen and validate receptor variant activity. Here, it suggests that the crystal structures of $A_{2A}R$ with agonists that have all been resolved with the $\Delta 316$ truncation may not represent the fully active state of the receptors; that is, the region following residue 316 is necessary for interaction with the G protein.

3.3 Τηε C-τερμινυς δοες νοτ πλαψ α ρολε ιν τηε σπεςιφιςιτψ οφ Γ_α ςουπλινγ

To understand the role of the C-terminus in the specificity of the GPCR-G_α interaction, we constructed an $A_1/A_{2A}R$ chimera with all seven transmembrane domains of A_1R (residues 1-290) and the C-terminus of the $A_{2A}R$ (residues 291-412) using homologous recombination. The crystal structures of $A_{2A}R$ have been resolved with a truncation at the 316^{th}residue, and therefore, a truncated chimera ($A_1/A_{2A}\Delta 316R$) consisting of the transmembrane domains of A_1R and the C-terminus of $A_{2A}R$ truncated at the 316^{th} residue was constructed. This truncated chimera contains the helix 8 residues of $A_{2A}R$. This $A_{2A}R$ truncation at residue 316 has been previously reported to have native-like binding to the agonist NECA and the antagonist ZM 241385 by Magnani and colleages (2008) at 32 nM and 12 nM, respectively, compared to 20 nM and 1 nM for the wild-type receptor reported previously (de Lera Ruiz et al., 2014). Full-length expression of the chimeras was observed using Western blot analysis (Figure 6A) and showed slightly improved membrane localization to the plasma membrane (Figure 6Bii) as compared to wild-type A_1 receptor (Figure 3Bi).

The downstream signaling response was evaluated in the inhibitory and stimulatory yeast reporter strains, and both the full-length and truncated chimeras, respectively, showed coupling with the inhibitory yeast strain, similar to wild-type A_1R (Figure 6C). The truncated chimera showed reduced MAPK signaling via lower mCherry levels than the full-length chimera. No signaling response was obtained in the stimulatory yeast strains for the A_1R variants (Figure 6D). This observation suggests that the presence of the $A_{2A}R$ C-terminus does not affect the interaction of the chimeric A_1/A_{2A} receptor with the native-like inhibitory G_α. This observation is consistent with previously published work with canine A_1R and $A_{2A}R$, where an A_1R chimera showed native coupling with $G_\alpha i/o$ (Tucker et al., 2000).

In our previous work (Jain et al., 2018), $A_1/A_{2A}R$ chimera expression in yeast showed exceptional yields of active receptors per cell as determined by radioligand binding (B_{max}), as compared to wild-type A_1R. The dissociation constant for NECA for the $A_1/A_{2A}R$ was similar to the reported values for A_1R (14 nM in (Stewart et al., 2009)), suggesting native-like affinity. Here, we explored whether the chimera showed similar efficacy in downstream signaling as compared to wild-type A_1R receptor in yeast. The dose-dependent mCherry fluorescence response for each receptor was comparable when the non-specific agonist, NECA, was added (Figure 6E).

To test the role of the $A_{2A}R$ C-terminus on A_1R signaling in mammalian cells, cAMP was measured in transiently transfected HEK-293 cells. The addition of the $A_{2A}R$ C-terminus to A_1R did not lead to constitutive activity of the receptor in the absence of ligand (Figure 7, blue open bars); therefore, 10 μM forskolin was used to enable a basal cAMP signal. When treated with the A_1R-specific agonist CPA (1 μM, in the presence of 10 μM forskolin), cells transfected with either $A_1/A_{2A}R$ or $A_1/A_{2A}\Delta 316R$ showed a moderate reduction in cAMP signaling (72±10% and 67±10%, respectively) compared to forskolin treatment, con-

sistent with the wild-type A_1R (62±3%), verifying that $A_1/A_{2A}R$ and $A_1/A_{2A}\Delta316R$ chimeras couple to $G_\alpha i$ (Figure 7, green hatched bars). When treated with the $A_{2A}R$-specific agonist CGS21680 (1 μM, in the presence of 10 μM forskolin) cells transfected with either $A_1/A_{2A}R$ or $A_1/A_{2A}\Delta316R$ showed a negligible change in cAMP signaling compared to forskolin treatment alone, verifying that $A_1/A_{2A}R$ chimeras do not bind $A_{2A}R$-selective agonist or couple to $G_\alpha s$ (Figure 7, blue solid bars). The results obtained are consistent with our observations in yeast. Taken together, these results suggest that the C-terminus of the adenosine receptors does not play a role in the specificity of the GPCR-G_α interaction but is necessary to produce a downstream signaling response.

3.4 Dimerization of $A_{2A}R$ is not necessary for the downstream signaling response

The C-terminus of $A_{2A}R$ has been hypothesized to be involved in oligomerization of the receptor (Navarro et al., 2018; Schonenbach et al., 2016). Thus, truncation of $A_{2A}R$ could result in loss of oligomerization, leading to the observed lack of downstream signaling. To test this possibility, we measured the downstream signaling response of three $A_{2A}R$ variants, S374A, C394S, and C394A, that are located on the $A_{2A}R$ C-terminus and have been shown previously to disrupt oligomerization (Borroto-Escuela et al., 2010; Schonenbach, 2017; Schonenbach et al., 2016). The $A_{2A}R$ S374A variant was shown to be incapable of forming $A_{2A}R$-Dopamine D_2R oligomers and to abolish $A_{2A}R$-mediated inhibition of D_2R signaling (Borroto-Escuela et al., 2010). Schonenbach and colleagues showed that a cysteine mutation at residue 394 led to a loss of dimer and higher oligomer formation in purified $A_{2A}R$ protein variants (Schonenbach et al., 2016). Here, the variants showed expression levels comparable to the wild-type receptor as measured by whole cell fluorescence of mCitrine-tagged receptor (data not shown). After the addition of 100 μM NECA, all the variants showed downstream signaling similar to wildtype $A_{2A}R$ in yeast (Figure 8). Furthermore the C394S and C394A variants had no apparent change in EC50 values as compared to the wild-type receptor (data not shown). Thus, an inability to oligomerize did not affect the signaling activity.

4. Discussion

Since the early nineties, the engineered yeast MAPK response pathway has been known as a useful tool to study human GPCR signaling and identify lead drug candidates by recapitulating native dose-response binding preferences (Fowlkes et al., 1997; King et al., 1990; Stewart et al., 2009). Both A_1R and $A_{2A}R$ have been shown previously to interact with yeast/human chimeric $G_\alpha$protein to produce downstream signaling responses in the engineered yeast (Bertheleme et al., 2013; Knight et al., 2016; Peeters et al., 2012; Stewart et al., 2009). Here, engineered yeast strains from different parental backgrounds successfully captured A_1R and $A_{2A}R$ downstream signaling via their corresponding native G_α proteins. Strains obtained from the Broach laboratory showed a higher signal to noise ratio than those from the Dowell laboratory under these conditions, indicating that other parental strain differences can impact the signal-to-noise obtained in cell-based assays.

One of the strengths of the engineered yeast is the capability of quantifying the GPCR- G_α interaction at a common endpoint of the signaling cascade. This allows direct comparison of the strengths of the activation for different G_α biased ligands. One such study performed by Stewart et al. (2009) identified a novel A_1R agonist with biased specificity for $G_\alpha i$ vs $G_\alpha o$ coupling. Efforts have been made to replicate this model of utilizing the last five amino acids of the C-terminus of the G_α protein into a mammalian system using $G_\alpha s$ or $G_\alpha q$ as templates (Conklin et al., 1993; Hsu et al., 2007). A study by Hsu and Lou (2007) implementing this approach in HEK-293 cells tested the interaction of A_1R with $G_\alpha s$ chimeras via a cAMP assay. The authors observed cAMP production for all G_α variants tested except $G_\alpha s$, suggesting the system was not effective in capturing the specificity of the interaction of A_1R with G_α proteins. In contrast, our results with native-like G_α coupling may have resulted from higher than native levels of G_α protein expressed (˜three-fold higher than mock transfected, native HEK) (Geppetti et al., 2015; Kostenis et al., 2005) and because additional GPCRs present in the HEK cells led to signal promiscuity (O'Hayre et al., 2013). In our study, all the signaling components in the engineered yeast were expressed under their native promoters and perhaps as a result, the yeast cell assay more effectively captured the specificity of GPCR-G_α interaction.

The long C-terminus of the $A_{2A}R$ (122 amino acids) is assumed to be highly flexible and disordered; thus,

crystallization of adenosine receptors has all focused on using truncated receptors. Here, we tested the ability of truncated receptors to couple with G_α protein to produce downstream signaling. Both for A_1R and $A_{2A}R$, a C-terminal truncation resulted in no downstream signaling. These observations in yeast were validated in transiently transfected mammalian cells. For A_1R, our observations are consistent with those of Pankevych et al (2003), who observed that the truncated rat A_1R receptor variants showed inefficient trafficking to the plasma membrane, reduced ligand binding, and downstream signaling, depending on the length of the C-terminus. We observed similar inefficient localization and loss of downstream signaling for the human $A_1\Delta 291R$.

Here we find that the $A_{2A}R$ C-terminus did not change the G-protein coupling preference from $G_\alpha i$ to $G_\alpha s$ for the $A_1/A_{2A}R$ variants. Our results were consistent with previously published work by Tucker et al. (2000) that found that a chimera of canine A_1R with a canine $A_{2A}R$ C-terminus showed no change in G-protein coupling behavior. The human $A_1/A_{2A}R$ chimera showed a dose-dependent fluorescent response similar to the wild-type receptor, suggesting there was no change in ligand binding or G-protein coupling behavior due to the presence of the $A_{2A}R$ C-terminus. Taken together with our previous results of exceptional yields of the chimera (Jain et al., 2018), these data suggest that $A_1/A_{2A}R$ could be an effective variant to study biophysical characteristics and ligand binding for the A_1 receptor.

The $A_{2A}R$ C-terminus is known to interact with many accessory proteins in the GPCR signaling pathways like G protein receptor kinases and β-arrestins that aid in receptor signaling and desensitization (Gsandtner et al., 2006; Keuerleber et al., 2011; Zezula et al., 2008) but, has previously been thought to be dispensable for G-protein signaling (Klinger et al., 2002; Palmer et al., 1997). Bennett et al (2013) showed that $A_{2A}\Delta 316R$ expressed by an inducible promoter was capable of coupling to $G_\alpha s$ in a receptor expression-level dependent manner; however, their data was normalized, and total cAMP levels not reported. We do see a small increase in ligand-dependent signaling for the $A_{2A}\Delta 316R$ truncation (Fig 4A), but the signal is over twenty fold less than wild type $A_{2A}R$, suggesting the truncation is responsible for the loss of G protein signaling.

The $A_{2A}R$ receptor has been shown to form homo-oligomers in native, mammalian systems, and in yeast (Canals et al., 2004; Ciruela et al., 2011; Ferre et al., 2007; McNeely, 2016; Vidi et al., 2008). The long C-terminus of the $A_{2A}R$ has been shown to interact with dopamine receptors and has been hypothesized to be involved in homo- oligomerization, which may impact signaling (Ciruela et al., 2011; Schonenbach, 2017). Our results show that the monomeric $A_{2A}R$ variants were still capable of native-like downstream signaling. Taken together, our results highlight the role of the C-terminus for $A_{2A}R$ and A_1R in G-protein coupling, but not in G-protein specificity.

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Figure legends

Figure 1. A) GPCR-mediated MAPK signaling cascade in yeast. In this engineered pheromone response signaling pathway, cells express a yeast/human chimeric G_α protein to enable human GPCRs to couple with the yeast signaling pathway. Upon activation of downstream signaling, cells express mCherry fluorescent protein, which acts as an indirect measure of receptor activation. B) Schematic represents approach to homologous recombination to include the mCherry gene along with the antibiotic resistance gene for clone selection in the Fus1 locus in yeast strains. C) Agonist (100 μM NECA, green hatched bars) mediated downstream signaling responses as compared to control (DMSO, red closed bars) for A_1R were measured in cells modified pheromone response pathway in yeast expressing Gpa1p-human G_α chimeras in GSK strains (A; mean ± S.D., for three independent experiments).

Figure 2. Agonist (100 μM NECA, green hatched bars) mediated downstream signaling responses as compared to control (DMSO, red closed bars) for A_1R (A) and $A_{2A}R$ (B) were measured in cells modified pheromone response pathway in yeast expressing Gpa1p-human G_α chimeras in Broach laboratory strains (mean ± 95% C.I., n=6, 3 independent transformants performed in duplicate).

Figure 3. Absence of the C-terminus for A_1R and $A_{2A}R$ resulted in a loss of the signaling response in yeast strains. A) Full-length expression of mCitrine tagged wild-type and truncated A_1R and $A_{2A}R$ was observed using Western blot analysis with an anti-GFP antibody. Full-length receptor is indicated by an arrow. Molecular weights were estimated using Precision Plus Protein Western C standards. The expected molecular weight of each receptor is as follows: A_1R, 63.4 kDa; $A_1\Delta291R$, 59.1 kDa; $A_{2A}R$, 71.6 kDa; and $A_{2A}\Delta316R$, 66.1 kDa. B) Representative confocal images yeast cells expressing i) A_1R, ii) $A_{2A}R$, iii) $A_1\Delta291R$ and iv) $A_{2A}\Delta316R$ show membrane trafficking of the receptor, as indicated. $A_{2A}R$ and its truncated receptor showed efficient trafficking to the plasma membrane whereas A_1R and its truncation showed intracellular puncta with some receptor localized at the membrane. MAP kinase response signaling of the full length and truncated receptor in (C) Gpa1p-G_αi1(5) and (D) Gpa1p-G_αs (5) strains. NECA (100 μM) shown as green hatched bars and DMSO as red bars. Data represents the mean ± 95% C.I. for experiments performed in duplicate for three independent transformants. Note that the signaling response

of A_1R and $A_{2A}R$ from Figure 2 are replotted in Fig C and D to facilitate comparison of the truncations to the full-length receptors across each G-protein chimera.

Figure 4. Transiently transfected HEK-293 cells were used to determine downstream signaling for chimeras. A) Agonist-mediated cAMP accumulation for transiently transfected cells with $A_{2A}R$ and $A_{2A}\Delta316R$ (no ligand shown in red filled bars, 1 μM CGS21680 in green hatched bars). Data represent mean ± S.E.M. for three independent transfections performed in triplicate (*p<0.001, Student's t-test). B) Western blot analysis of $A_{2A}R$ and its truncation from transiently transfected in HEK-293, as obtained from total cell lysate or membrane fractions. Precision Plus Protein Western C standards were used to determine molecular weight as indicated. Dimer and full-length receptor are indicated by an arrow for $A_{2A}R$. The $A_{2A}\Delta316R$ shows a smaller band visible at ˜30 kDa that is likely a proteolytic product, also indicated by an arrow. Expected molecular weights for $A_{2A}R$ is 44.7 kDa and $A_{2A}\Delta316R$ is 35.1 kDa, and molecular weight markers were estimated using Precision Plus Protein Western C standards.

Figure 5. Inhibition of cAMP production after forskolin stimulation in transiently transfected HEK-293 cells with full-length A_1R as compared to truncated A_1R (no ligand in blue open bars, 10 μM forskolin in red filled bars and 10 μM forskolin and 1 μM CPA in green hatched bars). Data represents mean ± S.E.M. for three independent transfections performed in triplicate (*p<0.001, Student's t-test).

Figure 6. Expression and downstream MAPK signaling response in yeast for $A_1/A_{2A}R$ chimera with full-length and truncated C-terminus show native A_1R-like behavior. A) Western blot images showing expression of mCitrine tagged receptors for full-length and truncated chimeric receptors. Precision Plus Protein Western C standards were used to determine molecular weight as indicated. B) Representative confocal images of yeast strains showing receptor localization of full length and truncated $A_1/A_{2A}R$ chimera. Both the full-length and truncated $A_1/A_{2A}R$ chimera produce signaling response in inhibitory Gpa1p-G_αi1(5) strain (C), but not in stimulatory Gpa1p-G_αs(5) strains (D). (E) Dose-response curve for $A_1/A_{2A}R$ chimera (blue squares) is similar to the native A_1R receptor (red circle). 100 μM NECA is shown as green hatched bars and DMSO in red filled bars. The signaling response for A_1R is replotted from Figure 2. Data represents the mean ± 95% C.I. for experiments performed in duplicate for three independent transformants.

Figure 7. Inhibition of cAMP production after forskolin stimulation in HEK-293 cells transiently transfected with full length and truncated A_1R (no ligand in blue open bars, 10 μM forskolin in red filled bars, 10 μM forskolin and 1 μM CPA in green hatched bars and 10 μM forskolin and 1 μM CGS21680 in blue filled bars). The signaling response for A_1R is replotted from Figure 5. Data represents the mean ± S.E.M. for three independent transfections performed in triplicate (*p<0.001 and #p<0.01, Student's t-test).

Figure 8. Dimerization of $A_{2A}R$ is not required for signaling. Three $A_{2A}R$ variants – S374A, C394A and C394S – previously reported to be incapable of dimer formation give MAPK signaling in yeast comparable to wild-type $A_{2A}R$ receptor. 100 μM NECA is shown in green hatched bars and DMSO (control) in red filled bars. The signaling response for $A_{2A}R$ is replotted from Figure 2. Data represent mean ± 95% C.I. for experiments performed in duplicate for three independent transformants.