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Regions on Adenylyl Cyclase VII required for selective regulation by the G_{13} pathway

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Running title: Regulation of Adenylyl cyclase VII by G_{13}

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Abbreviations:

AC, adenylyl cyclase; BMDM, bone marrow derived macrophages; BRET, bioluminescence resonance energy transfer; CAMYEL, cAMP BRET sensor using YFP-Epac-Luciferase; ISO, isoproterenol; S1P, sphingosine-1-phosphate; PGE_2, prostaglandin E_2;

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Abstract

Regulation of multiple adenylyl cyclases (AC) provides unique inputs to mediate the synthesis of cAMP, a ubiquitous 2nd messenger that controls many aspects of cellular function. Upon stimulation by G_s, the activities of ACs can be further selectively modulated by other pathways to ensure precise control of intracellular cAMP responses to specific stimuli. Recently, we reported that one of the AC isoforms, AC7, is uniquely regulated by the G_{13} pathway. To better understand the molecular mechanism of this regulation, we compared the regulation of AC7 with that of AC2 in bone marrow derived macrophages devoid of AC7. Although both enzymes could fully restore regulation of cAMP by Gβγ, activation of the G_{13} pathway preferentially synergized with AC7. Exchange of domains between the two isoforms indicates that the C1b domain and the N-terminus of the C1a domain are important for directing selective regulation of AC7 by the G_{13} pathway. A mutagenesis screen identified more specific regions of AC7 that differentially mediate its regulation by distinct pathways.

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Introduction

Regulation of cAMP metabolism plays important roles in many aspects of cellular function (Chin et al., 2002; Mosenden and Tasken, 2011; Pierre et al., 2009; Sadana and Dessauer, 2009). One of the ways to ensure the precise temporal and spatial control of this essential second messenger is through the regulation of its synthesis by adenylyl cyclases (ACs). While mammalian ACs 1-9 are all activated by G_s, the activities of specific isoforms are regulated by diverse signaling pathways such as the G_i, G_q/Ca^{++}, and G_{13} pathways (Cooper, 2003; Jiang et al., 2008; Jiang et al., 2007; Sunahara and Taussig, 2002). Thus, these enzymes serve as ideal integrators for translating inputs from multiple stimuli into coordinated cAMP responses.

The nine membrane associated ACs share a common topology, which consists of a short amino terminus, followed by six membrane spans (M1), a large cytoplasmic domain (C1), a second set of six transmembrane regions (M2), and another cytoplasmic domain (C2) (Patel et al., 2001; Sunahara et al., 1997; Tesmer et al., 1997). The C1 domain is further divided into subdomains a and b based on sequence homology. The C1a and C2 domains share 60% identity in amino acid sequence and are absolutely required for the expression of cyclase activity (Tang and Gilman, 1995). The C1b domain, diverse in sequence and dispensable for the enzyme activity, is important for modulating inputs from other pathways as evinced by identified sites for interaction of $G\alpha_i$, Ca^{++} and calmodulin (CaM), and $G\beta\gamma$ subunits on selected AC isoforms (Chen et al., 1997; Diel et al., 2006; Scholich et al., 1997; Wu et al., 1993).

A novel regulation of adenylyl cyclase by the G_{13} pathway was recently described (Jiang et al., 2008; Jiang et al., 2007). In macrophage and other hematopoietic cell lineages, stimulation from ligands that activate the G_{13} pathway greatly enhances intracellular cAMP in conjunction

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with stimulation of G_s. Although both AC2 and AC7 isoforms share high sequence identity and similar functional attributes (such as sensitivity to Gβγ stimulation but inhibition by G_i) (Sunahara and Taussig, 2002), we found the regulation from the G_{13} pathway requires the specific AC isoform, AC7. Furthermore, this regulation is distinct from that by Gβγ subunits as overexpression of the Grk2 PH domain failed to block stimulation of AC7 from the G_{13} pathway (Jiang et al., 2008).

To better understand the molecular mechanism of this regulation by the G_{13} pathway, we made use of bone marrow derived primary macrophage (BMDM) cells with the AC7 allele deleted genetically (Jiang et al., 2012). In wild type BMDMs, AC7 plays a crucial role in integrating cAMP responses from multiple G protein pathways (Jiang et al., 2008). BMDM cells deficient of the AC7 gene, although capable of generating intracellular cAMP responses upon stimulation of G_s, completely fail to respond to further stimulation from all other G protein pathways, including G_i (C5a), G_q (UDP), and G_{13} (sphingosine-1-phosphate, S1P). We used AC7 deficient BMDM cells to compare the activity of AC7 with that of AC2, an isoform highly homologous to AC7 in primary amino acid sequence and reported to be regulated by Gβγ *in vitro* (Diel et al., 2006). We found that exogenous expression of AC7 in AC7 deficient BMDMs rescued all of the defects in cAMP responses caused by absence of the isoform; however, overexpression of AC2 only rescued the cAMP response to the G_i/Gβγ pathway but not to the G_{13} pathway. Distinct response profiles from the two AC isoforms allowed us to identify regions on AC7 that are important for its regulation by different pathways using domain swapping and a mutagenesis screen.

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Materials and Methods

Reagents: Isoproterenol, complement C5a, and sphingosine-1-phosphate were purchased from Sigma and Avanti Polar Lipids. Reagents for molecular cloning were purchased from Life Technologies and New England Biolabs.

Molecular Constructs: Human AC7 cDNA was tagged with an N-terminal Flag epitope or a C-terminal myc epitope. Rat AC2 cDNA with the Flag epitope at the C terminus was a kind gift from Dr. Ronald Taussig. Chimeric AC2/AC7 cyclases were generated by sewing PCR. The boundaries of the C1a, C1b, and C2 domains are aa197-aa454, aa455-aa595, and aa864-aa1080, respectively, according to the human AC7 protein (NP_001105.1). The AC7-2C1aN construct was a kind gift from Dr. Masami Yoshimura (Yoshimura et al., 2006). The constructs containing the NAAIRS (Asn-Ala-Ala-Ile-Arg-Ser) sequence were generated by site-directed mutagenesis PCR of the targeted regions followed by cloning of the mutated fragments into pFBneo-AC7-myc vector using two unique restriction enzymes. The BRET (bioluminescent resonance energy transfer) sensor for cAMP, CAMYEL, was described previously (Jiang et al., 2007). All constructs were delivered into BMDMs via retroviral infection.

Isolation of BMDMs and retroviral infection: Knockout mice with a conditional deletion of AC7 in the myeloid lineage were generated in our laboratory (Jiang et al., 2012). Mice deficient of $G\alpha_{13}$ in the myeloid lineage were derived through backcross of mouse strain LysMcre; $G\alpha_{12}^{-/-}$; $G\alpha_{13}^{fl/fl}$, a kind gift from Dr. Nina Wettschureck (Moers et al., 2003), to C57BL/6J. All experimental procedures involving animals in this study were reviewed and approved by the Institutional Animal Care and Research Advisory Committee at the UT Southwestern Medical Center. Bone marrow derived primary macrophages were isolated from mouse femurs (mixed

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sex) and cultured as described (Takeshita et al., 2000). Procedures for retroviral infection of BMDMs were as described (Jiang et al., 2008). When the CAMYEL sensor and $G\alpha_{13}$ or AC7 constructs were both introduced into BMDMs, the constructs were co-transfected into the packaging cells and the retroviruses were produced simultaneously and subsequently used for infecting BMDMs.

Assay of BRET in live cells: BRET assays for measuring cAMP in live cells were done as described (Jiang et al., 2007). For reporting errors, an independent experiment refers to assays done using BMDMs isolated from a particular mouse, typically two assays per ligand condition; therefore an error from two or more independent experiments represents 4 or more individual assays.

Assessment of protein expression: Western blot analysis was used to assess the expression level of proteins. Antibodies used in the experiments include anti-$G\alpha_{13}$ (B-860) (Singer et al., 1994), anti-Flag (Sigma), and anti-myc (Cell Signaling Technology).

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Results

Stimulation of cAMP by sphingosine-1-phosphate in bone marrow-derived macrophage cells is mediated by $G\alpha_{13}$

The bioactive phospholipid, S1P, synergistically enhances increases in intracellular cAMP in conjunction with G_s-stimulation in several hematopoietic cell lines (Jiang et al., 2007). We showed that this synergy is mediated by $G\alpha_{13}$ in cultured cell lines using an RNAi approach. In the primary macrophage cells, BMDMs, we have inferred that this response is also mediated by $G\alpha_{13}$ because inactivation of the G_i pathway by pertussis toxin or removal of intracellular calcium by thapsigargin and EGTA did not change the effect of S1P on cAMP responses (Jiang et al., 2008). Here, we tested this directly using $G\alpha_{13}$ deficient BMDMs isolated from knockout mice deficient of $G\alpha_{13}$ specifically in the myeloid lineage (Moers et al., 2003). The efficiency of $G\alpha_{13}$ deletion was ~80% as measured by the amount of $G\alpha_{13}$ protein expressed in populations of isolated BMDMs (Supplementary Figure S1). As shown in Figure 1, $G\alpha_{13}$ deficient BMDMs display a normal cAMP response upon activation of the G_s pathway by stimulation of endogenous β-adrenergic receptors with isoproterenol (ISO). When C5a was used to stimulate the G_i pathway following the addition of ISO, a 2^{nd} peak of cAMP was elicited in both wild type and $G\alpha_{13}$ deficient BMDMs. Thus $G\alpha_{13}$ deficient BMDMs retained functional G_s and G_i pathways for regulation of cAMP. When S1P was added following stimulation with ISO, it provoked a robust 2^{nd} peak of cAMP in wild type BMDMs; however, the response was ablated in $G\alpha_{13}$ deficient BMDMs (Figure 1). Expression of exogenous wild type $G\alpha_{13}$ partially rescued the cAMP response to S1P without affecting the C5a response in $G\alpha_{13}$ deficient BMDMs. This result confirms that S1P regulates intracellular cAMP in BMDMs in a $G\alpha_{13}$ dependent manner.

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The activity of adenylyl cyclase VII is uniquely regulated by the $G\alpha_{13}$ pathway in BMDMs

When G_s is activated in BMDMs, stimulation with ligands that are coupled to the G_i or G_{13} pathways elicit synergistic increases in cAMP in an AC7 dependent manner. This is most easily shown as a burst of intracellular cAMP by sequential activation of G_i or G_{13} after the initial stimulus of Gs ((Jiang et al., 2008) and figure 1). BMDMs deficient in AC7 are devoid of these synergistic responses with only minor effects on stimulation of cAMP by G_s pathways. This apparent convergence of regulation on AC7 could be due to its abundant expression or its unique regulation in BMDMs. Gene expression profiling of AC isoforms in BMDMs by RT-PCR revealed abundant amplification of AC7 but weak expression of AC2 (Duan et al., 2010). AC2 and AC7 belong to the same subclass of ACs due to sequence homology and enhancement of their activities by $G\beta\gamma$ in conjunction with G_s stimulation (Diel et al., 2006; Sunahara and Taussig, 2002). We expect that if the convergence of cAMP regulation on AC7 is due to its abundance in BMDMs, overexpression of AC2 would be able to rescue at least the C5a/G_i pathway synergism missing in AC7 deficient BMDMs.

This idea was tested by expressing AC2 or AC7 in AC7 deficient BMDMs isolated from a conditional knockout mouse strain generated in our laboratory in which AC7 was specifically deleted in the myeloid lineage (Jiang et al., 2012). Stimulation of cAMP was measured in live cells using a BRET sensor. Expression of AC2 and AC7 was assessed indirectly as shown in Supplementary Figure S2A. Expression of AC7 using human AC7 cDNA rescued all of the cAMP response defects in AC7 deficient BMDMs. As shown in Figure 2, the response to the G_i pathway stimulus, C5a, is essentially restored to that observed with wild type cells; the response to the G_{13} pathway stimulus, S1P, is restored to ~50 % of wild type cells. The latter rescue is specific to the G13 pathway as overexpression of AC7 in $G\alpha_{13}$ deficient BMDMs failed to

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restore the S1P effect on cAMP response (Supplementary Figure S2B). These results confirm that the defects in cAMP responses present in AC7 deficient BMDMs are direct consequences of the loss of AC7 and that AC7 acts downstream of $G\alpha_{13}$ to mediate the stimulation of cAMP by S1P in BMDMs.

Overexpression of AC2 also rescued the 2nd peak of cAMP in response to C5a (Figure 2). This response is sensitive to pertussis toxin treatment and is presumably mediated by Gβγ upon activation of the G_i pathway. Therefore both AC2 and AC7 are capable of mediating Gβγ regulation from the G_i pathway in BMDMs. However, overexpression of AC2 in AC7 deficient BMDMs produced only a small response to the G_{13} pathway stimulus, S1P, about 20% of that induced by overexpression of AC7 in the same type of cells (Figure 2). These results indicate that the S1P/G_{13} pathway regulates cAMP responses preferentially via AC7, consistent with our previous assessment (Jiang et al., 2008).

The C1b domain and the N-terminus of the C1a domain are important for mediating regulation of AC7 by the G_{13} pathway

The two highly homologous AC isoforms, AC2 and AC7, appear to be equivalently regulated by Gβγ yet their abilities to mediate the regulation from the G_{13} pathway differ. To identify the domains required for regulation of AC7 by the G_{13} pathway, we utilized the distinct cAMP response profiles generated by expression of AC2 and AC7 in AC7 deficient BMDMs. We reasoned that regulatory regions could be identified by testing chimeras of these two homologous proteins for their abilities to mediate regulation by G_{13} in this system. Since both ACs are equally capable of mediating regulation of cAMP synthesis by Gβγ, all functional chimeric proteins should rescue the 2nd peak of cAMP stimulated by the C5a/G_i pathway. This

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response would also serve as a control for normalizing the expression level of the enzymes and provide a baseline for their functional efficacy.

The chimeric cyclase constructs shown in Figure 3A were expressed in AC7 deficient BMDMs. Their abilities to mediate enhancement of cAMP by the C5a/G_i and S1P/G_{13} pathways were assessed by the induction of a second peak of cAMP after initial stimulation with ISO. When the C2 domain of AC7 was replaced by the matching domain of AC2 (AC7-2C2), the chimeric cyclase behaved like wild type AC7 (Figure 3); it rescued both C5a and S1P induced cAMP responses in AC7 deficient BMDMs. When both the C1 and C2 domains of AC7 were replaced by the corresponding domains from AC2 (AC7-2C1-2C2), the resulting cyclase behaved like AC2; the C5a induced 2nd peak of cAMP was fully restored, and the S1P induced 2nd peak was small (about 25% of that induced by expression of wild type AC7) as expected for wild type AC2 (Figure 3). Replacing the C1 domain of AC7 with that of AC2 resulted in an inactive enzyme (data not shown and (Yoshimura et al., 2006)). Replacing the membrane domains of AC7 with those of AC2 yielded a functional cyclase, AC7-2M1-2M2. This cyclase rescued both C5a and S1P induced cAMP responses to the same extent; the peak response to either ligand is about 60% of that generated by expression of wild type AC7 (Figure 3; Supplementary Figure S3). The proportional reduction in both C5a and S1P responses could be caused by reduced expression or reduced cyclase activity. Importantly, this chimeric cyclase is capable of mediating regulation from both pathways. Together, these results indicate that regulation of AC7 activity by the G_{13} pathway is mediated through the intracellular domains of AC7 and suggest that the C1 domain is important for the selectivity of this regulation while the C2 domain contains no unique information.

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The C1 domain is divided into two subdomains based on sequence homology among AC isoforms (Sunahara and Taussig, 2002). The C1a domain is highly conserved and is essential to enzymatic activity, while the C1b domain is more divergent in sequence and has been proposed to mediate isoform specific regulation (Chen et al., 1997; Diel et al., 2006; Scholich et al., 1997; Wu et al., 1993). When the C1a domain of AC7 was replaced by the corresponding domain of AC2 (AC7-2C1a-2C2), the chimera fully rescued the C5a/G_i mediated cAMP response. However, its response to stimulation by S1P was reduced to half of that mediated by wild type AC7, but clearly greater than that restored by AC2 (Figure 3; Supplementary Figure S3). This result implies that both the C1a and C1b domains are important for regulation of AC7 by the G_{13} pathway. Replacing the C1b domain of AC7 by that of AC2 rendered the enzyme inactive, thus preventing a direct test of its regulatory impact. When ~70 amino acids at the N-terminus of the C1a domain (aa 197-269) were replaced by that of AC2, the resulting cyclase behaved similarly to AC7-2C1a-2C2, suggesting the importance of the N-terminus of C1a domain.

A mutagenesis scan in the C1b domain reveals specific amino acid residues important for the regulation of AC7 by different pathways

To directly assess the role of the C1b domain in the regulation of AC7 activity, a mutagenesis scan with the NAAIRS sequence was used to replace blocks of six amino acids at a time. The NAAIRS sequence is flexible enough to adopt various secondary structures without affecting the overall protein conformation and has been used to probe protein activities and their regulation including that of AC2 by Gβγ (Diel et al., 2006).

A total of 21 mutants covering the entire C1b domain were generated by NAAIRS replacement (Figure 4). Mutation in two regions elicited cAMP response profiles different from that of wild type AC7. The first region is around aa477-aa499. The four mutants across this

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region all showed an enhanced cAMP response to stimulation with ISO alone (Supplementary Figure S4), suggesting enhanced cyclase activation by G_s. However, their responses to stimulation with C5a/G_i and S1P/G_{13} were hampered. The AC7-477 mutant showed normal cAMP responses to S1P stimulation but its response to C5a was reduced by 40%. Two mutants, AC7-491 and AC7-494, displayed a 40-60% reduction in response to S1P stimulation with normal responses to C5a. AC7-485 displayed reduced responses both to C5a and more severely to S1P (Figure 4, Supplementary Figure S4). The second region is around aa564-aa569; the NAAIRS mutant in this region, AC7-564, also showed enhanced cAMP response to ISO but a dampened response to C5a and more severely to S1P (Figure 4, Supplementary Figure S4). A third region showed an equivalent reduction to stimulation with both the βγ and G_{13} pathways, likely reflecting reduced effective expression or activity of the two mutants. Collectively the mutations with NAAIRS confirm the importance of the C1b domain in the regulation of AC7 activity by the G_{13} pathway.

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Discussion

The novel regulation of AC7 by the G_{13} pathway was recently reported in several hematopoietic cell lines (Jiang et al., 2008; Jiang et al., 2007). In this study, we further confirmed this regulation in primary macrophages using BMDMs deficient of $G\alpha_{13}$ or AC7. BMDMs deficient of either protein failed to increase intracellular cAMP concentration in response to S1P and the phenotype can be rescued by re-expression of the respective missing protein. Moreover, overexpression of AC7 failed to rescue the S1P induced cAMP response in G_{13} deficient BMDMs, proving genetically that G_{13} acts upstream of AC7 to regulate its activity (Figures 1&2, Supplementary Figure S2).

Analyses of a series of chimeric cyclases generated through domain substitution between AC7 and the highly homologous isoform, AC2, identified two intracellular domains in AC7 that are important for mediating its regulation by the G_{13} pathway. The two domains, C1b and the N-terminus of C1a, are outside the essential catalytic core of the enzyme and display the greatest sequence divergence among the intracellular domains of AC isoforms. A mutagenesis screen using substitution with the NAAIRS sequence further identified specific regions in AC7 that are preferentially sensitive to disruption of regulation by the $G\beta\gamma$ or the G_{13} pathways. Several novel observations derive from these studies.

First, the sites of action for $G\beta\gamma$ regulation of AC7 and AC2 appear to differ. Based on studies of AC2, it has been proposed that $G\beta\gamma$ regulation of the type II cyclases (AC2, AC4, AC7) is mediated by the highly conserved PFAHL motif in their C1b domains. NAAIRS replacement of this region on AC2 rendered it insensitive to $G\beta\gamma$ stimulation in membrane based *in vitro* assays (Diel et al., 2006). However, identical mutations in AC7 yielded only a partial or

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no defect in stimulation by Gβγ. The PFAHL motif in AC7 corresponds to aa484-aa500. Of the three NAAIRS mutants encompassing this region, one (AC7-485) displayed ~50% reduction in response to stimulation with C5a, while the other two mutants (AC7-491 and AC7-494) at the C-terminal end of the motif showed no defect to Gβγ regulation (Figure 4 and Supplementary Figure S4). Mutation of the sequence immediately N-terminal to the PFAHL motif (AC7-477) also showed a partial reduction in response to the βγ pathway, similar to the equivalent mutation in AC2 (AC2.Δ490, (Diel et al., 2006)). At this point, it is not clear whether the differences in βγ recognition by AC7 are due to actual conformational differences in the two cyclases or the study of AC7 in living cells versus the analysis of AC2 *in vitro*. Nevertheless, our results suggest that the PFAHL motif is only one of several regions that are involved in mediating regulation of AC2 and AC7 by Gβγ and the relative contribution of each region to the regulation may be different between the two cyclases. This notion is consistent with a recent study reporting additional sites on AC2 for mediating its regulation by Gβγ (Boran et al., 2011). It is also consistent with the fact that the PFAHL motif alone could not render an AC isoform sensitive to Gβγ stimulation (Diel et al., 2006).

A key observation is that the site of action for G_{13} regulation of AC7 appears to be distinct from that of Gβγ. Four mutants in the C1b domain displayed preferential defects in response to stimulation by S1P/G_{13}. Three of these mutants are clustered at aa485-aa499, part of the PFAHL motif. While the overall signatures for recognition of the Gβγ or G_{13} pathways are unique in AC7, the overlap in the PFAHL region emphasizes its importance in regulating AC activities and the potential use of a common mechanism emanating from this region for mediating synergistic increases in activity. Differential recognition by regulatory pathways may then allow unique response profiles in the context of the specific AC isoforms. However the

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overlap in the sites for the βγ and G_{13} pathways in AC7 also suggests that an individual cyclase molecule could not interact simultaneously with both pathways.

A third observation is that the NAAIRS mutants that displayed large differential defects in response to stimulation with S1P/G_{13} vs. C5a/βγ could not explain the preferential coupling of the G_{13} pathway to the specific AC7 isoform by simple sequence differences. One likely reason is that multiple regions are involved for effective interaction as in the case of Gβγ regulation. Indeed a NAAIRS scan in the N-terminal C1a domain of AC7 indicated additional regions that may contribute preference to its regulation by the Gβγ or G_{13} pathways (Supplementary Figure S5). The collective signature of those regions residing in the 3D conformation of the enzyme then provides the selectivity or capability for differential regulation of AC7 and AC2. A more complete understanding of the selectivity for individual AC isoforms will require structural information on the C1b and the N-terminal C1a domains.

An alternative explanation for differential signaling in the *in vivo* experiments reported here is that the subcellular localization of AC7 is distinct from that of AC2 and this localization favors coupling of AC7 with the G_{13} pathway. This notion is supported by the fact that overexpression of AC2 in AC7 deficient BMDMs resulted in a small but significant response to S1P stimulation (Figure 2). Recent studies have demonstrated specific interactions between various AKAP proteins and AC isoforms as a mechanism of compartmentalized signaling (Delint-Ramirez et al., 2011; Dessauer, 2009). Although interactions between AKAP proteins and AC2 have been reported (Piggott et al., 2008), such coupling remains to be determined for AC7. Since AC7 responds well to the G_i pathway, this would suggest that AC2 has to be largely excluded from compartments with functional S1P/G_{13} signaling.

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Authorship contributions

Participated in research design: Jiang, Wang, and Sternweis.

*Conducted experiments :*Jiang and Wang

Performed data analysis: Jiang and Wang

Wrote or contributed to the writing of the manuscript: Jiang and Sternweis

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Footnotes

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FIGURE LEGENDS:

Figure 1. $G\alpha_{13}$ deficient BMDMs fail to generate a cAMP response to stimulation with S1P. Wild type or $G\alpha_{13}$ deficient BMDMs were infected with the cAMP BRET sensor, CAMYEL, alone or together with wild type $G\alpha_{13}$ cDNA as indicated. The cells were stimulated with 10 nM ISO at time 0 followed by addition of 50 nM C5a (A) or 4 nM S1P (B) at 120s; cAMP responses were measured by changes in BRET and calculated as described (Jiang et al., 2007). Error bars indicate the standard deviation of at least three independent experiments. Errors are similar for all conditions and only shown on one trace for clarity.

Figure 2. Rescue of cAMP responses in AC7 deficient BMDMs. Wild type or AC7 deficient BMDMs were infected with the CAMYEL sensor alone or together with wild type human AC7 or rat AC2 cDNA, as indicated. The cells were stimulated with 10 nM ISO at time 0 followed by addition of 50 nM C5a (A) or 4 nM S1P (B) at 120s as in Fig. 1 and cAMP responses were measured. Error bars for selected traces indicate the standard deviations from at least three independent experiments.

Figure 3. Domain mapping for the regulation of AC7 by G_{13}. (A). Schematic drawing of AC2/AC7 chimeric cyclases generated by domain substitution. (B-C). AC7 deficient BMDMs were infected with the CAMYEL sensor together with chimeric cyclases as indicated. The cells were stimulated with 10 nM ISO at time 0 followed by addition of 50 nM C5a (B) or 4 nM S1P (C) at 120s; cAMP responses were measured as described (Fig. 1). (D). Summary of cAMP responses to C5a and S1P generated by expression of AC2/AC7 chimeric cyclases in AC7 deficient BMDMs. The response to C5a or S1P is defined by the peak response to each ligand (at ~170-180s) less the concentration of intracellular cAMP prior to the addition of the 2^{nd} ligand (~110-120s). All responses were normalized to the responses generated by the expression of wild

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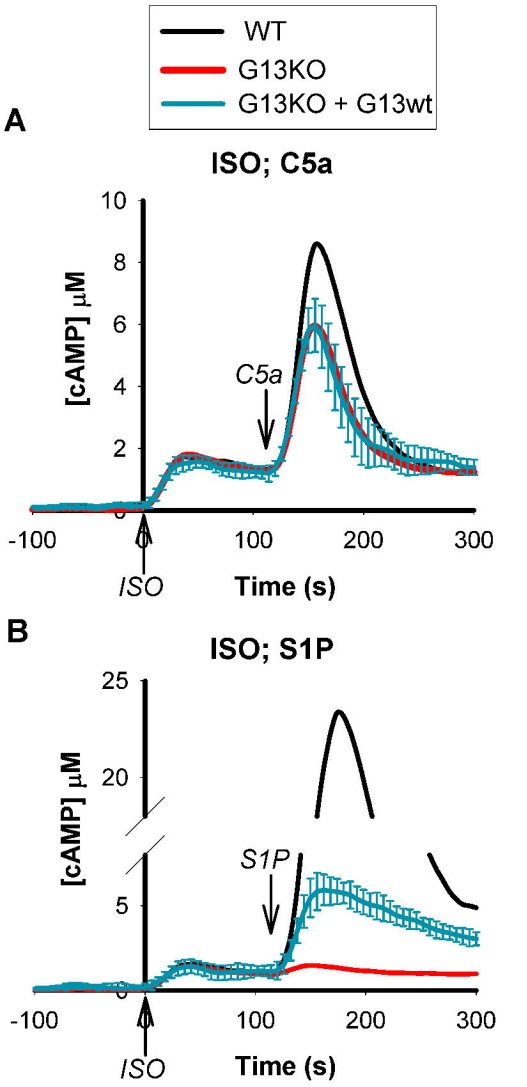
type AC7 in AC7 deficient BMDMs. Error bars for selected responses indicate the range of standard deviations from at least two independent experiments. Based on student t-test, the S1P response generated by hAC7-2C1-2C2 is significantly different from that of AC7 ($p<0.001$) but not different from that of AC2 ($p=0.6$); the S1P response generated by hAC7-2C1a-2C2 is significantly different from that of AC7 ($p<0.001$) and that of AC2 ($p<0.001$). There is no statistically significant difference between responses generated by hAC7-2C1a-2C2 and by hAC7-2C1aN.

Figure 4. Mutagenesis scan using NAAIRS replacement in the C1b domain. (A). Sequence of the C1b domain of human AC7. NAAIRS replacement mutants are indicated by underline; the number indicates the amino acid position of the first residue replaced. (B). Summary of cAMP responses to C5a and S1P generated by expression of AC7-NAAIRS mutants in AC7 deficient BMDMs. The response to C5a or S1P is defined by the peak response to each ligand as described in Fig. 3. All responses were normalized to the responses generated by expression of wild type AC7 in AC7 deficient BMDMs. Error bars indicate the standard deviation of two independent experiments. Black asterisks indicate that the response to C5a generated by expression of mutant AC7 is significantly smaller than wild type AC7 ($p<0.001$, t-test). Gray asterisks indicate that the response to S1P generated by expression of mutant AC7 is significantly smaller than wild type AC7 ($p<0.001$, t-test).

Figure 1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[cAMP] µM
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8
6
4
2
0
C5a
-100
100
200
300
ISO
Time (s)
B
ISO; S1P
[cAMP] µM
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20
5
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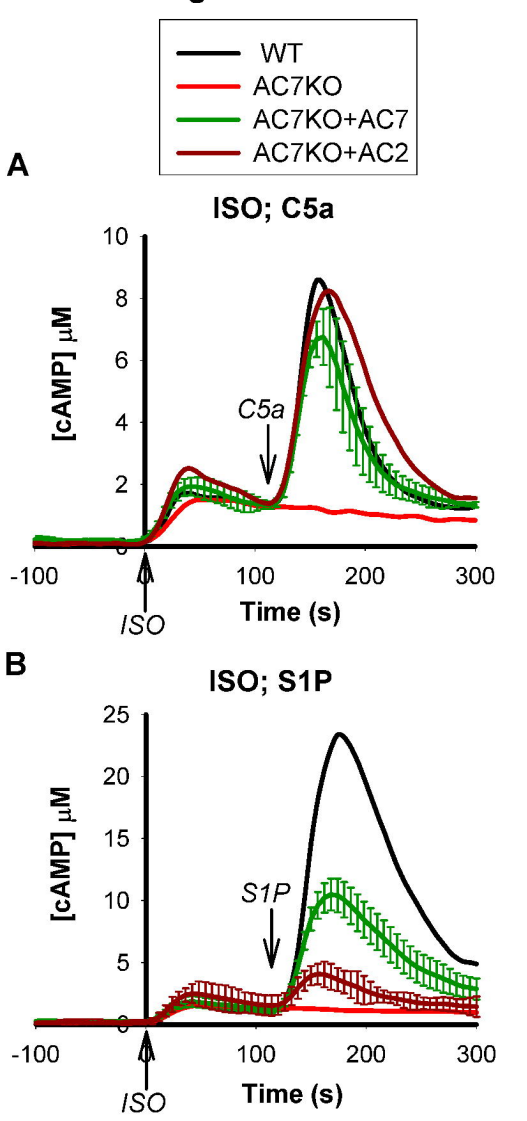
-100
100
200
300
ISO
Time (s)

Figure 2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AC7KO+AC7
AC7KO+AC2
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ISO; C5a
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C5a
ISO
Time (s)
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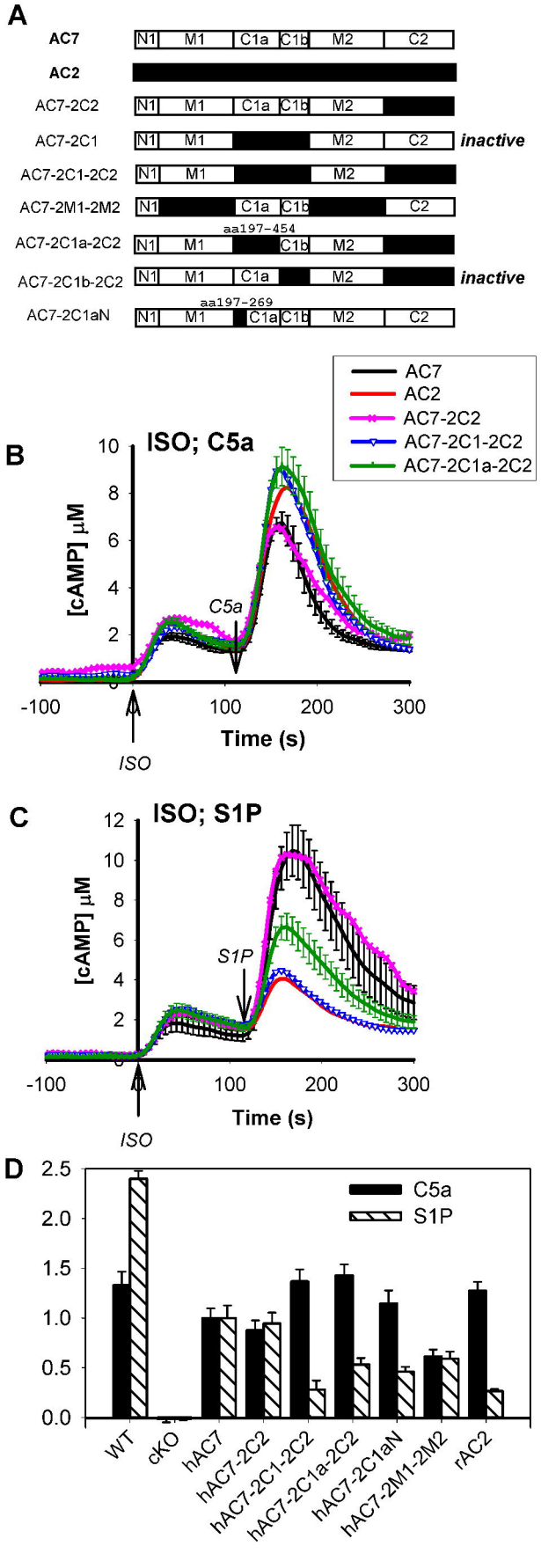
ISO; S1P
[cAMP] μM
S1P
ISO
Time (s)

Figure 3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AC2
AC7-2C2
AC7-2C1
AC7-2C1-2C2
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AC7-2C1b-2C2
AC7-2C1aN
N1
M1
C1a
C1b
M2
C2
inactive
inactive
aa197-454
aa197-269
B
ISO; C5a
AC7
AC2
AC7-2C2
AC7-2C1-2C2
AC7-2C1a-2C2
[cAMP] µM
C5a
ISO
Time (s)
C
ISO; S1P
[cAMP] µM
S1P
ISO
Time (s)
D
C5a
S1P
WT
cKO
hAC7
hAC7-2C2
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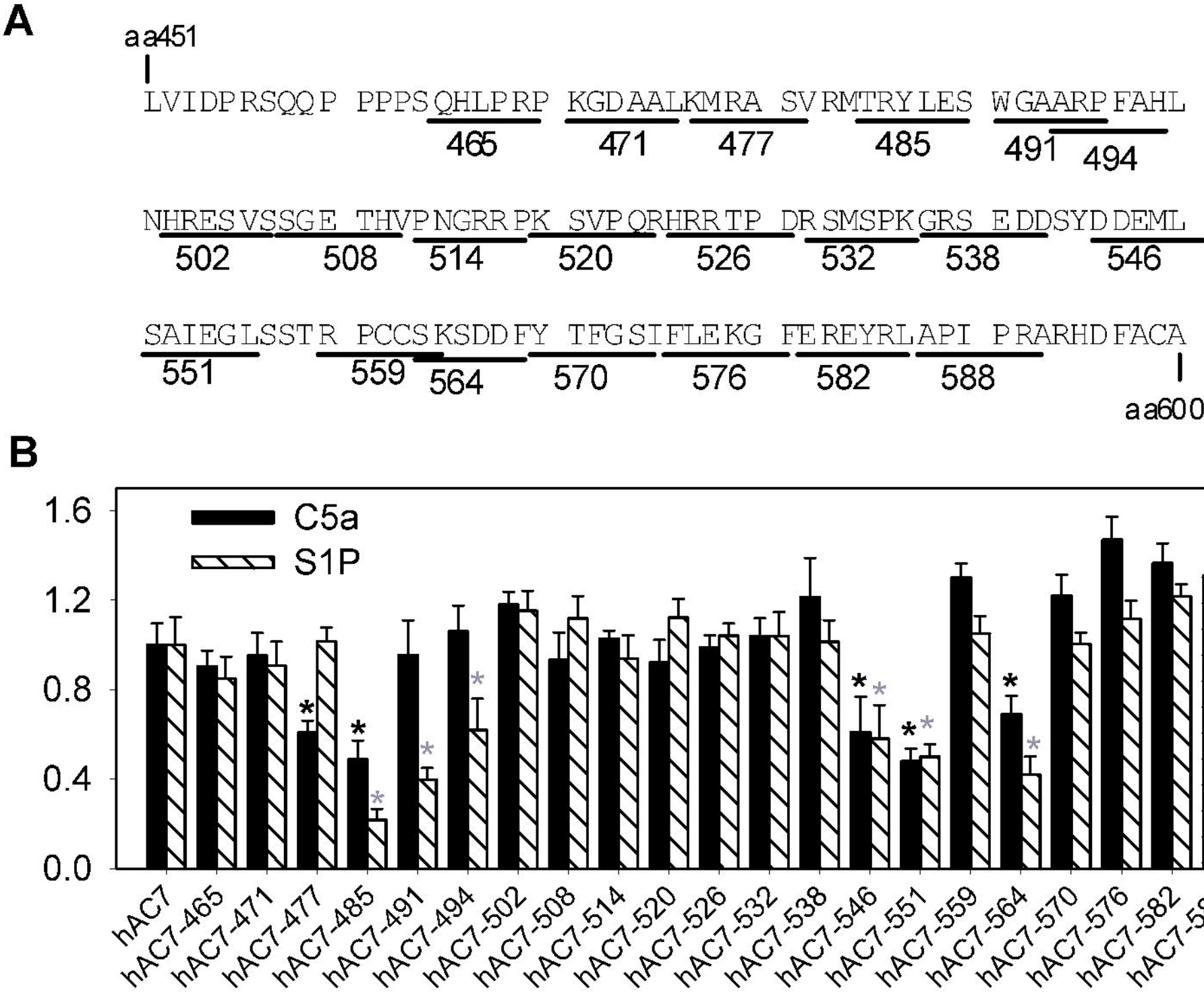
rAC2

Figure 4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546
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570
576
582
588
aa600
B
C5a
S1P
1.6
1.2
0.8
0.4
0.0
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hAC7-588