Regions on Adenylyl Cyclase VII Required for Selective Regulation by the G₁₃ Pathway

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Received September 14, 2012; accepted December 10, 2012

ABSTRACT

Regulation of multiple adenylyl cyclases (AC) provides unique inputs to mediate the synthesis of cAMP, a ubiquitous second messenger that controls many aspects of cellular function. On stimulation by Gₛ, 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 G₁₃ pathway. To understand more fully 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 G₁₃ 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 G₁₃ Pathway. A mutagenesis screen identified more specific regions of AC7 that differentially mediate its regulation by distinct pathways.

Introduction

Regulation of cAMP metabolism plays important roles in many aspects of cellular function (Chin et al., 2002; Pierre et al., 2009; Sadana and Dessauer, 2008; Mosenorden and Tasken, 2011). 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). Whereas mammalian ACs 1–9 are all activated by Gₛ, the activities of specific isoforms are regulated by diverse signaling pathways such as the Gₛ, Gₛ/⁰Ca²⁺, and G₁₃ pathways (Sunahara and Taussig, 2002; Cooper, 2003; Jiang et al., 2007, 2008). 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) (Sunahara et al., 1997; Tesmer et al., 1997; Patel et al., 2001). 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 (aa) sequence subdomains a and b based on sequence homology. The C₁a and C₂ domains share 60% identity in amino acid (aa) sequence around the critical C₂ domains share 60% identity in amino acid sequence. The C₁a domain and the N-terminus of the C₁b domain are important for directing selective regulation of AC7 by the G₁₃ Pathway.

ABBREVIATIONS: aa, amino acid; AC, adenylyl cyclase; BMDM, bone marrow derived macrophages; BRET, bioluminescence resonance energy transfer; CAMYEL, cAMP BRET sensor using YFP-Epac-Luciferase; ISO, isoproterenol; PCR, polymerase chain reaction; PFAHL, ProPheAlaHisLeu; S1P, sphingosine-1-phosphate.
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\textsubscript{13}/G\textsubscript{0} pathway but not to the G\textsubscript{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.

Materials and Methods

Reagents. Isoproterenol, complement C\textsubscript{5}a, and sphingosine-1-phosphate were purchased from Sigma-Aldrich (St. Louis, MO) and Avanti Polar Lipids (Alabaster, AL). Reagents for molecular cloning were purchased from Life Technologies (Grand Island, NY) and New England Biolabs (Ipswich, MA).

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 gift from Dr. Ronald Taussig. Chimeric AC2/AC7 cyclases were generated by sewing polymerase chain reaction (PCR). The boundaries of the C\textsubscript{1a}, C\textsubscript{1b}, and C\textsubscript{2} 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 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 bioluminescent resonance energy transfer (BRET) 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\textsubscript{13} in the myeloid lineage were derived through backcross of mouse strain LysMcre;G\textsubscript{13}\textsuperscript{fl/fl}, a gift from Dr. Nina Wettschureck (Moers et al., 2003). The efficiency of G\textsubscript{13} deletion was~80% as measured by the amount of G\textsubscript{13} protein expressed in populations of isolated BMDMs (Supplemental Fig. 1). As shown in Fig. 1, G\textsubscript{13}-deficient BMDMs display a normal cAMP response on activation of the G\textsubscript{13} pathway by stimulation of endogenous \(\beta\)-adrenergic receptors with isoproterenol (ISO).

Results

Stimulation of cAMP by Sphingosine-1-Phosphate in Bone Marrow-Derived macrophage Cells is Mediated by G\textsubscript{13}. The bioactive phospholipid S1P synergistically enhances increases in intracellular cAMP in conjunction with G\textsubscript{13} stimulation in several hematopoietic cell lines (Jiang et al., 2007). We showed that this synergy is mediated by G\textsubscript{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\textsubscript{13} because inactivation of the G\textsubscript{13} 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 by using G\textsubscript{13}-deficient BMDMs isolated from knockout mice deficient of G\textsubscript{13} specifically in the myeloid lineage (Moers et al., 2003). The efficiency of G\textsubscript{13} deletion was~80% as measured by the amount of G\textsubscript{13} protein expressed in populations of isolated BMDMs (Supplemental Fig. 1). As shown in Fig. 1, G\textsubscript{13}-deficient BMDMs display a normal cAMP response on activation of the G\textsubscript{13} pathway by stimulation of endogenous \(\beta\)-adrenergic receptors with isoproterenol (ISO).

Fig. 1. G\textsubscript{13}-deficient BMDMs fail to generate a cAMP response to stimulation with S1P. Wild-type (WT) or G\textsubscript{13}-deficient BMDMs were infected with the cAMP BRET sensor, CAMYEL, alone or together with wild-type G\textsubscript{13} cDNA as indicated. The cells were stimulated with 10 nM ISO at time 0, followed by the addition of 50 nM C\textsubscript{5}a (A) or 4 nM S1P (B) at 120 s; 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 shown on only one trace for clarity.
When C5a was used to stimulate the G\textsubscript{i} pathway following the addition of ISO, a second peak of cAMP was elicited in both wild-type and Ge\textsubscript{13}-deficient BMDMs. Thus, Ge\textsubscript{13}-deficient BMDMs retained functional G\textsubscript{s} and G\textsubscript{i} pathways for regulation of cAMP. When S1P was added after stimulation with ISO, it provoked a robust second peak of cAMP in wild-type BMDMs; however, the response was blunted in Ge\textsubscript{13}-deficient BMDMs (Fig. 1). Expression of exogenous wild-type Ge\textsubscript{13} partially rescued the cAMP response to S1P without affecting the C5a response in Ge\textsubscript{13}-deficient BMDMs. This result confirms that S1P regulates intracellular cAMP in BMDMs in a Ge\textsubscript{13}-dependent manner.

**The Activity of Adenylyl Cyclase VII is Uniquely Regulated by the Ge\textsubscript{13} Pathway in BMDMs.** When G\textsubscript{s} is activated in BMDMs, stimulation with ligands that are coupled to the G\textsubscript{i} or G\textsubscript{13} pathways elicits 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\textsubscript{i} or G\textsubscript{13} after the initial stimulus of G\textsubscript{s} (Jiang et al., 2008) (Fig. 1). BMDMs deficient in AC7 are devoid of these synergistic responses, with only minor effects on stimulation of cAMP by G\textsubscript{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 reverse transcriptase 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 because of their sequence homology and enhancement of their activities by G\textsubscript{bg}\textsubscript{Y} in conjunction with G\textsubscript{s} stimulation (Sunahara and Taussig, 2002; Diel et al., 2006). 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\textsubscript{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 Supplemental Fig. 2A. Expression of AC7 using human AC7 cDNA rescued all the cAMP response defects in AC7-deficient BMDMs. As shown in Fig. 2, the response to the G\textsubscript{i} pathway stimulus, C5a, is essentially restored to that observed with wild-type cells; the response to the G\textsubscript{13} pathway stimulus, S1P, is restored to \(\sim 50\%\) of wild-type cells. The latter rescue is specific to the G\textsubscript{13} pathway as overexpression of AC7 in Ge\textsubscript{13}-deficient BMDMs failed to restore the S1P effect on cAMP response (Supplemental Fig. 2B). 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 Ge\textsubscript{13} to mediate the stimulation of cAMP by S1P in BMDMs.

Overexpression of AC2 also rescued the second peak of cAMP in response to C5a (Fig. 2). This response is sensitive to pertussis toxin treatment and is presumably mediated by G\textsubscript{bg}\textsubscript{Y} on activation of the G\textsubscript{i} pathway. Therefore, both AC2 and AC7 are capable of mediating G\textsubscript{bg}\textsubscript{Y} regulation from the G\textsubscript{i} pathway in BMDMs. However, overexpression of AC2 in AC7-deficient BMDMs produced only a small response to the G\textsubscript{13} pathway stimulus, S1P, about 20\% of that induced by overexpression of AC7 in the same type of cells (Fig. 2). These results indicate that the S1P/G\textsubscript{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\textsubscript{13} Pathway.** The two highly homologous AC isoforms, AC2 and AC7, appear to be equivalently regulated by G\textsubscript{bg}\textsubscript{Y}, yet their abilities to mediate the regulation from the G\textsubscript{13} pathway differ. To identify the domains required for regulation of AC7 by the G\textsubscript{13} pathway, we used 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\textsubscript{13} in this system. Since both ACs are equally capable of mediating the regulation of cAMP synthesis by G\textsubscript{bg}\textsubscript{Y}, all
functional chimeric proteins should rescue the second peak of cAMP stimulated by the C5a/Gi pathway. This 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 Fig. 3A were expressed in AC7-deficient BMDMs. Their abilities to mediate enhancement of cAMP by the C5a/Gi and S1P/G13 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 (Fig. 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 second peak of cAMP was fully restored, and the S1P-induced second peak was small (about 25% of that induced by expression of wild-type AC7) as expected for wild-type AC2 (Fig. 3). Replacing the C1 domain of AC7 with that of AC2 resulted in an inactive enzyme (unpublished data; 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 (Fig. 3; Supplemental Fig. 3). 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 G13 pathway is mediated through the intracellular domains of AC7 and suggest that the C1 domain is important for the selectivity of this regulation, whereas the C2 domain contains no unique information.

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, and the C1b domain is more divergent in sequence and has been proposed to mediate isoform specific regulation (Wu et al., 1993; Chen et al., 1997; Scholich et al., 1997; Diel et al., 2006). When the C1a domain of AC7 was replaced by the corresponding domain of AC2 (AC7-2C1a-2C2), the chimera fully rescued the C5a/Gi-mediated cAMP response. However, its response to stimulation by S1P was reduced to half of that mediated by wild-type AC2 (Fig. 3; Supplemental Fig. 3). This result implies that both the C1a and C1b domains are important for regulation of AC7 by the G13 pathway.

S1P is defined by peak response to each ligand (at ~170–180s) less the concentration of intracellular cAMP before the addition of the second ligand (~110–120s). All responses were normalized to the responses generated by the expression of wild-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 hAC7-2C1a-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.
G₁₃ 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 assess the role of the C₁b domain in the regulation of AC7 activity directly, 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 Gbgβ (Diel et al., 2006).

A total of 21 mutants covering the entire C₁b domain were generated by NAAIRS replacement (Fig. 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 region all showed an enhanced cAMP response to stimulation with ISO alone (Supplemental Fig. 4), suggesting enhanced cyclase activation by Gbg. However, their responses to stimulation with C₅a/Gbg and S₁P/G₁₃ 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 (Fig. 4; Supplemental Fig. 4). 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 (Fig. 4; Supplemental Fig. 4). A third region showed an equivalent reduction to stimulation with both the βγ and G₁₃ 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 G₁₃ pathway.

**Discussion**

The novel regulation of AC7 by the G₁₃ pathway was recently reported in several hematopoietic cell lines (Jiang et al., 2007, 2008). In this study, we further confirmed this regulation in primary macrophages using BMDMs deficient of Gbg₁₃ 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 G₁₃-deficient BMDMs, proving genetically that G₁₃ acts upstream of AC7 to regulate its activity (Figs. 1 and 2; Supplemental Fig. 2).

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 G₁₃ 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 Gbgβ or the G₁₃ pathways. Several novel observations derive from these studies.

First, the sites of action for Gbgβ regulation of AC7 and AC2 appear to differ. Based on studies of AC2, it has been proposed that Gbgβ regulation of the type II cyclases (AC2, AC4, AC7) is mediated by the highly conserved ProPheAlaHisLeu (PFAHL) motif in their C₁b domains. NAAIRS replacement of this region on AC2 rendered it insensitive to Gbgβ stimulation in membrane-based in vitro assays (Diel et al., 2006). However, identical mutations in AC7 yielded only a partial or no defect in stimulation by Gbgβ. The PFAHL motif in AC7 corresponds...
to aa484–aa500. Of the three NAAIRS mutants encompassing this region, one (ACT-485) displayed ~50% reduction in response to stimulation with C5α; the other two mutants (ACT-491 and ACT-494) at the C-terminal end of the motif showed no defect to Gbgγ regulation (Fig. 4 and Supplemental Fig. 4). Mutation of the sequence immediately N-terminal to the PFAHL motif (ACT-477) also showed a partial reduction in response to the βγ pathway, similar to the equivalent mutation in AC2 (AC2Δ490) (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 the regulation of AC2 and AC7 by Gbgγ, 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 Gbgγ (Boran et al., 2011). It is also consistent with the fact that the PFAHL motif alone could not render an AC isoform sensitive to Gbgγ stimulation (Diel et al., 2006).

A key observation is that the site of action for G13 regulation of AC7 appears to be distinct from that of Gbgγ. Four mutants in the C1b domain displayed preferential defects in response to stimulation by SIP1/G13. Three of these mutants are clustered at aa485–aa499, part of the PFAHL motif. Whereas the overall signatures for recognition of the Gbgγ or G13 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 overlap in the sites for the βγ and G13 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 SIP1/G13 versus C5α/βγ could not explain the preferential coupling of the G13 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 Gbgγ regulation. Indeed, a NAAIRS scan in the C1α domain of AC7 indicated additional regions that may contribute preference to its regulation by the Gbgγ or G13 pathways (Supplemental Fig. 5). The collective signature of those regions residing in the three-dimensional 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 C1α 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 G13 pathway. This notion is supported by the fact that overexpression of AC2 in AC7-deficient BMDMs resulted in a small but significant response to SIP stimulation (Fig. 2). Recent studies have demonstrated specific interactions between various A-kinase anchoring proteins and AC isoforms as a mechanism of compartmentalized signaling (Dessauwa, 2009; Delint-Ramirez et al., 2011). Although interactions between A-kinase anchoring proteins and AC2 have been reported (Piggott et al., 2008), such coupling remains to be determined for AC7. Since AC7 responds well to the G1 pathway, this would suggest that AC2 has to be largely excluded from compartments with functional SIP1/G13 signaling.

Acknowledgments

The authors thank Dr. Ronald Taussig for the rat AC2 and human AC7 cDNAs, Dr. Masami Yoshimura for the AC7-2C1aN construct, and Dr. Nita Wentschuck for LysMere, Gα12–13, Gα12–13 knock-out mice. The authors thank Dr. Taussig for many stimulating discussions.

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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Molecular Pharmacology: Supplemental Material

Regions on Adenylyl Cyclase VII required for selective regulation by the G\textsubscript{13} pathway

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Supplementary Figure S1.

(A). Expression of G\textsubscript{\alpha 13} protein in G\textsubscript{\alpha 13} deficient BMDMs showed about 80% reduction when compared to wild type BMDMs as determined by western blot. (B). Absence of AC7 expression in AC7 deficient BMDMs was determined by qPCR.
Supplementary Figure S2.

S2A. Western blot assessment of expression of Flag-tagged hAC7 (at the N-terminus) and rAC2 (at the C-terminus) in the retrovirus packaging line, PlatE cells. After completion of viral production, similar samples of cells were lysed, separated by SDS-PAGE and blotted for the Flag epitope. Comparable expression of the two AC isoforms is shown. Expression of AC2 and AC7 in BMDMs was undetectable although sufficient enzymes was produced to restore cAMP response. This indirect assessment suggests that similar amounts of retrovirus was produced with a similar capacity to produce protein. Thus predicts that a similar percentage of BMDMs could be infected for each isoform.

S2B. Overexpression of wild type human AC7 does not rescue the defective cAMP response to S1P in G\(\alpha_{13}\) deficient BMDMs. Wild type, AC7 deficient, or G\(\alpha_{13}\) deficient BMDMs were infected with the cAMP BRET sensor, CAMYEL, alone or together with wild type human AC7 cDNA as indicated. The cells were stimulated with 10 nM ISO at time 0 followed by addition of 4 nM S1P at 120s; cAMP were measured in live cells using the BRET assay.
Supplementary Figure S3.

cAMP responses in AC7 deficient BMDMs expressing AC2/AC7 chimeric cyclases. 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 (A) or 4 nM S1P (B) at 120s; cAMP responses were measured by the BRET assay. Error bars indicate the standard deviation of at least two independent experiments.
Supplementary Figure S4.
cAMP responses in AC7 deficient BMDMs expressing selected AC7-NAAIRS mutants in the C1b domain. AC7 deficient BMDMs were infected with the CAMYEL sensor together with AC7-NAAIRS mutants as indicated. The cells were stimulated with 10 nM ISO at time 0 followed by addition of 50 nM C5a or 4 nM S1P at 120s; cAMP responses were measured by the BRET assay. Error bars indicate the standard deviation of two independent experiments.
Supplementary Figure S5.

cAMP responses in AC7 deficient BMDMs expressing selected AC7-NAAIRS mutants in the N-terminus of the C1a domain. (A).
Sequence of the N-terminus of the C1a domain of human AC7. NAAIRS replacement mutants are indicated by the underline; the number indicates the amino acid position of the first AC7 residue replaced. (B-C). AC7 deficient BMDMs were infected with the CAMYEL sensor together with AC7-NAAIRS mutants 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 by BRET. (D). 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 as 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.