Gβγ Activation Site in Adenylyl Cyclase Type II

ADENYLYL CYCLASE TYPE III IS INHIBITED BY Gβγ

Susanne Diel¹, Kathrin Klass¹, Burghardt Wittig¹, and Christiane Kleuss²³

From the Institute für Pharmakologie, Thielallee 67-73 and Institute für Molekularbiologie und BioInformatik, Arnimallee 22, Universitätsmedizin Berlin–Charité, 14195 Berlin, Germany

The Gβγ complex of heterotrimeric G proteins is the most outstanding example for the divergent regulation of mammalian adenylyl cyclases. The heterodimeric Gβγ complex inhibits some isoforms, e.g. ACI, and stimulates the isoforms ACII, -IV, and -VII. Although former studies identified the QHEA region located in the C2 domain of ACII as an important interaction site for Gβγ, the determinant of the stimulatory effect of Gβγ has not been detected. Here, we identified the C1β domain as the stimulatory domain using full-length adenylyl cyclase. The relevant Gβγ signal transfer motif in IIC1b was determined as MTRYLESWGAAKPFAHL (amino acids 493–509). Amino acids of this PFAHL motif were absolutely necessary for ACII to be stimulated by Gβγ, whereas they were dispensable for Gα or forskolin stimulation. The PFAHL motif is present in all three adenylyl cyclase isoforms that are activated by Gβγ but is absent in other adenylyl cyclase isoforms as well as other known effectors of Gβγ. The emerging concept of two contact sites on different molecule halves for effective regulation of adenylyl cyclase is discussed.

Adenylyl cyclase (AC), the enzyme that synthesizes the universal second messenger cAMP, is a key player in intracellular signaling pathways of hormones, neurotransmitters, odorsants, and chemokines. It is subject to coincident regulation by extracellular stimuli. Particulate mammalian ACs are represented by at least nine different isoforms (ACI–ACIX) that have been cloned and analyzed (1). All isoforms can be activated by the α subunit of the heterotrimeric stimulatory guanine nucleotide-binding protein (Gα) and with the exception of ACIX, also by the diterpene forskolin.

ACs are integral membrane proteins with a common topology (2) consisting of two sets of six transmembrane spans (M1 and M6) each followed by a cytosolic domain (C1 and C2). Both cytosolic domains can be subdivided on the basis of sequence similarity in domains Cα and Cβ. The C1α domain equals C2α in ~60% of amino acids, and both subdomains heterodimerize to form the pseudosymmetrical catalytic core. They can be expressed as independent polypeptides and when mixed form a heterodimer that exhibits catalytic activity as well as sensitivity to Gα and forskolin (3, 4). Outside of the catalytic core primary sequences significantly differ between the individual AC isoforms. The mechanisms of catalysis and activation by Gα were deduced from crystal structures of the soluble catalytic core bound to various regulators (5–7). However, little is known about the molecular mechanisms of isoform-specific regulation of ACs, i.e. by regulators other than Gα.

The Gβγ complex of heterotrimeric G proteins is a signal transfer motif (STY) specific to every adenylyl cyclase due to their common topology. Although former studies identified the QEHA region located in the C2 domain of ACII as an important interaction site for Gβγ, the determinant of the stimulatory effect of Gβγ has not been detected. Here, we identified the C1β domain as the stimulatory domain using full-length adenylyl cyclase. The relevant Gβγ signal transfer motif in IIC1b was determined as MTRYLESWGAAKPFAHL (amino acids 493–509). Amino acids of this PFAHL motif were absolutely necessary for ACII to be stimulated by Gβγ, whereas they were dispensable for Gα or forskolin stimulation. The PFAHL motif is present in all three adenylyl cyclase isoforms that are activated by Gβγ but is absent in other adenylyl cyclase isoforms as well as other known effectors of Gβγ. The emerging concept of two contact sites on different molecule halves for effective regulation of adenylyl cyclase is discussed.

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Known regulators of ACs include Ca2+- ions, Ca2+/calmodulin, Ca2+/calcineurin, AMP-dependent protein kinase, Ca2+-dependent protein kinase, the α subunits of Gα, Gβ, Gγ, and the G protein βγ complex (8). Gβγ is a conditional regulator, i.e. both activation and inhibition are best observed at the prestimulated AC. Gβγ has opposing effects on different subtypes of AC, it either activates (ACII, -IV, and -VII) or inhibits (ACI, -VIII, and presumably -V and -VI) the enzyme (9–13). The QHEA region (aa 956–982, located in the C2 domain of ACII) has been described as interacting with the Gβγ complex (14). However, we have shown that QHEA does not mediate the stimulatory effect of Gβγ to ACII (15). In the present work, we showed, on the full-length membrane-bound enzyme, the absolute requirement of the C1β domain in ACII for stimulation by Gβγ. In IIC1b, we identified a unique motif of 17 residues (PFAHL motif) comprising amino acids indispensable for ACII to be stimulated by Gβγ. However, the PFAHL motif on its own was not sufficient to transfer stimulation by Gβγ to every adenylyl cyclase due to the multiple interaction sites of Gβγ with distinct AC regions.

EXPERIMENTAL PROCEDURES

Generation of ACII Constructs—ACII with an N-terminal Myc tag and C-terminal HA tag was generated by PCR using rat cDNA encoding ACII as a template and primers: Myc-IIM1C1, 5'-GGAGGAACCTAGTACCATGGGAACAAATCTGATATCGGAAGAGACCTCGGCGGCGCGCACTACCTCGGACC-3'; and IIM2C2-HA, 5'-GGACGAAAGCTTATGCGTAGTCCGGCACGTCGTACGGATAGGATGCCAAATTTGCTCTGAGAAAAG-3'. PCR products were ligated into pBSKII+ (Strategene) and HindIII. The resulting ACII was indistinguishable from native, untagged ACII in terms of expression, intracellular localization, activity, and regulation and was used as the wild-type control throughout this work.

ACII.IC1b was generated by PCR and four-way ligation. Two sites for BsmBI were intermediately introduced into ACII cDNA at bp 1401 and bp 1659 by PCR. Three fragments were generated: 1) the replacing ACII fragment using ACII cDNA as a template and primers SD003 (5'-GCACGTCCTCATATTTTCCAGGGTCATTGTC-3') and SD004 (5'-GCACGTCCTCATATTTTCCAGGGTCATTGTC-3'); 2) ACII 5'-fragment; and 3) ACII 3'-fragment using ACII cDNA as a template and primers CK212 (5'-GCCATGATGAGAATATTAAATTTTAG-3'), CK213 (5'-GCACGTCCTCATATTTTCCAGGGTCATTGTC-3'), SD001 (5'-GCACGTCCTCATATTTTCCAGGGTCATTGTC-3'), and SD002 (5'-GCACGTCCTCATATTTTCCAGGGTCATTGTC-3'), respectively. The amplified ACII fragment was digested with BsmBI, the ACII 5'-fragment with NsiI and BsmBI, and the ACII 3'-fragment with BsmBI and Bsu36I. All fragments were ligated into Myc-ACII-HA in pBSKII+ between the intrinsic NsiI and Bsu36I restriction sites resulting in the plasmid coding for ACII.C1b. ACII.C1b comprised full-length ACII with the N-terminal Myc tag and C-terminal HA tag.
length ACII, N-terminally tagged with a Myc epitope, C-terminally tagged with a HA epitope, and C1b replaced by the C1b of ACI without introducing any additional codon. The ACII.C1b coding insert was then ligated into pFastBac1 (Invitrogen) using Spel and HindIII.

ACIII.C1b was generated by PCR and four-way ligation; the strategy was analogous to that applied for the mutant ACII.C1b. Three fragments were generated: 1) the replacing ACII fragment using ACII cDNA as a template and primers SD130 (5'-GCACGTCCTCGACGAGGAGTCTCCTAG-3'), SD131 (5'-GCACGTCTCAATTGAGTCTCTTCTTCGATCTCTGAGCTTCCCTCAG-3'), 2) the ACII 5'-fragment; and 3) the ACII 3'-fragment using ACII cDNA as a template and primers SD132 (5'-CGCCGTAACCTACTAGCTCTGTGG-3'), SD133 (5'-GCACGTCGTCACGCTTGCCCTGAGGGCAATGATG-3'), SD134 (5'-GCACGTCACGCTGATGATGATGATG-3'), and SD135 (5'-GTGTGGACAAAGGCTGCTCTGC-3'), respectively. The amplified ACII fragment was digested with BsmBI, the ACII 5'-fragment with KpnI and BsmBI, and the ACII 3'-fragment with BsmBI and BstEI. All fragments were ligated into ACII in pFastBac1 between the intrinsic KpnI and BstEII restriction sites resulting in the plasmid coding for ACIII.C1b.

For the IIC1b-NAAIRS screening, a construct encoding the ACII sequence from NsiI to Bsu36I was generated by PCR as a template and subcloned into pGEM TEasy (Promega). For the IIC1b-NAAIRS mutagenesis, a subclone from ACII using the restriction sites of Bsu36I and HindIII was generated as a template; the strategy was analogous to that applied for the IIC1b template.

All NAAIRS mutants were generated by QuiKChange site-directed mutagenesis using Pfu Turbo DNA polymerase (Stratagene) according to the instructions of the manufacturer. For each 6-amino-acid sequence selected for the replacement by asparagine-alanine-alanine-isoleucine-arginine-serine, two oligonucleotides were designed: one that contained the 15 nucleotides immediately upstream of the selected 6-codon sequence within ACII, followed by the nucleotide sequence 5'-AAATCTCGCAATTCCAGTCTCGC-3' coding NAAIRS, followed by the 15 nucleotides within ACII immediately downstream of the targeted 6-codon sequence. The second oligonucleotide was reverse-complementary (exact sequences are available upon request). Resulting NAAIRS constructs were ligated into the full-length tagged ACII DNA in pFastBac1 using NsiI and Bsu36I. All constructs were verified by sequencing. The resulting mutants contained NAAIRS substitutions in the following IIC1b sites: ACII.A472 (aa 472–477), ACII.A484 (aa 484–489), ACII.A490 (aa 490–495), ACII.A496 (aa 496–501), ACII.A503 (aa 503–508), ACII.A510 (aa 510–515), ACII.A522 (aa 522–527), ACII.A534 (aa 534–539), and ACII.A546 (aa 546–551) (see Fig. 3A for the final picture).

Protein Expression—Baculoviruses encoding the AC constructs were generated from the pFastBac1 constructs in Sf9 insect cells (Invitrogen). All AC constructs were expressed for 48–52 h after infection of cells with the virus. The cells were harvested, and plasma membranes were prepared by nitrogen cavitation as described previously (16). Goq for AC stimulation was applied as the constitutively active mutant Goq.Q213L derived from bovine Goq (short) with a C-terminal histidine tag (17). Recombinant bovine Gβγ was heterologously expressed in Sf9 cells and purified according to Kosaka and Gilman (18).

Adenyl Cyclase Assays—AC activity was measured as described by Smigel (19). In general, assays were performed for 7–10 min at 30 °C in a volume of 100 μl with the indicated amounts of recombinant Sf9 membranes in the presence of 10 mM MgCl2 and 500 μM ATP. When calmodulin (Calbiochem) was to be included, the AC-containing Sf9 membranes were washed with 1 mM EGTA prior to the AC assay and preincubated for 2 min at 30 °C before addition of the substrate.

Miscellaneous—Membrane proteins were quantified according to Bradford (34) with bovine serum albumin as the standard. Immunodetection of AC constructs was performed with commercially available antibodies: anti-c-Myc, 9E10 (Santa Cruz Biotechnology), anti-ACII, -III, C20 (Santa Cruz Biotechnology), and anti-HA, 12CA5 (Roche Applied Science).

RESULTS

Distinct regulatory regimes of individual AC isoforms allow them to play an interpretive role in signal transduction instead of being the read-out system of a linear pathway triggered by one G protein-coupled receptor. The ability of Gβγ subunits to stimulate ACII in the presence of activated Goq provides a mechanism by which disparate receptor systems can integrate their signals in the form of intracellular cAMP levels (20, 21). We aimed to identify the region on ACII that is responsible for the isoform-specific stimulation by Gβγ.

Domain Swap Mutant ACII.C1b—We generated the chimera ACII.C1b by substituting the C1b domain of ACII by the corresponding region of the Gβγ-inhibited subtype ACI. In contrast to many AC chimeras generated in the past, the domain swap mutant ACII.C1b contained solely amino acids of ACII (aa 485–604; numbering according to ACII) and ACII (aa 1–467 and aa 554–1087; numbering according to ACII) without any linker or unrelated amino acid artificially inserted for cloning. The chimera was heterologously expressed in insect cells to provide the most natural conformation of this particular enzyme with minimal background. Fig. 1A depicts that ACII.C1b was stimulated by forskolin and Goq. Although the catalysis rate of the domain swap mutant was reduced compared with ACII wild type, it exhibited activities clearly above the control levels (mock).
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**FIGURE 2. Test of NAAIRS in ACII.** A, localization in the AC structure. The structure of the catalytic core formed by the Cα region of ACV and the Cβ region of ACII was derived from Tesmer et al. (6). The NAAIRS-substituted stretch in the Cβ region (aa 948–953) is depicted in red. The four canonical, catalytically involved residues Lys-938, Asp-1018, Asn-1025, and Arg-1029 are shown in green. B, basic functional characterization. Membranes (20 μg of protein) from Sf9 cells expressing ACII wild type, ACIIΔ498, or β-galactosidase (mock) were incubated without regulator or with 100 μM forskolin or with 100 nM Goα. Values are means ± S.E. of three independent experiments performed in duplicate. C, Goα responsiveness. Membranes (5 μg of protein) from Sf9 cells expressing ACII wild type, ACIIΔ498, or β-galactosidase (mock) were incubated in the presence of 30 nM Goα, without or with 200 nM Gβγ. Data are representative of three similar assays performed in duplicate; the mean ± S.E. of the duplicates was <2%.

Sf9 cells that had been infected with an equal multiplicity of infection of a β-galactosidase-coding baculovirus. Interestingly, activities in the presence of forskolin were in the same order of magnitude for ACII wild type and the domain swap mutant, whereas Goα-stimulated activities significantly differed. In the presence of Goα, the domain swap mutant was as active as in the presence of forskolin, whereas ACII wild type was stimulated by Goα 5-fold better than by forskolin. Thereby, this domain exchange switched the forskolin-normalized high Goα responsiveness of ACII to the low responsiveness of ACI.

In contrast to ACII wild type, the domain swap mutant was completely insensitive to saturating concentrations of Gβγ (200 nM). The unresponsiveness of ACII.C1bΔ948 to Gβγ was not only observed in Goα (Fig. 1B) but also forskolin preactivation (not shown). Thereby, the activation profile of the domain swap mutant resembled that of the bisected C1b-deleted ACII (15) and established that C1b was required for the Gβγ stimulatory effect in the intact full-length ACII.

**Test of NAAIRS Substitution in ACII.** In a linear screening of the C1b domain, blocks of six amino acids were to be substituted by the hexapeptide NAAIRS (see "NAAIRS Screening of ACII.C1b"). This peptide has been shown to adopt various secondary structures in different proteins depending on the flanking motifs (22). Supposedly, this sequence is inherently flexible and can be used in substitution experiments with minimal impact to the overall molecule structure. We proved NAAIRS for conformational neutrality in ACII by replacing a sextet of amino acids in the catalytic core of the C1b domain (aa 948–953) (Fig. 2A, red) by NAAIRS, leaving the catalytically active amino acids unchanged (Fig. 2A, green). The resulting mutant ACIIΔ498 displayed AC activity under basal conditions and was activated by forskolin as well as Goα, (Fig. 2B). Furthermore, the isoform-specific activation of ACII by Gβγ persisted in ACIIΔ498 (Fig. 2C). Although this mutant displayed a diminished catalytic activity compared with ACII wild type, all basal (forskolin-, Goα-, and Gβγ-stimulated) activities were clearly above endogenous activities exhibited by mock-infected cells. These data provided strong evidence that the NAAIRS substitution neither abolished basic nor isoform-specific regulation of the ACII construct, revealing the structural flexibility of NAAIRS. The intact regulation pattern of ACIIΔ498 also indicated that NAAIRS neither interacted with Gβγ itself nor interfered with the Gβγ-mediated ACII stimulation. Taken together, we have proven NAAIRS to be a neutral substitution tool in ACII for the C1b screen.

**NAAIRS Screen of ACII.C1b.** In a series of subsequent experiments, we generated nine NAAIRS mutants with substitutions located in the C1b domain of full-length ACII (for exact localization, see Fig. 3A). After expression in Sf9 cells, all mutants of ACII localized to the plasma membrane. Integrity of the full-length protein was confirmed by detection with a Myc-specific antibody recognizing the extreme N-terminal tag of the mutants. A similar pattern was observed when using the ACII-specific antibody C20 (epitope aa 1071–1090) or the HA-antibody recognizing the extreme C-terminal hemagglutinin tag of the mutants (not shown). The activity of each of the nine mutants was significant for basal conditions (Fig. 3B). Furthermore, all mutants could be effectively stimulated by forskolin and Goα, although they exhibited differences in their catalytic activities. ACIIΔ472, ACIIΔ484, and ACIIΔ490 were stimulated by forskolin and Goα to the same extent as ACII wild type, whereas in other NAAIRS mutants (ACIIΔ496–ACIIΔ546), stimulatory efficacies of forskolin and Goα were reduced. Nevertheless, all ACII constructs generated in this study were catalytically active and responded in significant extent to forskolin and Goα stimulation. Even ACIIΔ496, with greatly diminished AC activities, was activated at least 4-fold by either stimulator. Most strikingly, NAAIRS mutants reacted individually to Gβγ, ranging between full stimulatory response to silence, depending on the site of NAAIRS substitution. Replacement of 6 amino acids between aa 496–508 by NAAIRS completely abolished stimulation by Gβγ (Fig. 4B, ACIIΔ496 and ACIIΔ503). Replacement of amino acids adjacent to this site resulted in mutants ACIIΔ490 (Fig. 4A) and ACIIΔ510 (see Fig. 4B) both being stimulated by Gβγ. ACIIΔ490 exhibited only modest stimulation by Gβγ, whereas ACIIΔ510 displayed an activation profile comparable with ACII wild type as well as...
the remaining mutants (Fig. 4C). Taken together, all mutants N-terminal to ACII.490 and C-terminal to ACII.503 were stimulated equally well by Gβγ as was ACII wild type.

Gβγ exhibited similar apparent affinities for 6 mutants and the ACII wild type (EC50 ~ 20 nM in the presence of 30 nM Gαs), diminished affinity for ACII.546 (EC50 70 nM), and apparently no affinity for the unresponsive mutants ACII.496 and ACII.503. Despite minor shifts in the activation profiles, all Gβγ-responsive constructs were maximally active at 300 nM Gβγ (saturating concentration). Therefore, the maximal activation of individual NAAIRS mutants at 300 nM Gβγ corresponded to the efficacy of Gβγ. The resulting activation profile of all mutants for Gβγ is depicted in Fig. 4D and elucidates the sharp loss of
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**FIGURE 5. Domain swap mutant ACIII.IIC1b**—A, basic characterization. Membranes (20 μg of protein) from Sf9 cells expressing ACIII wild type or the mutant ACIII.IIC1b were incubated without regulator, with 100 nm Gαs, or with 100 μM forskolin in the presence or absence of 2 μM calmodulin and 30 μM Ca2+. Data were corrected for the endogenous, particulate AC activity of Sf9 cells expressing β-galactosidase (basal, 0.007 nmol cAMP/min/mg protein). Gαs-stimulated, 0.03 nmol cAMP/min/mg protein; forskolin-stimulated, 0.05 nmol cAMP/min/mg protein; forskolin + calmodulin-stimulated, 0.3 nmol cAMP/min/mg protein. Values are means ± S.E. of three independent experiments performed in duplicate. B, Gβγ regulation. Membranes (20 μg of protein) from Sf9 cells expressing ACIII wild type or the mutant ACIII.IIC1b, were incubated with increasing concentrations of Gβγ in the presence of 100 μM forskolin, 2 μM calmodulin, and 30 μM Ca2+. Please note that AC is stimulated by Gβγ under these assay conditions (not shown). Data are representative of three similar assays performed in duplicate on independent membrane preparations; mean ± S.E. of duplicates was <2%.

Gβγ responsiveness of the three sequential mutants ACII.A490, ACII.A496, and ACII.A503, whereas Gβγ efficacies to stimulate all other mutants were close to wild-type level.

**Domain Swap Mutant ACIII.IIC1b**—The three NAAIRS mutants with low and lost Gβγ responsiveness covered amino acids 490–508, indicating that this region is necessary for mediating the stimulatory action of Gβγ to AC.

In an attempt to prove whether this region, on its own, sufficed in rendering an AC able to be stimulated by Gβγ, the complete IIC1b domain (aa 468–553; numbering according to ACII) was transferred into ACIII (aa 1–497 and aa 634–1145; numbering according to ACIII) replacing the homologous IIIC1b domain. We chose ACIII as receiving AC, because ACIII contains a QEHA equivalent region that enables Gβγ stimulation when substituted for QEHA in ACII (15). Furthermore, at the time of construction, ACIII was not described to be regulated by Gβγ. In Fig. 5, we could show for the first time that ACIII was not neutral but was inhibited by Gβγ, thereby completing the picture in the subfamily of AC I, III, and -VIII as being directly activated by calmodulin and inhibited by Gβγ. Gβγ was inhibitory when prestimulation was performed by calmodulin (Fig. 5B) as well as by forskolin (not shown), underscoring the regulatory mechanism that was already shown for ACI (9) in that inhibition is performed by Gβγ acting on AC and not by complexing the AC activator calmodulin.

The resulting domain swap mutant ACIII.IIC1b was activated by Gαs, forskolin and calmodulin (Fig. 5A) and inhibited by Gβγ (Fig. 5B). The Gβγ-mediated inhibition of ACIII.IIC1b was as efficient and as potent as in wild-type ACIII, indicating that even no partial compensation of the Gβγ inhibition on ACIII could be detected. This regulatory profile of the chimera ACIII.IIC1b indicated that aa 490–508 did not comprise an isolated motif that transferred Gβγ stimulation to every effector.

**PFAHL Motif**—However, the region covered by the three NAAIRS mutants with low and lost Gβγ responsiveness was highly conserved between those three AC isoforms that were stimulated by Gβγ (Fig. 6) and showed no similarity to the other AC isoforms or other Gβγ effectors, such as PLCβ2, PLCβ3, phosphatidylinositol 3-kinase γ, β-adrenergic receptor kinase, or various channels (K_ACh, N-type calcium, L-type calcium). A motif comprising 17 amino acids (aa 493–509) in this region is almost identical for the three isoforms: M-T-R-Y-L-E-S-W-G-A-A-(K/R)-P-F-A-H-L. The very last amino acid of this motif, Leu, was not covered by any NAAIRS mutant and is included in the motif simply based on similarity. According to the C-terminal amino acids this motif is named PFAHL.

**DISCUSSION**

Diversity among different AC isoforms reflects the broad range of regulatory susceptibilities at the effector level. The present study focused on ACII regulation by Gβγ. Although the mechanism for Gαs activation has been deduced from the landmarking data of the AC crystal, no models for stimulation by Gβγ or other regulators exist today. In this work, we identified the region on ACII that is responsible for the isoform-specific stimulation by Gβγ.

**Importance of the C1b Domain**—Theoretical considerations as well as experiments with deletion, domain swap, and point mutations have provided strong evidence that the C1b domain features crucial regulatory impact on AC activity. The C1b domains in ACV and -VI have been shown to alter the Gαs response profiles of these isoforms (23, 24). The primary structure of the C1b domain greatly differs in all AC isoforms and is therefore an appropriate target for isoform-specific regulators. For example, the C1b region in ACI binds Ca2+/calmodulin and is necessary for stimulation of the enzyme by this modulator (25), and the C1b domain in ACVI provides a cAMP-dependent protein kinase phosphorylation site that confers feedback inhibition by cAMP (24). Although C1b is dispensable for catalysis, it appears to be an intrinsic modulator of enzyme activity; the presence of C1b in model systems such as soluble or membrane-anchored proteins diminishes AC activity compared with a C1b-deleted enzyme (26–28). In a recent paper, Beeler et al. (26) deduced from circular dichroism data that the C1b domain may adopt various, ligand-dependent stable conformations, thereby altering the C1b–C2a constellation that impacts the catalysis rate either way, positively or negatively.

This project required maximal AC enzyme integrity. The Gβγ stimulation was sensitive to both the isoprenylation of the Gγ moiety and the particulate location of AC (not shown), whereas the mere interaction between Gβγ and AC was also observed with soluble AC constructs (15). Therefore, we applied a domain swap strategy to work on the intact, particulate, full-length AC with minimal perturbation of enzyme conformation. Indeed, the resulting mutant ACII.IIC1b could be stimulated by Gαs and forskolin, rendering the observed defect in Gβγ stimulation significant (see Fig. 1B).
The swapped C1b region that was chosen from ACI was that inhibited by Gβγ. Therefore loss of Gβγ regulation in ACII.IIC1b may also be interpreted as the result of supressing effects of Gβγ stimulation on ACII parts and Gβγ inhibition that might be mediated by the exchanged IC1b.

The Gβγ Stimulation Motif—We chose the hexapeptide NAAIRS for profound investigations of ACII. NAAIRS has been established as an appropriate tool for substitution experiments in several studies (29, 30) and is considered an omnistructural peptide. The “high risk” mutant ACII.A948 showed that NAAIRS was also a neutral substitution in ACII (see Fig. 2). The subsequent NAAIRS screen of IIC1b revealed that Gβγ stimulation in C1b is mediated by amino acids located in the stretch of aa 490–509. Based on some residual Gβγ responsiveness in ACII.A490 and local identity in the C1b domains of all three AC isoforms of the Gβγ-stimulated AC-subfamily, this region was confined to the PFAHL motif (aa 493–509) (see Fig. 6). The Gβγ stimulation on C1b is mediated by amino acids located in the stretch of aa 490–509. Based on some residual Gβγ responsiveness in ACII.A490 and local identity in the C1b domains of all three AC isoforms of the Gβγ-stimulated AC-subfamily, this region was confined to the PFAHL motif (aa 493–509) (see Fig. 6). Regarding the exclusive presence of this motif in ACII, -IV, and -VII, it was conceivable that the PFAHL motif (aa 493–509) (see Fig. 6). The PFAHL motif is located upstream of the region used to construct the first soluble C1b protein from ACVII (26). Unfortunately, constructs of Beeler et al. (26) embracing the PFAHL motif were unstable. The expressed PFAHL-devoid VIIIC1b construct could not re-establish Gβγ stimulation on soluble ACVII, corroborating our conclusion that, downstream of the PFAHL sequence, no other Gβγ stimulation site is present in C1b.

The PFAHL motif in C1b is clearly distinct from the QEHA domain in IIC, which was determined to be an interaction site for Gβγ (14) without mediating the stimulatory effect of Gβγ. Evidence for the QEHA region not being the stimulatory site for Gβγ was based on (i) substitution of the QEHA region in ACII by the corresponding regions of the Gβγ-inhibited AC and ACIII isoforms resulting in a mutant with conserved Gβγ-stimulated AC activity (15) and (ii) NAAIRS substitutions within the QEHA region resulting in mutants that were still inhibited by Gβγ (data not shown). In contrast, the PFAHL motif in IIC1b identified in the present study appeared to be the accurate Gβγ signal transfer site of C1b as substitution of IIC1b or the PFAHL motif by IC1b or NAAIRS, respectively, completely abolished Gβγ stimulation.

Although PFAHL is necessary for ACII to be stimulated by Gβγ, it is not sufficient. The domain swap mutant ACIII.IIC1b harbored the PFAHL-containing IIC1b domain but was still inhibited by Gβγ as well as the wild-type ACIII. This regulatory pattern pointed out two challenging details: (i) ACIII presents its Gβγ inhibitory sites outside of the swapped C1b region and (ii) the PFAHL motif mediates its stimulatory effect in ACII in concert with another site located outside of IIC1b.

Two Interaction Sites on AC—ACII provided two relevant Gβγ-affected sites: 1) PFAHL in IIC1b with profound regulatory significance and unique appearance only in Gβγ-stimulated AC isoforms and 2) QEHA containing the Gβγ binding motif QAXER in IIC, as a general Gβγ docking site with widespread appearance in several Gβγ effectors such as β-adrenergic receptor kinase, atrial potassium channel, or PLCβ3 (14). From the ACIII.IIC1b chimera (harboring both the PFAHL motif and a QEHA-equivalent region), it seemed unlikely that QEHA was the additional site needed for PFAHL being sufficient to transmit Gβγ stimulation. However, at least two interaction sites for each regulator appeared to be a general scheme for AC regulation. Gu and Cooper (31) discovered two relevant regions on ACVIII with regard to Ca2+/calmodulin modulation, one regulatory at the C terminus and one calmodulin-typical binding site at the N terminus. It is intriguing that both settings, ACII regulation by Gβγ and ACVIII regulation by Ca2+/calmodulin, harbor the two interaction sites on both enzyme halves. Assuming the simultaneous interaction of the regulator with both sites, these regulators might clamp the enzyme as already visualized for Goα in the crystal structure thereby affecting the conformation of the catalytic core and catalysis rate. However, this is the furthest that these regulatory schemes of ACs can be generalized. The known calmodulin interaction domains in ACVIII and ACI do not represent homologous sites in the individual enzymes (31, 32). For Gβγ interaction sites, we have shown no influence of the C1a domain on ACII stimulation, whereas Witttoph et al. (33) attributed to C1b on its own the Gβγ inhibitory effect in ACI. Furthermore, the complete loss of Gβγ regulation in the domain swap mutant ACIII.IIC1b on the one hand and the full maintenance of Gβγ inhibition in ACIII.IIC1b on the other hand pointed to differing usage of isoform-specific C1b domains by the respective ACs.

We have shown that the PFAHL motif in the IIC1b domain is essential for ACII regulation by Gβγ but does not suffice on its own to render a Gβγ-inhibited ACIII to a Gβγ-stimulated enzyme. PFAHL is unique to all three AC isoforms that are stimulated by Gβγ. Thereby, the PFAHL motif represents an ideal isoform-specific target to selectively modify the Gβγ-dependent branch of bifurcated cascades initiated by multiply coupling receptors or in complex dysfunctions involving Gβγ-activating receptor subtypes such as the dopamine receptor.

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