Persistent interactions between the two transmembrane clusters dictate the targeting and functional assembly of adenylyl cyclase

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Adenylyl cyclases possess complex structures like those of the ATP binding cassette (ABC) transporter family, which includes the cystic fibrosis transmembrane regulator, the P-glycoprotein, and ATP-sensitive K^+ channels [1–4]. These structures comprise a cytosolic N terminus followed by two tandem six-transmembrane cassettes, each associated with a highly homologous (ATP binding) cytosolic loop [5–8]. The catalytic domains, which are located in the two large cytoplasmic loops, are highly conserved and well studied. The crystal structure of these domains has even been described recently [9, 10]. However, nothing is known of the function or organization of the 12 transmembrane segments. In the present study we adopted a range of strategies including live-cell fluorescence resonance energy transfer (FRET) microscopy, coimmunoprecipitation, and functional assays of various truncated and substituted, fluorescently-tagged molecules to analyze the trafficking and activity of this molecule. When expressed as individual peptides, the two transmembrane domains – largely independently of any cytosolic region – formed a tight complex that was delivered to the plasma membrane. This cooperation between the two intact transmembrane domains was essential and sufficient to target the enzyme to the plasma membrane of the cell. The extracellular loop between the ninth and tenth transmembrane segments, which contains an N-glycosylation site, was also necessary. Furthermore, the interaction between the two transmembrane clusters played a critical role in bringing together the cytosolic catalytic domains to express functional adenylyl cyclase activity in the intact cell.

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Results and discussion

We considered the possibility that interactions occur between the transmembrane cassettes of adenyl cyclases (ACs) that might condense the molecule and facilitate its functions. To study the disposition of AC components in living cells, we engineered green fluorescent–protein (eGFP) tags on various constructs of the Ca^{2+}-stimulable AC type 8 (AC8) [11] and examined their expression (Figure 1). In HEK 293 cells, wild-type AC8 with an eGFP molecule at the N terminus (which behaved just like wild-type AC8, in terms of forskolin and Ca^{2+} stimulation; data not shown) displayed a localization pattern consistent with the plasma membrane (Figure 2a). To identify which region of AC8 was responsible for its plasma membrane location, the expression of eGFP-tagged AC8 truncations or discrete domains of AC8 (Figure 1) was examined. The expression of these proteins and their sizes were confirmed in Western-blotting experiments by the use of anti-eGPF antibody (not shown). GFP/AC8, GFP/8M1 (the truncated N terminus), and GFP/8M13 (the truncated N and C termini) [12] occurred mainly in the plasma membrane of the cells, and this finding indicates that both the N-terminal and C2b regions are superfluous for the plasma membrane location of the molecule (Figure 2a). Not surprisingly, the two cytosolic portions, GFP/8C1 and GFP/8C2, that lacked the transmembrane clusters were found in the cell cytosol (Figure 2a). However, fusion proteins with only one transmembrane cluster were all confined to a perinuclear network, most likely representing the endoplasmic reticulum (Figure 2a). Such fusion proteins include 8NTm1C1/GFP, 8NTm1/GFP, GFP/8Tm2C2, and GFP/8Tm2. These findings somewhat surprisingly suggest that no single domain of AC8 can migrate to the plasma membrane by itself. However, when the two halves of AC8, 8NTm1C1/GFP and GFP/8Tm2C2, were cotransfected, approximately 30% of the cells displayed eGFP fluorescence in the plasma membrane, which suggested that the two halves of the AC8 molecule could interact and traffic appropriately.

To analyze the apparent trafficking of the two halves of AC8 in more detail, we made three untagged constructs from the first half of AC8 (8NTm1C1, 8NTm1, and 8C1) and cotransfected them with GFP-tagged constructs from the second half of AC8 (GFP/8Tm2C2, GFP/8Tm2, and GFP/8C2) in nine combinations (Figure 2b). Cotransfections between 8NTm1C1 and GFP/8Tm2C2, between 8NTm1C1 and GFP/8Tm2, between 8NTm1 and GFP/8Tm2C2, and between 8NTm1 and GFP/8Tm2 resulted in the trafficking of GFP-tagged fragments to the plasma membrane of the cells (Figure 2b). This finding suggested

Figure 1

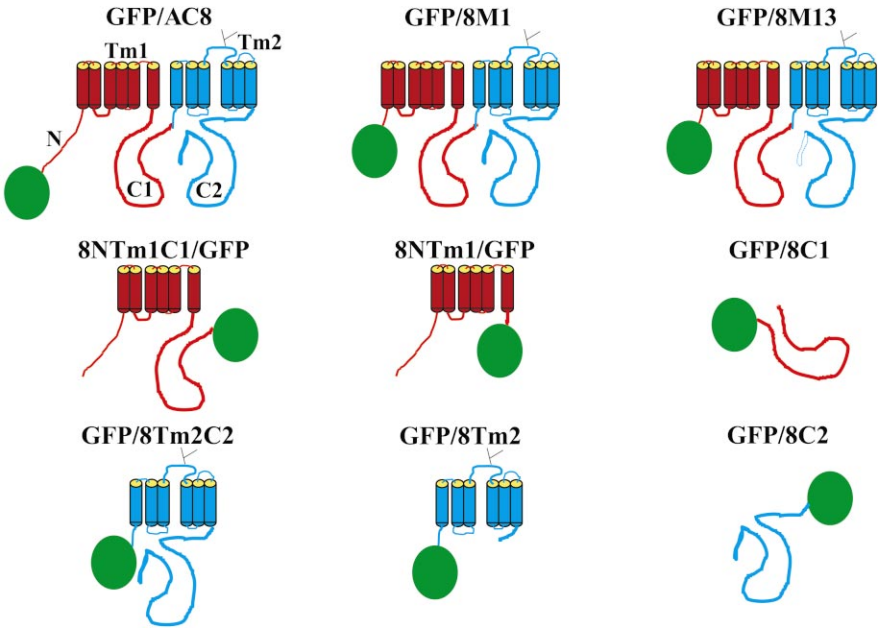


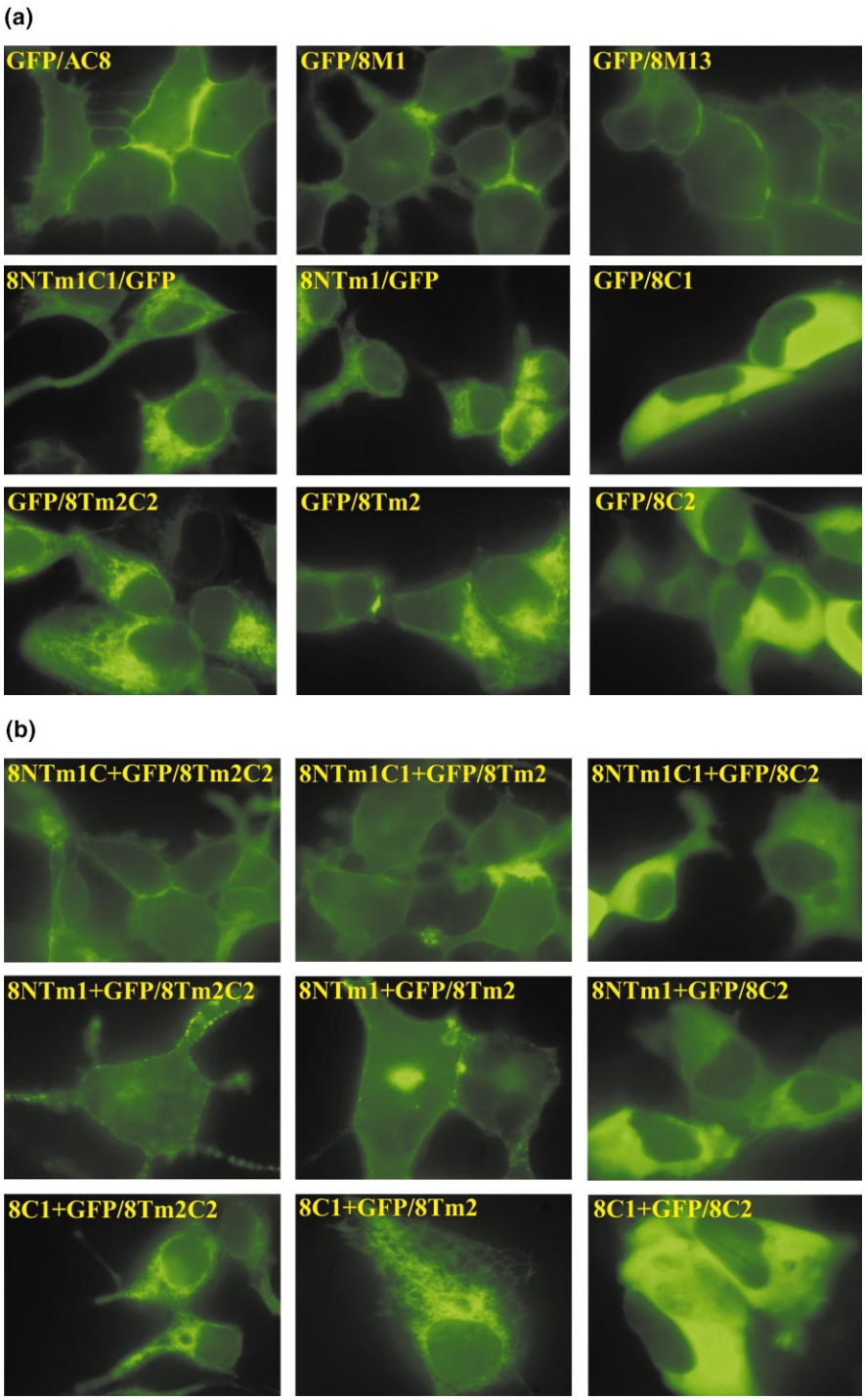
Diagram of eGFP fusion constructs. The green balls represent the eGFP molecule (GFP). The small cylinders are the transmembrane segments of AC8. The parts below and above the cylinders are inside and outside the cell, respectively. The red parts indicate the first half of AC8, which includes the N terminus (N), the first transmembrane cluster (Tm1), and the C1 loop (C1); the blue parts indicate the second half of AC8, which includes the second transmembrane cluster (Tm2) and the C2 loop (C2). The blue dotted line indicates the missing portion in GFP/8M13. The small branch on the top of the extracellular loop between the ninth and tenth transmembrane segments indicates the N-glycosylation site. In the construct names, "GFP" represents eGFP, a slash indicates the junction between the eGFP and the parts of AC8 (N, Tm1, C1, Tm2, and C2); viz., GFP/8C2 is N-terminally tagged, whereas 8NTm1/GFP is C-terminally tagged. These apply to all the cartoons and names for the rest of the figures and text.

that the two transmembrane clusters were necessary and sufficient for the whole molecule to traffic to the plasma membrane and that the C1 and C2 domains played no major role in targeting. The converse experiment – cotransfecting a tagged 8NTm1/GFP with untagged 8Tm2 – also resulted in appropriate plasma membrane expression, which confirms this conclusion (Table 1). Furthermore, the C1 loop alone could not change the ER location of either GFP/8Tm2C2 or GFP/8Tm2 (Figure 2b). In addition, the cytoplasmic distribution pattern of GFP/8C2 was not altered by cotransfection with any of the constructs from the first half of AC8 (Figure 2b).

The rescue of plasma membrane targeting by coexpression of the two transmembrane clusters suggested that the two clusters might interact. This conclusion is supported by coimmunoprecipitation (co-IP) assays (see Supplementary material available with this article on the internet). Interestingly, co-IP assays also revealed a strong homomeric interaction of 8Tm2. Dramatically decreased interactions were observed between the 8Tm1 and 8Tm2 domains of the B form of AC8. This B form is a naturally occurring splice variant of AC8 that lacks the extracellular loop between the ninth and tenth transmembrane segments, in which the only N-glycosylation site of AC8 resides [13]. The interaction between the Tm1 and Tm2 domains discerned by co-IP experiments is also specific for the isoform of adenylyl cyclase (see Supplementary material).

An unequivocal detection of protein-protein interactions in living cells is provided by FRET microscopy [14, 15]. We replaced the GFP in 8NTm1/GFP and in GFP/8Tm2 with CFP and YFP, respectively, cotransfected these constructs, and assessed whether FRET would occur. Since energy transfer between YFP and CFP occurs only at distances less than 5 nm, efficient FRET would indicate that the two termini were in close contact [14]. Cotransfecting the complimentary pairs 8NTm1/CFP and YFP/8Tm2 or 8NTm1/YFP and CFP/8Tm2 resulted in both constructs translocating to the plasma membrane (Figure 3a,b) where YFP and CFP fluorescence completely overlapped. However, more importantly, extremely efficient CFP-to-YFP energy transfer was detected. The apparent intensities of corrected FRET ($FRET^C$) signals were 4- to 5-fold higher than the $FRET^C$ signals measured by the identical technique for the EGFR-Grb2 interaction [15]. The strong FRET between the two cotransfected constructs clearly indicated that the two transmembrane clusters were closely associated (Figure 3a,b). FRET occurred in the plasma membrane as well as inside the cell (Figure 3a,b), and this finding suggested that a tight interaction occurs even after the complex is delivered to the plasma membrane. FRET analysis also reveals that 8Tm2 forms a homomultimer in the ER (Figure 3c), and this is in keeping with the co-IP results (see Supplementary material). 8NTm1 also forms homomultimers, but it does so much less tightly than does 8Tm2 (not shown). As negative controls, EGFR/YFP, which is expressed in the plasma membrane [15], as well as CFP/8Tm2 and 8NTm1 were cotransfected. YFP- and CFP-tagged proteins were colocalized in the plasma membrane, but FRET was not detected (Figure 3d). Cotransfection of the free CFP and YFP by themselves was performed as another negative control, which again showed colocalization (in the cytosol) but no

Figure 2



The cellular location of different domains of AC8. **(a)** Images captured from HEK 293 cells transfected with individual eGFP constructs, whose names are indicated on the pictures. HEK 293 cells were maintained as described previously [12]. **(b)** Images captured from nine combinations of the cotransfection of different domains from the first (nontagged) and second (eGFP-tagged) halves of AC8 in HEK 293 cells. The cDNA constructs for each cotransfection are indicated on each image. Large numbers of cells were counted in each cotransfection. In each cotransfection of the pairs (8NTm1C1+GFP/8C2, 8NTm1+GFP/8C2, 8C1+GFP/8C2, 8C1+GFP/8Tm2C2, and 8C1+GFP/8Tm2), no cell with eGFP fluorescence mainly in the plasma membrane was ever observed over three transfections. Cotransfections of the pairs 8NTm1C1+GFP/8Tm2C2, 8NTm1C1+GFP/8Tm2, 8NTm1+GFP/8Tm2C2, and 8NTm1+ GFP/8Tm2 were repeated at least three times, and each time at least 200 cells were counted. The percentage of cells expressing these constructs at the plasma membrane was 30%–40%. The cDNA ratio of the nontagged constructs to the eGFP constructs, which maximized the number of the cells with eGFP fluorescence in the plasma membrane, was 3:1.

FRET signals (Figure 3e). These studies clearly demonstrate that direct and persistent interactions occur between the two transmembrane clusters of AC in living cells.

To further identify the important regions for the interaction and trafficking in these two transmembrane clusters, we tested more constructs. 8Tm1 lacking the first 106 residues behaved just like 8NTm1, and this result confirmed that the N terminus of AC8 is not required for interaction and trafficking. However, $8Tm_{1-5}$, which lacked the sixth transmembrane segment, could not bring GFP/8Tm2 to the plasma membrane (Table 1). When

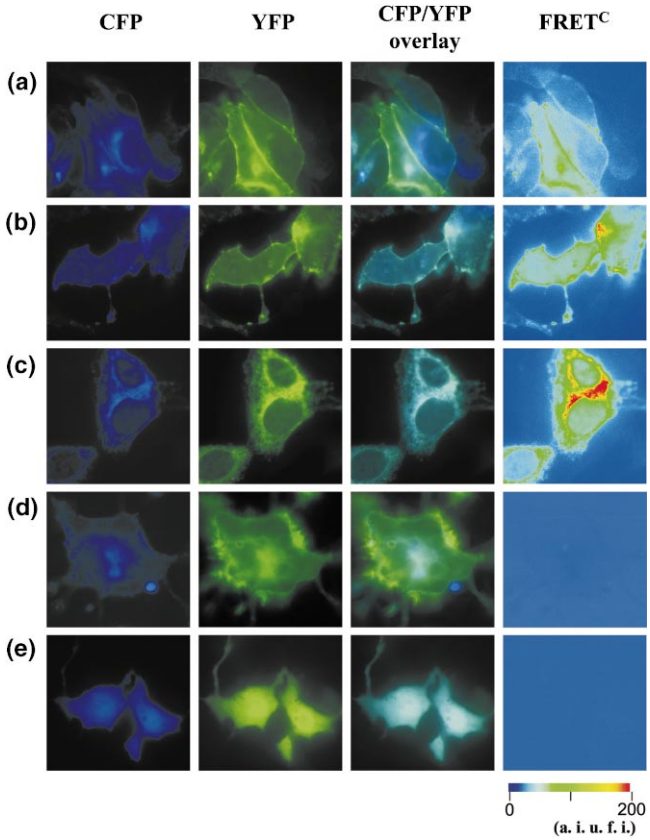
Table 1

GFP fluorescence imaging assay results

	GFP/ 8Tm2	GFP/ $8Tm_{7–9}$	GFP/ $8Tm_{10–12}$	GFP/ 8Tm2C2	GFP/ 8Tm2C2B	GFP/ 2Tm2C2	GFP/ 5Tm2C2	8NTm1/ GFP
8NTm1	+	–	–	+	–	–	–	–
8Tm1	+	–	–	+	–	ND	ND	ND
$8Tm_{1–5}$	–	–	–	–	ND	ND	ND	ND
8Tm2	–	ND	ND	ND	ND	ND	ND	+

eGFP fluorescence imaging assay results. The constructs in this table are described in detail in the Materials and methods section available with the Supplementary material. The subscript numbers indicate the transmembrane segments. Different combinations of cotransfection in HEK 293 cells were performed, with one construct eGFP tagged, the other not. eGFP fluorescence patterns were monitored. Trafficking (or not) of the molecule to the plasma membrane is indicated by plus or minus signs. "ND" indicates that the experiment was not performed because the results were expected to be negative.

Figure 3

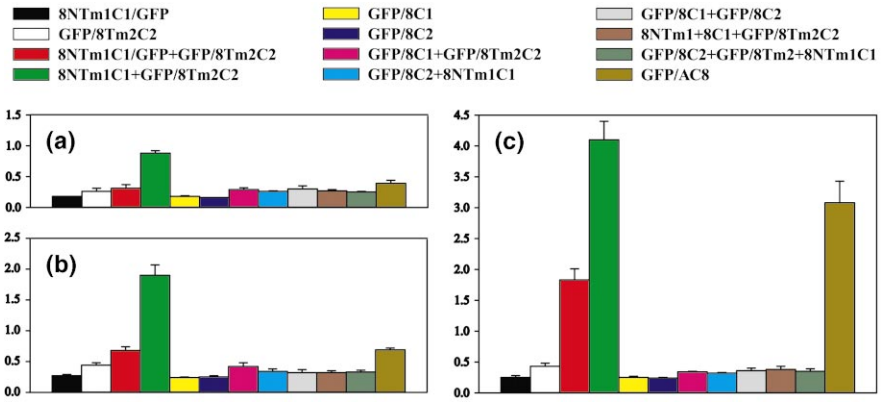


Interaction of the two transmembrane clusters of AC8 detected by FRET. CFP- and YFP-tagged cDNA constructs were cotransfected into HEK 293 cells. The pictures in each row were captured from the same cell. The pictures of the first and the second columns show the CFP and YFP fluorescence, respectively. The pictures of the third column are the overlay of the YFP and CFP images of the cell, which indicate colocalization of the YFP- and CFP-tagged constructs. The FRET images are presented in the fourth column. $FRET^C$ is displayed as a quantitative pseudocolor image. ALUFI stands for "arbitrary linear units of fluorescence intensity." Cotransfection of **(a)** 8NTm1/CFP and YFP/8Tm2; **(b)** 8NTm1/YFP and CFP/8Tm2; **(c)** CFP/8Tm2 and YFP/8Tm2; **(d)** EGFR/YFP, CFP/8Tm2, and 8NTm1; and **(e)** CFP and YFP. Each cotransfection was performed at least three times. As expected, transfection of 8NTm1/CFP, 8NTm1/YFP, CFP/8Tm2, or YFP/8Tm2 alone yielded an ER expression pattern (data not shown).

GFP/8Tm2 was dissected into two parts, GFP/$8Tm_{7–9}$ and GFP/$Tm_{10–12}$, both remained in the ER, even in the presence of 8NTm1 (Table 1). In agreement with co-IP data, GFP/8Tm2C2B also stayed in the ER when it was coexpressed with 8NTm1 (Table 1). In fact, the B form of AC8, eGFP-tagged at the N-terminal, was mainly located in the ER (data not shown), and this finding confirmed a previous speculation that the A and B forms of AC8 have different cellular localization [13]. These results suggested a role for N-glycosylation — or, more conservatively, the extracellular domain that includes the N-glycosylation site — in proper targeting. We also tested whether 8NTm1 could bring GFP/2Tm2C2 and GFP/5Tm2C2, respectively, to the plasma membrane. The imaging results were negative, which again was consistent with the co-IP data. This demonstrates the isotype selectivity for interactions among AC transmembrane domains (Table 1).

We next addressed the role of the interaction of the transmembrane clusters and catalytic activities of AC. Not surprisingly, given that AC activity needs both the C1 and C2 regions [16–19], transfection of any construct lacking either of the two cytoplasmic loops (C1 and C2) resulted in no catalytic activity above background activity (Figure 4). However, cotransfecting the two halves of AC8, such as in the cases of 8NTm1C1/GFP+GFP/8Tm2C2 and 8NTm1C1+GFP/8Tm2C2, resulted in forskolin- and Ca^{2+}-stimulable adenylyl cyclase activity in intact cells (Figure 4). A similar observation had been made when halves of AC1 (another Ca^{2+}-stimulable adenylyl cyclase) were expressed in Sf9 cells and the activity of membrane fractions was assessed [17]. The activities of the two cotransfected halves of AC8 are very similar to that of the full-length AC8 (Figure 4). Somewhat surprisingly, no activity was detected when either the C1 or C2 loop was transfected without its appropriate transmembrane cluster, such as in the cases of GFP/8C1+GFP/8C2, GFP/8C1+GFP/8Tm2C2, and GFP/8C2+8NTm1C1. This was also the case if the transmembrane cluster and the cytoplasmic loop were not covalently linked, such as in the cases of GFP/8C1+8NTm1+GFP/8Tm2C2 and GFP/8C2+GFP/8Tm2+8NTm1C1 (Figure 4). It would thus

Figure 4



In vivo adenylyl cyclase activities of coexpressed parts of AC8. Different transfections are shown by different colors, and the constructs in each transfection are indicated on the upper portion of the panel. The in vivo assays were performed as previously described [12]. The transfected HEK 293 cells were pretreated with thapsigargin (100 nM for 4 min) to activate the capacitative Ca^{2+} entry channel in the plasma membrane. The cAMP accumulation in the intact cells was measured for 1 min after the addition of **(a)** Vehicle (basal condition), **(b)** 20 μM forskolin, or **(c)** 20 μM forskolin plus 4 mM $CaCl_2$. In HEK 293 cells transfected with vector plasmid, the cAMP accumulation was similar to that of cells transfected with 8NTm1C1/GFP (not shown). Assays were performed three times with similar results.

appear that the C1/C2 interaction is not particularly strong, but that it is promoted by a much stronger interaction between the two transmembrane sections, which interaction effectively raises the relative concentrations of the C1 and C2 domains to very high levels. In in vitro experiments, purified proteins of the C1 and C2 loop of ACs can interact in a manner that is promoted by forskolin, but this could become inefficient in a living eukaryotic cell, in which far lower concentrations would be expected. Therefore, a further vital function of the interaction of the two transmembrane clusters is to enhance the interaction of the C1 and C2 catalytic loops.

Until the present studies, the function of the transmembrane domains of AC had been a puzzle. The live-cell FRET analysis along with the other techniques used here have convincingly demonstrated that (1) the two transmembrane clusters of AC interact with each other persistently; (2) this interaction is necessary and sufficient for the whole enzyme to traffic to the plasma membrane; (3) the extracellular loop between the ninth and tenth transmembrane segments containing the N-glycosylation site plays a requisite role; and (4) the interaction is critical to functionally assemble the two cyclase catalytic domains. The strong homomeric interaction of the second transmembrane cluster raises the possibility that adenylyl cyclases could adopt higher-order structures or interact with other membrane proteins. Future studies may address the mechanism for the interaction of the two transmembrane clusters and its role in the appropriate targeting of AC. For the present, the first light is shed on critical roles played by these hitherto puzzling and complex domains.

Supplementary material

Supplementary materials and methods are available with the electronic version of this article at http://current-biology.com/supmatin.htm.

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