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GTP-dependent binding of G_i, G_o and G_s to the γ-subunit of the effector of G_t†

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Abstract

The γ-subunit of the cGMP-phosphodiesterase (PDEγ) of retinal rods forms a tight complex with the activated α-subunit of transducin ($G_t\alpha GTP\gamma S$). We observe that while PDEγ is not the physiological effector of other Gα subtypes, it can still detectably interact with them. This interaction is strong with $G_{i1}\alpha$ and $G_{i3}\alpha$ ($K_d \approx 10$ nM) and weaker with $G_o\alpha$ and $G_s\alpha$ ($K_d \approx 1$ μM). For all these Gα subtypes, similar intrinsic fluorescence changes are observed upon PDEγ binding. Moreover, similar relative decreases in affinity are obtained when the GDP forms of $G_{i1}\alpha$, $G_{i3}\alpha$ or $G_t\alpha$ are used in lieu of the GTP forms. This points to a conserved GTP-dependent effector-interaction domain.

Key words: G-protein; Transducin; cGMP-phosphodiesterase; Tryptophan fluorescence

1. Introduction

Heterotrimeric G-proteins act as signal transducers at the cytoplasmic face of the cell's plasma membrane by interacting first with an activated receptor and then with an effector such as an enzyme or a channel [1,2]. The first interaction leads to the activation of the G protein, i.e. the switching of its α-subunit from an inactive, GDP-bound state to an active, GTP-bound state. The G-protein α-subunit (Gα), now bearing GTP, binds to and turn on an effector. The effector of transducin (G_t), the G-protein of the visual system is a cGMP-phosphodiesterase (PDE) [3]. This membrane-bound enzyme is composed of a large catalytic dimer, PDE$\alpha\beta$, whose activity is controlled by two small inhibitory subunits, PDEγ. $G_t\alpha GTP$ binds to PDEγ and relieves its inhibiton on PDE$\alpha\beta$ [4,5]. The PDEγ–$G_t\alpha GTP$ complex remains membrane-bound through weak interactions with PDE$\alpha\beta$ as well as with phospholipids [6, 7]. However, this complex can be easily solubilized and purified [8]. We have recently shown that, in vitro, a large fluorescence change accompanies the binding of isolated PDEγ to $G_t\alpha$ [9]. The signal depends on the conformation of $G_t\alpha$ and permits accurate determination of the affinity of PDEγ for $G_t\alpha$. PDEγ interacts tightly with $G_t\alpha GTP$ ($K_d < 0.1$ nM) but retains a substantial affinity for the inactive $G_t\alpha GDP$ ($K_d \approx 3$ nM). Using the same spectroscopic approach, we show here that four other Gα subtypes, $G_{i1}\alpha$, $G_{i3}\alpha$, $G_o\alpha$ and $G_s\alpha$ can also interact with PDEγ. As with $G_t\alpha$, the affinities of these interactions depend on the conformations of the Gα-subunits.

2. Materials and methods

2.1. Proteins

Recombinant PDEγ either in the wild-type form or carrying a single mutation at position 70 ($^{[W70F]}$PDEγ) was expressed in *E. coli* and purified as described previously [9]. $G_s\alpha$ (short form, 44 kDa; bovine), $G_{i3}\alpha$ (rat) and recombinant $G_o\alpha$ and $G_{i1}\alpha$ (expressed in *E. coli* [10]) were kindly provided by T. Higashijima and M. Linder (University of Texas, Southwestern Medical Center). Replacement of GDP by GTPγS was achieved by a 2-hour incubation at 25°C with a 10-fold excess of GTPγS.

2.2. Fluorescence measurements

All measurements were performed with a Shimadzu RF5000 fluorimeter. The magnetically stirred sample (500 μl) resided in a thermostated (25°C) cylindrical quartz cell (diameter 6 mm). Excitation was at 292 nm (± 2.5 nm). The buffer contained: HEPES, 20 mM (pH 7.5), KCl 120 mM, $MgCl_2$ 2 mM, DTT 1 mM, and 0.1% Thesit (Dodecylpoly(ethyleneglycolether)$_n$, Boehringer Mannheim). Emission spectra were recorded at a scan speed of 0.8 nm/s with a bandwidth of 5 nm. For time-scan recordings, the emission was measured at either 325 nm or 360 nm with a bandwidth of 30 nm.

2.3. Determination of Gα–PDEγ equilibrium constants (K_d)

Association of PDEγ to Gα was followed through the fluorescence change correlated with the formation of the complex [9]. The fluorescence cuvette initially contained 500 μl Gα at a concentration C_o of 200 or 500 nM. Small volumes (2 or 5 μl) from a concentrated stock solution of PDEγ were sequentially added to the cuvette and fluorescence emission was continuously monitored. As controls, the same experiments were done in the absence of Gα. All experiments were performed in duplicates and the maximal variation in the K_d value was 20%.

In the presence of Gα, the fluorescence, F, following the nth addition of PDEγ (concentration Δx) is:

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†This paper is dedicated to the memory of T. Higashijima.

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$$F = f_G C_0 + (f_{PG} - f_P - f_G)[\text{PDE}\gamma\text{–G}\alpha] + nf_P \Delta x$$

where f_P, f_G and f_{PG} are the molar fluorescences of PDEγ, Gα and PDEγ–Gα, respectively. $f_G C_0$ is the initial fluorescence level before the first addition of PDEγ and can be substracted out:

$$\Delta F = F - f_G C_0 = (f_{PG} - f_P - f_G)[\text{PDE}\gamma - \text{G}\alpha] + nf_P \Delta x$$

The fluorescence change due to PDEγ when it is alone is $\Delta F_{control} = nf_P \Delta x$ and can be subtracted out by using the control recordings:

$$\Delta F - \Delta F_{control} = (f_{PG} - f_P - f_G)[\text{PDE}\gamma - \text{G}\alpha]$$

Assuming the simple bimolecular association scheme:

$$\text{PDE}\gamma + \text{G}\alpha \overset{K_d}{\Leftrightarrow} \text{PDE}\gamma\text{–G}\alpha,$$

one obtains:

$$[\text{PDE}\gamma\text{–G}\alpha] = \tfrac{1}{2}\{(C_0 + n\Delta x + K_d) - \sqrt{(C_0 + n\Delta x + K_d)^2 - 4 \cdot C_0 \cdot n\Delta x}\}$$

$\Delta F - \Delta F_{control}$ is directly related to the fraction of complexed Gα. Thus, deriving the PDEγ–Gα binding curve from $\Delta F - \Delta F_{control}$ and fitting it with this expression for [PDEγ–Gα] yield K_d.

2.4. Determination of PDE activity

PDE activity was determined by measuring the pH change associated with cGMP hydrolysis. HoloPDE was extracted from bovine rod outer segments and reconstituted with large size unilamellar vesicles [9]. The buffer contained HEPES 10 mM (pH 7.5), KCl 120 mM, and $MgCl_2$ 2 mM.

3. Results

Fluorescence emission spectra of PDEγ and of $G_{i1}\alpha$GTPγS are shown in Fig. 1 (upper panel, dotted traces). If PDEγ did not interact with $G_{i1}\alpha$GTPγS, the spectrum of a mixture of the two proteins should equal the sum of the individual spectra of PDEγ and $G_{i1}\alpha$GTPγS measured separately. This is not the case: the spectrum of the mixture (thick trace) exhibits a blue shift with respect to the sum of the invidual spectra (thin trace). Consequently, the differential spectrum is biphasic: for $\lambda_{em} < 330$ nm, interaction between PDEγ and $G_{i1}\alpha$GTPγS gives a fluorescence increase, while a decrease is observed for $\lambda_{em} > 330$ nm. This differential spectrum closely resembles that observed when PDEγ interacts with its normal partner, $G_t\alpha$GTPγS [9]. Analogous experiments were performed with the other three Gα subunits. $G_{i3}\alpha$ is nearly identical to $G_{i1}\alpha$ in its interaction with PDEγ. $G_o\alpha$ and $G_s\alpha$ also interact to some extent with PDEγ. From the amino-acid sequences, these two α-subunits are more distantly related to G_t than the G_i's. Yet as shown in Fig. 1, a fluorescence blue shift is also observed for mixtures containing PDEγ and $G_s\alpha$GTPγS or $G_o\alpha$GTPγS.

For each Gα, the PDEγ–GαGTPγS binding curve was constructed and fitted as described in section 2 (Fig. 2). Except for $G_s\alpha$, the emission window was set at 360 ± 15 nm. The PDEγ–Gα interaction is thus better underscored as it gives fluorescence changes that differ in sign as well as in amplitude from the fluorescence increments

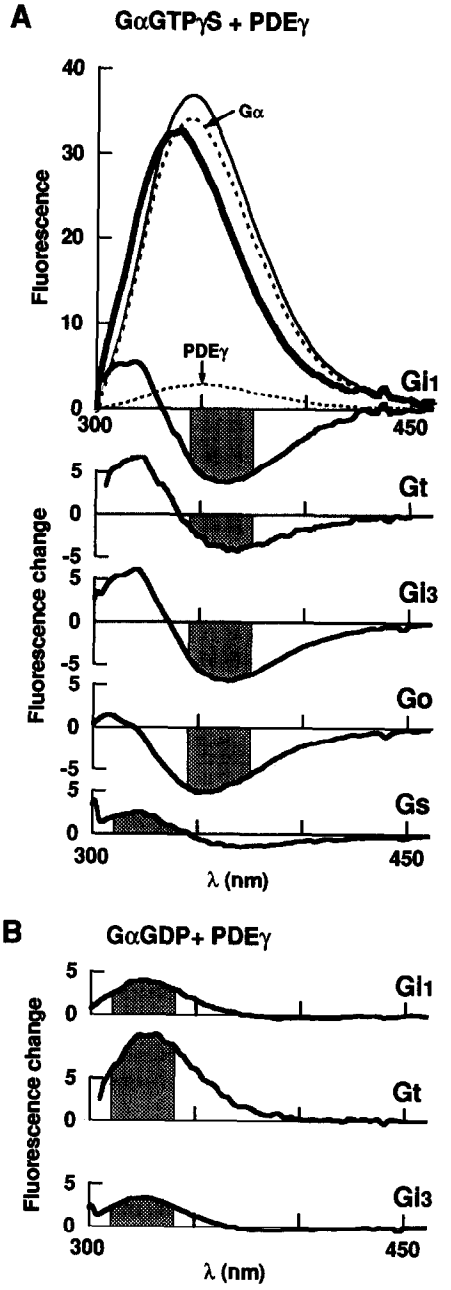

Fig. 1. Fluorescence changes induced by binding of PDEγ to various Gα-subtypes. (A) Individual fluorescence emission spectra of PDEγ (500 nM) or G_{i1}GTPγS (500 nM) are measured separately (dotted traces). The sum of the two spectra (thin trace) is markedly different from the spectrum of a mixture containing the two proteins (thick trace). The differential spectrum is obtained by subtracting the sum of individual spectra from the mixture spectrum. This difference shows up as the biphasic trace and reflects the interaction between PDEγ and G_{i1}GTPγS. This treatment is applied to all five GαGTPγS subunits, resulting in the five differential spectra shown here. Protein concentrations were: G_{i1}, 500 nM; G_t, 100 nM; G_{i3}, 750 nM; G_o, 500 nM; and G_s, 500 nM. For each Gα-subtype, [PDEγ] is made equal to [Gα]. The differential spectra were normalized according the amount of protein used. (B) For the interaction between PDEγ and GαGDP, differential spectra were detectable for only three subtypes, G_{i1}, G_t and G_{i3}. In both (A) and (B), differential spectra for $G_t\alpha$ are from [9]. The stippled areas show the two emission windows used in quantifying the binding of PDEγ to the five Gα subunits.

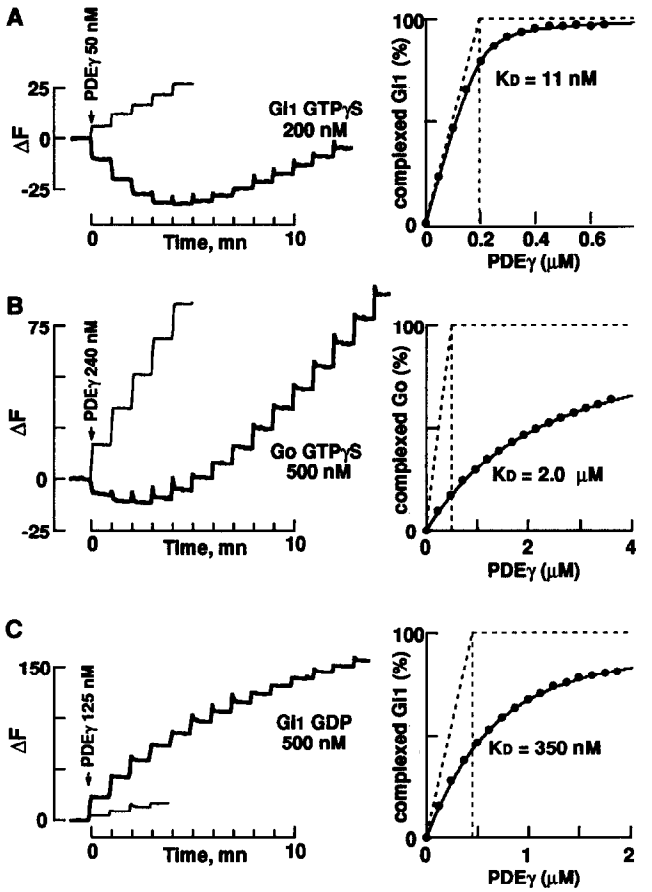

Fig. 2. Determination of the affinity of PDEγ for G_{i1}αGTPγS (A), G_oαGTPγS (B) and G_{i1}αGDP (C). The change in fluorescence emission from solutions containing 200 nM G_{i1}αGTPγS (A), 500 nM G_oαGTPγS (B) or 500 nM G_{i1}αGDP (C) was continuously monitored while equal amounts of PDEγ were added every minute to the fluorescence cuvette (thick traces). Identical experiments performed without Gα constitute the controls (thin traces). Emission was measured at 360 ± 15 nm in (A) and (B) or at 325 ± 15 nm in (C) (see Fig. 1). PDEγ steps were 50 nM (A), 240 nM (B) or 125 nM (C). At each concentration of PDEγ, the difference between the fluorescence change in the presence of Gα and that of the control is directly proportional to the concentration of Gα–PDEγ . The fraction of Gα complexed to PDEγ is calculated and plotted as a function of [PDEγ] added to give the binding curve shown on the right (see section 2). Continuous lines are best fits to the data. Dotted line corresponds to the fitting equation with K_d = 0 and indicate the stoechiometry of the complex.

due to additions of PDEγ to a cuvette with no Gα. In Fig. 2A, the first few additions of PDEγ (50 nM per step) to 200 nM G_{i1}αGTPγS cause fluoresence decreases. But on the fourth or fifth addition, the fluorescence jumps suddenly become positive and are about equal to those observed in the absence of Gα. This indicates the formation of a high affinity, one-to-one complex between G_{i1}αGTPγS and PDEγ. With G_oαGTPγS (Fig. 2B), the fluorescence steps change more gradually although higher amounts of proteins were used: PDEγ has a lower affinity for G_oα than for G_{i1}α. The binding curves of PDEγ for G_{i1}αGTPγS or G_oαGTPγS are shown in Fig. 2, right panels. Similar experiments were performed with G_{i3}α and G_sα. The K_d values for the four PDEγ–GαGTPγS complexes are: G_{i3}, K_d = 6 nM ; G_{i1}, K_d = 11 nM ; G_s, K_d = 1.7 μM ; G_o, K_d = 2.0 μM.

We next looked for fluorescence changes that would betray a possible interaction between PDEγ and the GDP-bound forms of these Gα subunits. No such signals were detectable for solutions containing micromolar amounts of PDEγ and G_oαGDP or G_sαGDP. This means that either no binding occurs or that it is fluorescently silent. On the other hand, a significant blue shift of 4 nm as well as an increase in fluorescence intensity were observed when PDEγ was mixed with G_{i1}αGDP or G_{i3}αGDP (Fig. 1B). These spectral changes are reminiscent of the interaction between G_tαGDP and PDEγ and are markedly different from those observed for the corresponding GTP-bearing Gα-subunits (compare A and B of Fig. 1). The binding curves for the two G_iαGDP's were determined using an emission window of 325 ± 15 nm. A typical experiment is shown in Fig. 2C. The K_d values for the PDEγ–G_{i1}αGDP and PDEγ–G_{i3}αGDP complexes are 0.36 μM and 0.22 μM, respectively.

Tryptophan 70 of PDEγ is involved in the interaction with G_tαGTPγS [9]. Its replacement by phenylalanine lowers the affinity between the two partners by a factor of at least 50 [9]. Does this mutation on PDEγ also weaken its interaction with the other Gα's? Competition experiments between wild-type and $^{[W70F]}$PDEγ for G_{i3}αGTPγS were carried out (data not shown). In the presence of equimolar amounts of the two PDEγ's, G_{i3}αGTPγS binds nearly completely to the wild type form. Hence, as in the case with G_t, the W70F mutation prevents or markedly weakens the interaction of PDEγ with G_{i3}.

As the affinity of PDEγ for the G_iα's is substantial, we wonder if G_{i1}αGTPγS can activate holoPDE in a reconstituted system. Addition of 100 nM G_tαGTPγS to a sample containing 25 nM PDEαβ$(\gamma)_2$ induced a dramatic enhancement of the PDE activity (Fig. 3). By contrast, no change was detected when 20 times as much G_{i1}αGTPγS was added. Moreover, G_{i1}αGTPγS could not compete with G_tαGTPγS: the rate of cGMP hydrolysis induced by 100 nM G_tαGTPγS was identical whether 2 μM G_{i1}αGTPγS was present or not. Since the affinities of PDEγ for G_tαGTPγS and for G_{i1}αGTPγS differ by at least two orders of magnitude, the inability of G_{i1} to activate holoPDE is not surprising. Analogously, the inactive form of transducin, G_tαGDP, though capable of binding to PDEγ with a K_d in the nanomolar range, does not activate holoPDE.

4. Discussion

As the two families of seven-α-helix receptors and heterotrimeric G-proteins display within themselves

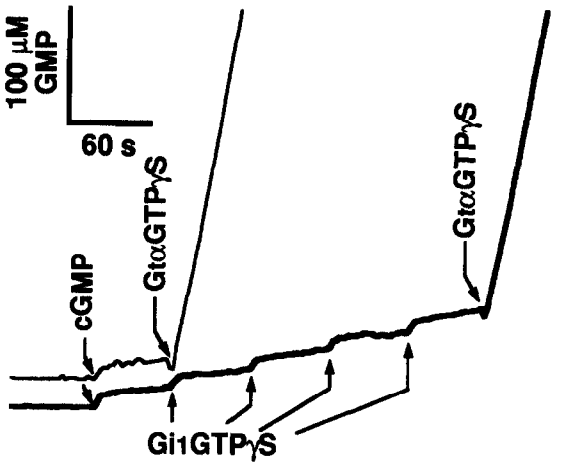

Fig. 3. G_{i1} does not activate holoPDE. PDE$\alpha\beta(\gamma)_2$ (25 nM) was mixed with phospholipid vesicles (1 mg/ml) and the hydrolysis of cGMP (2 mM) was continuously monitored. Four aliquots of $G_{i1}\alpha GTP\gamma S$ (0.5 μM each) were sequentially added to the sample with no detectable effect on PDE activity (thick trace). Addition of 100 nM $G_t\alpha GTP\gamma S$ alone (thin trace) or following the four G_{i1} aliquots induced the same PDE activities.

much structural homology, the conserved mechanisms through which these two types of protein interact can be no cause for surprise. The other protein–protein interaction in G-protein-mediated signalling, namely that between Gα and its intended effector, exhibits more complexity. In addition to the activation of PDE by G_t, two other G-protein–effector connections are molecularly well described: phospholipase Cβ1 is activated by $G_{q/11}\alpha$ [11,12] and adenylyl cyclase is activated by $G_s\alpha$ (for review see [1,2]) and inhibited by $G_i\alpha$ [13]. Structurally, no obvious homologies have been detected among these G-protein effectors. Functionally, aside from Gα, Gβγ can also act on some effectors [14,15]. Still, standing out against this backdrop of diversity is the varying degree of structural resemblance between $G_{i1}\alpha$, $G_{i3}\alpha$, $G_o\alpha$, $G_s\alpha$, and $G_t\alpha$, the five proteins studied here. We wonder how this commonality in form might relate to function, as may be assessed by applying the convenient tool of PDEγ binding.

We show here that in vitro, PDEγ also binds to the other four Gα-subtypes, in addition to G_t, its normal partner. Five features of this binding are particularly telling. First, binding tightness is related to the amino acid sequence closeness between the Gα-subtypes tested and $G_t\alpha$, as is summarized in Fig. 4A. Second, binding is greatly enhanced by the presence of GTP in the nucleotide site, as has already been described for G_t [9]. Third, for G_{i1}, G_{i3} and G_t where PDEγ binding to the GDP form is measurable, the ratios of affinities between the GTPγS and GDP states are all ≥ 30 (Fig. 4A). This indicates that for these three Gα-subtypes, the PDEγ binding energy increases by about the same amount as GTP replaces GDP in the nucleotide site. Fourth, for all five GαGTP's, the spectral changes incurred upon PDEγ binding are similar in nature. This also pertains to the three GαGDP's whose PDEγ binding is detectable. Fifth, the W70F mutation on PDEγ greatly loosens its interaction with $G_{i3}\alpha$, as is already known for G_t. Interaction between PDEγ and $G_t\alpha$ relies heavily on tryptophan 70 for the former [9] and tryptophan 207 for the latter [16]. W207 is conserved in all Gα-subtypes known to date and is solely responsible for the drastic fluorescence change observed as Gα switches from the GDP to the GTP state [16,17]. Replacement of either W70 or W207 by phenylalanine causes a 100-fold decrease in affinity between PDEγ and $G_t\alpha$ and greatly modifies the binding-induced fluorescence change.

Altogether, these five features, and especially the last three, strongly support the notion that PDEγ binds to an analogous site on $G_t\alpha$, $G_{i1}\alpha$ and $G_{i3}\alpha$, a site that includes the conserved tryptophan represented by W207 in G_t. As such, this site must reside somewhere on the switch do-

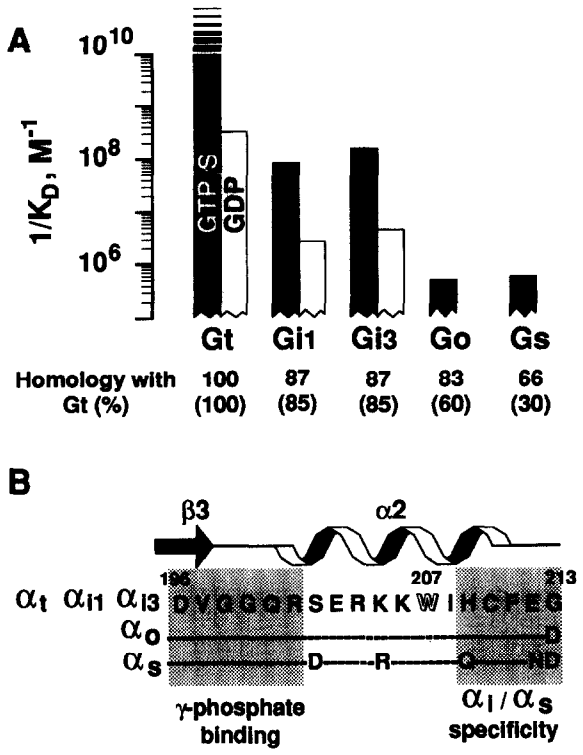

Fig. 4. Affinities of PDEγ for the five Gα-subtypes. (A) Affinities ($1/K_d$) of PDEγ for the five Gα-subtypes are shown in log scale. Given the logarithmic relationship between free energy and affinity ($\Delta G_0 = -2.3RT \log_{10}[1/K_d]$), this figure shows that the decrease in free energy of the PDEγ–Gα complex (or the increase in PDEγ binding energy) as GDP is replaced by GTP in the nucleotide site is the same for $G_t\alpha$, $G_{i1}\alpha$ and $G_{i3}\alpha$. The affinity value of $G_t\alpha GTP\gamma S$ with PDEγ is only a lower limit, as illustrated by the vanishing top end of the bar. Homologies between each Gα-subtype and $G_t\alpha$ are provided. Without parentheses are homologies in the C-terminal of the primary structure (starting from ^{196}D of $G_t\alpha$). Inside parentheses are homologies in the four effector-activating regions as defined by Berlot & Bourne [21]. (B) Sequence alignment of the switch II domain of G-protein α-subunits. The sequence is conserved among G_t, G_{i1} and G_{i3}. $G_s\alpha$ and $G_o\alpha$ differ from this conserved sequence at the residues shown.

main II (Fig. 4B), which includes helix α_2 [18] and exhibits three important features. (i) It is one of two domains that participate fully in the GDP-to-GTP conformational change [19]. (ii) It includes certain amino-acids that are involved in effector binding such as W207 [16]. (iii) It is well conserved within the Gα family and is strictly so among $G_t\alpha$, $G_{i1}\alpha$ and $G_{i3}\alpha$. Thus, the fact that for these three Gα-subunits, the GDP/GTP conformational change induces a similar enhancement in affinity for PDEγ, suggests that the switch II domain is a conserved GTP-dependent effector-interaction domain. However, since the K_d's of the PDEγ–Gα complexes studied here range over four orders of magnitude, much more specific amino acids of $G_t\alpha$ must be involved in the binding to PDEγ. It is interesting to note that without the extra tightness provided by such residues, no action on the effector is possible: $G_{i1}\alpha$GTP cannot activate holo-PDE, as $G_t\alpha$GTP can. Certain amino-acids in the C-terminal region of $G_t\alpha$ have been shown to contribute to PDE activation and are poorly conserved in other Gα-subunits [20]. In the case of $G_s\alpha$, four separate groups of amino-acids are specifically involved in the activation of Adenylyl Cyclase [21,22]. Two residues of the switch II domain that are specific to $G_s\alpha$ belong to one of these groups.

One is tempted to entertain the notion that G-protein-effector interaction calls upon at least two different classes of binding sites. One class, including the switch II domain and in which W207 and its cognates play a crucial role, provides the effector with information regarding the GDP/GTP conformation of Gα. This putative class would be at the root cause of the findings presented here. The other class of sites provides for specificity, in that it helps a Gα-subtype distinguish between an array of effectors. While functionally distinct, these two types of sites might well show partial overlap at the structural level.

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