The EMBO Journal vol.12 no.11 pp.4191-4198, 1993

Tryptophan W207 in transducin Tα is the fluorescence sensor of the G protein activation switch and is involved in the effector binding

Eva Faurobert, Annie Otto-Bruc, Pierre Chardin and Marc Chabre[1]

CNRS, Institut de Pharmacologie Moléculaire et Cellulaire, 660 route des Lucioles, Sophia Antipolis, 06560 Valbonne, France
[1]Corresponding author

Communicated by M.Chabre

We have produced a recombinant transducin α subunit (rTα) in sf9 cells, using a baculovirus system. Deletion of the myristoylation site near the N-terminal increased the solubility and allowed the purification of rTα. When reconstituted with excess Tβγ on retinal membrane, rTα displayed functional characteristics of wild-type Tα *vis à vis* its coupled receptor, rhodopsin and its effector, cGMP phosphodiesterase (PDE). We further mutated a tryptophan, W207, which is conserved in all G proteins and is suspected to elicit the fluorescence change correlated to their activation upon GDP/GTP exchange or aluminofluoride (AlFx) binding. [W207F]Tα mutant displayed high affinity receptor binding and underwent a conformational switch upon receptor-catalysed GTPγS binding or upon AlFx binding, but this did not elicit any fluorescence change. Thus W207 is the only fluorescence sensor of the switch. Upon the switch the mutant remained unable to activate the PDE. To characterize better its effector-activating interaction we measured the affinity of [W207F]TαGDP-AlFx for PDEγ, the effector subunit that binds most tightly to Tα. [W207F]Tα still bound in an activation-dependent way to PDEγ, but with a 100-fold lower affinity than rTα. This suggests that W207 contributes to the G protein effector binding.

Key words: cGMP phosphodiesterase/G protein/signal transduction/transducin/tryptophan fluorescence

Introduction

Transducin of vertebrate retinal rod is an archetype for the very large family of heterotrimeric G proteins that transmit signals from hormonal or sensory membrane receptors to intracellular effectors (Stryer and Bourne, 1986; Chabre and Deterre, 1989; Conklin and Bourne, 1993). The nucleotide binding subunit Tα shuttles between the G protein coupled receptor, rhodopsin and the effector, the cGMP phosphodiesterase (PDE), upon switching between an 'inactive' conformation with a bound GDP and an 'active' conformation with a bound GTP. In the inactive state, TαGDP is associated with the membrane-bound Tβγ subunit and has a high affinity for photoactivated rhodopsin (R*). R* catalyses the opening of the nucleotide binding site in Tα, allowing the rapid release of the otherwise permanently bound GDP. GTP can then bind in the empty site of the R*-bound Tα. The binding of GTP induces in Tα a change of conformation that results in the dissociation of TαGTP from Tβγ and from R* and its release from the membrane. TαGTP has the capacity to activate the membrane-bound cGMP PDE (PDEαβγ2). TαGTP binds to one of the PDEγ inhibitory subunits and displaces it from its inhibitory site on the PDEαβ catalytic complex. Tα has thus switched from a high affinity for R* and a low one for PDEγ, in the TαGDP 'inactive' state, to a low affinity for R* and a high one for PDEγ in the TαGTP 'active' state. Upon the GTPase step TαGTP will relax back to the TαGDP conformation which releases PDEγ and allows it to inactivate the PDE. As for the Gα subunit of all heterotrimeric G proteins, TαGDP can also be activated without the help of a receptor, and in the absence of GTP, by the action of fluoride. Fluoride anions form with various metal ions, in particular aluminum, complexes such as AlFx (x = 3−5) that act as high affinity phosphate analogues. AlFx binds next to the GDP in TαGDP and triggers the conformation switch to the active state by simulating the presence of the γ phosphate of an activating GTP (Bigay *et al.*, 1987; Antonny and Chabre, 1992).

No crystallographic structure of any heterotrimeric G protein α subunit is yet available, but three-dimensional models have been proposed (Masters *et al.*, 1986; Bourne *et al.*, 1991; Berlot and Bourne, 1992), based mostly, at least for the nucleotide binding domain, on sequence homologies with the small G protein $p21^{ras}$, whose crystal structure is known to high resolution (Milburn *et al.*, 1990; Pai *et al.*, 1990). Five conserved sequence motifs that form the guanine nucleotide binding domain in $p21^{ras}$ have recognizable analogues in all Gαs. Two flexible polypeptide loops near the second and the third nucleotide binding motif have been identified in ras as the 'switch I' and 'switch II' domains that undergo dramatic conformation changes between the GDP- and GTP-bound states (Wittinghofer and Pai, 1991; Stouten *et al.*, 1993). Analogous 'switch' regions have been suggested to exist in the α subunit of all heterotrimeric G proteins, Gsα being usually taken as the reference (Berlot and Bourne, 1992). For the identification of receptor and effector binding domains, much information has been gained recently from mutagenesis approaches in the various Gsα and Giα subunits. The main receptor-specific domain is very near the C-terminus (Hamm *et al.*, 1988; Conklin and Bourne, 1993). Effector binding domains are found on the C-terminal half of the Gα sequences (Rarick *et al.*, 1992). but effector-specific residues have also been detected in the domain equivalent in the heterotrimeric Gα subunits to the ras 'switch II' domain (Berlot and Bourne, 1992).

As in all Gα subunits, the conformational switch induced by receptor-catalysed GDP/GTP exchange or by AlFx binding, is correlated in Tα with a large increase in the protein intrinsic tryptophan fluorescence (Higashijima *et al.*, 1987, 1991; Philipps and Cerione, 1988; Antonny and Chabre, 1992; Antonny *et al.*, 1993). This fluorescence increase does not depend on the presence of Tβγ, neither on that of the receptor nor of an effector, and it is fully reversed upon the hydrolysis of the bound GTP or the elution

of AlFx. It thus depends strictly on the structure of Tα and reports the structural change in this subunit upon the 'activation' switch. The localization of the conformation-sensitive tryptophans may thus bring some insight into the switching mechanism. Tα contains only two tryptophan residues, which are strictly conserved in the homologous sequences of all heterotrimeric G protein α subunits. The first residue on the N-terminal side, W127 in Tα or W154 in Gsα, is located in a long insert between the first two guanine nucleotide binding motifs. This insert forms a domain of unknown function, and has no equivalent in the sequence of $p21^{ras}$. The other tryptophan, W207 in Tα or W234 in Gsα, is close to the third nucleotide binding motif, the γ-phosphate binding sequence, in a region that corresponds in $p21^{ras}$ to the 'switch II domain' whose conformation changes extensively upon activation (Stouten *et al.*, 1993). W207 in Tα is therefore the best candidate for a fluorescence sensor of the conformation switch. The strict conservation of this tryptophan in all G proteins could suggest that this residue is essential for either the correct folding of the switch domain or the switching mechanism. On the other hand this conservation among G proteins that activate very different types of effectors seemed to exclude a role of this tryptophan in the binding of the various G proteins to their specific effectors.

To delineate the exact roles of this conserved tryptophan, replaced it in transducin with another hydrophobic aromatic residue, phenylalanine, and we analysed the effects of this mutation on the spectral, structural and functional characteristics of Tα. Recombinant $^{[W207F]}$Tα was produced and purified from sf9 insect cells infected with a recombinant baculovirus. The mutation indeed suppressed the fluorescence signal observed upon activation in native Tα. Reconstitution assays showed that the mutation had not perturbed the interaction of the mutant with the receptor, nor the activation by GDP/GTPγS exchange or by AlFx, which induced in the mutant the same conformation switch as in native Tα. However, the effector-activating capacity of the 'activated' mutant on the PDE was severely hampered. Thus W207 is the fluorescence sensor of the activation switch in Tα and is also an effector binding residue of the activated G protein.

Results

Production and purification of recombinant Tα

We made numerous attempts at producing recombinant transducin Tα subunit in *Escherichia coli*, using various expression vectors carrying a cDNA or a synthetic gene coding for Tα, obtained respectively from B.K.K.Fung (Medynski *et al.*, 1985) and from T.Sakmar (Sakmar and Khorana, 1988). All our attempts ended up with large quantities of insoluble Tα in inclusion bodies and no correctly folded soluble protein. Transducin is indeed one of the few heterotrimeric G proteins whose α subunit has never been produced in a functional form by recombinant technology in *E.coli*. We thus resorted to production in sf9 insect cells infected with a recombinant baculovirus vector carrying the synthetic gene. In order to increase the solubility of the recombinant protein in the sf9 cells, N-terminal myristoylation of the recombinant G protein (Jones *et al.*, 1990; Mumby *et al.*, 1990; Neubert *et al.*, 1992) was prevented by the deletion of the myristoylation site

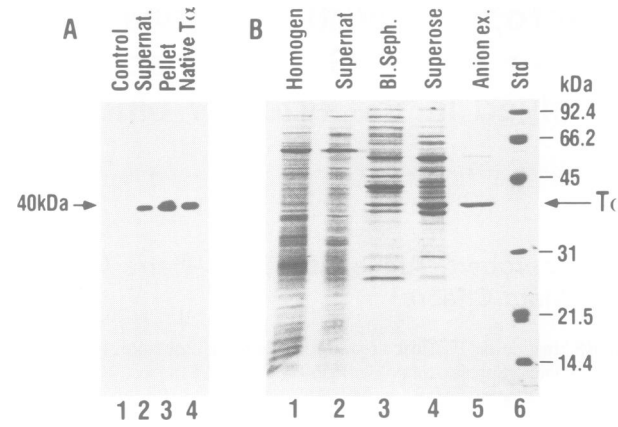

Fig. 1. Expression and purification of recombinant Tα.
(**A**) Immunoblot of cytosolic and particulate fractions of baculovirus-infected sf9 cells. Monolayer culture of 3×10^6 Sf9 cells was infected with the recombinant baculovirus (m.o.i = 5). At 48 h after infection, the cells were lysed and centrifuged at 400 000 *g* for 30 min to yield the cytosolic and particulate fractions. One percent of each fraction was resolved by SDS−PAGE/immunoblotting. Lane 1, cytosolic fraction of noninfected cells; lanes 2 and 3, cytosolic and particulate fractions of infected cells, respectively; lane 4, 200 ng of purified nTα. (**B**) Coomassie blue-stained SDS−polyacrylamide gels of transducin enriched fractions obtained during purification of rTα. Lane 1, sonicated cells, 15 μg protein; lane 2, 100 000 *g* supernatant, 10 μg protein; lane 3, Blue Sepharose chromatography eluate, 5 μg protein; lane 4, gel filtration eluate (Pharmacia superose 12 column), 5 μg protein; lane 5, ion exchange chromatography eluate (Pharmacia, polyanion SI column), 1 μg protein.

$_2GAGA_5$ and its replacement with an isoleucine residue. Soluble transducin, which represented 30% of the total recombinant Tα produced by sf9 cells 48 h after infection, was detected in the cell extracts (Figure 1A) and was purified by a succession of gel filtration and ion exchange chromatographies (Figure 1B), yielding 100 μg of 90% pure recombinant Tα per litre of cell culture. Hereafter we use rTα to denote recombinant transducin that is modified at its N-terminal but is otherwise unmutated. Myristoyl-free rTα has a lower affinity for Tβγ than native Tα. The effects related to the suppression of the myristoylation site in our recombinant transducins will be further discussed elsewhere. These effects have already been characterized in other G proteins and have been shown to affect slightly the binding of the Gα subunit to Gβγ and the coupling of the heterotrimeric G protein to agonist liganded receptor (Journot *et al.*, 1991; Linder *et al.*, 1991). The recombinant transducin which carries the additional mutation on W207 was obtained and purified with the same yields as for rTα. The characteristics of $^{[W207F]}$Tα will be compared with that of rTα and with that of native myristoylated transducin, purified from bovine retinal rods, which we denote nTα.

The $^{[W207F]}$Tα mutant binds with high affinity to R, and is released upon GDP/GTP exchange*

The high affinity binding of the mutant G protein to its coupled receptor and the ensuing nucleotide exchange process were studied by the classical binding assays of Kühn (1980). Purified recombinant transducin was added to illuminated retinal membrane that had been washed free of its native transducin and of all other soluble proteins, and supplemented with purified Tβγ subunits. In the absence of free guanine nucleotide, both $^{[W207F]}$Tα and rTα bound

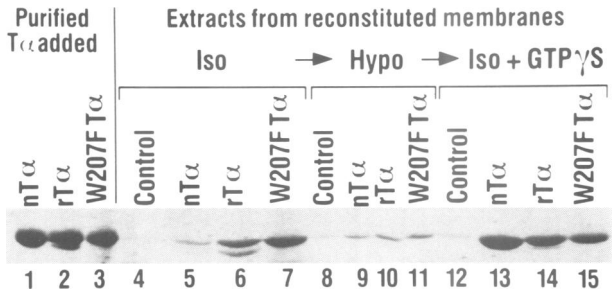

Fig. 2. High affinity binding of [W207F]TαGDP to R* and release by GTPγS. 0.75 μM of purified nTαGDP (lane 1), rTαGDP (lane 2), [W207F]TαGDP (lane 3) were incubated for 10 min at 25°C in 200 μl of isotonic buffer M (see Materials and methods) with illuminated and transducin-depleted membrane suspensions (50 μM rhodopsin) in the presence of 3.5 μM Tβγ. The membranes were then sedimented. The unbound Tα was assayed in aliquots of supernatants by SDS−polyacrylamide gel electrophoresis with Coomassie blue staining. Lane 4, control supernatant of transducin-depleted membranes; lane 5, nTα; lane 6, rTα; lane 7, [W207F]Tα. nTα was almost absent from this supernatant, it remained thus almost totally bound to the illuminated membrane. Equal amounts of [W207F]Tα and rTα (~30% of the initial amount) are detected. This slightly less efficient binding of [W207F]Tα and rTα to the illuminated membranes is probably due to the deletion of the myristoyl in the recombinants. [The purified rTα used here (lane 2) had kept a small contamination of slightly proteolysed rTα; see also Figure 3, lane 5, which shows a blot of the same preparation. This contaminant did not bind to the membrane, it remained entirely in the first supernatant, lane 6, and did not interfere with the result.] The membrane pellets were resuspended in hypotonic buffer L and sedimented. All the membrane-associated transducins remained resistant to elution in this low ionic strength buffer (Hypo supernatants, lanes 8−11). This is characteristic of high affinity binding of transducin to R* in the membrane. The pellets were then resuspended in buffer M supplemented with 100 μM GTPγS. This induced the release in solution of all the remaining R*-bound transducins (Iso + GTPγS supernatants, lanes 12−15). Thus [W207F]Tα and rTα are released from R* and become soluble upon binding GTPγS, like native transducin.

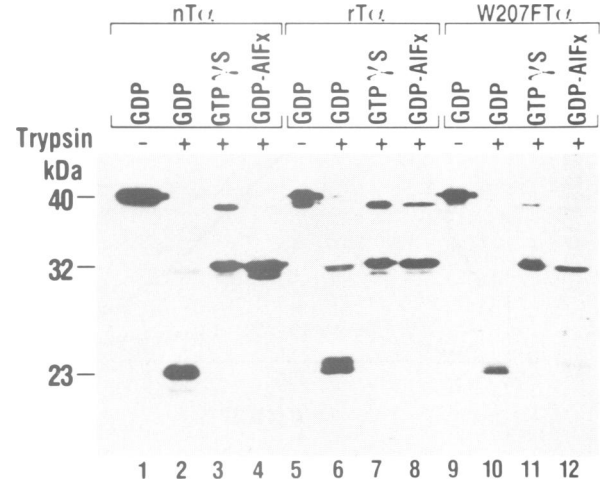

Fig. 3. Evidence from limited proteolysis for a conformation switch of [W207F]Tα upon activation by GTPγS or by AlFx. Native, recombinant and mutant transducin samples were activated either by GTPγS upon incubation with illuminated membranes, as shown in Figure 2, lanes 13−15, or were activated by AlFx after 15 min incubation with 5 mM NaF and 50 μM $AlCl_3$. All samples were incubated for 20 min at 20°C with trypsin (0.2 μg trypsin/μg transducin). Digestion was stopped by the addition of soybean trypsin inhibitor and the samples were analysed by immunoblotting with a purified anti-Tα polyclonal antibody (12% SDS−PAGE immunoblotting, 0.75 μg Tα per well). Native transducin, in the GDP-bound state, is cleaved down to a 23 kDa fragment that results from a proteolysis at the R204-K205 locus (lane 2). This proteolysis site is protected in the active conformation of nTαGTPγS and nTαGDP-AlFx, which are cleaved only down to 38 and 32 kDa fragments (lanes 3 and 4). Similar proteolytic patterns and similar GTPγS- and AlFx-dependent changes are observed for rTα (lanes 6−8) and for [W207F]Tα (lanes 10−12). Thus activation by GTPγS or by AlFx induces in [W207F]Tα the same type of conformational change as that observed in nTα.

equally well to these membranes. The binding was dependent on the presence of excess Tβγ, the illumination of the membrane and was resistant to extraction into low ionic strength buffer. This light-dependent binding was, however, slightly less efficient for the recombinant subunits than for purified native transducin nTα subunit (Figure 2). The difference is most likely due to the absence of myristoyl in the recombinant subunits, which reduces their affinity for Tβγ, hence their coupling to R*. R* catalysed the release of GDP from [W207F]Tα as well as from rTα and the nucleotide-depleted recombinant remained attached to R*, hence attached to the membrane. Upon the subsequent addition of GTP or GTPγS, the recombinant proteins were released from the illuminated membrane as well as native transducin (Figure 2). Thus the binding of GTPγS in the R*-bound transducin triggers in [W207F]Tα, as in native Tα, a change of conformation that induces the dissociation of [W207F]TαGTPγS from R* and from Tβγ, and its solubilization.

The [W207F]Tα mutant undergoes a change of conformation upon GDP/GTPγS exchange or AlFx binding

Proteolytic sensitivity of Tα to trypsin provides an assay for the inactive or active conformation of transducin (Fung and Nash, 1983; Bigay *et al.*, 1987). Native transducin in the TαGDP form is sensitive to proteolysis by trypsin at the locus R204-K205, which corresponds to locus R232-R233 in Gsα (Hurley *et al.*, 1984). This site is protected from trypsin action when the protein is kept permanently in the active conformation, with a bound GTPγS or with AlFx. This change of proteolytic sensitivity was observed upon the exchange of GDP for GTPγS in [W207F]Tα as well as in rTα and in nTα (Figure 3). This confirmed that the conformational switch that is typical of Tα activation occurred also upon nucleotide exchange in [W207F]Tα. It is noteworthy that the conformation-sensitive proteolytic site is only three three amino acids away from the mutated tryptophan. The change of proteolytic sensitivity to trypsin was also observed when [W207F]TαGDP had been incubated with AlFx in solution in the absence of rhodopsin, previous to the trypsin addition. This definitely shows that AlFx can bind in the nucleotide site of the mutant and induce the 'activation' conformational switch in [W207F]Tα as well as in native Tα.

The fluorescence increase triggered by AlFx binding in Tα is absent in [W207F]Tα

The W207F mutation modified only slightly the tryptophan fluorescence spectrum of TαGDP. The fluorescence yield of [W207F]TαGDP was decreased by 15 ± 5% and the fluorescence emission spectrum was slightly blue shifted with respect to that of rTαGDP (Figure 4A) or nTαGDP (not shown). Thus W207, which is suppressed in the mutant, must be weakly fluorescent and red shifted in Tα. This

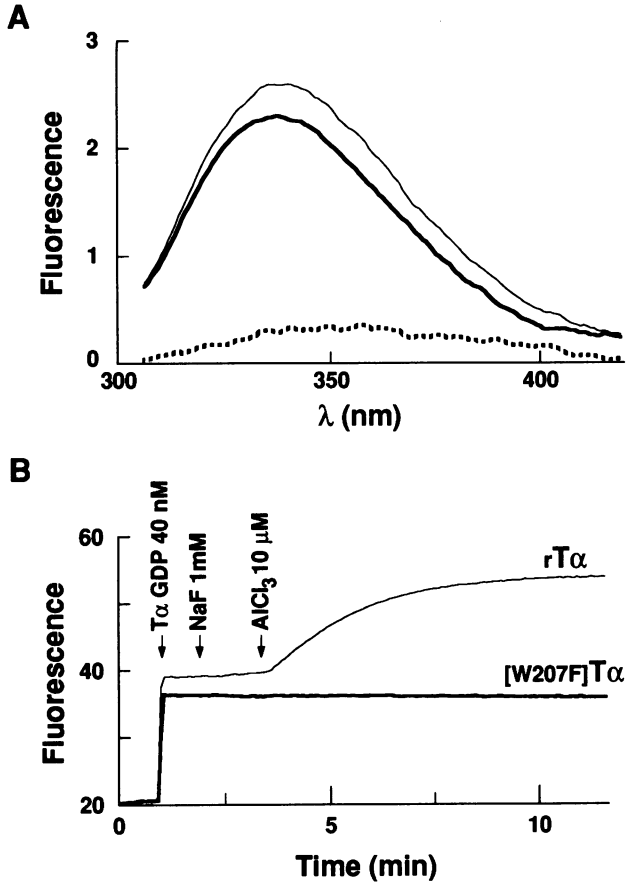

Fig. 4. Modification of the tryptophan fluorescence spectrum and suppression of the fluorescence change upon activation in [W207F]Tα. (**A**) Fluorescence emission spectra of rTαGDP (thin curve) and [W207F]TαGDP (thick curve); dotted curve, difference between rTαGDP and [W207F]TαGDP spectra. Excitation wavelength, 292 nm; excitation and emission bandwidths, 5 nm. (**B**) Fluorescence changes upon activation by fluoride complexes of 40 nM of rTαGDP or [W207F]TαGDP in 1.5 ml buffer (20 mM HEPES, pH 7,5, 120 mM KCl and 2 mM $MgCl_2$). 1 mM NaF and 10 μM $AlCl_3$ were injected as indicated. The fluorescence intensities were monitored at 340 nm (bandwith 30 nm), with excitation at 292 nm (bandwidth 5 nm). The fluorescence increase that monitors the activation of rTαGDP upon the slow binding of AlFx is totally suppressed in [W207F]TαGDP.

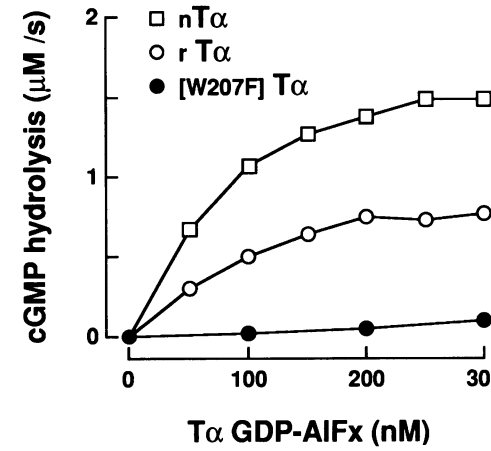

Fig. 5. Activation of PDE by native, recombinant and mutant transducins. Dose–response curves of PDE activation by nTαGDP-AlFx (□), rTαGDP-AlFx (○) [W207F]TαGDP-AlFx (●). Concentrated purified transducin preparations were pre-activated by 15 min incubation with 5 mM NaF and 50 μM $AlCl_3$. Successive injections of 50 nM pre-activated transducin were made into a pH metric cuvette containing 500 μl of reconstituted PDE (50 nM) on phospholipid vesicles (1 mg/ml) in an isotonic buffer (10 mM HEPES pH 7.5, 120 mM KCl and 2 mM $MgCl_2$) supplemented with 5 mM NaF and 50 μM $AlCl_3$. 1.5 mM cGMP was added and the PDE activity was monitored from the pH metric recording of the protons released upon the hydrolysis of cGMP. The maximal PDE activity elicited by rTα is 50% of that elicited by nTα, with comparable EC_{50} (70 nM). [W207F]Tα does not elicit a measurable PDE activity even when added at 300 nM concentration.

suggests that W207 is exposed to the solvent on the protein surface in the GDP conformation. A more dramatic effect of the mutation was the total suppression in [W207F]TαGDP of the fluorescence increase that is observed in nTαGDP or rTαGDP upon activation by GDP/GTP exchange or AlFx binding (Figure 4B). As the trypsin proteolytic test described above had confirmed that under those conditions [W207F]TαGDP bound AlFx and underwent a conformational switch, the total disappearance of a correlated fluorescence increase indicated that the fluorescence of W127, the only remaining tryptophan in [W207F]Tα, is unsensitive to this conformation switch. Thus in native Tα, W207 must be the exclusive fluorescence sensor of the conformational switch upon activation.

Upon binding AlFx and switching conformation, the [W207F]Tα mutant remains unable to activate significantly its effector, the PDE

It remained to be verified whether the W207F mutation in Tα interfered with its effector-activating power on the retinal cGMP PDE. Aliquots of native or recombinant Tα previously 'activated' by incubation with AlFx were added to suspensions of retinal extracted PDE that had been reassociated to lipid vesicles. PDE activity was measured by the pH metric method. The activity elicited by rTαGDP-AlFx was reduced by ~50% with respect to that observed for nTαGDP-AlFx (Figure 5). This slight decrease of activating efficiency of rTα is probably due to the absence of myristoyl in the recombinant protein, which may decrease its affinity for the membrane-bound PDE. But the W207F mutation induced a more drastic effect: the mutant [W207F]TαGDP-AlFx did not elicit a significant PDE activity even when added at concentrations at which native TαGDP-AlFx or rTαGDP-AlFx elicited maximal PDE activity. The point mutation apparently suppressed the effector-activating power of Tα.

[W207F]TαGDP-AlFx still binds its most specific target, the PDEγ subunit, but with a reduced affinity

The lack of PDE activation by [W207F]TαGDP-AlFx may suggest that the W207F mutation has totally suppressed the interaction between the G protein and its effector. But the activation of retinal cGMP PDE by transducin is a complex process and the stimulation of the cGMP hydrolytic activity may not be a straightforward monitor of the interaction of Tα with its specific target. TαGTPγS or TαGDP-AlFx activate the PDE via their strong binding to one of its two PDEγ inhibitory subunits, which they displace from a cGMP hydrolytic site on the catalytic complex PDEαβ. Under low ionic buffer conditions, the soluble TαGTPγS–PDEγ complex is released from the activated PDE, pin-pointing PDEγ as the most specific effector target of Tα (Deterre *et al.*, 1986, 1988). Thus the interaction of transducin with its effector can be assessed quantitatively, *in vitro*, on a simplified model by measuring the binding affinity of Tα

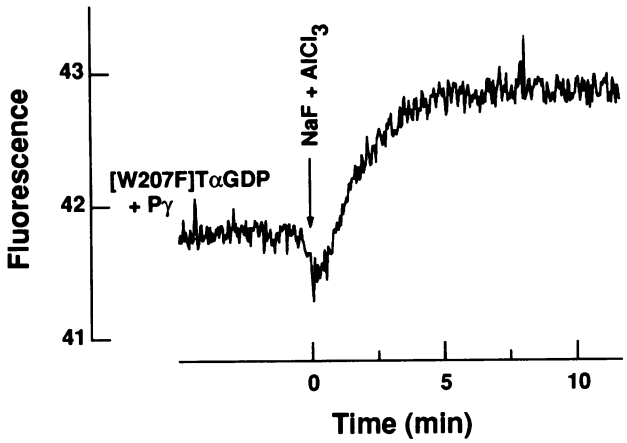

Fig. 6. Fluorescence monitoring of the binding of PDEγ to [W207F]TαGDP upon addition of AlFx. [W207F]TαGDP (50 nM) and excess PDEγ (300 nM) were mixed in 1.5 ml of buffer (20 mM HEPES pH 7.5, 120 mM KCl and 2 mM $MgCl_2$). Tryptophan fluorescence was monitored at 320 nm. At time 0, 1 mM NaF and 10 μM $AlCl_3$ were injected. The binding of PDEγ to [W207F]TαGDP-AlFx results in a 3% increase of the initial fluorescence level. The slow kinetics of the fluorescence increase is characteristic of the slow binding of AlFx in TαGDP (see Figure 4B).

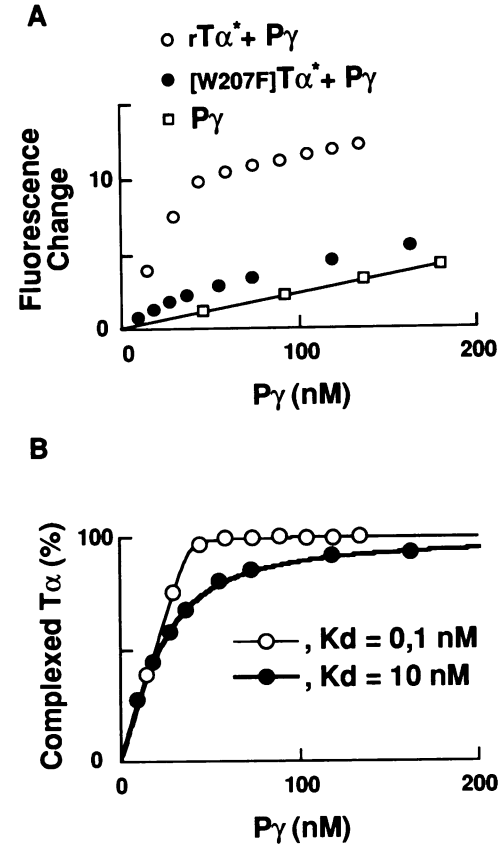

Fig. 7. Determination of the affinities of rTα and [W207F]Tα for PDEγ from fluorescence enhancements. (**A**) Fluorescence enhancements observed upon successive injections of aliquots of PDEγ in a cuvette containing 40 nM rTαGDP-AlFx (○) or 40 nM of [W207F]TαGDP-AlFx (●) in buffer supplemented with 1 mM NaF and 10 μM $AlCl_3$, and fluorescence of PDEγ alone injected in the same buffer (□). (**B**) Proportion of Tα complexed to PDEγ as computed from the fluorescence enhancements of panel A after subtracting the contribution of free PDEγ. The data are fitted assuming a simple association model Tα + PDEγ <=> Tα-PDEγ as done previously (Otto-Bruc *et al.*, 1993) and the corresponding values of dissociation constant are given.

for purified PDEγ in solution. This binding can be monitored from changes observed in the proteins' tryptophan fluorescence upon their association. This technique is very sensitive with native Tα because a large fluorescence change is correlated to its association to PDEγ. We showed recently by this technique that native transducin in the 'inactive' TαGDP form binds to recombinant PDEγ with a K_d of 3 nM (Otto-Bruc *et al.*, 1993). The K_d value decreases to 0.1 nM when transducin has switched to the 'active' TαGTPγS or TαGDP-AlFx form. The persistence of a fluorescence signal upon binding of the tryptophan-less [W70F]PDEγ mutant to native Tα indicated that part of this fluorescence change must originate from Tα and most probably from W207 in native Tα.

A small change of the proteins' tryptophan fluorescence was observable upon the addition of AlFx to a solution containing both inactive [W207F]TαGDP and recombinant PDEγ (Figure 6). This signal must be due to an interaction between the two subunits upon the binding of AlFx to [W207F]TαGDP, as no signal was observed when AlFx was added to [W207F]TαGDP alone. As the sensitive tryptophan W207 is suppressed from the mutant Tα, the small fluorescence change probably results here entirely from a perturbation on the tryptophan W70 in PDEγ, when it binds to [W207F]TαGDP-AlFx. The relatively slow kinetics of the fluorescence enhancement monitors the rate of binding of PDEγ to [W207F]TαGDP-AlFx, which is limited by the rate of binding of AlFx to [W207F]TαGDP. This rate is comparable with that observed under similar conditions for the binding of AlFx to native TαGDP (Antonny and Chabre, 1992). The binding rate of AlFx to transducin has been shown to be sensitive to perturbations of the binding site of the γ phosphate of GTP (Bornancin *et al.*, 1992). The fact that this rate is not strongly modified for the binding of AlFx to the [W207F]TαGDP mutant is an indication that the mutation has not perturbed the nucleotide site.

The affinity of [W207F]TαGDP-AlFx for PDEγ was assessed, as done previously for native Tα and PDEγ (Otto-Bruc *et al.*, 1993) by measuring the amplitudes of the fluorescence enhancements observed upon stepwise additions of PDEγ to a solution containing preactivated [W207F]TαGDP-AlFx or rTαGDP-AlFx for control (Figure 7A). The data were fitted assuming a simple association model, which yielded a K_d of 10 nM for the affinity of [W207F]TαGDP-AlFx for PDEγ, compared with a K_d of 0.1 nM for the affinity of the control rTαGDP-AlFx for PDEγ (Figure 7B). The affinity for PDEγ of the inactive [W207F]TαGDP form of the mutant was too low to be detected by this technique. Thus the W207F mutation did not suppress totally the interaction of the 'activated' form of Tα with PDEγ, but it reduced its strength by 100-fold. The 10 nM affinity for PDEγ of the 'activated' mutant [W207F]TαGDP-AlFx is indeed lower than the 3 nM affinity for PDEγ of native or recombinant TαGDP, which, in the absence of AlFx, is inactive on PDE. This accounts for the observation that [W207F]TαGDP-AlFx does not activate the PDE.

PDEγ binds to TαGDP in the proximity of W207

Isolated PDEγ binds also to native Tα, in its inactive TαGDP state, with an affinity of 3 nM (Otto-Bruc *et al.*, 1993). Using the proteolytic approach discussed above and shown in Figure 3, we observed (data not shown), that the site

R204-K205, which is very sensitive to proteolysis by trypsin in isolated TαGDP, is protected when a stoichiometric amount of PDEγ is added and forms TαGDP−PDEγ complexes. This suggests that PDEγ binds onto this proteolysis site of Tα, which is only two residues away from W207.

Discussion

Our structural and functional characterizations of the [W207F]Tα mutant demonstrate that W207 is, in native Tα, the tryptophan residue whose fluorescence changes upon the activation of the G protein. The W207F mutation totally suppressed the fluorescence change observed on native Tα upon activation by GTP or by AlFx and other assays verified that the activation process itself was not perturbed. The mutant Tα binds with high affinity to R* which catalyses a rapid GDP/GTP exchange and this triggers a conformational switch, revealed through the dissociation and solubilization of [W207F]TαGTP. With GTPγS, the conformation switch can also be detected through a change of proteolytic sensitivity, which is characteristic of transducin activation. AlFx binds in isolated [W207F]Tα, with the same kinetics as in native TαGDP and induces also the conformational switch. Thus W207 appears not to be essential for the interaction of the protein in the 'inactive' TαGDP state with Tβγ and with the receptor, nor for the capacity of the nucleotide site to exchange GDP for GTP or to bind AlFx, nor for the conformational switch upon binding GTPγS or AlFx. The conformation switch did not, however, appear to confer effector-activating potential to the mutant, which even in the GTPγS or AlFx-bound state remained unable to activate significantly the PDE. To characterize better the effector-activating interaction of the mutant, we measured its binding to PDEγ, its specific effector subunit. In its 'activated' form the mutant bound to PDEγ with the substantial affinity of 10^{-8} M. This affinity is, however, 100-fold lower than that of unmutated TαGTP for PDEγ. This suggests that the conformation switch releases the effector binding domain in the mutant, but that W207 has an important role for the binding of Tα to its effector. Independent evidence from proteolytic studies further suggests that PDEγ binds onto Tα in the proximity of W207.

Our results thus clearly identify W207 as the fluorescence sensor of the conformational switch upon the activation of Tα and suggest also that this tryptophan contributes to the binding of Tα with its effector. This residue is located, as discussed below, in a 'switch' region of Tα, close to the GTP γ-phosphate binding site, and is conserved at this locus in the α subunit of all heterotrimeric G proteins. Thus in Tα this 'switch' domain could also be an effector binding domain. This is very reminiscent of the case of the small G proteins $p21^{ras}$ whose 'switch II' domain is also involved in effector binding and activation (Polakis and McCormick, 1993).

The crystal structure of $p21^{ras}$ has already been used successfully as a model for the structure of heterotrimeric G protein α subunits (Berlot and Bourne, 1992). Figure 8A, adapted from Stouten *et al.* (1993), shows the crystal structures of $p21^{ras}$−GDP and $p21^{ras}$−GTP (Pai *et al.*, 1990; Wittinghofer and Pai, 1991) and stresses the change of conformation of the 'switch II region', near the γ-phosphate binding pocket. Sequence alignments of this region, which extends approximately from residues 55−75 in ras, with the corresponding ones in Gsα, as proposed by Bourne *et al.* (1991) and by Berlot and Bourne (1992), and with that of Tα which contains W207, are shown below (Figure 8B). Except for the well-identified γ-phosphate binding sequence 'G3' (57DTAGQE63 in ras) the sequence analogies are limited. But Valencia *et al.* (1991) have shown, from a comparison of the structures of the GTPase domain of EF-Tu and $p21^{ras}$, that limited sequence analogies suffice to conserve the structure of this region in GTP binding proteins of different families. It is thus reasonable to assume that in the proximity of the G3 region the secondary structures of the Gα chain of heterotrimeric G proteins resemble closely that of $p21^{ras}$. In this model, W207 in Tα aligns with R68 in ras, which is in the middle of the 'switch II' region, in helix α2. This helix is linked to the distal end of the very flexible loop L4 that includes the γ-phosphate binding sequence G3 responsible for triggering the switch. Stouten *et al.* (1993) have shown that upon the GDP/GTP exchange in $p21^{ras}$, helix α2 unwinds at its N-terminal end (residues 63−66), winds and extends at its C-terminal end

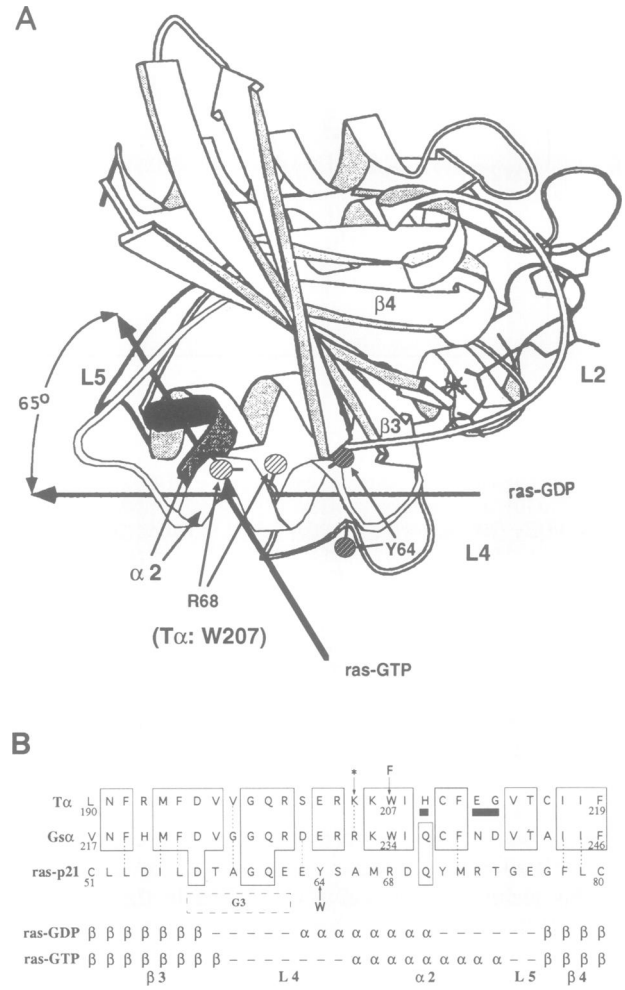

Fig. 8. (**A**) Crystal structure of $p21^{ras}$−GDP and $p21^{ras}$−GTP taken as models for the structures of transducin (adapted from Stouten *et al.*, 1993). (**B**) Alignments of the domains containing the conserved tryptophan (W234 in Gsα and W207 in Tα) with the 'switch II domain' of ras and secondary structure assignments (adapted from Berlot and Bourne, 1992).

(residues 71–74) and rotates about its own helical axis, and this axis rotates by 65°. The local conformation of the polypeptide chain of $p21^{ras}$ around R68 and the orientation of this residue, the equivalent of W207 in Tα, are thus considerably modified upon ras activation. When a tryptophan was introduced nearby in the sequence of $p21^{ras}$, by mutating the tyrosine Y64, the fluorescence of this tryptophan was highly sensitive to the activation state of $p21^{ras}$ (Antonny *et al.*, 1991). It thus does not come as a surprise that the fluorescence of W207 is sensitive to the conformation change upon transducin activation by GDP/GTP exchange or binding of AlFx. The total disappearance of the fluorescence change in the $^{[W207F]}$Tα mutant suggests indeed that W207 is the one and only fluorescence sensor, provided that the dissociation and solubilization of $^{[W207F]}$TαGTPγS and the limited proteolysis experiments had verified that this was not simply due to a misfunction of the switching process in the mutant. Although the activation-dependent proteolytic site is located in the switch II domain, between R204 and K205 (Figure 8B) and is only three residues away from W207, neither the sensitivity of this site to trypsin in the GDP-bound state nor its protection in the GTPγS-bound state were affected by the mutation. This provides further evidence that the mutation did not perturb extensively the conformation of the switch II domain in the GDP state, nor its refolding upon activation by GTPγS or AlFx.

A major effector-activating region of transducin is in the subterminal domain 293–314 (Rarick *et al.*, 1992), which coincides with one of the effector-activating regions of Gsα. Berlot and Bourne (1992) have presented evidence that the putative helix α2 of the switch II domain of Gsα is also on the surface of the protein that contacts the effector. By a scanning mutagenesis approach they have identified Q236, N239 and D240 (Figure 8B, black bars) in the C-terminal end of the helix α2, as residues that determine the specificity of the interaction of Gsα with its effector, adenylyl cyclase. Those are the only residues of helix α2 in Gsα that are not conserved in all Gα subunits. The equivalent residues in Tα are H209, E213 and G214. Other residues of helix α2 might also contribute to the effector contact, but their conservation precluded that they provide specificity in effector recognition, hence they could not be detected by the scanning mutagenesis approach. We suggest that the conserved tryptophan located in the middle of the helix α2, W234 in Gsα and W207 in Tα, which is only two residues away from the effector specific residue H209, contributes also to the effector binding. Replacing this tryptophan by a phenylalanine diminished by 100-fold the affinity of Tα for its effector, PDEγ. This mutation preserves and even increases the hydrophobicity of the residue. The loss of affinity corresponds to a decrease of ~3 kcal/mol of the effector binding energy, which is the average energy of a hydrogen bond in a protein–protein contact. The nitrogen indole ring of tryptophan is a hydrogen donor group. We may thus speculate that the conserved tryptophan in the switch II domain of Tα contributes to the binding of its effector PDEγ by its hydrogen bonding capacity rather than by its hydrophobicity. It may be noteworthy that the equivalent residue in the aligned sequence of the switch II domain of $p21^{ras}$, is an arginine, R68, which is also exclusively a hydrogen donor group, and is very well-conserved in the ras family.

Materials and methods

Construction of recombinant baculoviruses

The synthetic gene for Tα (Sakmar and Khorana, 1988) was kindly provided by T.Sakmar (MIT, Cambridge, MA). It was subcloned in an M13 mp18 vector using the *Eco*RI and *Hin*dIII restriction sites. In order to abolish the N-terminal myristoylation of rTα, the single-stranded template (M13-Tα) was mutagenized to replace the $_2GAGA_5$ sequence by an isoleucine residue. The N-terminal sequence of Tα, MGAGASAE, was thus changed to MISAE.

Site-directed mutagenesis of the tryptophan (TGG) at position 207 into phenylalanine (TTC) was performed by PCR amplification. The 180 bp restriction fragment *Msc*I–*Spe*I of Tα was amplified using a 5′ primer containing the mutation and recloned in the N-mutated Tα synthetic gene. The DNA sequence of this mutant was confirmed by DNA sequencing over the PCR-amplified fragment.

The *Eco*RI–*Hin*dIII fragment of both N-mutated Tα and $^{[W207F]}$Tα were subcloned in the baculovirus transfer vector pVL 1392 into *Eco*RI and *Bam*HI sites, after Klenow polymerase refilling of the noncompatible ends.

Recombinant baculoviruses were generated by cotransfection of Sf9 cells with the expression vectors described above and with BaculoGold linearized baculovirus DNA (Pharmingen, San Diego, CA) by the lipofection method (Kitts *et al.*, 1990). The BaculoGold linearized baculovirus DNA contains a lethal deletion. Recombination with the transfer vector complements the deletion and provides viable virus particles. This selection allows pure recombinants to be obtained after one plaque assay by picking virus plaques as described by Summers and Smith (1987). Recombinant protein expression was verified by SDS–PAGE of soluble and insoluble cell extracts after protein staining with Coomassie blue and by immunoblotting.

Production of recombinant Tα proteins

Cells were first infected at high density (10^7 cells/ml) for 1 h at 27°C at a multiplicity of infection of 5 p.f.u./cell. Infected cells were then seeded in a 1 l spinner Magna-flex (Wheaton) at a density of 10^6 cells/ml in TC100 medium supplemented with 10% heat-inactivated FCS, 0.33% lactalbumin hydrolysate and 1% pluronic acid. For correct oxygenation of infected cells, a maximal volume of 400 ml of culture medium was seeded per 1 l spinner. After 48 h of infection, cells were harvested and washed twice with phosphate buffered saline (PBS). The pellet of cells was frozen and kept at −70°C.

Purification of recombinant Tα

All purification steps were carried out at 4°C. All buffers and solutions contained 5 mM β-mercaptoethanol and 100 μM PMSF. Frozen cells were thawed in an ice-cold buffer containing 20 mM Tris–HCl pH 7.5, 2 mM $MgCl_2$, 50 μM GDP, 2 μg/ml aprotinin, 1 μg/ml pepstatin and 1 mM iodoacetamide. The cell suspension was homogenized with a cell disrupter (Vibra cell 72423, Bioblock Scientific) set at a potency of 5 W. The resultant homogenate was centrifuged for 20 min at 400 000 *g*. The supernatant was filtered (0.22 μm) and loaded onto a Pharmacia Blue Sepharose HiTrap (1 ml) at a flow rate of 1 ml/min using a Pharmacia FPLC system. The column was washed with 20 mM Tris–HCl pH 7.5, 2 mM $MgCl_2$ and 120 mM KCl, and eluted with a 120–600 mM KCl linear gradient (16 mM/ml) at 1 ml/min. rTα eluted between 240 and 360 mM KCl. The fractions containing rTα were pooled and concentrated to 200 μl on a Centricon 30 microconcentrator (Amicon). The concentrated proteins were loaded on a gel filtration column (Superose 12 HR 16/30 Pharmacia-LKB). The column was eluted at 0.5 ml/min with 20 mM Tris–HCl pH 7.5, 120 mM NaCl and 2 mM $MgCl_2$. Fractions containing rTα were pooled and diluted five times in 20 mM Tris–HCl pH 7.5, 2 mM $MgSO_4$ and applied to a Pharmacia Polyanion SI column HR 5/5 equilibrated in the same buffer. The recombinant protein was purified to near homogeneity by elution with a 0–300 mM Na_2SO_4 gradient (8.8 mM/min) at a flow rate of 0.5 ml/min and recovery at 120 mM Na_2SO_4. From each litre of culture, we routinely obtained 100 μg of purified rTα. Purity was near 90% as judged by Coomassie-stained SDS–polyacrylamide gels.

Extraction of proteins from retinal rod outer segments

Native TαGDP, Tβγ and PDE were extracted from illuminated ROS membranes according to their sensitivity to light and ionic strength (Kühn, 1980). Illuminated ROS were washed sequentially with an isotonic buffer M (20 mM Tris–HCl pH 7.5, 120 mM KCl and 100 μM $MgCl_2$), a hypotonic buffer L (5 mM Tris–HCl pH 7.5, 100 μM $MgCl_2$) and buffer M supplemented with 200 μM GTP. The first isotonic supernatants contained unidentified soluble proteins, the hypotonic supernatants contained PDE and the supernatants in buffer M supplemented with GTP contained Tα that had been eluted in the GTP-bound form and had later hydrolysed its bound

GTP. Using this procedure, extraction of ROS membranes at 100 μM rhodopsin yielded ~1 μM PDE, 3 μM Tα and 1.5 μM Tβγ with >80% purity. Tα and Tβγ were then purified on a Polyanion SI column (Pharmacia-LKB, Uppsala, Sweden) with 0.66 M Na_2SO_4 and 0.66 M $MgCl_2$ linear gradients, respectively, as previously described by Deterre *et al.* (1986). Protein concentrations were determined with a standard Bradford assay (Pierce, Rockord, IL). Recombinant PDEγ was obtained by expression in *E.coli*, as described previously by Otto-Bruc *et al.* (1993).

Reconstitution of Tα on illuminated membranes and solubilization

Illuminated membranes were washed successively with buffers M, L and L supplemented with 100 μM GTPγS, to extract all the endogenous transducin and PDE. Attachment of 0.75 μM of recombinants or native TαGDP to 50 μM R* was performed in the presence of 3.5 μM of purified Tβγ for 10 min at 20°C in buffer M. Membranes were then washed with buffer M to eliminate excess Tβγ. The high affinity R*-Tα binding was assayed by washing membranes with buffer L. Activation of Tα was monitored through its solubilization from membranes suspension in buffer M supplemented with 100 μM GTPγS. After sedimentation of the membrane pellets, the proteins in the supernatants were analysed by SDS–PAGE with 12% acrylamide.

Trypsin proteolysis assay

Native or recombinant transducins were incubated at 20°C in a low salt buffer (20 mM HEPES, pH 7.5 and 2 mM $MgCl_2$) for 20 min with TPCK (L-1-tosilamido-2-phenylethyl chloromethyl ketone)-treated trypsin from Sigma (0.2 μg trypsin/μg transducin). Digestion was stopped by the addition of soybean trypsin inhibitor (from Sigma, 5 μg/μg trypsin). Tryptic fragments were analysed by immunoblotting with a purified anti-Tα polyclonal antibody.

Fluorescence measurements

Intrinsic fluorescence of proteins was measured with a Shimadzu RF 5000 fluorimeter using a 1 × 1 cm quartz cuvette. The sample was vigorously stirred and thermostated at 25°C. The buffer contained 20 mM HEPES pH 7.5, 120 mM KCl, 2 mM $MgCl_2$ and 1 mM DTT. For emission spectra, the excitation was at 292 nm and the emission scan speed was 0.8 nm/s; the bandwidths were 5 nm for both excitation and emission. Time scan recordings were performed with excitation at 292 nm (5 nm bandwidth), emission at either 320 nm or 340 nm (bandwidth 30 nm). Fluorescence determination of Tα-PDEγ dissociation constant was done as described before (Otto-Bruc *et al.*, 1993). Stepwise additions of PDEγ were made to solutions containing 40 nM of purified rTα or $^{[W207F]}$Tα while the fluorescence was continuously recorded. As a control, the same stepwise additions of PDEγ were made to buffer alone.

PDE activation assay

Large unilamellar vesicles were prepared according to Szoda and Papahadjopoulos (1978). 10 mg of azolectin were dissolved in 3 ml diethyl ether and mixed with 500 μl of aqueous buffer A (10 mM HEPES pH 7.5, 120 mM KCl and 2 mM $MgCl_2$). The mixture was sonicated for 1–2 min at 0°C in a bath-type sonicator. The solvent was removed with a rotatory evaporator and the resulting aqueous suspension of vesicles was centrifuged and resuspended at 20 mg/ml in buffer A. Retinal extracted PDE (50 nM final concentration) was incubated for 5 min at 25°C in 500 μl buffer A containing 1 mg/ml of lipid vesicles, before the addition of cGMP and preactivated Tα. The pH variation due to the cGMP hydrolysis was recorded with a combined pH microelectrode (model U-402-M3-S7, Ingold Messtechnik GmbH, Steinbach/Ts, Germany). The PDE activity was deduced from the slope of the pH recordings.

Acknowledgements

We thank Dr P.Fafournoux for helpful advice on the construction of the baculovirus vector, Dr T.Sakmar for providing the synthetic gene for Tα, Dr B.Fung for providing the cDNA, Drs B.Antonny, J.Bigay and T.M. Vuong for helpful discussions and comments and I.Lenoir for efficient technical support. E.Faurobert was recipient of grants from the Ministère de la Recherche et de la Technologie (MRT) and from the Association pour la Recherche sur le Cancer (ARC). This work was supported by grants from INSERM (contract 910502), from ARC (contract 6103) and from the Human Frontier Science Program Organization.

References

Antonny,B. and Chabre,M. (1992) *J. Biol. Chem.*, **267**, 6710–6718.
Antonny,B., Chardin,P., Roux,M. and Chabre,M. (1991) *Biochemistry*, **30**, 8287–8295.
Antonny,B., Sukumar,M., Bigay,J., Chabre,M. and Higashijima,T. (1993) *J. Biol. Chem.*, **268**, 2393–2402.
Berlot,C.H. and Bourne,H.R. (1992) *Cell*, **68**, 911–922.
Bigay,J., Deterre,Ph., Pfister,C. and Chabre,M. (1987) *EMBO J.*, **6**, 2907–2913.
Bornancin,F., Franco,M., Bigay,J. and Chabre,M. (1992) *Eur. J. Biochem.*, **210**, 33–44.
Bourne,H.R., Sanders,D.A. and McCormick,F. (1991) *Nature*, **349**, 117–127.
Chabre,M. and Deterre,P. (1989) *Eur. J. Biochem.*, **179**, 255–266.
Conklin,B.R. and Bourne,H.R. (1993) *Cell*, **73**, 631–641.
Deterre,Ph., Bigay,J., Robert,M., Pfister,C., Kühn,H. and Chabre,M. (1986) *Prot. Struct. Funct. Genet.*, **1**, 188–193.
Deterre,Ph., Bigay,J., Forquet,F., Robert,M. and Chabre,M. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 2424–2428.
Fung,B.K.K. and Nash,C.R. (1983) *J. Biol. Chem.*, **258**, 10503–10510.
Hamm,H.E., Deretic,D., Arendt,A., Hargrave,P.A., Koenig,B. and Hofmann,K.P. (1988) *Science*, **241**, 832–835.
Higashijima,T., Fergusson,K.M., Sternweis,P.C., Ross,E.M., Smigel,M.D. and Gilman,A.G. (1987) *J. Biol. Chem.*, **262**, 752–756.
Higashijima,T., Graziano,M.P., Suga,H., Kainosho,M. and Gilman,A.G. (1991) *J. Biol. Chem.*, **266**, 3396–3401.
Hurley,J.B., Simon,M.I., Teplow,D.B., Robishaw,J.D. and Gilman,A.G. (1984) *Science*, **226**, 860–862.
Jones,T.L., Simonds,W.F., Merendino,J.J., Brann,M.R. and Spiegel,A.M. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 568–572.
Journot,L., Pantaloni,C., Bockaert,J. and Audigier,Y. (1991) *J. Biol. Chem.*, **266**, 9009–9015.
Kitts,P.A., Ayres,M.D. and Possee,R.D. (1990) *Nucleic Acids Res.*, **18**, 5667–5672.
Kühn,H. (1980) *Nature*, **283**, 587–589.
Linder,M.E., Pang,I.-H., Duronio,R.J., Gordon,J.I., Sternweis,P.C. and Gilman,A.G. (1991) *J. Biol. Chem.*, **266**, 4654–4659.
Masters,S.B., Stroud,R.M. and Bourne,H.R. (1986) *Protein Engng*, **1**, 47–54.
Medynski,D.C., Sullivan,K., Smith,D., Van Dop,C., Chang,F.H., Fung,B.K.K., Seeburg,P.H. and Bourne,H.R. (1985) *Proc. Natl Acad. Sci. USA*, **82**, 4311–4315.
Milburn,M.V., Tong,L., DeVos,A.M., Brünger,A., Yamaizumi,Z., Nishimura,S. and Kim,S.-H. (1990) *Science*, **247**, 939–945.
Mumby,S.M., Heukeroth,R.O., Gordon,J.I. and Gilman,A.G. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 728–732.
Neubert,T.A., Johnson,R.S., Hurley,J.B. and Walsh,K.A. (1992) *J. Biol. Chem.*, **267**, 1874–1877.
Otto-Bruc,A., Antonny,B., Vuong,T.M., Chardin,P. and Chabre,M. (1993) *Biochemistry*, in press.
Pai,E.F., Krengel,U., Petsko,G.A., Goody,R.S., Kabsch,W. and Wittinghofer,A. (1990) *EMBO J.*, **9**, 2351–2359.
Philipps,W.J. and Cerione,R.A. (1988) *J. Biol. Chem.*, **263**, 15498–15505.
Polakis,P. and McCormick,F. (1993) *J. Biol. Chem.*, **268**, 9157–9160.
Rarick,H.M., Artemyev,N.O. and Hamm,H.E. (1992) *Science*, **256**, 1031–1033.
Sakmar,T.P. and Khorana,H.G. (1988) *Nucleic Acids Res.*, **16**, 6361–6372.
Stouten,P.F.W., Sander,C., Wittinghofer,A. and Valencia,A. (1993) *FEBS Lett.*, **320**, 1–6.
Stryer,L. and Bourne,H.R. (1986) *Annu. Rev. Cell Biol.*, **2**, 391–419.
Summers,M.D. and Smith,E.G. (1987) *Tex. Agric. Exp. Stat. Bull.*, **1555**.
Szoda,F. and Papahadjopoulos,D. (1978) *Proc. Natl Acad. Sci. USA*, **75**, 4194–4198.
Valencia,A., Kjeldgaard,M., Pai,E.F. and Sander,C. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 5443–5447.
Wittinghofer,A. and Pai,E.F. (1991) *Trends Biochem. Sci.*, **16**, 382–387.

Received on July 1, 1993