Characterization of a Goα Mutant That Binds Xanthine Nucleotides*

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Bo Yu, Vladlen Z. Slepak†, and Melvin I. Simon§

From the Division of Biology, California Institute of Technology, Pasadena, California 91125

Several GTP binding proteins, including EF-Tu, Ypt1, rab-5, and FtsY, and adenylsuccinate synthetase have been reported to bind xanthine nucleotides when the conserved aspartate residue in the NKXD motif was changed to asparagine. However, the corresponding single Goa mutant protein (D273N) did not bind either xanthine nucleotides or guanine nucleotides. Interestingly, the introduction of a second mutation to generate the Goa subunit D273N/Q205L switched nucleotide binding specificity to xanthine nucleotide. The double mutant protein GoaD273N/Q205L (GoaX) bound xanthine triphosphate, but not guanine triphosphate. Recombinant GoaX (GodaD273N/Q205L) formed heterotrimers with βγ complexes only in the presence of xanthine diphosphate (XDP),1 and the binding to βγ was inhibited by xanthine triphosphate (XTP). Furthermore, as a result of binding to XTP, the Goa protein underwent a conformational change similar to that of the activated wild-type Goa. In transfected COS-7 cells, we demonstrate that the interaction between GoaX and βγ occurred only when cell membranes were permeabilized to allow the uptake of xanthine diphosphate. This is the first example of a switch in nucleotide binding specificity from guanine to xanthine nucleotides in a heterotrimeric G protein α subunit.

Materials and Methods

Mutagenesis and Expression of the Goa—Myristoylated recombinant mouse Goα was expressed in Escherichia coli. Conditions for growth, induction, and lysis of the Goa-expressing cells were described previously (24). The D273N mutation was introduced in both wild-type Goa and the activated mutant GoaQ205L by oligonucleotide-directed mutagenesis. The oligonucleotide TTTCGAACAGAGTATGATTG-GCGGAGATAAGAGTC was annealed to uracil-containing single-stranded DNA from the plasmids pGoa and pGoaQ205L. The resulting vectors were designated as pGoaD273N and pGoaX.

Expression and Purification of His-tagged Goa—We subcloned wild type and mutant Goa cDNAs into the E. coli expression vector pET15b (Novagen), which added a peptide of 20 amino acids MGSSHHHHHSSGLVPRGSAGLTVAGV. The resulting vectors were designated as pGoaD273N and pGoaX.

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Characterization of a Goa Mutant

The His6-tagged protein was eluted with a gradient of imidazole concentration (5–500 mM). The Goa and various mutant proteins eluted at about 250 mM imidazole. Proteins were then transferred to TED buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT) with 0.1 mM MgCl2 and 0.1 mM nucleotide diphosphate (GDP or XDP as appropriate) by gel filtration. Purified proteins were stored in 50% glycerol at -20 °C.

Synthesis of XTP—XTP was synthesized from XDP and ATP with nucleotidase-phosphohexose kinase (NDK) as described previously (25). To produce [35S]-labeled XTP, the reaction contained 10 μM XDP, 1 μM [35S]ATP, and 10 units NDK (Sigma) in 100 μl of NDK buffer (1 mM MgCl2, 5 mM DTT, 20 mM Tris-HCl, pH 8.0). The mixture was incubated at room temperature for 2 h. The resulting concentration of [35S]XTP was about 1 μCi (1 μCi/ml). The radiochemical purity of XTP was monitored by thin layer chromatography on Avicel/DEAE plates (Analtech) in 0.07 N HCl.

Nucleotide Binding—Binding of [35S]GTPγS and [35S]XTPγS to the recombinant Goa and the mutant proteins was performed as described (24). The binding reaction contained 0.5 μg of purified protein or 200 μg of crude E. coli protein in TED buffer with 0.1 mM MgCl2, 1 μM ATP, and 0.1 μM GTPγS or XTPγS (20,000 cpm/ml). For the time course experiments, 20-μl aliquots were withdrawn from a 200-μl reaction, diluted 10-fold with ice-cold TED buffer containing 0.1 mM MgCl2, filtered through a 0.45-μm nitrocellulose filter, washed, and dried. The amount of bound radioactivity was determined by scintillation counting.

Proteolysis with Trypsin—Approximately 0.1 μg of purified recombinant Goa was preincubated with nucleotide at room temperature for 30 min in the TED buffer. 10 ng of trypsin was then added to the mixture, and the reaction was terminated after 10 min by addition of an equal volume of 2 × SDS-PAGE sample buffer. Samples were resolved on SDS-PAGE. Gels were stained with Coomassie Blue, dried, and exposed to x-ray film.

Cell Culture and Transfection—COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. 10^6 cells/well were seeded in 12-well plates 1 day before transfection. All transfection assays contained a total amount of 1 μg of DNA; the plasmid pcIS encoding β-galactosidase was used to maintain a constant amount of DNA. To each well, 1 μg of DNA was mixed with 5 μl of lipofectamine (Life Technologies, Inc.) in 0.5 ml of Opti-MEM (Life Technologies, Inc.), and five hours later, 0.5 ml of 20% fetal calf serum in Dulbecco’s modified Eagle’s medium was added to the cells. After 48 h, cells were assayed for inositol phosphate levels as described previously (26, 27).

Permeabilization of COS-7 Cell Membranes—Transfected COS-7 cells were washed twice with phosphate-buffered saline and incubated in 200 μl of permeabilization solution consisting of 115 mM KCl, 15 mM NaCl, 0.5 mM MgCl2, 20 mM Hepes-NaOH, pH 7, 1 mM EDTA, 100 μM ATP, 0.37 mM CaCl2 (to give a free Ca2+ concentration of 100 mM), and 200 units/ml a-toxin with or without 0.1 μM XDP for 10 min at 37 °C. Then 2 μl of 1 M LiCl was added before the inositol phosphate assay.

RESULTS

To change the binding specificity of Goa from guanine nucleotides to xanthine nucleotides, we replaced Asp-273 by an asparagine residue, which was expected on the basis of structural analysis to coordinate with xanthine instead of guanine (Fig. 1b). This mutation was introduced into both the wild-type Goa subunit and the GTPase-deficient Goa mutant (Q205L). We chose Goa because myristoylated Goa can be expressed in E. coli, and it has been shown that many of the characteristics of the recombinant Goa protein are similar to those of the protein isolated from brain. To further characterize the function of XTP-bound Goa mutants, we purified these proteins in the form of non-myristoylated His6-tagged Goa by affinity chromatography on a Ni2+-NTA column. It has been shown that the non-myristoylated form of Goa has identical nucleotide binding properties compared with the myristoylated form, and it also forms trimeric complexes with βγ subunits although the affinity to βγ is much less than the myristoylated form (44).

Binding of GTPγS and XTPγS—The nucleotide binding of Goa, GoaD273N, and GoaX (GoaD273N/Q205L) was assayed with [35S]GTPγS and [35S]XTPγS. In E. coli crude extracts, Goa reached maximum binding of GTPγS in about 30 min (Fig. 2a). As expected, Goa showed no affinity for XTPγS. However, GoaX revealed a switch in nucleotide specificity. As shown in Fig. 2b, GoaX had high affinity for XTPγS but not for GTPγS. Interestingly, only the double mutant was active while GoaD273N did not bind either GTPγS or XTPγS (data not shown). Goa binds GTPγS very tightly in the presence of 1 μM Mg2+ (28, 29). Both Goa (Fig. 2c) and GoaX (Fig. 2d) did not exchange bound [35S]XTPγS when excess non-radioactive nucleotides were subsequently added.

The purified His6-tagged proteins in general retained the properties of the untagged myristoylated α subunits. However, we detected some differences in nucleotide binding. His6-tagged Goa or GoaX bound GTPγS or XTPγS, respectively, but the binding was less stable than with the untagged myristoylated protein. In the case of His6-tagged Goa, the bound GTPγS could be exchanged after excess non-radioactive GTPγS was added (Fig. 2c). Similar behavior was observed in the XTPγS binding of pure His6-tagged GoaX, which also showed distinct nucleotide exchange after non-radioactive XDP or XTP were added to the binding reaction (Fig. 2d). The decrease in nucleotide affinity was apparently the result of the presence of the His6-tag. Although the nucleotide binding of His6-tagged proteins was less stable, the specificity of binding was clearly maintained, and the mutant bound the xanthine nucleotides rather than the guanine nucleotides. As expected, the purified single mutant GoaD273N did not show any nucleotide binding activity (data not shown).

Activation Conformational Change as Assessed by Limited Proteolysis—Guanine nucleotides protect G protein α subunits, including Goa, from complete proteolytic degradation (30–32). The pattern of fragments derived from partial trypsic digestion can be used as an indicator of the conformation of the protein. In the presence of GDP, Goa is hydrolyzed by trypsin resulting in two products, a stable 25-kDa and an unstable 17-kDa peptide. Binding of non-hydrolyzable analogs of GTP can induce an active conformation of the Goa subunit, which is resistant to proteolytic degradation, and protects a stable 37-kDa polypeptide from further degradation. In the case of the activated mutant GoaQ205L, GTP can also protect the remaining 37-kDa polypeptide from complete proteolytic digestion by trypsin because GoaQ205L lacks GTPase activity. Fig. 3a shows that XTP protects GoaX from proteolysis by trypsin (lanes 4 and 5), whereas in the control experiment, GTPγS protected wild-type Goa (lane 8). This experiment indicates that GoaX binds XTP without hydrolyzing it. After binding to XTP, GoaX must have assumed a conformation similar to that of GTPγS-bound wild-type Goa. In this experiment, wild-type
Goa needed only 1 μM GTPγS to prevent complete proteolysis. Similarly, Goa was sufficiently protected in the presence of 1 μM XTP. It is noteworthy that GTPγS, but not GTP, was also able to protect Goa from complete tryptic digestion although this protection required GTPγS concentrations above 100 μM (lanes 1, 2, and 3). Thus, Goa has a much lower affinity for GTPγS than for XTP. We did not detect any of GTPγS binding activity of Goa in our nucleotide binding assay because the highest concentrations of [35S]GTPγS used in the reaction were micromolar. Consistent with the results of the nucleotide binding experiments, the single mutant GoaD273N was not protected by any nucleotides including GTP, GTPγS, and XTP up to millimolar concentrations (data not shown).

Pertussis Toxin-induced ADP-ribosylation—The interaction of Goa with the βγ complex can be assayed by ADP-ribosylation of the α subunit induced by pertussis toxin (PTX) because ADP-ribosylation requires the formation of the heterotrimeric complex(33, 34). Modification (by ADP-ribosylation) of recombinant Goa catalyzed by PTX is the same in the presence of GTP or GDP because of the GTPase activity of Goa. However, GTPγS strongly inhibits the modification since Goa cannot hydrolyze GTPγS. GTPγS binding thus promotes the dissociation of the trimeric αβγ complex and prevents the ADP-ribosylation of the Goa subunit. The activated GoaQ205L mutant lacks GTPase activity, and the effect of GTP on ADP-ribosylation is similar to that of GTPγS on the wild-type Goa. Therefore, PTX labeling can be used not only to examine βγ binding but also GTPase activity. Fig. 3b shows that purified Goa was ADP-ribosylated by pertussis toxin (lane 7), and the labeling was strongly inhibited by GTPγS (lane 6). In contrast, Goa was modified by pertussis toxin only in the presence of XDP (lane 4) but not with GDP (lane 5), and as expected, the reaction was strongly inhibited by XTP (lane 2), whereas GTP had no effect (lane 3). Therefore, only XDP-bound Goa can form trimeric complexes with βγ, and binding of XTP induces dissociation of the trimeric complex. As a control, we did not detect any ADP-ribosylation of Goa when GTPγS, GTP, or XTP alone was present (data not shown). Consistent with the results of trypsin digestion, this experiment indicated that XTP was not hydrolyzed by GoaX. The quantitation of [32P]ADP-ribose incorporation revealed that the labeling of GoaX was proportional to the amount of βγ used and reached a maximum at a GoaX:βγ ratio of 1:1, similar to wild-type Goa (data not shown). Interestingly, high concentrations (over 100 μM) of GTPγS also inhibited the ADP-ribosylation of GoaX (Fig. 3b, lane 1), offering further evidence that GoaX was able to bind GTPγS with low affinity. As expected, GoaD273N did not interact with βγ and was not modified by pertussis toxin in the presence of either GDP or XDP (data not shown).

XDP-dependent βγ Interaction in Transfected COS-7 Cells—In transfected COS-7 cells, β1γ2 is able to activate PLCγ2, and the activation of PLCγ2 can be inhibited by cotransfection with Goa because of competition for βγ (35). We cotransfected COS-7 cells with PLCγ2, β1γ2, and GoaD273N or GoaX and found that both Goa mutants did not inhibit PLCγ2 activity, whereas wild-type Goa did. This experiment indicates that both mutants do not bind βγ in COS-7 cells and is consistent with the in vitro experiments on PTX-induced ADP-ribosylation. GoaX bound βγ only in the presence of XDP, and because XDP concentration is negligible inside the cell, the
interaction did not occur. To deliver XDP into cells, we tried to permeabilize COS-7 cells by several methods including digitonin treatment, electroporation, and α-toxin (36). We found that only α-toxin gave us consistent results and had no effect on the PLCβ2 activities stimulated by bg. After incubating cells with α-toxin in the presence of XDP, we found that GoaX inhibited PLCβ2 activity, whereas GoaD273N was not affected by XDP (Fig. 4). In the control experiments, we found that adding GDP or GTP to the permeabilization buffer had no effect on the PLCβ2 activity of cells transfected with the Goaα mutants (data not shown). This experiment shows that the Goaα mutants behave similarly in vitro and in cultured cells; GoaX binds βγ only when exogenous XDP is available.

**DISCUSSION**

We engineered a mutant of Goa that switched nucleotide binding activity from guanine nucleotides to xanthine nucleotides. The mutation (D273N) was at a conserved residue of the NKXD motif that appears in all GTPase superfamily proteins. Crystal structures of transducin and Gi showed that this aspartic acid residue participated in hydrogen bonding to the guanine ring (Fig. 1a). The proposed interaction between the mutantized Asn and the xanthine ring is shown in Fig. 1b in which the hydrogen bond is “flipped” when compared with wild-type Goa. Similar single Asp→Asn mutations have been made in other GTP binding proteins, including EF-Tu (17, 18), Ypt1 (19), rab-5 (20, 21), and FtsY (22), and E. coli adenylsuccinate synthetase (23), resulting in active proteins regulated by xanthine nucleotides instead of guanine nucleotides. However, the similar D119N mutant of H-Ras induced transformation of NIH-3T3 cells with efficiency indistinguishable from wild-type H-Ras (16, 37). Although the mutant D119N Ras exhibited decreased affinity for GTP and increased affinity for XTP (by 2 to 3 orders of magnitude), the high intracellular concentration of XTP (millimolar) probably ensures that the protein is still bound to the guanine nucleotides in the cell. Interestingly, we found the corresponding D273N mutation in Goa did not result in binding of either GTPγS or XTPγS, whereas the D273N/Q205L double mutant, GoaX, switched nucleotide binding ability. When examining the crystal structure of transducin, it is not clear why the

**FIG. 3. Functional Regulation of Goa by Xanthine Nucleotides.** a, XTP protects the proteolysis of GoaX with trypsin. 0.1 μg of purified recombinant Goa or GoaX was incubated with indicated nucleotides at room temperature for 30 min. 10 ng of trypsin was then added to the mixture, and the reaction was terminated by addition of an equal volume of 2 × SDS-PAGE sample buffer. The proteolytic pattern was visualized by Western blot using an antibody against a C-terminal peptide of Goa. b, PTX-induced ADP-ribosylation of GoaX requires XDP and is inhibited by XTP. 0.1 μg of purified recombinant Goa or GoaX was mixed with 0.1 μg of purified bovine retinal βγ complex in the presence of indicated nucleotides (100 μM each, including the carry-over GDP or XDP from the protein storage buffer) and incubated for 10 min at room temperature. Then the reaction mixture containing 10 μg/ml pertussis toxin, 0.5 μM [32P]NAD (20,000 cpm/pmol), and other necessary components were added. Reactions were incubated for 30 min at room temperature and terminated by the addition of 10 μl of 5 × SDS-PAGE sample buffer. The samples were then resolved on a 10% SDS-polyacrylamide gel and visualized by autoradiography. The arrows indicate the positions of molecular mass markers.
Gln→Leu mutation (position 200 in transducin α), which is at the opposite side of the nucleotide binding pocket from the Asp→Asn mutation (position 268 in transducin α), rescued the xanthine nucleotide binding of GoaD273N. It is interesting to note that GoαX binds GTPγS at concentrations higher than 100 μM. In our nucleotide binding experiments, we could not observe this binding because the affinity was weak, requiring concentrations higher than 1 μM [35S]GTPγS, which was the highest concentration that we could use. The P–S bond of the γ phosphate in GTPγS is longer than the P–O bond in GTP, which not only prevents nucleotide hydrolysis when binding to G protein α subunits, it also results in qualitatively different interactions and different affinities.

In vitro experiments using limited trypsin digestion and PTX-induced ARB-ribosylation showed that GoαX retained the characteristic properties of wild-type Goα in the presence of XDP or XTP. In addition, our data confirm the assumption that the xanthine nucleotide binding of Goα is decreased by 1 μM [35S]GTPγS, which was the highest concentration that we could use. The P–S bond of the γ phosphate in GTPγS is longer than the P–O bond in GTP, which not only prevents nucleotide hydrolysis when binding to G protein α subunits, it also results in qualitatively different interactions and different affinities.

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