

Pertussis Toxin-Catalyzed ADP-Ribosylation of G_{α} with Mutations at the Carboxyl Terminus

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ABSTRACT: The guanine nucleotide-binding protein G_{α} has been implicated in the regulation of Ca^{2+} channels in neural tissues. Covalent modification of G_{α} by pertussis toxin-catalyzed ADP-ribosylation of a cysteine (position 351) four amino acids from the carboxyl terminus decouples G_{α} from receptor. To define the structural requirements for ADP-ribosylation, preparations of recombinant G_{α} with mutations within the five amino acids at the carboxyl terminus were evaluated for their ability to serve as pertussis toxin substrates. As expected, the mutant in which cysteine 351 was replaced by glycine (C351G) was not a toxin substrate. Other inactive mutants were G352D and L353Δ/Y354Δ. Mutations that had no significant effect on toxin-catalyzed ADP-ribosylation included G350D, G350R, Y354Δ, and L353V/Y354Δ. Less active mutants were L353G/Y354Δ, L353A/Y354Δ, and L353G. ADP-ribosylation of the active mutants, like that of wild-type G_{α} , was enhanced by the $\beta\gamma$ subunits of bovine transducin. It appears that three of the four terminal amino acids critically influence pertussis toxin-catalyzed ADP-ribosylation of G_{α} .

G_o ,¹ a heterotrimeric guanine nucleotide-binding protein abundant in neuronal tissues, shares some characteristics with several other signal-transducing proteins found in eukaryotic cell membranes. The physiologic function of G_{α} is incompletely understood, although it has been implicated in the regulation of neuronal Ca^{2+} channels (Hescheler et al., 1987) and identified as a component of the neural growth cone membrane (Strittmatter et al., 1990). Functional interactions of G_{α} include those with $\beta\gamma$ subunits, receptors, and effectors. The specificity of each of these interactions is determined by one or more domains of G_{α} . The carboxyl terminal region of G_{α} , like those of the other G_{α} proteins, is presumed to represent its site of interaction with receptors (Winslow et al., 1987). As examples, a mutant S49 lymphoma cell that exhibits an uncoupled phenotype, attributable to a defect in the ability of G_{α} to interact with receptors, has a proline substitution for an arginine near the carboxyl terminus of G_{α} (Rall & Harris, 1987). Transmission of signals from receptor to G-protein is blocked by pertussis toxin-catalyzed ADP-ribosylation of a cysteine four amino acids from the carboxyl terminus of G_{α} (C351) and of several other G_{α} subunits (West et al., 1985). Pertussis toxin-sensitive and insensitive G_{α} subunits are differentiated at present solely on the basis of the presence of a cysteine near the carboxyl terminus (see Table II in Results).

Although cysteine serves as the toxin target, ADP-ribosylation is at least partially dependent on protein domains distant in the linear sequence from the reaction site. Studies with α_s/α_i chimeras were consistent with the hypothesis that the α_i C-terminus is not sufficient for ADP-ribosylation by pertussis toxin (Osawa et al., 1990; Freissmuth & Gilman, 1989). Free cysteine appears to be a poor pertussis toxin

substrate (Lobban & van Heyningen, 1988). Proteolytic degradation at the carboxyl terminus, believed to result in the removal of the two terminal amino acids, appeared to inhibit ADP-ribosylation but not to affect association of α with $\beta\gamma$ (Neer et al., 1988).

Isolation and study of native G_{α} proteins from tissues is frequently hampered by the multiplicity of closely related entities that have neither been reliably separated from each other nor fully identified and by contamination with $\beta\gamma$ subunits, which poses a particular problem by virtue of the fact that they stimulate pertussis toxin-catalyzed ADP-ribosylation of G_{α} (Neer et al., 1988). The use of recombinant G_{α} expressed in a bacterial host to obviate some of these difficulties has been described (Linder et al., 1990). The study of single point and of minimal deletion mutants at the carboxyl terminus of recombinant G_{α} may provide information relevant to the mechanism of toxin-catalyzed ADP-ribosylation and of the signal transduction process.

As reported here, we used oligonucleotide-directed mutagenesis in association with polymerase chain reaction (PCR) amplification of G_{α} cDNA to generate a family of mutant G_{α} clones to test the effect of substitutions and/or deletions in the five carboxyl-terminal amino acids of G_{α} (G350, C351, G352, L353, and Y354) on its ability to serve as a pertussis toxin substrate.

EXPERIMENTAL PROCEDURES

Materials. Restriction endonucleases (*Hind*III and *Eco*RI) were purchased from Boehringer Mannheim (Indianapolis, IN); *Thermus aquaticus* DNA polymerase (Taq polymerase), polymerase chain reaction (PCR) buffer, and deoxynucleotides from Perkin-Elmer Cetus (Norwalk, CT); T4 DNA ligase from New England Biolabs (Beverly, MA); nylon plaque hybridization filters, [α -³⁵S]dATP (1000 Ci/mmol) and [*adenylate*-³²P]NAD (10–50 Ci/mmol) from New England Nuclear (Boston, MA); columns for plasmid purification from Qiagen (Studio City, CA); and T7 polymerase and Sequenase kits for DNA sequencing from U.S. Biochemicals (Cleveland, OH). The protein expression vector PRC-23

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¹ Abbreviations: G-protein, guanine nucleotide-binding protein; G_s and G_i , respectively, stimulatory and inhibitory G-proteins coupled to adenylyl cyclase; G_o , G-protein abundant in brain; G_{α} , G-protein from retina (transducin); $G_{\alpha s}$, $G_{\alpha i}$, and $G_{\alpha o}$, α subunits of the respective G-proteins; $G_{\beta\gamma}$, $\beta\gamma$ subunits of the G-proteins; $G_{i\beta\gamma}$, $\beta\gamma$ subunits of G_i ; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; UTR, untranslated region.

Table I: Primers Used in Construction of Mutants^a

mutant ^b	primer sequence	region of complementarity to G_{α} (base numbers)
Antisense Strand Primers		
G350D	AAGCCGAGTCCCGGAGG	1058 - 1041
G350R	GCCGCAGCGCCGGAGGT	1056 - 1039
C351G	ACAAGCCGCGCCCGGA	1060 - 1043
G352D	AGTACAAGTCGCAGCCCC	1063 - 1046
L353A, Y354A	GAGGTCAAGTACGCCGAGCC	1069 - 1048
Y354A	CAAGAGGTCAACAAAGCCG	1072 - 1052
Y354A, L353G	gggcccgcacgcgaagcttTCACCGCCGAGCCCCG	1062 - 1045
Y354A, L353A	gggcccgcacgcgaagcttTCACCGCCGAGCCCCG	1062 - 1045
Y354A, L353V	gggcccgcacgcgaagcttTCACCGCCGAGCCCCG	1062 - 1045
L353G	GTCAGTACCGCCGAGCCCC	1066 - 1047
P-2	gcgaagctTGGGTGCTAGGTTAGACAGGGGGC	1204 - 1179
Sense Strand Primers		
G350D	ACCTCCGGGACTGCGGCTTG	1040 - 1059
G350R	ACAACCTCCGGGCTGCGGC	1037 - 1056
L353A, Y354A	GGCTGCGGCTGATCTACTGACCT	1048 - 1068
Y354A	GCGGCTGTGATGACGCTCTT	1052 - 1071
L353G	GGGCTGCGGCGGTACTGAC	1047 - 1066
P-1	gaatTCTGTAAACAAGTCTCTC	761 - 780

^a Bases mutated are underlined. Bases that are not part of the targeting sequence are in lowercase letters. Oligonucleotides without mutant designation are not mutation-directing. ^b Δ, deletion of indicated amino acid. Single-letter code for amino acids is used.

(Crowl et al., 1985) and the *Escherichia coli* host RR1 pRK248cIts, which contains a plasmid encoding the temperature-sensitive λ CIAt2 repressor, were generously supplied by Dr. Robert Crowl, Roche Research Center, Nutley, NJ.

Oligonucleotides were made by an automated phosphoramidite method on an Applied Biosystems 380B DNA synthesizer and desalted on Sephadex G-50 (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). Some oligonucleotides were biotinylated at the 5' end using a biotinylated phosphoramidite compound (Du Pont, Wilmington, DE) according to protocols provided by the manufacturer.

Construction of G_{α} Expression Vector. The bovine retinal G_{α} cDNA clone λ GO9 (Van Meurs et al., 1987) was digested with *Eco*RI and *Nco*I. The excised insert was ligated into the *Eco*RI site of plasmid PRC-23; the product was blunt-ended with T4 DNA polymerase and ligated to yield a plasmid (PRC-4) containing the complete coding region of G_{α} cDNA together with 215 bp of 3'UTR and the initiator ATG codon positioned 7 bp from the Shine-Dalgarno sequence of the plasmid.

Site-Directed Mutagenesis. Ten mutant G_{α} cDNAs were prepared in the bacterial expression vector PRC-23 using mutation-directing oligonucleotides (Table I). Mutagenesis was performed by two different procedures. Mutants C351G and G352D were made by the Amersham system (Amersham Corp., Arlington Heights, IL) based on the method of Taylor et al. (1985). The other mutants were constructed using adaptations of the recombinant-PCR method of Higuchi et al. (1988). Briefly, a 200–400-bp segment of DNA containing a specific mutation was amplified from G_{α} cDNA using three PCRs. In PCR 1, an upstream sense oligonucleotide (primer P-1) was paired with a downstream antisense oligonucleotide complementary to the G_{α} carboxyl terminus but containing a sequence alteration that would result in a codon representing an amino acid alteration and/or a stop codon. In PCR 2, a

sense oligonucleotide complementary to the mutating oligonucleotide from PCR 1 was paired in amplification of G_{α} with an antisense oligonucleotide (primer P-2) complementary to PRC-23 adjacent to the *Hind*III restriction site flanking the insert to produce the segment of G_{α} immediately distal to the segment amplified in PCR 1 (with overlap in the area of the mutation-containing primers).

The products of PCRs 1 and 2 were mixed and excess primers were removed by ultrafiltration (Centricon 100 spin columns, Amicon Corp., Danvers, MA). The DNA was heat-denatured and then allowed to reanneal. The overlapping DNA strands were subsequently amplified in PCR 3 using the P-1 and P-2 primers. All PCRs were done in 50 mM KCl/10 mM Tris-HCl, pH 8.3/1.5 mM $MgCl_2$ /0.01% gelatin/0.1% Tween-20 with dNTPs (each 200 μ M), amplification primers (30 pmol of each), and 2.5 units of Taq polymerase (total volume 100 μ L) in a Perkin-Elmer/Cetus thermal cycler (1 min/95 °C, 30 s/50 °C, 45 s/72 °C, followed by extension for 7 min at 72 °C). PCRs 1 and 2 were carried out for 35 cycles and PCR 3 for 20 cycles. For four mutants that required alteration immediately adjacent to the TGA stop codon, it was found convenient to introduce the mutations and a *Hind*III restriction site into the amplified DNA segments in a single PCR. For this, primer P-1 was paired with the downstream antisense primer containing the desired alterations plus, at the 5' end of the oligonucleotide, extra bases to introduce a *Hind*III restriction site into the resulting PCR product. Mutated G_{α} cDNA segments produced by PCRs 1–3 were phenol-extracted, ethanol-precipitated, sequentially digested with *Hind*III and *Cla*I restriction endonucleases, and then substituted for the corresponding segments of the parent "wild-type" G_{α} -containing expression plasmid. Constructs were used to transform *E. coli* strain RR1 [pRK248cIts] made competent by $CaCl_2$ treatment (Maniatis et al., 1982) or by the Hanahan procedure (Hanahan, 1983). Cells prepared by the latter method were generously supplied by Mr. Joel Jessie of Bethesda Research Laboratories, Rockville, MD.

Sequences of G_{α} constructs were verified by PCR amplification and direct sequencing of plasmids from bacterial lysates using the biotin-affinity strand-separation method (Mitchell & Merrill, 1989). In some cases, sequence was also obtained by conventional sequencing of plasmid DNA using T7 polymerase.

Expression and Partial Purification of Recombinant G_{α} . Colonies containing plasmids of interest were inoculated into 25 mL of LB medium containing ampicillin (50 μ g/mL) and tetracycline (12.5 μ g/mL) and incubated at 37 °C overnight with shaking. A sample (5 mL) of this culture was added to 500 mL of LB broth with shaking until the A_{600} was 0.3. Plasmid-directed protein production was induced by raising the temperature to 42 °C and incubating cells for an additional 2 h. Cells were harvested by centrifugation in a GSA rotor at 4000g for 20 min. Cell pellets were suspended in 5 mL of 25% sucrose/5 mM EDTA/20 mM Tris-HCl, pH 8.1/5 mM DTT. Lysozyme (1 mg) was added to initiate cell lysis. After 15 min on ice, 50 μ L of 10% Lubrol PX (Sigma) was added, and suspensions were incubated an additional 10 min on ice before centrifugation (30000g, 40 min). Supernatants containing 5–10 mg of protein were collected and applied to columns (3 mL) of DEAE-Sephacel (Pharmacia), which were washed with 10 mL of 20 mM Tris-HCl, pH 7.0/1 mM EDTA/1 mM DTT before elution of G_{α} with 3 mL of the same buffer containing 0.25 M NaCl.

[³²P]ADP-Ribosylation of Recombinant G_{α} . Pertussis toxin (2 μ g/assay) was activated by incubation for 10 min at

30 °C in 75 mM glycine buffer, pH 8.0, with 90 mM DTT. Partially purified recombinant $G_{\alpha\alpha}$ (50 μ g) was incubated for 1 h at 30 °C (total volume 0.1 mL) in reaction buffer consisting of 50 mM potassium phosphate, pH 8.0/20 mM thymidine/120 μ M ATP/10 μ M [adenylate- 32 P]NAD (2 μ Ci, 10–50 Ci/mmol)/10 mM $MgCl_2$ /0.1% Lubrol PX/71 μ M GDP β S and pertussis toxin, 2 μ g, with $\beta\gamma$ subunits (2 μ g) as indicated. The reaction was terminated by addition of 1 mL of ice-cold 7.5% trichloroacetic acid and samples were stored overnight at 4 °C. Proteins were collected by centrifugation (14000g), suspended in 20 mM sodium phosphate, pH 7.5, separated by SDS-PAGE in 12% gels, and blotted to nitrocellulose for assessment of immunoreactivity and autoradiography.

Immunoblot Analysis. Anti- $G_{\alpha\alpha}$ polyclonal antibodies were kindly provided by Dr. S.-C. Tsai (Tsai et al., 1987). The antiserum was purified by affinity chromatography on a column containing *E. coli* proteins immobilized on CNBr-activated Sepharose 4B. To examine immunoreactivity, following [32 P]ADP-ribosylation, as described in the legend to Figure 1, the proteins were precipitated with trichloroacetic acid, suspended in 20 mM sodium phosphate, pH 7.5, separated by SDS-PAGE including prestained molecular weight markers (BRL), and blotted to nitrocellulose. The blots were exposed to X-ray film (Kodak XAR) for 6–18 h and then incubated with anti- $G_{\alpha\alpha}$ polyclonal antibodies using a peroxidase-based detection system (Halpern et al., 1986).

Transducin was purified from bovine retinas by the procedure of Kühn (1980). $G_{i\alpha}$ and $G_{i\beta\gamma}$ subunits were separated by Blue Sepharose CL-6B (Pharmacia) affinity chromatography (Shinozawa et al., 1990). $G_{i\alpha}$ contaminating the $\beta\gamma$ preparation was ADP-ribosylated using 2 μ g of pertussis toxin and 20 μ M nonradioactive NAD in 2-fold concentrated reaction buffer (total volume 50 μ L) overnight at 4 °C. [adenylate- 32 P]NAD (2 μ Ci) and recombinant $G_{\alpha\alpha}$ were then added and the mixture (total volume 100 μ L) was incubated for 1 h at 30 °C. Two independent ADP-ribosylation assays were carried out for each of the two mutants, G350D and G352D. For the wild-type protein and each of the other eight mutants, four or more assays were performed.

RESULTS AND DISCUSSION

Description of the $G_{\alpha\alpha}$ mutants and comparison with pertussis toxin-sensitive and insensitive G_{α} subunits are given in Table II. Results of Western immunoblotting and ADP-ribosylation assays are presented in Figure 1 for mutations of amino acids 350–352 and in Figure 2 for mutations of residues 353 and 354.

Expression of normal and mutant recombinant $G_{\alpha\alpha}$ in the bacterial host was evaluated by immunoreactivity on Western blots. Each product exhibited a band in a position similar to that of brain $G_{\alpha\alpha}$. Assuming equal immunoreactivity, it appears that levels of expression of the recombinant proteins were comparable except for the mutant L353 Δ ,Y354 Δ , expression of which was somewhat lower.

The mutants G350R (Figure 1, left panel) and G350D (Figure 1, right panel) were ADP-ribosylated by pertussis toxin to the same extent as r $G_{\alpha\alpha}$ and the reaction was markedly enhanced by $\beta\gamma$ subunits. Aspartic acid is, in fact, the fifth amino acid from the carboxyl end in $G_{i\alpha1}$, $G_{i\alpha2}$, $G_{i\alpha1}$, and $G_{i\alpha2}$ (Table II). On the other hand, the mutant protein G352D was not a pertussis toxin substrate, either in the presence or in the absence of $\beta\gamma$. As expected, the mutant C351G was likewise not ADP-ribosylated (Figure 1, left panel). Pertussis toxin-insensitive α subunits are all believed to have an alternative amino acid in place of cysteine (Table II).

Table II: Carboxyl-Terminal Sequences of G_{α} Subunits

G-protein	sequence	ADP-ribosylation	ref
(I) Pertussis Toxin-Insensitive G_{α} Subunits			
$G_{s\alpha}$	LR Q Y E LL	–	<i>a</i>
G_{za}	LK Y I G LC	–	<i>a</i>
G_{i4}	LR E F N LV	–	<i>b</i>
$G_{\alpha q}G_{\alpha 11}$	LK E Y N LV	–	<i>a</i>
$G_{\alpha 12}$	LK D I M LQ	–	<i>c</i>
$G_{\alpha 13}$	LK Q L M LQ	–	<i>c</i>
$G_{\alpha 15,16}$	LD E I N LL	–	<i>b,d</i>
(II) Pertussis Toxin-Sensitive G_{α} Substrates ^e			
$G_{i1\alpha}$	LK D <u>C</u> G LF	+	<i>f</i>
$G_{i2\alpha}$	LK D <u>C</u> G LF	+	<i>f</i>
$G_{i3\alpha}$	LK E <u>C</u> G LY	+	<i>f</i>
$G_{i1\alpha}$	LK D <u>C</u> G LF	+	<i>f</i>
$G_{i2\alpha}$	LK D <u>C</u> G LF	+	<i>f</i>
$G_{\alpha 1,2}$	LR G <u>C</u> G LY	+	<i>g</i>
(III) $G_{\alpha\alpha}$ Mutants			
M $G_{\alpha\alpha}$	LR G G G LY	–	
M $G_{\alpha\alpha}$	LR G C D LY	–	
M $G_{\alpha\alpha}$	LR G C G - ^h	–	
M $G_{\alpha\alpha}$	LR D C G LY	+	
M $G_{\alpha\alpha}$	LR R C G LY	+	
M $G_{\alpha\alpha}$	LR G C G L-	+	
M $G_{\alpha\alpha}$	LR G C G V-	+	
M $G_{\alpha\alpha}$	LR G C G G-	●	
M $G_{\alpha\alpha}$	LR G C G A-	●	
M $G_{\alpha\alpha}$	LR G C G GY	±	

^a Simon et al., 1991. ^b Wilkie et al., 1991. ^c Strathmann & Simon, 1991. ^d Amatruda et al., 1991. ^e The cysteine ADP-ribosylated by toxin is underlined. ^f Price et al., 1990. ^g Tsukamoto et al., 1991. ^h Hyphens indicate amino acid deletions.

ADP-ribosylation of wild-type $G_{\alpha\alpha}$ was markedly stimulated by bovine retinal $G_{i\beta\gamma}$ (Figure 1B, both panels; Figure 2, right panel). To evaluate the effect of $G_{i\beta\gamma}$ on ADP-ribosylation, different exposure times were used for autoradiography in order to see the low intensity of samples minus $\beta\gamma$. Relative activity was compared to wild-type controls (Figure 2B, left and center panels). To demonstrate that a $\beta\gamma$ effect occurred, identical 48-h exposures were analyzed (Figure 2, legend). The mutant Y354 Δ was a pertussis toxin substrate (Figure 2, left and center panels) and reaction was enhanced by $\beta\gamma$ subunits. The mutant L353 Δ ,Y354 Δ was not ADP-ribosylated, with or without $\beta\gamma$. Mutants L353G and L353G,Y354 Δ (Figure 2, left and center panels) were only minimally ADP-ribosylated by pertussis toxin. The mutant L353A,Y354 Δ was slightly more effective but still much less so than wild-type r $G_{\alpha\alpha}$ or L353V,Y354 Δ (Figure 2, right panel), consistent with the closer similarity of Leu to Val. With all of the active mutants, addition of $\beta\gamma$ subunits enhanced ADP-ribosylation.

The assay, as performed in these studies, is largely qualitative and is not suited for strict quantitative comparison of substrates. Nevertheless, since the assays contained equivalent amounts of immunoreactive protein, major differences of intensity among bands corresponding to the ADP-ribosylated substrates reflect their relative reactivities. The mutants may then be divided into three categories: (1) those that are modified to an extent similar to the normal protein, (2) those that are not detectably ADP-ribosylated, and (3) those with greatly diminished reactivity. The $\beta\gamma$ subunits invariably had an enhancing effect on modification of normal r $G_{\alpha\alpha}$ and those carboxyl-terminal mutants that were substrates for pertussis toxin. With apparently nonreactive mutants, however, addition of $\beta\gamma$ had no discernible effect, indicating that the carboxyl-terminal sequence adjacent to C351 is an overriding determinant of the ability to serve as an ADP-ribose acceptor in the pertussis toxin-catalyzed reaction.

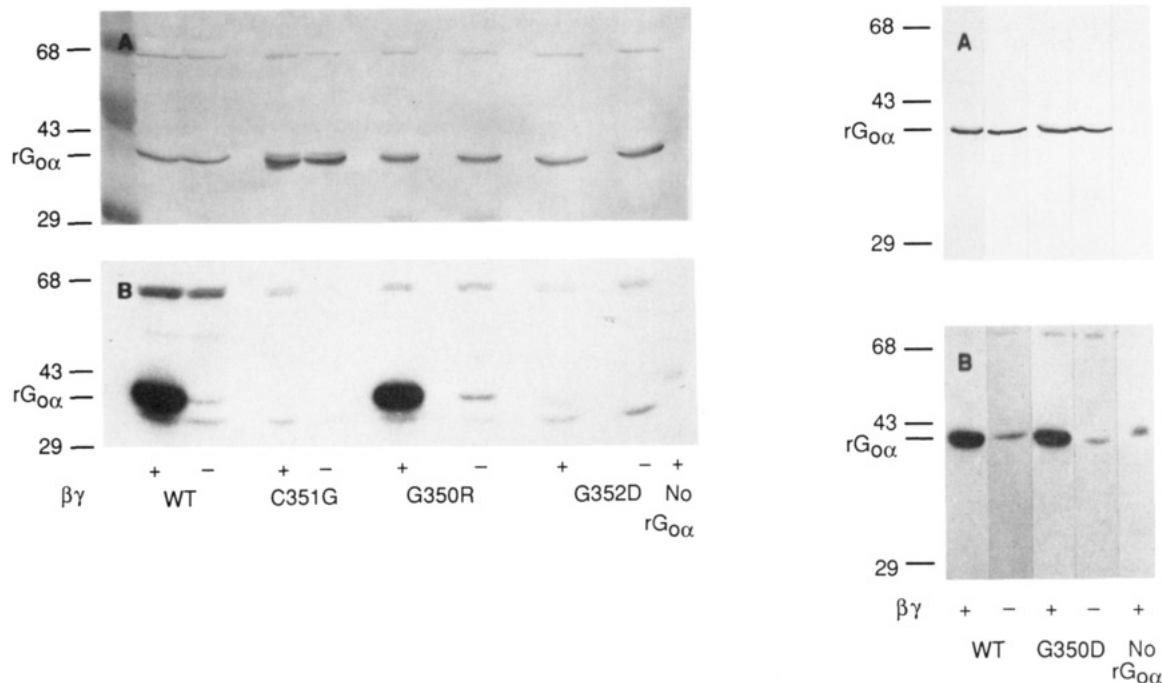


FIGURE 1: ADP-ribosylation of mutants of amino acids 350–352. (A) Immunoblots incubated with anti- $G_{\alpha\alpha}$ antiserum. (B) Autoradiograms of $[^{32}\text{P}]$ ADP-ribosylated substrates. Fifty micrograms of partially purified recombinant protein was ADP-ribosylated by pertussis toxin with or without $G_{i\beta\gamma}$. Reaction mixture with $G_{i\beta\gamma}$ was preincubated with the toxin and nonlabeled NAD overnight at 4 °C. Recombinant protein and $[\text{adenylate-}^{32}\text{P}]\text{NAD}$ were added and the samples further incubated at 30 °C for 1 h. Left panel, left to right: wild type, C351G, G350R, G352D, $G_{i\beta\gamma}$ only. Right panel, left to right: wild type, G350D, $G_{i\beta\gamma}$ only.

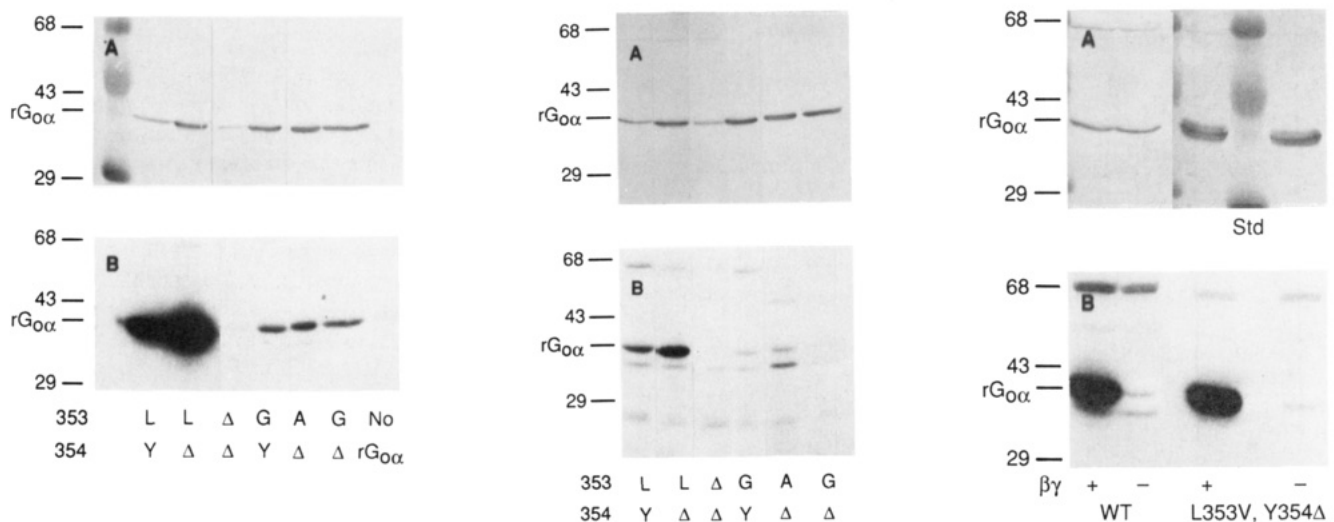


FIGURE 2: ADP-ribosylation of $rG_{\alpha\alpha}$ with mutations of amino acids 353 and 354. (A) Immunoblots incubated with anti- $G_{\alpha\alpha}$ antiserum. (B) Autoradiograms of $[^{32}\text{P}]$ ADP-ribosylated substrates. Procedures were as described for Figure 1. Amino acids at positions 353 and 354 are as indicated. (L353,Y354 is wild type.) Densitometric determinations were made for experiments in Figure 2, left and center panels, with autoradiograms exposed for the same times (48 h). Identical exposure times facilitate comparisons of $[^{32}\text{P}]$ ADP-ribosylation in the presence or absence of $\beta\gamma$; the analysis revealed 27.8- and 14.4-fold effects for $\beta\gamma$ with the wild-type and Y354 Δ $rG_{\alpha\alpha}$, respectively. Left panel: ADP-ribosylation in the presence of $G_{i\beta\gamma}$; film exposure for 18 h. Center panel: ADP-ribosylation in the absence of $G_{i\beta\gamma}$; film exposure for 3 days. Right panel: ADP-ribosylation with or without $G_{i\beta\gamma}$, as indicated; film exposure for 6 h.

Recombinant α -subunits of several G-proteins that were expressed in *E. coli* (Linder et al., 1990; Jones et al., 1990) interacted with guanine nucleotides and $\beta\gamma$ subunits and were ADP-ribosylated by pertussis toxin. The degree to which the recombinant $G_{\alpha\alpha}$ subunits synthesized in *E. coli* interact with $\beta\gamma$ subunits, as assessed by pertussis toxin-catalyzed ADP-ribosylation, was less than the interaction of bovine brain $G_{\alpha\alpha}$ with $\beta\gamma$, presumably due to the fact that mammalian $G_{\alpha\alpha}$ is cotranslationally modified by N-myristoylation of the terminal glycine (after removal of the initiator methionine). With higher affinity between the α and $\beta\gamma$ subunits, there is more $\alpha\beta\gamma$, the preferred substrate, leading to increased ADP-ri-

bosylation (Linder et al., 1991). The present study demonstrates that ADP-ribosylation occurs even in the absence of $\beta\gamma$ with those recombinant $G_{\alpha\alpha}$ products that are able to serve as acceptors. The sizable effect of $\beta\gamma$ on the extent of the reaction observed with most mutants agrees with the response reported by Linder et al. (1991). The extent of ADP-ribosylation of a nonmyristoylated substrate reached a value equal to that seen with the myristoylated preparation under conditions of equimolar or higher ratio of $\beta\gamma$ to $G_{\alpha\alpha}$ (Linder et al., 1991).

The carboxyl terminus of G_{α} subunits is believed to be involved in interaction with receptors [see review (Bourne et

al., 1988)] and contributes, therefore, to their functional specificity. Since ADP-ribosylation of the carboxyl-proximal cysteine is known to interfere with interactions of G-proteins with receptors, it is reasonable to assume that at least some similar features of protein configuration are required for both of these processes and that pertussis toxin-catalyzed modifications of G_{α} mutants may reflect their potential for functional competence. The present results demonstrate the basic requirement for the three penultimate amino acid residues in the carboxyl-terminal region of G_{α} , -CGL, for pertussis toxin-catalyzed ADP-ribosylation. This amino acid sequence is present in all the G_{α} proteins that are known to serve as substrates for pertussis toxin (Table II). The three $G_{i\alpha}$ and the two $G_{t\alpha}$ subunits are identical in the sixth, fourth, third, and second positions from the carboxyl terminus. There is some variability, however, in the fifth position, as well as in the carboxyl-terminal position, where either phenylalanine or tyrosine is found [see review (Price et al., 1990)]. Hsu et al. (1990) tabulated sequences of a number of G_{α} proteins including some that result from alternative splicing of a single transcript. In a total of six peptides derived from four widely divergent species (hamster, *Xenopus laevis*, *Drosophila melanogaster*, and *Locusta migratoria*), the sequences of the eight carboxyl terminal amino acids are identical. Consequently, it is likely that the carboxyl-terminal amino acid sequence of G_{α} required for optimal pertussis toxin-catalyzed ADP-ribosylation derived by mutational analysis reflects part of the conserved structure mandated by the biological functions of the protein.

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