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Guanine nucleotide-binding proteins that enhance choleragen ADP-ribosyltransferase activity: Nucleotide and deduced amino acid sequence of an ADP-ribosylation factor cDNA

(cholera toxin/adenylate cyclase)

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ABSTRACT **Three (two soluble and one membrane) guanine nucleotide-binding proteins (G proteins) that enhance ADP-ribosylation of the $G_{s\alpha}$ stimulatory subunit of the adenylyl cyclase (EC 4.6.1.1) complex by choleragen have recently been purified from bovine brain. To further define the structure and function of these ADP-ribosylation factors (ARFs), we isolated a cDNA clone (λARF2B) from a bovine retinal library by screening with a mixed heptadecanucleotide probe whose sequence was based on the partial amino acid sequence of one of the soluble ARFs from bovine brain. Comparison of the deduced amino acid sequence of λARF2B with sequences of peptides from the ARF protein (total of 60 amino acids) revealed only two differences. Whether these are cloning artifacts or reflect the existence of more than one ARF protein remains to be determined. Deduced amino acid sequences of ARF, $G_{o\alpha}$ (the α subunit of a G protein that may be involved in regulation of ion fluxes), and c-Ha-*ras* gene product p21 show similarities in regions believed to be involved in guanine nucleotide binding and GTP hydrolysis. ARF apparently lacks a site analogous to that ADP-ribosylated by choleragen in G-protein α subunits. Although both the ARF proteins and the α subunits bind guanine nucleotides and serve as choleragen substrates, they must interact with the toxin A1 peptide in different ways. In addition to serving as an ADP-ribose acceptor, ARF interacts with the toxin in a manner that modifies its catalytic properties.**

Choleragen (cholera toxin), a secretory product of *Vibrio cholerae*, is responsible in part for the fluid and electrolyte abnormalities characteristic of clinical cholera. The toxin exerts its effects on intestinal cells by activating adenylyl cyclase [adenylate cyclase; ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1], thereby increasing intracellular cyclic AMP (1, 2). Activation results from the toxin-catalyzed ADP-ribosylation of $G_{s\alpha}$, the α subunit of the guanine nucleotide-binding protein (G protein) G_s. $G_{s\alpha}$ is the stimulatory regulatory subunit of the cyclase system (3, 4). $G_{s\alpha}$ binds guanine nucleotides and functions physiologically to couple hormone or neurotransmitter receptors to the cyclase catalytic unit (3, 5, 6). ADP-ribosylation of $G_{s\alpha}$ increases its sensitivity to activation by GTP by inhibiting its intrinsic GTPase activity (4, 7–9).

ADP-ribosylation of $G_{s\alpha}$ and activation of adenylyl cyclase by choleragen is enhanced by membrane and soluble factors (10–20). One such factor, a protein termed ARF (for ADP-ribosylation factor) was purified from bovine brain membranes and shown to bind guanine nucleotides (17). Subsequently, two soluble ARF-like proteins (sARF I and sARF II) were purified from bovine brain (20). The membrane and soluble ARFs are all ≈19-kDa proteins that act by directly stimulating the ADP-ribosyltransferase activities of the toxin A1 protein (19, 20). The stimulatory activity of the ARF proteins is dependent on the presence of GTP or nonhydrolyzable GTP derivatives; GDP, GDP analogues, and adenine nucleotides are inactive (19, 20). To define the structure–function relationships in the ARF molecule, a cDNA clone was isolated from a bovine retinal cDNA library by use of a synthetic oligonucleotide probe based on amino acid sequence of peptides from sARF II, which was purified from bovine brain (20). The characterization and complete coding sequence of this ARF clone is reported here[§] and compared to those of other G proteins.

MATERIALS AND METHODS

Materials. Bacteriophage T4 polynucleotide kinase and *Eco*RI restriction endonuclease were purchased from Bethesda Research Laboratories; [γ-^{32}P]ATP (6000 Ci/mmol; 1 Ci = 37 GBq), [α-^{32}P]dATP (3000 Ci/mmol), and [α-[^{35}S]-thio]dATP (500 Ci/mmol) from New England Nuclear; Bluescript "phagemid" vectors from Stratagene; kits for dideoxynucleotide sequencing with Sequenase from United States Biochemical and for random primed DNA labeling from Boehringer Mannheim; and custom oligonucleotides from Pharmacia.

Purification and Amino Acid Sequence Analysis. sARF II was purified from the 105,000 × *g* supernatant of bovine brain homogenate as described by Tsai *et al.* (20) and further purified by reverse-phase high-performance liquid chromatography (HPLC) (model 1090 liquid chromatograph; Hewlett–Packard, Sunnyvale, CA). In brief, sARF II was applied to a Vydac C_4 column (214TP54, 4.6 × 250 mm; The Separations Group, Hesperia, CA) equilibrated with 0.14% trifluoroacetic acid/0.1% *N*-methylmorpholine, pH 4.3, and then was eluted with an acetonitrile gradient (9.1–70.0%) over 70 min at a flow rate of 0.5 ml/min. Protein concentration was monitored by absorbance at 280 nm and 220 nm (model 1040A diode-array detection system; Hewlett–Packard). Samples of fractions with absorbance at 280 nm were subjected to $NaDodSO_4$/16% polyacrylamide gel electrophoresis to iden-

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Abbreviations: G protein, guanine nucleotide-binding protein; G_s, stimulatory G protein coupled to adenylyl cyclase; $G_{s\alpha}$, α subunit of G_s; G_o, G protein that may regulate ion fluxes; $G_{o\alpha}$, α subunit of G_o; ARF, ADP-ribosylation factor (ARF is also used to refer to the protein encoded by clone λARF2B); sARF II, ARF protein purified from bovine brain supernatant.

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§The sequence reported in this paper is being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03794).

tify sARF II. The sARF II peak fractions were pooled and stored at −20°C.

An attempt to determine the NH_2-terminal sequence of sARF II on an Applied Biosystems model 470A gas-phase sequenator equipped with on-line phenylthiohydantoin analyzer model 120 (Applied Biosystems, Foster City, CA) (21) yielded no identifiable phenylthiohydantoin amino acid derivative. For cleavage with CNBr, HPLC-purified sARF II (200 μg) was dissolved in 200 μl of 50% (vol/vol) formic acid and incubated with 1 mg of CNBr at 22°C overnight. After 5-fold dilution with water and lyophilization, the residue was suspended in 200 μl of 0.1% trifluoroacetic acid and the peptides were separated by reverse-phase HPLC on the same Vydac C_4 column used for sARF II purification, which was equilibrated with 0.1% trifluoroacetic acid. Peptides were eluted with a shallow acetonitrile gradient (28–50%) for 50 min at a flow rate of 0.5 ml/min. Fractions from well-separated peaks were pooled, partially lyophilized, and applied to the gas-phase sequencer described above. The standard protocol and reagents for sequencing were supplied by Applied Biosystems.

Cloning and Sequencing of sARF cDNA. A bovine retinal cDNA library in λgt10, kindly provided by Jeremy Nathans (Stanford University), was screened by plaque hybridization with a 17-base mixed oligodeoxyribonucleotide probe, 5′-GCYTGDATRTACCARTT-3′ (Y = C or T; D = A, G, or T; R = A or G). This sequence was selected by computation of the least redundant oligonucleotide probe based on reverse translation of amino acid sequences of CNBr peptides from purified bovine brain sARF II. Replicate filters were prehybridized in 5× NET (1× = 0.15 M NaCl/1 mM EDTA/30 mM Tris·HCl, pH 8.0)/5× Denhardt's solution (1× = 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin)/0.5% $NaDodSO_4$ for 6 hr at 42°C and then hybridized for 16 hr at 42°C with probe that had been labeled at the 5′ end with ^{32}P (22). Filters were washed once with 5× NET/0.5% $NaDodSO_4$ and twice with 2× NET/0.5% $NaDodSO_4$, each at 42°C for 20 min, before exposure to Kodak XAR film overnight at −80°C with intensifier screens.

A 1.5-kilobase insert, excised from clone λARF2B by using *Eco*RI, was subcloned into the polylinker region of the Bluescript KS(+) phagemid vector. The nucleotide sequence was determined by sequencing both DNA strands of the plasmid by the dideoxynucleotide method using Sequenase and synthetic 18-base oligonucleotide primers. Sequence analyses were performed using the PC Gene software package (IntelliGenetics, Mountain View, CA) in conjunction with Bionet. The deduced amino acid sequence of ARF was aligned with that of the bovine retinal G-protein α subunit $G_{o\alpha}$ (23) by the method of Needleman and Wunsch (24), as modified by Dayhoff (25) and Feng *et al.* (26), and by inspection. Alignment of ARF with the sequence of the *ras*-encoded protein p21 (27) was based in part on the alignment between G-protein α subunits and *ras* protein by Masters *et al.* (28).

RNA Gel Blot Analysis. Total bovine retinal RNA was isolated according to Chirgwin *et al.* (29). Poly(A)$^+$ RNA was purified by oligo(dT)-cellulose chromatography, fractionated by electrophoresis in a 1% agarose/2.2 M formaldehyde gel, and transferred to nitrocellulose. The RNA blot was prehybridized for 6 hr at 42°C in 40% (vol/vol) formamide/5× Denhardt's solution/5× SSC (1× = 0.15 M NaCl/0.015 M sodium citrate, pH 7.5)/10 mM Tris·HCl, pH 7.5/10% (wt/vol) dextran sulfate/denatured salmon sperm DNA (100 μg/ml) and then hybridized at 42°C for 16 hr with the [^{32}P]cDNA insert labeled by using random primers (30). After one wash with 2× SSC/0.5% $NaDodSO_4$ and one wash with 0.5× SSC/0.5% $NaDodSO_4$ (each for 30 min at 65°C), the blot was exposed to Kodak XAR film at −80°C with intensifier screens.

RESULTS AND DISCUSSION

Screening of ≈1.4 × 10^6 clones identified 13 positive clones. These were isolated and one, λARF2B, was selected for sequencing. λARF2B contains an open reading frame coding for a protein of 181 amino acids with a molecular weight of 20,700 (Fig. 1). Although this is consistent with the apparent size of the ARF proteins, proof that the coding region is complete is lacking. There are only 7 nucleotides upstream of the putative initiating ATG codon, which is not immediately preceded by the ACC sequence commonly found in eukaryotic mRNAs (31). Of ≈1000 nucleotides in the 3′ untranslated region, 110 were sequenced and no potential polyadenylylation signals were noted.

Comparison of the deduced amino acid sequence of λARF2B with the sequences of two CNBr peptides from purified bovine brain sARF II (a total of 60 amino acids) revealed near identity with only two differences (Fig. 2).

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                                                                -7  AGT AACA

 1  ATG GGG AAC GTT TTT GAA AAA TTG TTT AAA AGT CTC TTT GGG AAA AAG GAG ATG CGG ATT
 1  MET GLY ASN VAL PHE GLU LYS LEU PHE LYS SER LEU PHE GLY LYS LYS GLU MET ARG ILE

61  CTT ATG GTG GGT TTG GAT GCC GCT GGA AAA ACC ACC ATC TTG TAC AAA CTG AAG CTG GGA
21  LEU MET VAL GLY LEU ASP ALA ALA GLY LYS THR THR ILE LEU TYR LYS LEU LYS LEU GLY

121 GAG ATT GTG ACT ACC ATC CCC ACG ATA GGT TTC AAC GTG GAG ACA GTA GAA TAT AAA AAC
41  GLU ILE VAL THR THR ILE PRO THR ILE GLY PHE ASN VAL GLU THR VAL GLU TYR LYS ASN

181 ATC AGC TTC ACA GTC TGG GAT GTC GGC GGC CAG GAC AAA ATC AGA CCT CTG TGG CGA CAT
61  ILE SER PHE THR VAL TRP ASP VAL GLY GLY GLN ASP LYS ILE ARG PRO LEU TRP ARG HIS

241 TAT TTC CAG AAC ACA CAA GGT CTG ATT TTC GTG GTC GAC AGT AAT GAC AGA GAG CGG GTC
81  TYR PHE GLN ASN THR GLN GLY LEU ILE PHE VAL VAL ASP SER ASN ASP ARG GLU ARG VAL

301 AAT GAA GCC CGA GAA GAA CTA ACC AGA ATG TTA GCT GAA GAC GAG CTC AGA GAT GCG GTC
101 ASN GLU ALA ARG GLU GLU LEU THR ARG MET LEU ALA GLU ASP GLU LEU ARG ASP ALA VAL

361 TTA TTG GTG TTT GTA AAT AAA CAG GAT CTT CCC AAT GCT ATG AAC GCA GCA GAG ATA ACA
121 LEU LEU VAL PHE VAL ASN LYS GLN ASP LEU PRO ASN ALA MET ASN ALA ALA GLU ILE THR

421 GAC AAG CTT GGC TTA CAT TCC CTC CGC CAG AGA AAC TGG TAC ATT CAG GCT ACT TGC GCC
141 ASP LYS LEU GLY LEU HIS SER LEU ARG GLN ARG ASN TRP TYR ILE GLN ALA THR CYS ALA

481 ACC AGT GGA GAT GGG CTT TAT GAA GGC CTG GAC TGG CTC TCC AAC CAG CTC AAA AAC CAG
161 THR SER GLY ASP GLY LEU TYR GLU GLY LEU ASP TRP LEU SER ASN GLN LEU LYS ASN GLN

541 AAG TGA CGGG AGGT GCTG CGCT CCCC TGCG TCGT GGCA AAGT CAGC TGGC CTCT TGTG TGTG CGTG TGTG CGTG TGAG
181 LYS STOP

619 GAGC CGAG TGTG GGTG TGTG GCGG GCGT GGGA GCAG CTT
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FIG. 1. Nucleotide and deduced amino acid sequence of λARF2B. Nucleotides and amino acids are numbered relative to the first nucleotide of the start codon ATG or the initiating methionine. The 3′ untranslated region of the clone was only partially sequenced.

```
                          10                  20
PEPTIDE 1    L A E D E L R D A V L L V F A N K Q D L P N A
                                        * * * * * *
DEDUCED      - - - - - - - - - - - - - - V - - - - - - - -
(111-133)
______________________________________________________________

                          10                  20
PEPTIDE 2    N A A E I T D K L G L H S L R H R N W Y I Q A T C
                                            * * * * * *
DEDUCED      - - - - - - - - - - - - - - - Q - - - - - - - - -
(135-159)

                 30
PEPTIDE 2    A T S G D G L Y E G L D

DEDUCED      - - - - - - - - - - - -
(160-171)
```

FIG. 2. Comparison of sequences of peptides from sARF II and deduced amino acid sequence for λARF2B. Sequences are given in conventional one-letter amino acid symbols (see legend to Fig. 3). CNBr peptides from sARF II were prepared, purified, and sequenced as described in *Materials and Methods*. The corresponding deduced amino acid sequences are shown below the peptide sequences. Numbers in parentheses indicate positions in the deduced sequence. Dashes indicate identity of peptide and deduced amino acid sequence. Sequences of peptides used for construction of oligonucleotide probes are marked with asterisks.

Peptide 1 contains an alanine corresponding to valine in position 125 of the deduced sequence. A mixed oligonucleotide probe (5′-TCYTGYTTRTTNGCRAA-3′ where N = A, C, G, or T) representing the sequence -Phe-Ala-Asn-Lys-Gln-Asp- in peptide 1 hybridized only weakly to the clone, even under very low stringency. The weak hybridization probably can be explained by the presence of guanine in position 13 of the probe, which would be occupied by adenine in an oligonucleotide complementary to the clone. In peptide 2 there is a histidine instead of the glutamine that is found in position 150 of the deduced sequence. Both of the differences between the peptide and deduced amino acid sequences could result from single nucleotide substitutions, which may have occurred during cloning. Alternatively, the differences may reflect the existence of more than one ARF protein.

Although three forms of ARF have been purified from bovine brain, two soluble (20) and one membrane (17, 19, 20), it is not known whether these result from different posttranslational modifications of a single protein or are products of different mRNAs; i.e., λARF2B does not encode sARF II but rather another closely related ARF protein. When the ≈1.5-kilobase λARF2B cDNA was labeled and used to probe a blot of bovine retinal poly(A)$^+$ RNA, a single mRNA ≈2.6 kilobases was detected (data not shown).

The deduced amino acid sequences of ARF from λARF2B, c-Ha-*ras* p21 (27), and bovine retinal $G_{o\alpha}$ (23), a G-protein α-subunit thought to be involved in the regulation of ion flux (32), are compared in Fig. 3. G_o is one of a family of trimeric G proteins that are composed of α, β, and γ subunits. The α subunits bind and hydrolyze GTP. Putative functional domains in $G_{o\alpha}$ and other G-protein α subunits have been assigned based on alignment of their deduced amino acid sequences with that of translational elongation factor EF-Tu or *ras* p21 and related to models of the GDP-binding domains of the latter proteins (28, 33, 34).

Similarities between ARF and $G_{o\alpha}$ or the *ras*-encoded protein are, for the most part, restricted to sequences believed to be involved in guanine nucleotide binding and hydrolysis (28). The region corresponding to positions 16–34 in ARF is presumed to participate in GTP hydrolysis. A

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                                                               * * * * * * * * * * * * * *
Go    M G C T L S A E E R A A L E R S K A I E K N L K E D G I S A A K D V K L L L L G A G E S G :45
ARF                                 M G N V F E K L F K S - - L F G K K E M R I L M V G L D A A G :29
RAS                                                               M T E Y K L V V V G A G G V G :15

      * * * * *
Go    K S T I V K Q M K I I H E D G F S G E D V K Q Y K P V V Y S N T I Q S L A A I V R A M D T :90
ARF   K T T I L Y K L K L G E I V T T - - - - - - - - - - - - - - - - - - - - - - - - - - - - - :45
RAS   K S A L T I Q - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - :22

Go    L G I E Y G D K E R K A D A K M V C D V V S R M E D T E P F S P E L L S A M M R L W G D S :135
ARF   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
RAS   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -

Go    G I Q E C F N R S R E Y Q L N D S A K Y Y L D S L D R I G A A D Y Q P T E Q D I L R T R V :180
ARF   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - I :46
RAS   - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - L I Q N H F V D E Y D P T I :36

                                    * * * * * * *                                 * *
Go    K T T G - I V E T H F T F K N L H F R L F D V G G Q R S E R K K W I H C F E D V T A I I F :224
ARF   P T I G F N V E T V E - Y K N I S F T V W D V G G Q D K I R P L W R H Y F Q N T Q G L I F :90
RAS   E D S Y - R K Q V V I D G E T C L L D I L D T A G Q E E Y S A M R D Q Y M R T G E G F L C :80

      * * * * * * *
Go    C V A L S G Y D Q V - - - - - - - - - - - - - L H E D E T T N R M H E S L M L F D S I C N :256
ARF   V V D S N D R E R V - - - N E A R E E L T R M L A E D E L R - - - - - - - - - - - - - - - :117
RAS   V F A I N N T K S F E D I H Q Y R E Q I K R V K D S D D V P - - - - - - - - - - - - - - - :110

                  * * * * * * * * * * * *
Go    N K F F I D I S I I L F L N K K D L F G E K I K K S P L T I C F P E Y T G S N T Y E D A A :301
ARF   - - - - - D A V L L V F V N K Q D L P - - - - - - - - - - - - - - - - - - - - - - - - - - :131
RAS   - - - - - - - M V L V - G N K C D L A A R T V - - - - - - - - - - - - - - - - - - - - - - :125

Go    A Y I Q A Q F E S K N R S P N K E I Y C H T M C A T D T N N I Q V V F D A V T D I I I A N :346
ARF   N A M N A A E I T D K L G L H S L R Q R N W Y I Q A T C A T S G D G L Y E G L D W L S N Q :176
RAS   E S R Q A Q D L A R S Y G I P Y I E T S A K T R Q G V E D A F Y T L V R E I R Q H K L R K :170

Go    N L R G C G L Y                                                                         :354
ARF   L K N Q K                                                                               :181
RAS   L N P P D E S G P G C M S C K C V L S                                                   :189
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FIG. 3. Comparison of deduced amino acid sequences of bovine $G_{o\alpha}$ (Go), λARF2B (ARF), and human c-Ha-*ras*-encoded p21 (RAS). Gaps, indicated by hyphens, were introduced to obtain maximal similarity. Asterisks above the sequences mark regions presumed to be involved in guanine nucleotide binding and GTP hydrolysis (28, 33, 34). Identical amino acids are boxed with conservative substitutions. Categories of conservative substitutions are as follows: cysteine (C); serine (S), threonine (T), proline (P), alanine (A), and glycine (G); asparagine (N), aspartic acid (D), glutamic acid (E), and glutamine (Q); histidine (H), arginine (R), and lysine (K); methionine (M), isoleucine (I), leucine (L), and valine (V); phenylalanine (F), tryptophan (W), and tyrosine (Y).

lysine corresponding to that in position 30 of ARF is found in all of the known G-protein α subunits as well as in *ras* p21 and EF-Tu. It is thought to interact with the β phosphate of GDP (28, 33). In ARF, Asp-26 corresponds to Gly-12 in the *ras* proteins, replacement of which results in diminished GTPase activity (34). Deduced sequences of the G-protein α subunits each contain a glycine in the cognate position. Replacement of this glycine by aspartic acid in ARF would be consistent with the reported absence of detectable GTPase activity (17). Amino acids in positions 121–130 in ARF are very similar to those in corresponding regions of *ras* p21, $G_{o\alpha}$ (as well as the other G-protein α subunits), and EF-Tu. This includes the sequence -Asn-Lys-Xaa-Asp-, which is conserved in all of these proteins. It has been suggested that the asparagine and aspartic residues interact with the guanine ring of the bound nucleotide (28, 33, 34).

Outside of the limited regions where similarities between ARF and several other GTP-binding proteins are considerable, the amino acid sequences appear rather divergent. For example, positions 141–181 in ARF seemingly have little similarity to $G_{o\alpha}$ or *ras* p21. This COOH-terminal region of the G-protein α subunits is believed to be responsible for interaction with receptor (23, 28, 35, 36). These proteins (with the exception of $G_{s\alpha}$) contain cysteine as the fourth amino acid from the COOH terminus. This cysteine is the site of ADP-ribosylation by pertussis toxin, which results in effective uncoupling from receptor (4). The COOH-terminal 69 amino acids of the *ras* protein appear to be critical for biological activity (37). c-Ha-*ras*-encoded p21 terminates with the sequence -Cys-Val-Leu-Ser. The cysteine is acylated with palmitic acid, which is important for attachment to the cell membrane and transformation (38, 39).

The G-protein α subunits have blocked NH_2 termini (40, 41) and some of these, as well as several other proteins, are known to be myristoylated, apparently on an NH_2-terminal glycine (40, 41). The NH_2 terminus of sARF II appeared blocked to Edman degradation, and the deduced amino acid sequence of ARF contains glycine next to the initiating methionine. It does not, however, contain a hydroxy amino acid in position 5 (glycine = residue 1), which is found in many, but not all, myristoylated proteins (42).

Choleragen modifies the activities of $G_{s\alpha}$ and transducin α subunit by catalyzing the ADP-ribosylation of an arginine (43) that corresponds to Arg-179 in $G_{o\alpha}$. To obtain the alignment in Fig. 3, a gap that overlaps this region of $G_{o\alpha}$ was introduced into the ARF sequence, and ARF apparently lacks an analogous site for choleragen-catalyzed ADP-ribosylation. We conclude that although both the ARF proteins and the G-protein α subunits bind guanine nucleotides and serve as choleragen substrates, they must interact with the toxin A1 peptide in rather different ways. In addition to serving as an ADP-ribose acceptor, ARF interacts with the toxin in a manner that modifies its catalytic properties.

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