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Posttranslational modification of $G\alpha_{o1}$ generates $G\alpha_{o3}$, an abundant G protein in brain

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ABSTRACT **$G\alpha_o$, the most abundant G protein in mammalian brain, occurs at least in two subforms, i.e., $G\alpha_{o1}$ and $G\alpha_{o2}$, derived by alternative splicing of the mRNA. A third $G\alpha_{o1}$-related isoform, $G\alpha_{o3}$, has been purified, representing about 30% of total G_o in brain. Initial studies revealed distinct biochemical properties of $G\alpha_{o3}$ as compared with other $G\alpha_o$ isoforms. In matrix-assisted laser desorption/ionization peptide mass mapping of gel-isolated $G\alpha_{o1}$ and $G\alpha_{o3}$, C-terminal peptides showed a difference of +1 Da for $G\alpha_{o3}$. Nanoelectrospray tandem mass spectrometry sequencing revealed an Asp instead of an Asn at position 346 of $G\alpha_{o3}$. Gel electrophoretic analysis of recombinant $G\alpha_{o3}$ showed the same mobility as native $G\alpha_{o3}$ but distinct to $G\alpha_{o1}$. The conversion of ^{346}Asn→Asp changed the signaling properties, including the velocity of the basal guanine nucleotide-exchange reaction, which points to the involvement of the C terminus in basal guanosine 5′-[γ-thio]triphosphate binding. No cDNA coding for $G\alpha_{o3}$ was detected, suggesting an enzymatic deamidation of $G\alpha_{o1}$ by a yet-unidentified activity. Therefore, Gα heterogeneity is generated not only at the DNA or RNA levels, but also at the protein level. The relative amount of $G\alpha_{o1}$ and $G\alpha_{o3}$ differed from cell type to cell type, indicating an additional principle of G protein regulation.**

Heterotrimeric G proteins are known as membrane-tethered cellular switches linking a diverse set of heptahelical receptors to dozens of cellular effectors (1–3). Specificity within this signaling network depends on a structural diversity originating from multiple genes (4). Alternative splicing of transcripts increases heterogeneity, and at the protein level distinct $\alpha\beta\gamma$ compositions of G proteins allow further specificity and selectivity in signaling. Regulation of G protein activity is accomplished through interaction with heptahelical receptors, G protein-regulated effectors with GTPase-activating activity, or the recently identified regulators of G protein signaling. In addition, the function of an individual G protein is further regulated by posttranslational modifications induced by bacterial toxins, cellular kinases, or lipidation (5–9).

Many functions of G proteins have been elucidated despite poor expression of some isoforms. However G_o proteins, which have been known for a long time as abundantly expressed proteins, still lack definition of a clear-cut function. In brain they are a major constituent in cells, making up 0.5–1% of the plasma membrane proteins and 10% of the membrane proteins in growth cones (10–12). Expression of G_o is restricted to neuronal and endocrine systems and the heart (13). Transcription of the gene results in alternatively spliced products (14, 15) coding for $G\alpha_{o1}$ and $G\alpha_{o2}$, of which $G\alpha_{o1}$ predominates (16). In view of the limited knowledge about their biological roles G_o proteins were predicted to be a storage form of $G\beta\gamma$ complexes (17). Disruption of the $G\alpha_o$ gene in mice has pointed to a significant participation in the muscarinic regulation of Ca^{2+} channels in heart and a discrete involvement in neurotransmission in the nervous system (18, 19). Furthermore, a large body of evidence has implicated a role for G_o proteins in modulation of voltage-gated calcium channels. It has been demonstrated that the inhibitory actions of G_o on calcium channels are mediated by $G\beta\gamma$ (20–24). G_o proteins also have been detected on endomembranes, suggesting specific roles in vesicular function (25). Recently, we have identified $G\alpha_{o2}$ as a modulator of the monoamine uptake system in granules of chromaffin cells (26).

Interestingly, further heterogeneity of $G\alpha_o$ proteins is evident at the protein level (27–31). In addition to $G\alpha_{o1}$ and $G\alpha_{o2}$, a third $G\alpha_o$ isoform, $G\alpha_{o3}$ or $G\alpha_{oc}$, has been found in mammals (16, 31). This unknown protein represents the second most abundant $G\alpha_o$ isoform, comprising about 30% of total $G\alpha_o$ expressed in mammalian brain (16, 32).

This isoform diversity correlates with functional differences between the three $G\alpha_o$ proteins. Thus, activation of $G\alpha_{o3}$ and $G\alpha_{o1}$, but not of $G\alpha_{o2}$, was observed on depolarization of rat brain synaptoneurosomes (56), whereas activated $G\alpha_{o2}$, but neither $G\alpha_{o1}$ nor $G\alpha_{o3}$, inhibited the vesicular monoamine transporter system in PC-12 cells (26). In contrast to $G\alpha_{o1}$ and $G\alpha_{o2}$, infusion of purified $G\alpha_{o3}$ failed to rescue receptor-induced calcium channel inhibition in pertussis toxin (PT)-treated GH_3 cells (33).

Hitherto several fruitless attempts have been undertaken to identify the structural basis of $G\alpha_{o3}$ (32, 34, 35). In the present study, the primary structures of $G\alpha_o$ isoforms were characterized by using matrix-assisted laser desorption/ionization (MALDI) and nanoelectrospray ionization tandem mass spectrometry (MS) (36). $G\alpha_{o3}$ contains a single amino acid exchange (Asn→Asp) in the extreme C terminus as compared with $G\alpha_{o1}$. This amino acid conversion is likely to explain the observed differences in the signal transduction properties of $G\alpha_{o1}$ and $G\alpha_{o3}$. Because we did not detect corresponding genetic information, we speculate that deamidation is an additional mechanism for regulation of G protein activity.

MATERIALS AND METHODS

Purification of G Proteins. Isolation of G proteins and their $G\alpha$ and $G\beta\gamma$ subunits from bovine brain membranes was reported previously (32). Final resolution of $G\alpha_o$ isoforms was achieved by ion exchange chromatography in fast protein liquid chromatography (FPLC) using a Mono Q HR 5/5 column (Amersham Pharmacia). Subsequent to an additional

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Abbreviations: GTPγS, guanosine 5′-[γ-thio]triphosphate; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; PT, pertussis toxin.

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heptylamine-Sepharose chromatography step $G\beta\gamma$ complexes finally were purified by FPLC as for $G\alpha_o$ subunits (37).

Digestion of Purified Proteins and HPLC Separation of Peptides. For in-gel protein digestion of $G\alpha_o$, bands of resolved proteins were cut from the gel, incubated in the presence of trypsin, chymotrypsin (Sigma), or Asp-N endoproteinase (Calbiochem) and prepared for MS as described (38). Peptides derived from $G\alpha_{o1}$ and $G\alpha_{o3}$ isoforms were separated by using an Applied Biosystems model 140B HPLC pump equipped with a 2.1 mm-by-150 mm reversed-phase column (Vydac C18, The Separations Group). Elution buffers consisted of (A) 0.1% trifluoroacetic acid and (B) 0.07% trifluoroacetic acid in 90% acetonitrile. A 120 μl/min linear gradient from 5% to 60% B was used. Peptide elution was monitored by UV absorption at 214 nm, and peptide-containing fractions were collected and subsequently subjected to MALDI MS analysis.

MS. Mass analysis of crude peptide mixtures generated by in-gel digestion and of HPLC-purified peptides was accomplished by delayed extraction MALDI on a Bruker REFLEX time-of-flight mass spectrometer (Bruker-Franzen Analytik, Bremen) (38). Similarly, aliquots from extracted peptide mixtures or HPLC fractions were analyzed by MALDI. HPLC fractions were screened by using an automated MALDI data acquisition system developed at the European Molecular Biology Laboratory (38). Peptide sequencing was performed by nanoelectrospray tandem MS (36).

Analysis of $G\alpha_o$ mRNA. Total RNA was isolated from rat brains by using the InViSorb RNA kit II (InViTek, Berlin). cDNAs were synthesized by SuperScript II RNAseH$^-$ Reverse Transcriptase (Gibco/BRL) using an oligo(dT) primer. Sequences encoding the C-terminal fragment of $G\alpha_{o1}$ and $G\alpha_{o3}$ were amplified by PCR. Primers (ARK Scientific Biosystems, Dieburg, Germany) used were 5′-G TCA CCC TTG ACC ATC TGC TTT CC-3′ and 5′-TCA GTA CAA GCC ACA GCC CCG GAG-3′, corresponding to nucleotides 1549–1572 and 1771–1748, respectively. PCR was performed in a final volume of 50 μl containing 20 mM Tris·Cl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 200 μM of each dNTP, 100 pmol of each primer, and 250 milliunits of *Taq* DNA polymerase. The amplified DNA fragments were ligated into pGEM-T Easy (Promega). A total of 101 clones were isolated and analyzed on a model 377 sequencer (Perkin–Elmer).

Specific Cleavage of cDNA. In three parallel experiments total mRNA from rat brains was amplified by reverse transcription–PCR. The oligonucleotide 5′-TC CTC AAC AAG AAA GAC CTC-3′, corresponding to nucleotides 1509–1528, was used as a forward primer in all cases. The reverse primers were designed to generate additional restriction cleavage sites when extended by $G\alpha_{o1}$ (*Acl*I used) and $G\alpha_{o3}$ (*Aat*II and *Eco*RV used) sequences. Accordingly, for amplification of $G\alpha_{o1}$-encoding sequences the reverse primer was 5′-A GTA CAA GCC ACA GCC CCG GAG AAC GT-3′ (primer 1, nucleotides underlined: changed nucleotides to generate a new cleavage site), corresponding to nucleotides 1769–1743 in $G\alpha_{o1}$-coding DNA. Because two different oligonucleotides were used to amplify possible cDNA sequences encoding $G\alpha_{o3}$, primers 2 and 3 were 5′-A GTA CAA GCC ACA GCC CCG GAG GAC GT-3′ and 5′-A GTA CAA GCC ACA GCC CCG GAG GAT-3′, respectively, corresponding to nucleotides 1769–1745. Reactions were performed in 20 mM Tris·Cl, pH 8.8/10 mM KCl/10 mM $(NH_4)_2SO_4$/2 mM $MgSO_4$/0.1% Triton X-100/0.1 mg/ml BSA/200 μM of each dNTP/100 pmol of each primer/125 milliunits of *Pfu* DNA polymerase (Stratagene). The generation of cleavage sites by primer extension in PCR cycles was verified by a restriction digest (1 h, 37°C) with the appropriate enzymes. DNA amplified by primer 1 was digested with *Acl*I, while for primer 2- and primer 3-derived PCR products *Aat*II and *Eco*RV, respectively, were used. Internal controls added were pcDNA3 for PCR products obtained with primers 1 and 3 and pQE60 in the experiment using primer 2.

Expression and Purification of Recombinant $G\alpha_{o1}$ and $G\alpha_{o3}$. For expression of recombinant proteins we used the cDNA of murine $G\alpha_{o1}$, which is highly homologous to those of other mammalian species (39). No differences in splicing between murine and bovine $G\alpha_{o1}$ transcripts were observed. Additionally, on the protein level murine and bovine $G\alpha_{o1}$ and $G\alpha_{o3}$ show similar apparent molecular masses and pI values (16, 32) although the deduced amino acid sequence of murine $G\alpha_{o1}$ differs from bovine $G\alpha_{o1}$ in six positions. Wild-type murine $G\alpha_{o1}$ in the pCIS vector was a gift from M.I. Simon, CalTech, Pasadena, CA. PCR was performed as described above with primer 5′-GCG CAG CCC GCG AAT TCA GAT-3′, corresponding to nucleotides 830–850 of the pCIS vector, i.e., 41–21 nt upstream the $G\alpha_{o1}$ insert, and reverse primer 5′-GCG TAA GCT TCA GTA CAA GCC GCA-3′, corresponding to nucleotides 1771–1757 of $G\alpha_{o1}$, i.e., its C terminus. Asn at position 346 in $G\alpha_{o1}$ was mutated to Asp by site-directed mutagenesis (italic letters) using the primer 5′-GCG TAA GCT TCA GTA CAA GCC ACA GCC CCG GAG ATT ***GTC*** GGC AAT GAT G-3′, corresponding to nucleotides 1771–1732. All clones constructed were confirmed by DNA sequencing. The amplified DNA fragments were used for construction of recombinant baculoviruses as described (37). $G\alpha_o$ isoforms were expressed together with $G\beta_1$- and $G\gamma_2$-His_6 and purified from Sf9 cell membranes as detailed elsewhere (40).

SDS/PAGE and Immunoblotting. SDS gels contained 6 M urea. Resolved proteins were transferred to poly(vinylidene difluoride) membranes (Immobilon P, Millipore) and detected with subtype-specific rabbit antisera (32).

^{35}S-Guanosine 5′-[γ-Thio]Triphosphate (GTPγS) Binding and ADP Ribosylation of G Proteins by PT. Purified G proteins were depleted from aluminium fluoride by dialyzing the samples against a buffer consisting of 20 mM Tris·Cl (pH 8.0), 1 mM EDTA, 20 mM 2-mercaptoethanol, 100 mM NaCl, 12 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, and 10 μM GDP. For analysis of differences in the time course of ^{35}S-GTPγS (NEN) binding to $G\alpha_o$ isoforms we followed a published protocol (41). The time course of binding reactions was monitored at 25°C. $G\alpha_o$ isoforms were ADP-ribosylated in the presence of various concentrations of purified $G\beta\gamma$ complexes by using PT. $G\alpha$ and $G\beta\gamma$ were incubated at indicated concentrations in 25 mM Tris·Cl, pH 8.0/1 mM EDTA/0.2% (wt/vol) Lubrol (Sigma)/2.5 mM $MgCl_2$/2.5 mM ATP/30 μM ^{32}P-NAD (5×10^6 cpm; NEN)/7.4 μg/ml activated PT (Calbiochem) for 30 sec at 32°C. Under these conditions ADP ribosylation was linear as a function of time and toxin concentration. For maximal ADP ribosylation, reactions were allowed to proceed for 3 h at 32°C or 2 days at 5°C (42). Samples were subjected to urea-SDS/PAGE. For quantification of ^{32}P-incorporation, dilutions of the reaction mixture were spotted on nitrocellulose membranes and dried. Gel slabs and membranes were autoradiographed by using a BAS 1500 Fuji-imager (Raytest, Straubenhard, Germany).

Statistics. A paired Student's *t* test was used to compare results within individual experiments. Values quoted are means ± SD.

RESULTS AND DISCUSSION

Analysis of Purified Monomeric $G\alpha_o$ and Heterotrimeric G_o Proteins. Previous work has identified $G\alpha_{o3}$ as a major G protein present in mammalian brain of different species. It exhibits an apparent molecular weight similar to $G\alpha_{o1}$ in standard SDS/PAGE, whereas addition of urea (4–6 M) results in distinct gel electrophoretic mobilities of these two $G\alpha$ isoforms. Regardless of the species tested $G\alpha_{o3}$ proteins exhibit a more acidic pI value and a slower mobility in urea-SDS/PAGE than $G\alpha_{o1}$ and $G\alpha_{o2}$ (16), allowing purification of this $G\alpha$ isoform by conventional chromatographic approaches (32). Analysis of the three $G\alpha_o$ isoforms with specific antibodies excluded that $G\alpha_{o3}$ represents a

variant of the spliced isoform $G\alpha_{o2}$. When purified as heterotrimers, $G\alpha_o$ isoforms have been reported to possess individual $G\gamma$ subunit profiles (31). To identify the $G\beta$ isoforms copurifying with $G\alpha_o$ proteins, we separated heterotrimeric $G_{i/o}$ proteins by fast protein liquid chromatography using Mono Q columns (Fig. 1). In accordance with previous reports, G_{o2} eluted in front of G_{o1} followed by G_{o3} (43–45). This pattern matches the previously communicated $G\gamma$ profile of the individual $G\alpha_o$ isoforms (31). However, the $G\beta$ composition also reproducibly differed between the three purified G_o proteins. Although $G\alpha_{o1}$ and $G\alpha_{o2}$ associated with $G\beta_1$ and $G\beta_2$ isoforms, $G\alpha_{o3}$ almost exclusively copurified with $G\beta_2$ (Fig. 1).

MS Analysis of $G\alpha_o$ Primary Structure. Our previous work has indicated that native as well as denatured $G\alpha_{o3}$ represents a more acidic and a slightly more hydrophilic protein compared with $G\alpha_{o1}$ (refs. 16 and 32; T.E. and B.N., unpublished work). Although a difference in pI values of 0.15 units may suggest that $G\alpha_{o3}$ could represent an ADP-ribosylated variant of $G\alpha_{o1}$, the PT-sensitive cysteine at the C terminus was not modified. Subsequent biochemical studies also confirmed the absence of other posttranslational modifications such as phosphorylation, glycosylation, or lipidation. These initial results with $G\alpha_o$ proteins were observed among all species tested. Bovine brain membranes were chosen as the source for large-scale purification of $G\alpha_o$ proteins used in this study.

To identify the structural difference between the two proteins we enzymatically digested $G\alpha_{o1}$ and $G\alpha_{o3}$ and used MS. Tryptic and chymotryptic peptide mass maps of purified $G\alpha_{o1}$ and $G\alpha_{o3}$ were obtained by MALDI. As expected from previous work (32, 35) the tryptic peptide mass maps predominantly displayed peptides from the N-terminal domain of $G\alpha_{o1}$ and $G\alpha_{o3}$ and did not reveal any differences between the two isoforms in this region (underlined in Fig. 2). For both isoforms, a mass consistent with the myristoylated but unpalmitoylated N-terminal tryptic peptide was detected. In contrast, the chymotryptic peptide mass maps displayed peptides from the central and C-terminal domain of $G\alpha_{o1}$ and $G\alpha_{o3}$ (italicized in Fig. 2). A close inspection of the MALDI peptide mass maps revealed that the only significant difference was a mass increase of +0.95 Da of a peptide signal at m/z 1978.07 in $G\alpha_{o1}$ to m/z 1979.02 in $G\alpha_{o3}$ (Fig. 3 *A* and *B*). This peptide mass was assigned as the chymotryptic peptide DAVTDIIIANNLRGCGLY (predicted m/z 1978.01) generated from the extreme C terminus of $G\alpha_{o1}$ by cleavage after ^{336}Phe.

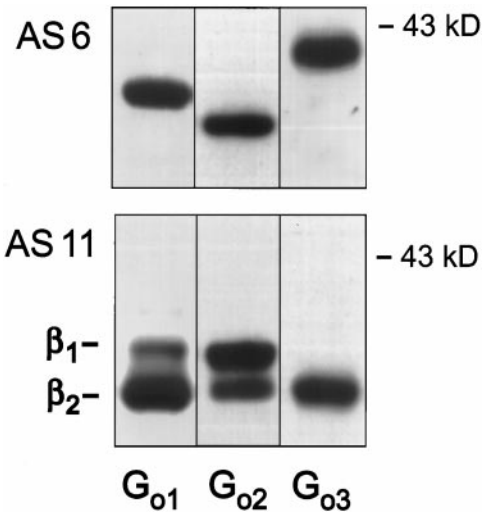

FIG. 1. Immunostain of G_o proteins purified from bovine brain membranes. Proteins were resolved on urea-SDS/PAGE and immunoblotted with an anti-$G\alpha_o$ common antiserum (AS 6) (*Upper*) or a $G\beta_1$- and $G\beta_2$-detecting antiserum (AS 11) (*Lower*).

GCTLSAEERAALERSKAIEKNLKEDGISAAKDVKLLLLGAGESGKSTIVKQMKIIHEDGF

SGEDVKQYKPVVY*SNTIQSLAAIVRAMDTLGIEY*GDKERKADAKMVCDVVSRMEDTE

PFSPELLSAMMRLWGDSGIQECF*NRSREYQLNDSAKY*YLDSLDRIGAADYQPTEQDI

LRTRV*KTTGIVETHFTFKNLHF*RLF*DVGGQRSERKKWIHCFEDVTAIIFCVALSGYDQV*

*LHEDETTNRMHESLMLFDSICNNKFFIDISIILFLNKKDLFGEKIKKSPLTICFPEY*TGSN

TYEDAAAYIQAQF*ESKNRSPNKEIY*CHMTCATDTNNIQVVF*DAVTDIIIA*$\frac{D}{N}$*NLRGCGLY*

FIG. 2. Amino acid sequence of $G\alpha_{o1}$ and $G\alpha_{o3}$ covered by tryptic peptides (underlined) and chymotryptic peptides (italicized) assigned by MALDI peptide mass mapping or by MALDI analysis of HPLC fractions.

To analyze the structural differences of the respective $G\alpha_{o1}$ and $G\alpha_{o3}$ chymotryptic peptides, we used nanoelectrospray tandem MS for peptide sequencing. It was not possible to detect the C-terminal $G\alpha_{o3}$ peptide by direct nanoelectrospray mass analysis of the peptide mixture. Instead, the chymotryptic peptides were separated by narrow-bore reverse-phase HPLC followed by offline analysis by automated MALDI MS to identify the fractions containing the native and modified C-terminal peptide. The m/z 1978 peptide was detected in a minor fraction eluting at 87.1 min (Fig. 3*A*) whereas the m/z 1979 peptide from $G\alpha_{o3}$ was detected in a fraction eluting at 87.7 min (Fig. 3*B*). Isolated peptides from $G\alpha_{o1}$ and $G\alpha_{o3}$ were sequenced by nanoelectrospray tandem MS. In the $G\alpha_{o1}$-derived peptide, a series of y-ion signals corresponding to cleavage of amide bonds confirmed that this chymotryptic peptide originated from the C terminus of $G\alpha_o$ with the predicted sequence (Fig. 3*C*, *Inset*). In the $G\alpha_{o3}$-derived peptide, a mass shift of +1 Da was observed for all peptide fragment ions containing the amino acid in position 346, thereby localizing and identifying the modification as ^{346}Asn→Asp (Fig. 3*C*). The finding that $G\alpha_{o3}$ had an additional anionic residue was consistent with a difference in HPLC elution time between the two peptides (see above). A mass difference of +1 Da was observed exclusively in two distinct C-terminal peptide pairs of which one was obtained after cleavage with trypsin and one after digestion with chymotrypsin. Correspondingly, results from peptide mass mapping and determination of the total masses of $G\alpha_{o1}$ and $G\alpha_{o3}$ made additional variations between the two $G\alpha$ isoforms unlikely.

Generation of $G\alpha_{o3}$. Thus far, we have shown that $G\alpha_{o3}$ differs from $G\alpha_{o1}$ by conversion of ^{346}Asn→Asp. This amino acid exchange might have resulted from an artificial deamidation of $G\alpha_{o1}$ during purification and analysis of the proteins. To address such an instability of the γ-amide bond, we rechromatographed isolated $G\alpha_{o1}$ devoid of $G\alpha_{o3}$ on a Mono Q column by using the identical conditions as for purification of $G\alpha_{o3}$. As seen in Fig. 4*A* only a single band was visible on immunoblots of the eluted fractions, excluding a purification artifact. Although a mass difference of +1 Da was seen exclusively in two distinct C-terminal peptide pairs after cleavage with different enzymes, this observation does not exclude the presence of an additional variation between $G\alpha_{o1}$ and $G\alpha_{o3}$. However, peptide mass mapping covered the entire sequence with the exception of two amino acids for both isoforms (Fig. 2). In addition we could exclude major differences in molecular weight by nanoelectrospray MS analysis of intact proteins of $G\alpha_{o1}$ and $G\alpha_{o3}$, which showed similar total masses for the two isoforms. To test the effect of the observed amino acid exchange we replaced the coding triplet 346 (AAC) of the murine cDNA of $G\alpha_{o1}$ by GAC using site-directed mutagenesis. This replacement would result in translation of Asp instead of Asn at position 346. $G\alpha_{o1}$- and $G\alpha_{o3}$-encoding cDNAs were used to generate recombinant baculoviruses. After infection with these viruses, Sf9 cells overexpressed $G\alpha_o$-immunoreactive proteins that showed electrophoretic properties identical to their respective native murine counterparts (Fig. 4*B*). To confirm the presence of ^{346}Asp instead of Asn in recombinant $G\alpha_{o3}$ we subjected the purified protein to

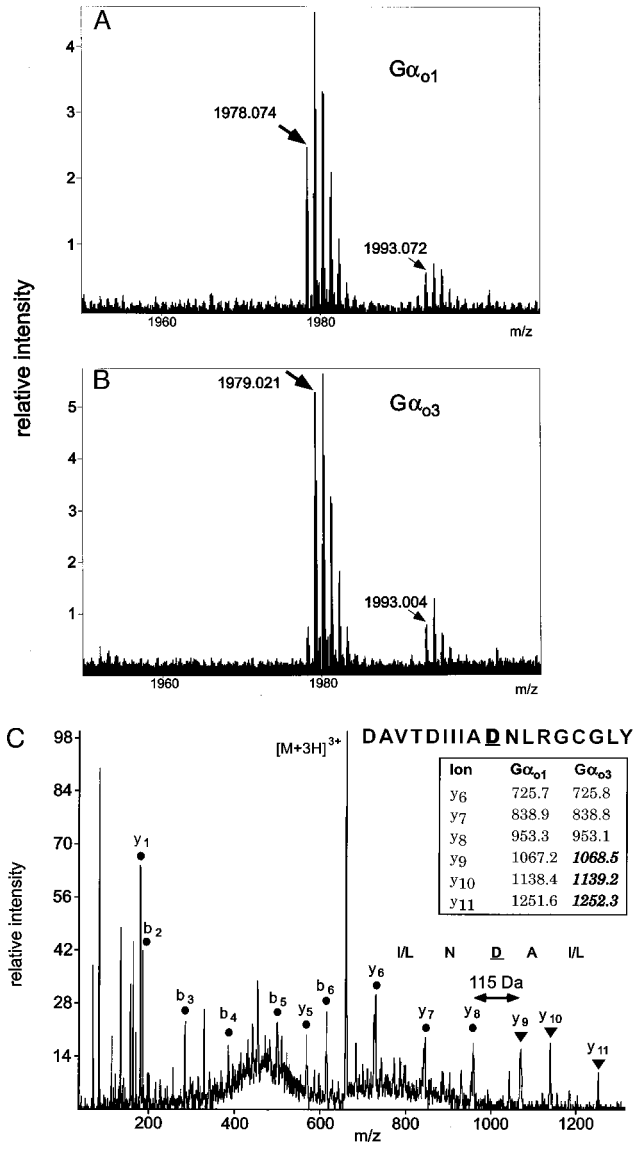

FIG. 3. Identification of the modified amino acid residue by MS. Part of MALDI MS peptide mass map obtained after chymotryptic digestion of $G\alpha_{o1}$ (*A*) and $G\alpha_{o3}$ (*B*). Peptides at m/z 1978.074 ($G\alpha_{o1}$) and 1979.021 ($G\alpha_{o3}$) were assigned as the normal and modified octadeca C-terminal peptide, respectively. Signals at m/z 1993.0 are of unknown origin but appear identical in both MALDI MS peptide mass maps. (*C*) Nanoelectrospray tandem mass spectrum obtained from the HPLC-isolated chymotryptic peptide from the $G\alpha_{o3}$ isoform. Tandem MS of the triply charged peptide ions generated C-terminal (y type) and N-terminal (b type) peptide fragment ions. All y-type fragment ions containing amino acid residue 346 of $G\alpha_{o3}$ displayed a mass shift of +1 Da as compared with $G\alpha_{o1}$ (*Inset*). This finding identifies the modification as an Asn to Asp conversion in position 346, consistent with the amino acid sequence DAVTDIIIA**D**NLRGCGLY where C is S-carbamidomethylcysteine.

digestion by chymotrypsin and endoprotease Asp-N, and monitored these reactions by MS. Inspection of the MALDI peptide mass maps after chymotrypsin treatment of recombinant $G\alpha_{o3}$ revealed a peptide signal at m/z 1979.0 instead of m/z 1978. As for native $G\alpha_{o3}$ this peptide mass was assigned as the chymotryptic peptide DAVTDIIIA**D**NLRGCGLY generated from the C terminus of recombinant $G\alpha_{o3}$. We also confirmed that endoprotease Asp-N cleaved between ^{345}Ala and ^{346}Asp of recombinant $G\alpha_{o3}$ to generate a peptide signal at m/z 1067.6 corresponding to the C-terminal peptide ^{346}DNLRGCGLY (data not shown). Hence, an exchange of a single amino acid, i.e., ^{346}Asn→Asp, by site-directed mutagenesis resulted in expression of a recombinant protein with gel

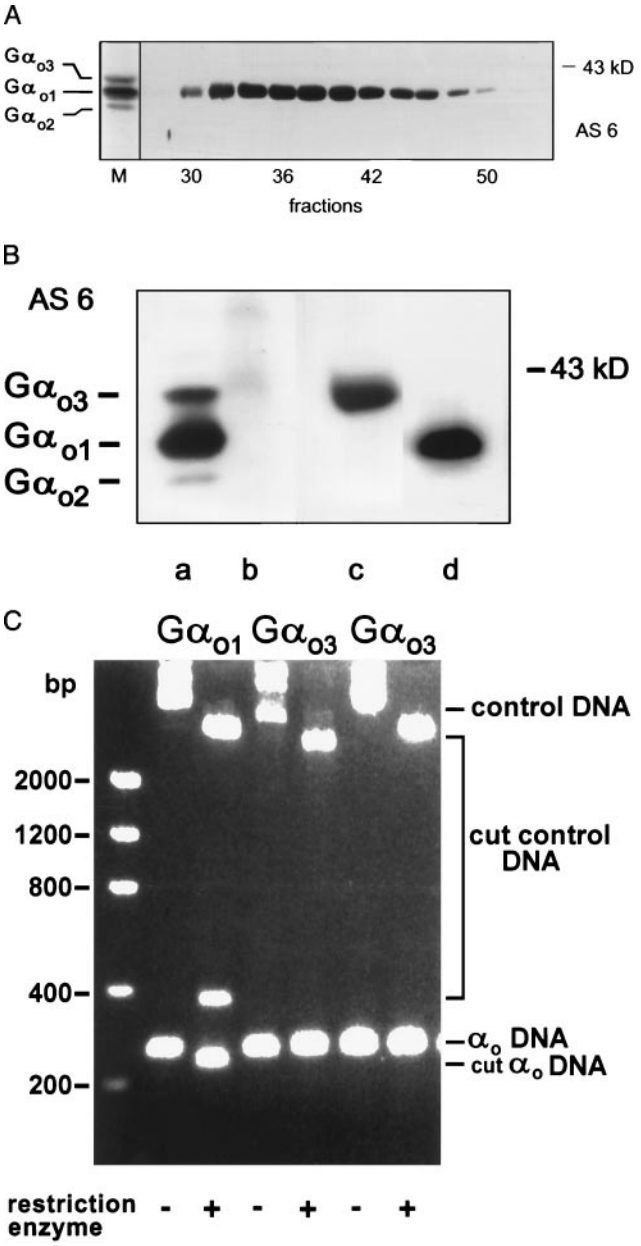

FIG. 4. (*A*) Immunoblot of fractions containing $G\alpha_{o1}$ but not $G\alpha_{o3}$ collected after rechromatography of purified $G\alpha_{o1}$ using antiserum AS 6. The migration pattern of a purified mixture of three $G\alpha_o$ isoforms is shown on the left (M). (*B*) AS 6-stained immunoblot showing the mobilities of native murine brain $G\alpha_o$ isoforms (lane a) in comparison to recombinant murine $G\alpha_{o1}$ (lane d) and $G\alpha_{o3}$ (lane c). Lane b shows the absence of proteins after wild-type baculovirus infection of Sf9 cells. Apparent molecular mass of a marker protein is indicated. (*C*) Cleavage of $G\alpha_o$-specific PCR products and control DNA by restriction enzymes. Total mRNA from rat brains was amplified by reverse transcription–PCR. While the forward primer was identical in all three experiments, reverse primers differed to generate additional restriction cleavage sites for *Acl*I in the case of $G\alpha_{o1}$, or *Aat*II and *Eco*RV for the two possible $G\alpha_{o3}$-specific sequences. The presence of cleavage sites generated by primer extension in PCR cycles was verified by a restriction digest with the appropriate enzymes. The amplified cDNAs and resulting cleavage products were separated on agarose gels and visualized by ethidium bromide staining. In the case of $G\alpha_{o1}$ the intact PCR product (−; α_o DNA) was 260 bp in length, whereas the cleaved product (+; cut α_o DNA) had a length of 233 bp. The internal controls (control DNA) added were pcDNA3 for PCR products obtained with primers 1 and 3 and pQE60 in the experiment using primer 2. Note that cleavage of pcDNA3 by *Acl*I resulted in an additional product of 373 bp (third lane from left). Positions of DNA standards are shown on the left.

electrophoretic properties similar to native $G\alpha_{o3}$ but distinct from both native and recombinant $G\alpha_{o1}$ (Fig. 4*B*).

Having confirmed the sequence of a major G protein in brain we sought to identify the basis for its generation. Previous analysis of the genomic sequences has revealed the existence of transcripts encoding only $G\alpha_{o1}$ and $G\alpha_{o2}$ (39, 46). Disruption of this gene in mice resulted in complete loss of $G\alpha_o$-specific immunoreactivity in homozygous knockout animals (18, 19). Therefore, the genetic information for $G\alpha_{o3}$ must be located on this $G\alpha_o$ gene. Accordingly, we performed reverse transcription–PCR on total mRNA obtained from rat brain. Guided by the sequence of $G\alpha_{o1}$ we generated cDNAs encoding C-terminal regions of $G\alpha_{o1}$ and $G\alpha_{o3}$ to identify a $G\alpha_{o3}$-specific transcript. However, cloning and sequencing of 101 of these constructs yielded only nucleotide sequences coding for the C terminus of $G\alpha_{o1}$ but failed to detect any $G\alpha_{o3}$-coding cDNA. In a second independent and complementary approach $G\alpha_{o1}$- and $G\alpha_{o3}$-specific primers were used to generate additional restriction cleavage sites when extended by $G\alpha_{o1}$ and $G\alpha_{o3}$ sequences. Because of a degeneration for the Asp code two alternative triplets had to be considered for $G\alpha_{o3}$. Analysis of the cleavage sites formed in the PCR products again showed no $G\alpha_{o3}$-specific cDNA (Fig. 4*C*). Only the $G\alpha_{o1}$-specific product was cleaved whereas both $G\alpha_{o3}$-specific products remained unaffected. In contrast, plasmid DNA supplied in the same tube as the PCR product was cut by the respective enzymes. Therefore, processes such as allelic variations, alternative splicing, or mRNA editing are unlikely to generate $G\alpha_{o3}$ at the transcriptional level.

Functional Consequences of $G\alpha_{o1}$ Deamidation. The ^{346}Asn→Asp exchange observed between $G\alpha_{o1}$ and $G\alpha_{o3}$ alters the charge of the C terminus. C-terminal regions of $G\alpha$ were found important for receptor and effector coupling (3). Therefore deamidation of $G\alpha_{o1}$ may affect biochemical and cellular signaling properties of this isoform. It has been observed that infusion of $G\alpha_{o1}$ and $G\alpha_{o2}$, but not $G\alpha_{o3}$, restored receptor-mediated calcium channel inhibition in cultured cells after stimulation with various agonists (32, 33). Alterations in the C terminus also could affect the sensitivity of $G\alpha$ toward PT, as PT ADP-ribosylates ^{351}Cys close to position 346. We previously have noticed that under standard conditions G_{o3} appeared to be less well ^{32}P-ADP-ribosylated by PT than G_{o1} (32). Therefore, we studied initial rates of PT-mediated ADP ribosylation of $G\alpha_{o1}$ and $G\alpha_{o3}$ (Fig. 5*A*). Short-term incubation revealed significant differences in the velocity of ADP ribosylation between $G\alpha_{o1}$ and $G\alpha_{o3}$ (Fig. 5*A*, *Left*) whereas no differences in maximal incorporation of ^{32}P-ADP into $G\alpha_{o1}$ and $G\alpha_{o3}$ were observed after prolonged incubation of $G\alpha$ in the presence of equimolar amounts of $G\beta\gamma$ complexes (data not shown). Because we observed that the three purified G_o proteins differed in their $G\beta$ composition (Fig. 1), we could not exclude that the difference in velocity of ADP ribosylation was caused by a different interaction of $G\alpha_{o1}$ and $G\alpha_{o3}$ with $G\beta\gamma$ dimers. Thus, differences in PT-mediated ^{32}P-ADP ribosylation could be secondary to $\alpha\beta\gamma$ heterotrimer formation rather than representing an immediate consequence of the different C termini. Hence, we carried out a series of experiments in which $G\alpha_{o1}$ and $G\alpha_{o3}$ were incubated with increasing concentrations of $G\beta\gamma$ complexes. In the presence of excess $G\beta\gamma$, we observed similar maximum rates of ^{32}P-ADP ribosylation between $G\alpha_{o1}$ and $G\alpha_{o3}$ (Fig. 5*A*, *Right*). Thus, increasing the $G\beta\gamma$ concentration abolished differences in reaction velocity, indicating that $G\alpha_{o1}$ and $G\alpha_{o3}$ have different affinities to $G\beta\gamma$. Accordingly, the EC_{50} values were 60 nM and 280 nM $G\beta\gamma$ for $G\alpha_{o1}$ and $G\alpha_{o3}$, respectively. Therefore, we speculate that the different rates of PT-mediated ADP ribosylation observed between $G\alpha_{o1}$ and $G\alpha_{o3}$ are the result of differences in affinity to $G\beta\gamma$. Evidence from cross-linking studies suggest an interaction of the C terminus of $G\alpha$ with $G\gamma$ (47).

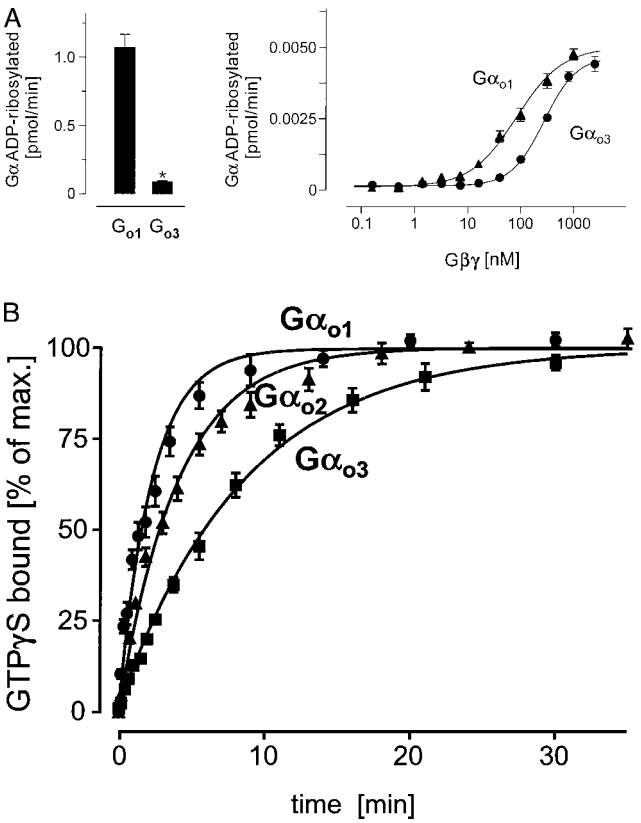

FIG. 5. (*A*) PT-mediated ADP ribosylation. $G\alpha_o$ isoforms (350 nM, *Left*; 4 nM, *Right*) were ADP-ribosylated in the presence of stoichiometric (*Left*) or increasing concentrations (*Right*) of $G\beta\gamma$ complexes using PT. The reactions were stopped by addition of an equal volume of 2× concentrated electrophoresis sample buffer and subjected to urea-SDS/PAGE. For quantification of ^{32}P-incorporation dilutions of the reaction mixture were spotted on nitrocellulose membranes and dried. Gel slabs and membranes were autoradiographed and analyzed by using a PhosphorImager. Data shown are mean values ± SD ($n = 3$) from one typical experiment of three (*, $P < 0.01$). (*B*) Time course of ^{35}S-GTPγS binding to three purified $G\alpha_o$ isoforms. The appropriate $G\alpha$ was added to the reaction mixture in a final concentration of 3–5 nM in the presence of 1 mM EDTA at 25°C. The binding reaction was carried out at 50 nM ^{35}S-GTPγS, yielding a total binding of 110,000 to 180,000 cpm. The reaction was stopped at the indicated time points by diluting samples with ice-cold buffer followed by filtration through nitrocellulose. Filters were washed and counted in a liquid scintillator counter. Nonspecific binding was less than 5% of the total (3,600 to 7,800 cpm). Shown are mean values ± SD ($n = 3$) from one typical experiment of three.

It is well established that changes in the C-terminal sequence affect the specificity of G protein-receptor coupling and activation kinetics (48). In the activated GTPγS-bound state, the extreme C terminus has been found to contact the switch II region of $G\alpha$ (49). We therefore compared the activation reactions of the $G\alpha_o$ isoforms. Previously, it was reported that heterotrimeric G_{o1} and G_{o2} differed in their GTPγS binding rates (41). We confirmed these results (not shown) and extended them by testing $G\alpha$ monomers of all three $G\alpha_o$ isoforms (Fig. 5*B*). $G\alpha_{o1}$ and $G\alpha_{o2}$ still exhibited a faster GTPγS binding than $G\alpha_{o2}$, whereas $G\alpha_{o3}$ bound GTPγS with the slowest rate. This observation is in agreement with suggestions that the C terminus of $G\alpha$ is functionally involved in conformational changes of $G\alpha$ during its activation on GTP binding. The C terminus contains the α5 helix, which is partially unwound during activation (50, 51) and has been proposed to function as an internal GDP dissociation inhibitor (52). Interestingly, ^{346}Asn, as the last residue of the α5 helix in the inactive GDP-bound state, contributes to the stability of the helix. Because Asn, but not Asp, is known as a favored amino acid for this position, the substitution by a carboxylate for the amide is

likely to alter the stability of this α5 helix, affecting the GDP/GTP-exchange reaction (53). In this context it was interesting to notice significant differences in the time course of GTPγS binding between $G\alpha_o$ isoforms (Fig. 5*B*). Our findings support the idea that closely related G protein isoforms display significant differences in the signal transduction cascade caused by differences in activation kinetics.

The ^{346}Asn→Asp exchange changes the physicochemical and functional properties of $G\alpha_{o1}$, increasing diversity in G_o-dependent cellular signaling. Moreover, we obtained preliminary evidence that deamidation is not restricted to $G\alpha_{o1}$. In this context, the observation that *Escherichia coli* cytotoxic necrotizing factor-1 deamidates ^{63}Gln of Rho proteins, yielding a constitutively active Rho, may be of relevance (54, 55). Our data suggest that mammals also express deamidases, which convert $G\alpha_{o1}$ to $G\alpha_{o3}$. This hypothesis is supported by the observation that cell lines generate $G\alpha_{o1}$ and $G\alpha_{o3}$ in varying relative amounts. For instance, HIT cells express $G\alpha_{o1}$ and $G\alpha_{o3}$ in roughly equal amounts whereas RIN or GH_3 cells express only $G\alpha_{o1}$ (16, 33). Moreover, although the relative concentrations of all $G\alpha_o$ isoforms have been found to be roughly constant among total brain membrane preparations of various species (16), more detailed analysis has shown that relative expression levels of $G\alpha_{o1}/G\alpha_{o3}$ varied considerably among cells or in distinct regions of the brain (28–30). Furthermore, the $G\alpha_{o1}/G\alpha_{o3}$-concentration ratios changed on development of Alzheimer's disease, suggesting different functional control of $G\alpha_o$ isoforms (K. Kolasa, personal communication). The reported differences in functional properties of $G\alpha_{o3}$ encourage further studies to establish deamidation as an additional regulatory mechanism of G protein function.

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