Interactions between the Amino- and Carboxyl-Terminal Regions of Gα Subunits: Analysis of Mutated $G\alpha_o/G\alpha_{i2}$ Chimeras†

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ABSTRACT: Receptors activate the Gα subunits of heterotrimeric G proteins by binding to the C-terminus and reducing their affinity for bound GDP, therefore promoting exchange of GDP for GTP. Although this general mechanism is the same for all Gα subunits, different Gα subunits vary in nucleotide binding and hydrolysis even though the residues that make up the guanine nucleotide binding site are virtually identical. We have shown previously that truncation of 14 amino acids from the C-terminus of $G\alpha_o$ decreased the apparent affinity for GDP and permitted us to see an activated conformation with GTP [Denker, B. M., et al. (1992) *J. Biol. Chem. 267,* 9998−10002]. To test whether mutations in the receptor binding region lead to different phenotypes in closely related Gα subunits, we made the equivalent deletions in $G\alpha_{i2}$, synthesized the proteins in vitro in a rabbit reticulocyte lysate and used the pattern of native tryptic proteolysis as an index of conformation. The phenotype of truncated $G\alpha_{i2}$ was different from that of truncated $G\alpha_o$: GDP affinity was reduced, but we could not detect an activated conformation with GTP (although GTPγS activated normally). Analysis of shorter deletions showed that loss of three hydrophobic residues (between 11 and 13 residues from the C-terminus) was responsible for the phenotypes. To define the regions of $G\alpha_o$ and $G\alpha_{i2}$ that were responsible for their different phenotypes, we used a conserved *Bam*HI site (codon 212) to make chimeras. Each chimera truncated at the C-terminus had the phenotype of the donor of the amino-terminal portion. Both truncated chimeras were activated by GTPγS-like wild-type proteins, and both had decreased apparent affinity for GDP. Full-length chimeric subunits behaved like wild-type proteins. The crystal structure of $G\alpha_t$ and $G\alpha_{i1}$ shows that the three hydrophobic amino acids we have identified make contact with residues in the N- and C-terminal portions of the protein. Our studies point to the importance of the contacts in the N-terminal region (start of β strands 1 and 3) that may stabilize the C-terminal α helix, affect nucleotide binding, and determine the characteristic features of different Gα subunits.

The heterotrimeric G proteins, made up of Gα and Gβγ subunits, transmit signals from plasma membrane receptors to a variety of intracellular effector enzymes and ion channels. Agonist-liganded hormone receptors cause Gα subunits to release GDP. The Gα subunits then bind GTP, become activated, and remain activated until their intrinsic GTPase activity hydrolyzes GTP to GDP [reviewed in Neer and Clapham (1988), Bourne et al. (1990, 1991), and Simon et al. (1991)].

Recently, the crystal structures of $G\alpha_t$ (transducin α) and $G\alpha_{i1}$ have been solved (Noel et al., 1993; Coleman et al., 1994). The amino acid sequences of $G\alpha_t$ and $G\alpha_{i1}$ subunits are similar to those of the other Gα subunits, especially those of the $G\alpha_o/G\alpha_i$ family (about 68% identity). $G\alpha_o$ is 69% identical to $G\alpha_{i2}$, while $G\alpha_{i1}$ and $G\alpha_{i2}$ are 88% identical. There is only a single amino acid difference in the binding site between $G\alpha_{i1}$ and $G\alpha_t$ (Leu instead of Val) at position 273 (Coleman et al., 1994). All 14 amino acids that directly contact the bound guanine nucleotide, and the 6 residues that interact with it indirectly, are identical in $G\alpha_o$, $G\alpha_{i1}$, and $G\alpha_{i2}$. Despite the conservation of amino acids comprising the nucleotide binding site, there are differences in both the structure of the binding site and the guanine nucleotide binding characteristics. For example, Arg-179, which is important for cleavage of the γ phosphate (Figure 1), is oriented differently in $G\alpha_t$ and $G\alpha_{i1}$ (Noel et al., 1993; Coleman et al., 1994). Such differences may have important effects on nucleotide binding. In the G_o/G_i family, $G\alpha_o$ binds GTPγS[1] faster than $G\alpha_{i2}$ (K_{app} = 0.27 versus 0.063 min^{-1}) and releases GDP faster (k_{off} = 0.19 versus 0.072 min^{-1}). The steady-state GTPase activity of $G\alpha_o$ is faster than $G\alpha_{i2}$ (turnover number = 0.27 versus 0.095 min^{-1}) (Ferugson et al., 1986; Linder et al., 1990) although the catalytic constant (k_{cat}) is identical (Linder et al., 1990). There are significant differences in nucleotide binding even within the $G\alpha_i$ subfamily where the proteins are 85−90% identical. $G\alpha_{i2}$ binds GTPγS faster and releases GDP faster than $G\alpha_{i1}$ or $G\alpha_{i3}$ (Carty et al., 1990; Linder et al., 1990). Since the residues that contact the guanine nucleotide are probably the same in these Gα subunits, it is likely that these functional differences arise from subtle variations in the positioning of the residues with respect to the nucleotide.

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[1] Abbreviations: G proteins, guanine nucleotide binding proteins; GTPγS, guanosine 5′-(γ-thio)triphosphate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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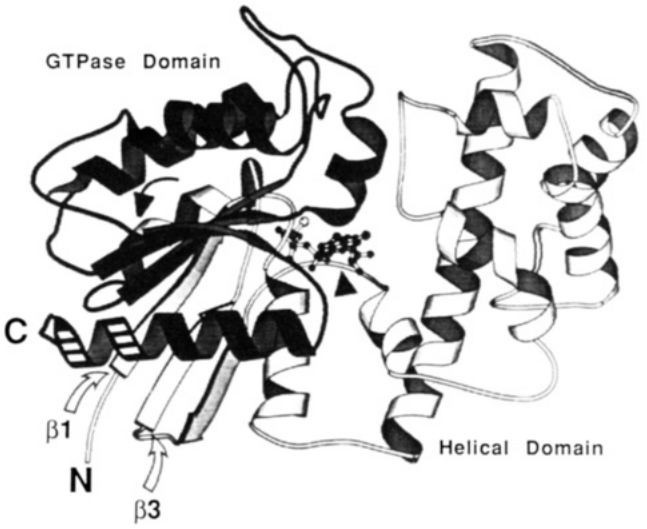

FIGURE 1: Model of Gα subunit. The coordinates for the crystal structure of $G\alpha_t$ (Noel et al., 1993) were used to generate a three-dimensional model with the program Molescript (Kraulis, 1991). The C-terminal portion (amino acids 208−349 of transducin) is shaded darker, and the GTPase and helical domains are noted. The N- and C-terminus are marked with N and C, respectively. The crystal structure does not include the first 26 amino acids of transducin or the last amino acid. The β1 and β3 sheets are marked by open arrows. The curved solid arrow indicates the chimera site in helix α 2. The site of tryptic cleavage for GDP-liganded Gα subunits is a few amino acids amino-terminal to the chimera site. Cleavage at this site generates approximately 25- and 17-kDa fragments. The solid triangle indicates linker 2, which contains Arg-179, which is essential for GTP hydrolysis. The bound GTP can be seen as a stick and ball structure just above linker 2. The site of the 14 amino acid C-terminal deletion is noted with white stripes in the C-terminal helix.

We previously reported that deletion of 14 amino acids from the C-terminus of $G\alpha_o$ decreases GDP affinity with little or no change in GTPγS affinity (Denker et al., 1992b). Like a receptor-activated Gα subunit, the truncated $G\alpha_o$ rapidly exchanges GDP for GTP and can be activated by GTP. Normally, GTP does not activate the Gα subunits in solution because the GDP that is rapidly formed from hydrolysis of GTP remains bound to the active site. We now report that an equivalent deletion of 14 amino acids from the C-terminus of $G\alpha_{i2}$ also reduces apparent GDP affinity but does not lead to a detectable activated state. Analysis of shorter C-terminal truncations suggests that removal of three well-conserved hydrophobic residues 11−13 amino acids from the C-terminus causes the observed phenotypes of $G\alpha_o$ and $G\alpha_{i2}$. This result gave us the opportunity to segregate the contributions of different parts of $G\alpha_o$ and $G\alpha_{i2}$ to the final phenotype of the truncated molecule by analysis of $G\alpha_o$ and $G\alpha_{i2}$ chimeras. We deleted 14 amino acids from the C-terminus of each chimera and asked whether the phenotype was determined by the "donor" of the N-terminal or the C-terminal portion. A convenient way to make chimeras is to divide $G\alpha_o$ and $G\alpha_{i2}$ at a highly conserved *Bam*HI site in the cDNA at codon 212. The *Bam*HI site divides the molecule nearly in half (60% of the sequence from the N-terminal and 40% from the C-terminal portion).[2] It divides the residues that make up the guanine nucleotide binding site (see Figure 1) and segregates the two major regions of sequence difference between $G\alpha_o$ and $G\alpha_{i2}$ (amino acids 61−175 and 300−317). The region immediately surrounding the *Bam*HI site is highly conserved with 12 identical amino acids on the N-terminal side and 5 identical amino acids on the C-terminal side (a single conservative difference is then followed by another 11 identical amino acids) in $G\alpha_o$ and $G\alpha_{i2}$.

We used the native tryptic cleavage patterns of [^{35}S]-methionine-labeled $G\alpha_o$ and $G\alpha_{i2}$ made in rabbit reticulocyte lysates as a tool to assay the overall conformation of the Gα subunit. This approach allows rapid detection of labeled tryptic peptides and has been extensively utilized for Gα subunit analysis (Denker et al., 1992a). Although in vitro translation is not suitable for some kinds of biochemical analysis, the fact that the protein is radiolabeled makes other analysis simpler. For example, tryptic cleavage patterns of in vitro translated proteins are very sensitive indicators of the conformation of Gα subunits. There are over 40 potential tryptic cleavage sites in $G\alpha_o$ and $G\alpha_{i2}$, most of which are inaccessible in the correctly folded molecule. When GTPγS (a nonhydrolyzable analogue of GTP) is bound to the $G\alpha_o$ subunit, proteolysis by trypsin only occurs near lysine 21 and yields a stable 37-kDa fragment. In the presence of GDP or of GTP (which is cleaved to GDP, which then persistently occupies the nucleotide binding site), the 37-kDa fragment is further cleaved by trypsin to approximately 25- and 17-kDa peptides (Fung & Nash, 1983; Hurley et al., 1984; Winslow et al., 1986). Stabilization of the 37-kDa form is an index of the activated (or GTP-liganded) conformation of $G\alpha_o$ and $G\alpha_{i2}$, while production of the 25- and 17-kDa products reflects the inactive, GDP-liganded state. The tryptic sites that are accessible in the GDP-liganded form are near Arg-209 in a region that changes conformation on GDP binding and is within a few amino acids of the junction site of the chimeric molecules (see Figure 1). Our analysis of the properties of wild-type Gα subunits and of full-length and truncated chimeras suggests that although the truncations are at the C-terminus, the resultant phenotype is largely determined by the N-terminal portion (amino acids 1−212). Because the crystal structures of Gα subunits are available, we know which residues in the N-terminal portion of Gα contact the three critical residues in the C-terminus whose removal leads to the phenotypes we observe. Our studies allow us to suggest which of these residues are most important for normal guanine nucleotide exchange.

EXPERIMENTAL PROCEDURES

Mutagenesis and Construction of Chimeras. Deletions in rat $G\alpha_o$ and rat $G\alpha_{i2}$ were generated by the polymerase chain reaction. All PCR products were subcloned into the *Eco*RV site of Bluescript (Stratagene) by blunt-end ligations. The 5′ and 3′ primers for $G\alpha_{i2}$ included *Hin*dIII restriction sites. The cDNAs for these subunits were kindly provided by Dr. R. Reed, The Johns Hopkins University, Baltimore, MD. All mutated cDNAs were sequenced by the dideoxy method (Sanger et al., 1977). The complete amino acid sequences were verified for $G\alpha_o$-14 and $G\alpha_{i2}$-14 and were identical to wild-type rat clones outside the mutation.

Chimeras of $G\alpha_o$ and $G\alpha_{i2}$ were made using a conserved *Bam*HI site in codon 212 of the amino acid sequence. $G\alpha_{i2}$ contains a single *Bam*HI site, but $G\alpha_o$ has an additional site in codon 136. $G\alpha_{i2}$ and $G\alpha_{i2}$-14 were excised from Blue-

[2] The numbering of amino acids used throughout this paper will be for $G\alpha_o$. $G\alpha_o$ is 354 amino acids; $G\alpha_{i2}$ and $G\alpha_{i1}$ are 355 amino acids, and transducin is 350 amino acids.

script with *Hin*dIII and then digested with *Bam*HI, generating N- and C-terminal fragments. To obtain the correct *Bam*HI fragments from $G\alpha_o$ and $G\alpha_o$-14, they were excised from Bluescript with *Eco*RI and *Sal*I. The insert of ~1.1 kb was gel purified and digested with *Bam*HI (8 units/μL) at 1 unit/μg of $G\alpha_o$ cDNA or 0.5, 0.25, or 0.125 unit/μg DNA for 1 h at 37 °C. For $G\alpha_o$ and $G\alpha_o$-14, five different-sized fragments were generated, and the two corresponding to the expected size of the N- and C-terminal portions were isolated from agarose gels. Chimeras were made by ligating the appropriate fragments together with either *Hin*dIII/*Sal*I-cut Bluescript or *Hin*dIII/*Eco*RI-cut Bluescript. Chimeras were confirmed by dideoxy nucleotide sequencing from the N- and C-terminii and by *Bam*HI digests.

In Vitro Translation. mRNA was transcribed from the cDNA, and the proteins were made by in vitro translation using a rabbit reticulocyte lysate as described previously (Denker et al., 1992b). Some proteins were in vitro translated from the cDNA using a single-step method [TNT in vitro kit (Promega)] which allows transcription and translation to occur in the same reaction tube. Labeled proteins were analyzed by SDS−PAGE (Laemmli, 1970); gels were soaked in Enhance (Du Pont-New England Nuclear), and autoradiograms were exposed for 1−10 days with Fuji XAR film and two enhancing screens.

Tryptic Digestion. Samples of reticulocyte lysate (5−10 μL) were incubated with 0−20 pmol of L-1-(tosylamido)-2-phenylethyl chloromethyl ketone-treated trypsin (Cooper Biomedical) at 30 °C for 10 min. Samples were preincubated with either GTPγS, GDP, or GTP at the concentrations stated in the figure captions for 10 min at 30 °C. All experiments with GTPγS and GTP were done by adding nucleotide directly to the translation mixture. The reticulocyte lysate contained ~50 μM GTP [information from Promega, Inc. (Surgenor, 1974)]. Experiments with no nucleotide added to the reticulocyte lysate gave tryptic patterns nearly identical to those from experiments done with 100 μmol of GTP added (about 150 μM total). GTP was added to minimize variations in lysate GTP concentrations. For experiments with GDP, the nucleotides and creatine phosphate in the translation mixture were removed by Sephadex G-50 (Pharmacia LKB Biotechnology, Inc.) column chromatography in 50 mM Tris-HCL, pH 7.6, 1 mM dithiothreitol, 1 μM GDP, 1 mM EDTA, and 6 mM $MgCl_2$ at 4 °C. Proteolytic reactions were stopped by the addition of SDS−PAGE sample buffer and heating to 100 °C for 3 min. Samples were analyzed by SDS−PAGE as described above.

Sucrose Density Gradient Centrifugation. Sucrose density gradients (5−20%) were made up in 50 mM Tris-HCl, pH 7.6, 1 mM EDTA, 1 mM dithiothreitol, 0.3% Triton X-100, 1 mM $MgCl_2$, and 100 μM GDP. All gradients contained 100 μg of bovine serum albumin ($s_{20,w}$ = 4.3), ovalbumin ($s_{20,w}$ = 3.5), and carbonic anhydrase ($s_{20,w}$ = 2.9) as markers. $G\alpha_o$ and $G\alpha_{i2}$ were in vitro translated as described above, incubated with 100 μM GDP for 10 min at 30 °C, and then digested with 2 pmol of trypsin for 10 min at 30 °C. Trypsin was inactivated with the addition of 0.1 μmol of benzamidine. Trypsinized $G\alpha_o$ and $G\alpha_{i2}$ were mixed with undigested in vitro translated wild-type proteins and markers and were centrifuged at 52 000 rpm for 16 h at 4 °C as previously described (Denker et al., 1992b). Gradient fractions were analyzed by SDS−PAGE as described above. The positions of the peaks of marker and radiolabeled proteins were determined by laser densitometry of autoradiograms or stained gels.

Cross Linking. The cross-linking reagent BMH [1,6-bis-(maleimido)hexane] was prepared fresh as a 50 mM stock in dimethyl sulfoxide. Dithiothreitol (DTT) was removed from samples translated in vitro by Sephadex G-50 column chromatography. One-milliliter columns were equilibrated with 50 mM Hepes, pH 8, 6 mM $MgCl_2$, 75 mM sucrose, 1 mM EDTA, and 0.1% Triton X-100. Twenty-microliter samples were cross-linked in 2 mM BMH on ice for 20 min and then quenched by addition of DTT to 20 mM. Bovine brain purified $\beta\gamma$ was added to all samples, and controls had DTT added to the mixture prior to addition of BMH. Samples were analyzed by SDS−PAGE and autoradiography as described above.

RESULTS

Deletion of 14 Amino Acids from the C-Terminus Produces a Different Phenotype in $G\alpha_o$ and $G\alpha_{i2}$. As we have previously shown (Denker et al., 1992b), deletion of 14 amino acids from the C-terminus of $G\alpha_o$ decreases GDP affinity. We can detect the activated conformation (determined by stabilization of the 36-kDa peptide)[3] of $G\alpha_o$-14 with both GTPγS and GTP (Figure 2a,b). The stabilization of the 36-kDa peptide of $G\alpha_o$-14 with GTP reflects the reduced affinity for GDP and the relatively high GTP concentration in reticulocyte lysate (see Experimental Procedures) (Surgenor, 1974). However, the proteolytic patterns of $G\alpha_o$-14 and $G\alpha_{i2}$-14 with GTP differ strikingly. The 36-kDa band does not form from $G\alpha_{i2}$-14 in GTP at any trypsin concentration (Figure 2d) and cannot be detected even with 10 mM GTP (data not shown). With GTP, trypsin apparently degrades $G\alpha_{i2}$-14 to small peptides. The failure of $G\alpha_{i2}$-14 to be stabilized by GTP is not likely to be due to protein misfolding or to inability to bind GTP since the protein is stabilized by GTPγS (Figure 2c). We see somewhat less GTPγS-stabilized fragment arising from $G\alpha_{i2}$-14 than from $G\alpha_o$-14 at higher amounts of trypsin. This difference probably refects the fact that wild-type $G\alpha_{i2}$ is intrinsically more sensitive than $G\alpha_o$ to tryptic proteolysis (data not shown). Both wild-type $G\alpha_{i2}$ and $G\alpha_{i2}$-14 translated in vitro can interact with $\beta\gamma$ to form the expected $\alpha-\beta$ cross-linked products that migrate with apparent molecular mass of 122 and 140 kDa as previously described for $G\alpha_{i1}$ and for $G\alpha_o$ (Thomas et al., 1993; Yi et al., 1991) (Figure 3). The concentration range over which GTPγS stabilizes the 36-kDa peptide is similar for $G\alpha_o$, $G\alpha_{i2}$, $G\alpha_o$-14, and $G\alpha_{i2}$-14 (1−100 μM). Although $G\alpha_o$-14 is partially and $G\alpha_{i2}$-14 is almost totally degraded by trypsin with GTP, the characteristic 25-kDa band produced from GDP-liganded Gα subunits (see Figures 6 and 7 for examples of wild-type cleavage patterns with GDP) is not seen unless the GDP concentration is increased to 10 mM (Denker et al., 1992b). The faint bands seen with $G\alpha_o$-14-GDP [shown in Denker et al. (1992b)] are also seen with $G\alpha_{i2}$-14-GDP (data not shown). Therefore, we conclude that both $G\alpha_o$-14 and $G\alpha_{i2}$-14 have a significantly reduced ability to bind GDP. Both proteins with C-terminal deletions can bind GTPγS as demonstrated

[3] Since about 1 kDa has been removed from the C-terminus, the stable GTPγS and GTP-liganded product is now 36 kDa instead of 37 kDa.

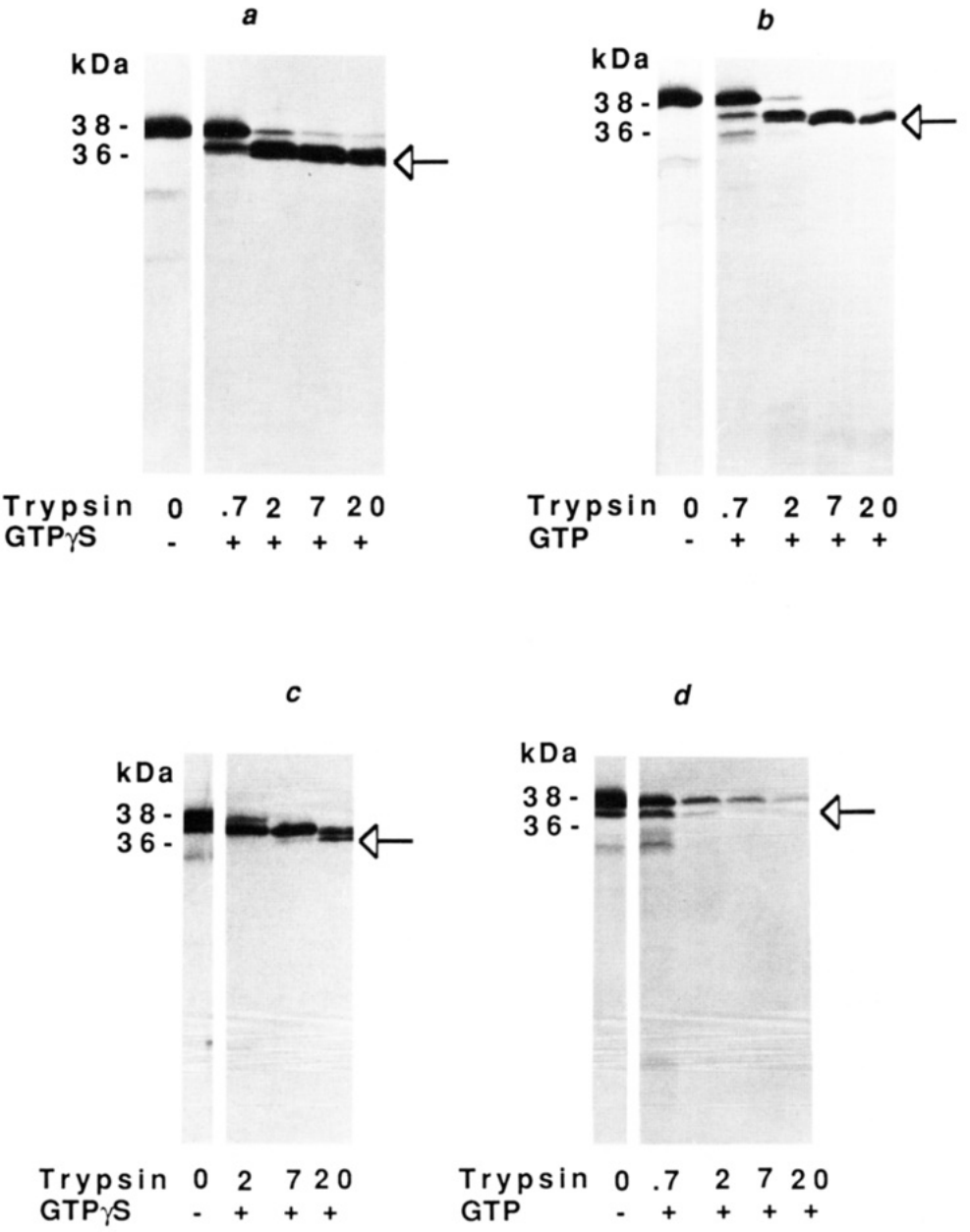

FIGURE 2: Patterns of tryptic digestion of $G\alpha_o$-14 and $G\alpha_{i2}$-14 translated in vitro. In vitro translated $G\alpha_o$-14 (a, b) and $G\alpha_{i2}$-14 (c, d) were digested for 10 min at 30 °C with increasing amounts of trypsin (0−20 pmol) after incubation with GTPγS (100 μM) (a, c) or GTP (100 μM) (b, d) as described in Experimental Procedures. The arrows mark the location of stabilized 36-kDa peptide. The smaller band underneath the starting material of $G\alpha_{i2}$-14 (c, d) is most likely an internal initiation site. $G\alpha_{i2}$ has methionine at amino acid 18 (Ala in $G\alpha_o$) which would give rise to a labeled protein of this molecular weight. We identify this band in all $G\alpha_{i2}$ cDNAs, including those modified at the C-terminus: $G\alpha_{i2}$ (Figure 6b), $G\alpha_{i2}/G\alpha_o$ (Figure 6d), $G\alpha_{i2}/G\alpha_o$-14 (Figure 5b), and $G\alpha_{i2}$-10 and -13 (Figure 4c,d). These observations make proteolytic digestion or premature termination unlikely explanations for the origin of this band.

by protection of the 36-kDa peptide (Figure 2a,c), but only one of them ($G\alpha_o$-14) shows an activated state with GTP.

Three Hydrophobic Amino Acids in the C-Terminal Portion Are Important for the Phenotype of Truncated $G\alpha_o$ and $G\alpha_{i2}$. To narrow the C-terminal amino acids essential for these phenotypes, we deleted 10 and 13 amino acids from the C-terminus. Table 1 shows the C-terminal sequences of $G\alpha_o$ and $G\alpha_{i2}$ and the sites of the C-terminal deletions. Tryptic proteolytic patterns with GTP of $G\alpha_o$-10 and $G\alpha_{i2}$-10 are similar to those of full-length subunits (Figure 4a,c compared with Figure 6a,b). However, both $G\alpha_o$-13 and $G\alpha_{i2}$-13 have tryptic patterns with GTP that are indistinguishable from those of proteins missing the last 14 amino acids (Figure 4b,d compared with Figure 2b,d). The tryptic patterns of the 13 and 14 amino acid C-terminal deletions with GTPγS and GDP are also indistinguishable (data not shown). Both $G\alpha_o$-13 and $G\alpha_{i2}$-13 are stabilized with 10−100 μM GTPγS and attain the GDP-liganded conformation poorly only in 10 mM GDP. Therefore, we conclude that the phenotype of both $G\alpha_o$ and $G\alpha_{i2}$ is caused by removal of residues 11−13 from the C-terminus (see Table 1). These three hydrophobic residues (11−13 from the C-terminus) are highly conserved in all Gα subunits.

The N-Terminal Portion (Amino Acids 1−212) Determines the Phenotype of C-Terminal Truncated Gα Chimeras. To determine whether the phenotypes of $G\alpha_o$-14 and $G\alpha_{i2}$-14 were due to differences in the N- or C-terminal portions of the molecules, we made C-terminal deletions in chimeric $G\alpha_o$ and $G\alpha_{i2}$ subunits. The tryptic pattern observed with proteins missing 14 amino acids from the C-terminus could arise from

Table 1: Amino Acid Sequences of C-Terminus of $G\alpha_o$ and $G\alpha_{i2}$ and Carboxyl-Terminal Deletions[a]

$G\alpha_o$	.	.	.	.	.	.	A	V	T	D	I	I	I	A	N	N	L	R	G	C	G	L	Y
$G\alpha_o$-14	.	.	.	.	.	.	A	V	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$G\alpha_o$-13	.	.	.	.	.	.	A	V	T	D	-	-	-	-	-	-	-	-	-	-	-	-	-
$G\alpha_o$-10	.	.	.	.	.	.	A	V	T	D	I	I	I	-	-	-	-	-	-	-	-	-	-
$G\alpha_{i2}$	.	.	.	.	.	.	A	V	T	D	V	I	I	K	N	N	L	K	D	C	G	L	F
$G\alpha_{i2}$-14	.	.	.	.	.	.	A	V	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-
$G\alpha_{i2}$-13	.	.	.	.	.	.	A	V	T	D	-	-	-	-	-	-	-	-	-	-	-	-	-
$G\alpha_{i2}$-10	.	.	.	.	.	.	A	V	T	D	V	I	I	-	-	-	-	-	-	-	-	-	-

[a] Dots represent continuation of sequence and dashes represent deleted amino acids.

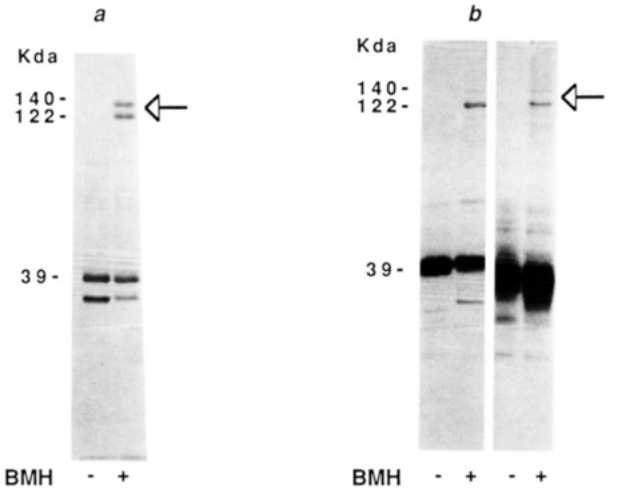

FIGURE 3: Cross-linking of $G\alpha_{i2}$ and $G\alpha_{i2}$-14 to $\beta\gamma$ subunits. In vitro translated $G\alpha_{i2}$ and $G\alpha_{i2}$-14 were prepared as described in Experimental Procedures. Samples including bovine brain $\beta\gamma$ (0.3 μg) were incubated with 2 mM BMH (+) or 20 mM DTT followed by addition of 2 mM BMH (−) on ice for 20 min as described in Experimental Procedures. Samples were analyzed by SDS−PAGE and autoradiography as previously described. Panel a: cross-linked bovine brain purified $G\alpha_o\beta\gamma$ stained with Coomassie blue. Panel b: In vitro translated and cross-linked $G\alpha_{i2}$ (left) and $G\alpha_{i2}$-14 (right). The gel was exposed for 24 h at −70 °C with two enhancing screens. The characteristic bands that migrate with apparent molecular masses of 122- and 140-kDa are marked with the arrow. Cross-linking efficiency with in vitro translated proteins is less than that with pure proteins. This is probably due to the high concentration of proteins in rabbit reticulocyte lysate competing for cross-linking reagent.

disruption of amino acid interactions with either the N- or C-terminal portion of the molecule. Like wild-type $G\alpha_o$ and $G\alpha_{i2}$, both chimeras with C-terminal truncations bind GTPγS and can attain the activated conformation (Figure 5, sets a, b, and c, last lane of each set), showing that the truncated chimeras are functional by this criterion. With GTP, each chimera behaved similarly to the amino-terminal donor; $G\alpha_o/\alpha_{i2}$-14 is activated by GTP for a sufficient time to detect a substantial amount of protein in the GTP-liganded conformation (36-kDa band, Figure 5c). We detect only a very faint 36-kDa band for $G\alpha_{i2}/\alpha_o$-14 in the GTP-liganded conformation (Figure 5b). The band is similar in size to a minor band in the starting material that is probably due to an internal start site (see the caption to Figure 2). However, it is stable to tryptic digestion and may represent a very small amount of GTP-liganded $G\alpha_{i2}/G\alpha_o$-14. Since both C-terminal truncated chimeras can bind GTPγS and give tryptic cleavage patterns similar to those of wild-type proteins, neither chimera is grossly misfolded (last lane in each set). In addition, like wild-type truncated proteins, both truncated chimeras form faint 25-kDa bands with high (10 mM) GDP (not shown). The truncated chimeras can be cross-linked to $\beta\gamma$ subunits to form the same products as wild-type proteins and nonchimeric truncated proteins (described above and in Figure 3), giving further evidence that they are not grossly misfolded. These results show that the N-terminal donor to the chimera determines the phenotype of proteins with C-terminal deletions.

Exchanging the C-Terminal Region Does Not Significantly Affect the Structure of the N-Terminal Region. To ensure that chimera construction per se did not alter the pattern of tryptic proteolysis, cleavage patterns of full-length chimeras $G\alpha_o/G\alpha_{i2}$ and $G\alpha_{i2}/G\alpha_o$ were compared with wild-type patterns (Figure 6). Tryptic cleavage patterns of full-length proteins without added nucleotide reflect the GDP-liganded conformation because GTP in the rabbit reticulocyte lysate (see Experimental Procedures) is hydrolyzed to GDP, which remains tightly bound to the active site. Trypsin cleaves near Arg-209, generating 25- and 17-kDa peptides (see Figure 1). The tryptic cleavage pattern of $G\alpha_o$-GDP (Figure 6a) differs from that of $G\alpha_{i2}$-GDP (Figure 6b). $G\alpha_o$-GDP gives a doublet at 25 kDa, and the lower band of the doublet increases with increasing trypsin concentrations (Figure 6a). For α_{i2}-GDP, increasing the trypsin concentration causes cleavage of the 25-kDa band to a doublet at 20 kDa (Figure 6b). A faint 17-kDa band is visible for both $G\alpha_o$ and $G\alpha_{i2}$ at the lower trypsin amounts (see Figure 6a,b). The 17-kDa

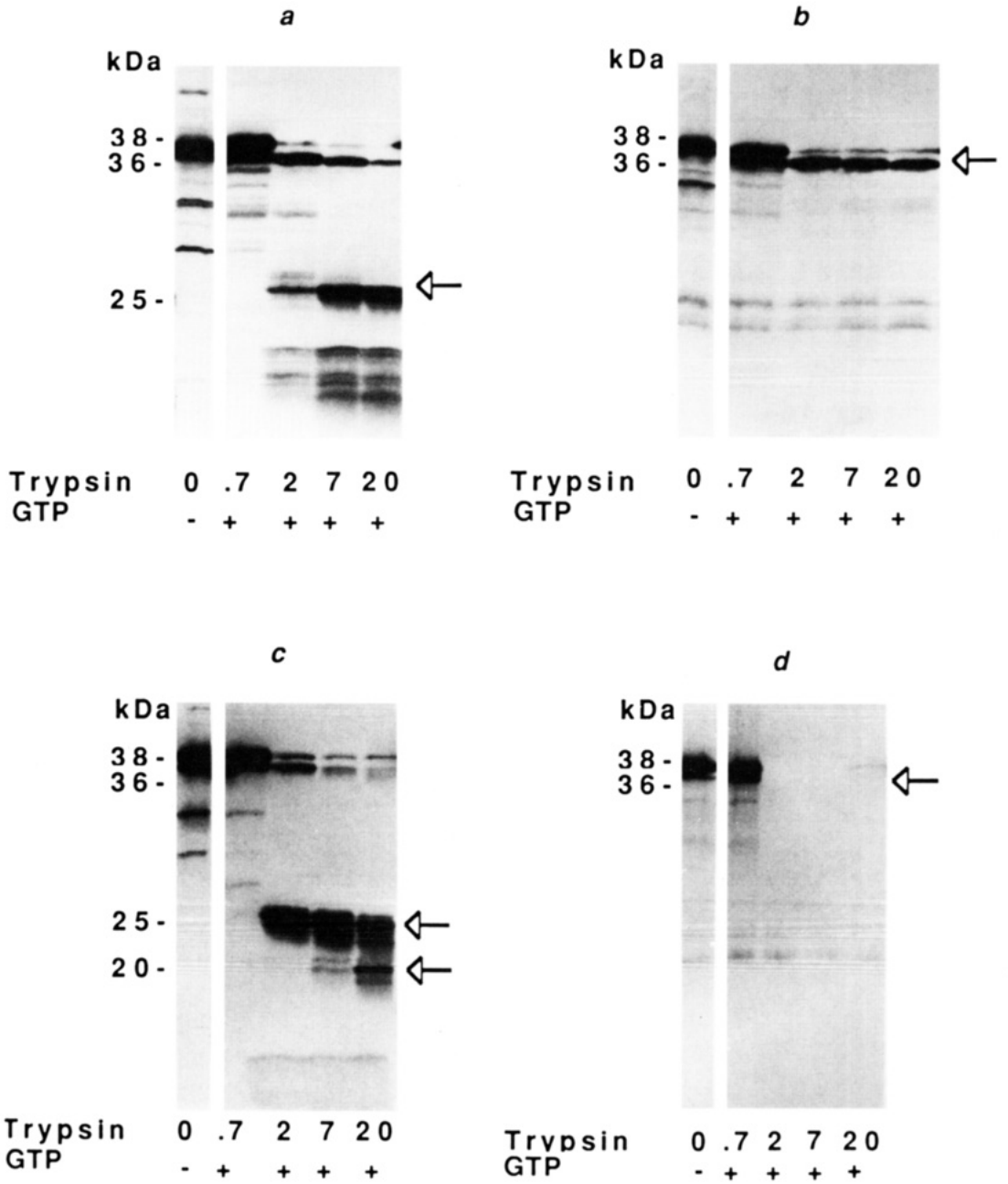

FIGURE 4: Comparison of tryptic patterns for $G\alpha_o$ and $G\alpha_{i2}$ with 10 and 13 amino acids deleted from the C-terminus. In vitro translated $G\alpha_o$-10 (a), $G\alpha_o$-13 (b), $G\alpha_{i2}$-10 (c), and $G\alpha_{i2}$-13 (d) were incubated with 100 μM GTP followed by tryptic proteolysis as previously described. $G\alpha_o$-13 forms the protected 36-kDa band [arrow in (b)] similar to $G\alpha_o$-14. The arrow in (d) marks the location expected for stabilized 36-kDa peptide, which is not seen for $G\alpha_{i2}$-13 (similar to $G\alpha_{i2}$-14).

band is difficult to visualize because it has only three methionines (versus six in the 25-kDa peptide of $G\alpha_o$ and five in $G\alpha_{i2}$) and is more sensitive to further tryptic proteolysis than the 25-kDa band [even with purified protein from bovine brain (Winslow et al., 1986)].

Despite the strongly interactive N- and C-terminal regions, exchanging C-terminal regions (amino acids 212−354) does not significantly affect protein folding of the amino terminus (25-kDa bands) as detected by tryptic proteolysis. The $G\alpha_o$/$G\alpha_{i2}$ tryptic pattern is indistinguishable from that of $G\alpha_o$ (Figure 6a,c), and $G\alpha_{i2}$/$G\alpha_o$ was nearly identical to wild-type $G\alpha_{i2}$ (Figure 6 b,d). The characteristic tryptic patterns of the wild-type proteins are seen in the chimeric molecules containing the wild-type donor of its amino terminus (Figure 6c,d). This is to be expected since the 25-kDa fragments come from the amino terminus, but it is reassuring that addition of a heterologous C-terminus does not cause gross changes in the folding of the N-terminal region.

G Protein α Subunit Tryptic Fragments Remain Associated after Tryptic Digestion. The stabilization of fragments by GDP suggests that the guanine nucleotide binding site of Gα subunits is still intact after tryptic proteolysis. There are 20 amino acids that interact with either the phosphates or the guanine ring of bound nucleotide; 14 are located in the 25-kDa fragment, while six are in the 17-kDa fragment. Sucrose density gradient analysis shows that the 25- and 17-kDa tryptic peptides remain associated because they cosediment with undigested, in vitro translated wild-type protein (Figure 7). The $s_{20,w}$ values are the same as we previously reported for wild-type in vitro translated $G\alpha_o$ ($3.1 + 0.2$, $n = 4$) for the 39-, 25-, and 17-kDa peptides) (Denker et al., 1992b). The pattern for $G\alpha_{i2}$ is similar to that of $G\alpha_o$ with

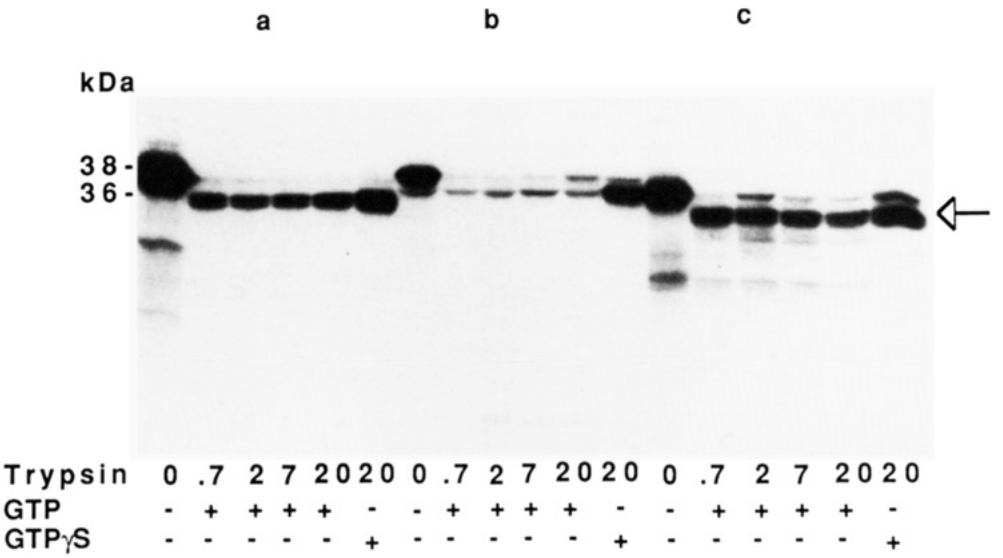

FIGURE 5: Comparison of tryptic digestion of chimeric Gα subunits with C-terminal truncations. In vitro translated $G\alpha_o$-14 (a), $G\alpha_{i2}/\alpha_o$-14 (b), and $G\alpha_o/\alpha_{i2}$-14 (c) were digested with increasing amounts of trypsin after preincubation with 100 μM GTP as previously described. The last lane of each group shows tryptic digestion with 20 pmol of trypsin after preincubation with 100 μM GTPγS. The arrow marks the position of the protected 36-kDa peptide which is seen for $G\alpha_o$-14 (a) and $G\alpha_o/\alpha_{i2}$-14 (c) but not for $G\alpha_{i2}/\alpha_o$-14 (b). The faint band seen in this position arises from the internal start site that was described in the Experimental Procedures. Note that $G\alpha_{i2}/\alpha_o$-14 is stabilized with GTPγS (last column in panel b).

an $s_{20,w}$ value of 3.3 + 0.2 ($n = 6$) for the wild-type protein and the 25- and 17-kDa peptides. These results are consistent with our results and the crystal structure of $G\alpha_t$ and $G\alpha_{i1}$, which shows that tryptic sites do not define distinct domains of the Gα subunit and that the N- and C-terminal regions of the molecule are strongly interactive through extensive hydrogen bonding (see Figure 1) (Noel et al., 1993).

DISCUSSION

Our findings that identical mutations in $G\alpha_o$ and $G\alpha_{i2}$ give different phenotypes allow us to begin to study regions of difference among Gα subunits that are likely to explain some of their distinct functions. Figure 1 shows the three-dimensional structure of $G\alpha_t$ with the portion C-terminal to the junction site of the chimeras shaded. The tryptic site accessible in the GDP-liganded conformation occurs several amino acids upstream from the junction site of the chimera (Figure 1). The crystal structure of $G\alpha_t$ and $G\alpha_{i1}$ reveals a network of hydrogen bonds that link one functional region to another. Our observation that the 25- and 17-kDa tryptic fragments sediment as a complex on sucrose density gradients is consistent with the integrated structures of transducin and $G\alpha_{i1}$ and, by inference, of $G\alpha_o$ and $G\alpha_{i2}$. In principle, the phenotypes of $G\alpha_o$-14 and $G\alpha_{i2}$-14 could have been determined by the N-terminal region, the C-terminal region, or both. Our analysis of chimeric truncated molecules did not reveal an intermediate phenotype but showed that the phenotype of the chimera matched that of the amino-terminal donor.

We have previously shown that truncation of 14 residues from the C-terminus of $G\alpha_o$ decreases its apparent affinity for GDP and allows the GTP-liganded state to exist long enough to be detected by the tryptic proteolysis assay, probably because of increased GTP/GDP exchange (Denker et al., 1992b). We now show that the same truncation of $G\alpha_{i2}$ decreases the apparent affinity for GDP (because the characteristic tryptic fragments only appear at very high GDP concentration), but the GTP-liganded form cannot be detected even at 10 mM GTP. Both truncated forms of $G\alpha_o$ and $G\alpha_{i2}$ bind GTPγS similarly since they can be stabilized over a similar range of GTPγS concentration (1−100 μM). Although most of our studies were done with Gα subunits and chimeras from which 14 amino acids were removed, we have shown that removal of 13 amino acids is sufficient to elicit the full phenotype in both $G\alpha_o$ and $G\alpha_{i2}$. Removal of 10 amino acids from either wild-type $G\alpha_o$ or $G\alpha_{i2}$ had only a small effect on the apparent GDP affinity and gave a tryptic proteolytic pattern similar to wild type (Denker et al., 1992b). The three amino acids that are 11, 12, and 13 residues from the C-terminus (Ile-Ile-Ile in $G\alpha_o$ and Val-Ile-Ile in $G\alpha_{i2}$; see Table 1) are conserved in virtually all Gα subunits.

The crystal structures of transducin and $G\alpha_{i1}$ suggest how loss of these residues may affect the GDP affinity. In transducin, these three hydrophobic residues interact with Val-30, Leu-190, Cys-216, and Tyr-316. They are equivalent to Val-34, Leu-195, Ala-221, and Tyr-321 in $G\alpha_o$, $G\alpha_{i1}$, and $G\alpha_{i2}$. Two of these contact residues fall in the N-terminal, and two in the C-terminal, portion of our chimeras. Since each chimera behaves like the N-terminal donor, our study suggests that disruption of the N-terminal contacts is a major contributor to the final phenotype. The N-terminal contacts are more important than the C-terminal contacts. The two in the amino-terminal portion occur at the start of the β1 and β3 strands (see Figure 1). It is likely that the interaction of the C-terminal helix with these two residues constrains the flexibility of the helix. Eliminating this interaction may make the helix more flexible, causing a conformational change that is propagated to the loop at the end of the α helix. This loop is important for stabilizing the binding of the guanine ring. Such a mechanism is responsible for the activated phenotype of $G\alpha_s$-A366S, a mutation that leads to precocious puberty in humans (Iiri et al., 1994). We have previously shown that mutation of a cysteine in this loop to alanine ($C325AG\alpha_o$) decreases GDP affinity of $G\alpha_o$ by about an order of magnitude (Thomas et al., 1993). Val-34 that

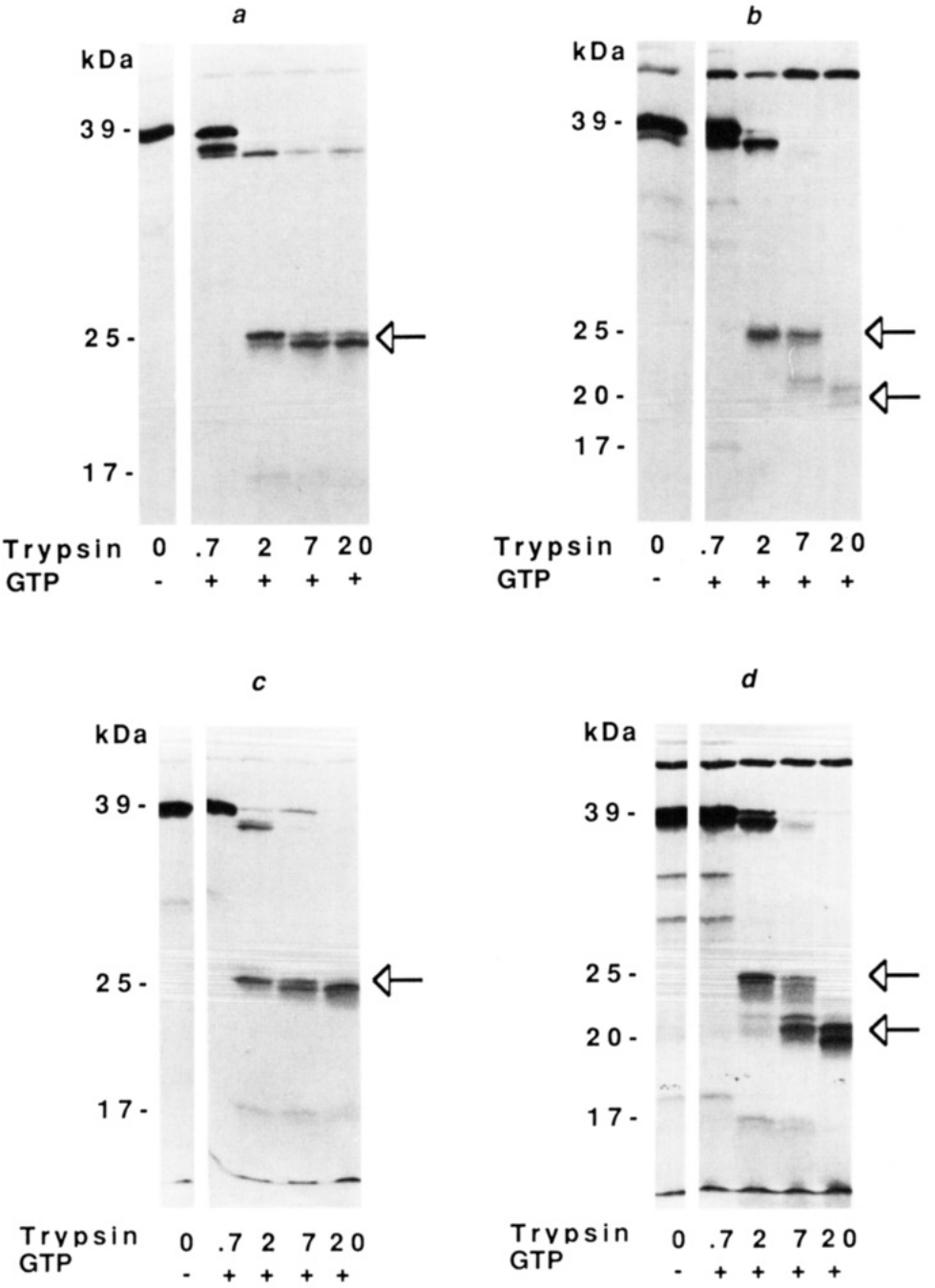

FIGURE 6: GTP-liganded patterns of tryptic digestion for wild-type $G\alpha_o$, $G\alpha_{i2}$, $G\alpha_o/G\alpha_{i2}$, and $G\alpha_{i2}/G\alpha_o$. $G\alpha_o$ (a), $G\alpha_{i2}$ (b), $G\alpha_o/G\alpha_{i2}$ (c), and $G\alpha_{i2}/G\alpha_o$ were digested with increasing amounts of trypsin after incubation with GTP (100 μM) as previously described. Panels a and b show the characteristic 20−25-kDa peptides generated from GDP-liganded $G\alpha_o$ and $G\alpha_{i2}$ subunits. The chimeric subunits (c, d) have the same pattern as the donor of the amino terminus. A band at 45 kDa is occasionally seen even in controls without added mRNA (panel b). Its intensity varies with the manufacturer of the [^{35}S]methionine used to label the translated protein, and it is usually absent when we use [S^{35}]methionine from Amersham (Arlington Heights, IL).

starts β strand 1 (Figure 1) has been mutated to alanine (Slepak et al., 1993). $G\alpha_o$ with this point mutation bound GTPγS and interacted normally with $\beta\gamma$, but there is no information about GDP binding. We would predict that mutating both Val-34 and Leu-195 to bulky polar amino acids would lead to a decrease in GDP affinity. If the contacts with bound nucleotide are really the same in $G\alpha_o$ and $G\alpha_{i2}$ as in transducin and $G\alpha_{i1}$, then all the residues that are predicted to contact the deleted hydrophobic residues are identical in $G\alpha_o$ and $G\alpha_{i2}$. The different phenotypes therefore are likely to result from subtle differences in folding within the amino terminus between $G\alpha_o$ and $G\alpha_{i2}$ that position identical amino acids somewhat differently.

We had originally explained the activation of $G\alpha_o$-14 with GTP by rapid exchange of GDP for GTP so that the GTP-liganded state existed for a larger fraction of the time than the wild-type $G\alpha_o$. Like $G\alpha_o$-14, $G\alpha_{i2}$-14 also binds GDP poorly, but it does not show an activated state with GTP. Therefore, the difference in phenotype between $G\alpha_o$-14 and $G\alpha_{i2}$-14 may reflect an additional differential effect of the truncation on the GTPase activity. The catalytic constant (k_{cat}) is the same for both wild-type $G\alpha_o$ and $G\alpha_{i2}$ (Linder et al., 1990). The C-terminal truncation could decrease the intrinsic GTPase activity of $G\alpha_o$, keeping GTP in the active site long enough to be detected by the trypsin assay, or increase the GTPase activity of $G\alpha_{i2}$-14, shortening the time

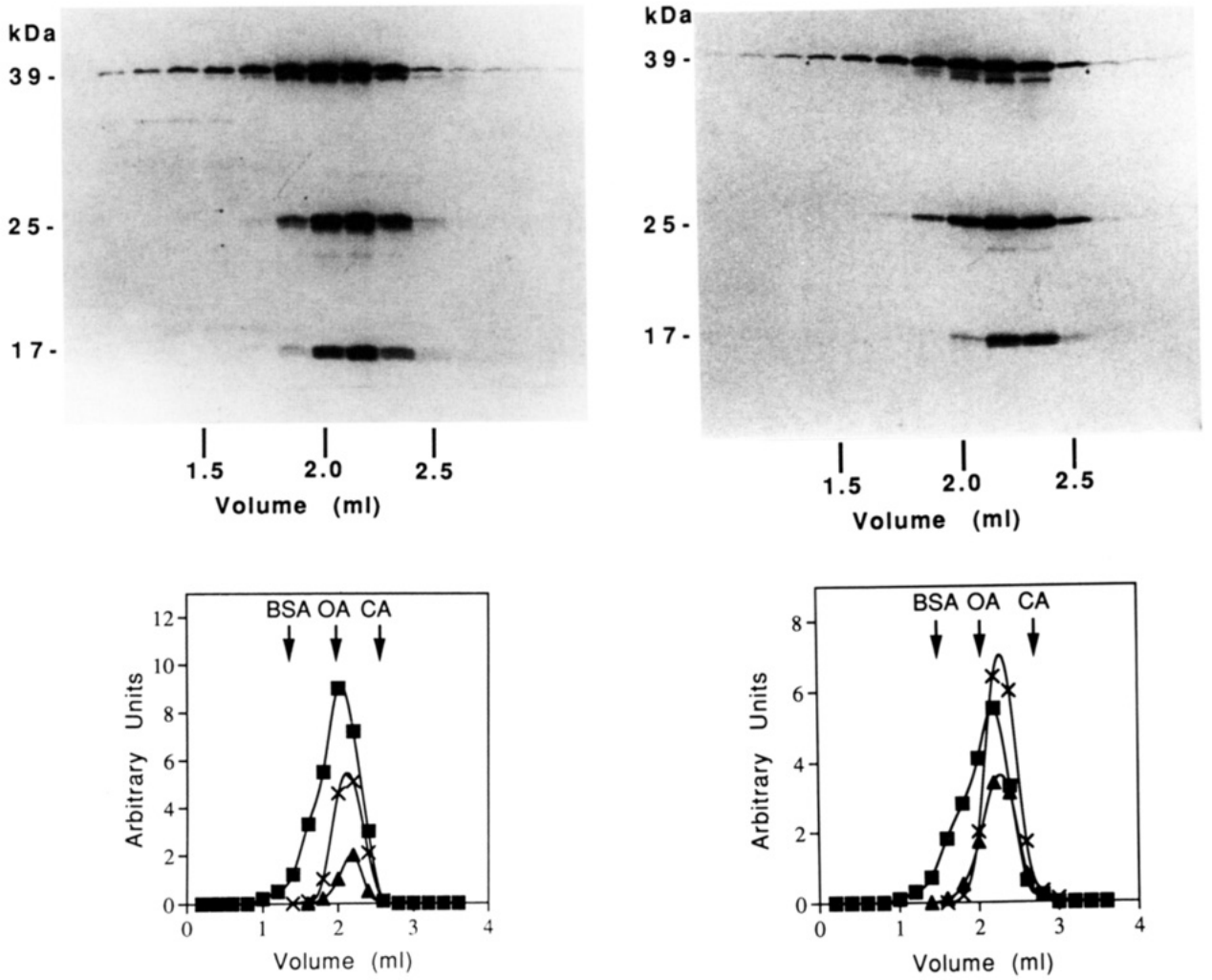

FIGURE 7: Sucrose gradient sedimentation profile of $G\alpha_o$ translated in vitro and trypsinized. In vitro translated $G\alpha_o$ (a, left two panels) and $G\alpha_{i2}$ (b, right two panels) were digested with 2 pmol of trypsin after preincubation with 100 μM GDP as described in Experimental Procedures. The reaction was stopped by the addition of 0.1 μmol of benzamidine, and the sample was mixed with uncleaved $G\alpha_o$ or $G\alpha_{i2}$ translated in vitro and 3 μg of $G\alpha_o$ subunit (purified from bovine brain). The mixture of trypsinized and untrypsinized subunits was centrifuged through 5−20% sucrose gradients containing 100 μM GDP. Marker proteins (100 μg) were included in the sample: bovine serum albumin (BSA), carbonic anhydrase (CA), and ovalbumin (OA). Upper panels: Autoradiograms of fractions from a sucrose density gradient analysis of in vitro translated $G\alpha_o$ (a) or $G\alpha_{i2}$ (b). Lower panels: Densitometry of the autoradiograms shown above. (■) 39-, (×) 25-, and (▲) 17-kDa peaks. Sedimentation positions of marker proteins are shown by arrows.

GTP is bound and preventing detection by this assay. Ultimately, purified proteins will be needed to evaluate these different potential mechanisms. It is possible to use immunoprecipitation of in vitro translated proteins to measure the amount of bound GDP. However, most precipitating antibodies are against epitopes in the C-terminus and would not be useful for these studies. Furthermore, these measurements would be confounded by endogenous $G\alpha_{i2}$ in the reticulocyte lysate.

In summary, our studies have shown that the N- and C-terminal portions of $G\alpha_o$ and $G\alpha_{i2}$ have sufficient interactions to remain associated when cleaved by trypsin. The network of hydrogen bonds that stabilize the structure allows conformational changes to be transmitted throughout the molecule. A functional consequence of such an integrated structure is that the phenotype of deletions in the C-terminal portion is determined by the structure of the N-terminal portion. Agonist-liganded hormone receptors interact with the C-terminus of Gα subunits to reduce GDP affinity (Ui et al., 1984; Van Dop et al., 1984; West et al., 1985; Hamm et al., 1987, 1988; Sullivan et al., 1987; Masters et al., 1988; Spiegel et al., 1990; Okamoto et al., 1994). Hormone receptors are thought to affect the GTPase activity of Gα subunits by decreasing GDP affinity and not by changing k_{cat}, although the evidence is indirect (Landis et al., 1989). It is possible that the motion of the C-terminal α helix induced by receptors may also cause subtle changes in the GTPase activity of Gα subunits. Such differences would lead to variations in the lifetime of the activated state. Our studies suggest that three hydrophobic amino acids near the C-terminus are especially important for inducing the conformational change that allows GDP affinity to fall and Gα activation to occur. We predict that mutations of these amino acids would lead to a phenotype similar to those of the C-terminal truncations. The subtle structural differences between $G\alpha_o$ and $G\alpha_{i2}$ have significant functional consequences. Therefore, although all the Gα subunits are extremely similar, each has its individual character that is likely to suit it for a specific intracellular role.

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