Crystal Structures of the Scaffolding Protein LGN Reveal the General Mechanism by Which GoLoco Binding Motifs Inhibit the Release of GDP from Gaα

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Background: GoLoco (GL) motif binds to Ga and inhibits its guanine nucleotide dissociation.

Results: Crystal structures of LGN-GL3(4)-Gaαi complexes reveal a double Arg finger-mediated GDP recognition mechanism, which is distinct from that shown in the RGS14-Gaαi complex.

Conclusion: LGN-GL/Gaα interaction might represent a general binding mode between GoLoco motifs and Gaα.

Significance: Our findings shed new light on the GoLoco motif-mediated G protein signaling regulation.

GoLoco (GL) motif-containing proteins regulate G protein signaling by binding to Ga subunit and acting as guanine nucleotide dissociation inhibitors. GLs of LGN are also known to bind the GDP form of Gaα during asymmetric cell division. Here, we show that the C-terminal GL domain of LGN binds four molecules of Gaα-GDP. The crystal structures of Gaα-GDP in complex with LGN GL3 and GL4, respectively, reveal distinct GL/Gaα interaction features when compared with the only high resolution structure known with GL/Gaα interaction between RGS14 and Gaα1. Only a few residues C-terminal to the conserved GL sequence are required for LGN GLs to bind to Gaα-GDP. A highly conserved “double Arg finger” sequence (ΨΨ(Δ/E)(Δ/D)EQR) is responsible for LGN GL to bind to GDP bound to Gaα. Together with the sequence alignment, we suggest that the LGN GL/Gaα interaction represents a general binding mode between GL motifs and Gaα. We also show that LGN GLs are potent guanine nucleotide dissociation inhibitors.

The α subunit of the heterotrimeric G proteins (Gaα) is a critical component of the G protein signaling pathway, in which Gaα cycles between the GDP- and GTP-bound states (1). In the canonical signaling model, ligand-mediated activation of G protein-coupled receptors (GPCRs)4 catalyzes the exchange of GDP for GTP in binding to Gaα and subsequently results in the dissociation of Gaα-GTP from Gaαγ heterodimer (2, 3). The dissociated Gaα-GTP binds to and activates downstream effectors, thus transducing signals from GPCR (4–6). Because Gaα has intrinsic GTPase activity, the Gaα subunit subsequently returns to the Gaα-GDP form, which marks the termination of the GPCR signaling. Many proteins have been discovered as regulators of the GTP- and GDP-bound forms of the Gaα reaction cycle. Among these, GoLoco motif proteins were discovered to bind specifically to GDP-loaded Gaα or Gaαγ and inhibit the spontaneous release of GDP from Gaα. These GoLoco proteins are referred to as guanine nucleotide dissociation inhibitors (GDIs) (7–11).

The GoLoco motif (8, 12, 13) was first identified as a conserved sequence of 19 amino acids, occurring singly or as tandem repeats in a variety of signaling proteins across the animal kingdom (7). Our understanding of the molecular mechanism of the GDI function of GoLoco proteins is mainly based on the crystal structure of RGS14 GoLoco bound to Gaαi-GDP (14), which shows that the conserved GoLoco motif and its variable C-terminal tail interact with the Ras-like and all-helical domains of Gaαiγ, respectively. A so-called “arginine finger” formed by the highly conserved D/EQR triad in the conserved GoLoco motif extends into the GDP-binding pocket and directly contacts the α- and β-phosphates of GDP (14). This structure and the subsequent mutagenesis and structural studies (14–18) suggested an appealing hypothesis: the highly variable C-terminal sequences following the conserved GoLoco motifs and the all-helical domain of Gaα subunits are likely the specificity determinants of interactions between GoLoco motifs and different Gaα subunits. However, because there are no structures of GoLoco motifs in complex with Gaα other than the

4 The abbreviations used are: GPCR, G protein-coupled receptor; GL, GoLoco; GDI, guanine nucleotide dissociation inhibitor; TPR, tetratricopeptide; aa, amino acids; ITC, isothermal titration calorimetry.
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\( \alpha_i \)-RGS14 complex are available to date, the above hypothesis remains untested.

LGN is a multidomain scaffolding protein containing eight tetratricopeptide (TPR) repeats in its N-terminal region, a flexible linker sequence in the middle, and four GoLoco motifs in the C-terminal end (19, 20). LGN is an evolutionarily conserved protein (Pins in Caenorhabditis elegans) that plays crucial roles in regulating spindle orientations during asymmetric cell division (19, 21) and can be considered as an example member of the multiple GoLoco motif protein family. It forms a ternary protein complex with nuclear mitotic apparatus protein NuMA (Mud in Drosophila and Lin5 in C. elegans) and cortical membrane-bound GoLoco motifs of LGN via its TPR repeats and GoLoco motifs, respectively (22–28). The central linker of LGN binds to the guanlyate kinase domain of the DLG family scaffold protein in a phosphorylation-dependent manner (29–31). In Drosophila neuroblast, loss of Pins or GoLoco affects cell polarity as well as mitotic spindle orientation (32). In mammals, overexpression or removal of LGN results in dramatic spindle rocking in metaphase and improper spindle pole organization (19, 21, 33). The binding of GoLoco through the GoLoco motifs was shown to regulate the cortical localization of LGN (33). Thus, the LGN GoLoco motifs can be viewed as scaffolding modules in tethering the TPR repeat partners (e.g. NuMA/Mud and mInsc/Insc) of LGN to the cell cortex via binding to membrane-attached GoLoco. Interestingly, the GoLoco motifs of LGN can directly bind to TPR repeats intramolecularly, thus keeping LGN in an autoinhibited conformation (22). GoLoco-GDP binding to GoLoco motifs releases the autoinhibited conformation of LGN and renders LGN TPR repeats capable of binding to NuMA (22, 34), although the mechanistic basis of the LGN autoinhibition is unknown.

In this study, we performed detailed biochemical and structural analyses of the interactions between LGN GoLoco motifs and GoLoco-GDP. We demonstrate that in contrast to the RGS14/\( \alpha_i \)-GDP interaction, only a few residues of the highly variable sequences C-terminal to the conserved GoLoco motifs of LGN are involved in binding to GoLoco-GDP. The structures of two LGN GoLoco motifs in complex with GoLoco reveal a double Arg finger sequence (RP(D/E)(D/E)/QR) within the GoLoco motif that is specifically involved in the GDP coordination. We further show that the LGN GoLoco/GoLoco-GDP interaction observed in this study likely represents a general mode of GoLoco motif-mediated Go binding. We further demonstrate that the LGN GoLoco motifs are potent GDIs. Thus, the LGN GoLoco motifs can function as a Go/LGN/NuMA/Insc scaffold as well as a regulator of Go\( \alpha_i \) signaling in asymmetric cell division.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—The human GoLoco, Go\( \alpha_i \), mouse LGN GL fragments were individually cloned into a modified version of pET32a vector. All the mutations were created using the standard PCR-based method and confirmed by DNA sequencing. Recombinant proteins were expressed in *Escherichia coli* BL21 (DE3) host cells at 16 or 37 °C and were purified by using a Ni\(^{2+}\)-nitriotriaetic acid-agarose affinity chromatography followed by size exclusion chromatography. For *in vitro* biochemical analysis, LGN GLs were expressed as the GST-fused proteins and purified by GSH-Sepharose affinity chromatography.

**Isothermal Titration Calorimetry Measurements**—ITC measurements were performed on an ITC200 Microcalorimeter (MicroCal) at 25 °C. All protein samples were dissolved in the buffer containing 50 mM Tris, pH 8.0, 100 mM NaCl, and 1 mM EDTA. The titrations were carried out by injecting 40 \( \mu \)l of GoLoco-GDP aliquots (0.2 mM) into LGN GLs fragments fused to the C-terminal end of thioredoxin (0.02 mM) at time intervals of 2 min to ensure that the titration peak returned to the base line. The titration data were analyzed using the program Origin7.0 from MicroCal.

**Fluorescence Polarization Assay**—Fluorescence polarization assay were performed on a PerkinElmer LS-55 fluorimeter equipped with an automated polarizer at 25 °C. Commercial synthesized peptides were labeled with fluorescein 5-isothiocyanate (Invitrogen) at the N termini. In a typical assay, the FITC-labeled peptide (≈ 1 \( \mu \)M) was titrated with binding partners in a 50 mM Tris pH 8.0 buffer containing 100 mM NaCl, 1 mM DTT, and 1 mM EDTA. The free \( K_D \) values were obtained by fitting the titration curves with the classical one-site binding model, with or without invoking the Hill coefficient model.

**GST Pulldown Assay**—For GST pulldown assay, GST or GST-tagged proteins (60 \( \mu \)l from 1 mg/ml stock solutions) were first loaded to 40 ml GSH-Sepharose 4B slurry beads in an assay buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM DTT, and 1 mM EDTA). The GST fusion protein-loaded beads were then mixed with potential binding partners, and the mixtures were incubated for 1 h at 4 °C. After three times washing, proteins captured by affinity beads were eluted by boiling, resolved by 15% SDS-PAGE, and detected by Coomassie Blue staining.

**Analytical Gel Filtration Chromatography**—Analytical gel filtration studies were carried out on an AKTA FPLC system (GE Healthcare). Proteins at concentration of 10–20 \( \mu \)M in a volume of 100 \( \mu \)l were loaded on a Superose 12 10/300 GL column (GE Healthcare) equilibrated with the buffer containing 50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM DTT, and 1 mM EDTA. Protein elution was detected by absorbance at 280 nm.

**GDI Activity Assay**—Measurements of AlF\(_4^-\)-induced increase of intrinsic tryptophan fluorescence were performed on the PerkinElmer LS-55 spectrometer with excitation at 292 nm and emission at 342 nm. Purified GoLoco-GDP protein was diluted in 2-ml cuvettes to 200 nm in a preactivation buffer (100 mM NaCl, 100 \( \mu \)M EDTA, 2 mM MgCl\(_2\), 20 \( \mu \)M GDP, 20 mM Tris-HCl, pH 8.0) and incubated at 30 °C. At the time points 400 and 500 s after GoLoco-GDP dilution, 2 mM NaF and 30 \( \mu \)M AlCl\(_3\) (final concentrations), respectively, were added to the reaction mixture, and fluorescence intensity changes as a function of time were recorded. The GDI activities of GL peptides were assayed by repeating the above procedure except that the reaction mixtures contained defined concentrations of specific peptides.

The measurements of GTP\(_{\gamma}\)S binding were also performed on PerkinElmer LS-55 spectrometer with excitation at 485 nm and emission at 530 nm (slit widths each at 2.5 nm). BODIPY FL-GTP\(_{\gamma}\)S was diluted to 1 \( \mu \)M in buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 10 mM MgCl\(_2\)) and equilibrated to 30 °C in 2-ml cuvettes. Purified GoLoco was diluted to 100 nm in the buffer (100 mM NaCl, 100 \( \mu \)M EDTA, 2 mM MgCl\(_2\), 20 \( \mu \)M GDP, 20 mM
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RESULTS

Mapping the Minimal Goα<sub>1</sub>-GDP Binding Sequences in LGN GoLoco Motifs—The C-terminal region of LGN contains four GoLoco motifs, each of which consists of a conserved 19-residue fragment followed by a stretch of variable amino acid residues with different lengths (Fig. 1A). We define the full-length GoLoco motif to be the conserved 19-residue fragment plus all of the following C-terminal sequence before the start of the next GoLoco motif core. With this definition, each GL1, 2, 3, and 4 motif of LGN consists of 54, 51, 34, and 51 residues, respectively (Fig. 1A). Previous structural study of the Goα<sub>1</sub>-RGS14-GoLoco complex showed that the 16-residue sequence C-terminal to the GoLoco core motif make extensive contacts with Goα<sub>1</sub> and thus are essential for the interaction between Goα<sub>1</sub> and RGS14 (14). To understand the interaction between LGN and Goα<sub>1</sub> we set out to map the minimal Goα<sub>1</sub>-GDP binding sequence of each LGN GL. We first used GST-fused LGN GL with different lengths to pull down purified Goα<sub>1</sub>-GDP in our binding assay. This assay showed that each GL containing only the 19-residue core displayed only a background level of binding to Goα<sub>1</sub>-GDP (Fig. 1B). Obvious binding of Goα<sub>1</sub>-GDP to GL1 and GL4 was observed by extending the conserved 19-residue GL core by two residues (Fig. 1B). We next measured the quantitative binding affinities of each of the four GLs to Goα<sub>1</sub>-GDP using isothermal titration calorimetry or fluorescence spectroscopy. Such quantitative binding assays revealed that the four full-length GLs share similar affinities (K<sub>d</sub> = 54–96 nM) in binding to Goα<sub>1</sub>-GDP (Fig. 1C). In agreement with the results derived from the pulldown binding assay, each LGN GL with a length of 25 residues has an essentially same binding affinity compared with the corresponding full-length motif (Fig. 1C). This finding is in sharp contrast to the interaction

TABLE 1

Statistics of x-ray crystallographic data collection and model refinement

| Data sets | Goα<sub>1</sub> GL4 | Goα<sub>1</sub> GL4 | Goα<sub>1</sub> GL3 | Goα<sub>1</sub> GL3 |
|-----------|-----------------|-----------------|-----------------|-----------------|
| Space group | P6<sub>2</sub>,2 | P6<sub>2</sub>,2 | P6<sub>2</sub>,2 | P6<sub>2</sub>,2 |
| Unit cell (Å) | a = 207.3, c = 236.6 | a = 207.4, c = 236.7 | a = 209.6, c = 237.2 | a = 209.7, c = 235.5 |
| No. of unique reflections | 66,825 | 66,971 | 39,388 | 35,265 |
| Resolution limit (Å) | 50.00–2.90 (2.95–2.90) | 50.00–2.90 (2.95–2.90) | 50.00–3.50 (3.56–3.50) | 50.00–3.60 (3.66–3.60) |
| Redundancy | 10.8 (11.2) | 9.4 (9.7) | 4.4 (4.5) | 9.2 (9.4) |
| Completeness (%) | 100 (100) | 100 (100) | 98.8 (99.9) | 99.7 (100) |
| I/σ(I) | 27.5 (3.6) | 25.1 (3.3) | 14.7 (1.8) | 37.6 (5.8) |
| R<sub>rmerge</sub> (%) | 9.8 (76.2) | 9.6 (73.5) | 11.0 (75.7) | 7.3 (39.6) |

Structure refinement

- Resolution range (Å) 43.04–2.90 (3.00–2.90) 47.69–2.90 (3.00–2.90) 49.65–3.50 (3.61–3.48) 39.63–3.62 (3.75–3.62)
- Redundancy 10.8 (11.2) 9.4 (9.7) 4.4 (4.5) 9.2 (9.4)
- Completeness (%) 100 (100) 100 (100) 98.8 (99.9) 99.7 (100)
- I/σ(I) 27.5 (3.6) 25.1 (3.3) 14.7 (1.8) 37.6 (5.8)
- R<sub>rmerge</sub> (%) 9.8 (76.2) 9.6 (73.5) 11.0 (75.7) 7.3 (39.6)

| No. of unique reflections | 66,825 | 66,971 | 39,388 | 35,265 |
| No. of reflections | 63256 (6246) | 63457 (6234) | 37222 (3584) | 33318 (3272) |
| Ramachandran plot<sup>a</sup> | Favored (%) 99.5 | 99.8 | 92.2 | 90.1 |
| Allowed (%) | 4.5 | 4.2 | 7.4 | 8.2 |
| Outliers (%) | 0 | 0 | 0.4 | 1.7 |

<sup>a</sup> R<sub>rmerge</sub> = Σ|I<sub>obs</sub> − I<sub>calc</sub>|/Σ|I<sub>obs</sub>| where I<sub>obs</sub> is the intensity of measured reflection, and I<sub>calc</sub> is the intensity of calculated reflection. The numbers in parentheses represent the value for the highest resolution shell.
between Gαi3-GDP and the RGS14 GoLoco motif, which requires a total length of 35 residues (14). Consistent with earlier studies (40), the LGN GLs bind to Gαi3-GTPγS with a ~100-fold weaker affinity than to Gαi3-GDP (data not shown).

**Gαi3-GDP Can Simultaneously Bind to All Four LGN GLs**—We next asked whether Gαi3-GDP can simultaneously bind to the multiple GLs of LGN. We first tested the interaction between Gαi3-GDP and the LGN GL34 tandem (aa 587–650), because the intervening sequence between the core sequences of GL3&4 is the shortest (15 residues to be exact; Fig. 1A). According to the structure of Gαi-RGS14 complex (14), two successive GL core sequences separated by a 15-residue linker cannot bind to two Gαi because the bound Gαi molecules would crash into each other. We examined the binding stoichiometry between LGN-GL34 and Gαi3-GDP using analytical gel filtration chromatography. Upon addition of 2 or 3 molar ratios of Gαi3 to GL34, a peak corresponding to a (Gαi3-GDP)2-GL34 complex was detected (Fig. 2A), indicating that the two GLs in GL34 can simultaneously bind to Gαi3-GDP. To further substantiate that the elution peak at ~11.60 ml in Fig. 2A represents the 2:1 stoichiometric complex formed between Gαi3-GDP and GL34, we used two GL34 mutants (L594E and I628E), in which either the Gαi3-binding site on GL3 (the L594E mutant) or on GL4 (the I628E mutant) was disrupted. On gel filtration column, the 1:2 mixtures of the two GL34 mutants with Gαi3-GDP were eluted at a volume significantly larger than the wild type GL34, and a large portion of free Gαi3-GDP was also detected (Fig. 2B); presumably the GL34 mutants only formed 1:1 stoichiometric complex with Gαi3-GDP. This result also confirms that the wild type GL34 can form a 1:2 stoichiometric complex with Gαi3-GDP. Further lengthening of the linker between GL3 and GL4 by inserting 10 flexible residues (five GS repeats, referred to as GL34Ins5GS) did not alter the elution profile of its complex with Gαi3-GDP (data not shown), indicating that the 15-residue intervening sequence between GL3 and GL4 is sufficiently long for two molecules of Gαi3-GDP to bind simultaneously to GL34. Similarly, two molecules of Gαi3-GDP are capable of binding to LGN-GL12 (aa 483–586) or GL23 (aa 537–620). (Fig. 2, C and D). Additionally, three molecules of Gαi3-GDP were found to bind simultaneously to GL123 (aa 483–620) or GL234 (aa 537–650) of LGN (Fig. 2, E and F).

To characterize the binding stoichiometry more precisely, ITC analyses were performed. The titration profiles of Gαi3-GDP to GL23 and GL34 can be well fitted with the model using one set of identical sites, yielding overall stoichiometries of 1:9:1 and 1:8:1, respectively (Fig. 3A and B, and Table 2), consistent with the binding stoichiometry derived from the gel filtration analyses. The apparent binding affinity of GL23 was similar to those of the individual GoLocos, whereas GL34 had a weaker binding affinity than that of GL3 or GL4 (Table 2). The ITC titration profile of Gαi3-GDP to the triple-GoLoco-containing protein GL234 was also fitted with the model with one set of binding sites, giving a weaker binding affinity of ~358 nM and a binding stoichiometry of 3:1:1 (Fig. 3C and Table 2). The purified GL234 protein underwent slight degradation, which might affect the accuracy of the binding affinity measurement. The titration profile of Gαi3-GDP to GL12, however, was best fitted with the model that assumes two sets of binding sites (Fig. 3D), yielding one strong site \(K_D \approx 11 \text{ nM}\) and one weak site \(K_D \approx 188 \text{ nM}\) (Table 2). The ITC titration profile of Gαi3-GDP to GL123 was also fitted with the ‘two sets of binding sites’ model, giving rise to two strong sites \(K_D \approx 4 \text{ nM}\) and one weak site \(K_D \approx 186 \text{ nM}\) (Fig. 3E and Table 2). Similar atypical profiles of ITC titrations were also observed in the analyses of AGS3-GLs/Gαi3-GDP interaction (41). It is worth

![Crystal Structures of LGN GoLoco-Gαi Complex](image)
Crystal Structures of LGN GoLoco-Gαi Complex

FIGURE 2. Gαi-GDP binding to multiple GL containing fragments of LGN analyzed by analytical gel filtration chromatography. A, the binding of LGN-GL34 to different molar ratios of Gαi3-GDP. B, the binding of LGN-GL34(L594E) and LGN-GL34(I628E) to Gαi3-GDP. C, the binding of LGN-GL12 to Gαi3-GDP. D, the binding of LGN-GL23 to Gαi3-GDP. E, the binding of LGN-GL123 to Gαi3-GDP. F, the binding of LGN-GL234 to Gαi3-GDP.
noting that these data analyses do not represent the complete description of the thermodynamics of the interactions between tandem LGN-GoLoco repeats and $\alpha_{i3}$-GDP, in which intersite cooperativity likely exists. Because the full-length GoLoco region of LGN, i.e., GL1234, suffers from severe degradation, we did not analyze the binding property of GL1234 directly. However, the ITC titration data, consistent with the gel filtration analyses, strongly suggested that the full-length LGN binds $\alpha_{i3}$-GDP with a stoichiometry of 1:4. The four GoLoco motifs of LGN have intrinsically similar binding affinities to $\alpha_{i3}$-GDP. To explore the molecular details of the binding, we proceeded to determine the crystal structure of the $\alpha_{i3}$-LGN-GoLoco complex.

Overall Crystal Structures of GL3 and GL4 in Complex with $\alpha_{i3}$-GDP—Extensive efforts have been put to screen various constructs of the four LGN GLs in complex with GDP-loaded $\alpha_{i3}$ or $\alpha_{i1}$, and we succeeded in obtaining well diffracting crystals for synthetic GL4 (E216DEDFFSLILRSQAKRMDEQRV-LLQRD645) and GL3 (E287DEDFFDLVKCQGSRLDDQRCAPP$^{611}$) peptides in complex with $\alpha_{i3}$-GDP. The $\alpha_{i3}$-GL4, $\alpha_{i3}$-GL4, and $\alpha_{i3}$-GL3 complexes diffracted to 2.9, 3.5, and 3.6 Å resolutions, respectively (Table 1). According to a previous structure-based protein design study, point mutations on $\alpha_{i1}$ (E116L, Q147L, and E245L, respectively) can enhance its binding affinity to various GLs (15). We therefore constructed such three $\alpha_{i3}$ mutants, hoping that the mutants might have higher affinities in binding to LGN GLs and thus yield better quality complex crystals. Opposite to our expectation, none of these mutants showed obviously enhanced binding to LGN GLs (data not shown). Nonetheless, the Q147L-$\alpha_{i3}$ mutant-GL4 complex yielded better diffracting crystals (2.9 Å) than the wild type $\alpha_{i3}$-GL4 complex.

FIGURE 3. ITC analyses of the binding of tandem GoLoco motifs to $\alpha_{i3}$-GDP. ITC measurements of binding of $\alpha_{i3}$-GDP to LGN-GL23 (A), LGN-GL34 (B), LGN-GL24 (C), LGN-GL12 (D), and LGN-GL13 (E). The titration data were fitted with the models with one set of binding sites and two sets of binding sites. The derived thermodynamic parameters are shown in Table 2.
The structures of Goαi1(33)GL4 and Goαi2GL3 were solved by molecular replacement using the Goαi1RGS14 structure as the search model (Protein Data Bank code 1KJY) (14). The Goαi2-GDP structure is well defined, and 21–22 amino acids of the GL3 or GL4 peptide are ordered in the structures of complexes (Fig. 4, A and B). The structures of Goαi in the Goαi1(33)GL4 and Goαi2GL3 complexes are highly similar to that in the Goαi1RGS14 complex (root mean square deviation of 0.67 Å), except for the Switch II region, which is shifted further away from the LGN-GL peptides because of the presence of two bulky hydrophobic residues in the GL peptide (Fig. 5, A and B). The GL peptides in the three complexes adopt highly similar structures (Fig. 4, C). The N-terminal 10 residues of each LGN GL peptide (aa 623–632 of GL4 and aa 589–598 of GL3), which corresponds to the first half of the conserved 19-residue core, forms an α-helix that occupies the cleft between Switch II and α3 of Goαi (Fig. 4A). The following eight residues of the GL core (aa 633–640 of GL4 and aa 599–606 of GL3) forms a “lid” in covering GDP. Only three or four residues C-terminal to the core (aa 633–640 of GL4 and aa 599–606 of GL3) forms a “lid” core, forms an all-helical domain of G sterile GL peptides because of the presence of two (root mean square deviation of 0.67 Å), except for the Switch II region, which is shifted further away from the LGN-GL peptides because of the presence of two bulky hydrophobic residues in the GL peptide (Fig. 5, A and B). The GL peptides in the three complexes adopt highly similar structures (Fig. 4, C). The N-terminal 10 residues of each LGN GL peptide (aa 623–632 of GL4 and aa 589–598 of GL3), which corresponds to the first half of the conserved 19-residue core, forms an α-helix that occupies the cleft between Switch II and α3 of Goαi (Fig. 4A). The following eight residues of the GL core (aa 633–640 of GL4 and aa 599–606 of GL3) forms a “lid” in covering GDP. Only three or four residues C-terminal to the GL core (aa 641–643 of GL4 and aa 607–610 of GL3) were found to bind to the all-helical domain of Goαi (Fig. 4). The structures of the LGN GL peptides in complex with Goαi are entirely consistent with our biochemical data, showing that extending of the conserved GL core at the C-terminal end by three or four residues is necessary and sufficient for LGN GLs to bind to Goαi (Figs. 1 and 2). The structures of the complexes also indicate that LGN GLs should function as GDIs by directly stabilizing the bound GDP as well as the interaction between the Ras-like domain and the all-helical domain of Goαi (Fig. 4A).

A General Interaction Mode Revealed by the LGN GLs in Complex with Goαi—Although the structures of Goαi bound to the GLs of RGS14 and LGN are highly similar, the conformation of Goαi-bound GLs of RGS14 and LGN are distinctly different (Fig. 5). First, a 16-residue fragment C-terminal to the conserved GL core of RGS14 is required for binding to Goαi, and this 16-residue fragment forms ordered structure and has extensive interactions with the all-helical domain of Goαi (14). In LGN-GL4/GL3, in contrast, only three or four residues C-terminal to the GL core are required for binding to Goαi (Fig. 4A). Second, the orientation of the variable C-terminal tail of the RGS14 GL peptide is opposite to that of the LGN GL peptides (Fig. 5A). In the LGN GL4-Goαi complex, the hydrophobic side chains of Val641, Leu642, and Leu643 interact with Val72 and Tyr69 from the αA helix of the Goαi all-helical domain; thus the C-terminal end of GL4 extends toward the N-terminal end of Goαi αA (Fig. 5C). The residue corresponding to Val641 in the RGS14 peptide is Gly517 (Fig. 5D and Fig. 6A). The backbone carbonyl oxygen of Gly517 in the RGS14 peptide forms two hydrogen bonds with side chains of Ser75 and Gin79 from Goαi αA. The unique backbone dihedral angles (ϕ = 78°, ψ = −171°) of Gly517, which are not allowed by other amino acids, enable the C-terminal tail of the RGS14 GL peptide to take a sharp turn at this position and extend to the C-terminal end of Goαi αA (Fig. 5, A and D). Sequence alignment of all known GLs from mammals reveals that only the GLs of RGS14 and RGS12 contain a Gly right after the conserved core motif, and the C-terminal residues of these two GLs share the identical sequence (Fig. 6A). The above structure-based amino acid sequence analysis suggests that the LGN GL/Goαi interactions observed in this study represent the general mode of the interactions between GoLoco proteins and Goαi. RGS14 and RGS12, instead, may represent a special subclass of GoLoco proteins in terms of Goαi binding.

The Double Arg Finger-mediated GDP Binding of LGN GLs—The structure of the Goαi1RGS14 GL complex shows that a highly conserved (D/E)QR triad at the C-terminal end of the conserved GL core plays a critical role in binding to Mg2+-GDP (14). Similar to the Goαi1RGS14 GL interaction, the side chain of Arg640 (Arg606) of GL4 (GL3) in the (D/E)QR triad, which is equivalent to Arg516 of RGS14, is inserted into the GDP-bind-

**TABLE 2**

Thermodynamic parameters of the bindings of LGN GoLoco motifs to Goαi-GDP determined by ITC titration

The titration data of GL12 and GL123 were fitted with the two sets of binding sites model, whereas the other data were fitted with the one set of binding sites model. N denotes the number of binding sites in each model.

|     | N  | K<i><sub>p</sub> | ΔH | ΔS | ΔG |
|-----|----|----------------|-----|-----|-----|
| GL12 |    |                |     |     |     |
| Site 1 | 0.97 | 11.27 | −16.69 | −18.7 | −11.12 |
| Site 2 | 0.89 | 188.32 | −22.80 | −44.4 | −9.57 |
| GL123 |    |                |     |     |     |
| Site 1 | 1.63 | 4.69 | −12.55 | −3.97 | −11.37 |
| Site 2 | 1.0  | 186.22 | −15.46 | −21.0 | −9.20 |
| GL23  | 1.86 | 87.72 | −17.05 | −24.0 | −9.90 |
| GL34  | 1.81 | 173.61 | −20.97 | −37.2 | −9.88 |
| GL34  | 3.08 | 358.42 | −5.12 | 11.9  | −8.67 |

The structures of Goαi1(33)GL4 and Goαi2GL3 were solved by molecular replacement using the Goαi1RGS14 structure as the search model (Protein Data Bank code 1KJY) (14). The Goαi2-GDP structure is well defined, and 21–22 amino acids of the GL3 or GL4 peptide are ordered in the structures of complexes (Fig. 4, A and B). The structures of Goαi in the Goαi1(33)GL4 and Goαi2GL3 complexes are highly similar to that in the Goαi1RGS14 complex (root mean square deviation of 0.67 Å), except for the Switch II region, which is shifted further away from the LGN-GL peptides because of the presence of two bulky hydrophobic residues in the GL peptide (Fig. 5, A and B). The GL peptides in the three complexes adopt highly similar structures (Fig. 4, C). The N-terminal 10 residues of each LGN GL peptide (aa 623–632 of GL4 and aa 589–598 of GL3), which corresponds to the first half of the conserved 19-residue core, forms an α-helix that occupies the cleft between Switch II and α3 of Goαi (Fig. 4A). The following eight residues of the GL core (aa 633–640 of GL4 and aa 599–606 of GL3) forms a “lid” in covering GDP. Only three or four residues C-terminal to the GL core (aa 641–643 of GL4 and aa 607–610 of GL3) were found to bind to the all-helical domain of Goαi (Fig. 4). The structures of the LGN GL peptides in complex with Goαi are entirely consistent with our biochemical data, showing that extending of the conserved GL core at the C-terminal end by three or four residues is necessary and sufficient for LGN GLs to bind to Goαi (Figs. 1 and 2). The structures of the complexes also indicate that LGN GLs should function as GDIs by directly stabilizing the bound GDP as well as the interaction between the Ras-like domain and the all-helical domain of Goαi (Fig. 4A).
The Double-arginine Fingers Are Critical to the GDI Activities of LGN-GLs—To confirm the functional importance of the two Arg in the double-arginine finger in LGN GLs, we performed point mutations of the two arginines and tested the $\Gamma_0$-GDP binding affinities and GDI activities of these mutants. Single substitution mutations (R635G, R635A, and R640A) caused a 50-fold decrease in GL4 binding to $\Gamma_0$-GDP, and the double mutation (R635A/R640A) led to a 500-fold $\Gamma_0$-GDP binding affinity decrease (Fig. 6C). Similar results were also obtained from the other LGN GLs, indicating that the two conserved arginine fingers are critical for binding of $\Gamma_0$-GDP to LGN-GLs. This finding is in contrast to the RGS14 GL, in which the substitution of the Arg in the finger with Ala or Leu did not decrease the binding affinity of RGS14 to $\Gamma_0$-GDP (14). Careful examination of the crystal structures of $\Gamma_0$ in complex with LGN GL peptides revealed that the side chains of the two Arg residues also form hydrogen bonds with Val179 and Thr181 from $\Gamma_0$, (Fig. 6B). In contrast, the side chain of Arg516 in RGS14 GL interacts exclusively with GDP (14).

The GDI activities of LGN GLs were evaluated by AlF$_4^−$-induced increase of intrinsic tryptophan fluorescence of $\Gamma_0$ and by direct binding of BODIPY-GTPS to $\Gamma_0$. In agreement with the previous studies (40), the four GLs exhibited similar GDI activities (data not shown). Moreover, comparison of the GDI activities of GL peptides with different lengths showed that the 25-residue minimal $\Gamma_0$-binding GL fragments shown in Fig. 1 are also sufficient for their GDI activities (data not shown). Further quantification of the GDI activities using the association rate of BODIPY-GTPS binding revealed IC$_{50}$ values of a few μM for LGN GLs, which is slightly weaker than that of GDP.
At a saturated concentration of GL peptide (GL, 200 μM; G, 0.2 μM), the wild type LGN-GL4 showed a complete inhibition of GDP dissociation from G (Fig. 6D). The R635G-GL4 or the R635A-GL4 displayed obviously weakened GDI activities, whereas the R640A-GL4 and R635,640A-GL4 had essentially no detectable GDI activity (Fig. 6D). Substitution of the first Arg (Arg601) in the double-arginine finger of GL3 with Ala or Gly also diminished its GDI activity (data not shown). Thus, we conclude that both arginines in the double-arginine finger of LGN GoLoco motifs are important for their GDI activity.

DISCUSSION

Both the binding to GDP-loaded Gα subunits and the GDI activity of GL require residues beyond the 19-residue conserved core sequence (14, 41). Because the C-terminal flanking sequences of GLs are highly diverse among GoLoco proteins (7), it has been hypothesized that the variable C-terminal tail sequences of GLs are the specificity determinants governing GL/Gαi interactions. In the present study, we demonstrate that only a few residues (3–4 aa) C-terminal to the conserved GL core are required for LGN GLs to bind to and to inhibit GDP dissociation of Gαi GDP (Fig. 6D). Substitution of the first Arg (Arg601) in the double-arginine finger of GL3 with Ala or Gly also diminished its GDI activity (data not shown). Thus, we conclude that both arginines in the double-arginine finger of LGN GoLoco motifs are important for their GDI activity.

RGS14 GL (data not shown). At a saturated concentration of GL peptide (GL, 200 μM; G, 0.2 μM), the wild type LGN-GL4 showed a complete inhibition of GDP dissociation from Gαi (Fig. 6D). The R635G-GL4 or the R635A-GL4 displayed obviously weakened GDI activities, whereas the R640A-GL4 and R635,640A-GL4 had essentially no detectable GDI activity (Fig. 6D). Substitution of the first Arg (Arg601) in the double-arginine finger of GL3 with Ala or Gly also diminished its GDI activity (data not shown). Thus, we conclude that both arginines in the double-arginine finger of LGN GoLoco motifs are important for their GDI activity.

DISCUSSION

Both the binding to GDP-loaded Gα subunits and the GDI activity of GL require residues beyond the 19-residue conserved core sequence (14, 41). Because the C-terminal flanking sequences of GLs are highly diverse among GoLoco proteins (7), it has been hypothesized that the variable C-terminal tail sequences of GLs are the specificity determinants governing GL/Gαi interactions. In the present study, we demonstrate that only a few residues (3–4 aa) C-terminal to the conserved GL core are required for LGN GLs to bind to and to inhibit GDP dissociation of Gαi GDP, a finding that is in sharp contrast to that of RGS14 GL. Sequence alignment analysis suggests that the conformation of the GL peptide in the Gαi-RGS14 structure is likely a unique example of GL/Gαi interaction. The LGN GL/Gαi interaction described in the current study instead is likely a general binding mode between GLs and Gαi. The structures of LGN GLs in complex with Gαi-GDP also suggest that...
in response to the binding of Goαi-GDP and NuMA/Mud (34, 43). In addition to this, the multiple GLs in LGN (Pins) also function as a scaffold in regulating the localization of related protein complexes and organizing signaling pathways mediating spindle orientations. The detailed characterization of interactions between LGN-GLs and Goαi-GDP in this work demonstrate that in its open state the four LGN GLs have equal capacity to bind to Goαi-GDP (Fig. 6E). In another word, the stoichiometry of LGN/Goαi-GDP complex in vivo likely depends on the concentration of Goαi-GDP, which in turn regulates the cortical localization of LGN-bound proteins, such as NuMA. Recently, it was found that the extrinsic GPCR Tre1 signaling determines the orientation of cortical polarity in the asymmetric cell division of Drosophila neuroblast (44). Tre1 was shown to activate Goαo, and the GTP form Goαo can specifically associate with the first GL of Pins (44). Thus, the presence of multiple GLs allows Pins to function as a scaffold to simultaneously engage Goαo- and Goαi-mediated signaling events during asymmetric cell division.

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