Structure and Biochemical Characterizations of LGN
GoLoco/Gαi Interaction

By

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and have found that it is complete and satisfactory in all respects,
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the thesis examination committee have been made.

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Structure and Biochemical Characterizations of LGN
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Abstract

GoLoco motif (GL)-containing proteins regulate G-protein signaling by binding to Gα subunit and acting as guanine nucleotide dissociation inhibitors (GDI). GLs of LGN are also known to tether its N-terminal TPR repeats targets (e.g. NuMA and mlnsc) to cell cortex via binding to membrane bound Gαᵢ₀ during asymmetric cell division. The molecular basis underlying both G-protein signaling and scaffolding roles of LGN GLs is unclear. Here, we show that each of the four GLs of LGN binds to Gαᵢ·GDP independently and with comparable affinities ($K_D \sim 100$ nM). The crystal structures of Gαᵢ·GDP in complex with LGN GL3 and GL4 peptides, respectively, reveal distinct GL/Gαᵢ interaction features when compared to the only known GL/Gαᵢ interaction between RGS14 and Gαᵢ₁: only a few residues C-terminal to the conserved GL motif sequence are required for LGN GLs to bind to Gαᵢ·GDP; the C-terminal part of LGN GL peptide occupies a much smaller surface area on the helical-domain of Gαᵢ in contrast to that occupied by the RGS14 GL; a highly conserved double Arg-finger sequence ("R-Ψ-D/E-D/E-Q-R") is responsible for LGN GL/Gαᵢ recognition. We demonstrate that the LGN GL/Gαᵢ interaction represents a general binding mode between GL motifs and Gαᵢ. Finally, we show that the LGN GL motifs are potent GDIs. Thus the GLs of LGN may function both as the Gα/LGN/NuMA/mlnsc complex scaffold and a Gα signaling regulator during asymmetric cell division.
Chapter 1

Introduction

1.1 Gα signaling

The α subunit of the heterotrimeric G proteins (Gα) is a critical component of the G protein signaling pathway, in which Gα cycles between the GDP and GTP bound states (Malbon, 2005). In the canonical signaling model, ligand-mediated activation of G-protein coupled receptors (GPCR) catalyzes the exchange of GDP for GTP in binding to Gα and subsequently results in the dissociation of Gα-GTP from Gβγ heterodimer (Gilman, 1987; Sprang, 1997). The dissociated Gα-GTP binds to and activates downstream effectors, thus transducing signals from GPCR (Kozasa et al., 1998; Singer et al., 1997; Sunahara et al., 1996). Since Gα has intrinsic GTPase activity, the Gα subunit subsequently returns to the Gα·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 Gα reaction cycle. Among these, GoLoco motif proteins are recently discovered to bind specifically to GDP-loaded Gαi or Gαo and inhibit the spontaneous release of GDP from Gα. These GoLoco proteins are referred to as guanine nucleotide dissociation inhibitors (GDI) (Bernard et al., 2001; Kimple et al., 2001; Natochin et al., 2001; Siderovski et al., 1999; Willard et al., 2004).

1.2 GoLoco motif

GoLoco (GL) motif 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 (Willard et al., 2004). 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 Gαi1·GDP (Kimple et al., 2002), which shows that the conserved GoLoco motif and its variable C-terminal tail interact with the Ras-like and all-helical domains of Gαi1, respectively. A so-called “arginine finger” within the highly conserved ‘D/E-Q-R’ triad in the conserved GoLoco motif extends into the GDP binding pocket and directly contacts the α- and β-phosphates of GDP (Kimple et al., 2002). This structure and the subsequent
mutagenesis studies (Bosch et al., 2011; Kimple et al., 2002; Sammond et al., 2007) suggested an appealing hypothesis: the highly variable C-terminal sequences following the conserved GoLoco motifs and the all-helical domain of Gα subunits are likely the specificity determinants of interactions between GoLoco motifs and different Gα subunits. However, since there is no other structures of GoLoco motifs in complex with Gα other than the Gαi/RGS14 complex, the above hypothesis remains untested.

1.3 LGN and asymmetric cell division

LGN is a multi-domain scaffolding protein containing seven tetratricopeptide (TPR) repeats in its N-terminal region, a flexible linker sequence in the middle, and four GoLoco motifs in the C-terminal end (Du et al., 2001). LGN is an evolutionarily conserved protein (Pins in Drosophila, and GPR1/2 in C. elegans) that plays crucial roles in regulating spindle orientations during asymmetric cell division (Du et al., 2001; Du et al., 2002), 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) on mitotic spindles and cortical membrane-bound Gαi/o with its TPR and GoLoco domains, respectively (Bowman et al., 2006; Colombo et al., 2003; Du and Macara, 2004; Gotta et al., 2003; Izumi et al., 2006; Siller et al., 2006; Srinivasan et al., 2003). The central linker of LGN binds to the guanylate kinase domain of DLG family scaffold protein in a phosphorylation-dependent manner (Bellaiche et al., 2001; Sans et al., 2005; Zhu et al., 2011). In Drosophila neuroblast, loss of Pins or Gαi affects cell polarity as well as mitotic spindle orientation (Yu et al., 2006). In mammals, overexpression or RNAi-mediated knockdown of LGN results in dramatic spindle rocking in metaphase and improper spindle pole organization (Du et al., 2001; Du et al., 2002; Kaushik et al., 2003). The binding of Gαi through the GoLoco motifs was shown to regulate the cortical localization of LGN (Kaushik et al., 2003). Thus the LGN GoLoco motifs can be viewed as scaffolding modules in tethering LGN’s TPR-repeats partners (e.g. NuMA/Mud and mInsc/Insc) to cell cortex via binding to membrane attached Gαi. Interestingly, the GoLoco motifs of LGN can directly bind to TPR repeats intra-molecularly, thus keeping LGN in an auto-inhibited conformation (Du and Macara, 2004). Gαi-GDP binding to GoLoco motifs releases the auto-inhibited conformation
of LGN and renders LGN TPR repeats capable of binding to NuMA (Du and Macara, 2004; Nipper et al., 2007), although the mechanistic basis of the LGN auto-inhibition is unknown.

1.4 Objectives of my research

In this study, we performed detailed biochemical and structural analyses of the interactions between LGN GoLoco motifs and Ga\textsubscript{i}-GDP. We demonstrate that the highly variable sequences C-terminal to the conserved GoLoco motifs of LGN are not involved in binding to Ga\textsubscript{i}-GDP, thus the specificity of GoLoco/Ga\textsubscript{i} interaction does not depend on these sequences. The complex structures of two LGN GoLoco motifs in complex with Ga\textsubscript{i} reveal a “double Arg-finger” sequence (“R-Ψ-D/E-D/E-Q-R”) within the GoLoco motifs that is specifically involved in the GDP coordination. We further show that the LGN GoLoco/Ga\textsubscript{i}-GDP interaction observed in this study, instead of the previously characterized RGS14/Ga\textsubscript{i}-GDP complex, represents a general mode of GoLoco motif-mediated Ga\textsubscript{i} bindings. We further demonstrate that the LGN GoLoco motifs are potent GDIs. Thus the LGN GoLoco motifs can function as a Ga\textsubscript{i}/LGN/NuMA/Insc scaffold as well as a regulator of Ga\textsubscript{i} signaling in asymmetric cell division.
2.1 Protein Expression and Purification

The human $\alpha_{i3}$, $\alpha_{i1}$, mouse LGN GoLoco motif 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$^{2+}$-NTA agarose affinity chromatography followed by size-exclusion chromatography. For *in vitro* biochemical analysis, LGN-GoLocos were expressed as the GST-fused proteins and purified by GSH-Sepharose affinity chromatography.

2.2 Isothermal Titration Calorimetry Measurements

ITC measurements were performed on an ITC200 Micro calorimeter (MicroCal) at 25°C. All protein samples were dissolved in a buffer containing 50 mM Tris, pH 8.0, 100 mM NaCl, and 1 mM EDTA. The titration was carried out by injecting 40 $\mu$L $\alpha_{i3}$-GDP aliquots into LGN-GoLoco fragment fused to the C-terminal end of thioredoxin at time intervals of 2 minutes to ensure that the titration peak returned to the baseline. The titration data were analyzed using the program Origin7.0 from MicroCal.

2.3 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, Molecular Probe) at 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 $K_D$ values were obtained by fitting the titration curves with the classical one-site binding model, with or without invoking the Hill coefficient model.
2.4 GST Pull-Down Assay

For GST pull-down assay, GST or GST-tagged proteins (60 µl 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 hr 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.

2.5 Analytical Gel Filtration Chromatography

Analytical gel filtration chromatography was carried out on an AKTA FPLC system (GE Healthcare). Proteins were loaded on to a Superose 12 10/300 GL column 20 (GE Healthcare) equilibrated with a buffer containing 50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM DTT, and 1 mM EDTA.

2.6 GDI activity Assay

Measurements of intrinsic tryptophan fluorescence were performed on the PerkinElmer LS-55 spectrometer with excitation at 292 nm and emission at 342 nm. Purified Gαi3 protein was diluted in 2-ml cuvettes to 200 nM in a pre-activation buffer (100 mM NaCl, 100 µM EDTA, 2 mM MgCl2, 20 µM GDP, 20 mM Tris-HCl, pH 8.0) and incubated at 30 °C. At the time points 400 and 500 sec after Gα dilution, 2mM NaF and 30 µM AlCl3 (final concentrations) were added respectively to the reaction mixture, and fluorescence intensity changes as a function of time were recorded. The GDI activities of GoLoco peptides were assayed by repeating the above procedure except that the reaction mixtures contained defined concentrations of specific peptides.

The measurements of GTPγS binding were also performed on PerkinElmer LS-55 spectrometer with excitation at 485 nm and emission at 530nm (slit widths each at 2.5nm). BODIPY FL-GTPγS was diluted to 1 µM in buffer (20 mM Tris-HCl, pH 8.0, 1 mM EDTA and 10 mM MgCl2) and equilibrated to 30°C in 2-ml cuvettes. Purified Gαi3 was diluted to 100nM in the buffer (100 mM NaCl, 100 µM EDTA, 2 mM MgCl2, 20 µM GDP, 20 mM Tris-HCl, pH 8.0) and pre-incubated with GL peptides (with different concentrations) at 30°C for 10 min before the addition to the cuvette. Relative fluorescence levels were set to zero at the average fluorescence reading over the first 70 s, and Gαi3/GL mixtures were added at the
time point of 100 sec.

2.7 Crystallography

Crystals of the Gαi1(3) in complex with GL3/4 (diluted to 7.5 mg/ml in 50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM Mg\(^{2+}\), 20 μM GDP buffer) were obtained by the hanging drop vapor diffusion method at 18 °C. The crystals were grown in buffer containing 0.5 M ammonium sulfate, 1.0 M lithium sulfate monohydrate, 0.1 M sodium citrate tribasic dihydrate, pH 5.6. Crystals were soaked in crystallization solution containing a higher concentration (1.5 M) of lithium sulfate for cryoprotection. The diffraction data were collected at Shanghai Synchrotron Radiation Facility at wavelength of 0.9793 Angstrom and were processed and scaled using HKL2000 (Otwinowski and Minor, 1997). Molecular replacement was used to solve the structure of Gαi1(3)/GL4(3) with the program Molrep (Vagin and Teplyakov, 1997). The initial model was rebuilt manually and then refined using REFMAC (Murshudov et al., 1997) and PHENIX (Adams et al., 2010) against the whole data set. Further manual model building and adjustment were completed using COOT (Emsley and Cowtan, 2004). The final refinement statistics are summarized in Table 3.1. All structure figures were prepared by PyMOL (http://www.pymol.org/).
Chapter 3
RESULTS

3.1 Mapping the minimal $\Gamma_{i}\cdot$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. 3.1A). We refer each full-length GoLoco motif as the conserved 19-residue plus all of the following C-terminal sequence before the start of the next GoLoco motif core. With this definition, the full-length GoLoco1, 2, 3 and 4 domains of LGN consist of 53, 51, 34 and 51 residues respectively (Fig. 3.1A). Previous structural study of the $\Gamma_{i}\cdot$RGS14-GoLoco complex showed that a stretch of 16-residue sequence C-terminal to the GoLoco core motifs make extensive contacts with $\Gamma_{i}$ and thus are essential for the interaction between $\Gamma_{i}$ and RGS14 (Kimple et al., 2002). To understand the interaction between LGN and $\Gamma_{i}$, we set out to map the minimal $\Gamma_{i}\cdot$GDP binding sequence of each LGN GoLoco motif. We first used GST-fused LGN GoLoco motifs with different lengths to pull down purified $\Gamma_{i}\cdot$GDP as a binding assay. This assay showed that each of the LGN GoLoco motif containing only the 19-residue core displayed only a background level binding to $\Gamma_{i}\cdot$GDP (Fig. 3.1B1). Obvious bindings of $\Gamma_{i}\cdot$GDP to GoLoco1 and GoLoco4 were observed by extending the conserved 19-residue GoLoco core by 2 residues (Fig. 3.1B2). Any of the LGN GoLoco motifs with length equal to or longer than 25 residues displayed comparable bindings compared to the corresponding full-length motif (Fig. 3.1B3). We next measured the quantitative binding affinities of each of the four LGN GoLoco motifs to $\Gamma_{i}\cdot$GDP using isothermal titration calorimetry or fluorescence spectroscopy. Such quantitative binding assays revealed that the four full-length LGN GoLoco motifs share similar affinities ($K_D$: 54 ~ 96 nM) in binding to $\Gamma_{i}\cdot$GDP (Fig. 3.1C). In agreement with result derived from the pull down-based assays, each of the LGN GoLoco motifs with a length of 25 residues has an essentially same binding affinity compared to the corresponding full-length motif (Fig. 3.1C), indicating that each of the 25-residue LGN GoLoco motif contains the complete $\Gamma_{i}\cdot$GDP binding sequence. This finding is in sharp contrast to the
interaction between Goα3-GDP and the RGS14 GoLoco motif, which requires a total length of 35 residues (Kimple et al., 2002). Consistent with earlier studies (McCudden et al., 2005), the LGN GoLoco motifs bind to Goα3-GTPγS with a ~100-fold weaker affinity than to Goα3-GDP (Fig. 3.2).

3.2 Goα3-GDP can simultaneously bind to all four GoLoco motifs of LGN

We next asked whether Goα3-GDP can simultaneously bind to the multiple GoLoco motifs of LGN. We first tested the interaction between Goα3-GDP and the LGN GoLoco34 tandem (aa...
Figure 3.2. The binding affinity of LGN-GL1 peptide (30 aa) to GTP bound or GDP·AlF₄⁻ bound Gαᵢ₃ measured with fluorescence-based assay.

587-650), as the intervening sequence between the core sequences of GoLoco3&4 are the shortest (15 residues to be exact, Fig. 3.1A). According to the structure of Gαᵢ₃/RGS14 complex (Kimple et al., 2002), two continuous GoLoco motif core sequences separated by 15-residues cannot bind to two Gαᵢ as the bound Gαᵢ molecules will crash into each other. We examined the binding stoichiometry between LGN-GL34 and Gαᵢ₃·GDP using analytical gel-filtration chromatography. Upon addition of saturating molar ratios of Gαᵢ₃·GDP (e.g. 2 or 3 molar ratios of Gαᵢ₃ to GL34), a complex peak corresponding to a (Gαᵢ₃·GDP)₂/GL34 complex was detected (Fig. 3.3A), indicating that the two GoLoco motifs in GL34 can simultaneously bind to Gαᵢ₃·GDP. In the presence of sub-stoichiometric amount of Gαᵢ₃·GDP (e.g. 0.5 or 1 molar ratio of Gαᵢ₃ to GL34), we detected co-existence of a (Gαᵢ₃·GDP)₂/GL34 complex and a 1:1 Gαᵢ₃·GDP/GL34 complex (the peak should at an elution volume ~12.5 ml in Fig. 3.3A, also see Fig. 3.3B). To further substantiate that the elution peak at ~11.60 ml in Fig. 3.3A represents the 2:1 stoichiometric complex of
Figure 3.3. $G_{o_i}$-GDP binding to multiple GoLoco containing fragments of LGN analyzed by analytical gel-filtration chromatography. (A) The bindings of LGN-GL34 to different molar ratios of $G_{o_i}$-GDP. (B) The bindings of LGN-GL34(L594E) and LGN-GL34(I628E) to $G_{o_3}$-GDP. (C) The bindings of LGN-GL12 to $G_{o_3}$-GDP. (D) The bindings of LGN-GL23 to $G_{o_3}$-GDP. (E) The bindings of LGN-GL13 to $G_{o_3}$-GDP. (F) The bindings of LGN-GL24 to $G_{o_3}$-GDP.
Figure 3.4. Characterization of the bindings between Ga\textsubscript{13}-GDP and LGN GoLoco motifs. (A) GST-fusion protein-based pull down assay showing that the L594E and I628E mutations effectively disrupted the bindings of GL3 or GL4 to Ga\textsubscript{13}-GDP. (B) Analytical gel-filtration-based analysis of the bindings of LGN-GL34Ins5GS to Ga\textsubscript{13}-GDP. (C) Analytical gel-filtration-based analysis of the bindings of LGN-GL13 (Q554A) to Ga\textsubscript{13}-GDP. (D) Analytical gel-filtration-based analysis of the bindings of LGN-GL24 (Q605A) to Ga\textsubscript{13}-GDP.

Ga\textsubscript{13}-GDP/GL34, we used two GL34 mutants (L594E and I628E), in which either the Ga\textsubscript{13}-GDP binding site on GL3 (the L594E mutant) or on GL4 (the I628E mutant) was disrupted (Fig. 3.4A). On gel-filtration column, the 1:2 mixtures of the two mutants with Ga\textsubscript{13}-GDP were eluted at a volume significantly larger than the wild type GL34 and a large portion of free Ga\textsubscript{13}-GDP was also detected (Fig. 3.4B), presumably that the GL34 mutants only formed 1:1 stoichiometric complex with Ga\textsubscript{13}-GDP. This result also confirms that the wild type GL34 can form a 1:2 complex with Ga\textsubscript{13}-GDP. Further lengthening of the linker between GL3 and GL4 by inserting ten flexible residues (five GS repeats, referred to as
“GL34Ins5GS”) did not alter the elution profile of its complex with Ga\textsubscript{i3}-GDP (Fig. 3.4B), indicating that the 15-residue intervening sequence between GL3 and GL4 is sufficiently long for two molecules of Ga\textsubscript{i3}-GDP to bind simultaneously to GL34. Similarly, two molecules of Ga\textsubscript{i3}-GDP are capable of binding to LGN-GL12 (aa 483-586) or GL23 (aa 537-620). (Fig. 3.3C&D). Additionally, three molecules of Ga\textsubscript{i3}-GDP were found to bind simultaneously to GL123 (aa 483-620) or GL234 (aa 537-650) of LGN (Fig. 3.3E&F and Fig. 3.4C&D). We were not able to obtain any purified LGN C-terminal fragment containing GL1234 to test its binding stoichiometry with Ga\textsubscript{i3}-GDP. Nonetheless, the panels of binding data shown in Fig. 3.3 strongly indicate that the four GoLoco repeats of LGN can bind as many as four molecules of Ga\textsubscript{i}-GDP, and each GoLoco motif functions autonomously in binding to Ga\textsubscript{i}-GDP. To explore the molecular details of the binding, we further pursued to determine the crystal structure of Ga\textsubscript{i}/LGN-GoLoco complex.

3.3 Overall crystal structures of GL3 and GL4 in complex with Ga\textsubscript{i}

Extensive efforts have been put to screen various constructs of the four LGN GoLoco motifs in complex with GDP-loaded Ga\textsubscript{i3} or Ga\textsubscript{i1}, and we successfully obtained well-diffracting crystals for GL4 (aa 621-645) and GL3 (aa 587-611) in complex with Ga\textsubscript{i1/3}-GDP. The Ga\textsubscript{i1}/GL4, Ga\textsubscript{i3}/GL4 and Ga\textsubscript{i3}/GL3 complexes diffract to 2.9, 3.5 and 3.6 Å resolutions, respectively (Table 3.1). According to a previous structure-based protein design study, point mutations on Ga\textsubscript{i} (E116L, Q147L and E245L, respectively) can enhance its binding affinity to various GoLoco motifs (Bosch et al., 2011). We therefore constructed such three Ga\textsubscript{i3} mutants, hoping that the mutants may have higher affinity in binding to LGN GoLoco motifs and thus give rise to better quality crystals of the complexes. In contrast to the previous study (Bosch et al., 2011), none of these mutants showed enhanced binding LGN GoLoco motifs (Fig. 3.5). Nevertheless, the Q147L-Ga\textsubscript{i3} mutant/GL4 complex gave better diffracting crystals (2.9 Å) than the wild type Ga\textsubscript{i3}/GL4 complex.

The structure of Ga\textsubscript{i1/3}/GL4 and Ga\textsubscript{i3}/GL3 were solved by molecular replacement using the Ga\textsubscript{i1}/RGS14 structure as the searching model (PDB ID: 1KJY) (Kimple et al., 2002). The structure of the Ga\textsubscript{i}-GDP is well defined, and 21~22 amino acids of the GL3 or GL4 peptides
Figure 3.5. Binding affinities of LGN-GL peptides with G\(\alpha_{i3}\) mutant Q147L derived from fluorescence-based assay.

are ordered in the complex structures (Fig. 3.6A, B, Fig. 3.7). The structures of G\(\alpha_i\) in the G\(\alpha_{i1}(3)/\)GL4 and G\(\alpha_{i3}/\)GL3 complexes are very similar with that in the G\(\alpha_{i1}/\)RGS14 complex (RMSD of 0.67 Å), except for the Switch II region, which is shifted further away from the LGN-GoLoco peptides due to the presence of bulky two hydrophobic residues in the GL peptides (Fig. 3.8A&B). The GoLoco peptides in three complex structures adopt highly similar structures (Fig. 3.6C). The N-terminal 10 residues of each LGN GoLoco peptide (aa 623-632 of GL4 and aa 589-598 of GL3), which corresponding to the first half of the conserved 19-residue GoLoco motif core, forms an \(\alpha\)-helix which occupies a cleft between the Switch II and the \(\alpha3\) helix of G\(\alpha_i\) (Fig. 3.6). The following 8 residues from the GoLoco motif core (aa 633-640 of GL4 and aa 599-606 of GL3) forms a “lid” in covering GDP. Only 3-4 residues C-terminal to the GoLoco motif core (aa641-643 of GL4 and aa607-610 of GL3) were found to bind to the helical domain of G\(\alpha_i\) (Fig. 3.6). The structures of the LGN GoLoco
Figure 3.6. Crystal structures of $\text{G}_{\alpha 3}$ in complex with GL4 and GL3, respectively. (A) Ribbon diagram showing the crystal structure of LGN-GL4 in complex with $\text{G}_{\alpha 11}$-GDP. GDP is shown in the ball-and-stick model. All-helical domain and Ras-like domain of $\text{G}_{\alpha 11}$ is shown in wheat and light grey, respectively. The three switches are shown in violet, and the GoLoco peptide is shown in cyan. (B) The Fo-Fc density maps of GoLoco peptides are shown in green and contoured at 3.0 $\sigma$. (C) Comparison of the structures of the $\text{G}_{\alpha 11}$/GL4, $\text{G}_{\alpha 3}$/GL4 and $\text{G}_{\alpha 3}$/GL3 complexes by superimposing the backbone atoms in the three structures. $\text{G}_{\alpha 11}$ complex with GL4 are shown the same as panel A, while $\text{G}_{\alpha 3}$ complex with GL3 (RMSD of 0.76 Å) and GL4 (RMSD of 0.51 Å) are not shown. GL3 is shown in blue and GL4 peptides complex with $\text{G}_{\alpha 11}$ and $\text{G}_{\alpha 3}$ are shown in red and green respectively.
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**Structure Refinement**

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Numbers in parentheses represent the value for the highest resolution shell.

a. Rmerge = Σ |I_i - <I>| / Σ I_i, where I_i is the intensity of measured reflection and <I> is the mean intensity of all symmetry-related reflections.

b. Rcryst = Σ |F_cal - F_obs| / Σ F_obs, where F_obs and F_cal are observed and calculated structure factors.

c. R_free = Σ |T* F_cal| - |F_obs| / Σ F_obs, where T is a test data set of about 5% of the total unique reflections randomly chosen and set aside prior to refinement.

d. B factors and Ramachandran plot statistics are calculated using MOLPROBITY (Richardson et al., 2010).
Figure 3.7. Crystal structures of LGN-GoLoco4(3) in complex with Gαi(3). The F₀-Fc density maps of GoLoco peptides are shown in green and contoured at 3.0 σ. (A) LGN-GL4 in complex with Gαi₁. (B) LGN-GL4 in complex with Gαi₃ Q147L. (C) LGN-GL4 in complex with Gαi₃. (D) LGN-GL3 in complex with Gαi₃.

peptides in complex with Gαi are entirely consistent with our biochemical data showing that extending of the conserved GoLoco motif core at the C-terminal end by 3-4 residues is necessary and sufficient for LGN GoLoco motifs to bind to Gαi (Figs. 3.1&3.3). The complex structures also indicate that the LGN GoLoco peptides function as GDIs by directly stabilizing the bound GDP as well as by stabilizing the interaction between the Ras-like domain and the helical domain of Gαi (Fig. 3.6A).

3.4 A general GoLoco/Gαi interaction mode revealed by the LGN GoLoco motif in complex with Gαi₃

Although the structures of Gαi when bound to the GoLoco motifs of RGS14 and LGN
Figure 3.8. Comparison of the crystal structures of Gαi1/GL4 and Gαi1/RGS14 complexes. (A) Comparison of the crystal structure of Gαi1/GL4 (cyan) with that of Gαi1/RGS14 (green). The helical domain and Ras-like domain of Gαi are colored wheat and light grey, respectively. The Switch I, II and III regions of Gαi1 in complex with LGN-GL4 and with RGS14 are highlighted with violet and light blue, respectively. (B) Comparison of the structural details of the α-helical region of LGN-GL4 and RGS14-GoLoco, showing that the larger hydrophobic side chains of LGN-GL4 result in the shift of the Switch II of Gαi1. (C) Structure details of the C-terminus of LGN-GL4, showing that two backbone hydrogen bonds stabilize the C-terminal conformation. (D) Structure details of the sharp turn at G517 of RGS14 peptide. Hydrogen bonds formed between RGS14 and Gαi1 are shown with dashed lines.

are highly similar, the conformation of Gαi-bound GoLoco motifs of RGS14 and LGN are distinctly different (Fig. 3.8). First, a 16-residue fragment C-terminal to the conserved
GoLoco motif core of RGS14 is required for binding to Gαi, and this 16-residue fragment forms ordered structure and has extensive interactions with the all-helical domain of Gαi1 (Kimple et al., 2002). In LGN-GL4/GL3, in contrast, only 3-4 amino acids C-terminal to the GoLoco motif core are required for binding to Gαi (Fig. 3.6A). Second, the orientation of the variable C-terminal tail of the RGS14 GoLoco peptide bound to the Gαi helical domain is opposite to that of the LGN GoLoco peptides (Fig. 3.8A). In the LGN GL4/Gαi complex, the hydrophobic side chains of V641, L642 and L643 interact with V72 and Y69 from the αA helix of the helical domain of Gαi, thus the C-terminal end of GL4 extends towards the N-terminal end of Gαi αA (Fig. 3.8C). Residue corresponding to V641 in the RGS14 peptide is G517 (Fig. 3.8D and Fig. 3.9A). The backbone carbonyl oxygen of G517 forms twohydrogen bonds with sidechains of S75 and Q79 from Gαi αA. The unique backbone dihedral angles ($\phi = 78^\circ$, $\psi = -171^\circ$) of G517, which are not allowed by other amino acids, enable the C-terminal tail of the RGS GoLoco peptide to take a sharp turn at this position and extend to the C-terminal end of Gαi αA (Fig. 3.8A&D). Sequence alignment of all known GoLoco motifs from mammals reveals that only the GoLoco motifs of RGS14 and RGS12 contain a Gly right after the conserved core motif, and the C-terminal residues of these two GoLoco motifs share the identical sequence (Fig. 3.9A). The above structure-based amino acid sequence analysis suggests that the LGN GoLoco/Gαi interactions observed in this study represent the general mode of the interactions between GoLoco proteins with Gαi. RGS14 and RGS12, instead, may represent a special sub-class of GoLoco proteins in terms of Gαi binding.

3.5 The “double Arg-finger”-mediated GDP binding of LGN GoLoco motifs

The structure of the Gαi1/RGS14 GoLoco complex shows that a highly conserved “D/E-Q-R” triad at the C-terminal end of the conserved GoLoco motif core plays a critical role in binding to Mg$^{2+}$-GDP (Kimple et al., 2002). Similar to the Gαi1/RGS14 GoLoco interaction, the side chain of R640 (R606) of GL4 (GL3) in the ‘D/E-Q-R’ triad inserted into
Figure 3.9. The double arginine fingers in GoLoco motifs of LGN play a crucial role in GDP coordination and GDI activity. (A) Sequence alignment of the GoLoco motifs in mammalian GoLoco proteins. Absolutely and highly conserved residues are highlighted in red and yellow respectively. The residue right behind D/E-Q-R which determines the C-terminal direction is highlighted with blue triangle. The residues involved in the interactions with Go\(\alpha_i\) are labeled with a red star on the top. The di-arginine fingers are highlighted with black boxes. (B) Stereoview of the structural details of the GDP binding pocket in the Go\(\alpha_{i1}/GL4\) complex. Polar interactions are shown with dashed lines. The color scheme is the same as in Fig. 3.6A. (C) Binding affinities of the GL4 mutants with single or double substitutions of its two arginine residues to Go\(\alpha_{i3}\)-GDP derived from fluorescence-based assays. (D) GDI activities of the wild type and mutant GL4 peptides measured with AlF\(^4^-\) induced increase of intrinsic tryptophan fluorescence.
Figure 3.10. The binding affinities of LGN-GL1 peptide (30aa) to GDP bound Ga\(_{i3}\) with or without Mg\(^{2+}\) measured by fluorescence-based assay. For the binding assay in the presence of Mg\(^{2+}\), 2 mM Mg\(^{2+}\) was included in the assay buffer. Conversely, 1 mM EDTA was included in the assay buffer in order to ensure the complete absence of Mg\(^{2+}\) in the assay buffer.

the GDP binding pocket and together with R178 of Ga\(_i\) from the “Switch I” bound to \(\alpha\)-phosphate of GDP (Fig. 3.9B). However, there is a distinct feature of GL4/GL3 in GDP binding with respect to RGS14 GoLoco. Another highly conserved Arg at five residues upstream of the Arg in the “D/E-Q-R” triad in LGN GoLoco peptides (R635 in GL4 and R601 in GL3) binds to the \(\alpha\) and \(\beta\) phosphates of GDP (Fig. 3.8B). In RGS14 GoLoco, the residue corresponding to this second Arg is a Gly, and a Mg\(^{2+}\) ion was found to be necessary to stabilize the \(\beta\) phosphates of GDP (Kimple et al., 2002). Therefore, different from RGS14, LGN GoLoco motifs use two Arg residues instead of one to bind to and stabilized GDP. The structure of the LGN GoLoco motif in complex with Ga\(_i\) further indicate that the LGN
GoLoco motifs can bind to GDP-bound Gαi independent on the presence of Mg²⁺. This structure-based prediction is confirmed by direct binding experiment (Fig. 3.10). Sequence alignment analysis reveals that, except for RGS14 GoLoco, the rest of known GoLoco motifs all contain a “R/K-X-D/E-D/E-R”, GDP binding sequence (Fig. 3.9A), and we refer this sequence as the “double Arg-finger”. This sequence analysis further supports that the LGN GoLoco motif/Gαi interactions represent the general mode of GoLoco motif-mediated binding to Gα.

3.6 The double-arginine fingers are critical to the GDI activities of LGN-GoLocos

To confirm the functional importance of the two Arg in the double-arginine finger in LGN GoLoco motifs, we performed point mutations of the two arginines, and tested the Gαi-GDP binding affinities (using fluorescence-based binding assays) and GDI activities of these mutants. Single substitution mutations (R635G, R635A, and R640A) caused ~50-fold decrease in GL4’s binding to Gαi-GDP, and the double mutation (R635,640A) led to ~500-fold Gαi-GDP binding affinity decrease (Fig. 3.9C). Similar results were also obtained for the other GoLoco motifs of LGN (data not shown), indicating that the two conserved arginine fingers are critical to the Gαi-GDP binding of LGN-GoLocos. This finding is in contrast to the RGS14 GoLoco, of which the substitution of the Arg in the finger with alanine or leucine did not decrease the binding affinity of RGS14 to Gαi1-GDP (Kimple et al., 2002). Careful examination of the crystal structures Gαi in complex with LGN GoLoco peptides revealed that the side chains of the two arginines also form hydrogen bonds with V179 and T181 from Gαi (Fig. 3.9B). In contrast, the side chain of R516 in RGS14 GoLoco interacts exclusively with GDP (Kimple et al., 2002).

The GDI activities of LGN-GoLocos were evaluated by AlF₄⁻ induced increase of intrinsic tryptophan fluorescence of Gαi and by direct binding of BODIPY-GTPγS to Gαi. In agreement with the previous studies (McCudden et al., 2005), the four GoLoco domains exhibited similar GDI activities (Fig. 3.11). Moreover, comparison of the GDI activities of GoLoco peptides with different lengths showed that the 25-residue minimal Gαi–binding GoLoco fragments shown in Fig. 3.1 are also sufficient for their GDI activities (Fig. 3.11A&B
Figure 3.11. GDI activities of the LGN GoLoco motifs. GDI activities of LGN-GoLoco peptide with 25 (A) and 30 (B) residues were measured by AlF₄⁻ induced increase of intrinsic tryptophan fluorescence. (C) Quantification of GDI activity of different GoLoco peptides using BODIPY-GTPγS binding assay. (D) GDI activities of GL3 fused to the C-terminal end of thioredoxin and its mutant were measured by AlF₄⁻ induced increase of intrinsic tryptophan fluorescence.

). Further quantification of the GDI activities using the association rate of BODIPY-GTPγS binding revealed IC₅₀ values of a few μM for LGN-GoLocos, which is slightly weaker than that of RGS14 GoLoco (Fig. 3.11C). At a saturated concentration of GoLoco peptide (GL:Goα₃ = 2000:1), the wild type LGN-GL4 showed complete inhibition of GDP dissociation. The R635G-GL4 or the R635A-GL4 displayed obviously weakened GDI activity, whereas the R640A-GL4 and R635, 640A-GL4 had essentially no detectable GDI activities (Fig. 3.9D). Substitution of the first Arg (R601) in the double-arginine finger of GL3 with Ala or Gly also diminished its GDI activity (Fig. 3.11D). Thus, we conclude that both Arg in the double-arginine finger of LGN GoLoco motifs are important for their GDI activity.
Chapter 4
DISCUSSION

4.1 LGN GoLocos/Gαi interactions represent a general binding mode between GoLoco motifs and Gα subunits

GoLoco motif was first identified and defined as a conserved core of 19 amino acids (Willard et al., 2004). However, the binding to GDP loaded Gα subunits and the GDI activity of GoLoco proteins require residues beyond the conserved core (Adhikari and Sprang, 2003; Kimple et al., 2002). Since the C-terminal flanking sequences of GoLoco motifs are highly diverse among GoLoco proteins (Willard et al., 2004), one may wonder whether the Gαi1/RGS14 structure represents a general diagram of GoLoco/Gα interaction, in which a long C-terminal tail of ~15 residues binds specifically to the all-helical domain of Gαi1 (Kimple et al., 2002). In the present study, we demonstrate that a few residues (3-4 aa) C-terminal to the conserved GoLoco core are necessary and sufficient for LGN GoLoco motifs to bind to and to inhibit GDP dissociation of Gαi•GDP, a finding which is in sharp contrast to the case of RGS14. The structures of LGN GoLoco motifs in complex with Gαi•GDP also suggest that the short variable C-terminal sequences of LGN-GoLocos are unlikely to determine their binding selectivity to Gα subunits. Consistently, previous studies have shown that LGN-GoLocos bind to all three forms of Gαi (i1, i2, i3) bound with GDP. As for the Gαo•GDP binding, discrepancies exist in the literatures. An early study by McCudden et al. reported that GoLoco domain of LGN selectively bind to Gαi•GDP, but not to Gαo•GDP or Gαo•GDP (McCudden et al., 2005). Recently, Kopein et al. found that LGN, as well as its Drosophila homolog Pins can bind robustly to both GDP loaded Gαo and Gαi (Kopein and Katanaev, 2009). Sequence alignment suggests that the conformation of the GoLoco peptide in Gαi1/RGS14 structure is likely a unique example of GoLoco/Gα interaction. The LGN GoLocos/Gαi interactions described in the current study may represent a more general binding mode between GoLoco motifs and Gα subunits.
Figure 4.1. Comparison of $G\alpha_{i1}/GL4$ structure with the structure of the fully activated $G\alpha$ conformation derived from the $\beta_2$-AR-$G\alpha_\beta\gamma$ structure. The all helical domain, Ras-like domain and three switches of GPCR bound $G\alpha_s$ are shown in lightblue, pink and yellow, respectively. Part of the GPCR ($\beta_2$-AR) is shown in orange. The coloring of the $G\alpha_{i1}/GL4$ complex is the same as in Fig. 3.6A.

4.2 The structural basis of GDI activities of GoLoco motifs

We have demonstrated in this study that every one of the four GoLoco motifs of LGN can bind to $G\alpha_i$-GDP with high affinity. Additionally, although the LGN GoLoco peptides are much shorter than their counterpart from RGS14, LGN GoLocos also act as potent GDIs. The structure of the LGN GL3 and GL4 in complex with $G\alpha_i$ suggest that both the double-arginine finger and the short variable tail of the GoLoco peptides are important for their GDI activities. The di-arginine finger makes extensive salt bridges with the phosphates of GDP, which can make contacts with both the Ras-like and all-helical domains. This will fix the GDP coordination as well as the relative orientation of the two domains of $G\alpha_i$ (Fig. 4.1).
Figure 4.2. Structural model of the LGN/Gαi-GDP complex. TPR domain, the TPR-binding NuMA peptide and the GoLoco motifs responsible for Gαi-GDP binding are shown in blue, red and cyan respectively.

Additionally, the hydrophobic interactions between the variable C-terminal tail of GoLoco peptides with the all-helical domain of Gαi further stabilize the closed conformation of Gαi (i.e. by restricting the opening of the helical domain and subsequent dissociation of GDP from Gαi, Fig. 4.1).

4.3 Scaffolding role of LGN GoLoco motifs

The characteristic multiple GoLoco motifs in LGN and its Drosophila homolog Pins have been implicated to play a role in regulating their intra-molecular interactions between TPR repeats and GoLocos in response to the binding of Gαi-GDP and NuMA/Mud (Nipper et al., 2007; Smith and Prehoda, 2011). Besides this, the multiple GoLoco motifs in LGN (Pins) also function as a scaffold in regulating the localization of related protein complex and organizing signaling pathways of spindle orientations. The detailed characterizations of interactions between LGN-GoLocos and Gαi-GDP in this work demonstrate that in its open state the four GoLoco motifs of LGN have equal capacity to bind to Gαi-GDP (Fig. 4.2). In another word, the stoichiometry of LGN/Gαi-GDP complex in vivo likely depends on the concentration of Gαi-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 (Yoshiura et al., 2011). Tre1 was shown to activate Gαo, and the GTP form Gαo can specifically associate with the first GoLoco motif of Pins (Yoshiura et al., 2011). Thus, the presence of multiple GoLoco motifs allows Pins to function as a scaffold to simultaneously engage Gαo- and Gαi-mediated signaling events during asymmetric cell division.
Reference


generates multiple Pins activation states to link cortical polarity and spindle orientation in Drosophila neuroblasts. Proc Natl Acad Sci U S A 104, 14306-14311.


