Crystallization and preliminary crystallographic studies of GRASP65 GRASP domain from *Rattus norvegicus*

Xinxin Li, Yanbin Feng and Xinqi Liu*

State Key Laboratory of Medicinal Chemical
Biology and College of Life Sciences, Nankai
University, 94 Weijin Road, Tianjin 300071,
People's Republic of China

Correspondence e-mail:
liu2008@nankai.edu.cn

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GRASP65 and GRASP55 were classified as Golgi reassembly stacking proteins which play crucial and complementary roles in the stacking of Golgi cisternae. They also participate in vesicle tethering, mitotic progression, the disassembly and reassembly of the Golgi apparatus during mitosis and unconventional secretory pathway regulation. In this study, the expression, crystallization and preliminary crystallographic analysis of the GRASP65 GRASP domain from *Rattus norvegicus* are presented. The crystals diffracted to 2.0 Å resolution and belonged to space group $P2_12_12$, with unit-cell parameters $a = 44.99$, $b = 104.29$, $c = 37.93$ Å, $\alpha = \beta = \gamma = 90^\circ$. Furthermore, molecular replacement was employed to determine the structure of the GRASP65 GRASP domain from *R. norvegicus*.

1. Introduction

The Golgi apparatus is an important organelle which serves in protein modification, processing, intracellular sorting and transport. The formation of stacks relies on the existence of Golgi reassembly stacking proteins (GRASPs), which facilitate the accurate localization and function of local enzymes. In mammalian cells, Golgi reassembly stacking proteins 65 and 55 (GRASP65 and GRASP55) are homologous proteins which belong to the same family. They are localized to medial and *cis*-Golgi cisternae, respectively. GRASP65 depletion by RNAi not only results in Golgi ribbon unlinking, but also causes an arrest in cell division followed by spindle defects and cell death, with a reduced number of cisternae in the stacks (Sütterlin *et al.*, 2005; Tang *et al.*, 2010). GRASP65 is the key target of a signalling pathway. It reorientates the Golgi during cell migration and breaks down the Golgi during apoptosis (Vinke *et al.*, 2011). The Golgi apparatus disassembles during mitosis and reassembles at the onset of interphase. Specific phosphorylation of GRASP65 at the G2 phase leads to Golgi ribbon unlinking, a process that is crucial for cells to enter mitosis (Rabouille & Kondylis, 2007; Nickel & Rabouille, 2009). Perturbing the activity of GRASP65 inhibits Golgi fragmentation at the G2/M transition and the lack of Golgi breakdown prevents cells from progressing through mitosis (Sütterlin *et al.*, 2005; Levi & Glick, 2007). In addition to being a target during mitosis, GRASP65 also appears as the site for regulatory kinases binding during both interphase and mitosis (Lowe & Barr, 2007; Levi & Glick, 2007).

GRASP65 is essential for Golgi structure formation. It forms stable homodimers which residing on adjacent Golgi membranes form oligomers (Wang *et al.*, 2003, 2005; Xiang & Wang, 2010). The N-terminus of the protein (amino acids 1–202) is named the GRASP domain and is highly conserved between GRASP65 and GRASP55 in various mammalian species (Shorter *et al.*, 1999; Barr *et al.*, 1998; Wang *et al.*, 2005). The formation of GRASP domain dimers also facilitates trans-oligomerization. These trans-oligomers are capable of holding the cisternal membranes together in stacks (Wang *et al.*, 2003, 2005; Xiang & Wang, 2010). The C-terminus of the protein (amino acids 203–446) is a serine/proline-rich (SPR) domain that could be phosphorylated by mitotic kinases (Wang *et al.*, 2005). The phosphorylation of this region inhibits the oligomerization of GRASP65 and promotes cisternal unstacking during mitosis.

The GRASP domain is composed of two circularly permuted PDZ domains, which are common protein-binding domains found in postsynaptic density 95 (PSD95), disk large tumour suppressor (Dlg) and zonula occludens protein 1 (ZO-1)-like domain (Nakamura, 2010). Biochemical research presented a possible mechanism for GRASP65 in Golgi stacking. For GRASP65, PDZ2 is targeted to the C-terminus of *cis*-localized GM130, which presents a PDZ ligand. In contrast, PDZ1 is likely to bind to a similar GRASP65 molecule on an adjacent membrane, thereby forming a *trans*-complex that bridges the two membranes. Tethering occurs when PDZ1 and PDZ2 present the binding pocket and surface-exposed internal ligand, respectively (Truschel *et al.*, 2011).

GRASP65 plays important roles in the normal function of Golgi apparatus, yet previous research has failed to uncover the structure in order to understand its effects on homotypic membrane tethering and other functions performed by the GRASP proteins. To investigate this question, we have constructed and purified the GRASP65 GRASP domain and determined the crystal structure. Here, we report the preliminary crystallographic studies of this domain. Our preliminary crystallographic study of multifunctional GRASP65 provides important evidence for deciphering the molecular mechanism of Golgi ribbon formation and membrane tethering.

2. Materials and methods

2.1. Protein expression and purification

The DNA fragment encoding residues 1–228 of the rat GRASP65 GRASP domain was amplified by PCR using designed primers. The fragment was subcloned into expression vector pET-30a(+) (Novagen). A tobacco etch virus (TEV) protease cleavage site (ENLYFQSN) was inserted between an N-terminal 6×His tag and the target gene. The sequence was verified by DNA sequencing.

The pET-30a(+) plasmid containing the GRASP65 GRASP domain was transformed into *Escherichia coli* Rosetta (DE3) strain and grown in Luria–Bertani medium containing 50 mg ml^{−1} kanamycin at 310 K. Fully grown overnight pre-cultures were subsequently diluted in fresh medium (at a 1:100 ratio) and induced by IPTG at a final concentration of 0.1 mM when the optical density

(OD₆₀₀) reached 0.6–1.0. After induction, cells were grown at 289 K for 13–15 h. The cells were harvested by centrifugation at 4000g for 10 min at 277 K. Pellets were resuspended in buffer A (50 mM Tris pH 8.0, 500 mM NaCl, 10 mM imidazole, 1% Triton X-100) and lysed by sonication.

The soluble proteins in the supernatant were isolated by centrifugation at 18 000g for 40 min at 277 K. The GRASP65 GRASP domain was then purified to homogeneity using a three-step procedure. Firstly, the supernatant was loaded onto an Ni-NTA affinity column (Qiagen) which had been pre-equilibrated with buffer A. The column was washed by washing buffer (50 mM Tris–HCl pH 8.0, 500 mM NaCl, 20–50 mM imidazole). The recombinant protein was eluted with elution buffer (50 mM Tris–HCl pH 8.0, 500 mM NaCl, 250 mM imidazole). The eluate was concentrated using Amicon Ultra-10 (Millipore, USA) against buffer consisting of 50 mM Tris–HCl pH 8.0, 100 mM NaCl. Secondly, an anion-exchange Q-Sepharose column was applied using an ÄKTA FPLC system (GE Healthcare). The protein was eluted using buffer (50 mM Tris–HCl pH 8.0) containing a linear NaCl gradient (0.1–1 M NaCl). Thirdly, the protein was further purified on a HiLoad 16/600 Superdex 200 gel-filtration column (GE Healthcare) equilibrated with buffer B (50 mM Tris–HCl pH 8.0, 500 mM NaCl) (Fig. 1). The peak position was consistent with the predicted molecular weight of a monomer (27 kDa) of the GRASP65 GRASP domain. The purified protein was concentrated to 5 mg ml^{−1} using an Amicon Ultra-10 (Millipore, USA) and stored at 193 K until use. At every stage, the purity of GRASP65 was monitored by SDS–PAGE.

2.2. Crystallization and data collection

Initial crystallization screening was carried out by the sitting-drop vapour-diffusion method at 293 K with the following screening kits: Crystal Screen, Crystal Screen 2, Index, Natrix and SaltRX (Hampton Research). The protein drops consisted of 1 µl protein solution (5 mg ml^{−1} in 50 mM Tris–HCl pH 8.0, 500 mM NaCl) and 1 µl mother solution and were equilibrated against 100 µl reservoir solution at 293 K. The first hit was obtained in Index kit condition No. 42 with small crystals (0.1 M bis-tris pH 5.5, 25% PEG 3350). The initial crystallization condition was optimized by changing the concentration of protein, the gradient of the precipitant and the pH and by the use of additives. Finally, a 2 µl protein drop (5 mg ml^{−1}) mixed with an equal amount of mother liquor and equilibrated against 500 µl reservoir solution (0.1 M bis-tris pH 5.5, 25% PEG 3350) yielded crystals that were suitable for data collection (Fig. 2).

Figure 1
Size-exclusion column profiles of the GRASP65 GRASP domain using a HiLoad16/600 Superdex 200 gel-filtration column (GE Healthcare). The corresponding purified protein from the peak was analysed by SDS–PAGE gel (12%). Lane M contains molecular-weight markers (labelled in kDa).

Figure 2
Crystal of GRASP65 GRASP domain.

Table 1

Data-collection and processing statistics of the GRASP65 GRASP domain.

Values in parentheses are for the outermost resolution shell.

X-ray source	SSRF, China
Wavelength (Å)	0.9793
Temperature (K)	100
Crystal-to-detector distance (mm)	200
Rotation range per image (°)	1.0
Total rotation range (°)	180
Space group	$P2_12_12$
Unit-cell parameters (Å, °)	$a = 44.99$, $b = 104.29$, $c = 37.93$, $\alpha = \beta = \gamma = 90.00$
Resolution (Å)	2.0 (2.18–2.00)
Matthews coefficient V_M (Å ³ Da ^{−1})	1.92
Solvent content (%)	36
Total No. of reflections	115787 (10292)
No. of unique reflections	12841 (1906)
Multiplicity	9.0 (5.4)
$\langle I/\sigma(I) \rangle$	18.5 (2.5)
Completeness (%)	99.5 (97.0)
$R_{\text{merge}}^{\dagger}$ (%)	6.8 (31.7)
$CC_{1/2}$	0.811 (0.373)

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the i th observed diffraction-intensity value of unique reflection hkl and $\langle I(hkl) \rangle$ is the mean value of i observations of reflection hkl .

2.3. Data collection and processing

The average dimensions of the crystals were $0.2 \times 0.1 \times 0.1$ mm. To avoid the presence of ice rings, the crystals were soaked in paraffin oil for 2 s prior to flash-cooling in liquid nitrogen for data collection. The crystals were mounted on a goniometer in a nitrogen stream at 100 K. Data collection was performed at 100 K using a wavelength of 0.9793 Å at the Shanghai Synchrotron Radiation Facility (SSRF), Shanghai, China. The distance between the crystal and the detector was 200 mm. 180° of data were collected with an exposure time of 1.2 s per frame with 1.0° oscillation. The best data set reached a resolution of 2.0 Å (Fig. 3).

All data sets were indexed, integrated and scaled with the *HKL-2000* program suite (Otwinowski & Minor, 1997). Molecular replacement was performed by the *Phaser* program in the *CCP4* package (Winn *et al.*, 2011), using the structure of the human GRASP55 GRASP domain (PDB entry 3rle; Truschel *et al.*, 2011) as the search model. The separate PDZ domains were used as ensem-

bles owing to the relative movement between the two PDZ domains compared with those in GRASP55. An initial solution with a log-likelihood gain (LLG) of 220 was obtained and used for further refinement.

Model building and refinement were performed using *PHENIX* (Adams *et al.*, 2010) and *Coot* (Emsley & Cowtan, 2004), respectively. Detailed data-collection statistics are summarized in Table 1.

3. Results

3.1. Protein expression and purification

The GRASP65 GRASP domain was efficiently expressed in *E. coli* and finally purified by gel filtration with a HiLoad 16/600 Superdex 200 column to homogeneity (Fig. 1). The GRASP65 GRASP domain was monomeric in solution as judged by the elution volume from a gel-filtration column. Interestingly, the GRASP55 GRASP domain purified by Truschel *et al.* (2011) also appears as a monomeric form in solution and no functional dimer could be identified in their crystal structures. Both the full-length GRASP65 and GRASP55 appeared as nonspecific aggregates in solution when purified on a large scale from expression in *E. coli*. All of these observations cast doubt on the physiological oligomerizations of these molecules.

3.2. Crystallization and data collection

The concentrated recombinant GRASP65 GRASP domain was crystallized using the sitting-drop and hanging-drop vapour-diffusion methods at 293 K. X-ray diffraction data were collected from rhombic-shaped crystals grown in drops consisting of 0.1 M bis-tris pH 5.5, 25% PEG 3350 in one week (Fig. 2). The crystals belonged to space group $P2_12_12$, with unit-cell parameters $a = 44.99$, $b = 104.29$, $c = 37.93$ Å, $\alpha = \beta = \gamma = 90^\circ$. Each asymmetric unit was estimated to contain one molecule, with a Matthews coefficient of 1.92 Å³ Da^{−1} (Matthews, 1968). This corresponded to a solvent content of 36%. The GRASP65 GRASP domain possesses high sequence similarity to the fragment of the GRASP55 GRASP domain (69% identity). Therefore, the latter (PDB entry 3rle; Truschel *et al.*, 2011) was used as the search model in molecular replacement. A reliable initial

Figure 3

The diffraction pattern of the GRASP65 GRASP domain.

solution with an LLG of 220 was obtained and further structural refinement is ongoing.

4. Discussion

GRASP65 is a peripheral membrane protein tightly attached to the Golgi membrane. GRASP proteins play an important role in the stacking of Golgi cisternae, vesicle tethering, mitotic progression and unconventional secretory pathway regulation (Levi & Glick, 2007). GRASP65 stacks Golgi membranes by forming oligomers through its N-terminal GRASP domain (Xiang & Wang, 2010). Previous research suggested that the tethering interface of each self-interaction involved a deep binding pocket in PDZ1 and an internal ligand from PDZ2 (Truschel *et al.*, 2011). The dimerization and oligomerization has long been suggested to be essential for the function of GRASPs in Golgi reassembly.

Initially, we cloned and expressed GRASP domain consisting of residues 1–207, but no crystals could be produced. This length was used in the crystallization of the GRASP55 GRASP domain, which successfully yielded good crystals. Other constructs expressing residue ranges 1–202 or 1–217 also failed to produce crystals. Only the longer fragment from residues 1 to 228 could generate high-quality diffracting crystals. This fragment contains the important phosphorylation sites (Thr220 and Thr224) following the canonical GRASP domain. We believe that the residues following the GRASP domain, *i.e.* residues 203–228, may be involved in crystal packing and/or in the molecule–molecule interaction. Phosphorylation is an important way to regulate the disassembly of GRASPs. It will be interesting to see how the phosphorylation sites interact with GRASP domains from crystallographic data.

GRASP65 and GRASP55 connect membranes at distinct locations in Golgi stacks. The differential distribution implies that GRASP65 and GRASP55 may act collaboratively as stacking factors in different regions of the Golgi (Pfeffer, 2001). These two stacking factors interact dissimilarly with their respective receptors, GM130 and Golgin45. Additionally, as the key target of signalling events, they are phosphorylated by various kinases. In summary, all of these distinctions may imply differential mechanisms in membrane tethering and trans-oligomerization modulation. Recent work has

speculated on the mechanism of the PDZ interaction of GRASP55 in membrane tethering in medial Golgi cisternae, yet what determines the difference between GRASP65 and GRASP55 is still uncertain. Since no crystal structures have been reported for GRASP65, we hope that our study on the GRASP65 GRASP domain will provide clues to clarify the underlying molecular mechanisms of this important protein. The crystal structure of the GRASP65 GRASP domain will not only reveal the structural basis for ligand specificity, but also provide a structure-based method to regulate PDZ-mediated interaction. Further studies on how the GRASP65 GRASP domain functions in the Golgi membrane will be explored in the future.

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