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Disorder-to-order transitions in the molten globule-like Golgi Reassembly and Stacking Protein

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ABSTRACT

Background: Golgi Reassembly and Stacking Proteins (GRASPs) are widely spread among eukaryotic cells (except plants) and are considered as key components in both the stacking of the Golgi cisternae and its lateral connection. Furthermore, GRASPs were also proved essential in the unconventional secretion pathway of several proteins, even though the mechanism remains obscure. It was previously observed that the GRASP homologue in *Cryptococcus neoformans* has a molten globule-like behavior in solution.
Methods: We used circular dichroism, synchrotron radiation circular dichroism and steady-state as well as time-resolved fluorescence.
Results: We report the disorder-to-order transition propensities for a native molten globule-like protein in the presence of different mimetics of cell conditions. Changes in the dielectric constant (such as those experienced close to the membrane surface) seem to be the major factor in inducing multiple disorder-to-order transitions in GRASP, which shows very distinct behavior when in conditions that mimic the vicinity of the membrane surface as compared to those found when free in solution. Other folding factors such as molecular crowding, counter ions, pH and phosphorylation exhibit lower or no effect on GRASP secondary structure and/or stability.
General significance: To the best of our knowledge, this is the first study focusing on understanding the disorder-to-order transitions of a molten globule structure without the need of any mild denaturing condition. A model is also introduced aiming at describing how the cell could manipulate the GRASP sensitivity to changes in the dielectric constant during different cell-cycle periods.

1. Introduction

The Golgi complex is a multifunctional organelle responsible for receiving a large number of proteins from the Endoplasmic Reticulum (ER) to further processing and sorting for transport to their final destination [1]. The mammalian Golgi is polarized, consisting of three particular regions: one close to the ER called *cis* Golgi network, a second called Golgi stacks (which is divided into medial and trans Golgi) and the *trans* Golgi network, where the secreted vesicles are released [2,3]. The correct assembly of the cisternae is important for proper protein glycosylation and sorting [4,5,6]. A family of proteins is responsible for keeping the arrangement of the cisternae and for correctly changing it according to the cell needs [7,8,9]. These proteins are called Golgi Reassembly and Stacking Proteins (GRASPs) and are responsible for the organization of the cisternae into stacks and ribbons [10,11], for facilitating the conventional protein secretion in some cases [12], for Golgi dynamics during mitotic and apoptotic periods [13], for Golgi remodeling during cell migration [14], and for unconventional protein secretion [15,16].

GRASP is a peripheral membrane protein, with an N-terminus structured into two PDZ domains exhibiting a protozoa PDZ folding, at least in vertebrates (GRASP domain) [17,18]. A second region in the GRASP structure, usually larger than the GRASP domain, consists of a non-evolutionary conserved domain that is rich in serine and proline (SPR domain) with regulatory function [19,20] and intrinsically disordered characteristics [21]. Two GRASP paralogues (GRASP55 and 65) are usually found in higher eukaryote organisms: GRASP65, which binds to GM130 C-terminus mostly in the *cis* Golgi [22], and GRASP55, which binds to golgin-45 mainly in the medial-Golgi [23]. Trans-oligomerization of GRASP55 and 65 is mitotically regulated and has

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complementary functions in maintaining the stacking of the cisternae's membrane [24].

In a previous work, we showed that the GRASP from the fungal pathogen *Cryptococcus neoformans* (CnGRASP) [25] presents features usually observed for a molten globule state, even in the absence of any mild denaturing conditions [21]. That work was the first (and so far the only) structural characterization of a full length GRASP in solution. The classification of CnGRASP as a member of the intrinsically disordered protein (IDP) family brings unexplored possibilities regarding the protein function *in vivo*. IDPs are frequently observed as main components of the cellular signaling machinery [26], commonly functioning as central hubs [27], especially because of their unique structural plasticity [28]. It has been also observed that particular Prion-forming sequences, which are especially enriched in asparagine, promote molten globule-like structures, where amyloid-nucleating contacts can be made [29,30]. It is then becoming clear that the understanding of the molten globule structural behavior in solution is of pharmacological interest.

Nevertheless, how the fungus, or any other GRASP-containing cell, could exploit this particular molten-globule feature remains elusive at this point. An appealing possibility is that different cell conditions could induce disorder-to-order transitions in GRASP allowing it to interact with multiple protein partners, thus taking different roles in cell processes. Moreover, IDPs are usually very sensitive to changes in the physicochemical parameters of the bulk solution, which raises concern about the precipitant conditions usually found in crystallization assays. Particular conditions might trap specific conformations that could not be representative of the whole ensemble.

Protein misfolding disorders such as Alzheimer disease, type 2 diabetes and inherited cataracts have been increasing their occurrence all around the world and, for most of them, there is no effective medical treatment yet [31]. Especially for the amyloidogenic diseases, misfolded intermediates, such as the molten globule and pre-molten globule states, play a key role in the amyloid formation in several human diseases [32,33,34,35]. Because these intermediate states are of transient nature and characterized by a large conformational heterogeneity, it is very challenging to study them in solution using standard techniques. Besides, so far in the literature, all the studies focusing on molten globule structures rely on artificial molten globules induced by extreme or mild denaturing conditions like low pH, the presence of chaotropic agents and/or mutations [36,37,38].

In this paper, we investigated the effects on the GRASP structure, and consequently on a molten globule-like structure, induced by changes in the physicochemical properties of the medium without the need of any mild denaturing condition. These changes are intended to resemble particular conditions found in the cell. Using a multi-technique approach, which includes conventional and synchrotron radiation circular dichroism along with steady state and time-resolved fluorescence techniques, we showed that regions of CnGRASP structure undergo multiple disorder-to-order transitions under certain conditions. Changes in the dielectric constant and local dehydration seem to be the main regulators of GRASP structure, suggesting that the protein undergoes a significant structural rearrangement when going from the bulk solvent to the membrane-bound state.

2. Materials and methods

2.1. Protein expression and purification

Samples of CnGRASP were prepared as described previously [21] with a minor change in the working buffer, which now contains 25 mM Hepes/NaOH, 150 mM NaCl, 5 mM β-mercaptoethanol, 5% glycerol v/v, pH 7.4. For the molecular crowding assays, glycerol was removed prior to the experiments by extensive dialyses. The CnGRASP + Histag construct, i.e., a 6xHis-tag attached to the protein N-terminus, was obtained after a subcloning procedure to a pET28a plasmid (Novagen). The purification step was the same used for the pETSUMO GRASP but without the N-terminal tag removal procedure [21].

The phosphomimetic mutants (T217D, S220D, S224D, S241D and S245D) were constructed using a variation of the Quick-change protocol [39] developed by us to decrease the negative impact of oligonucleotide dimers on the amplification process. The protocol consisted of two "quick-change reactions", where the traditional Quick-change conditions were used, but with only one of each oligonucleotide in each reaction tube (Fig. S1). After the single strand amplification was finished, both reactions were combined together and an annealing step was performed. Once this reaction was finished, the final DNA was obtained (Fig. S1 and Tables S1, S2, and S3). The product was treated with Dpn1 to eliminate the parental non-mutated plasmid and then transformed in *E.coli* DH5α. The success of each mutation was checked by DNA sequencing. We observed a higher efficiency using this protocol to perform mutation than any other protocol tested so far. All mutants were expressed and purified using the same protocol used for the native protein [21].

2.2. Liposome preparation

The phospholipids 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) (POPG), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dioleoyl-*sn*-glycero-3-[(*N*-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt) (18:1 DGS-NTA(Ni)) and 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol) (DPPG) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Large unilamellar vesicles (LUVs) were prepared by extruding a multilamellar lipid suspensions in 10 mM sodium phosphate buffer, 10 mM NaCl, pH 7.4 (buffer B) through a 100 nm Nuclepore Track-Etched membrane filters (Whatman) using an Avanti Polar Lipids mini-extruder.

2.3. Synchrotron radiation circular dichroism (SRCD)

SRCD experiments were performed in the UV-CD12 beamline of ANKA – KIT synchrotron (Karlsruhe, Germany). Protein concentration was ca. 18 μM in a 25 mM sodium phosphate, 50 mM NaCl, 1 mM β-mercaptoethanol, 10% glycerol, pH 7.4 buffer. Three scans were collected over the wavelength range from 280 to 180 nm with 0.5 nm bandwidth, 1.5 s dwell time in a 100 μm path length demountable quartz cell (Hellma Ltd., UK), at 20 °C; the baseline for each sample consisted of the buffer and all components present in the sample other than the protein. The results were processed using CDTools [40], including averaging, baseline subtraction and normalization to $\Delta\varepsilon$.

A dried film of CnGRASP was formed on the surface of a Suprasil quartz circular plate by the deposition of a protein solution (1 mg/mL, 20 μL) followed by evaporation of the solvent. SRCD spectra was collected over the wavelength range from 280 to 160 nm, with 0.5 nm step size, at 20 °C, at four different rotation angles on the plate (increment by 90°). The final spectrum reported (mdeg units) is an average of all the scans, after the subtraction of the SRCD signal of the plate.

2.4. Circular dichroism (CD)

Far-UV (190–260 nm) CD experiments were carried out in a Jasco J-815 CD spectrometer (JASCO Corporation, Japan) equipped with a Peltier temperature control and using a quartz cell with a path length of 1 mm. The experimental parameters were: scanning speed of 50 nm·min^{-1}, spectral bandwidth of 1 nm, response time of 0.5 s and temperature of 25 °C. The protein samples for the alcohol titration were based on a protein dilution in aqueous solution containing the appropriate amount of pure alcohol (methanol, ethanol or isopropanol), from a high concentrated protein stock solution (dilution of < 5% for all experiments). The following dielectric constant values (ε) for the pure solvents were used [41]: 78.3 (water), 33.1 (methanol), 25.3 (ethanol), and 20.2 (isopropanol). The dielectric constant of the alcohol/water

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