

Golgi protein FAPP2 tubulates membranes

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The Golgi-associated four-phosphate adaptor protein 2 (FAPP2) has been shown to possess transfer activity for glucosylceramide both in vitro and in cells. We have previously shown that FAPP2 is involved in apical transport from the Golgi complex in epithelial MDCK cells. In this paper we assign an unknown activity for the protein as well as providing structural insight into protein assembly and a low-resolution envelope structure. By applying analytical ultracentrifugation and small-angle x-ray scattering, we show that FAPP2 is a dimeric protein in solution, having a curved shape 30 nm in length. The purified FAPP2 protein has the capability to form tubules from membrane sheets in vitro. This activity is dependent on the phosphoinositide-binding activity of the PH domain of FAPP2. These data suggest that FAPP2 functions directly in the formation of apical carriers in the trans-Golgi network.

membrane tubulation | PH domain | phosphatidylinositol 4-phosphate | trans-Golgi network | small-angle x-ray scattering (SAXS)

Outgoing traffic from the Golgi complex diverges into different directions. In epithelial cells, one major route is to the apical membrane. The machinery responsible for the formation of apical carriers is poorly understood. Several proteins have been identified that play a role (1–3). One such protein is the Golgi-associated four-phosphate adaptor protein 2 (FAPP2) (4–6). FAPP2 is a cytosolic protein consisting of the following: an N-terminal pleckstrin homology (PH) domain recognizing the Golgi marker, phosphatidylinositol 4-phosphate [PI (4)P], followed by a central proline-rich region, and a glycolipid transfer protein (GLTP)-like domain toward the C terminus (4). Recent studies have shown that FAPP2 has transfer activity for glucosylceramide (GlcCer) both in vitro and in cells (7, 8). Knocking down FAPP2 by RNAi reduces the conversion of (GlcCer) to lactosylceramide (LacCer) and to downstream complex glycolipids and gangliosides. Evidence suggesting that FAPP2 regulates membrane transport from the Golgi by its glycolipid transfer function was also brought forward. However, the two papers give different directions for the transfer. D'Angelo et al. (7) favor a transfer of GlcCer from the *cis*-Golgi to the trans-Golgi, whereas Halter et al. (8) suggest that FAPP2 takes GlcCer from the trans-Golgi membrane to the endoplasmic reticulum. In the latter case, GlcCer would be flopped after its transfer to the luminal side to be transported to the Golgi complex, where it would function as a precursor for glycolipid biosynthesis. Thus, there is no consensus as to how FAPP2 would control exit of proteins from the trans Golgi network to the cell surface.

In this paper we explored other possible properties of FAPP2 and discovered that purified FAPP2 tubulates lipid membranes in a PI (4)P-dependent fashion. This finding would fit well with properties previously attributed to the protein (4) and adds an important missing function in the machinery responsible for apical transport. In addition, analytical ultracentrifugation (AUC) and small-angle x-ray scattering (SAXS) studies provide structural insights into the dimeric solution state and molecular shape of the full-length FAPP2 protein.

Results

Previous studies by Godi et al. have shown that expression of FAPP2 in Cos7 cells led to the formation of tubules from the

trans-Golgi network (4). To check whether this is also the case in MDCK cells, we expressed FAPP2 in these cells and could demonstrate that FAPP2 was indeed present in tubules forming from the trans-Golgi (Fig. S1C).

To explore this phenomenon further, we tested whether purified FAPP2 had tubulation activity in vitro. We first produced full-length canine FAPP2 having a glutathione S-transferase (GST) fusion at the N terminus in *E. coli*. The protein was purified to high-purity (Fig. S3B, control lanes) for analysis of its effects on membranes. In addition, an N-terminally tagged mCherry-FAPP2 protein was produced to monitor the location of FAPP2 on membranes.

Membrane Tubulation by FAPP2. The effect of FAPP2 on lipid membranes sheets was monitored in real time by light microscopy (9). A mix of POPC:PI (4)P:GlcCer (96:2:2 mol%) was spotted on a coverslip surface and dried. Injection of the soluble protein to the rehydrated membrane sheets induced growth of membrane tubules at the edges or on the top of the most superficial layer of the lipid sheets (Fig. 1A). Tubules coated with mCherry-FAPP2 grew away from the membrane lipid sheets, implying that the binding of FAPP2 to the membrane sheet induced positive curvature of the membrane (Fig. 1E).

Because FAPP2 binds PI (4)P via its PH domain and GlcCer through its GLTP domain, we investigated which lipids are required for membrane tubulation. Full-length FAPP2 tubulated membrane sheets in a PI (4)P-dependent manner, consistent with the capability of its PH domain to bind specifically to this phosphoinositide (10). Only PI(4)P and not GlcCer is required, as tubulation occurred with POPC and PI (4)P (98:2 mol %) (Fig. 1B), but not with either POPC alone (Fig. 1C) or with a mixture of POPC and GlcCer (98:2 mol%) (Fig. 1D).

As the ability of FAPP2 to tubulate membrane sheets appeared to be dependent on its PH domain and PI (4)P, we examined whether mutation of Arg-18 to a leucine residue (R18L) in the PH domain, which has been described to abolish Golgi localization of FAPP2 (4), would affect tubulation. Purified FAPP2-R18L mutant protein did not induce any detectable membrane tubulation (Fig. 2A, Table S1, and Movie S1), suggesting an inability to associate with, insert into, or bend membranes. When WT-FAPP2 was injected into the same chamber, tubules grew out from the membrane sheet immediately, indicating a “recovery” of activity at the membrane surface (Fig. 2B and Movie S2). Together this indicates that tubulation activity requires a functionally intact PH domain that can bind to PI (4)P.

The potential role of GlcCer in the tubulation activity was also

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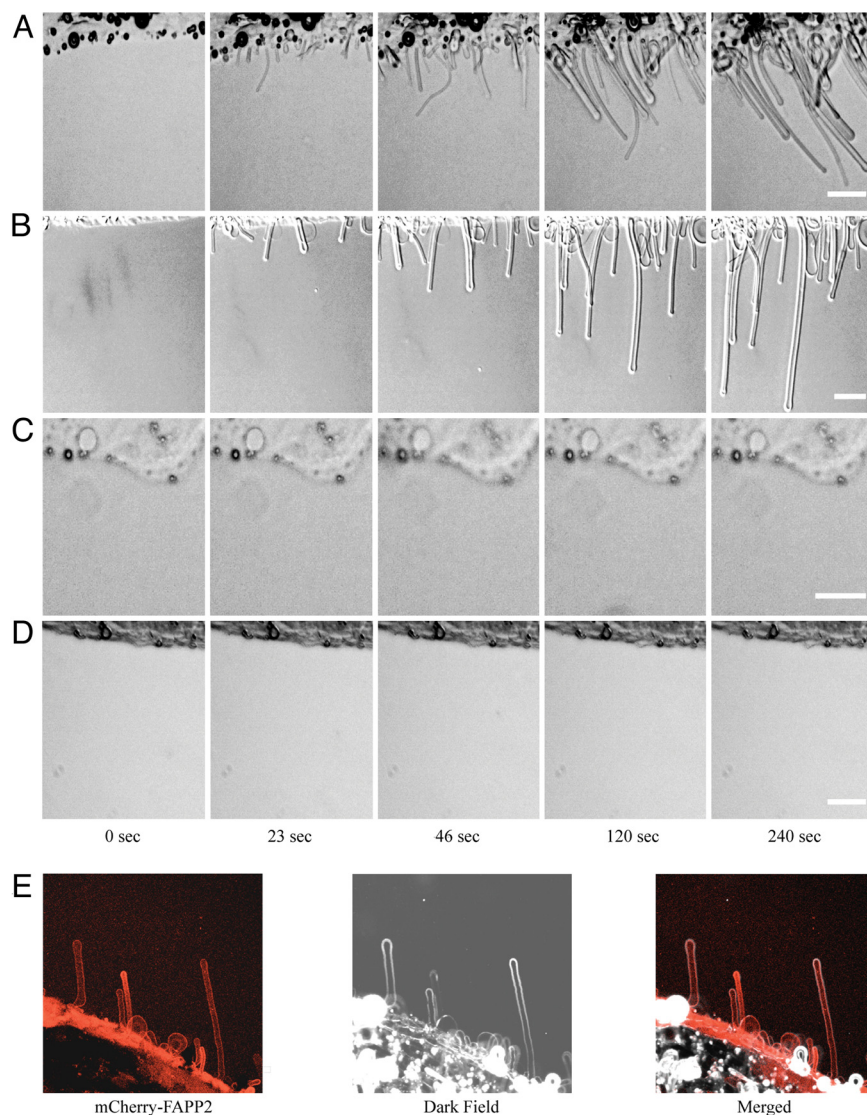


Fig. 1. FAPP2-mediated tubulation of flat membrane sheets. Tubulation activity of FAPP2 on membrane sheets with different compositions was followed by DIC. Lipids membrane sheets consisted of the following: (A) POPC:PI (4)P:GlcCer (96:2:2 mol%); (B) POPC:PI (4)P (98:2 mol%); (C) POPC; and (D) POPC:GlcCer (98:2 mol%). (E) Fluorescence and dark field images of tubules generated on membrane sheet containing POPC:PI (4)P (98:2 mol%) by mCherry-FAPP2. Tubulation was initiated by injection of 5 μ l FAPP2 (1 mg/ml) into the reaction chamber. Bars, 40 μ m.

assessed by mutating an essential Trp in the GLTP domain of FAPP2, disabling the protein to bind to GlcCer (7). The W407A mutant protein generated tubules from the membrane sheets, consistent with our observation that GlcCer was not required for bilayer tubulation (Fig. 2C and Movie S3).

As an independent approach and also to measure the lipid-binding properties the FAPP2 protein, we measured the surface pressure changes ($\Delta\Pi$) on lipid monolayers containing POPC and PI (4)P (98:2 mol%) after continuous addition of FAPP2 (Fig. S4). For WT-FAPP2, a constant increase in pressure was noted up to 1.5 mN/m and at a protein concentration of 10 μ g/ml. At this point, surface pressure began to drop and reached a level well below the starting pressure value, indicating the removal of lipid by FAPP2. The FAPP2-R18L mutant, which is deficient in PI (4)P binding, behaved in the same way as the FAPP2 on POPC alone. FAPP2 associated linearly with the monolayer as the protein concentration was increased but caused no drop in pressure, indicating that although weak/transient binding did occur, no removal of lipids could be observed. Taken together,

also here the FAPP2-PH domain appears to be required for membrane penetration and tubulation in vitro.

Solution State of FAPP2. A combined approach of analytical ultracentrifugation and chemical cross-linking was used to get insights into the solution state of FAPP2. The ability of the GST protein to dimerize can influence the hydrodynamic properties of GST fusion proteins, complicating structural characterization. We therefore decided to produce an additional FAPP2 protein, which is tagged by 3myc and His₆ at the N- and C-termini. To define the relevant solution state of the FAPP2 proteins, size exclusion chromatography (SEC) and analytical ultracentrifugation (AUC) analysis of 3myc-FAPP2-His₆ and GST-FAPP2 were performed.

The SEC results revealed that the 3myc-FAPP2-His₆ protein eluted as one symmetric peak and was stable and monodispersed. The GST-FAPP2 protein formed additional, larger species, consistent with the ability of GST to dimerize (Fig. S2A). These states were stable in that they could be separated from each other over repeated SEC experiments to enrich for the

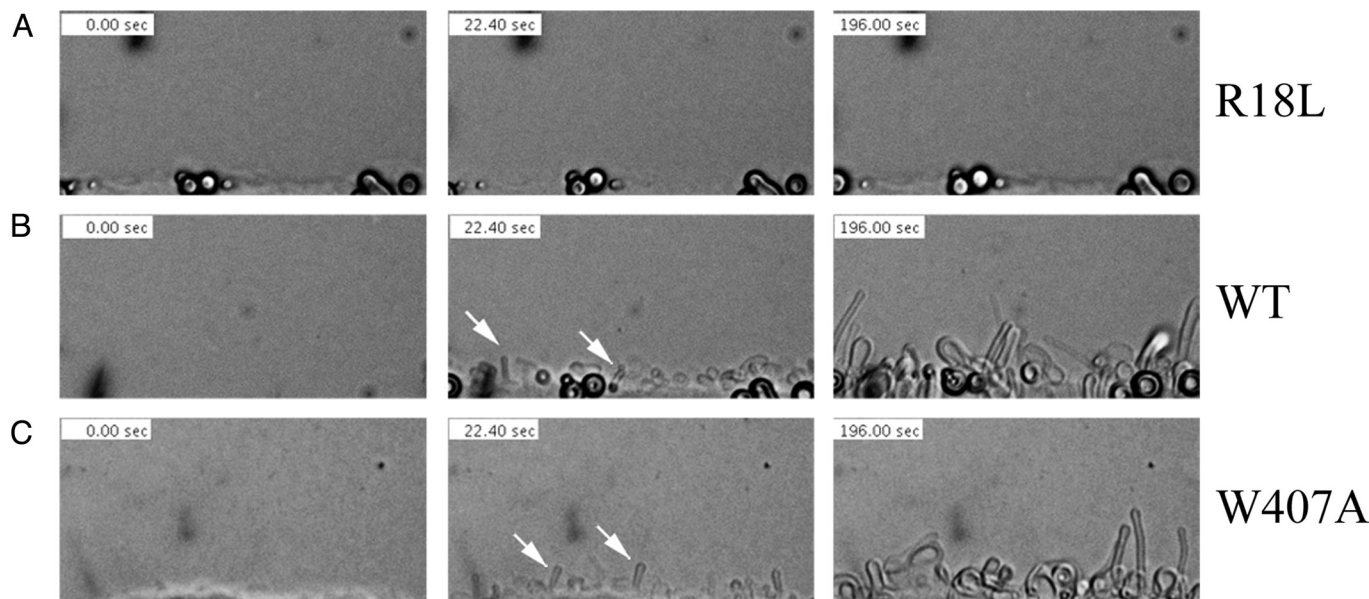


Fig. 2. Membrane tubulation activity of FAPP2 is PI(4)P dependent. Screen shots were taken from [Movies S1–S3](#). (A) PI(4)P binding-deficient FAPP2-R18L lacks tubulation activity, whereas addition of WT-FAPP2 rescues membrane tubulation (B). (C) FAPP2-W407A, lacking GlcCer binding, displays tubulation activity as WT-FAPP2. Fast and narrow tubules, indicated by arrows, are appearing at early time point as in Fig. 1 A but grow back toward the lipid sheet. In all tubulation assays, a lipid mixture consisting of POPC:PI(4)P:GlcCer (96:2:2 mol%) was used. Tubulation was initiated by injection of 5 μ l FAPP2 (1 mg/ml) into the reaction chamber.

main species. Both purified proteins were monodispersed based on sedimentation velocity centrifugation (Fig. S3A). The molecular weights were estimated from sedimentation equilibrium data, and showed that the 3myc-FAPP2-His₆ and GST-FAPP2 proteins were 129 and 166 kDa, respectively, over a broad concentration range (Table S2). As the theoretical molecular mass of the respective monomers is 64 kDa and 84 kDa, the centrifugation data showed that both FAPP2 constructs are dimeric in solution. The dimeric state of each FAPP2 construct was confirmed by chemical cross-linking experiments using bis(sulfosuccinimidyl)-suberate (BS³). Both proteins yielded only one additional band on SDS/PAGE gradient gels with measured molecular weights of 159 and 205 kDa for 3myc-FAPP2-His₆ and GST-FAPP2, respectively, consistent with covalently modified dimeric states (Fig. S3B).

To account for any possible tag effects on the membrane tubulation activity, we also applied the 3myc-FAPP2-His₆ protein to our membrane sheet tubulation assay and could show that the membrane tubulation activity was retained (Movie S4).

Low-Resolution Structure of FAPP2. Given the novel membrane activity of FAPP2 and evidence of its dimeric solution state, we used SAXS to provide insights into the overall size, shape and modular architecture. The GST-FAPP2 protein was not considered for SAXS analysis because of the bulky GST tag. Instead, the stable double-tagged version of FAPP2 (3mycFAPP2-His₆) was used (see [SI Materials and Methods](#)). Inspection of the Guinier plots at low angles indicated good data quality and no protein aggregation. The distance distribution function $p(r)$ was calculated from the scattering intensities $I(s)$ using a reduced angular range from 0.07 nm^{-1} to 3 nm^{-1} , and the maximal particle size (D_{max}) was estimated to 30 nm (Fig. 3B). The radius of gyration (R_g) of 3myc-FAPP2-His₆ was found to be 8.37 nm. Together with the maximum molecular dimension of 30 nm, FAPP2 appears to be an extended protein assembly. This was also supported by the calculated prolate frictional ratio of 1.71, based on analytical ultracentrifugation data (Table S2). A low-resolution envelope model of FAPP2 was derived by ab initio

shape modeling (see [Materials and Methods](#)). The molecular shape is a well extended and curved assembly. To assign the possible location of the FAPP2 protein domains, global rigid body modeling (see [Materials and Methods](#)) against the obtained scattering data indicated the GLTP and PH domain to be at either end of the dimeric FAPP2.

Discussion

Our data demonstrate that FAPP2 has the capability to form tubules from membrane sheets in vitro. This activity is dependent on the PI(4)P-binding activity of the PH domain of FAPP2. FAPP2 can thus be included in the growing list of proteins that can bend membranes to generate tubules.

Mechanism of Tubulation. Two principal mechanisms are used by proteins to induce membrane curvature. BAR domain-containing proteins are “banana-shaped” and thus confer curvature by direct membrane scaffolding (11–13). They bind to membranes by their positively charged concave face and therefore are able to sense, stabilize, and generate membrane curvature (14). The other mechanism relies on insertion of a small amphipathic or hydrophobic wedge to induce membrane asymmetry resulting in curvature (11, 15). These proteins include N-BAR domain-containing proteins having curvature-sensing surfaces with additional N-terminally located amphipathic wedges, inbuilt to generate membrane deformation and tubulation. BAR and N-BAR domains can be extended additionally at their ends by lipid binding modules, such as PH and PX domains to localize the proteins at their intended compartment or by protein–protein interacting modules, like SH3 or PDZ domains. Our results suggest that FAPP2 also should be included in the growing repertoire of tubulating proteins. Stahelin et al. showed, in monolayer assays, that the FAPP1-PH domain also can penetrate into lipid monolayers and decrease the surface pressure (16). These data are in line with our monolayer assays and membrane sheet tubulation results. Altogether, these studies suggest that both membrane binding and tubulation is a property of the PH domain, which seems to be conserved in FAPP1 and

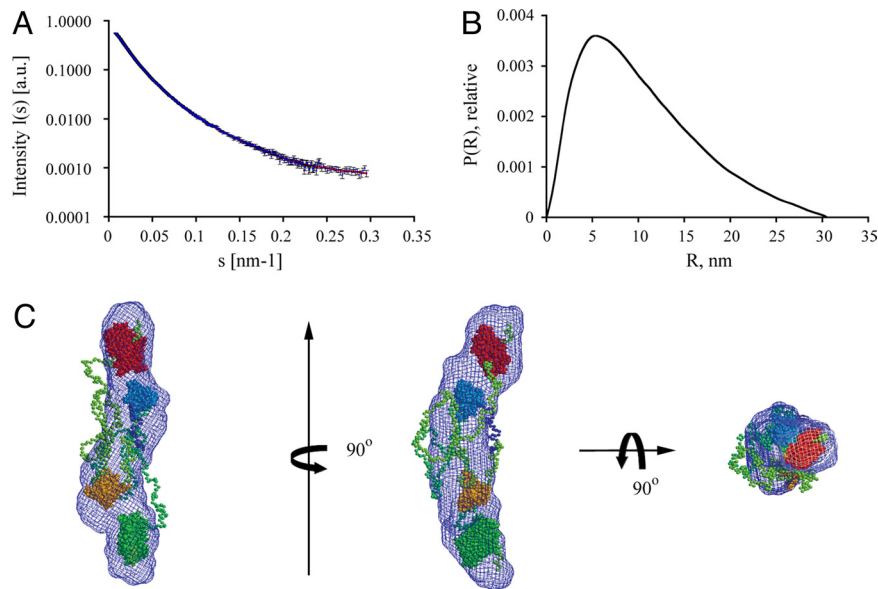


Fig. 3. SAXS analyses of dimeric 3myc-FAPP2-His₆ protein. (A) Experimental scattering curve (blue) and theoretical scattering curve of the modeled envelope structure (red). (B) Distance distribution function shows 3myc-FAPP2-His₆ to be an extended molecule ≈ 30 nm in length. (C) Low-resolution envelope of 3myc-FAPP2-His₆ calculated by ab initio modeling (blue mesh surface). Rigid body modeling was used to calculate the possible locations of the PH- (blue and orange) proline-rich (light green) and GLTP (dark green and red) domain of FAPP2.

FAPP2. The combined analytical centrifugation and SAXS analyses indicate an extended curved shape of the dimeric protein structure of 30 nm length. Because of the low resolution of the FAPP2 structure, it is unclear to what extent the curved shape of FAPP2 contributes to the described tubulation activity and/or to curvature-sensing activity.

FAPP2 in the Cell Biology Context. Whether FAPP2 exerts membrane tubulating activity in cells is not yet known. However, Godi et al. reported that in Cos7 cells overexpressing GST-tagged FAPP2, the protein is present on tubular extensions emanating from the TGN. We confirmed these results in MDCK cells. The carriers transporting protein cargo to the plasma membrane from the TGN are known to be pleiomorphic in shape, forming tubulovesicular structures and tubules of different lengths (17, 18). FAPP2 is also reported to bind the small GTPase, Arf1, another protein recently assigned a membrane-tubulating activity (19–21). It is possible that FAPP2 and Arf1 may act together, inducing membrane deformations leading to tubulation at the TGN. Our data demonstrate that the GLTP domain and its lipid target GlcCer do not play a direct role in the tubulation activity *in vitro*.

Thus the function of the GLTP domain in FAPP2 remains unclear. It could be involved in transferring GlcCer to the cellular site, where GlcCer can be translocated across the membrane to function as a precursor luminally for complex glycolipid synthesis either at the TGN or in the ER as suggested previously (7, 8). However, whether the membrane trafficking function could be explained solely by the glycolipid-transfer activity of FAPP2 seems unlikely because reduction of glycolipid biosynthesis has not previously been demonstrated to have such inhibitory effects on TGN to plasma membrane transport (22–24). It is also possible that FAPP2 functions as a sensor for regulating glycolipid levels in the cell. The presence of GlcCer on the cytoplasmic side of the TGN membrane could serve as a signal for FAPP2 to bind. It would do so by coincidence, binding to PI (4)P, Arf1, and potentially other factors. This ensemble would contribute to the formation and tubulation of transport carriers, which exit from the TGN to deliver both its protein and

(glyco)lipid cargo to the cell surface. A feedback mechanism would limit GlcCer translocation from the cytosolic to the luminal leaflet when LacCer and other downstream glycolipids accumulate in the luminal leaflet of the TGN. Such a function would be in keeping with the proposition that lipid transfer proteins in general could function as biosensors regulating lipid levels in the cell (24–26). Obviously this speculative hypothesis would need to be tested experimentally. Nevertheless the demonstration that FAPP2 has membrane tubulation activity brings in a new dimension in the mechanistic dissection of FAPP2 function in membrane trafficking.

Materials and Methods

In Vitro Tubulation of Flat Membrane Sheets. A system similar to that described in Roux et al. was used (9). Briefly, two 1- μ l droplets of a 10 mg/ml lipid stock solution with indicated composition (see *Results*) were spotted on each coverslip and allowed to dry. To remove any trace chloroform, lipid-coated coverslips were further dried under vacuum for at least 1 h. Lipids were then rehydrated for 20–30 min in an incubator (37 °C, 10% CO₂, and 100% humidity). A small reaction chamber was built by placing the coverslip over a glass slide with strips of double-sided tape as spacers. Subsequently, lipids were fully rehydrated by injecting 15–20 μ l buffer (20 mM Hepes-NaOH, pH 7.4, 100 mM NaCl, 1 mM MgCl₂, 1 mM DTT), containing 0.1 mg/ml casein (C7078, Sigma) into the chamber. A 5- μ l quantity of protein solution (1 mg/ml) was then injected and the deformation of membrane sheets was recorded by DIC microscopy on a Zeiss Axioplan 2 microscope (Carl Zeiss Jena) equipped with EC Plan-Neofluar 10x/0.3 objective.

X-Ray Scattering Experiments and Data Analysis. SAXS data were collected at the updated X33 beamline (27–29) using a MAR345 image plate detector (MarResearch, Norderstedt, Germany) located at the EMBL Hamburg Outstation located on a bending magnet (sector D) on the storage ring DORIS III of the Deutsches Elektronen Synchrotron (DESY). A single photon counting pixel detector system (PILATUS 500k) was used as detector. A sample-detector distance of 2,675 mm was used, covering the range of momentum transfer $0.1 < s < 5 \text{ nm}^{-1}$ ($s = 4\pi \sin(\theta)/\lambda$, where θ is the scattering angle and $\lambda = 0.1504$ nm is the x-ray wavelength). The s -axis was calibrated by the scattering pattern of Silver-behenate salt (d -spacing 5.84 nm). The scattering patterns from 3myc-FAPP2-His₆ protein was measured at protein concentrations of 3.87 and 7.74 mg/ml. Protein sample was prepared in Dulbecco's PBS and 20 mM DTT as radical quencher. Four repetitive measurements of 30 s at 15 °C of the same protein solution were performed to check for radiation damage. All initial

