scientific report

Facilitated aggregation of FG nucleoporins
under molecular crowding conditionsSigrid Milles, Khanh Huy Bui, Christine Koehler, Mikhail Eltsov, Martin Beck⁺ & Edward A. Lemke⁺⁺

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Intrinsically disordered and phenylalanine–glycine-rich nucleoporins (FG Nups) form a crowded and selective transport conduit inside the NPC that can only be transited with the help of nuclear transport receptors (NTRs). It has been shown *in vitro* that FG Nups can assemble into two distinct appearances, amyloids and hydrogels. If and how these phenomena are linked and if they have a physiological role still remains unclear. Using a variety of high-resolution fluorescence and electron microscopic (EM) tools, we reveal that crowding conditions mimicking the NPC environment can accelerate the aggregation and amyloid formation speed of yeast and human FG Nups by orders of magnitude. Aggregation can be inhibited by NTRs, providing a rationale on how the cell might control amyloid formation of FG Nups. The superb spatial resolving power of EM also reveals that hydrogels are enlaced amyloid fibres, and these findings have implications for existing transport models and for NPC assembly.

Keywords: intrinsically disordered protein; amyloid fibres; electron microscopy; fluorescence spectroscopy; nuclear pore complex

EMBO reports (2013) 14, 178–183. doi:10.1038/embo.2012.204

INTRODUCTION

Nuclear pore complexes (NPCs) facilitate the exchange of proteins and RNA between the nuclear and cytoplasmic compartments of all eukaryotic cells [1]. They are assembled from several copies of more than 30 different proteins called nucleoporins (Nups) [2], which can be roughly categorized into two classes. First, scaffold Nups are the key architectural elements that stabilize the membrane gap, leaving a central channel of about 50–60 nm in diameter [3,4]. Second, intrinsically disordered and phenylalanine–glycine-rich Nups (FG Nups) line the central channel that tightly controls nucleocytoplasmic transport [5]. FG motifs occur in several copies per single FG Nup and are, on

average, spaced apart by about 20 amino acids by rather hydrophilic linkers [6,7]. FG Nups form a permeability barrier that can be overcome by nuclear transport receptors (NTRs) through interaction with the FG repeats [5,8]. Owing to the size and complexity of the NPC, the *in vivo* structure of the central transport channel remains elusive. Thus, many studies focus on studying the FG repeat domains under purified biochemical conditions [6,7,9–13]. Although FG Nups are generally accepted to be intrinsically disordered proteins meaning that they lack stable secondary structure [9], they have been found to adopt at least three distinct states *in vitro*: First, ensemble and single-molecule studies have shown that many FG domains can adopt a collapsed state [7,13]. Second, the yeast Nup100, which is enriched in GLFG repeats, has been shown to form amyloid fibres by a variety of classical assays for amyloid detection [14,15]. However, the observed amyloid formation of this protein was rather slow and its physiological relevance thus remains questionable [14]. Third, concentrated protein solutions of the human Nup153 FG domain (hNup153FG), the human Nup98 FG domain and a few yeast FG Nups have been reported to form macroscopic hydrogels [6,13,16,17]. As such hydrogels obey NPC-like permeability barrier properties, that is, they accumulate NTRs much faster than inert cargo [6,12], they were used as model systems for the NPC transport mechanism and were previously utilized to experimentally test certain properties of the NPC barrier *in vitro* [6,16,18]. A structural model of FG hydrogels was put forward suggesting that FG Nups link into a structure similar to a fisher net. In this model, the ropes are individual FG Nups that are tied together by knots made from crosslinks between FG repeats. These interactions were suggested to be specifically melted by NTRs when penetrating the hydrogel. In such a model, the mesh size of the network is largely defined by the amino-acid spacing between neighbouring FG repeats (~4 nm) [6,12]. In addition, solid state NMR and X-ray scattering techniques suggested the existence of ‘amyloid-like’ β -sheet folds with inter- β -strand distances of up to 1.3 nm [19,20]. However, such spectroscopic techniques permit only an indirect visualization of structural properties whereas the spatial picture over different length scales remains still unknown.

In this work, we combined fluorescence-based assays on the single-molecule (pM protein concentrations) and ensemble level (nM protein concentrations) with classical aggregation assays and

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Received 13 June 2012; revised 19 November 2012; accepted 21 November 2012; published online 14 December 2012

electron tomography to study the aggregation behaviour of hNup153FG under molecular crowding conditions that mimic the interior of the NPC. We found that molecular crowders dramatically enhance the aggregation of hNup153FG by orders of magnitude. Using kinetic measurements, we show that NTRs inhibit aggregate/amyloid formation, pointing to a possible biological mechanism that potentially enables cells to cope with the high aggregation propensity. The remarkable aggregation propensity prompted us to further investigate the molecular ultrastructure of hydrogels formed by FG Nups. Here, we used electron microscopy (EM) to directly image the three-dimensional ultrastructure of FG Nup hydrogels from the nanometre to the micrometre scale. We determined that hydrogels formed by the FG domains of hNup153 and the yeast Nup49 (yNup49FG) *in vitro* have a backbone structure that is build from amyloid fibres of several nm in diameter. Those fibres interlace into each other yielding a variable mesh size ranging up to 40 nm, that is, as large as the diameter of the entire central channel of the NPC, a finding that comprises unique insights into the architecture of these supramolecular structures.

Taken together, our findings reveal new biochemical properties of FG Nups linking the three known phenotypes: Intrinsically disordered FG Nups can aggregate, fold into β -sheets and stack onto each other to form highly ordered and elongated amyloid fibrils. This process is dramatically accelerated under crowding conditions and provides a rational for how such amyloids can grow into networks to adapt the macroscopic phenotype of a hydrogel.

RESULTS AND DISCUSSION

Molecular crowding accelerates FG Nup aggregation

We previously showed that under single-molecule conditions, the intrinsically disordered hNup153FG adopts a collapsed conformation in solution [13]. Such a compaction can be mediated by attractive intrachain interactions, and such forces can also favour attractive intermolecular interactions at higher protein concentrations, leading to structured amyloids [21,22]. Amyloids are high molecular weight aggregates originating from proteins being densely stacked onto each other typically using β -sheet folds to assemble into elongated filamentous structures of several nm in width and μ m in length [23]. In fact, many intrinsically disordered proteins, such as prions or α -synuclein, can form amyloids [23,24]. We thus first tested if hNup153FG can also form amyloids. We performed a thioflavin T (ThT) assay, the classical assay for *in vitro* amyloid detection on the basis of the incorporation of the initially non-fluorescent ThT into amyloid fibres, where it then turns fluorescent [25]. An increase of ThT fluorescence by orders of magnitudes as well as the prototypical time dependence of amyloid formation showing a lag phase, a burst phase and finally a saturation phase (also illustrated in supplementary Fig S1a online) is commonly used for amyloid detection [26]. hNup153FG clearly followed both requirements (Fig 1A) in the ThT assay, and the formation of amyloids with a typical amyloid twist was also confirmed using EM (Fig 1B,C) [27]. As human and yeast FG Nups share only little sequence similarity, we subjected yNup49FG to the same test, and determined the same phenotype as for hNup153FG (supplementary Fig S1 online). Our data indicate that the ability to form structured amyloid fibres might be common to many FG Nups and also conserved across species. This observation is

also strengthened by the recent finding that also the yeast GLFG Nup100, which shares limited sequence similarity with our two tested proteins, can form amyloids [14].

As the NPC constitutes a highly crowded environment in which 60–120 MDa of proteins are squeezed into a small nanometre-sized volume [28], we wanted to further investigate the influence of various cosolutes (that is, osmolytes, osmoprotectants and classical crowders), in this work summarized as molecular crowders, onto FG Nup aggregation into amyloids. Cosolutes can, for example, geometrically restrict the available space for proteins, can have a considerable effect on protein stability, structure and function, and can also influence the aggregation behaviour of proteins [29]. To mimic the molecular crowding of the cell *in vitro*, we first used high concentrations of serine in the buffer, an amino acid also enriched in the nuclear pore—possibly even locally at molar concentrations (supplementary Material online). The ThT assay reported dramatically fastened amyloid formation kinetics of hNup153FG in buffer containing serine. Increasing serine concentrations even led to instantaneous aggregation of the protein (Fig 1A). To further characterize this surprisingly rapid aggregation, we developed a fluorescence assay that can robustly report on fast formation of early protein aggregates that usually occur in the beginning of amyloid formation kinetics. Two batches of hNup153FG were labelled with two different dyes, such that FRET can occur between a Donor (D, Alexa488) and an Acceptor (A, Alexa594) dye only if two molecules come in close proximity. D- and A-labelled hNup153FG were mixed with serine and immediately a clear FRET signal became apparent that rose with increasing serine concentrations in a sigmoidal fashion (Fig 1D,E). This aggregation occurred even at the single-molecule level (pM protein concentrations, see Fig 1F; supplementary Fig S2 online) and is thus extremely high under molecular crowding conditions. As enhanced aggregation also occurred in the presence of other cosolutes such as lysine, trimethylaminoxid and the large molecule polyethylene glycol 20,000, we conclude that the aggregation accelerating effect is not owing to a specific chemical functionality but rather a general phenomenon, like space restriction or sequestering of water. A similar phenomenon was observed for yNup49FG (supplementary Fig S1 online), indicating that also crowding-induced aggregation might be a common property of FG Nups and conserved across species. An all F $\rightarrow$ A mutant of hNup153FG neither showed aggregation in our FRET assay, nor under crowding conditions, nor formed amyloid fibres in a ThT assay (Fig 1). This observation points towards a role of the FG motifs also in the high amyloid formation propensity.

Importin β can prevent aggregation of FG Nups

The nucleus and in particular the NPC is a highly crowded environment, and uncontrolled fibrillation of FG Nups is likely not beneficial. The question thus arises how this property can be controlled. NTRs are very abundant proteins in the nucleocytoplasmic transport machinery, and we tested if the presence of NTRs can modulate the aggregation propensity of FG Nups. As shown in Fig 2, we incubated saturating concentrations of Importin β together with a 1:1 mixture from separate batches of Alexa488- and Alexa594-labelled hNup153FG, and then transferred this mixture to molecular crowding conditions. No FRET signal between the dyes was observed, indicating that Importin β prevents hNup153FG molecules to come into such proximity that

Fig 1 | Amyloid formation and enhanced aggregation of hNup153FG under molecular crowding conditions. (A) ThT assays of 1 mg/ml hNup153FG in the absence (black circles) and presence of 20% ($\square$), or 30% serine ($\triangle$) and of hNup153AG (grey circles). (B,C) Negative-staining electron micrographs of hNup153FG fibres assembled in PBS. Arrows indicate amyloid twists in the fibres. (D) Fluorescence spectrum of hNup153FG-Alexa488 and hNup153FG-Alexa594 mixed in PBS (dashed line) or 40% TMAO in PBS (solid line) in a 1:1 ratio on excitation of Alexa488. (E) Titration of a 1:1 mixture of hNup153FG-Alexa488 and hNup153-Alexa594 with different concentrations of cosolutes (circle, serine; triangle, lysine; diamond, PEG2000; square, TMAO). (F) Titration of 50 pM hNup153FG-Alexa488 mixed with 50 pM hNup153FG-Alexa647 at the sm level with serine reveals sigmoidal dependence. Corresponding data for yNup49FG are shown in supplementary Fig S1 online. ThT, thioflavin T; TMAO, trimethylaminoxid; PEG, polyethylene glycol; sm, single molecule.

the two dyes can undergo FRET. Even if Importin β was added after aggregation was induced, it could immediately stop further increase of the FRET signal compared with a sample that did not contain the transport receptor (Fig 2B). In agreement with these results, Importin β could also prevent amyloid formation of both hNup153FG and yNup49FG, as reported in the ThT assay (Fig 2C; supplementary Fig S1a online), likely by binding to and thus shielding of the FG repeats.

Fibrous meshworks can form hydrogels

FG Nup hydrogels have the interesting ability to mimic biochemical properties of the NPC permeability barrier. While during the above described experiments, crowding was induced through high concentrations of chemically inert molecular crowding reagents; it is possible that FG hydrogels also constitute a form of crowded environment because they form at very high protein concentrations. As elucidating the structure of the

hydrogel is a crucial prerequisite for a better understanding of its potential role in the NPC transport mechanism, we analysed the structure of plastic-embedded hNup153FG and yNup49FG hydrogels using electron tomography. This technique has the power to resolve volumes across various length scales (nanometre to micrometre scale) and thus reveals a very comprehensive picture. Figure 3 and supplementary Movie S1 online show that hNup153FG hydrogels are formed from interlaced fibres that build up a meshwork giving rise to the supramolecular architecture of a hydrogel (see supplementary Fig S3 online; supplementary Movie S2 online for yNup49FG). The fibres have a diameter of several nanometres and are thus similar to the amyloid fibres formed in solution (Fig 1). In the amyloid meshwork, we frequently observe holes of several tens of nanometres in diameter between the interlacing fibres, and the hole size depends on the interconnectivity of this meshwork. In support of this conclusion, hydrogels formed at lower protein concentrations maintain the same overall

Fig 2 | Inhibition of FG Nup aggregation and fibre formation by Importin β . (A) hNup153FG labelled with Alexa488 or Alexa594 were mixed in a 1:1 ratio in the presence or absence of Importin β . The samples were then diluted into 10% serine or 0% serine in PBS. Only hNup153FG without Importin β indicates aggregation (FRET) in the 10% serine buffer. (B) Importin β was added to a hNup153FG Alexa488/Alexa594 mixture (as described before) either before transferring into 10% serine buffer (red circle, red line) or after 1 h of aggregation (red circle, dashed red line). Time of Importin β addition is indicated with an arrow. Sample (black circle, black line) did not contain any Importin β . The development of the FRET signal (aggregation of hNup153FG) was monitored over time for all samples. Higher FRET values indicate larger amounts of aggregated proteins. (C) ThT assay of 1 mg/ml hNup153FG in the absence (black circle) and presence (red circle) of Importin β . ThT, thioflavin T.

Fig 3 | Composition of hNup153FG hydrogels from amyloid fibres and corresponding transport assays. (A) Isosurface rendering of a plastic-embedded hNup153FG hydrogel (~ 200 nm thickness). Scale bar, 200 nm. (B) Estimation of the mesh size in hNup153FG amyloid networks (hydrogels). The segmented image shows only meshes with sizes larger than 1,250 nm² (equivalent to 40 nm in diameter) as colour patches on the basis of two-dimensional projections of the corresponding image processed tomogram (50 nm thickness). A schematic of the NPC is drawn to scale. Scale bar, 200 nm. (C) Scanning electron microscopy image of an hNup153FG hydrogel. Scale bar, 200 nm. (D) Gel entry of Importin β labelled with tetramethylrhodamine dye (lower part) and exclusion of dextran labelled with fluorescein isothiocyanate dye (upper part, filtered with a median filter with 1 pixel radius) 0 and 60 min after addition of the Importin β /dextran mix. Scale bar, 50 μ m. (E) Corresponding relative gel entries of Importin β and dextran into an hNup153FG hydrogel (right panel; black, Importin β ; grey, dextran). Corresponding data for γ Nup49FG are shown in supplementary Fig S3 online. NPC, nuclear pore complex.

architecture and thickness of amyloid fibres, while the hole size is growing (supplementary Fig S4 online). To exclude artifacts that might arise from sample preparation, we confirmed these results using alternative preparation and imaging methods, namely

scanning electron microscopy of heavy metal-shaded samples (Fig 3; supplementary Fig S3 online), as well as transmission electron microscopy of frozen-hydrated cryosections (supplementary Fig S5 online). We consistently observed fibrous

networks within the hydrogels. We next performed a standard fluorescence microscopy assay to confirm that these hydrogels can still mimic the permeability barrier of the pore (Fig 3 for hNup153FG and supplementary Fig S3 online for yNup49FG): labelled Importin β rapidly enriched inside the gel in contrast to labelled dextran, a standard inert cargo, which is in agreement with the previous literature [6,13,16]. It is interesting to note that even isolated FG Nup amyloids maintain the ability to bind NTRs (supplementary Fig S6 online) and it is therefore conceivable that preferred entry of NTRs into the hydrogel is owing to their affinity towards the fibres. If the holes are only water filled, the macromolecular phenomenon of a hydrogel might be unrelated to the *in vivo* pattern inside the NPC. However, the resolution of electron tomography is not sufficient to determine if the holes contain less electron dense, proteinaceous material that could give rise to a barrier inside the hole as envisioned by for example, the entropic brush [10,11], reduction of dimensionality [30], virtual gate [31] as well as the selective phase model [6,12,20]. Future experiments will thus be necessary to elucidate the properties of these *in vitro* hydrogels in more detail, in particular taking the dimensions of these fibre meshworks into account.

Our study reveals a new property of FG nucleoporins: dramatically enhanced aggregate formation when in an environment that mimics the crowded interior of the cell. We showed that FG Nup amyloid fibres can grow into dense networks and give rise to the supramolecular architecture of a selective hydrogel. The high-resolution structure provided by EM shows that hydrogels and amyloids share a similar molecular origin. As amyloid fibres are typically made from stacked β -sheets [32] and the entire backbone structure of the hydrogel is composed of amyloids, these observations also provide a rationale for the previous detection of amyloid-like folds in hydrogels with spectroscopic techniques [19,20]. In general, FG Nups have a very heterogeneous sequence composition. It is thus conceivable that only parts of the protein contribute to the fibre core, whereas other parts remain disordered, as has been observed for other amyloid-forming proteins [23]. The yeast Nsp1, of which one domain can, the other cannot form hydrogels, might be such a candidate [19,20]. Moreover, FG Nups are heavily posttranslationally modified and modifications such as phosphorylation or glycosylation [33] might influence formation and appearance of hydrogels. It is interesting to note that fibrous hydrogels are very commonly studied by the material science field and are frequently formed from FG rich peptides [34,35].

While the functional relevance of FG Nup hydrogels and the significance of their composition in light of the different transport models remain speculative and subject to further investigations, accelerated amyloid formation of FG Nups is a fundamental biochemical property for the cell to contend with. Our observation that NTRs can block FG Nup aggregation *in vitro* provides a rationale for a possible *in vivo* mechanism that would allow the cell to control and prevent rapid amyloid formation. Geometrical constraints inside the NPC (for example, anchoring of Nups inside the pore), as well as posttranslational modifications, might add more elements to control amyloid formation propensity. Furthermore, it is conceivable that the cell might in fact exploit this high FG Nup aggregation propensity, as it can provide a driving force to facilitate FG Nup association during NPC assembly.

METHODS

Protein expression, purification and labelling. The recombinant hNup153FG, hNup153AG, yNup49FG and Importin β were expressed, purified and labelled essentially as described previously [13,36]. See SI Materials and Methods for more detailed information.

Aggregation FRET assay. hNup153FG with amino-acid position Cys 8 labelled with Alexa488 was mixed with hNup153FG Cys 8 labelled with Alexa594 1:1 to a final concentration of 1 μ M (10 nM for single molecule experiments) in 4 M guanidinium hydrochloride (GdmCl) in PBS, pH 7.4, and then diluted to 10 nM (100 pM) protein into PBS pH 7.4 with 2 mM dithiothreitol and 2 mM magnesiumacetate and various concentrations of osmolytes. Immediately after mixing, fluorescence spectra were recorded on a PTI Quantamaster (Seefeld, Germany) with an excitation wavelength of 488 nm and emission from 500 to 650 nm. Ensemble FRET was calculated from the intensity Alexa594/(Alexa488 + Alexa594). Single-molecule data were recorded and analysed as described in SI Materials and Methods.

ThT assay. Lyophilized proteins were taken up in 7 M GdmCl in 20 mM Hepes or PBS, pH 7.4, and then diluted into 20 mM Hepes with 150 mM NaCl, or PBS pH 7.4 (GdmCl <0.5 M) containing various concentrations of osmolytes with 2 mM dithiothreitol, 2 mM magnesiumacetate and 200 μ M ThT. ThT fluorescence (excitation at 450 nm and emission at 480 nm) was recorded over time. ThT time traces were fit with a sigmoidal curve.

Hydrogel formation. hNup153FG and yNup49FG hydrogels were formed at a concentration of 50 mM FG repeat in H₂O + 0.1% trifluoroacetic acid. Transmission electron microscopy of FG hydrogels was performed on plastic-embedded hydrogels or cryosections of hydrogels. Scanning electron microscopy was performed of metal-shaded FG Nup hydrogels. Transport assays were performed essentially as described previously. Briefly, entry of labelled Importin β and a 70 kDa dextran was observed by confocal fluorescence microscopy. Their relative gel entry rates were determined by an automated procedure (SI Materials and Methods). A detailed description of further hydrogel handling for the individual methods can be found in the SI Materials and Methods.

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

ACKNOWLEDGEMENTS

We thank Drs Iain Mattaj, Virginia VanDelinder, Carsten Sachse and Katja Beck for helpful discussions. This study was technically supported by the Advanced Light Microscopy and Protein Expression core facilities of The European Molecular Biology Laboratory. We gratefully acknowledge support from The European Molecular Biology Laboratory's electron microscopy core facility and the CellNetworks electron microscopy core facility of the University of Heidelberg, in particular from Uta Haselmann-Weiss and Dr Ingrid Hausser. S.M. is a fellow of the Boehringer Ingelheim Fonds. K.H.B. was supported by the Swiss National Fonds and a long-term fellowship of the European Molecular Biology Organization. E.A.L. acknowledges funding by the Emmy Noether programme of the Deutsche Forschungsgemeinschaft.

Author contributions: S.M., K.H.B., C.K. and M.E. performed experiments; S.M. and K.H.B. analysed the data; S.M., K.H.B., M.B. and E.A.L. conceived or designed the experiments; and S.M., M.B. and E.A.L. wrote the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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