Multiple Recognition Motifs in Nucleoporin Nup159 Provide a Stable and Rigid Nup159-Dyn2 Assembly

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Abstract

Dyn2 is the yeast ortholog of the molecular hub LC8, which binds disordered proteins and promotes their self-association and higher order assembly. Dyn2 is proposed to dimerize and stabilize the Nup82-Nsp1-Nup159 complex of the nuclear pore assembly through its interaction with nucleoporin Nup159. Nup159 has six LC8 recognition motifs separated by short linkers. NMR experiments reported here show that the Dyn2 binding domain of Nup159 is intrinsically disordered and that binding of one equivalent of Dyn2 dimer aligns two Nup159 chains along the full Dyn2 binding domain to form a bivalent scaffold that promotes binding of other Dyn2 dimers. Isothermal titration calorimetry of Dyn2 to Nup constructs of increasing lengths determine that the third LC8 recognition motifs does not bind Dyn2. A new approach to identifying active LC8 recognition motifs based on NMR-detected β-sheet propensities is presented. Isothermal titration calorimetry experiments also show that, due to unfavorable entropy changes, a Nup-Dyn2 complex with three Dyn2 dimers is more stable than the wild-type complex with five Dyn2 dimers. The calorimetric results argue that, from a thermodynamics perspective, only three Dyn2 dimers are needed for optimal stability and suggest that the evolutionary adaptation of multiple tandem LC8 recognition motifs imparts to the complex other properties such as rigidity and a kink in the rod-like structure. These findings extend the repertoire of functions of intrinsically disordered protein to fine-tuning and versatile assembly of higher order macromolecular complexes.

Intrinsically disordered proteins, a class of proteins lacking a well defined structure, are often at the organizational center of biological complexes where they act as flexible scaffolds presenting multiple binding domains and promoting spatial orientations that facilitate protein–protein interactions (reviewed in Ref. 1). Intrinsically disordered proteins in such complexes have some regions that fold upon binding (2), whereas other regions retain their disordered structure, a phenomenon referred to as fuzziness (3). Dynamic and adaptable intrinsically disordered protein-partner complexes require chaperones or regulators that aid the coordination of assembly and disassembly of their higher order structures.

Nucleoporin Nup159, a subunit at the cytoplasmic end of the yeast nuclear pore complex, functions both as a structural component of the nuclear pore complex and as a docking site for transiently associated nuclear transport factors (4). In studies of the assembly of Nup159-Nsp82-Nsp1 nucleoporin subcomplex, Hurt and co-workers (5) identified Dyn2, the yeast ortholog of LC8, as a binding partner of Nup159. Dyn2 and Drosophila melanogaster LC8 are very closely related, with 48% sequence identity and essentially the same three-dimensional structure (6, 7). LC8, a dynein light chain that also binds numerous other non-dynein partners, has been identified as a versatile hub protein that in diverse systems binds to disordered protein segments and assists folding, assembly, and regulation of multiprotein complexes (8). As a dimer with two symmetrical binding grooves, LC8 has an inherent tendency to bind two chains of the same protein and create a bivalent scaffold that promotes either self-association or docking of other bivalent ligands or both (9, 10). For example, binding of LC8 to the intrinsically disordered N-terminal domain of dynein intermediate chain (IC)2 promotes both IC self-association and increased affinity for the other dimeric light chains (9, 11, 12). This binding enhancement involving multiple bivalent dimers is termed “polybivalency” (9, 10) and the IC-light chain assemblage is a canonical example of polybivalency in intrinsically disordered proteins. A common feature among LC8 binding partners, including dynein IC, is that the LC8 recognition motif is disordered in the apo form but is ordered in the LC8-bound form where it packs as a β-strand that spans the length of LC8 dimer interface (6, 13–17).

Several binding partners of LC8 have multiple LC8 recognition motifs, as is the case for Nup159. Hurt and co-workers (22) localized the Nup159 region that binds Dyn2, termed DID for...
Dyn2 interacting domain, to six putative LC8 recognition motifs, five of which bind Dyn2 by pepscan analysis. Fig. 1 summarizes the Nup159 domains, namely, six presumptive LC8 recognition motifs in tandem flanked by an N-terminal predicted β-propeller domain (18) followed by a predominantly unstructured Phe-Gly-rich segment (19) and a C-terminal coiled-coil domain (20).

Other LC8 binding partners that have multiple LC8 recognition motifs include the following: the C-terminal domain of ATM-interacting protein 18 SQ/TQ LC8 recognition motifs spanning a 400-amino acid stretch, of which 11 bind LC8 by pepscan analysis (21); the protein Kibra (22) with two LC8 binding motifs separated by >600 amino acid residues (23); the membrane-associated protein Bassoon with three LC8 recognition motifs in tandem (24), and the spoke phosphoprotein RS protein 3 with five LC8 recognition motifs spanning a 110-amino acid stretch, three of which bind simultaneously to LC8 (25).

There are notable similarities in domain organization between the more extensively studied LC8 binding partner, dynein IC, and Nup159. Similarities include a β-propeller domain at the N-terminal of Nup159 and the C-terminal of IC, a middle unstructured segment, and a predicted coiled-coil at the N-terminal of IC and the C-terminal of Nup159. However, although Nup159 binds five Dyn2 dimers, D. melanogaster IC binds one LC8 dimer and an LC8-like dimer (Tctex1) (9), and yeast IC (Pac11) binds 2 Dyn2 dimers (26). This raises interesting questions, such as why multiple Dyn2 dimers associate with Nup159. Are all needed for complex stability, or similar to IC, are two sufficient? Equally relevant is why Dyn2 binds only five of the six LC8 consensus recognition motifs; bringing to the fore the question of how recognition motifs are specified and whether the presence of such a motif implies an actual binding event. In this study, we use NMR and isothermal titration calorimetry to identify which Nup159 recognition motifs indeed bind Dyn2, to characterize the collapsed and aligned Nup-Dyn2 complex at low levels of Dyn2, and to rationalize the function of multiple recognition motifs in Nup159.

**EXPERIMENTAL PROCEDURES**

*Construct Design and Cloning*—Recombinant Dyn2 and constructs of Nup159 that include residues 891–1264 (hereafter referred to as QT1–6CC1), 1154–1210 (QT5–6), 1166–1210 (QT6) were gifts (Ed Hurt, Biochemie-Zentrum der Universitat, Heidelberg, Germany). QT1–6CC1 was used as the template in polymerase chain reactions to generate amplified DNA fragments corresponding to residues 1075–1115 (QT1), 1075–1128 (QT1–2), 1075–1140 (QT1–3), 1075–1153 (QT1–4), 1075–1165 (QT1–5), and 1075–1178 (QT1–6), which were subsequently cloned into a pMCSG9 vector (27) using the ligation-independent cloning protocol of Eschenfeldt et al. (28). QT1–6CC1 was subcloned in a PET Sumo vector (Invitrogen), and QT5–6 and QT6 were subcloned in PET 15b vectors (Novagen). Recombinant vectors were transformed into BL21(DE3) or Rosetta cell lines for protein expression.

*Protein Expression and Purification*—Recombinant proteins were produced by growing cell lines at 37 °C in Luria broth or 13C or 12C glucose/15N ammonia-enriched MJ9 media. At a cell density (A600) of 0.6–0.8, protein expression was induced for 3 h by adding 0.1–0.5 mM isopropyl 1-thio-β-D-galactopyranoside. Cells were harvested, lysed, centrifuged to remove all cell debris, and soluble recombinant proteins purified using Qiagen nickel-nitrilotriacetic acid affinity chromatography protocol (Qiagen, Valencia, CA).

The His₆ maltose-binding protein fusion, part of the pMCSG9 vector of each Nup159 construct was removed with tobacco etch virus protease. All proteins were further purified on a Superdex™ 75 (GE Healthcare) size exclusion chromatography column, resulting in purity > 95%. For constructs used in NMR studies, an additional anion exchange chromatography purification step resulted in purity > 98%.

Proteins were dialyzed against appropriate buffers, and their concentrations were determined from absorbances at 280 nm and computed (ExPasy) molar extinction coefficient values (QT1, 1490 m⁻¹ cm⁻¹; QT1–2, QT1–3, QT1–4, QT1–5, QT1–6, and QT1–6CC1, 2980 m⁻¹ cm⁻¹; QT5–6 and QT6, 5960 m⁻¹ cm⁻¹; Dyn2, 12950 m⁻¹ cm⁻¹). Reported concentrations are those for the monomeric proteins. The computed Dyn2 molar extinction coefficient takes into account the polyhistidine sequence from the expression vector. The proteins were subsequently stored at 4 °C and used within a week.

**NMR Data Collection and Analyses**—NMR samples were prepared in 50 mM sodium phosphate, 50 mM NaCl, pH 6.0 buffer containing 1 mM DSS, 10% D₂O, and a protease inhibitor mixture (Roche Applied Science). Data were acquired on a Bruker Avance 600 MHz spectrometer equipped with a cryogenic ¹H/¹³C/¹⁵N TCI probehead with the z axis gradient coil at 25 °C. Five-dimensional HN(CA)CONH and HabCabCONH experiments (29) and an auxiliary three-dimensional HNCO experiment (30) recorded with non-uniform sampling of the indirectly detected dimensions were used for sequential assignments of 500 µM ¹³C,¹⁵N-uniformly labeled QT1–6. Interaction of unlabeled Dyn2 with isotopically labeled QT1–6 was characterized by collecting HSQC spectra with Dyn2 at final molar ratios (Nup:Dyn2) of 1:0.8, 1:2, and 1:6.

The three-dimensional HNCO spectrum was processed using Multidimensional Fourier Transform (31), and the non-uniformly sampled five-dimensional HN(CA)CONH and HabCabCONH spectra were processed with Sparse Multidimensional Fourier Transform (32–34) (the software for data processing is available online at the Warsaw University Laboratory). Titration data were processed with NMRpipe (35). All spectra were analyzed with the graphical NMR assignment and integration software Sparky 3.115 (36).

**Circular Dichroism Spectroscopy**—The CD spectrum of QT1–6 was acquired at 25 °C, on a JASCO 720 spectropolarimeter, using a 1-mm path length cell. The protein, at a concentration of 20 µM was prepared in 10 mM sodium phosphate, 10 mM NaCl, pH 6.0.

**Isothermal Titration Calorimetry**—ITC experiments using Microcal VP-ITC microcalorimeter (North Hampton, MA), were performed at 25 °C (unless otherwise stated) in buffer composed of 50 mM sodium phosphate, 50 mM NaCl, 5 mM β-mercaptoethanol, 0.5 mM sodium azide, pH 7.5. A typical experiment involved an initial 2 µl injection, followed by 26
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10-μl injections of Dyn2 to Nup159 constructs in the concentration range of 9–100 μM, accompanied by stirring at a constant rate of 351 rpm. Added Dyn2 was in the concentration range of 0.4–1.2 mM. The heats of dilution were measured by titrating Dyn2 into buffer and subtracting the resulting enthalpies from the data set. Data were analyzed using Origin (version 7.0) and fit to a single-site binding model. Recorded data are the average of two independent experiments, with the uncertainty reported as the difference between the experimental value and the average.

RESULTS

Construct Design—The carboxyl-terminal half of Nup159 includes a Dyn2 binding domain (DID in Fig. 1) adjacent to a predicted coiled-coil domain. Within the DID are six putative LC8 recognition motifs (QT) each composed of a 10-residue sequence ending in QTX where X is any amino acid. Nup159 constructs were designed to include one (QT1), two (QT1–2), three (QT1–3), four (QT1–4), five (QT1–5), last two (QT5–6), last (QT6), or all six (QT1–6, QT1–6CC1) recognition motifs. QT1–6CC1 includes the first part of the predicted coiled-coil domain (Fig. 1).

Solution Studies of Nup159-DID Domain—The DID domain QT1–6 is a monomer by multi-angle laser light scattering criteria (observed and calculated molecular masses of 11.97 kDa and 11.69 kDa, respectively). A 1H,15N HSQC spectrum (Fig. 2A) shows limited amide proton dispersion characteristic of a predominantly unstructured protein. Backbone assignments for all 101 non-proline residues were determined using five dimensional HN(CA)CONH and HabCabCONH experiments (29) optimized for predominantly unstructured proteins. A CD spectrum characteristic of a protein with limited secondary structure (Fig. 2B) confirms the disorder observed by NMR. Local conformational propensities of residues were experimentally derived from the deviation of Cα and CB chemical shifts from standard random coil values (37). Fig. 2C identifies secondary structure propensities with β-strand propensities for residues 1101–1109, 1150–1156, 1159–1166, and 1168–1174, and some helical propensity for residues 1126–1134, also shown in hatched arrows and a bar below the sequence in Fig. 3E. For comparison, β-sheet propensities predicted by sequence-based algorithms are shown in Fig. 3E as open arrows, whereas the rest of the sequence is predicted as disordered.

NMR Studies of the Nup159-Dyn2 Interaction—Plots of normalized intensities of “random coil” peaks with Dyn2 added to 1H,15N or 13C,15N-labeled QT1–6 at molar ratios (Nup:Dyn2) of 1:0.8, 1:2, and 1:6 are shown in Figs. 3, A–C, respectively. Fig. 3E shows which residues have peaks that fully disappear at the various molar ratios. At the lowest concentration of Dyn2 (Fig. 3A), peaks that completely disappear are denoted by blue bars above the plot; they correspond to residues 1109–1117 (a stretch that includes the last four residues of QT1, the three residues between QT1 and QT2, and the first two residues of QT2), 1142–1149 (all within QT4) and 1168–1169. The majority of the other peaks lose more than half of their intensities. Increasing the concentration of Dyn2 to twice the concentration of QT1–6 (Fig. 3B) results in the complete disappearance of the majority of the peaks (green portions of the bar above the plot). With a 6-fold molar excess of Dyn2 (Fig. 3C), the only visible peaks correspond to N-terminal residues 1075–1100, which precede the first recognition motif (red peaks in Fig. 3D), whereas all others in the sequence 1101–1178 disappear (denoted magenta in the bar above the plot in Fig. 3C).

Why do Nup “random coil” peaks disappear when QT1–6 and Dyn2 interact? Random coil peaks are reporters of NH groups in residues whose local disorder is equivalent to the same residue in a random coil. When proteins interact, peak disappearance can be due to line broadening associated with intermediate exchange processes and/or to longer rotational correlation time and correspondingly faster transverse relaxation as molecular mass increases (38) as it does in a complex of QT1–6 and Dyn2.

The structural implications of the variation in the “random coil” peak intensities when about one equivalent of Dyn2 is added to six equivalents of QT motifs (i.e., one equivalent of QT1–6) are the following. First, the high intensity of a few of the “random coil” peaks in Fig. 3, A and B, cannot arise from some fraction of apo QT1–6 because any apo QT1–6 will have the same intensity of “random coil” peaks for all residues. The contrary, other than the few random coil peaks with high intensity, located primarily in stretches at the N termini of QT1 and QT6, the intensity of other peaks in Fig. 3A varies widely. We conclude that at a molar ratio of about one Dyn2 to 6 QT motifs, NMR spectra (Fig. 3A) indicate that the two proteins form a dynamic complex. In this weakly associating complex, a few residues of the Nup chain are highly flexible as indicated by the high intensity of their “random coil” peaks, whereas other Nup residues are conformationally restricted due to interactions with Dyn2 or with a second Nup chain. Second, the total disappearance of peaks at more than one QT motif in Fig. 3A indicates that the dynamic Nup:Dyn2 complex is not only collapsed but also that the Nup chains are aligned. Third, peaks that retain partial intensity in Fig. 3A do so without detectable broadening, which indicates that the corresponding NH groups are in slow chemical exchange between conformational microstates separated by significant energy barriers (38). The observation that these peaks disappear as the relative concentration of Dyn2 is increased (Fig. 3, B and C) implies that the final complex with multiple Dyn2 dimers is significantly less flexible, and of proportionally lower entropy, than the dynamic collapsed complex formed at the lowest Dyn2 concentration.
Thermodynamics of Nup159-Dyn2 Interactions—Isothermal titration calorimetry was used to determine the binding energetics of Dyn2 with Nup159 constructs containing an increasing number of recognition motifs. Representative plots are shown for Dyn2 interaction with QT1 (Fig. 4B), QT1–2 (Fig. 4C), QT1–3 (Fig. 4D), QT1–4 (Fig. 4E), QT1–5 (Fig. 4F), QT1–6 (Fig. 4G), QT5–6 (Fig. 4H), QT6 (Fig. 4I), and QT1–6CC1 (Fig. 4J). The weak interaction of Dyn2 with QT1 (Fig. 4B) was not fit to a binding model and is not included in the summary of the thermodynamic parameters of Table 1. A stoichiometry of 2 is determined for both QT1–2 and QT1–3, indicating that the QT3 motif does not bind Dyn2, but a 2-fold increase in binding affinity is observed with the longer QT1–3 construct. Although there appears to be this slight increase in binding affinity, binding is still relatively weak compared with the QT1–4 construct (compare $K_d$ 12 and 6 $\mu$M with 0.8 $\mu$M, Table 1). Compared with the QT1–4 construct, there is a slight decrease in binding affinity to the QT1–5, QT1–6, and QT1–6CC1 constructs (compare $K_d$ 0.8 $\mu$M (QT1–4) with 1.8 $\mu$M, 2.9 $\mu$M, and 2.5 $\mu$M respectively), and a corresponding decrease in entropic terms. Comparable binding affinities are observed for constructs that include both the 5th and 6th consensus sequences (compare $K_d$ 2.9 $\mu$M for QT5–6 and QT1–6, and 2.5 $\mu$M for QT1–6CC1).

DISCUSSION

The DID Domain of Nup159 Is Intrinsically Disordered with Interspersed Segments That Have Secondary Structure Propensity—A common property of several partner proteins of the Dyn2 ortholog LC8 (also known as DYNLL) is intrinsic disorder in their LC8 binding region (8) indicated by sequence-
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**Figure 3.** Residue-specific titrations of Nup159-DID. Relative intensity (Int.) versus residue numbers of non-proline NH peaks in NMR spectra of Dyn2-bound QT1–6 at Nup:Dyn2 molar ratios of 1:0.8 (A), 1:2 (B), and 1:6 (C). Peak intensities are relative to the intensity of the same peak in apo QT1–6, which is taken as one. Adding Dyn2 at 0.8-, 2-, or 6-fold molar excess results in the disappearance of peaks for residues shown in blue, green, and magenta, respectively. D, overlay of $^1$H-$^15$N HSQC spectra of apo (black) and Dyn2-bound (red) $^15$N,$^13$C Nup159 QT1–6, with unlabeled Dyn2 added in 6-fold molar excess. Peaks visible in the bound spectrum are labeled. E, a summary of results of titration experiments in frames A, B, and C mapped onto residues 1101 through 1178, which lose peak intensity as Dyn2 is added to Nup159 QT1–6. Below the sequence are secondary structure propensities of apo QT1–6 (arrows, strand; bar, helix) based on sequence prediction algorithms (open arrows) and NMR chemical shift data (hatched arrows or bar). F, comparison of experimentally determined binding segments (yellow highlights) to outputs from two disorder-based predictors of binding sites, MoRFpred (46), and ANCHOR (47). MoRFpred, which identifies short molecular recognition features located within intrinsically disordered regions that bind to protein partners via disorder-to-order transitions, identified the 5th binding segment as a putative binding region (red highlighted regions). ANCHOR, which finds disordered regions that become ordered when bound to a globular partner identified the 1st, 2nd, part of the 4th, and the 5th binding segments (black bars) as potential binding sites. The experimentally determined binding segments highlighted in yellow correspond to Nup159 residues 1103–1112, 1116–1125, 1141–1150, 1153–1162, and 1165–1174.

Based prediction and experimental measurement of secondary structure. The DID domain of Nup159 is no exception: both CD and limited amide proton dispersion of $^1$H-$^15$N NMR spectra are consistent with a disordered polypeptide having limited secondary structure (Fig. 2). NMR-detected secondary structure propensities of Nup159-DID show that of the six consens 

Based prediction and experimental measurement of secondary structure. The DID domain of Nup159 is no exception: both CD and limited amide proton dispersion of $^1$H-$^15$N NMR spectra are consistent with a disordered polypeptide having limited secondary structure (Fig. 2). NMR-detected secondary structure propensities of Nup159-DID show that of the six consensus sequences QT1, QT5, and QT6 have β-strand tendencies, QT2 and QT4 show no preference for secondary structure, and a segment of small helical propensity joins QT2 and QT3 and extends into QT3 (Figs. 2C and 3E). All high resolution struc-
tures of bound LC8 complexes to date show partner proteins adopting a sixth β-strand in an LC8 antiparallel β-sheet (6, 7, 13, 16, 17), and the Nup-Dyn2 complexes are expected to have the same (6, 7); thus the QT3 motif containing a segment with helical propensity is less likely to favor acquisition of a β-strand conformation at the binding interface with Dyn2.

Binding of Dyn2 to Nup159 DID Forms a Collapsed, Aligned Assembly—The NMR titration experiments in Fig. 3 offer new insights into Nup-Dyn2 binding and into repeat recognition motifs in general, namely that the initial binding of Dyn2 aligns the two Nup chains in a flexible, collapsed state. Addition of Dyn2 even at the low molar ratio of Fig. 3A results in formation of a loosely packed dimeric complex composed of one Dyn2 dimer with two Nup chains. The complex is a dynamic ensemble (39) of interconverting conformations in which two Nup molecules are attracted to each other and to a Dyn2 molecule but not stably bound to either. The complete disappearance of peaks at QT4 and at sites near the N and C termini of QT1–6 suggests that Dyn2 “hops” along the chain, reversibly binding at more than one location. The complete loss of these peaks, along with the 50% loss of intensity for peaks of most other residues along the DID chain, imply that the two Nup molecules are essentially aligned after binding one Dyn2 equivalent to five QT equivalents. It is important to note that, even when saturation of all QT sites and all the peaks within the six QT motifs disappear, the peaks N-terminal to QT1 do not lose intensity, meaning that these residues retain random coil flexibility.

Polybivalency in Dyn2-Nup159 Interactions—We introduced the term polybivalency to describe assembly of intrinsi-

### TABLE 1

| Construct | \(n\) | \(K_d\) | \(\Delta H^o\) | \(\Delta S^o\) | \(\Delta G^o\) |
|-----------|-------|---------|----------------|----------------|----------------|
| QT1–2     | 1.9 ± 0.03 | 12.2 ± 0.06 | −2.3 ± 0.04 | 4.4 ± 0.02 | −6.7 ± 0.03 |
| QT1–3     | 1.9 ± 0.04 | 6.0 ± 0.04 | −2.5 ± 0.02 | 4.6 ± 0.04 | −8.1 ± 0.04 |
| QT1–4     | 2.8 ± 0.02 | 0.8 ± 0.1 | −3.4 ± 0.1 | 4.9 ± 0.2 | −8.3 ± 0.2 |
| QT1–5     | 3.8 ± 0.10 | 1.8 ± 0.2 | −3.5 ± 0.07 | 4.3 ± 0.1 | −7.8 ± 0.1 |
| QT1–6     | 4.8 ± 0.04 | 2.9 ± 0.06 | −4.4 ± 0.03 | 3.1 ± 0.03 | −7.6 ± 0.03 |
| QT3–6     | 2.0 ± 0.01 | 2.9 ± 0.05 | −6.3 ± 0.04 | 1.2 ± 0.05 | −7.5 ± 0.04 |
| QT6       | 1.0 ± 0.01 | 2.0 ± 3 | −5.8 ± 0.01 | 0.65 ± 0.1 | −6.5 ± 0.1 |
| QT1–6CC*  | 5.1 ± 0.1 | 2.5 ± 0.4 | −6.1 ± 0.1 | 1.6 ± 0.1 | −7.7 ± 0.1 |

* Data for QT1–6CC were recorded at 30 °C.
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cally disordered proteins in dynamic complexes, in particular those that bind LC8 (9, 40). This assembly mode is manifested in the association of Dyn2 with the Nup159 DID domain discussed above: the first bound Dyn2 forms a bivalent scaffold with significant ordering in the center motif and considerable loss of random coil flexibility along the full length of DID and with increased affinity for additional Dyn2.

This increased affinity is demonstrated by the ITC-determined binding thermodynamics with Nup constructs having incremental numbers of QT motifs (Fig. 4 and Table 1). The QT1 motif binds Dyn2 very weakly when alone, but when followed by QT2 in the construct QT1–2, the average affinity for two Dyn2 dimers is significantly higher than with QT1 alone and is more than 2-fold higher when compared with the affinity of a short peptide containing only QT2 (6).

The 2-fold increase in binding affinity observed with QT1–3 although the QT3 motif does not bind Dyn2 could be attributed to self-association of the helices. Such increased affinity due to self-association is also observed for the LC8-dynein intermediate chain interaction (10). The absence of Dyn2 binding at QT3 may explain the kink observed in electron micrographs of the wild-type Nup-Dyn2 complex but not in an engineered DID domain that binds six Dyn2 dimers; the latter forms a more extended complex (41). It is tempting to speculate that the evolutionary adaptation of a QT motif to helix-helix self-association is to promote a kink in the rod-like structure of the complex, thus relieving the steric strain present in a complex with six Dyn2 dimers bound in close proximity on two Nup strands.

A considerable increase in average Dyn2 binding affinity is observed when three Dyn2 dimers bind to QT1–4. Similarly, with QT6, binding is weak, but with QT5–6, the average binding is considerably enhanced.

In summary, the thermodynamics of Nup-Dyn2 assembly in DID constructs of increasing lengths illustrates significant enhancement of a second Dyn2 binding event (or of self-association) and further enhancement with a third binding event such that the average binding affinity is higher than the average of the first two. Interestingly, this enhancement trend stops at QT5 as the average binding affinity decreases in QT1–5 and QT1–6, suggesting that Dyn2 binding of QT5 and QT6 is not required for complex stability but rather results in a somewhat less stable assembly. In the longest natural construct, QT1–6CC1, the average binding affinity plateaus at about a $K_d$ of 2–3 µM.

What Are the Roles of QT5 and QT6?—Why does the DID domain contain five binding motifs when the first three (QT1–4) or the last two (QT5–6) alone are stable assemblies? That is, why is a Nup-Dyn2 complex containing the first three Dyn2-binding motifs more stable than a complex containing all motifs? One answer is that binding of Dyn2 to both QT5 and QT6 imparts rigidity to the assembled complex (Fig. 5). A considerable loss of degrees of freedom when the last two motifs are bound is inferred from the increased order (decreased entropy) of the system that accompanies their binding (Table 1). Comparing QT1–5 and QT1–6 to QT1–4, an increase in unfavorable entropy results in decreased stability even though the enthalpic contribution is more favorable, and, this effect is far greater for QT1–6 than for QT1–5.

Nup159 is the only reported example containing multiple recognition motifs for LC8-like proteins with short linkers separating the motifs and the only one that stacks five LC8 dimers in the fully bound complex. In single particle electron micrographs (EM), the Nup-Dyn2 complex forms an elongated structure described as “beads on a string,” which has been proposed to project the N-terminal Phe-Gly repeats into the cytoplasm where they are accessible to nuclear transport proteins at the cytoplasmic end of the nuclear pore (5). A Nup construct containing only the first three motifs is too flexible to be observed by EM, supporting the idea that the last two motifs impart rigidity. In spoke stalks present in axonemes, rigidity is also apparently imparted by multiple binding of LC8 (25).

In summary, the decreased entropy of the Nup-Dyn2 system appears to reflect a process that could be critical to its function,
namely formation of a rod-like complex with restricted flexibility. The presence of five recognition sites apparently provides entropy/enthalpy balance to a stable yet entropically unfavorable complex with moderate affinity. Formation of a rigid Nup-Dyn2 complex is consistent with the conclusion that polybivalency, essentially an idea about enhancement of binding affinity of multiple ligands for disordered proteins arising from entropic effects and from local effective concentrations of reactants, confers both binding enhancement and energetic compensation for entropically unfavorable interactions. In this respect, Nup-Dyn2 assembly highlights the importance of entropic considerations in disordered proteins in fine-tuning assembly of versatile complexes.

Recent studies from the Hurt lab (42) demonstrate that assembly of Nup159 with the nuclear pore complex occurs before Dyn2 recruitment. The requirement of Dyn2 late in the assembly process argues that Dyn2 is not essential for Nup82 assembly. Rather, binding of all five Dyn2 molecules may be required to promote formation of a structure that is relatively stiff, rod-like, and composed of beads between two strings, perhaps for subsequent recruitment of cargo molecules at the cytoplasmic end. To the extent that this is true, it underscores the importance of motifs QT5 and QT6 whose Dyn2 binding promotes this rigidity.

Ubiquitylation of Nup159 at Lys-897 N-terminal to the Dyn2 recognition motifs appears to control the anchoring of Dyn2 to the nuclear pore complex (43). Although it is clear that binding of Dyn2 does not require ubiquitylation of Nup159 (this work and Ref. 5), it is possible that ubiquitylation of Nup159 may increase its binding affinity to Dyn2 so that it is more tightly anchored to the nuclear pore complex. Hayakawa et al. (43) also report that preventing ubiquitylation alters the nuclear segregation and the spindle positioning at the onset of mitosis, roles that are attributed to the cytoplasmic dynein complex. However, it is important to note that when Dyn2 is bound to Nup159, it is not part of the cytoplasmic dynein complex and the properties attributed to Dyn2 do not necessarily extrapolate to those of the dynein complex. Dyn2/LC8 is a hub protein (8), and as such, its role in Nup159 bears no relationship to its role in dynein (26), as it uses the same binding sites for dynein assembly and Nup159 assembly and cannot bind both simultaneously (23).

Recognition by LC8/Dyn2 of Multiple Motif Variants—LC8 is a hub protein because it recognizes diverse partners with varying recognition motif sequences. Many binding partners of LC8 or its orthologs, including Nup159, carry variants of the characteristic consensus K/RXTQT motif (44). Although there are other less common motifs (13) and some binding partners lacking the motif (16), the (K/R)XTQT motif is the most prevalent both in nature and in a directed evolution study (8, 45). We and others (23, 45) have proposed that entropy/enthalpy compensation associated with the flexibility of LC8 binding clefts that dock the motif expands the spectrum of proteins recognized by LC8. The results reported here illustrate another determinant of specificity, that is, the propensity of the residues preceding the motif to form an extended β-strand, a tendency that favors orientation of this segment as a β-strand in the hydrophobic cleft at the LC8 dimer interface. NMR spectroscopic techniques that identify segments with a propensity to form β-strand or helical structure in unfolded proteins are a powerful augmentation of sequence-based secondary structure prediction algorithms (Fig. 3E) or disorder-based predictors of binding sites (Fig. 3F). As it is evident for Nup159, not all apparent recognition sequences for Dyn2 actually bind Dyn2, and it is likely that the same is true for other arrays of presumptive recognition motifs for LC8 and its orthologs. For these cases, as for Nup159-Dyn2 sites, strong helical propensity may override similarity to the characteristic QT sequence, whereas β-strand propensity indicates most likely recognition motifs.

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