Interactions of Yeast Dynein with Dynein Light Chain and Dynactin

GENERAL IMPLICATIONS FOR INTRINSICALLY DISORDERED DUPLEX SCAFFOLDS IN MULTIPROTEIN ASSEMBLIES*

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Intrinsically disordered protein (IDP) duplexes composed of two IDP chains cross-linked by bivalent partner proteins form scaffolds for assembly of multiprotein complexes. The N-terminal domain of dynein intermediate chain (N-IC) is one such IDP that forms a bivalent scaffold with multiple dynein light chains including LC8, a hub protein that promotes duplex formation of diverse IDP partners. N-IC also binds a subunit of the dynein regulator, dynactin. Here we characterize interactions of a yeast ortholog of N-IC (N-Pac11) with yeast LC8 (Dyn2) or with the intermediate chain-binding subunit of yeast dynactin (Nip100). Residue level changes in Pac11 structure are monitored by NMR spectroscopy, and binding energetics are monitored by isothermal titration calorimetry (ITC). N-Pac11 is monomeric and primarily disordered except for a single α-helix (SAH) at the N terminus and a short nascent helix, LH, flanked by the two Dyn2 recognition motifs. Upon binding Dyn2, the only Pac11 residues making direct protein-protein interactions are in and immediately flanking the recognition motifs. Dyn2 binding also orders LH residues of Pac11. Upon binding Nip100, only Pac11 SAH residues make direct protein-protein interactions, but LH residues at a distant sequence position and L1 residues in an adjacent linker are also ordered. The long distance, ligand-dependent ordering of residues reveals new elements of dynamic structure within IDP linker regions.

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Cytoplasmic dynein is an essential microtubule-based motor that controls diverse cellular processes including centrosome separation and movement, mitotic spindle assembly and orientation, and axonal transport (1, 2). In higher eukaryotes, cytoplasmic dynein is a 1.6-MDa multisubunit protein complex that moves along microtubules using its motor domain and transports cargos associated with its cargo attachment domain. The motor domain, part of the heavy chain subunit, includes the ATPase activity that generates force to drive dynein motion. The cargo attachment domain functions in loading of cargo, maintenance of stability, and modulation of dynein activity (3). In Drosophila the primary chains that compose the cargo domain are intermediate chains (IC), light chains LC7, LC8, and Tctex1, and light intermediate chains.

Dynein processivity and activity require interactions with various regulatory proteins (4), including dynactin, an essential multisubunit complex (5). Dynactin mediates cargo recruitment (6) and increases dynein processivity along microtubules (7, 8). Because Saccharomyces cerevisiae dynein has structural and functional differences from Drosophila dynein, we sought in these studies to compare and contrast the consequences of dynactin and light chain binding on intermediate chain structure in the two species. In yeast, the single nonessential function of dynein is positioning the mitotic spindle into the mother/bud neck during mitosis (9). Also, the yeast cargo attachment domain has a simpler subunit composition. Although higher eukaryotes have one copy each of the three different light chains, yeast has two copies of one light chain, Dyn2, the LC8 ortholog. Disrupting Dyn2 binding impairs dynactin recruitment (10).

In Drosophila, the specific binding of dynein to dynactin involves protein-protein interactions of IC and p150Glued, a domain of the largest subunit of dynactin. A predicted coiled-coil domain of p150Glued associates with the N-terminal domain of dynein IC, also predicted to be a coiled coil (11). Specifically, p150Glued binds IC at a multiregion interface corresponding to residues 1–40 and 46–75 (12). Tctex1 and LC8 bind the disordered IC segment 110–138, which is separated from the p150Glued binding residues by a flexible linker. Because

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‡ The abbreviations used are: IC, intermediate chain; SAH, single α-helix; IDP, intrinsically disordered protein; HSQC, heteronuclear single quantum coherence; LH, linker helix, or short nascent helix; TROSY, transverse relaxation optimized spectroscopy.
the light chains are dimeric, their binding affinity for IC is thought to be enhanced by mutual bivalency effects (10, 13). In yeast, Nip100 is the dynactin subunit that binds yeast intermediate chain Pac11. The proximity of the binding sites for Dyn2 and Nip100 in the Pac11 sequence allows measurement of Pac11 binding to each partner individually and both partners simultaneously.

A combined analysis of high resolution NMR data, binding energetics, and gel filtration profiles delineate for the first time residue level changes in Pac11 structure and dynamics upon binding Nip100 or Dyn2, show clear differences in the energetics of light chain binding to Pac11 versus Drosophila IC, and identify within Pac11 new structural domains formed when either Dyn2 or Nip100 is bound. Although linker residues remain disordered in the Pac11 complex with Dyn2 or Nip100, they are not featureless “random coils” but rather sequences containing distinctive elements of dynamic structure.

Sequence analysis of Pac11 indicates that, contrary to commonly expressed assumptions (10, 11, 14), the N-terminal residues predicted to be coiled coils are instead a single α-helix (SAH). This conclusion is supported by the strongly helical NMR-detected secondary structure of apo Pac11 residues 1–24. Analysis of IC sequences from other species support our hypothesis that a SAH domain at or near the N terminus is a conserved structural feature among IC homologs.

**Experimental Procedures**

**Protein Preparation**—Pac11 construct containing residues 1–87 was generated by PCR and subsequently cloned into a modified pET15da (Novagen) expression vector with an N terminus His	extsubscript{6} tag followed by a tobacco etch virus protease cutting site. Pac11 1–87 Δ66–73 was cloned into the same vector by the Gibson assembly method. A Nip100 construct containing residues 214–389 (Nip100 CC1B) was generated by PCR and subsequently cloned into a pET15b (Novagen) expression vector with a C terminus His	extsubscript{6} tag. DNA sequences were verified by automated sequencing. The recombinant vectors were transformed into Rosetta (DE3) cell lines for protein expression. Dyn2 was prepared as described elsewhere (15) but with modifications that allow the removal of the fused SUMO tag.

Cells were grown in LB medium at 37 °C to an optical density ($A_{600}$) of 0.6–0.8 followed by protein induction for 3–16 h with 0.1–0.4 mM isopropyl β-D-thiogalactopyranoside. For Pac11 1–87 WT and Δ66–73, both of which form inclusion bodies under the above conditions, the cells were lysed using denaturing buffer (100 mM sodium phosphate, pH 8, 10 mM Tris, 8 mM urea) followed by affinity chromatography purification on nickel-nitrilotriacetic acid resin (Qiagen) and elution in imidazole denaturing buffer (20 mM sodium phosphate, pH 8, 500 mM sodium chloride, 350 mM imidazole, 1 mM sodium azide, 6 mM urea). The proteins were refolded by stepwise dilution in refolding buffer (20 mM sodium phosphate, pH 8, 500 mM sodium chloride, 1 mM sodium azide) to a urea concentration of 1 mM followed by dialysis into the same buffer with 0 mM urea. The His	extsubscript{6} tag was cleaved using tobacco etch virus protease followed by purification on a Superdex 75 (GE Healthcare) size exclusion chromatography column, resulting in purity of >95%. Dyn2 and Nip100 CC1B were purified under native conditions. The SUMO tag was cleaved from Dyn2 using SUMO protease (Cornell University). Nip100 CC1B was lysed in the affinity buffer containing 2 M urea to prevent aggregation and improve its interactions with nickel-nitrilotriacetic acid resin. Protein concentrations were determined from absorbances at 280 nm using molar extinction coefficient values (Pac11 1–87, 2980 M⁻¹ cm⁻¹; Pac11 1–87 Δ66–73, 1490 M⁻¹ cm⁻¹; Dyn2, 12950 M⁻¹ cm⁻¹; Nip100 CC1B, 8940 M⁻¹ cm⁻¹) computed with the ProtParam tool at the ExPASy website.

Protein complex formation was analyzed by assayable size exclusion chromatography (Superdex 200 10/300; GE Healthcare) at 0.2 ml/min in buffer containing 50 mM sodium phosphate, 100 mM sodium chloride, 1 mM sodium azide, pH 7.2. In each run, 100-μl protein samples were loaded at concentrations of 100 μM for Nip100 CC1B, Pac11 1–87, and Pac11 1–87 Δ66–73, and of 200 μM for Dyn2. Protein (complex) markers were selected to facilitate comparison to expected complexes of similar molecular masses; markers show elution volumes for the 114-kDa complex of IC$_{T_{11}}$Tctex1-LC8-LC7 (16), 35 kDa for β-lactoglobulin, and 13.7 kDa for ribonuclease A. Computed molecular masses of assigned complexes were derived using the molecular masses of Pac11 1–87 monomer (10.6 kDa), Dyn2 dimer (20.8 kDa), and Nip100 CC1B dimer (42.8 kDa). Experimental molecular masses were determined using multi-angle light scattering (MALS). SDS-PAGE was used to examine protein content in the eluted fractions.

**Circular Dichroism Spectroscopy**—Spectra were recorded on a JASCO 720 spectropolarimeter using a 1-mm cell at a concentration of 20 μM in 10 mM sodium phosphate, pH 6, and at 25, 15, and 5 °C.

**Isothermal Titration Calorimetry**—ITC experiments of Pac11 with Dyn2 and Nip100 CC1B were performed using a Microcal (North Hampton, MA) VP-ITC microcalorimeter at 25 °C in buffer composed of 50 mM sodium phosphate, pH 7.2, 50 mM sodium chloride, 0.5 mM sodium azide. Experimental conditions were identical for Pac11 1–87 and for Pac11 1–87 Δ66–73, in which residues 66–73 are deleted. In Dyn2 experiments, an initial 2-μl injection was followed by 26 injections of 10-μl Dyn2 samples (250–400 μl) into 15–28 μM Pac11 1–87 (see Fig. 2A) or into Pac11 1–87 Δ66–73 (see Fig. 2B) in the sample cell. An initial 2-μl injection was followed by 26 injections of 10 μl of Pac11 1–87 (350–500 μl) into Nip100 CC1B (36–50 μl) in the sample cell. All experiments were accompanied by a constant stirring rate of 351 rpm. Protein samples and buffer were degassed prior to data collection. The data were processed using Origin 7.0 (Microcal) and fit to a single-site binding model. Stoichiometry was computed for number of binding sites; for example, on one Pac11 monomer, there is one Nip100 or Dyn2 partners simultaneously.

**NMR Experiments**—Doubly labeled 15N/13C- and 15N-labeled proteins were prepared in buffer composed of 10 mM sodium phosphate at pH 6.0 with 50 mM sodium chloride, 10 mM Arg$^+$, 10 mM Glu$^-$, 1 mM sodium azide, 8% H$_2$O, a prote-
ase inhibitor mixture (Roche Applied Science), and 2,2-dimethyl-
silapentane-5-sulfonic acid for \(^{1}H\) chemical shifts refer-
cencing. For backbone assignments, NMR spectra of Pac11 1–87
were obtained at 15 °C and a concentration of 300 \(\mu\)M. The
dynamics data of \(^{15}N\)-labeled Pac11 1–87 were collected for the
apo, for Pac11-Dyn2 complex at a molar ratio (Pac11: Dyn2) of
1:2.2, and for Pac11-Nip100 CC1B complex at a molar ratio
(Pac11: Nip100 CC1B) of 1:1.2. Native PAVE and \(^{1}H\)-\(^{15}N\)
HSQC spectra were used to assay sample integrity before and
after every NMR data collection. All reported data are from
HSQC spectra that remain unchanged during data collection.

Backbone resonance assignments for apo Pac11 1–87 were
determined from a set of \([^{15}N,^{1}H]\) TROSY-based (17) triple-
resonance HN(CO)CACB and HN(CAC)O experiments featuring
BEST (band selective excitation short transient) methodology
(18) to reduce measurement times. A TROSY-type HAHBHN-
(CACO)NH (19) experiment was collected to determine proton
assignments. These experiments were carried out with a cryo-
probe on a Bruker Avance 900-MHz spectrometer, whereas the
remaining experiments (HN(CO)CACB, HNCO) employed a
room temperature xyz gradient probe on a Bruker Avance 500-
MHz spectrometer. Backbone resonance assignments for
Dyn2-bound Pac11 1–87 (Pac11:Dyn2 at a molar ratio of 1:2.2)
were determined using a similar HN(CO)CACB experiment
collected on a Bruker Avance 600-MHz spectrometer.

Longitudinal (\(T_1\)) and transverse (\(T_2\)) \(^{15}N\) relaxation times
were determined at 600-MHz \(^{1}H\) frequency using standard
HSQC-based pulse sequences (20) at 15 °C. For each series,
spectra with ten different relaxation periods (three in duplicate
for error estimation) were recorded in an interleaved manner.
Their duration varied from 10 ms to 1.3 s in \(T_1\) measurements,
from 23.2 ms to 371.2 ms in \(T_2\) measurements of the free Pac11
1–87, and from 20 ms to 200 ms in \(T_2\) measurements of the
complex with Dyn2 or Nip100. Steady-state \(^{15}N\)-\(^{1}H\) NOE val-
ues resulted from application of TROSY-based pulse sequences
(21) using a total recovery delay of 8 s. A train of 180°\(^{1}H\) pulses
spaced by 22 ms was applied to protons for 6 s in the saturation
experiment.

NMR titrations of \(^{15}N\)-labeled Pac11 with unlabeled Dyn2 and
Nip100 CC1B were acquired on a Bruker Avance 700 MHz
spectrometer at 25 °C. A series of BEST HSQC spectra of \(^{15}N\)
labeled Pac11 1–87 were collected with Dyn2 at final molar ratios
(Pac11: Dyn2) of 1:0.1, 1:0.3, 1:0.5, 1:0.7, 1:1.3, and 1:2.2
and with Nip100 CC1B at final molar ratios (Pac11: Nip100
CC1B) of 1:0.1, 1:0.3, 1:0.5, 1:0.7, and 1:1.2. Titration experi-
ments with Nip100 CC1B were performed at pH 7.0. The spec-
tra of fully bound Pac11-Dyn2 (molar ratio of Pac11:Dyn2 =
1:2.2) and Pac11-Nip100 (molar ratio of Pac11:Nip100 = 1:1.2)
were also collected on a Bruker Avance 900 MHz spectrometer.

**NMR Data Analysis**—All spectra were processed with Top-
Spin (Bruker) and analyzed using Sparky (22) and NMRView
(23). \(C_\alpha\) and \(C_\beta\) chemical shifts were used to determine the
secondary structure by calculating the difference from the ran-
dom coil values at specified temperature and pH (24). For
Pac11-Dyn2 interactions, per-residue absolute chemical shift
change was calculated using both \(^{15}N\) and \(^{1}H\) chemical shift
changes in spectra of free Pac11 and Dyn2-bound Pac11 col-
lected at 900 MHz. A scaling factor of 0.17 was used on \(^{15}N\)
chemical shift to eliminate the differences in the \(^{15}N\) and \(^{1}H\)
chemical shift ranges. For titration and dynamics experiments,
peak intensities were measured as peak heights. To account for
differences in concentration across the titration series, a nor-
malization factor was determined from the signal to noise ratio
of residue 1 in Pac11-Dyn2 titration or residue 87 in Pac11-
Nip100 titration. Changes in peak intensities \(I_{\text{bound}}/I_{\text{free}}\) were
culated as the ratios of signal intensity in spectra of the com-
plex and free Pac11. \(T_1\) and \(T_2\), and steady-state NOE values were
determined as described previously (12, 25).

**Chemical Shift-based Structures**—An ensemble of structures
for apo Pac11 1–87 was generated using the program CS-Roset-
ta (26) and chemical shift assignments for the \(^{1}H_\alpha\), \(^{1}H_\beta\),
\(^{13}C_\alpha\), \(^{13}C_\beta\), and \(^{15}N\) atoms. The CS-Rosetta calculations
were carried out on the server at the Biological Magnetic Res-
one Bank using the chemical shifts data. The average root
mean square deviation for the 10 structural models that have
the lowest energy structures is 16.2 Å. This large deviation is
due to the expected differences in the disordered regions. All 10
structures have similar N-terminal \(\alpha\)-helix spanning residues
1–24 with relatively lower average root mean square deviation
of 2.3 Å.

**Sequence Analysis**—Domain boundaries of Pac11 were deter-
mined using NCBI BLAST. Coiled-coil domains were predicted
by the Coils program (27), and secondary structure was pre-
dicted by Psipred v3.3 (28).

**Results**

**Construct Design and Characterization**—The *S. cerevisiae*
dynein intermediate chain Pac11 1–87 construct includes the
sequence-predicted coiled-coil domain (1–24), as well as two
Dyn2 recognition motifs, QT1 (45–54) and QT2 (75–84) (Fig.
1, A and D). Pac11 1–87Δ66–73 includes the same domains
and is used to determine the effect of the 66–73 deletion on
Dyn2 and Nip100 binding affinities. The Nip100 214–389,
Nip100 CC1B, contains the coiled-coil domain identified in
mammalian homolog p150Glued as sufficient to bind dynein
intermediate chain (11). Pac11 1–87 is primarily disordered
with a small percentage of CD-detected helical structure indi-
cated by negative ellipticity at 222 nm (Fig. 1B) and is mono-
ermic with a MALS-determined molecular mass of 12.3 kDa
(calculated MW is 10.6 kDa). Nip100 CC1B and Dyn2 are stable
dimers as determined by MALS.

**Resonance Assignments and Secondary Structure of Pac11 1–87**—The \(^{15}N\)-\(^{1}H\) HSQC spectrum of apo Pac11 1–87 (Fig. 1C)
exhibits a limited range of amide proton chemical shifts
(7.90 – 8.85 ppm), indicating a predominantly disordered pro-
tein. Backbone assignments were determined for 82 of 84 non-
proline residues. Secondary chemical shifts indicate high helix
propensity for residues 1–24 and lower helix propensity for
residues 66–73 (Fig. 1D). These propensities match the
sequence-based algorithmic predictions of helix (28) for apo
Pac11, except the NMR-detected 66–73 helix is slightly longer
and considerably weaker than the predicted 65–69 helix (Fig.
1D, top panel).

Although N-terminal residues of Pac11 1–87 are predicted
to form a coiled coil, they lack the hydrophobic seam found in
coiled coils. Instead, the 1–24 segment is rich in highly charged
residues (65.2% Arg, Lys, and Glu) (Fig. 1D), suggesting helix-stabilizing electrostatic interactions between residues $i$ and $i + 4$ (e.g. Lys5–Glu9 and Lys15–Glu19) and $i$ and $i + 3$ (e.g. Glu8–Arg11), characteristic of a single α-helix (SAH) domain (29). Hereafter, we refer to helix 1–24 as SAH and nascent helix 66–73 as LH (for linker helix). The linker region (helix 25–44) between SAH and QT1 is L1, and the linker region (helix 58–65) between QT1 and LH is L2.

**Pac11-Dyn2 and Pac11-Nip100 CC1B Interactions**—Pac11 1–87 binds dimeric Dyn2 with a stoichiometry of 1:2 (n value of 2) and an apparent $K_a$ value of 0.04 μM (Fig. 2A and Table 1). Because the stoichiometry is computed for binding sites, 1:2 means two Pac11 monomers bind two Dyn2 dimers. In comparison, Dyn2 binding to a Pac11 peptide containing only one Dyn2 binding motif (QT2) has a $K_d$ value of 0.62 μM and a stoichiometry of 1:1 (30). Deletion of LH residues 66–73 in Pac11 does not significantly affect Dyn2 binding affinity (Table 1).

Pac11 binding affinity for Nip100 is much lower than for Dyn2. The Pac11-Nip100 CC1B interaction measured by ITC has a stoichiometry of 1:1 (n value of 1; two Pac11 monomers bind one Nip100 dimer) and an apparent $K_a$ value of 5.6 μM (Fig. 2C and Table 1). The absence of LH in Pac11 1–87 significantly reduces Nip100 binding affinity (Fig. 2, C and D).

**NMR Studies of Dyn2 Binding to Pac11**—In NMR spectra of 15N-labeled Pac11 1–87, taken before and after addition of unlabeled Dyn2 at varying molar ratios, two titration effects are readily apparent for peaks in the amide NH spectral region (Fig.
FIGURE 2. Thermodynamic measurements of interactions of Pac11 1–87 and Pac11 1–87Δ66–73 with Dyn2 and with Nip100 CC1B. In each panel, representative results of ITC experiments are shown as thermograms (top panels) and binding isotherms (bottom panels) for the titrations of Pac11 1–87 with Dyn2 (A), Pac11 1–87Δ66–73 with Dyn2 (B), Nip100 CC1B with Pac11 1–87 (C), and Nip100 CC1B with Pac11 1–87Δ66–73 (D). The data were collected at 25 °C in 50 mM sodium phosphate, 50 mM sodium chloride, 0.5 mM sodium azide, pH 7.2, and fit to a single-site binding model. The weak binding of Nip100 CC1B with Pac11 1–87Δ66–73 cannot be reliably fit at these concentrations.

TABLE 1

Thermodynamic parameters for association of Pac11 1–87 and Pac11 1–87Δ66–73 with Dyn2 and Nip100 CC1B

| Protein | Ligand | $n$ | $K_a$ | $\Delta \mu$ | $\Delta H^0$ | $-T\Delta S$ |
|---------|--------|-----|-------|-------------|--------------|--------------|
| Pac11$^a$ | Dyn2 | 2.0 ± 0.1 | 0.04 ± 0.01 | -10.1 ± 0.1 | -7.2 ± 0.6 | -2.9 ± 0.7 |
| Pac11Δ66–73$^a$ | Dyn2 | 1.8 ± 0.2 | 0.04 ± 0.01 | -10.1 ± 0.1 | -6.8 ± 0.2 | -3.3 ± 0.04 |
| Nip100$^b$ | Pac11 | 0.97 ± 0.005 | 5.6 ± 0.07 | -7.1 ± 0.02 | -8.0 ± 0.5 | 0.8 ± 0.5 |

$^a$ The data are the averages of duplicate ITC runs. Pac11 denotes Pac11 1–87.
$^b$ The data are the averages of duplicate ITC runs. Nip100 denotes Nip100 CC1B.

3A); some NH peaks are attenuated, but their lost intensity is observed in a new peak at a different chemical shift, whereas other NH peaks lose intensity that is not detected elsewhere in the spectrum. Resonance assignments of all peaks were determined using standard three-dimensional experiments. Secondary chemical shifts of observable peaks in the spectrum of Dyn2-bound Pac11 (Fig. 3B, red) are the same as for apo Pac11 (Fig. 3B, black), indicating that, for residues that can be detected in Dyn2-bound Pac11, there is no change in secondary structures compared with apo Pac11 (e.g. SAH peaks 1–24).

The first titration effect is illustrated in Fig. 3A, which overlays HSQC spectra of Pac11 1–87 without Dyn2 (black) and with a Pac11:Dyn2 molar ratio of 1:2.2 (red). With Dyn2, peaks of Pac11 residues 58–67 in linker region L2 are observed at a new chemical shift as shown in Fig. 3C, where chemical shift changes are quantified. Although the 58–67 chemical shifts change with added Dyn2, the combined intensity of the two peaks for each NH does not change. A second NH peak at a different chemical shift, with no loss in combined intensity, indicates slow exchange between apo and Dyn2-bound ensembles in which L2 residues experience different local environments and hence different average chemical shifts. For both apo and Dyn2-bound Pac11, L2 peaks are sharp and within the spectral region considered random coil. We conclude that, in Dyn2-bound Pac11, the L2 domain is an ensemble of conformations that still retain a high degree of local flexibility but are, on average, slightly more ordered than in apo Pac11, as evidenced from the small increase in steady-state NOEs (see Fig. 6).

The second titration effect, attenuation of peak intensity as Dyn2 is added, is quantified in Fig. 3D, where bar heights indicate normalized peak intensity, and bar colors code the Pac11: Dyn2 ratio. Peak intensity that is first diminished and then completely lost as Dyn2 concentration is increased is observed for residues 44–57 and 68–87, two segments that encompass residues in or immediately flanking binding motifs QT1 and QT2 and LH (Fig. 3, D and E).

The titration of peak intensity as apo Pac11 is converted to the Pac11-Dyn2 complex is explained by one or both of two scenarios. In the first, these residues interact with a large, collapsed domain and so tumble with that domain; the consequent increase in their rotational correlation time and the associated decrease in NMR relaxation time render their peaks undetectable. A second possible explanation for the total loss of peak intensity at saturating Dyn2 concentration is intermediate exchange between two or more conformational ensembles of Dyn2-bound Pac11 forms. The QT1 and QT2 domains best fit the first explanation as their motifs are incorporated into the three-dimensional fold of the Dyn2 dimer (20) with which they would tumble. Although LH peaks disappear in a manner similar to residues in the QT$^*$ domains, their tight integration into the nearby QT2 is not consistent with their NMR spectral behavior when Nip100 is added to apo Pac11 (Fig. 4) discussed below, nor with the unchanged binding energetics of Dyn2 to Pac11 versus Pac11Δ66–73 (Table 1).

NMR Studies of Nip100 CC1B Binding to Pac11—NMR spectra of $^{15}$N-labeled Pac11 1–87, taken before and after addition of unlabeled Nip100 CC1B (Fig. 4), identify large segments of contiguous Pac11 residues for which NH peaks are attenuated as Nip100 is added. Comparing the results of titration experiments in Figs. 3D and 4B, it is striking that the overall patterns of titrated peak intensity show a rough reciprocity; in one graph the two segments of tall red bars (relative peak intensity unchanged at highest ligand concentration) tend to be in regions that in the other graph have no red bars (all intensity lost at highest ligand concentration). Peaks that markedly decrease in intensity with added Nip100 are those of N-terminal residues 2–46 and residues 63–73.
Peak attenuation caused by direct interactions (line broadening associated with slower tumbling time) versus attenuation caused by conformational exchange (line broadening caused by intermediate exchange) may be distinguished by comparing Pac11 spectra of free versus Nip100-bound samples collected at 700 MHz (Fig. 4C) and at 900 MHz (Fig. 4D). Broadening caused by conformational exchange is sensitive to field strength. At higher field there is an increased $\Delta H_{900} - \Delta H_{700}$, the difference in Hz between two exchange peaks, but no change in $k_{ex}$, the molecular exchange rate between conformations. At 700 MHz, a conformational exchange rate on the order of $\Delta \omega$ would cause line broadening of an NH peak. At 900 MHz, the same conformational exchange rate may be smaller than $\Delta \omega$, and by definition in the slow exchange regime, with the result that 900 MHz spectra have less broadened peaks for the same NH.

The data in Fig. 4 (B–D), taken together, differentiate three structural ensembles affected by Nip100 binding: SAH, 2–24, L1, 25–46, and LH, 63–73. In Fig. 4B, SAH peak intensities have a distinctive attenuation pattern: a fairly uniform and greater loss of intensity at substoichiometric Nip100 concentrations (e.g., gray and yellow bars are <50%). L1 peaks differ in having relatively nonuniform and higher intensity at the same Nip100 concentrations, consistent with SAH being the Pac11 region that interacts directly with Nip100. Further, the intensities of SAH and L1 residues in apo Pac11 spectra have different field strength dependences (black bars in Fig. 4, C and D); the intensities of L1 peaks relative to SAH peaks are lower at 700 MHz as compared with 900 MHz. This implies that SAH and L1 residues form different ensembles, each having distinctive conformational dynamics, consistent with the conclusion that in apo Pac11, only residues 2–24 form a single $\alpha$-helix.

The dynamic ensemble structure of LH is clearly distinguishable from both SAH and L1. The significantly higher intensities of LH peaks observed in 900 MHz spectra of Nip100-bound Pac11 (residues 63–73; red bars in Fig. 4, C and D) imply slow exchange at the higher field, between LH conformations separated by a significant energy barrier. Although peaks 63–73 in 700-MHz spectra are apparently exchange-broadened, in 900-MHz spectra, they are more clearly resolved.
MHz spectra they are sharpened and more intense because of a shift from an intermediate to a slow exchange regime, with a possible contribution from increased sensitivity.

Effect of Dyn2 on Nip100 Binding to Pac11—

Gel filtration elution profiles (Fig. 5A) indicate that the presence of Dyn2 increases the stability of the Pac11 1–87-Nip100 CC1B complex. When a sample of Pac11 1–87 and Nip100 CC1B at equimolar concentrations (two Pac11 monomers to one Nip100 dimer) was loaded on an analytical column, the two elution peaks (Fig. 5A, red) correspond to Nip100 CC1B dimer (42.8 kDa) and to Pac11 1–87 monomer (10.6 kDa), which migrates earlier than expected for a globular protein of similar size. The absence of detectable Pac11-Nip100 complex is consistent with their low binding affinity given in Table 1.

When a sample of a complex of Pac11 1–87, Nip100 CC1B and Dyn2, at a molar ratio of 1:1:2 was loaded, the major feature of the elution profile (Fig. 5A, blue) is a single intense peak with a MALS-determined molecular mass of 127 kDa, consistent with a Pac11-Dyn2-Nip100 ternary complex, which has an expected molecular mass of 110 kDa (two chains of Pac11 1–87, two Dyn2 dimers, and one Nip100 CC1B dimer). The ternary complex is confirmed in lane a of the SDS-PAGE results in Fig. 5C.

Similar elution profiles are obtained for Pac11 1–87Δ66–73, a Pac11 variant lacking LH residues (Fig. 5B). Whether with Nip100 alone or with both Nip100 and Dyn2, no difference in elution volume of the resultant complexes is observed. The ternary complex with Pac11 1–87Δ66–73 in the high molecular mass peak is confirmed in lane b of Fig. 5C. Apparently LH residues do not contribute to the relative stability of Pac11-Nip100 versus Pac11-Nip100-Dyn2.

NMR-detected Dynamics of Pac11 1–87—Backbone dynamics of Pac11 1–87 were determined from $^{15}$N $T_1$, $T_2$, and steady-state heteronuclear NOE experiments (Fig. 6). The $T_1$ values are fairly uniform throughout the sequence with an overall average of 0.64 s (Fig. 6A). The $T_2$ values, in contrast, show highly heterogeneous dynamics. The heterogeneity in $T_2$ relaxation with residues in the SAH and LH domains evincing low $T_2$ values (average $T_2$ value of 0.11 and 0.21 s, respectively) compared with the overall average value of 0.25 s is indicative of nonrandom structure for these segments (Fig. 6B). Steady-state heteronuclear NOE measurements, which provide indications of ordered structure on the nanosecond to picosecond time scale, suggest order in residues 1–24 and to a lesser extent in residues 65–81, segments that have positive NOE values with averages of 0.45 and 0.17, respectively (Fig. 6C). The rest of the
Pac11-Dyn2-Nip100 Interactions

To examine the effect of Dyn2 binding, similar dynamics experiments were carried out for the Pac11-Dyn2 complex. Because peaks for residues 44–57 and 68–87 disappear in spectra of the bound form, comparison of the dynamics was assessed only for the remaining detectable peaks. Except for a pronounced dip around residue 31 in $T_1$ and a significant drop in $T_2$ for residues 1–24, support the conclusion that this segment is an ordered SAH domain of the type first identified in murine myosin 10 (33), where it serves as a stiff, processive extension of the myosin lever (34).

Several characteristics of Pac11 and IC in higher eukaryotes support the generalization that an N-terminal SAH domain is a conserved feature of dynein intermediate chain. The domain architecture is similar for apo forms of S. cerevisiae Pac11 and Drosophila IC, as illustrated in Fig. 1A. At the N terminus, each has a sequence predicted by standard algorithms to be coiled coil. However, as noted here for IC and Pac11 and in Ref. 33 for murine myosin, the predicted coiled-coil sequence lacks coiled-coil-stabilizing hydrophobic residues and has residues characteristic of a SAH domain conducive to helix-stabilizing electrostatic interactions. Similar to Pac11, in its first 40 residues Drosophila IC favors $\alpha$-helical structure with restricted motions detected by NMR dynamics experiments (12). Significantly, the yeast Pac11 N-terminal SAH domain binds dynactin (via essentially the same as for free Pac11. Interesting to note are the more negative NOE values and slightly higher $T_2$ values for residues in L2 and at the C-terminal end distant from binding, suggesting increased flexibility in this region upon Nip100 binding. Increased flexibility on both sides of LH makes the peak attenuation observed in LH even more striking.

Discussion

High resolution methods for monitoring structural changes of large, partially disordered, flexible, macromolecular assemblies are few. Our approach—production of appropriate model polypeptides amenable to NMR spectroscopy and titration calorimetry and further development of NMR ligand titration techniques (12, 15, 25, 31, 32)—has been extremely useful in characterization of dynein intermediate chain complexes and is generally applicable to characterization of other large dynamic complexes.

The N-terminal 87-residue segment of Pac11, containing two Dyn2 recognition motifs (QT1 and QT2) and the binding site for Nip100, is primarily monomeric and disordered except for a SAH (residues 1–24) and a short nascent helix (LH, residues 66–73) in the 20-amino acid segment separating the Dyn2 recognition motifs. Recognition sites and changes in structure upon binding to either Dyn2 or to Nip100 are inferred from NMR titration results (Figs. 3 and 4), based on peak attenuation patterns and/or changes in chemical shift with added Dyn2 or Nip100 and on the field strength dependence of peak attenuation. Increased ordered structure in Pac11 complexes is monitored at the residue level by comparison of $^{15}$N relaxation data for apo Pac11 and for Pac11-Dyn2 and Pac11-Nip100 complexes.

An N-terminal SAH Domain Is a Conserved Structural Feature of Dynein Intermediate Chain Responsible for Binding Dynactin—Three models of apo Pac11 1–87 corresponding to the lowest energy structures are presented as an overlay in Fig. 7A. The ordered SAH domain aligns well in all three conformations (long red ribbon); in one conformation LH is a short segment exhibiting some helical propensity (short red ribbon); in the rest of the chain, all three conformations have considerable disorder. Dynamics measurements (Fig. 6), in particular the significantly lower $T_2$ values for residues 1–24, support the conclusion that this segment is an ordered SAH domain of the type first identified in murine myosin 10 (33), where it serves as a stiff, processive extension of the myosin lever (34).

For the Pac11-Nip100 CC1B complex, although many peaks disappear, relaxation parameters for the remaining peaks are
Nip100) as demonstrated here, and Drosophila and mammalian IC N-terminal SAH domains bind dynactin (via p150Glued) (11, 12). An elongated SAH may favor binding of this relatively short sequence to the coiled-coil domain of Nip100 or p150Glued. In summary, although the N-terminal 40–50-amino acid segment in IC of higher eukaryotes is commonly assumed to be a coiled coil based on prediction algorithms, we conclude instead that they form a single /H-helix to which a subunit of dynactin binds.

Dyn2 Binding Orders LH Residues as Well as QT1 and QT2 Domains—The aligned IDP duplex formed by two Pac11 1–87 chains and two Dyn2 dimers is illustrated in Fig. 7B. Incorporation of Pac11 QT recognition motifs into Dyn2 β-sheets is evident in an x-ray structure for Dyn2 bound to a short peptide corresponding to QT2 (30), other LC8 partner peptide structures with similar motifs (35, 36), and the NMR titration results in Fig. 3D. A complete loss of peak intensity of residues in and around QT1 and QT2 sequences 44–54 and 75–84 indicates the expected effect of protein-protein interactions at the Pac11-Dyn2 interfaces (Fig. 3D).

However, along with residues in and near QT1 and QT2, also attenuated are residues 68–73 within LH, which in apo Pac11 forms a nascent helix 66–73 (Fig. 3E). Although LH residues lose all peak intensity in the complex (Fig. 3D), they are likely not incorporated into the well packed QT2 interface of Pac11-Dyn2 because deletion of LH affects neither Dyn2 binding affinity (Table 1) nor Dyn2 stabilization of the Pac11-Nip100-Dyn2 ternary complex (Fig. 5). An explanation for LH peak attenuation is that ordering of LH helices accompanies Dyn2 binding. LH residues in the Pac11-Dyn2 complex, shown in Fig. 7B as separate red ribbons, may tend to self-associate. Such interchain self-association of LH sequences between QT recognition motifs is reminiscent of the Ana2 (the anastral spindle-2 centriole duplication factor) system involving two LC8 recognition motifs flanking a 34-amino acid predicted helix. The self-association of this helix was proposed to be involved in
formation of four-helix bundle resulting in higher order oligomerization of Ana2 (37). Similarly for Pac11, it is possible that binding of two Dyn2 at two somewhat separated motifs promotes formation of ordered self-associated helices as a new surface for binding other partners.

Dyn2 binding has no effect on the structure and dynamics of the distant SAH domain, which comprises the Nip100 site. This observation implies that the increased binding of Pac11 to Nip100 upon Dyn2 binding (Fig. 5) is due solely to bivalency.

Nip100 Binding to Pac11 SAH Domain Orders Adjacent L1 and Distal LH Residues—Dimeric Nip100 CC1B binds two chains of Pac11 1–87 (Table 1) as illustrated in Fig. 7C. Although NH peaks of SAH (2–24), L1 (25–46), and LH (63–73) are all diminished with added Nip100, their attenuation patterns and field strength dependence imply that these three regions form local dynamic conformational ensembles having different responses to Nip100 binding. The rest of the Pac11 backbone remains highly disordered in the complex.

We conclude that direct interactions of Nip100 coiled-coil domain with Pac11 are localized to residues in the SAH. Attenuation of NH peaks of proximal L1 and of the more distant LH is primarily due to self-organization of these segments into domains that are somewhat more ordered but still flexible. A dynamic L1 domain is visualized in Fig. 7C as brown backbone chains that are somewhat collapsed. A dynamic LH domain is visualized as favoring helical conformations, possibly self-associated helices. Residues within these domains could transiently interact with Nip100 or pack against the SAH domain (Fig. 7C, right panel), consistent with diminished binding of Nip100 to a Pac11 variant having LH residues deleted (Fig. 2D).

The salient outcome of these conclusions is that Pac11 contains domains that form elements of local dynamic structure, which respond differently to ligand binding and duplex formation. Functional implications include new conformations that may selectively bind additional ligands that are not necessarily bivalent. Such species could arise from an ensemble shift in conformational populations associated with complex formation. For example, dynein assembly of intermediate chains and light chains transiently bind other partners, such as Zw10 subunit of the RZZ complex in higher eukaryotes (38), and Num1 (39), which might bind newly favored conformations. Further, in the IC sequences of higher eukaryotes the LH domain preceding QT2 is replaced by an extended motif for binding dynein light chain Tctex1, suggesting the possibility of a ligand that binds Tctex1 in higher eukaryotes and Pac11 LH in yeast.

IDP Duplex Scaffolds—This work identifies residue-specific binding effects of dimeric proteins Nip100 and Dyn2 on apo Pac11, a monomeric, IDP. The Pac11-Dyn2 complex is one example of a more general class of IDP systems in which two IDP monomers bind one or more LC8-like proteins to form a duplex scaffold composed of two IDP chains in parallel alignment (15, 31, 40, 41). Along the scaffold are multiple bivalent sites for assembly of various essential bivalent proteins. In the functional assembly, the scaffold does not fold into an arrangement of packed macromolecules; rather the overall complex retains an inherent flexibility in IDP “linker” sequences between bound proteins. The developing insight is that, in these disordered linker sequences, the duplex often self-organizes new domains in response to binding partner ligands. The new domains may self-associate and/or offer new binding surfaces for additional ligands, not necessarily bivalent. A “new domain” in this sense is not a folded unit but rather a dynamic ensemble in which the relative populations of local, interconverting, and widely varying conformations can be shifted when the duplex binds partner ligands. In other words, a new domain is a sequence element of local dynamic structure in which con-
formational populations may shift when the IDP chain is constrained by binding partner ligands. In apo IDPs, these domains may or may not be experimentally detected by a tendency to form secondary structure. However, when the IDP is incorporated into a duplex by binding a cross-linking ligand, the domains are detected as contiguous residues that, for example, are self-associated (one segment from each chain), that display increased secondary structure, and/or that have NMR peaks that are sensitive to changes in field strength. New elements of dynamic structure identified for the IDP duplex scaffold of Pac11 are LH domains in response to Pac11 binding of Dyn2 or Nip100 and an L1 domain in response to binding of Nip100.

These results reinforce several generalizations about IDP duplex scaffolds and underscore the interplay of intrinsic disorder, bivalency, and propensity to form local dynamic structure in propagating long range effects of ligand binding to IDP scaffolds. First, binding contacts of bivalent partner proteins are limited to IDP residues in and near the recognition motif. Second, the flexible linkers that retain disorder in the duplex are not featureless peptide sequences connecting binding motifs; rather they contain elements of dynamic structure from which conformations specific for self-association and/or for binding additional ligands may be selected. Last, the biological relevance of IDP duplex scaffolds extends beyond dynein; an array of similar IDP scaffolds is expected for assemblies that function in an astonishingly broad range of biological systems (42, 43), including interactions with the DNA damage response protein ASC1ZU, tumor suppressor protein KirbA, mitotic spindle assembly protein Chica and in rabies virus (44–47).

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