Intrinsic Disorder in Dynein Intermediate Chain Modulates Its Interactions with NudE and Dynactin

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Background: NudE and dynactin regulate dynein through their interaction with the intermediate chain subunit (IC).

Results: NudE binds one region of the bi-segmental IC binding footprint of p150Glued to form a heterotetrameric complex.

Conclusion: NudE and p150Glued bind IC with similar affinity but with distinct binding modes.

Significance: These distinct binding modes elucidate crucial new insight into dynein regulation.

The functional diversity of cytoplasmic dynein is in part attributed to multiple interactions between noncatalytic dynein subunits and an array of regulatory proteins. This study focuses on the interaction between the dynein intermediate chain subunit (IC) and a dynein regulator protein (NudE). We use isothermal titration calorimetry and NMR spectroscopy to map their interacting sections to their respective N-terminal domains, which are predicted to form dimeric coiled-coils. Interestingly, the specific residues within IC that interact with NudE are a subset of the bi-segmental binding region reported for p150Glued, a subunit of the dynein activator protein dynactin. Although the IC binding domains of both NudE and p150Glued form dimeric coiled-coils and bind IC at a common site, we observe distinct binding modes for each regulatory protein: 1) NudE binds region 1 of the bi-segmental binding footprint of p150Glued, whereas p150Glued requires regions 1 and 2 to match the binding affinity of NudE with region 1 alone. 2) Compared with unbound IC, NudE-bound IC shows a slight increase in flexibility in region 2, in contrast to the increase in ordered structure observed for p150Glued-bound IC (Morgan, J. L., Song, Y., and Barbar, E. (2011) J. Biol. Chem. 286, 39349–39359). 3) Although NudE has a higher affinity for the common binding segment on IC, when all three proteins are in solution, IC preferentially binds p150Glued. These results underscore the importance of a bi-segmental binding region of IC and disorder in region 2 and flanking linkers in selecting which regulatory protein binds IC.

Cytoplasmic dynein, a microtubule-associated minus-end directed molecular motor, is responsible for many aspects of intracellular transport (2), including chromosome segregation (3), mitotic spindle positioning and assembly (4, 5), and regulation of the spindle assembly checkpoint (6). Emerging evidence suggests that the functional diversity of the dynein complex is in part due to an array of accessory regulatory proteins that are not stoichiometric components of the dynein complex (7). The regulatory proteins interact with one or more dynein subunits, namely the heavy chains, intermediate chains, light intermediates, and light chains, and target the complex to specific cellular cargoes or locations.

The intermediate chain subunit (IC),2 is an essential component of the dynein complex (8) and plays a key role in its cargo recognition, assembly, and regulation. It links several putative dynein cargo molecules including huntingtin, adenovirus, the CIC-2 chloride channel, and PLAC 24 to the dynein complex (9–12), directly interacts with the light chains (Tctex1, LC8, LC7) (13, 14), and associates with the dynein regulatory proteins NudE, dynactin, NudC, and zeste white 10 (reviewed in Ref. 7). IC is a 74-kDa protein with a predominantly unstructured N-terminal domain (15) and a WD-rich C-terminal domain. Within the predominantly unstructured N-terminal domain of Drosophila melanogaster IC, residues 3–36 are helical (1), consistent with the coiled-coil secondary structure predicted for residues 1–40 from sequence analysis (16), residues 48–60 have nascent helical propensity (1), and residues 222–231 constitute a self-association domain (17). The N-terminal domain binds all three light chains. For D. melanogaster IC the light chain-binding sites are mapped to residues 110–122 (Tctex1), 126–138 (LC8), and 221–258 (LC7) (15, 18, 19).

The best characterized of the dynein regulatory proteins is dynactin; a multiprotein assembly that is essential for most cellular functions of the cytoplasmic dynein complex (20). Dynactin links dynein to cellular cargo (21) and increases dynein processivity (22). Interactions between the two complexes are mediated by dynein IC and the p150Glued subunit of dynactin (23). Another dynein regulatory protein, NudE (Ndel1), and its closely related homolog NudEL (Ndel1), share ~55% amino acid sequence identity and modulate several dynein functions including kinesochrome and centrosome migration, transport of vimentin, organization of the Golgi complex, centrosome duplication and mitotic spindle positioning, and membrane transport (24–30). NudE, first identified in Aspergillus nidulans as a protein required for even distribution of nuclei along the hyphae (31), has a predicted N-terminal coiled-coil domain and a largely unstructured C-terminal domain. The NudE N-terminal domain is the site of interaction with Lis1, a protein

2The abbreviations used are: IC, 74-kDa dynein intermediate chain corresponding to gene Cdc12b; NudE, isoform A of the DnudE gene; p150Glued, the 150-kDa polypeptide corresponding to the Glued gene; ITC, isothermal titration calorimetry; HSQC, heteronuclear single-quantum coherence; SUMO, small ubiquitin-like modifier protein; GdnCl, guanidine chloride.
associated with lissencephaly (31), whereas the unstructured C-terminal domain associates with CENP-F, a nuclear matrix component required for kinetochore-microtubule interactions (32).

Studies using recombinant and/or endogenous p150\textsuperscript{Glued} and NudE/EL from various organisms, and large peptides corresponding to various domains, are beginning to provide structural detail and functional insight into these dynein regulatory proteins. In studies of \textit{D. melanogaster} proteins by NMR spectroscopy and titration calorimetry, the Barbar group showed that the binding site of p150\textsuperscript{Glued} on IC corresponds to two segments: region 1 composed of residues 1–41, a sequence predicted to have coiled-coil secondary structure, and region 2, composed of residues 46–75, a predominantly unstructured segment observed to have nascent helical propensity (1) (Fig. 1). Consistent with the \textit{Drosophila} IC observations, Vallee and coworkers (33) found that a \textit{Rattus norvegicus} (DynC1I2) construct composed of IC residues 1–70 binds p150\textsuperscript{Glued}, and similarly showed that NudE also binds within the same IC construct. It is worth noting that the sequence of rat IC residues 1–67 is also predicted to form a coiled-coil, making the rat IC predicted coiled-coil region much longer than that of \textit{D. melanogaster} IC (resides 1–40). Thus, whereas the rat IC(1–70) construct includes the predicted coiled-coil segment corresponding to \textit{Drosophila} region 1 of the p150\textsuperscript{Glued} binding footprint on IC, it does not include all of the primarily disordered segment corresponding to region 2. For NudE/EL, the IC binding site was mapped to a region N-terminal to the Lis1 binding site, but still within the N-terminal predicted coiled-coil domain (34, 35). The C-terminal domain of NudE was also reported to bind IC and the LC8 subunit of cytoplasmic dynein (36).

The observation that rat NudE and p150\textsuperscript{Glued} share a common binding site on IC (33) creates a paradox because dynactin, NudE, and dynein co-localize in many cellular compartments such as the kinetochores and the lysosomes (37). NudE is also required for localization of p150\textsuperscript{Glued} at the nuclear envelope in prophase (reviewed in 37). How does p150\textsuperscript{Glued} binding to IC affect NudE binding to IC, and vice versa, and how does dynein select between different regulators? The current study was initiated to elucidate 1) at the residue level the IC binding site for NudE, specifically whether region 2 of the p150\textsuperscript{Glued} binding site is involved; 2) the solution properties of the NudE domain involved in the interaction; and 3) how regulation of dynein by NudE and dynactin may be coordinated.

We use ITC to identify domains of recombinant \textit{D. melanogaster} proteins that are sufficient for the IC-NudE interaction, and solution NMR to identify specific IC residues involved in the interaction and changes in flexibility associated with binding. Although IC binds NudE and p150\textsuperscript{Glued} with similar but moderately weak affinity, binding of p150\textsuperscript{Glued} is favored over that of NudE when both are present in solution. These results suggest that NudE and p150\textsuperscript{Glued} are associated with distinct dynein pools having specific functions. For the case when both are present in the same compartments, we propose novel processes for selection between NudE and p150\textsuperscript{Glued}.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Recombinant NudE Proteins—** The gene encoding \textit{Drosophila} NudE (isoform A) was obtained from the \textit{Drosophila} Genomics Resource Center, Bloomington, IN (fly.bio.indiana.edu). Constructs encoding amino acids 1–317 (the full-length construct, NudE), 1–174 (N-terminal domain, nNudE), and 179–317 (C-terminal domain, cNudE) were prepared by PCR. The PCR products were cloned into the Champion\textsuperscript{TM} pET SUMO expression vector (Invitrogen), which has an N-terminal polyhistidine sequence adjacent to a sequence encoding a small ubiquitin-like modifier protein (SUMO). After DNA sequencing, clones with the desired insert were transformed into BL21-DE3 cell lines for protein expression.

The transformed BL21-DE3 cell lines were grown in LB media at 37 °C to an optical density (\(A_{600}\)) of \(\sim 0.6\) followed by protein induction for 3 h with 0.1 mM isopropyl 1-thio-\(\beta\)-d-galactopyranoside. Cells were harvested, lysed, and centrifuged to remove cell debris. The soluble fractions were incubated with nickel-nitrilotriacetic acid resin for purification. The region encoding the polyhistidine and SUMO sequences were removed by incubating the proteins with SUMO protease (Invitrogen). Final purification on a Superdex\textsuperscript{TM} 75 (16/60) gel filtration column (GE Healthcare) resulted in pure fractions of recombinant proteins, which were stored at 4 °C and used within 1 week, or at \(-20\) °C and used within 1 month.

Cloning, expression, and purification of intermediate chain constructs IC(1–143), IC(1–87), IC(84–143), IC(1–40), the dynein subunit LC8, and a construct of p150\textsuperscript{Glued} (residues 221–509, referred hereafter as p150\textsubscript{CC1}) followed previously published protocols (1, 15, 18).

**Protein Concentrations—** Concentrations were determined from absorbances at 280 nm using molar extinction coefficients (NudE, 30,940; nNudE, 18,450; cNudE, 12,490; IC(1–143), 2,980; IC(84–143), 2,980; p150\textsubscript{CC1}, 1,490; LC8, 14,565 M\(^{-1}\) cm\(^{-1}\)) computed with the protparam algorithm (EXPASY). The concentrations of IC(1–40) and (1–87) were determined by comparing their intensities on a Coomassie Blue-stained polyacrylamide gel with similar intensities of predetermined concentrations of lysozyme.

**Circular Dichroism and Fluorescence Measurements—** CD experiments were recorded on a JASCO 720 spectropolarimeter. Proteins were prepared in buffer composed of 10 mM sodium phosphate, 10 mM NaCl, pH 7.5. Data on the native protein were collected at 25 °C, using a 1-mm cell, and a protein concentration of 10 \(\mu\)M. Temperature denaturation data were acquired in the temperature range of 5–80 °C, using a 1-cm cell, and a protein concentration of 3 \(\mu\)M. Temperature denaturation data were acquired in the temperature range of 5–80 °C, using a 1-cm cell, and a protein concentration of 3 \(\mu\)M. Temperature denaturation data were acquired in the temperature range of 5–80 °C, using a 1-cm cell, and a protein concentration of 3 \(\mu\)M. Temperature denaturation data were acquired in the temperature range of 5–80 °C, using a 1-cm cell, and a protein concentration of 3 \(\mu\)M. Temperature denaturation data were acquired in the temperature range of 5–80 °C, using a 1-cm cell, and a protein concentration of 3 \(\mu\)M.
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scanned from 310 to 380 nm, with excitation and emission bandwidths set to 2.8 nm each. The protein concentration was 3 μM in a reaction buffer composed of 10 mM sodium phosphate, 10 mM NaCl, pH 7.5. GndCl unfolding experiments used the same buffer with concentrations of GndCl in the 0 – 6 M range, and protein concentrations of 0.5, 1.5, or 3 μM. Data were collected using a batch type experiment, where each protein sample was equilibrated for 20 h with the appropriate concentration of GndCl prior to data collection. Reported data are the normalized intensities at 341 nm (λ_max of native protein) as a function of the GndCl concentrations.

The fraction of unfolded protein U was calculated from the equation: 

\[ U = \frac{Y_f - Y_{obs}}{Y_f - Y_u} \]

where \( Y_{obs} \) is the CD or fluorescence signal at 222 or 341 nm, respectively, \( Y_f \) and \( Y_u \) are the intensities for the folded and unfolded conformations. The values for \( Y_f \) and \( Y_u \) were obtained by extrapolating the base lines for the pre- and post-transition regions.

Isothermal Titration Calorimetry—Binding of IC constructs or LC8 to NudE was recorded at 25 °C using Microcal's VP titration calorimeter (Microcal, Northampton, MA). Protein samples at concentrations of 400–500 μM (IC or LC8), or 30–40 μM (NudE) in buffer composed of 50 mM sodium phosphate, 50 mM NaCl, 0.5 mM sodium azide, pH 7.5, were degassed prior to data acquisition. Each run consisted of 27 10-μL injections of IC or LC8 into 1.3 ml of NudE. The heats of dilution determined from similar runs with IC or LC8 titrated into buffer were subtracted from the reaction heats before data analyses using the Origin™ software. Each binding isotherm is the average of duplicate runs recorded under similar conditions.

NMR Experiments—NMR data were acquired on a Bruker Avance 600 spectrometer. Samples were prepared in buffer composed of 10 mM sodium phosphate, 50 mM NaCl, 1 mM sodium azide, pH 7.3, a protease inhibitor mixture (Roche Applied Science), and 2,2-dimethylsilapentene-5-sulfonic acid for 1H chemical shifts referencing. Two-dimensional 1H-15N HSQC experiments using echo-antiecho phase discrimination of 200 increments and 1024 points were collected at 5 °C. Experiments to identify IC residues directly involved in binding to nNudE used 500 μM 15N-labeled IC(1–87) unbound or bound to 100 μM, 500 μM, or 1.5 mM unlabeled nNudE. At these concentrations, the molar ratios of IC to NudE were 1:0.2, 1:1, and 1:3, respectively. To rule out contributions from nonspecific interactions associated with the high protein concentrations, similar experiments were performed with 150 μM IC bound to 900 μM nNudE.

Experiments to monitor competition between p150CC1 and nNudE for binding to IC used 500 μM 15N-labeled IC(1–87) and 1.5–2 mM p150CC1 or nNudE. Data were acquired on the p150CC1-IC(1–87) complex (molar ratio 3:1), the nNudE-IC(1–87) complex (molar ratio 3:1), the p150CC1-IC(1–87) complex titrated with nNudE (molar ratio 3:1:4), and the nNudE-IC(1–87) complex titrated with p150CC1 (molar ratio 3:1:4).

Steady-state 1H-15N heteronuclear NOE, and \( T_2 \) experiments were recorded at 20 °C on 15N-labeled IC(1–87) unbound, or bound to a 3-fold molar excess of unlabeled nNudE. Spectra with proton saturation utilized a 3-s period of saturation and an additional delay of 1.5 s. \( T_2 \) relaxation data were acquired using relaxation delays of 15.4, 30.9, 46.3, 61.8, 77.2, 92.6, 123.5, and 139.0 ms.

NMR Data Analysis—NMR spectra were processed with NMRPipe (38) and analyzed with NMRView (39), or Sparky (40). The protein backbone was assigned by comparing backbone chemical shifts with previously published assignments for D. melanogaster IC residues 1–143 and 84–143 (1, 18). The peak corresponding to residue 87 was used as an internal reference to correct for small changes in peak intensities across spectra. The relative intensity of each peak (measured as peak heights) was calculated as the ratio of the intensity of the peak in the spectrum of the bound IC to the intensity of the same peak in the spectrum of the unbound IC.

Steady-state NOE values were obtained from the ratios of peak intensities in the presence and absence of amide proton saturation. Standard deviations were determined from the intensities of the baseline noise as described elsewhere (18). \( T_2 \) values were determined by fitting the measured peak heights versus time profiles to the relationship: 

\[ I = I_0 \exp(-t/T_2) \]

where \( t \) is the relaxation delay, \( I_0 \) is the intensity of the peak measured at time \( t \), and \( I_0 \) is the initial intensity of the peak. Curve fitting was done with the rate analysis program in NMRview (39).

Native Gel Electrophoresis—Protein complexes were prepared by incubating 20 μM IC(1–40) or IC(1–87) with 25 μM nNudE, p150CC1, or a mixture of equimolar concentrations of p150CC1 and nNudE. After a 1-h incubation period at room temperature, complexes were resolved on a 15% native gel prepared without SDS (41). Electrophoresis was done at a constant current of 10 mA, using buffer composed of 25 mM Tris, 19.5 mM glycine, pH 7.3. Bands were visualized by Coomassie Brilliant Blue staining.

RESULTS

Constructs Used in this Work—For IC, we use IC(1–87), which contains both region 1 and region 2 of the p150Glued binding segment, IC(1–40), which contains only region 1, and IC(84–143), which contains the unstructured region C-terminal to region 2 (1) (Fig. 1). These constructs were chosen to facilitate direct comparison to the binding studies performed with p150Glued. They do not include the weak self-association domain (IC residues 222–231) (17), which is considerably distant from the residues involved in direct interaction with NudE and p150Glued and may have only limited effect on their binding affinities. For NudE, sequence-based structural prediction algorithms (16, 42) identify two domains: an N-terminal predominantly helical domain (residues 1–174), with coiled-coil propensity, and a largely unstructured C-terminal domain (residues 181–317). Consistent with prediction, limited proteolysis experiments of the recombinant NudE protein show susceptibility of the C-terminal region to proteolytic degradation, whereas a domain corresponding to the N-terminal region is less susceptible to proteolysis (data not shown). Based on these observations, constructs nNudE (corresponding to residues 1–174) and cNudE (corresponding to residues 179–317) were designed to include, respectively, the predicted helical/coiled-coil domain, and the unstructured domain (Fig. 1). For
p150\textsubscript{Glued}, a construct p150\textsubscript{CC1} (residues 221–509) containing the IC binding site and corresponding to the most strongly predicted coiled-coil residues was used.

**Solution Properties of nNudE**—The structure and stability of nNudE was characterized by CD and fluorescence spectroscopy. The negative ellipticity at 208 and 222 nm of the CD spectrum is characteristic of predominantly helical proteins (Fig. 2A). The wavelength of maximum tryptophan fluorescence ($\lambda_{\text{max}}$) at 341 nm is consistent with partial burial of either or both of the two tryptophans at positions 17 and 28 (Fig. 2B). In predictions of coiled-coil from sequence analysis, these residues are at core positions “d” and “a,” respectively. The temperature unfolding profile has a single transition midpoint ($T_{\text{m}}$) of 48 °C (Fig. 2C). The GndCl unfolding profile shows two-step unfolding (Fig. 2D). The first transition midpoint at 0.5 M GndCl is accompanied by a shift in $\lambda_{\text{max}}$ from 341 to 344 nm and a decrease in the fluorescence signal, whereas the second transition midpoint at 2.2 M GndCl has a corresponding shift in $\lambda_{\text{max}}$ to 348 nm, and a decrease in fluorescence intensity. In 6 M GndCl, the decrease in fluorescence signal is accompanied by a shift in $\lambda_{\text{max}}$ to 357 nm (Fig. 2D, inset). The intermediate state, accompanied by a shift in $\lambda_{\text{max}}$ from 341 to 344 nm suggests partial unfolding in the vicinity of one or both tryptophans (Trp-17 and Trp-28). Similar two-step unfolding profiles were observed for less concentrated samples as low as 0.5 M (fluorescence). Thermal and chemical unfolding profiles of nNudE monitored by CD ($\Delta G$) in the temperature range of 5–80 °C, and fluorescence ($\Delta G$) in the GndCl concentration range of 0–6 M. GndCl-induced unfolding is accompanied by a decrease in the intensity of the fluorescence signal, and a shift in $\lambda_{\text{max}}$ from 341 to 357 nm signal (inset).

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**Dynein-NudE Interaction Monitored by ITC**—Representative thermograms of the interaction of nNudE with IC constructs are shown in Fig. 3: IC(1–143) (Fig. 3A), -(1–87) (Fig. 3B), -(84–143) (Fig. 3C), and -(1–40) (Fig. 3D). IC(1–143), -(1–87), and -(1–400) bind nNudE with stoichiometries of 1:1, and apparent $K_d$ values of 2.2, 2.5, or 1.7 μM, respectively. Associated enthalpic and entropic contributions are reported in Table 1. No ITC-detected interaction is observed between IC(84–143) and nNudE (Fig. 3C). Similar titration experiments between LC8 and full-length NudE show no measurable binding (Fig. 3E). Because interactions between IC and NudE were detected only when both interacting proteins included the respective predicted coiled-coil domains, subsequent experiments used constructs IC(1–40) or IC(1–87), and nNudE.

**IC(1–87)-nNudE Interactions Monitored by NMR Spectroscopy**—NMR titration experiments of isotopically labeled IC(1–87) with unlabeled nNudE, are shown in Fig. 4 as superimposed $^{1}$$H$, $^{15}$$N$ heteronuclear single quantum spectra of $^{15}$$N$-labeled IC(1–87) (black) with the nNudE-bound protein (red), at IC:nNudE molar ratios of 1:0.2, 1:1, and 1:3. The limited amide proton chemical shift dispersion of IC(1–87) is characteristic of a predominantly unstructured protein. 75 of the 86 nonproline residues were assigned; the missing resonances are within the 1–40 segment, which shows relatively broader peaks and overlapping resonances possibly due to intermediate exchange between the predominantly helical conformation and a minor coiled-coil population.

At a molar ratio of 1:0.2 (Fig. 4A), a plot of the relative intensities of peaks in the bound spectrum (plot below in Fig. 4A) shows a >50% decrease in the intensities of most peaks corresponding to IC residues 1–40. Spectra of unbound and nNudE-bound IC at a molar ratio of 1:1 show for the bound protein the disappearance of peaks corresponding to residues within the 1–40 segment (Fig. 4B). The broadening of resonances at this molar ratio can either be due to exchange between bound and free IC, or to structural plasticity at the binding interface.

In the final titration, a 3-fold molar excess of unlabeled nNudE was added to IC. Here, all peaks corresponding to IC residues 41–87 are still observed in the spectrum of the nNudE-bound protein (Fig. 4C, red peaks). The intensities of peaks corresponding to residues 54–63 on average decrease by 45%.
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To determine whether the observed reduction in the intensities of these peaks is due to specific binding or to an artifact of the high protein concentrations, a 6-fold molar excess of nNudE was added to 150 μM 15N-labeled IC(1–87) (~3-fold dilution of the previous concentration). At this IC concentration and high IC:nNudE molar ratio, only peaks corresponding to residues 1–40 disappear; peaks corresponding to residues 54–63 do not significantly change (supplemental Fig. S1). In summary, adding a molar excess of nNudE to IC at protein concentrations of 150 or 500 μM results in the disappearance of peaks corresponding to IC residues 1–40, indicating that the binding interface is localized to residues 1–40.

Dynamics of the nNudE-IC Complex—Steady-state heteronuclear NOE, and transverse relaxation experiments were used to compare the dynamics of IC residues 1–87 when unbound, and bound to nNudE. Because peaks at the binding interface are not visible in the spectrum due to resonance broadening, a direct comparison of the unbound and nNudE-bound IC is only made for residues 41–87. For unbound IC, average NOE values of 0.4 ± 0.04 (residues 1–40), −0.4 ± 0.05 (residues 41–47), 0.2 ± 0.05 (residues 48–61), and −0.6 ± 0.08 (residues 62–87) were observed (Fig. 5A). In the nNudE-bound protein, residues 41–47 and 62–87 become slightly more ordered with average NOE values of −0.3 ± 0.05 and −0.5 ± 0.02, respectively, whereas residues 48–61 become slightly more disordered with average NOE values of 0.1 ± 0.02. These data suggest that the nascent helix observed in this region in the unbound IC is not stabilized upon binding but on the contrary becomes slightly more disordered.

Transverse relaxation measurements of unbound and nNudE-bound IC show a similar average T2 value for residues 41–87 of 0.14 (Fig. 5B). The overall dynamics of this region of IC do not change upon NudE binding except for small changes localized to the segment corresponding to residues 48–61 with average T2 values of 0.16 (unbound) and 0.12 (bound).

IC-nNudE-p150CC1 Competition Experiments Monitored by NMR Spectroscopy and Native Gel Electrophoresis—A different pattern of peak disappearance is observed for the p150CC1-bound IC binary complex when compared with the nNudE-bound IC. Peaks corresponding to residues 1–40 completely disappear in both, whereas the intensities of peaks corresponding to residues 46–75 are on average reduced by 80% in the p150CC1-bound IC (supplemental Fig. S2) but only somewhat reduced in the nNudE-bound IC spectrum (Fig. 4C). To determine whether p150CC1 and nNudE compete for binding to IC, we use binary complexes of nNudE-IC(1–87) (Fig. 4C, red) or p150CC1-IC(1–87) (supplemental Fig. S2A, blue). The binary complexes were prepared by adding a 3-fold molar excess of unlabeled nNudE or p150CC1 to 15N-labeled IC(1–87), and then titrated with a 4-fold molar excess of the reciprocal protein. In the nNudE-IC spectrum, only peaks corresponding to residues 41–87 are observed (red spectrum in supplemental Fig. S3A), but when excess p150CC1 is added, more peaks disappear and only peaks corresponding to residues 42–45 and 76–87 (green spectrum in supplemental Fig. S3A) remain. The resulting spectrum is apparently similar to the spectrum of the p150CC1-IC binary complex (supplemental Fig. S2) but with reduced intensities for peaks corresponding to residues 46–74. Superimposed spectra with a subset of peaks corresponding to residues 41–87 are shown in Fig. 6A. A plot of the relative intensities of peaks after the addition of p150CC1 to p150CC1-bound IC (supplemental Fig. S3A) shows that when p150CC1 is in excess, it can partially displace nNudE.

In the reciprocal experiment where nNudE is added to the p150CC1-IC complex, the intensities of peaks corresponding to residues 46–53 and 66–75 that are reduced by ~70–80% in the p150CC1-IC binary complex (supplemental Fig. S2) are only 5–10% more intense when nNudE is present. In contrast, peaks corresponding to residues 54–64 are even less intense than in the p150CC1-IC binary complex (~94% instead of 85%), with peaks corresponding to residues 58 and 60–64 disappearing completely (Fig. 6, B and D). As there is no indication of significant signal enhancement for IC residues 46–75 as one would expect if nNudE were to displace p150CC1, these results indicate that excess nNudE cannot displace p150CC1.

TABLE 1

| Titrant          | n  | Kd (μM) | ΔG° (kcal/mol) | ΔH° (kcal/mol) | T3S° |
|------------------|----|---------|---------------|---------------|------|
| IC(1–143)        | 1.00 | 2.2    | −7.8          | −4.2          | 3.6  |
| IC(1–87)         | 1.01 | 2.5    | −7.6          | −3.2          | 4.4  |
| IC(1–40)         | 1.03 | 1.7    | −7.9          | −3.6          | 4.3  |

FIGURE 3. Cytoplasmic dynein-NudE interactions monitored by ITC. Representative isothermal titration plots of nNudE with IC(1–143) (A), IC(1–87) (B), IC(84–143) (C), and IC(1–40) (D). Titration of NudE with the dynein subunit LC8 is shown in E. Experiments were performed at 25 °C in 50 mM sodium phosphate, 50 mM sodium chloride, 0.5 mM sodium azide, pH 7.5. Data were fit to a single site binding model (A + B ⇌ AB) in Origin (Microcal).
To determine whether IC simultaneously interacts with both binding partners, that is, whether a ternary nNudE-IC-p150CC1 complex is formed, pre-formed complexes of nNudE-IC(1–40/1–87), p150CC1-IC(1–40/1–87), and nNudE-p150CC1-IC(1–40/1–87) were resolved on a nondenaturing polyacrylamide gel (Fig. 6E). At the experimental pH of 8.4–8.8, unbound nNudE (pI 4.8), p150CC1 (pI 5.0), and IC(1–87) (pI 6.3) migrate as discrete bands, whereas unbound IC(1–40) (pI 9.9) does not migrate into the gel. For mixtures containing only IC and nNudE or p150CC1, slower migrating bands corresponding to the binary complexes, in addition to bands with similar migration as unbound nNudE or p150CC1 are observed. The mixture of all three proteins shows bands with similar migration to unbound nNudE, the nNudE-IC complex, and the p150CC1-IC complex. Under the conditions of this experiment and using purified recombinant proteins, there is no detected band for a ternary complex.

**DISCUSSION**

*NudE Binds IC Region 1 but Not Region 2*—Based on NMR analyses we conceptually partition the N-terminal domain of IC into several sequence elements. The N-terminal helix predicted to form a coiled-coil is region 1 (residues 1–40), the nascent helix followed by a disordered segment is region 2 (residues 46–75), the short disordered segment separating region 1 and region 2 is linker 1 (L1) (residues 42–45), and the long disordered segment C-terminal to region 2 is linker 2 (L2) (Fig. 7). Our studies are performed on *D. melanogaster* IC but due to similarity in sequence and structure prediction we anticipate that the results apply to mammalian IC as well. For the *D. melanogaster* NudE-dynein interaction, ITC-detected binding experiments indicate that the binding site on IC is within the first 40 residues (region 1) because IC constructs 1–40, 1–87, and 1–143 all bind NudE with comparable affinities (Table 1). Furthermore, NMR experiments to elucidate the residue level details of the NudE-IC interactions identify IC residues 1–40 as the full NudE consensus sequence, detected as those residues whose peaks disappear when NudE is present. Taken together, results from NMR and ITC experiments clearly show that IC region 1 is necessary and sufficient for NudE binding, in contrast to the multiregion IC recognition segment for p150Glued binding (1), as discussed below.

The IC Binding Domain of NudE Is a Dimeric Coiled-coil—The residues primarily involved in the interaction between dynein IC, dynactin p150Glued, and NudE include for all three proteins segments whose secondary structure is predicted by standard sequence-based algorithms to be coiled-coil (16). Solution studies of apo IC(1–143), however, show that whereas residues 3–36 are helical, there is no detectable population of stable coiled-coil, and the predominate conformations of unbound IC are disordered (1, 43). In contrast, p150CC1 (residues 221–509) is a dimeric coiled-coil as determined from a
CD-detected molar ellipticity ratio $\theta_{222/208}$ value of 1.07, multiangle light scattering and covalent cross-linking (data not shown). Similarly, nNudE(1–174) is a dimeric coiled-coil based on the following observations. First, the $\theta_{222/208}$ of 1.06 is essentially the same as that reported for the N-terminal domain of NudEL (1.07), a structural homologue of NudE shown by x-ray crystallography to form a parallel dimeric coiled-coil (44). Second, nNudE unfolds with a transition midpoint of 48 °C, similar to a value of 49 °C reported for the coiled-coil domain of NudEL (44). Third, chemical unfolding experiments detected by fluorescence spectroscopic techniques (Fig. 2D) show no protein concentration dependence of unfolding, indicating a stable nNudE dimer in the concentration range used. Taken together, these solution properties support coiled-coil formation for nNudE as predicted from its sequence. The existence of a stable dimeric conformation at the low protein concentrations of fluorescence experiments suggests that the dimeric coiled-coil is the biologically relevant state in vivo, and that its interaction with IC does not likely require stabilizing interactions with partners such as Lis1.

Exchange broadening observed in the $^1$H-$^{15}$N HSQC spectrum of apo-IC is indicative of equilibrium between a major population of monomeric helix conformations and a minor population of dimeric coiled-coil conformations. We postulate that the coiled-coil interacting domain that is common to both nNudE and p150Glued could bind the minor population of the IC dimeric coiled-coil conformation and drive the formation of a nNudE/IC or p150Glued/IC hetero-tetrameric coiled-coil complex. Such a heterotetrameric coiled-coil structure is not uncommon in the assembly of large dynamic complexes. In the Ndc80 complex, for example, a heterotetrameric complex formed from two subcomplexes each containing a coiled-coil linked end to end organizes the complex. This type of linkage is proposed to maintain tension between the spindle and chromosomes (45, 46).

Different Modes of Binding of NudE and p150Glued to IC—Although NudE and p150Glued bind IC at a common site and possibly form a tetrameric coiled-coil, there are clear differences in the details of their interactions. First, the IC binding domain of NudE, nNudE, binds to region 1 only, although the IC binding domain of p150Glued, p150Glued, requires regions 1 and 2 to match the binding affinity of nNudE observed with region 1 alone. Second, the dynamic structures of the tetrameric complexes differ. Although they have similar dynamics on the mil-

![Figure 6](https://example.com/figure6.png)
lisecond-microsecond time scale, evidenced by disappearance of peaks in the spectra of both complexes, nNudE-bound IC has increased flexibility on the faster time scale in region 2, evidenced by lower heteronuclear NOE values relative to apo-IC. These results are distinct from p150CC1-bound IC, which by similar criteria show an increased ordered structure in region 2 (1). Third, there are different effects on the affinity of one protein due to pre-binding of the other. Spectra of IC(1–87) when p150CC1 is titrated in a pre-formed nNudE-IC(1–87) binary complex show that p150CC1 can displace nNudE resulting in even more pronounced peak disappearance than with p150CC1 alone. In contrast, in the reciprocal experiment, nNudE in excess does not appear to compete with p150CC1. This indicates that whereas both nNudE and p150Glued bind IC with similar affinity, IC appears to have a higher affinity for p150Glued (50), and could selectively bind NudE (right). All three proteins are dimeric, but for simplicity are drawn as half-dimers.

FIGURE 7. A, sequence alignment of IC from D. melanogaster (DM) and from R. norvegicus IC isoforms 1A and 2C (IC 1A and IC 2C). Binding sites for NudE (region 1) and p150Glued (regions 1 and 2) are shown above the sequence. The two regions are followed by flexible linkers (L1 and L2). Residues within the IC DM and IC 2C sequences with coiled-coil propensities are underlined. Potential phosphorylation sites that could modulate binding of p150Glued are highlighted. B, model illustrating possible selective regulation of dynein by NudE and dynactin when both are present. Dynactin p150Glued binds dynein IC regions 1 and 2 making the dynactin-bound dynein the major product (left). When IC is phosphorylated at sites within the unstructured region L2, it has less affinity for p150Glued (50), and could selectively bind NudE (right). All three proteins are dimeric, but for simplicity are drawn as half-dimers.

Protein Disorder Underlies Versatility in Dynein Regulation—Dynactin and NudE are among a growing number of proteins proposed to control diverse dynein functions including recruitment of cargo, and localization and regulation of dynein at the kinetochores, centrosomes, cell cortical regions, and the nuclear envelope (37). There is clear evidence of functional redundancy such that, for example, in the absence of dynactin, NudE performs roles similar to dynactin allowing dynein to retain its ability to recruit lysosomes, larval membranes, and adenovirus particles (9, 47, 48). There is also ample evidence that both NudE and dynactin are present during these processes, and presumably required for most of them.

Our findings reported here begin to explain how regulation of IC by dynactin versus NudE is coordinated when both are present in the same compartment. Briefly, our findings are: apo IC binds both NudE and p150Glued with moderately weak affinity; whereas p150Glued binds two IC regions that are separated by a disordered 4-residue linker, NudE binds only one of these regions. Taken together, the ability of p150Glued to displace nNudE, but not vice versa, suggests that in vivo binding of NudE is diminished by the presence of dynactin. To the extent that binding to IC implies regulation of dynein, processes that require binding of dynactin to IC take precedence over those dependent on NudE binding to IC. It is possible that this hierarchy is altered, even inverted, if IC is phosphorylated or present in differently spliced isoforms, either of which may preferentially bind NudE (see below).

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**Dynein-NudE Interactions**

Collectively, the results immediately suggest that events that modify region 2 but do not significantly affect region 1 could interfere with p150Glued binding but have a limited effect on NudE binding. Such events could be phosphorylation and/or alternative splicing. In fact, in apo-IC, disordered segments in region 2 and in linker 2 are rich in potential phosphorylation and alternative splicing sites (Fig. 7), and in one case phosphorylation indeed results in reduced binding to p150Glued (49). Thus, residue-specific structural changes in and near region 2 likely may diminish or enhance dynactin binding with limited effect on NudE binding. An interesting consequence is that a modification localized to a short stretch in IC can modulate selection among different regulators of dynein function. Intrinsic disorder of IC likely facilitates modifications in and near region 2 that has a long disordered segment; it is known that phosphorylation and alternative splicing are more common in disordered segments of proteins than in ordered segments (50). Furthermore, the 4-residue disordered linker between regions is expected to minimize effects on the conformational ensemble of region 1 arising from modifications or truncation in region 2.

But, in unmodified IC, what could explain why, in the presence of both p150Glued and NudE, binding of the former predominates even though the IC affinity of the latter is somewhat higher. Again, intrinsic disorder of IC, implying an ensemble of conformations and minimal three-dimensional contact in apo-IC between regions 1 and 2 suggests a rationale. When bound, some population of the IC-NudE ensemble will have apo-IC between regions 1 and 2 suggests a rationale. When conformations and minimal three-dimensional contact in higher. Again, intrinsic disorder of IC, implying an ensemble of alternative splicing sites (Fig. 7), and in one case phosphorylation and splicing; IC disorder in general promotes and alternative splicing. In fact, in apo-IC, disordered segments in NudE binding. Such events could be phosphorylation and/or

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