Light Chain-dependent Self-association of Dynein Intermediate Chain*\#\$\&

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Dynein light chains are bivalent dimers that bind two copies of dynein intermediate chain IC to form a cargo attachment subcomplex. The interaction of light chain LC8 with the naturally disordered N-terminal domain of IC induces helix formation at distant IC sites in or near a region predicted to form a coiled-coil. This fostered the hypothesis that LC8 binding promotes IC self-association to form a coiled-coil or other interchain helical structure. However, recent studies show that the predicted coiled-coil sequence partially overlaps the light chain LC7 recognition sequence on IC, raising questions about the apparently contradictory effects of LC8 and LC7. Here, we use NMR and fluorescence quenching to localize IC self-association to residues within the predicted coiled-coil that also correspond to helix 1 of the LC7 recognition sequence. LC8 binding promotes IC self-association of helix 1 from each of two IC chains, whereas LC7 binding reverses self-association by incorporating the same residues into two symmetrical, but distant, helices of the LC7-IC complex. Isothermal titration experiments confirm the distinction of LC8 enhancement of IC self-association and LC7 binding effects. When all three light chains are bound, IC self-association is shifted to another region. Such flexibility in association modes may function in maintaining a stable and versatile light chain-intermediate chain assembly under changing cellular conditions.

Cytoplasmic dynein is a 1.2-MDa protein complex responsible for organization and maintenance of the Golgi apparatus, nuclear migration, formation of the mitotic spindle, viral transport, and positioning and transport of various organelles (1–4). The dynein complex is comprised of two functional subcomplexes; that is, the motor domain, responsible for ATP hydrolysis and movement along microtubules, and the cargo attachment subcomplex. The motor domain consists of two heavy chain subunits (DYNCL1H1). The cargo attachment subcomplex is variably composed of light intermediate chains (DYNC1LI), intermediate chains, IC2 (DYNC1I1), and three dimeric light chain subunits, LC8 (DYNLL), Tctex1 (DYNLT), and LC7, also known as km23 or Roadblock (DYNLR). IC is essential to dynein cargo attachment function as it directly interacts with several putative dynein cargoes (5–7) and indirectly by linking cytoplasmic dynein to dynactin, another multisubunit complex that targets dynein to specific cargoes and increases dynein processivity (8).

Although IC may well serve as a cargo adaptor, linking cargo molecules to dynein, such a role for the light chains is not easily reconciled with binding data. For Tctex1, peptides of IC compete with the G protein βα subunit (9), an indication that both peptides bind at the same location and, therefore, that Tctex1 cannot be simultaneously bound to βα subunit and to IC (its link to the dynein complex). LC7 binds several non-dynein proteins (10, 11), but whether it can bind both IC and the non-dynein proteins simultaneously is not clear. For LC8, several structural and thermodynamics studies call into question a cargo adaptor role (12, 13). In crystal structures of LC8 bound to peptides of IC and Drosophila melanogaster Swallow (14), the peptides occupy the same groove at the LC8 dimer interface and have different binding affinities (15). Taken together, these studies support a role other than that of a cargo adaptor for the light chains.

The idea that light chains are instead involved in regulating dynein complex assembly is gaining credence from structural and binding studies of light chains and the primarily unstructured N-terminal domain of the intermediate chain (N-IC) (16–19). N-IC is central to dynein assembly as it binds all three bivalent light chain subunits (16, 20–22) and includes a dimerization domain proposed in Rattus norvegicus from deletion mutations and co-immunoprecipitation (23) and a predicted coiled-coil, proposed in D. melanogaster to be the site of self-association that is enhanced by LC8 binding (17, 18) (Fig. 1). When either LC8 or Tctex1, whose recognition sequences are separated by a 3-residue linker, is bound to a 60-residue IC construct, the binding affinity of the other light chain is increased (19). When LC8 is bound to a 300-residue D. melanogaster N-IC construct, it induces helix formation at a site distant from the LC8 recognition sequence (17). This, together with NMR evidence for apoIC of a nascent helix for a construct of IC corresponding to residues 123–260 but lacking residues 143–198; 219, disulfide cross-linked IC154C, IC204C, IC214C, IC219C, IC224C, IC228C, and IC241C, mutants of IC7 with single cysteine substitutions at residues 154, 204, 214, 219, 224, 228, and 241 respectively; IC7-3.

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1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S5.
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3 The abbreviations used are: IC, the 74-kDa dynein intermediate chain corresponding to gene Dync1lli, intermediate chains, IC2 (DYNC1I1), and three bivalent light chain subunits, LC8 (DYNLL), Tctex1 (DYNLT), and LC7, also known as km23 or Roadblock (DYNLR). IC is essential to dynein cargo attachment function as it directly interacts with several putative dynein cargoes (5–7) and indirectly by linking cytoplasmic dynein to dynactin, another multisubunit complex that targets dynein to specific cargoes and increases dynein processivity (8).
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residues in the predicted coiled-coil domain has fostered the hypothesis that LC8 binding of two IC chains at residues 126–138 promotes formation of an interchain helical structure, probably coiled-coil, in predicted coiled-coil residues 207–237 (18). Such a coiled-coil promoting role for LC8 is likely in other systems (24–26) and has led to the paradigm shifting idea that LC8 acts as a dimerization hub for disordered monomeric binding partners (12).

Although LC8 and possibly Tctex1 appear to promote self-association of IC in the predicted coiled-coil region, a recently reported crystal structure of the LC7-IC complex (27) shows that the IC segment bound to LC7 includes about half of the residues of the predicted coiled-coil and that LC7 orients the IC chains away from each other, preventing IC self-association in this region. Thus, the LC7-IC crystal structure is not compatible with a simple model of LC8-induced IC self-association of predicted coiled-coil residues.

Based on these intriguing data for various interactions of IC with the light chains, we use here a combination of techniques including NMR, fluorescence quenching, chemical cross-linking, and isothermal titration calorimetry to identify at the residue level the IC self-association sequence, to test the LC8-induced IC self-association model, and to investigate in solution and, in the presence of all the light chains, the interplay between LC8 and LC7 binding and IC self-association.

**EXPERIMENTAL PROCEDURES**

**Protein Preparation**—Site-directed mutagenesis of Asn-154, Thr-204, Val-214, Asn-219, Glu-224, Met-228, and Arg-237 of ICTL7 (lacking residues 92–122 and 143–198), was performed using the Stratagene QuikChange™ kit (Agilent Technologies) following the manufacturer’s protocol. After verifying the sequences of the resulting mutants (ICTL7154C, ICTL7204C, ICTL7214C, ICTL7219C, ICTL7224C, ICTL7228C, and ICTL7241C) by DNA sequencing, each mutant was transformed into *Escherichia coli* Rosetta™ cell lines (Novagen) for protein expression. For NMR studies, a deletion mutant of ICTL7, ICTL7Δ (lacking residues 92–122 and 143–198), was generated following the two-step PCR reaction of Horton et al. (28). The PCR product was subcloned into the Champion™ pET SUMO protein expression system (Invitrogen) and transformed into *E. coli* BL21-DE3 cell lines for protein expression.

Recombinant cells were grown in LB or minimal media at 37 °C to an *A*<sub>600</sub> of ~0.6. Minimal media were supplemented with [15N]ammonium chloride and [12C]- or [13C]glucose as the sole nitrogen and carbon sources, respectively (Cambridge Isotopes). Protein expression and purification under denaturing conditions followed previously published protocols (16) with slight modifications; denatured proteins were eluted from the Ni<sup>2+</sup>-nitrilotriacetic acid affinity column (Qiagen) with a 50–350 mM imidazole gradient. The SUMO tag was removed following the Invitrogen protocol. All proteins were further purified on a Superdex<sup>™</sup> 75 (16/60) gel filtration column (GE Healthcare) with a running buffer composed of 50 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.4 M NaCl, 1 mM NaN<sub>3</sub>, pH 7.5. Under these conditions a peak corresponding to the disulfide dimer was observed for all the cysteine mutants except ICTL7204C and ICTL7204C and was easily purified from the reduced monomer. Expression and purification of LC8, Tctex1, and LC7 followed procedures described elsewhere (16, 27, 29). The association states of the proteins were determined from size exclusion chromatography and multiance light-scattering (MALS) (mini-DAWN, Wyatt Technology) placed in line with UV and refractive index detectors.

**Circular Dichroism Spectroscopy**—Samples for circular dichroism (CD) measurements were prepared at pH 7.5 in 10 mM Na<sub>2</sub>HPO<sub>4</sub> buffer containing 10 mM NaCl. Data were collected at 25 °C on a Jasco 720 spectropolarimeter using a 1-mm path length cell. Protein concentrations of 10–12 μM were determined from extinction coefficient values and absorbance at 280 nm. For GdnCl unfolding studies, IC<sub>L7</sub>Δ at 50 μM was prepared in 10 mM Na<sub>2</sub>HPO<sub>4</sub> and 10 mM NaCl buffer, pH 6.3, with 0.1, 0.3, 0.5, 0.7, 0.9, and 1.1 M GdnCl. Samples were equilibrated at 5 °C for 1 h before data acquisition at the same temperature.

**Labeling of Proteins for Fluorescence Studies**—Tetramethylrhodamine-5-iodoacetamide (TMR) (Molecular Probes, Eugene, OR) dissolved in 50 μl DMSO was added at a final concentration of 250 μM to 10–20 μM concentrations of each purified IC<sub>L7</sub> cysteine mutant in 50 mM Tris-Cl, 50 mM NaCl, 10 mM tris(2-carboxyethyl) phosphine, pH 7.5. The conjugation reaction was performed at 4 °C in the dark for 12–16 h. For “lightly labeled” IC<sub>L7</sub> cysteine mutants, TMR was added to a final concentration of 20 μM, and the conjugation reaction performed at 4 °C in the dark was allowed to proceed for 2 h. After the conjugation reaction, a 5-fold molar excess β-mercaptoethanol was added to react with any unconjugated dye, thus, ensuring that no reactive species were present during the subsequent dialysis steps. Unreacted dye molecules were removed by extensive dialysis against 50 mM Na<sub>2</sub>HPO<sub>4</sub>, 50 mM NaCl, 1 mM NaN<sub>3</sub>, pH 7.5. Concentrations of the protein-dye conjugate were determined using the equation

\[
\frac{A_{280} - (A_{max} \times CF)}{\epsilon} \times DF
\]  
(Eq. 1)  

where \(\epsilon\) is the molar extinction coefficient for the protein, \(A_{280}\) is the absorbance of the protein-dye conjugate at 280 nm, \(A_{max}\) is the absorbance of a dye solution measured at 555 nm (the \(A_{max}\) for TMR absorbance), \(CF\) is the correction factor, which adjusts for the absorbance of the dye at 280 nm (a value of 0.3 was used for TMR), and \(DF\) is the dilution factor of the protein-dye conjugate. The labeling efficiency of 0.6 to 0.8 (or 0.03 in the case of the lightly labeled mutants) was determined using the equation

\[
\frac{A_{max} \times DF}{\epsilon} \times \text{protein concentration (M)}
\]  
(Eq. 2)  

where \(\epsilon\) is the molar extinction coefficient of TMR. Labeled proteins were stored in the dark at 4 °C and used within a week.

**Fluorescence Quenching**—Fluorescence data were acquired on a Fluorolog-3 spectrofluorometer (Horiba Scientific). All experiments were collected at room temperature in a 0.5-cm
quartz cuvette using 2 μM TMR-labeled IC cysteine mutants in 50 mM Na₂HPO₄, 50 mM NaCl, pH 7.5, with or without 6 μM LC8, Tctex1, or LC7 prepared in the same buffer. The excitation wavelength was set to 555 nm, and the emission was recorded between 560 and 650 nm using a scan speed of 1 nm/s and excitation and emission slit widths of 1 or 5 nm. Experiments to monitor binding to LC8 and Tctex1 or to all three light chains were recorded in duplicate. Reported values are the average readings expressed as

\[
\text{Change in intensity} = \frac{I_b - I_u}{I_u} \times 100 \quad \text{(Eq. 3)}
\]

where \(I_b\) and \(I_u\) are the emission intensities at 573 nm of the bound and unbound proteins, respectively.

**NMR Data Collection and Analysis**—Two-dimensional \(^1\text{H}, ^{15}\text{N}\) heteronuclear single-quantum coherence (HSQC) experiments of \(^{15}\text{N}\)-labeled ICL7-Δ were collected at 5 °C in 50 mM Na₂HPO₄, 50 mM NaCl, 1 mM NaN₃, pH 6.3, protease inhibitor mixture (Roche Applied Science) and in the 150–400 μM protein concentration range. A temperature of 5 °C and a pH of 6.3 were used to maintain protein solubility. Experiments were collected on ICI,Δ unbound or bound to 3-fold molar excess unlabeled LC8 or LC7 with or without 0.1, 0.3, or 0.5 M GdnCl. HSQC experiments used echo-anticorrelation phase discrimination of 256 increments and 1024 points. Spectra were processed with NMRPipe (30) and analyzed with NMRView (31). \(^1\text{H}\) chemical shifts were referenced with internal 2,2-dimethylsilapentene-5-sulfonic acid at 0 ppm. Backbone resonances were determined from published assignments for IC residues 84–143 and 198–237 (18) and confirmed by CBCA(CO)NH (32) and HNCA triple resonance experiments (33) collected on \(^{13}\text{C}, ^{15}\text{N}\)-labeled ICI,Δ.

**Isothermal Titration Calorimetry**—Thermodynamics of binding were determined at 25 °C using a VP-isothermal titration calorimeter (MicroCal, Northampton, MA). Protein concentrations were typically 180–250 μM for the titrant (LC8) and 20–25 μM for the macromolecule (IC). In experiments with Tctex1-bound IC or LC7-bound IC, the concentrations of Tctex1 or LC7 were typically 3–5-fold higher than the concentration of IC. Reactions were performed at pH 7.5 in buffer composed of 50 mM Na₂HPO₄, 50 mM NaCl, and 1 mM NaN₃. Data were fit to a single-site binding model using the ORIGIN software provided by the manufacturer. Heats of dilution, estimated to be equal to the average of the last five enthalpies of injection, were subtracted from the binding data before fitting. Each interaction gave a binding stoichiometry of 1. Data reported are an average of two experiments performed under similar conditions using proteins from two different preparations.

**RESULTS**

**Design of IC Mutants**—To investigate the interplay between LC8 and LC7 binding and IC self-association, we use a construct of *D. melanogaster* N-IC, ICTL7, that corresponds to residues 92–260 and includes binding regions for Tctex1, LC8, and LC7 (Fig. 1). Based on sequence alignment, residues 149–250 of *D. melanogaster* ICTL7 correspond to the deletion-mapped dimerization domain (residues 151–211) of *R. norvegicus* (23). ICTL7, also includes the segment that gains helical structure upon LC8 binding (17) and residues 207–237, which are predicted to form a weak coiled-coil (34) (PrCC in Fig. 1).

For fluorescence experiments, we took advantage of the absence of native cysteines in ICTL7 to introduce single cysteine mutations at seven positions: position 154, which is immediately downstream of the LC8 binding region; positions 204, 214, and 219, which precede helix 1 of the LC7 binding region; positions 224 and 228, which are within helix 1; position 241, which is within helix 2 of the LC7 binding region. Residues 214, 219, 224, and 228 are also within the predicted coiled-coil of *D. melanogaster* IC (Fig. 1). The mutations allow labeling with a cysteine-specific fluorophore to be used as a reporter of self-association of various IC segments upon binding of unlabeled LC8/Tctex1 and/or LC7.

For NMR experiments, we use a deletion mutant of ICTL7, ICTL7-Δ, which lacks most of the intervening sequence between the LC8 binding region and the predicted coiled-coil but retains the binding regions for LC8 and LC7 (Fig. 1). Although deleting the intervening sequence does not affect the isothermal titration calorimetry-measured binding affinity for the light chains (Kₐ of 10 μM, similar to ICTL7) (supplemental Fig. S1), it does make the construct more amenable for residue-specific NMR studies of the effect of the light chain(s) binding on IC self-association.

**Average Structure and Association States of IC Mutants**—The CD spectrum of ICTL7, or its mutants (supplemental Fig S2A) is that of a predominantly unstructured protein with little regular secondary structure. The K2D2 algorithm of Andrade et al. (35) estimates helical contents of 7% for ICTL7 and 7.8% for ICTL7-Δ, less than the 16% (ICTL7) (supplemental Fig. S1) of ICTL7-Δ calculated using sequence-based helical prediction of residues 222–230 (helix 1) and 232–250 (helix 2) (36). This estimate is similar to previously determined helical content of the full-length N-terminal domain of IC (17).

ICTL7 is primarily monomeric with a MALS-determined molecular mass of 21.3 ± 0.3 kDa (calculated monomeric molecular mass of 20.5 kDa) in the protein concentration range up to 180 μM (higher concentrations were not possible). The association state of ICTL7-Δ, however, is concentration-dependent. At a protein concentration of 400 μM, ICTL7-Δ has a MALS-determined molecular mass of 16.3 ± 0.2 kDa (calculated dimeric molecular mass of 18.6 kDa). At this concentration, the single size-exclusion peak migrates as two bands resolved by non-denaturing polyacrylamide gel electrophoresis (data not shown). Upon dilution to concentrations less than 150 μM, the MALS-determined molecular mass becomes 11.8 ± 0.6 kDa, slightly higher than the calculated and mass spectrometry-determined monomeric molecular mass of 9349.5 Da. The change in MALS-determined molecular mass toward a monomer is an indication that self-association observed at high protein concentration is reversible.

Under reducing conditions, all ICTL7 cysteine mutants elute as single peaks with elution times similar to wild type ICTL7 (data not shown), an indication that the reduced proteins are primarily monomers. Under oxidizing conditions, an earlier
peak with a MALS-determined molecular mass of 48 kDa (theoretical dimer is 41 kDa) is observed for ICTL7214C, ICTL7219C, ICTL7224C, ICTL7228C, and ICTL7241C mutants but not for ICTL7154C and ICTL7204C (supplemental Fig. S2B). This second peak corresponds to an intermolecular disulfide IC dimer as verified by non-reducing denaturing polyacrylamide gel electrophoresis (data not shown). Thus, mutants showing a disulfide dimer peak under oxidizing conditions apparently have their single cysteine in or near the self-association region.

**Self-association Monitored by Fluorescence Quenching**—The overall strategy is to label IC with the dye TMR at various residue positions along its sequence and then measure the position dependence of TMR fluorescence intensity. Each of the seven marker variants of ICTL7 has a single TMR group. A decrease in the fluorescence intensity (quenching) can arise from homotransfer between two such TMR moieties in close proximity (37, 38). With optimal orientation, TMR dyes in close proximity can form non-covalently stacked dimers, further decreasing the fluorescence quantum yield (39–41). The degree of quenching is influenced by the orientation and location of the TMR group. Quenching is not optimal if the TMR groups point away from each other even when on a residue in a self-association domain (where the dyes are expected to be in close proximity) or if the bulky TMR group disrupts the weak packing interaction in the self-association domain. Additionally, local environmental changes in three-dimensional packing may also affect fluorescence intensity. Given these limitations, the data are interpreted qualitatively with a focus on the relative degree of quenching in light chain(s)-bound IC as compared with unbound IC. All TMR labels are distant from the LC8/Tctex1 binding sites, but some overlap with the LC7 binding site. The differences in quenching between unbound ICTL7 and light chain(s)-bound ICTL7 for each specifically positioned dye-marker are presented in Fig. 2 with the fluorescence intensity of unbound ICTL7 set as the reference and normalized to 1.

The differences in fluorescence emission intensities for the TMR-labeled IC(T for Tctex1, L for LC8, and 7 for LC7) is N-IC residues 92–260, which contains the recognition sequences for Tctex1, LC8, and LC7. A, in the domain map of ICTL7, the orange, green, and blue regions correspond to binding sites for Tctex1 (109–120), LC8 (126–138), and LC7 (221–258), respectively. PrCC is a predicted coiled-coil region. Numbered residues in ICTL7 are those replaced by cysteine to make ICTL7154C, ICTL7204C, ICTL7214C, ICTL7219C, ICTL7224C, ICTL7228C, and ICTL7241C and then either covalently attached to a fluorescent dye or, in the case of ICTL7219C, oxidized to make the disulfide cross-linked ICTL7219CDimer. Construct IC7Δ (N-IC residues 123–260) lacks the Tctex1 binding site and residues 143–198. B, in the sequence alignment of the N-terminal region of D. melanogaster IC (AF 263371.1) and the R. norvegicus IC-2C isoform (AAA 89165.1), identical residues (*), conserved residues (:), and semi-conserved substitutions (.) are designated. Binding segments for light chains (yellow for Tctex1, green for LC8, and blue for LC7) are highlighted. PrCC residues are shown in bold. Secondary structure is predicted with Psipred (36), and the structural elements are shown above the sequence (arrows for β-strands, bars for helices, and dotted lines for disordered segments). The dashed line corresponds to residues that are absent in R. norvegicus IC sequence and underlined residues correspond to the deletion-mapped dimerization domain as identified in R. norvegicus IC.
LC-dependent Self-association of Dynein IC

Effects of light chain binding on fluorescence emission spectra of TMR-labeled IC

The differences in fluorescence emission intensities for TMR-labeled IC_T17 mutants unbound or bound to LC7 are shown in Fig. 2, C, D, and G. Although no difference in emission intensity was observed for IC_T17_154C-TMR (Fig. 2C), binding of LC7 resulted in increases in intensity in the range of 2.6–11% for the other mutants (Fig. 2G). The increase in emission intensity for IC_T17_219C-TMR (4.5%), IC_T17_224C-TMR (11%), and IC_T17_228C-TMR (10%) was accompanied by blue shifts of 1 nm, consistent with burial of these residues at the IC/LC7 interface (Fig. 2D and data not shown) and as observed in the crystal structure of the IC/LC7 complex (27).

When the three light chains are simultaneously bound to IC_T17, both an increase and decrease in intensity were observed depending on the proximity of the label to the light chain binding region (Fig. 2, E, F, and G). As with LC8/Tctex1 binding alone, a decrease in emission intensity was observed for 154, 204, and 214, but of higher magnitude (~11, 25, and 11%, respectively) (Fig. 2, E and G). As with LC7 binding alone, an increase in emission intensity was observed for 219, 224, 228, and 241 (6, 19, 7, and 8%, respectively) (Fig. 2, F and G). The largest change in intensity was observed for IC_T17_204C-TMR (Fig. 2E).

Self-association Sequence Mapped by NMR Spectroscopy—The 1H,13N HSQC spectrum of IC_A shows backbone assignments for all non-proline residues within the 123–142 and 200–235 segments (Fig. 3). The limited amide proton chemical shift dispersion is characteristic of a predominantly unstructured or helical protein. Because IC_A has a propensity to dimerize at high protein concentration, spectra used to map the self-association domain were collected at increasing concentrations of denaturant to shift the equilibrium toward the monomeric population. Spectra recorded in 0.1, 0.3, or 0.5 M GdnCl show a decrease in amide chemical shift dispersion (supplemental Fig. S5A) with in-

of increasing concentrations of GdnCl, an increase in the fluorescence intensity is expected if the fraction of non-fluorescent rhodamine dimer population is converted to monomers (39). A small increase in fluorescence intensity for unbound IC_T17_219C-TMR, and a larger increase for LC8/Tctex1-bound IC_T17_219C-TMR were observed (supplemental Fig. S3).

To assess contributions from the local environment to changes in the fluorescence quantum yield in the self-associated IC, binding of LC8 and Tctex1 to lightly labeled (labeling efficiencies 3%) IC_T17_154C-TMR and IC_T17_219C-TMR were monitored. Under these conditions, 97% of the IC chains are not covalently attached to a TMR moiety; therefore, quenching arising from homotransfer and/or stacked dimers is expected to be minimal. The fluorescence emission spectra of the unbound and LC8/Tctex1-bound to lightly labeled mutants show no change in fluorescence emission intensity for lightly labeled IC_T17_154C-TMR, but a 14% increase in emission intensity for the lightly labeled IC_T17_219C-TMR (supplemental Fig. S4). The increase in fluorescence intensity is consistent with burial of the single TMR moiety when IC self-associates at/near marker 219; that is, self-association between one IC chain that is covalently attached to a dye moiety and one that is not.

The changes in intensity for all the ICTL7-TMR-labeled mutants (7% for 154, 204, and 214; 11% for 224 and 228, 14% for 241, and 38% for 219) are shown in Fig. 2G. Changes in the intensities of IC_T17_224C-TMR and IC_T17_228C-TMR are also accompanied by a 1-nm red shift, consistent with TMR dimer formation (42). In the presence
creasing concentrations of denaturant. A graph of the weighted changes in chemical shifts for ICL7Δ with added 0.3 M GndCl is shown in Fig. 3B. Residues 124–139, which are within the LC8 binding region, on average undergo minimal chemical shift changes (<0.05 ppm). Residues 222–231, which define helix 1 of the LC7 binding region, on average undergo changes >0.05 ppm, with residues 222–223 and 228–231 showing the greatest changes. Other residues, 207, 213, 214, and 216, also shift by more than 0.05 ppm, but on average the change in chemical shift for residues within helix 1 of the LC7 binding region is greater than the preceding residues 207–221 of the predicted coiled-coil. Multiple resonances in slow/intermediate exchange were observed for residues 222, 229, and 230. The spectral regions that include residues 222–224 and 229–230 of ICL7Δ in 0, 0.1, 0.3, and 0.5 M GndCl are shown in Fig. 4A. These residues show the highest amide proton chemical shift toward the center of the spectrum and multiple peaks with increasing denaturant concentration.

ICL7Δ spectra in 0.1 or 0.3 M GndCl were also recorded in the presence of excess unlabeled LC8. Fig. 4B shows peaks for residues in 0 or 0.3 M GndCl overlaid with the same residue peaks when ICL7Δ in 0.3 M GndCl is bound to LC8. LC8 binding reverses the shift (away from the center of the spectrum) for residues 222, 223, 229, and 230. Furthermore, for residue 223, there was a change from a broad peak, indicative of intermediate exchange between two states, to two resolved peaks in the LC8-bound protein, consistent with an increase in population of the state corresponding to the peak farther from the center of the spectrum (the dimer state) (Fig. 4B, middle panel). Similarly, only the peak farther from the center of the spectrum (the dimer state) was observed for residues 229 and 230 in spectra of the LC8-bound protein. Fig. 4C shows regions of the spectra of unbound ICL7Δ with or without 0.1 M GndCl superimposed with the LC8-bound protein in 0.1 M GndCl. As in 0.3 M GndCl, LC8 binding reverses the direction of the chemical shift for residues 223 and 229, resulting in peaks that have chemical shift values closer to those observed for unbound IC without GndCl. Residue 230, at the end of helix 1, undergoes a significant loss in peak intensity. Peaks for residues 124, 131, and 133 that are close or within the LC8 binding region (126–138) undergo exchange broadening and are not observed in the spectrum of the bound protein (Fig. 4C, left panel). The disappearance of these peaks is an indication that LC8 binding is not perturbed at low concentrations of GndCl. Peaks for residues 211, 217, and 219 that are within the predicted coiled-coil but not part of helix 1 of the LC7 binding region undergo minimal chemical shift changes (Fig. 4C, left panel).

ICL7Δ spectra in 0.3 M GndCl were also recorded in the presence of excess unlabeled LC7. As with LC8 binding, peaks for residues involved in binding to LC7 (IC residues 221–255) are not observed due to exchange broadening. Unlike LC8 binding, however, peaks for residues distant from the LC7 binding region show no chemical shift changes (data not shown).

In summary, the ICL7Δ dimer population is decreased by the addition of GndCl in the concentration range 0–0.5 M. Varying the ICL7Δ dimer population in low concentrations of GndCl affects the chemical shift and exchange broadening of peaks of some residues but not others. Those affected are assigned to the IC self-association sequence. From the data in Figs. 3 and 4, we conclude that the self-association sequence of ICL7Δ is localized to residues 222–231 and that LC8 binding enhances self-association in this region.

**Thermodynamics of LC8-IC Interactions—Isothermal titration calorimetry data were collected for binding of LC8 with ICL7Δ with ICL7Δ prebound to Tctex1 (ICL7Δ-Tctex1) and with ICL7Δ prebound to LC7 (ICL7Δ-LC7). Data were also collected for LC8 with the ICL7Δ-219C disulfide dimer (219C_{dimer}), used here as a model for the self-associated IC, and the 219C_{dimer}-Tctex1 binary complex. Representative plots for each titration are shown in Fig. 5, and a summary of the thermodynamic parameters is given in Table 1. LC8 binds to ICL7Δ with an affinity of 9.8 μM. Prebinding with Tctex1 results in enhanced binding affinity of LC8 to ICL7Δ of about 40-fold (K_{d} of 0.24 μM), whereas no change in binding affinity was observed upon prebinding with LC7 (K_{d} of 10.4 μM). For the 219C_{dimer} binding affinity was enhanced 6-fold to 1.7 μM, and prebinding Tctex1 to 219C_{dimer} resulted in a higher binding affinity of 0.27 μM.
It is interesting to note that binding of LC8 to all constructs and binary complexes of IC$_{TL7}$ results in a similar change in enthalpy but varying changes in entropy (Table 1). Thus, observed increases in binding affinity relative to unbound IC$_{TL7}$ apparently arise from entropic processes.

**FIGURE 4.** Chemical shift changes of selected residues in apoIC$_{TL7}$ and in LC8-bound IC$_{TL7}$ upon the addition of GdnCl. A, apoIC$_{TL7}$ residues in 0 M (black), 0.1 M (green), 0.3 M (blue), and 0.5 M (magenta) GdnCl are shown. Changes in chemical shift with increasing denaturant concentration are shown for residues 222–224 and 229–230; all are within IC helix 1 of the LC7 binding region. The upfield or downfield direction of shifts caused by addition of denaturant is indicated by black arrows. B, shown is a comparison of peaks of the same residue in spectra of samples in 0.3 M GdnCl of apoIC$_{TL7}$ (blue) and of LC8-bound IC$_{TL7}$ (red). The corresponding apoIC$_{TL7}$ peaks of samples without GdnCl (black) are also shown. The direction of peak shifts after the addition of 0.3 M GdnCl to apoIC$_{TL7}$ and of LC8 to IC$_{TL7}$ in 0.3 M GdnCl is indicated by blue and red arrows, respectively. C, shown is comparison of peaks of the same residue in spectra of samples in 0.1 M GdnCl of apoIC$_{TL7}$ (green) and of LC8-bound IC$_{TL7}$ (red). The corresponding apoIC$_{TL7}$ peaks of samples without GdnCl (black) are also shown. The direction of peak shifts after the addition of 0.1 M GdnCl to apoIC$_{TL7}$ and of LC8 to IC$_{TL7}$ in 0.1 M GdnCl is indicated by green and red arrows, respectively. Peaks of IC$_{TL7}$ residues 124, 131, and 133, close to the LC8 binding site, and of 211, 217, and 219, within PrCC but outside the LC7 binding site, have similar chemical shifts.

**FIGURE 5.** Representative isothermal titration calorimetry plots. Isothermal titration calorimetry data with thermograms (top panels) and isotherms (bottom panels) for LC8 binding to IC$_{TL7}$ (A), IC$_{TL7}$-Tctex1 complex (B) IC$_{TL7}$-LC7 complex (C) IC$_{TL7}$-219C-dimer (D), and IC$_{TL7}$-219C-dimer-Tctex1 complex (E) are shown. The experiments were performed at 25 °C in buffer composed of 50 mM sodium phosphate, 50 mM sodium chloride, 1 mM sodium azide, pH 7.5. Solid lines correspond to the non-linear least squares fit for an A + B to AB binding model.

**DISCUSSION**

We present here the first demonstration of distinct roles of dynein light chains in IC self-association. Specifically we identify the sequence on IC with propensity for self-association, verify the LC8-induced IC self-association model, show that
LC7 binding reverses IC self-association, and propose that the function of native disorder and bivalency in IC is to provide conformational versatility that is adapted to the variable requirements of the cellular environment. The solution data reported here, from fluorescence, NMR, and calorimetric experiments, are fully consistent with the recently determined crystal structure of LC7 in complex with an IC peptide (27) as well as the LC8-induced IC self-association model.

D. melanogaster IC Self-association Is Localized to Residues 222–231—For mapping the self-association sequence, we employ ICTL7Δ, which with a 27-residue linker between the LC8 and LC7 binding regions, is closer in length to mammalian IC (42-residue linker compared with 83-residue linker for D. melanogaster). ICTL7Δ is primarily a dimer in the 150–400 μM protein concentration range used for NMR and in the absence of GndCl. NMR titrations with GndCl allow assignment of residues at the IC-IC dimer interface to those that show distinctive behavior with increasing GndCl. Changes in individual peaks are interpreted to reflect changes in the local environment of that residue as the dimeric population shifts toward a more monomeric population. Assignments of monomer peaks were verified by comparison to those of a smaller IC construct that ends at residue 237 and is a monomer (18). This construct has chemical shifts that perfectly match the IC construct that ends at residue 237 and is a monomer (18). (IC-IC dimer) is primarily a dimer in the 150–400 μM protein concentration range used for NMR and in the absence of GndCl. NMR titrations with GndCl allow assignment of residues at the IC-IC dimer interface to those that show distinctive behavior with increasing GndCl. Changes in individual peaks are interpreted to reflect changes in the local environment of that residue as the dimeric population shifts toward a more monomeric population. Assignments of monomer peaks were verified by comparison to those of a smaller IC construct that ends at residue 237 and is a monomer (18). This construct has chemical shifts that perfectly match the IC construct that ends at residue 237 and is a monomer (18).

| Macromolecule | K_d (μM) | ΔG° (kcal/mol) | ΔH° (kcal/mol) | ΔS° (kcal/mol) |
|---------------|---------|----------------|----------------|---------------|
| ICTL7Δ        | 9.8 ± 0.2 | −6.8 ± 0.02 | −14.0 ± 0.2 | 7.2 ± 0.2 |
| ICTL7Δ-Tctex1 | 0.24 ± 0.04 | −9.1 ± 0.1 | −14.9 ± 0.9 | 5.9 ± 0.8 |
| ICTL7Δ-LC7   | 10.4 ± 0.3 | −6.8 ± 0.02 | −14.7 ± 0.2 | 7.9 ± 0.2 |
| 219C_dimer   | 1.7 ± 0.01 | −7.9 ± 0.03 | −14.3 ± 0.1 | 6.4 |
| 219C_dimer-Tctex1 | 0.27 ± 0.06 | −9.0 ± 0.1 | −13.2 ± 1.3 | 4.2 ± 1.1 |

What then accounts for the high quenching associated with marker 219 even though it is not part of the 222–231 self-association sequence identified by NMR of ICTL7Δ? One likely explanation is that because 219 immediately precedes the 222–231 self-association sequence, its high quenching reports an increase in IC interchain interactions involving these residues when LC8 is bound. Furthermore, because 219 is within a segment with random coil propensity and 224 and 228 are within segments with helical propensity (36) (Fig. 1), the high quenching associated with 219 and the intermediate quenching associated with markers at 224 and 228 (even though they are within the 222–231 self-association domain) is consistent with the view that the location and orientation of the TMR moiety influences the fluorescence intensity.

Enhancement of self-association upon LC8 binding is also observed by NMR as an increase in the dimer population of ICTL7Δ in 0.1 and 0.3 M GndCl (Fig. 4). As LC8 is added, there is a clear shift toward dimer reflected in either an increase in intensities of dimer peaks or a change in chemical shift toward dimer or both. All changes are localized to residues 222–231. The limited chemical shift changes observed for 219 is a further indication that this residue is not part of the self-association sequence, even though it shows the highest fluorescence quenching upon LC8 binding.

With LC7, increased fluorescence intensity for markers 219, 224, 228, and 241 (decreased quenching) supports conclusions inferred from the crystal structure that any IC-IC contacts in the vicinity of residue 219 is not likely (36). Residue 219 immediately precedes the LC7 binding site, and 224, 228, and 241 are within it. Increased intensity upon LC7 binding may be attributed to burial of the dye at the IC-LC7 interface and to separation of residue 219 from 219′, 224 from 224′, 228 from 228′, and 241 from 241′ in LC7-bound even more than in the average structure of the unbound IC. The minimal change in LC residues 154, 204, and 241 (farther from the LC7 consensus sequence) indicates that in the LC7-ICTL7 complex dye markers on 154, 204, and 241 are on average considerably more distant from each other than in the Tctex1-LC8.IC-ICTL7 complex.

**IC Self-association Is Enhanced by LC8 Binding but Reversed by LC7 Binding**—As a measure of changes in IC self-association upon light chain binding, we monitor the TMR self-quenching in ICTL7Δ at or near a marker residue that is dye-labeled. When IC is bound to Tctex1/LC8, some dye-dye quenching is observed for all marker residues; however, quenching is high when the TMR moiety is on residue 219, intermediate when on residues 224, 228, and 241, and small when on residue 154, 204, and 214 (Fig. 2). The small changes in fluorescence intensities associated with 154, 204, and 214 are consistent with the observation from NMR of ICTL7Δ that these residues are not part of the self-association sequence.
In summary, fluorescence quenching effects on light chain-bound IC relative to apoIC are in opposite directions for LC8 versus LC7. Decreased fluorescence intensity (increased quenching) arising from Tctex1/LC8 binding to IC (Fig. 2) is interpreted due to increased interchain contacts in the 222–231 self-association sequence identified by NMR (Fig. 4). In contrast, increased fluorescence intensity arising from LC7 binding (Fig. 2) is interpreted as due to decreased self-association in the 222–231 sequence.

Model for Dynein IC Light Chain Cargo Attachment Subcomplexes—The N-terminal domain of IC and its associated light chains is a unique example of a natively disordered protein complex and the best characterized representative of this new category of elongated and aligned multiprotein assembly system. Fig. 6 is a pictorial summary of all our data on D. melanogaster IC subcomplex. When unbound, IC is essentially disordered except for nascent helices in segments that tend to self-associate (Fig. 6, left). When bound to Tctex1 and LC8, IC becomes ordered at the light chain-IC interface sequences, remains highly flexible in its disordered, unbound regions, and is partially ordered in its self-association sequence. Self-associated conformations make interchain helical contacts between residues 222–231 (Fig. 6, upper complex). Our structure of LC7-IC\textsubscript{1,7} (Fig. 6, lower complex) is based on the fluorescence and NMR evidence of the absence of long range IC-IC contacts and the crystal structure of an IC peptide bound to LC7 (19). Outside the LC7-bound IC sequence, the IC chains (indicated by a red aperiodic line) are oriented away from each other, and their self-association is hindered.

In the quaternary assembly of Tctex1, LC8 and LC7 bound to IC\textsubscript{1,7}, and for residues preceding the self-association sequence, IC-IC contacts that are slightly populated with Tctex1/LC8 and completely absent with LC7 alone become considerably populated (Fig. 6, right). Evidence for this includes the moderate quenching of residue 204 (Fig. 2F), suggesting a degree of self-association in the quaternary complex at an IC region N-terminal to the predicted coiled-coil.

How does binding of LC7 to Tctex1/LC8-IC subcomplex reverse IC self-association in the 222–231 sequence and promote self-association at a new site? The answer lies in the inherent disorder in unbound regions of the light chain-IC complex and in the self-association sequence, for both of which the ensemble structure samples multiple, flexible, interconverting conformations that may be selected by a variety of binding partners. For example, IC segment 222–231 may self-associate by packing two helices, one from each IC chain when LC8 but not LC7 is present, or if LC7 is present, the same residues bind to LC7 as two separated helices, one from each IC chain (as in the crystal structure (27)). When LC7 binds to a Tctex1/LC8-IC complex, it likely interacts initially with helix-like forms in the conformational ensemble of residues 232–255. Presumably, these interactions out-compete IC-IC interactions in the 222–231 region, and mass action converts the IC 221–258 segment to the LC7-bound helix-turn-helix, bringing the region at or near 204 into close proximity (Fig. 6, right).

**Functional Implications of IC Self-association**—IC self-association is modulated by the length of the linker separating the LC8 consensus sequence and the self-association sequence and by interactions with the light chains. The degree of self-association varies among species. In D. melanogaster, apoIC\textsubscript{1,7} is primarily monomeric, as determined by MALS (this study) and analytical ultracentrifugation (16), and requires LC8 binding for its association. In contrast, IC from R. norvegicus appears to be a dimer without LC8 (13, 23). The linker in R. norvegicus (42 residues) and IC\textsubscript{1,7}\Delta (27 residues) is considerably shorter than in D. melanogaster (83 residues). In addition, the R. norvegicus sequence has a high occurrence of prolines immediately C-terminal to the LC8 binding region (Pro-140, -153, -155, -156, and -159) that can impose constraints on polypeptide conformations and dynamics and favor conformations suitable for oligomerization (43).

The thermodynamic coupling between LC8 binding and IC self-association suggests that a small population of self-associated dimer in apoIC will enhance LC8 binding affinity, which at the right cellular concentration may initiate the assembly process. As LC8 binding enhances IC self-association and this enhances binding affinity of other bivalent IC ligands, the resulting complex will be quite stable but readily reversible. This speculation assumes that in vivo IC binds LC8 before LC7, a reasonable assumption consistent with the findings that prebinding IC with LC8 simplifies the LC7 binding pro-
cess (19). This would explain the puzzling and apparently contradictory effects of LC8 and LC7 on IC self-association.

Polybivalency in Assembly of Disordered Protein Complexes—Although binding enhancement due to multivalency effects is common in biological systems (44), multivalency in disordered complexes is a newly described phenomenon and was first quantified in the assembly of IC with Tctex1 and LC8 (19). When the first dimeric light chain binds two IC chains, it increases the effective concentration of bivalent IC, for which a second light chain has higher affinity than for monovalent IC. Likewise, self-associated bivalent IC, which is populated to varying extents in different species and is modeled here by an engineered disulfide cross-link at residue 219 (81 residues apart from the LC8 recognition sequence), has a 6-fold binding enhancement relative to monovalent IC (Fig. 5 and Table 1). Binding enhancement is primarily of entropic origin, and thus, it is expected that any bivalent ligand binding to IC will enhance the second bivalent binding event.

However, not every bivalent ligand results in binding enhancement. Dimeric LC7 binds IC at a similar distance from LC8 as the disulfide cross-link without providing any discernable binding enhancement of IC to LC8. The most likely explanation for the absence of enhancement with LC7 is accompanying negative interactions (the loss of IC self-association within the 222–231 segment is one possibility) that offset the 6-fold gain from multivalency, seen with the cross-linked dimer.

Thus, assembled IC is an elongated, flexible polybivalent scaffold that is modulated by long range coupling between IC self-association and LC8 binding and by binding of any of its multiple bivalent ligands. Like IC, many LC8 binding partners appear to assemble by forming polybivalent scaffolds. With Nup159, for example, five LC8 dimers bind to two disordered Nup159 chains and apparently promote coiled-coil formation distant from binding (12, 24). Likewise with Swallow, LC8 dimer binds to a disordered Swallow segment and promotes coiled-coil formation, also at a distant site (45). We speculate that formation of a polybivalent scaffold is not limited to complexes involving LC8 but may be general for assembly of disordered bivalent protein complexes.

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