

Interplay of disorder and sequence specificity in the formation of stable dynein-dynactin complexes

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Condensed Running Title: *Nascent helices in dynein interactions*

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Abstract

Cytoplasmic dynein is a eukaryotic motor protein complex that, along with its regulatory protein dynactin, is essential to the transport of organelles within cells. The interaction of dynein with dynactin is regulated by binding between the intermediate chain (IC) subunit of dynein and the p150^{Glued} subunit of dynactin. Even though in the rat versions of these proteins this interaction primarily involves the single α -helix (SAH) region at the N-terminus of IC, in *Drosophila* and yeast ICs the removal of a nascent helix (H2) downstream of the SAH considerably diminishes IC-p150^{Glued} complex stability. We find that for ICs from various species, there is a correlation between disorder in H2 and its contribution to binding affinity, and that sequence variations in H2 that do not change the level of disorder show similar binding behavior. Analysis of the structure and interactions of the IC from *Chaetomium thermophilum* demonstrates that the H2 region of *C. thermophilum* IC has a low helical propensity and establishes that H2 binds directly to the coiled-coil 1B (CC1B) domain of p150^{Glued}, thus explaining why H2 is necessary for tight binding. Isothermal titration calorimetry, circular dichroism, and NMR studies of smaller CC1B constructs localize the region of CC1B most essential for a tight interaction with IC. These results suggest that it is the level of disorder in H2 of IC along with its charge, rather than sequence specificity, that underlie its importance in initiating tight IC-p150^{Glued} complex formation. We speculate that the nascent H2 helix may provide conformational flexibility to initiate binding, whereas those species that have a fully folded H2 have co-opted an alternative mechanism for promoting p150^{Glued} binding.

Statement of Significance

The intrinsically disordered N-terminal domain of the dynein intermediate chain is thought to be central to the function of this motor protein because it specifically binds cargo and also regulates the assembly and function of the whole complex through its binding to the p150^{Glued} subunit of dynactin. We have found that the structural propensity of the helix 2 (H2) region of the intermediate chain correlates with a decrease in the strength of the binding interaction with p150^{Glued} and that the H2 region interacts directly with p150^{Glued}, rather than indirectly via the single α -helix region. One possible model that explains our results is that H2 makes multiple nonspecific contacts with p150^{Glued} before the single α -helix locks into a more specific binding interaction.

Introduction

Cytoplasmic dynein is a 1.6 MDa microtubule-based motor protein complex that is essential for centrosome separation during mitosis, mitotic spindle assembly, axonogenesis, and the retrograde transport of organelles and other cellular cargo (1–3). The dynein complex is composed of pairs of subunits that segregate the roles of generating motion and cargo attachment; the heavy chain subunits contain the microtubule binding domains and generate motion via ATPase activity, whereas the intermediate chain (IC), light IC, and light chain subunits play roles in assembly and stabilization of the dynein complex, cargo attachment, and modulating dynein activity (4–6). Dynein carries out its functions by binding to various cofactors and regulatory proteins (2), including dynactin, a 1.2 MDa protein complex composed of more than 20 subunits, the largest of

which is p150^{Glued} (7). p150^{Glued} is present in two copies in the dynactin complex and forms coiled-coil domains at multiple locations, one of which, coiled-coil 1B (CC1B), directly interacts with the dynein IC (8–10). Because dynein is involved in numerous critical cellular processes, inhibition of dynein severely disrupts normal functioning of cells and, in particular, disruption of the interaction between dynein and dynactin causes a motor neuron degeneration pathology resembling amyotrophic lateral sclerosis (11). Axonal transport, autophagy, and the clearance of aggregated proteins are also affected by defective dynein regulation, which suggests that dynein misregulation plays a role in the development of several neurodegenerative diseases, including Huntington's, Parkinson's, and Alzheimer's diseases (12).

The locus of dynein's interaction with p150^{Glued} is the N-terminal region of the IC, a region that contains α -helical segments flanked by intrinsically disordered linkers (13). Although cryo-electron microscopy structures of the entire dynactin-dynein-BicD2 complex exist (14, 15), the p150^{Glued}-IC portion in those structures is invisible due to the disorder inherent in the system. Therefore, the p150^{Glued}-IC interaction is one of the final missing pieces for a complete description of the complex. Intrinsically disordered proteins (IDPs) like the N-terminal region of IC (16–18) and the C-terminal region of dynein light intermediate chain (19, 20) maintain partial disorder even when interacting with partners, providing flexibility and functional versatility.

The interactions between IC and p150^{Glued} from rat, *Drosophila*, and yeast primarily depend on a highly conserved, N-terminal single α -helix (SAH) of IC (8, 13, 21), and a coiled-coil region (CC1B) of p150^{Glued} (9, 10). Results from nuclear magnetic resonance (NMR) spectroscopy have identified a second helix (H2) (8, 21) that is nascent in *Drosophila* and yeast IC, but fully formed in rat IC (9, 13) (Figure 1A, 2B). Isothermal titration calorimetry (ITC) studies showed that whereas removing H2 did not significantly affect the binding of rat p150^{Glued} to IC (13), its removal weakened this interaction in yeast (21) and *Drosophila* (8) proteins (Table S1 in the Supporting Materials). These observations suggest that although a fully folded H2 does not contribute to binding of p150^{Glued}, a nascent H2 does play a role, although it is unclear what this role is in any of these species.

In this study, we set out to address three questions about the interaction between the IC and p150^{Glued}: Is the structural propensity of the H2 region species-dependent? Is there direct interaction between H2 and p150^{Glued}? And where on the CC1B region does IC bind? Using a helical propensity prediction algorithm, we observed that the length and location of H2 is conserved in a wide range of species (Figure 2). To explore the function and contributions of H2 in p150^{Glued} binding, we focused on proteins from the model organism *Chaetomium thermophilum* (CT), a thermophilic fungus that grows at temperatures as high as 60 °C (22). In addition to its thermostability, we selected this system because, as seen in Figure 2A, Agadir predicts only a very low level of helicity for the H2 region. ITC, together with circular dichroism (CD) and NMR, was used to establish the importance of a nascent H2 helix in the interaction between dynein and dynactin and to define the locus of IC binding on the p150^{Glued} CC1B region.

Materials and Methods

Helix prediction of ICs using Agadir

Sequences for the dynein intermediate chain from a range of species were obtained from the UniProt protein database (23). *Rattus norvegicus* (rat, UniProt: Q62871-3), *Drosophila melanogaster* (fruit fly, UniProt: Q24246-11), and *Saccharomyces cerevisiae* (yeast, UniProt: P40960-1) were selected because of the availability of biophysical data from previous studies (8,

13, 21). Additionally, six other species were selected to provide phylogenetic diversity: *Homo sapiens* (human, UniProt: O14576-2), *Danio rerio* (zebrafish, UniProt: A1A5Y4-1), *Callorhinchus milii* (Australian ghostshark, UniProt: V9KAN3-1), *Octopus bimaculoides* (Californian two-spot octopus, UniProt: A0A0L8HM30-1), *Caenorhabditis elegans* (nematode, UniProt: O45087-1), and *Chaetomium thermophilum* (thermophilic fungus, UniProt: G0SCF1-1). The first 100 amino acids of IC from each species were scored using the Agadir algorithm, which outputs a prediction for percent helicity at the residue level (24–28).

Protein expression and purification

The CT IC₈₈ (residues 1–88), IC₃₅ (residues 1–35), p150_{ABC} (residues 478–680), p150_{AB} (residues 478–622), p150_{BC} (residues 542–680), p150_A (residues 478–541), p150_B (residues 542–622), and p150_C (residues 623–680) constructs were prepared by PCR and cloned into a pET-24d vector with an N-terminal His₆ tag using the Gibson Assembly protocol (29, 30). In addition, an IC₈₈ construct with the 11 amino acid residues of the H2 region replaced by 11 residues from the H2 region of rat IC was generated by the same method. The gene for CT IC_{37–88} (residues 37–88) was synthesized and inserted into a pET-24a(+) vector by GeneScript (Piscataway, NJ). For all constructs, an internal tobacco etch virus (TEV) protease cleavage site was included to allow the removal of the His₆ tag. IC_{37–88} was engineered so that after cleavage with TEV protease, no non-native residues were present, whereas for the other constructs a non-native GAHM sequence remained at the N-terminus of the proteins after cleavage. DNA sequences were verified by Sanger sequencing. The recombinant plasmids were transformed into Rosetta (DE3) *E. coli* cells (Merck KGaA, Darmstadt, Germany) for protein expression.

Bacterial cultures for expression of unlabeled proteins were grown in lysogeny broth (LB) at 37°C to an optical density (A_{600}) of 0.6–0.8, whereas cultures for expression of isotopically-labeled (¹⁵N or ¹⁵N/¹³C) proteins were grown in MJ9 minimal media (31) at 37°C to an A_{600} of 0.8. Protein synthesis was then induced with 0.4 mM isopropyl- β -D-1-thiogalactopyranoside (IPTG), and expression proceeded overnight at 26°C. Cells were harvested from the cultures by centrifugation at 2500 rcf, lysed by sonication, and then centrifuged a second time at 24,000 rcf to clarify the lysate. The resulting supernatant was purified by immobilized metal affinity chromatography using TALON Metal Affinity Resin (Takara Bio USA, Mountain View, CA), after which the His₆ tag was cleaved using TEV protease. Proteins were further purified using a Superdex 75 (Cytiva, Marlborough, MA) size-exclusion chromatography (SEC) column or, for IC_{37–88}, a HiPrep 16/60 Sephacryl S-100 HR SEC column (Cytiva, Marlborough, Massachusetts) SEC column.

Protein concentrations were determined by measuring the absorbance at 280 nm for proteins with nonzero molar extinction coefficients at this wavelength. Molar extinction coefficients measured at 280 nm were computed for p150_{ABC}, p150_{AB}, p150_{BC}, p150_A, and p150_C, using the ProtParam tool on the ExPASy website (32). The concentrations of proteins that lack aromatic residues, including IC₈₈, IC₃₅, IC_{37–88}, and p150_B, were determined using a Bradford assay (Bio-Rad, Hercules, California), absorbance at 205 nm, and/or by comparing their band sizes on SDS polyacrylamide gels against standards. Molar extinction coefficients for absorbance measurements at 205 nm were computed with a Protein Calculator tool (33). All purified proteins were stored at 4°C with a protease inhibitor mixture of pepstatin A and phenylmethanesulfonyl fluoride and used within one week. Gels run before the use of the samples showed no evidence of proteolysis when stained with Coomassie blue, and NMR confirmed that samples were stable over the period of at least a week because additional peaks from proteolyzed fragments were not observed over this time period.

Circular dichroism measurements

CD measurements were made using a JASCO (Easton, MD) J-720 CD spectropolarimeter. Samples consisted of protein at concentrations of 5–20 μM in 10 mM sodium phosphate buffer (pH 7.5). All experiments were done using a 300 μL cuvette with a path length of 0.1 cm. The data shown are the average of three scans. Thermal unfolding data were collected in increments of 5°C over a temperature range of 5–50°C. Fractional helicity estimations based on the CD measurements were made using the following equation:

$$\text{Fractional Helicity} = \frac{(\theta_{222}^{\text{exp}} + 3000 \text{ deg cm}^2 \text{ dmol}^{-1})}{-36,500 \text{ deg cm}^2 \text{ dmol}^{-1}} \quad (1)$$

where $\theta_{222}^{\text{exp}}$ is the experimentally observed mean residue ellipticity at 222 nm (34).

Isothermal titration calorimetry

ITC experiments were conducted using a MicroCal VP-ITC microcalorimeter (Malvern Panalytical, Malvern, UK). All experiments were performed at 25°C with the protein samples in a buffer containing 50 mM sodium phosphate (pH 7.5), 50 mM sodium chloride, and 1 mM sodium azide. Samples were degassed at 25°C for 15 min before loading. Each experiment was started with a 2 μL injection and followed by 27 to 33 injections of 10 μL . In every experiment, IC constructs at a concentration of 200–300 μM were titrated into p150^{Glued} constructs at a concentration of 20–30 μM .

Analytical ultracentrifugation

To form the IC₈₈-p150_{ABC} complex, p150_{ABC} was combined with an excess of IC₈₈, after which the complex was purified using SEC on a Superdex 200 column (GE Healthcare Life Sciences, Pittsburgh, PA). The buffer conditions for the final purification were 20 mM Tris-HCl, 50 mM NaCl, 1 mM sodium azide, 5 mM DTT (pH 7.4). Samples were checked for purity and a lack of proteolysis using SDS-PAGE.

Sedimentation velocity analytical ultracentrifugation (SV-AUC) experiments with p150_{ABC} at a concentration of 80 μM and the IC₈₈-p150_{ABC} complex at a concentration of 60 μM were performed with a Beckman Optima XL-A analytical ultracentrifuge (Beckman Coulter Life Sciences, Indianapolis, IN). The buffer conditions were the same as the final purification, except that the 5 mM DTT was replaced with 5 mM tris(2-carboxyethyl)phosphine. The complex was loaded into a standard double-sector cell with quartz windows and a 1.2 cm pathlength and centrifuged at 42,000 rpm at 20°C using an An-60 Ti analytical rotor. 300 scans were acquired at 280 nm with no interscan delay. Data were fit to a c(s) distribution using the software SEDFIT (35).

NMR measurements and analysis

NMR samples were buffer exchanged into a 50 mM sodium chloride, 50 mM phosphate (pH 6.5) buffer that included 5% D₂O, 1 mM sodium azide, 0.2 mM 2,2-dimethylsilapentane-5-sulfonic acid (DSS), and 1× cOmplete™ protease inhibitor cocktail (Roche, Basel, Switzerland). All NMR spectra were collected with a sample temperature of 15°C using a Bruker (Billerica, MA) Avance III HD 800 MHz spectrometer with a TCI cryoprobe or, for IC_{37–88}, a Bruker Avance NEO 600 MHz spectrometer with a room-temperature TXI probe. Band-selective excitation short transient (BEST) variants of TROSY-based triple resonance sequences (HNCO, HN(CA)CO, HNCACB, HN(CO)CACB) were used for backbone assignment (36), whereas side-chain assignments were performed using ¹⁵N-TOCSY-HSQC and HCCH TOCSY experiments.

For IC₈₈, transverse (R_2) and longitudinal (R_1) ^{15}N relaxation rates, as well as $\{^1\text{H}\}$ - ^{15}N heteronuclear nuclear Overhauser effect enhancements (NOEs), were measured for amide nitrogens using TROSY-based experiments (37). The longitudinal relaxation measurement used delay times of 20, 60, 100, 200, 400, 600, 800, and 1200 ms; the experiments with 60 and 600 ms delay times were collected in triplicate. The transverse relaxation measurement repeated a 17 ms CPMG block 1, 2, 4, 8, 10, 12, 14, and 16 times; the experiments with 2 and 12 repetitions of the CPMG block were collected in triplicate. For the HetNOE experiment, ^1H spins were saturated by 120° radiofrequency pulses every 5 ms during the 6 s relaxation delay before the initial ^{15}N pulse. For all three dynamics experiments, the data were collected in an interleaved fashion.

^{15}N -separated NOESY-TROSY (120 ms mixing time) and ^{13}C -separated NOESY-HSQC (200 ms mixing time) experiments were used to generate NOE-based distance constraints for IC₈₈. $^3J_{\text{HNH}\alpha}$ values for IC₈₈ were estimated by measuring the intensity ratio between the cross-peaks and the diagonal peaks in a three-dimensional (3D) HNHA experiment. The accessibility of IC₈₈ amide protons to exchange with the solvent was determined from peak volumes in a Fast-HSQC spectrum with a 50 ms CLEANEX-PM mixing period (38).

Residual dipolar couplings ($^1D_{\text{HN}}$) for IC₈₈ were determined by first measuring scalar couplings ($^1J_{\text{HN}}$) for an isotropic ^{15}N IC₈₈ sample by the difference in position between peaks in TROSY and semi-TROSY spectra. These spectra were then repeated for an aligned sample to measure the sum of the residual and dipolar couplings ($^1J_{\text{HN}} + ^1D_{\text{HN}}$). IC₈₈ was aligned by soaking it into a cylindrical 6% polyacrylamide gel that was then compressed from a 5.4 mm diameter to a 4.2 mm diameter using a gel press (New Era, NJ), resulting in a gel sample that was stretched along the axis of the magnet field. Under these conditions, the splitting of the HOD peak in the ^2H spectrum was 6.4 Hz.

For measuring chemical exchange between different structural forms of p150_B, a series of TROSY-based ^{15}N ZZ-exchange experiments (39) were acquired with mixing times of 10, 50, 100, 200, and 500 ms.

NMR data were processed using TopSpin 4.0.7 (Bruker) and NMRPipe (40). For 3D experiments that employed nonuniform sampling, the spectra were reconstructed using SCRUB (41). Peak assignment and relaxation analysis were performed using CCPN Analysis 2.5.0 (42).

NMR titrations

NMR titrations were performed by combining ^{15}N -labeled IC₈₈ with unlabeled p150_{ABC}, p150_{AB}, p150_{BC}, p150_A, p150_B, and p150_C. A small stoichiometric excess (1:1.2) was used when titrating with p150_{AB}, p150_{BC}, and p150_C; the other combinations used a 1:1 ratio of binding partners. The IC₈₈ concentration was in the range of 100–150 μM for these samples.

Structure calculation

Structures were calculated using ARIA 2.3 (43), using chemical shift-matched NOESY peaks lists, backbone dihedral angle restraints generated using TALOS-N (44), residual dipolar couplings ($^1D_{\text{HN}}$), and $^3J_{\text{HNH}\alpha}$ couplings as inputs. $^1D_{\text{HN}}$ values were only used for the most rigid part of the SAH region (residues 5–28). The alignment tensor was determined to have an axial component (D_a) of -28.1 Hz and a rhombicity (R) of 0.066 by using REDCAT (45) and a preliminary structure of the SAH region that was calculated without RDCs. Approximately 25% of the NOE restraints (all of which corresponded to intraresidue or sequential contacts) were manually assigned, the remainder were automatically assigned starting from chemical shift-matched restraint lists using the ARIA protocol. The high degree of manual NOE restraint assignment was needed to achieve good convergence in the structure calculations because of the poor chemical shift dispersion for

many of the amino acid types. Without manual assignments, the high degree of degeneracy in the side-chain chemical shifts occasionally resulted in erroneous structures in which IC₈₈ was bent into a hairpin because of the ARIA protocol assigning some NOE restraints as long-distance interactions when they could be more parsimoniously interpreted as short-range restraints. The standard ARIA protocol was used except that 30 structures were calculated in each iteration (other than for iteration 8, in which 50 structures were calculated), the log-harmonic potential was enabled (46), the spin diffusion correction was enabled, and the number of simulated annealing steps were tripled in the two cooling periods (cool1 and cool2). The 20 structures with the lowest energy from iteration 8 were selected for water refinement and used to generate the structural ensemble.

Statistics describing the restraints used for the calculations and the resulting ensemble are provided in Table S2 in the Supporting Materials. Chemical shifts and restraints were deposited at the Biological Magnetic Resonance Bank (BMRB: 30633) and atomic coordinates were deposited in the Worldwide Protein Data Bank (PDB: 6PQT).

Results

Predicted H2 structural propensity varies among species

To determine how the level of helicity varies among species, we used the Agadir algorithm (24–28) for helical prediction. As the Agadir algorithm does not account for long-range or tertiary interactions, it is most useful for predicting helicity in short peptides as well as helices in otherwise intrinsically disordered regions such as the N-terminus of the IC. Predictions for the selected species illustrate a highly conserved helix in the SAH region, whereas the H2 region varies in its helical propensity (Figure 2A and 2B). Human, rat, and zebrafish IC are predicted to have a completely helical H2 region, whereas the H2 regions from Australian ghostshark and *C. elegans* are predicted to have intermediate helicity. For octopus, *C. thermophilum*, *Drosophila*, and yeast, only a very small amount of helicity (i.e., a nascent/transient helix) is predicted for the H2 region, a result that on its own is not necessarily meaningful as these values are smaller than the standard deviation for Agadir helix predictions ($\pm 6\%$). Nevertheless, NMR secondary chemical shift data for rat, *Drosophila*, and yeast are consistent with the Agadir predictions (Figure 2B) for both the SAH and H2 regions, suggesting that in the case of ICs, Agadir is able to accurately predict the location of helical regions and their degree of helicity.

In chordates, the H2 region is highly conserved, with a high degree of helicity, whereas the H2 region in non-chordates shows little conservation in either sequence or position (Figure 2C). Interestingly, despite the lack of conservation in sequence, a nascent H2 helix is predicted in all species analyzed here, suggesting that this feature is in some way functionally important.

Structural characterization of IC from *Chaetomium thermophilum*

We selected CT IC as a promising system for determining the importance of this region and its disorder in interactions with p150^{Glued}. We designed a CT IC construct that included only the SAH region (IC₃₅, containing residues 1–35) and another construct that included the SAH, the potential H2, and the intrinsically disordered linker regions (IC₈₈, containing residues 1–88; Figure 1B).

The double minima observed at 222 and 208 nm (34) in CD spectra of IC₃₅ (Figure 3A) are consistent with completely helical structure. In contrast, the shift of the first minimum to 205 nm in the CD spectra of IC₈₈ (Figure 3B) suggests that it contains a mixture of helical and disordered

regions. Analysis of the mean residue ellipticity (MRE) at 222 nm indicates that IC₃₅ and IC₈₈ have fractional helicities of approximately 80% and 35%, respectively, results that are consistent with the Agadir predictions (Figure 2A).

We used NMR spectroscopy to confirm the location of the SAH and H2 regions for IC₈₈, to establish the degree of helical propensity of these regions, and to generate a structure of the ordered regions. Complete backbone and side-chain resonance assignments for IC₈₈ (Figure 4A) show evidence for both α -helical and random-coil regions. Secondary chemical shift analysis (Figure 4C) clearly indicates α -helical structure for residues 1–30 (the SAH region) and a nascent α -helix for residues 50–64 (the H2 region). Other residues with secondary chemical shift values close to zero are intrinsically disordered.

Three-bond H_N-H _{α} scalar coupling constants ($^3J_{\text{HN-H}\alpha}$) for IC₈₈ (Figure 4D) are in the range of 4–6 Hz for residues 1–30, which implies that this region has α -helical secondary structure. The remaining residues have coupling values that are, for the most part, between 6 and 8 Hz, which is consistent with these residues lacking a fixed secondary structure. Likewise, measurements of one-bond H-N residual dipolar couplings ($^1D_{\text{HN}}$, Figure 4E) show a cluster of positive values for residues 1–30 consistent with this region forming a stable α -helix and do not show values indicative of structural propensity elsewhere. Both the $^3J_{\text{HN-H}\alpha}$ and the $^1D_{\text{HN}}$ coupling values support our prediction that the H2 region forms a nascent, rather than a fully formed, α -helix.

Using a combination of NOESY, chemical shift-based backbone dihedral angle restraints, residual dipolar coupling restraints ($^1D_{\text{HN}}$), and scalar coupling restraints ($^3J_{\text{HNH}\alpha}$, which constrains the backbone dihedral angles), we generated the structural ensemble shown in Figure 4G (see also Table S2). As expected for a protein with no tertiary structure, the NOE contact map from the final cycle of the ARIA protocol (Figure 4F) shows that most of the NOE restraints correspond to short-range intra-residue or sequential ($i\pm 1$) contacts. For the SAH region, the pattern of medium-range $i\pm 3$ and $i\pm 4$ contacts for residues 5–32 is due to contacts between protons that are one turn of helix apart from one another and is a pattern expected for an α -helical region. A similar, but weaker, pattern of medium-range contacts is seen for some residues in the H2 region.

The structures that make up the ensemble shown in Figure 4G were aligned based on residues 5–25 (within the SAH region) and show consistency in the SAH length. The portion of the H2 region that is regular enough to be rendered as a helix varies considerably from structure to structure, a result consistent with a nascent, rather than a stable, helix. As expected, the ensemble does not reveal any evidence of tertiary structure, consistent with the residues outside of the SAH and H2 regions being intrinsically disordered.

For proteins studied at high magnetic fields, longitudinal (R_1) ^{15}N relaxation rates decrease and transverse (R_2) ^{15}N relaxation rates increase for amides in more structured regions of proteins because of the increase in the effective correlation time. For IC₈₈, the decrease in R_1 (Figure 5A) and the increase in R_2 (Figure 5B) for residues 1–30 are indicative of this region having a high structural propensity relative to the rest of the protein. For residues 50–60, a smaller increase in R_2 (Figure 5B) is observed compared with residues 1–30. The large R_2 -values for 54E, 58D, and 61L (all of which are on the same face of the H2 helix) are most likely due to conformational exchange on the micro- to millisecond timescale. $\{^1\text{H}\}$ - ^{15}N heteronuclear NOE values (Figure 5C) are greatest for residues 1–30 and are somewhat increased for residues 50–60 in comparison to the more dynamic parts of the protein. The information conveyed by the R_1 - and R_2 -values is highlighted by the ratio of these rates (Figure 5D), as large values indicate less flexible (more structured) regions and values approaching unity indicate dynamic regions. Taken together, the

NMR dynamics data support a stable α -helix for the SAH region and a nascent helix for the H2 region.

Measurement of chemical exchange between the solvent and amide protons made using CLEANEX-PM mixing provides insight into the accessibility of amide protons; amide protons involved in stable hydrogen bonds exchange more slowly with the solvent and have peaks of lower intensity than protons that are not protected from exchange. The stretches of near-zero values for residues 3–30 and 51–62 in the chemical exchange data shown in Figure 5E are consistent with these regions being involved in α -helices, and the nonzero values for amides in other parts of the protein indicate that these regions are relatively disordered.

Structural characterization of CT p150^{Glued} CC1B

Studies of mammalian p150^{Glued} have shown that the site at which the IC binds is located within the CC1B region [(9); Figure 1C]. Alignment of human, *Drosophila*, yeast, and CT p150^{Glued} sequences and analysis of the coiled-coil propensity of these sequences (Figure S1 in the Supporting Materials) was used to identify residues 478–680 of CT p150^{Glued} as the CC1B region, which in the following sections we will refer to as p150_{ABC} (Figure 1D). Given that the region of the IC that binds p150^{Glued} has fewer than 80 amino acids, we presumed that only a portion of the ~200 amino acid CC1B region is actually involved in IC binding. To test this hypothesis, we engineered a series of shorter constructs that contained only portions of the p150^{Glued} CC1B region. Analysis of p150_{ABC} using COILS (47) with a window width of 21 residues revealed that the coiled-coil contains two short stretches of decreased coiled-coil propensity (Figure 1D). This prediction was used to subdivide p150_{ABC} into three smaller regions (A, B, and C), which formed the basis of the p150_{AB}, p150_{BC}, p150_A, p150_B, and p150_C constructs.

CD spectra for the CT p150^{Glued} constructs (Figure 6A) show that the full-length p150_{ABC} construct is largely α -helical but that some of the shorter constructs have less secondary structure. Analysis of the MRE at 222 nm reveals that p150_{ABC}, p150_{AB}, and p150_A are largely helical with fractional helicities of 80%, 90%, and 70%, respectively. The remaining constructs have a greater mixture of disorder and helix, with a fractional helicity of approximately 60% for p150_{BC} and less than 5% (i.e., mostly disordered) for p150_B and p150_C. These results roughly match the predicted coiled-coil propensities for these regions of CC1B (Figure 1D), which suggests that the “A” region of p150^{Glued} helps to stabilize the coiled-coil dimer. Therefore, constructs lacking this region tend to fray (p150_{BC}) and, if shortened even more, almost completely unravel (p150_B and p150_C).

We expressed ¹⁵N- and/or ¹³C/¹⁵N-labeled p150_{AB}, p150_B, and p150_{BC} samples for NMR spectroscopy. ¹H-¹⁵N correlation spectra of p150_{AB} and p150_{BC} (Figure S2 in the Supporting Materials, left and right spectra) showed far fewer peaks than what we would expect based on the number of residues. One possible explanation is that the p150_{AB} and p150_{BC} constructs form long coiled-coils that tumble anisotropically. As the N-H bond vectors for coiled-coils are nearly parallel to the long axis of the coiled-coil structure, the effective correlation time for nuclei in the folded regions of these proteins is relatively long, resulting in rapid relaxation and peak broadening (48). For both p150_{AB} and p150_{BC}, the peaks observed in the spectra are assigned to amides at the dynamic N- and C-termini of these constructs. For p150_{AB}, amide peaks for only the first ~4 and last ~20 amino acids are observable, whereas in the case of p150_{BC}, a larger number of peaks (the first ~25 and the last ~15 amino acids) appear in the ¹H-¹⁵N spectrum, a result that correlates with the greater disorder and lower helicity observed in the CD spectrum of p150_{BC} (see Figure 6A).

Peaks from residues 480–600 for the p150_{AB} construct and 570–665 for the p150_{BC} construct are not readily observed, implying that these regions form the coiled-coil structure. As these regions overlap, this suggests that the reason that we see peaks for the C-terminal amides of p150_{AB} and for the N-terminal amides of p150_{BC} is because the coiled-coil structure has been destabilized at the termini of the constructs and not because these residues are intrinsically disordered in the full-length construct. This implies that in the p150_{ABC} construct we would only observe peaks from the disordered ~4 amino acids at the N-terminus and ~15 amino acids at the C-terminus.

Attempts were made to further study the p150_{BC} construct by generating a ²H/¹³C/¹⁵N-labeled sample. Although many additional peaks with greater chemical shift dispersion (data not shown) were observed in the ¹H-¹⁵N TROSY spectrum for this deuterated sample, these peaks were relatively broad and not observable in 3D backbone assignment experiments. Because p150_{BC} is structurally unstable at higher temperatures, increasing the sample temperature to narrow the peaks was not successful.

NMR spectra of the shorter p150_B construct were readily assigned using TROSY-based 3D backbone experiments. Analysis of the backbone chemical shifts indicated that all of p150_B was disordered rather than α -helical, a result that is consistent with the degree of disorder predicted from its CD spectrum (Figure 6A). Interestingly, a second set of peaks for p150_B was observed with somewhat greater chemical shift dispersion (labeled in red, Figure S2, center), suggesting that this minor conformation has more helical propensity. ¹⁵N ZZ-exchange spectra established the time-scale for interconversion between these two forms to be on the order of 100 ms (data not shown). This more structured minor conformer of p150_B explains why an interaction was observed between p150_B and IC₈₈ in the NMR titration and ITC data (see following section) despite the major conformer of p150_B being disordered.

Characterization of the IC-p150^{Glued} interaction

Previous ITC studies on rat IC and p150^{Glued} (13) reported a dissociation constant (K_d) on the order of 10 μ M (Table S1), whereas our results for the CT versions of these proteins revealed binding that is apparently stronger and substantially more complex than in the mammalian system (Figure 7A). In comparison, the thermogram for IC₃₅ binding to p150_{ABC} (Figure 7A, middle) showed that removing H2 and the disordered linker regions surrounding it (residues 30–50 and 60–88) drastically diminished the second step. These differences suggest that some combination of the H2 and linker regions is important for strong interactions with p150^{Glued}. Fitting results for the ITC curves in Figure 7A are provided in Table S1, but we caution that the numerical values are model dependent and should be treated with the usual caveats for interpreting multiphasic ITC fits (49).

We also characterized binding using SV-AUC. SV-AUC experiments clearly show a peak for the p150_{ABC}-IC₈₈ complex that is significantly shifted from the peak for free p150_{ABC} (Figure 7C) consistent with the tight binding of the complex observed with ITC as well as the existence of only a single oligomeric state.

To probe whether the contribution to the IC-p150^{Glued} interaction by H2 is sequence specific, we generated a chimeric IC₈₈ construct (Figure 7B), referred to in the following as IC_{88C}, in which 11 amino acids of the CT H2 sequence were replaced by 11 (out of 15) amino acids from the rat H2 sequence. Although in rat IC, the H2 region is an ordered helix, this helix is destabilized in the chimeric construct; the CD spectrum for IC_{88C} is nearly identical to the spectrum for the native IC₈₈ construct (data not shown), indicating that the level of helicity for the H2 region is the same for both IC₈₈ and IC_{88C}. As shown to the right of Figure 7A, the thermogram for IC_{88C} binding to

p150_{ABC} is almost identical to the thermogram for the native IC₈₈ sequence (Figure 7A, left), a result that implies that the interaction between H2 and p150^{Glued} may depend on the charge distribution or the helical propensity of H2, rather than its specific sequence. Although this binding behavior could also be due to the disordered linker regions flanking H2, our NMR data strongly suggest that it is H2, and not the linkers, that plays the more significant role (as explained in the following section and shown in Figure 7D).

ITC was also used to broadly determine the regions necessary for binding between IC₈₈ and the fragments of p150_{ABC} (Figure 6B). p150_{BC} is the only construct that bound IC₈₈ in a similar two-step manner as p150_{ABC}, whereas for p150_{AB} and p150_A, only weak binding was observed (Table S1). Further isolation of the region of p150_{BC} involved in binding IC was not successful because shortening the sequence to form the p150_B and p150_C constructs completely destabilized their coiled-coil structures, leading to weak (p150_B) or negligible (p150_C) binding at the concentrations used for ITC. The persistence of a weak interaction between IC₈₈ and p150_B despite the disorder of the p150_B construct is most likely due to the presence of the minor, partially folded conformer that we observed in the NMR spectra (Figure S2, middle).

H2 of IC directly interacts with p150^{Glued}

NMR spectra of ¹⁵N-labeled IC₈₈ with excess of the p150^{Glued} constructs identify the regions of CT IC involved in binding (Figure 6C). Peak volume ratios close to unity indicate regions of IC₈₈ that are mostly unaffected by the addition of the p150^{Glued} constructs, whereas decreased or zero values indicate regions that are either directly involved in binding or are adjacent to regions that bind. Upon addition of p150_{ABC}, p150_{AB}, and p150_{BC}, peaks from IC₈₈ amides in both the SAH and H2 regions, as well as the disordered linker between them, completely disappear in the NMR spectra. Addition of the smaller (and more weakly binding) p150_A and p150_B constructs show similar disappearances of peaks, except for the interhelix linker region, which shows only reduced peak intensity. For samples that we tested with a substoichiometric ratio of the binding partners (i.e., an excess of IC₈₈), the disappearance of the peaks was intermediate between the unbound and fully bound samples, indicating that the binding process occurs on a timescale that is fast compared with the NMR experiment (data not shown). Furthermore, although they have different binding affinities, the protein concentrations in the NMR samples are significantly above the K_d for all of them, and therefore, we would not expect to see a difference in the spectra of the bound proteins. Intriguingly, whereas ITC showed no measurable interaction between p150_C and IC₈₈, and CD showed that p150_C is almost completely disordered, the higher protein concentration used for the NMR experiments revealed a weak interaction between p150_C and IC₈₈, resulting in the disappearance of peaks from the H2 region but not the SAH region. This result suggests that it is the H2 region and not the neighboring linkers that interacts with p150^{Glued} and that this interaction is direct rather than mediated by the SAH region. Whether the H2 region of IC₈₈ interacts with unfolded p150_C or with a more folded minor conformation populated at NMR concentrations cannot be established with the data that we have at present, but the fact that peaks for the H2 region disappear in all of the NMR titrations suggests that it plays an important role in binding p150^{Glued}.

To confirm that the H2 region does actually interact directly with p150^{Glued} and not in an indirect manner dependent on the SAH region, we investigated a CT IC construct consisting of only residues 37–88 (IC_{37–88}). This construct contains the H2 region and the neighboring linker regions but does not contain the SAH region. Except for residues near the N-terminus, the peaks in the spectra for this construct were at identical chemical shifts as for IC₈₈, indicating that the structure of the H2 and linker regions was unchanged relative to the larger construct. An NMR

titration of ^{15}N -labeled IC_{37–88} with unlabeled p150_{ABC} (Figure 7D) shows a complete loss of signal for the H2 region and that signal for the linker regions diminishes depending on how close the linker residues are to the H2 region. This confirms that the H2 region directly interacts with p150_{ABC} and strongly suggests that reduced peak volumes for residues in the linker regions (as seen in Figures 6C and 7D) are a result of the reduced mobility of these residues due to their proximity to regions that bind p150_{ABC} rather than due to a direct interaction between these residues and p150_{ABC}.

To elucidate the importance of H2 to the stability of the p150^{Glued}-IC complex, we performed temperature dependence experiments on IC₃₅, IC₈₈, and p150_{ABC} as well as complexes of these proteins (Figure 8). The CD spectra for individual proteins show a rapid decrease in helicity as the temperature is increased (222 nm becomes less negative, Figure 8A–D). On the other hand, complexes of p150_{ABC} with IC₃₅ or IC₈₈ have increased thermal stability (Figure 8E,F) indicating that these proteins are co-stabilizing, with the IC₈₈-p150_{ABC} complex, which contains the H2 region of IC, showing higher stability. A comparison of the temperature dependent 222 nm signal between the complexes and free proteins (Figure 8A) clearly illustrates that the IC₈₈-p150_{ABC} complex has the highest stability. Although these CD data alone cannot rule out a role for the disordered linker regions of IC₈₈ in stabilizing the complex, together with the NMR data, they strongly suggest that the H2 region promotes the formation of a more stable IC-p150^{Glued} complex.

Discussion

The 300 amino acid N-terminal domain of IC is primarily disordered except for a short SAH within residues 1–40, which is the primary binding site to dynactin p150^{Glued}. Linked to the SAH by a short, disordered linker is helix H2, which in rat IC is fully ordered but does not contribute to binding of p150^{Glued} (13), whereas in yeast and *Drosophila* ICs (8, 21), H2 is disordered and its removal drastically diminishes IC binding to p150^{Glued}. In this work we use the IC from *Chaetomium thermophilum* as a tractable model for ICs with disordered H2 regions to establish the importance of H2 in IC-p150^{Glued} interactions.

The structural propensity of H2 varies among species

Across a wide range of eukaryotes, the N-terminal region of IC has a well-defined helical region (SAH) and a secondary helix (H2) that varies in structural propensity. Although the location, sequence, and size of H2 varies among non-chordate species, structural propensities for this region based on predictions and NMR data are consistent with a nascent helix. Along with this, binding to p150^{Glued} in rat is largely unaffected by the removal of the folded H2 region (13) whereas binding to p150^{Glued} drastically weakens in *Drosophila*, yeast (8, 21) and, as shown here, CT when the nascent H2 region is removed. Binding to p150_{ABC} is significantly stronger for IC₈₈ than it is for the IC₃₅ construct, which only contains the SAH region (Figure 7A), suggesting that disorder in H2 is species dependent and that this disorder is correlated with its contribution to binding affinity.

H2 is involved in a direct interaction with p150^{Glued}

Whereas previous work with *Drosophila* and yeast IC indicated that H2 is somehow important for tighter binding between the IC and p150^{Glued}, it was never clear whether H2 was involved in direct interactions with p150^{Glued}, or packed against the SAH region (8, 21), as shown in the binding model of Figure 9A. The existence of a p150^{Glued} construct (p150_C) that binds the H2

region, but not the SAH region provides, for the first time, to our knowledge, evidence that in CT IC, the H2 region binds directly to p150^{Glued}, leading us to propose a new model for IC-p150^{Glued} binding (Figure 9B).

Interestingly, the H2 region is affected in titrations with all six p150^{Glued} constructs (Figure 6C), despite regions “A”, “B”, and “C” having no obvious sequence homology. Likewise, replacing the CT H2 region with the rat H2 region does not diminish binding to p150^{Glued} (Figure 7A, right). Taken together, these results indicate that the interaction between H2 and p150^{Glued} is not sequence-specific. Most likely, this nonspecific interaction is electrostatic in nature because over 40% of both the CC1B region of p150^{Glued} and the H2 region of CT and rat IC consists of charged residues (D, E, L, K). The dominance of electrostatic interactions in driving CT IC-p150^{Glued} binding is consistent with the observation that mammalian IC-p150^{Glued} complex formation is sensitive to salt, temperature, and pH (9). Interestingly, despite the nonspecific nature of the interaction between the H2 region and p150^{Glued}, the H2 region is not completely promiscuous in binding to coiled coils; when we titrated ¹⁵N-labeled IC₈₈ with SWADIMER(205–275), a stable 71-residue parallel homodimeric coiled-coil with a similar percentage of charged residues (50), no change in the IC₈₈ NMR spectrum was observed (data not shown). This shows that there is some degree of specificity to the “nonspecific” interaction between the H2 region of CT IC and the various p150^{Glued} constructs that we have studied.

One possible model for IC-p150^{Glued} binding is that the H2 region is involved in scanning the CC1B region via relatively nonspecific electrostatic interactions (Figure 9C) until the SAH region locks into a more specific interaction with p150^{Glued} (Figure 9B) in a manner analogous to how DNA binding proteins slide or hop along the DNA double helix until locking onto a specific binding site (51, 52). We speculate that the nascent H2 helix provides conformational flexibility that supports this function, whereas other organisms (chordates) have developed or co-opted an alternative mechanism for promoting p150^{Glued} binding that does not depend on the H2 region having a low structural propensity.

Regions “B” and “C” of p150^{Glued} are the main locus of IC binding

The majority of the binding interface is located within the “B” and “C” regions, as shown by the ability of the p150_{BC} construct to replicate almost the entirety of the IC-p150_{ABC} thermogram (Figure 6B). The main difference, an initial increase in heat magnitudes seen in the p150_{BC} thermogram, may represent the stabilization of the coiled-coil structure of p150_{BC} in the final complex. We hypothesize that the “A” region of p150^{Glued} is essential for stabilizing the coiled-coil structure of the CC1B region, as the CD data show that it is the only one of the shorter p150^{Glued} constructs that is helical by itself, a result that is consistent with the coiled-coil propensity predictions for the regions of p150^{Glued} shown in Figure 1D.

The IC binding site on p150^{Glued} is unlikely to include the entire CC1B region, but evidence points to a majority of the region being required for strong binding in CT. The “BC” region is sufficient to replicate the binding of the full construct, whereas p150_A and p150_{AB} only show weak binding with CT IC even though they are fully folded. This suggests that although it is likely that there are a number of nonspecific electrostatic interactions that occur before the formation of a stable complex, the B and C regions contain the majority of the binding sequence that is essential for formation of a tight IC-CC1B complex.

Conclusions

The N-terminal region of the CT IC contains a helical SAH region, consistent with all ICs studied to date, as well as a nascent helix in the H2 region. Our predictions for the structural propensity of H2, along with experimental structural characterization of ICs, suggests that the extent of helicity is species-dependent and that low helical propensity is correlated with its importance in promoting tight complex formation with the CC1B region of dynactin p150^{Glued}. Our results suggest that the CT IC binding site on p150^{Glued} is within regions “B” and “C” (and most likely in the range of residues 550–650, which roughly corresponds to residues 410–510 in human p150^{Glued}). The intriguing observation that the H2 region in CT interacts with all subsections of CC1B suggests that the disorder in H2 may form initial nonspecific contacts with p150^{Glued} before the SAH region locks into a specific interaction with CC1B. Organisms with folded H2 regions of IC, however, could have evolved alternative mechanisms to initiate and stabilize IC-p150^{Glued} binding. Indeed, for rat IC, which has a folded H2, its interaction with p150^{Glued} is regulated by phosphorylation (13, 53), making it tantalizing to suggest that disorder in H2 underlies regulation of binding in the absence of phosphorylation.

One intriguing aspect of the CT IC interactions with p150^{Glued} is the uncharacteristically high affinity observed when compared with mammalian IC-p150^{Glued} interactions. In the latter, even when in the full complex, dynactin and dynein required an adaptor such as BicD2, Rab11-FIP3, Spindly, or Hook3 to enhance the stability of the complex that dynein forms with dynactin, leading to highly processive motility (54). In contrast, yeast dynein shows directional motion on its own, and its processivity is enhanced upon binding dynactin (55). Based on the very tight binding observed in CT, we propose that adaptors such as BicD2, which add another layer of regulation in the mammalian system, may not be needed when the disordered H2 is involved in a direct interaction with p150^{Glued}.

Author Contributions

N.M.L., S.S., N.E.J., and E.B. designed the study. N.M.L., S.S., N.E.J., and K.J. performed the research and analyzed the data. N.M.L., S.S., and E.B. wrote the manuscript with input from all authors. E.B. oversaw the study.

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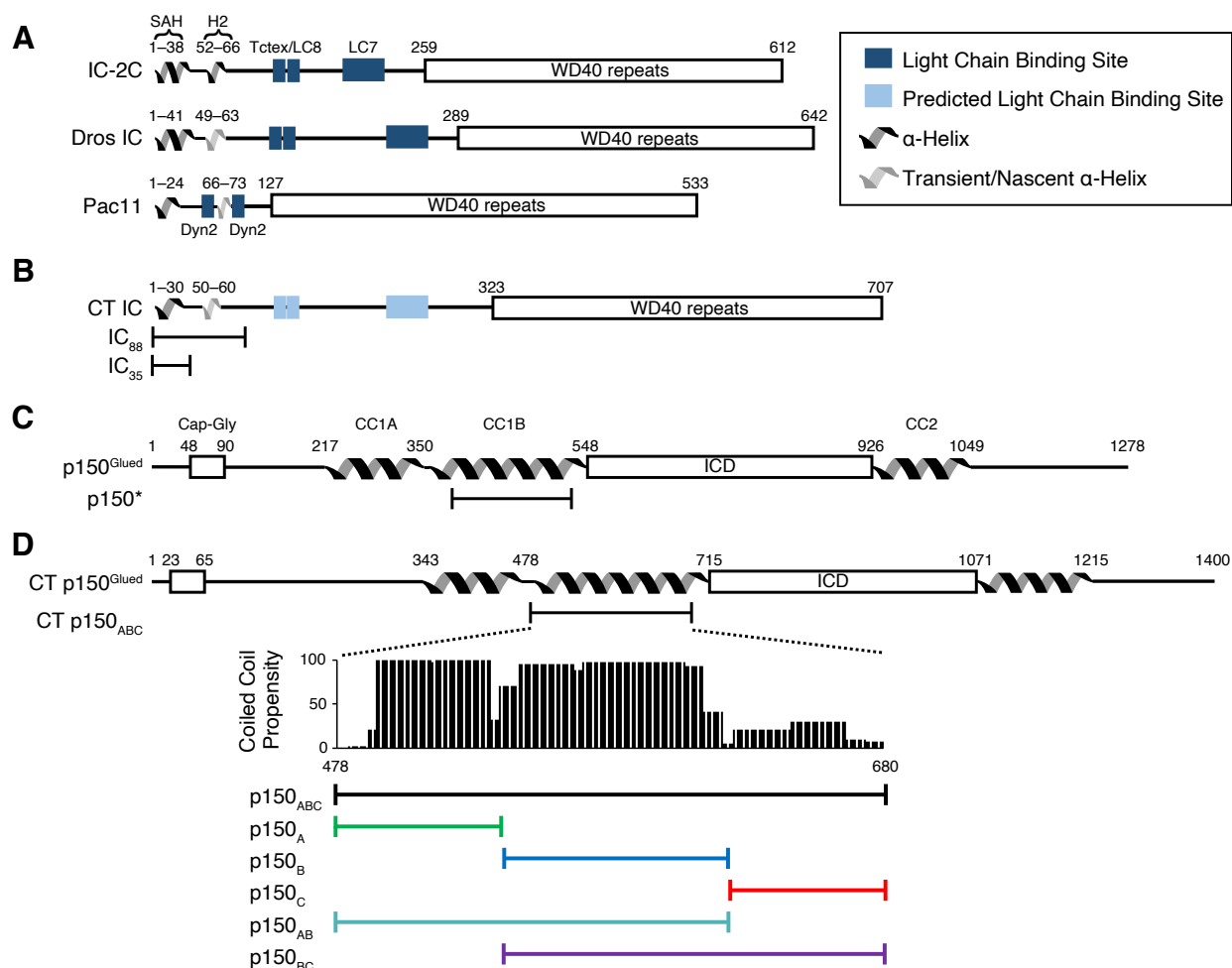


Figure 1: Dynein intermediate chain and dyactin p150^{Glued} domain architecture

(A) ICs from rat (IC-2C), *Drosophila* (Dros IC), and yeast (IC homolog Pac 11) have an N-terminal single α -helix (SAH) as well as a second helix [H2, (8, 13, 21)]. H2 is either fully formed (black helix) or transient/nascent (grey helix). Rat and *Drosophila* IC have binding sites for three light chains (Tctex, LC8, and LC7), whereas the yeast IC has binding sites for two copies of an LC8 homolog (DYN2). The C-terminal region for all ICs studied is predicted to contain seven WD40 repeat domains.

(B) The IC from *Chaetomium thermophilum* (CT IC) is predicted to have an α -helical SAH region and a nascent helix in the H2 region. Based on sequence motifs, the Tctex, LC8, and LC7 binding sites are predicted to be in regions similar to Dros IC. IC₈₈ and IC₃₅ are the constructs used in this work.

(C) Mammalian p150^{Glued} has a Cap-Gly domain near the N-terminus, and two coiled-coil domains, CC1 and CC2, that are separated by an intercoil domain (ICD). CC1 is further divided into two regions called CC1A and CC1B. The construct p150* was used in previous studies.

(D) CT p150^{Glued} is predicted to have similar domains as mammalian p150^{Glued}. The COILS Server prediction tool was used to predict the coiled-coil propensity of each residue in the CC1B region (p150^{ABC}), which informed the selection of the smaller p150^{Glued} constructs.



(A) Residue-level percent helicity predictions generated using the Agadir algorithm for the first 100 amino acids of IC from *Homo sapiens* (human), *Danio rerio* (zebrafish), *Callorhinchus milii* (Australian ghostshark), *Octopus bimaculoides* (Californian two-spot octopus), *Caenorhabditis*

elegans (nematode), and *Chaetomium thermophilum* (thermophilic fungus). The insets for *O. bimaculoides* and *C. thermophilum* show detail for the predicted H2 region.

(B) The top graphs show the percent helicity prediction for the ICs from *Rattus norvegicus* (rat), *Drosophila melanogaster* (fruit fly) and *Saccharomyces cerevisiae* (yeast). The insets for *Drosophila* and yeast show detail for the predicted H2 region. The bottom graphs show NMR secondary chemical shift data for IC constructs from rat, *Drosophila*, and yeast from prior studies (8, 13, 21).

(C) Sequence alignments of ICs from chordates (human, rat, zebrafish, and shark) and from nonchordates (octopus, fruit fly, CT, and yeast) using the MAFFT alignment program (56–58). Highlighted residues show predicted (light blue) and experimentally determined (dark blue) H2 regions. Identical (asterisk), strongly similar (colon), and weakly similar (period) residues are shown at the bottom of each alignment.

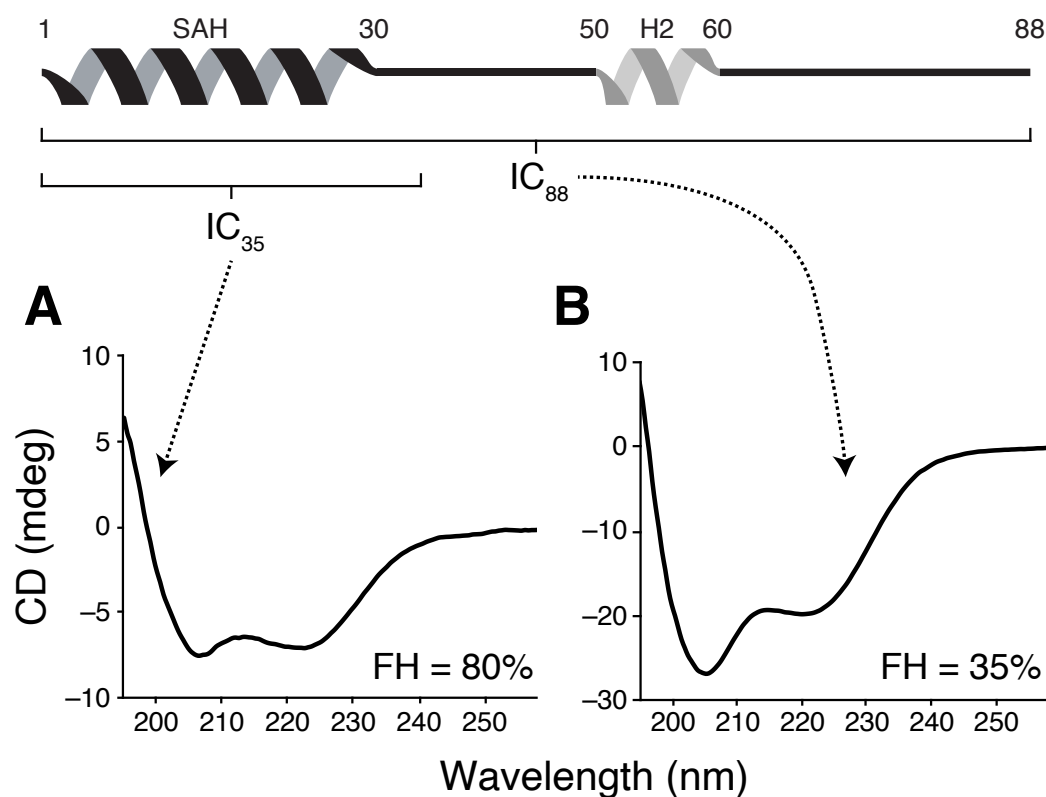


Figure 3: CD spectra of IC₃₅ and IC₈₈ at 15°C.

The CD spectrum of IC₃₅ (A) shows double minima at 222 and 208 nm consistent with a completely helical structure, whereas the shift of the first minimum for IC₈₈ (B) to 205 nm suggests that its structure consists of a mixture of helical and disordered regions. FH indicates the fractional helicity estimated at 222 nm using Eq. 1.

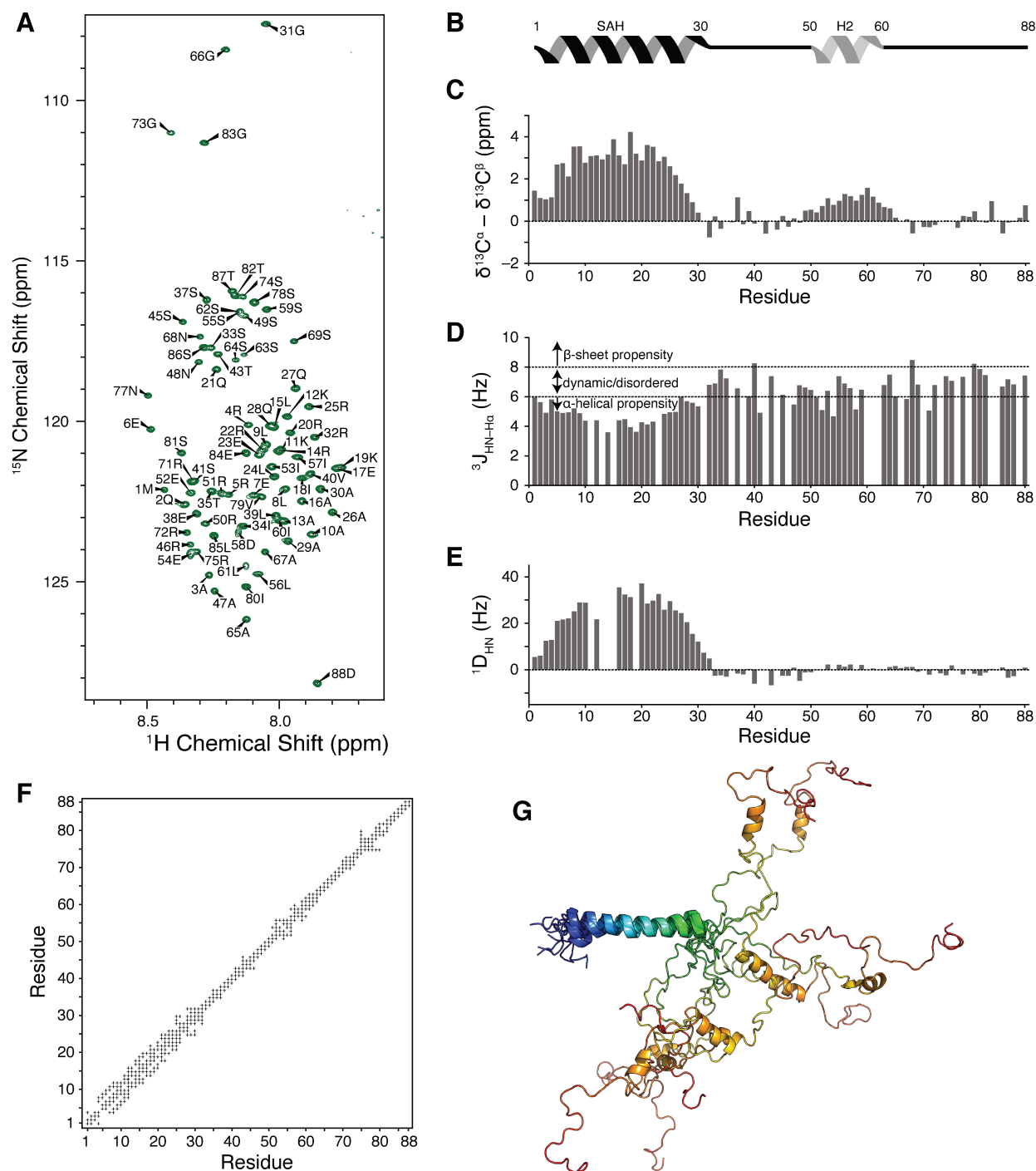


Figure 4: NMR assignments, secondary chemical shifts, couplings, and structural ensemble for IC₈₈

(A) ^{15}N - ^1H TROSY-HSQC spectrum of IC₈₈ acquired at 800 MHz for ^1H showing assignments for the amide resonances. (B) Schematic structure for IC₈₈ showing the location of the α -helical SAH region and the nascent α -helix H2 region. (C) Secondary chemical shifts for IC₈₈; clusters of residues with positive values correspond to α -helical regions, whereas values close to zero

correspond to intrinsically disordered regions. (D) Three-bond H_N - H_α scalar coupling constants for IC₈₈ calculated using cross-peak/diagonal peak intensity ratios from a 3D HNHA experiment. The predominance of coupling values between 6 and 8 Hz for residues 30–88 is consistent with those residues being dynamic or disordered. (E) One-bond H-N residual dipolar couplings measured using an IC₈₈ sample in a stretched gel. The cluster of positive values for residues 1–30 is consistent with this region forming a stable α -helix. (F) NOE contact map for IC₈₈ after using ARIA to assign NOE peaks. Most of the NOE contacts are either intra-residue or sequential ($i\pm 1$). The pattern of medium-range contacts ($i\pm 3$ and $i\pm 4$) observed in the SAH region (residues 1–30) is consistent with α -helical secondary structure. (G) IC₈₈ structural ensemble calculated using a combination of NOE restraints, scalar coupling-based torsion angle restraints, residual dipolar couplings, and chemical shift-based torsion angle restraints. The 20 lowest-energy structures from 50 structures calculated in the final iteration of the ARIA protocol were then refined in explicit water and used to generate the ensemble. The N-terminus is colored blue, whereas the C-terminus (residue 88) is colored red. For purposes of clarity, only 10 of the 20 structures used to define the structural ensemble are shown here with residues 5–25 aligned.

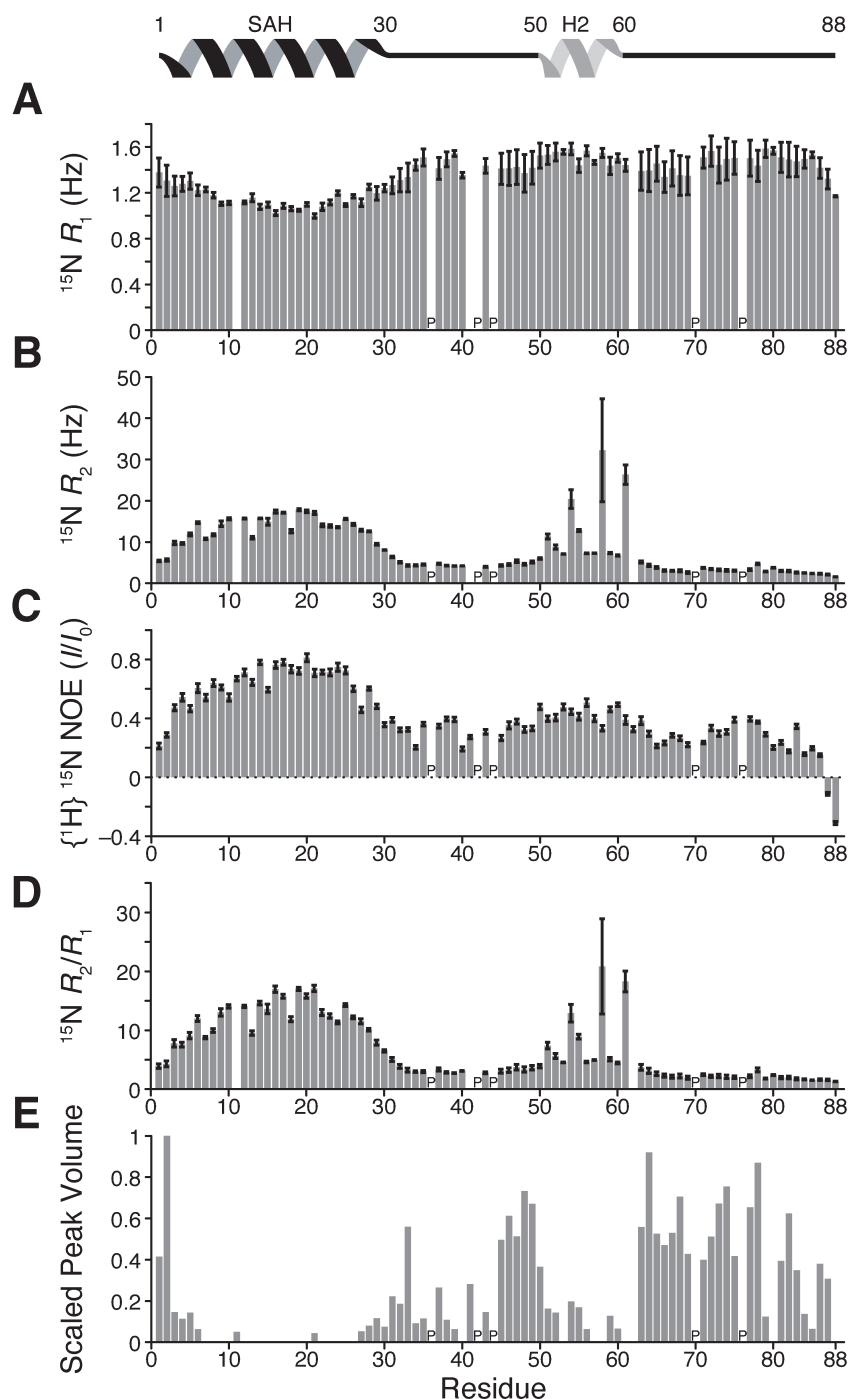


Figure 5: NMR relaxation and chemical exchange data for IC₈₈

IC₈₈ NMR relaxation data acquired at 800 MHz for ^1H includes (A) ^{15}N longitudinal relaxation rates, (B) ^{15}N transverse relaxation rates, and (C) $\{^1\text{H}\}$ ^{15}N heteronuclear nuclear Overhauser effect enhancements. The location of proline residues, which do not provide a signal in these experiments, are indicated by “P”. The ratio of the transverse and the longitudinal relaxation rates (R_2/R_1) shown in (D) is expected to be close to unity for portions of IC₈₈ that have a short

correlation time (i.e., regions that are highly dynamic), whereas larger values for this ratio are observed for more rigid regions. (E) Chemical exchange data for IC₈₈ measured using CLEANEX-PM; positive values indicate amide protons that exchange with the solvent, whereas zero or near-zero values indicate amide protons that are protected from exchange. The stretches of near-zero values for residues 3–30 and 50–60 are consistent with α -helical secondary structures in these regions. For (A) and (B) the error bars are the standard errors of the R_1 and R_2 values derived from fitting the data; for (C) the error bars are from propagating the root-mean-square deviations of the noise floors of the saturated and control spectra; for (D) the error bars are from propagating the errors in the R_1 and R_2 values.

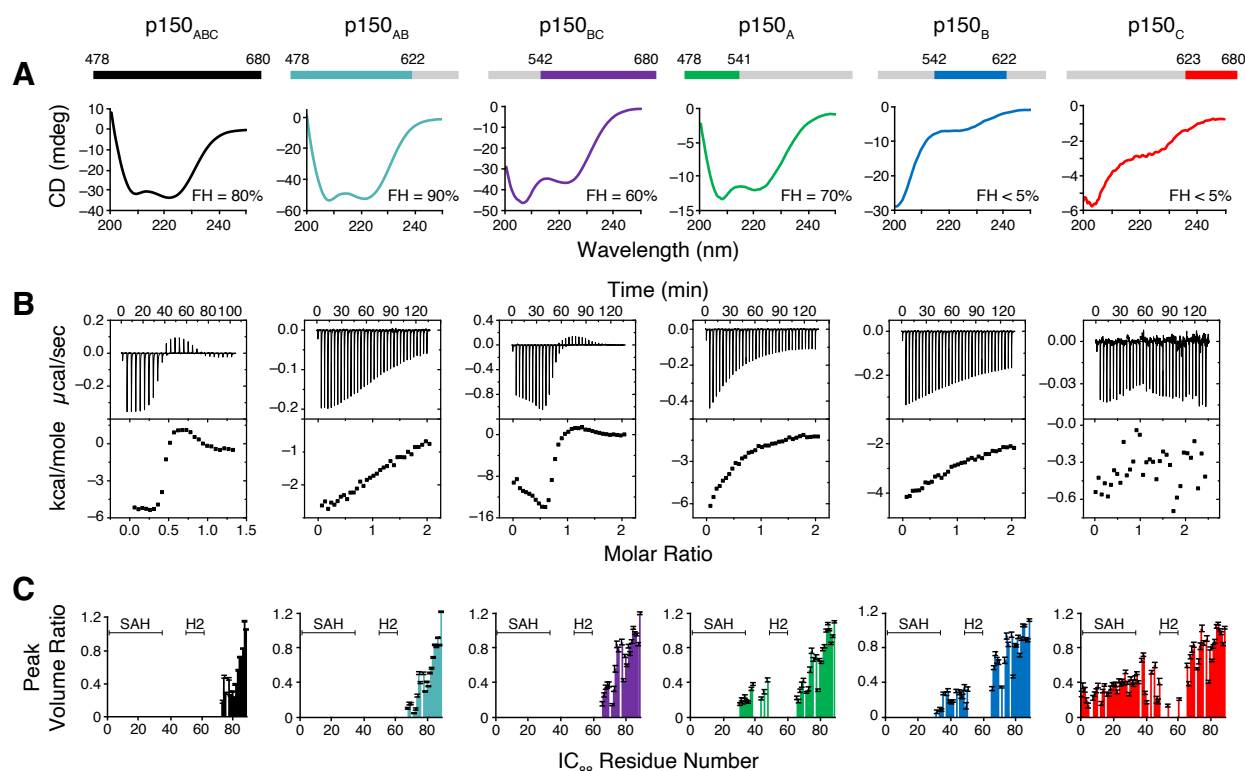


Figure 6: Identification of minimal sequence requirements for folding and binding using CD, ITC, and NMR titrations

(A) CD spectra at 25°C for p150_{ABC}, p150_{AB}, p150_{BC}, p150_A, p150_B, and p150_C; FH indicates the fractional helicity estimated at 222 nm using Eq. 1. The colored bars above the CD spectra graphically represents the portion of the CC1B region (residues 478–680) used for each construct.

(B) ITC thermograms (top) and binding isotherms (bottom) from titrations of p150_{ABC}, p150_{AB}, p150_{BC}, p150_A, p150_B, and p150_C with IC₈₈.

(C) ¹H-¹⁵N spectra peak volume ratios from titrations of ¹⁵N-labeled IC₈₈ with unlabeled p150_{ABC}, p150_{AB}, p150_{BC}, p150_A, p150_B, and p150_C. All data were acquired at 800 MHz for ¹H. Samples for p150_{ABC}, p150_A, and p150_C were at an IC to ligand molar ratio of ~1:1. Samples for p150_{AB}, p150_{BC}, and p150_B were at an IC to ligand molar ratio of 1:1.2.

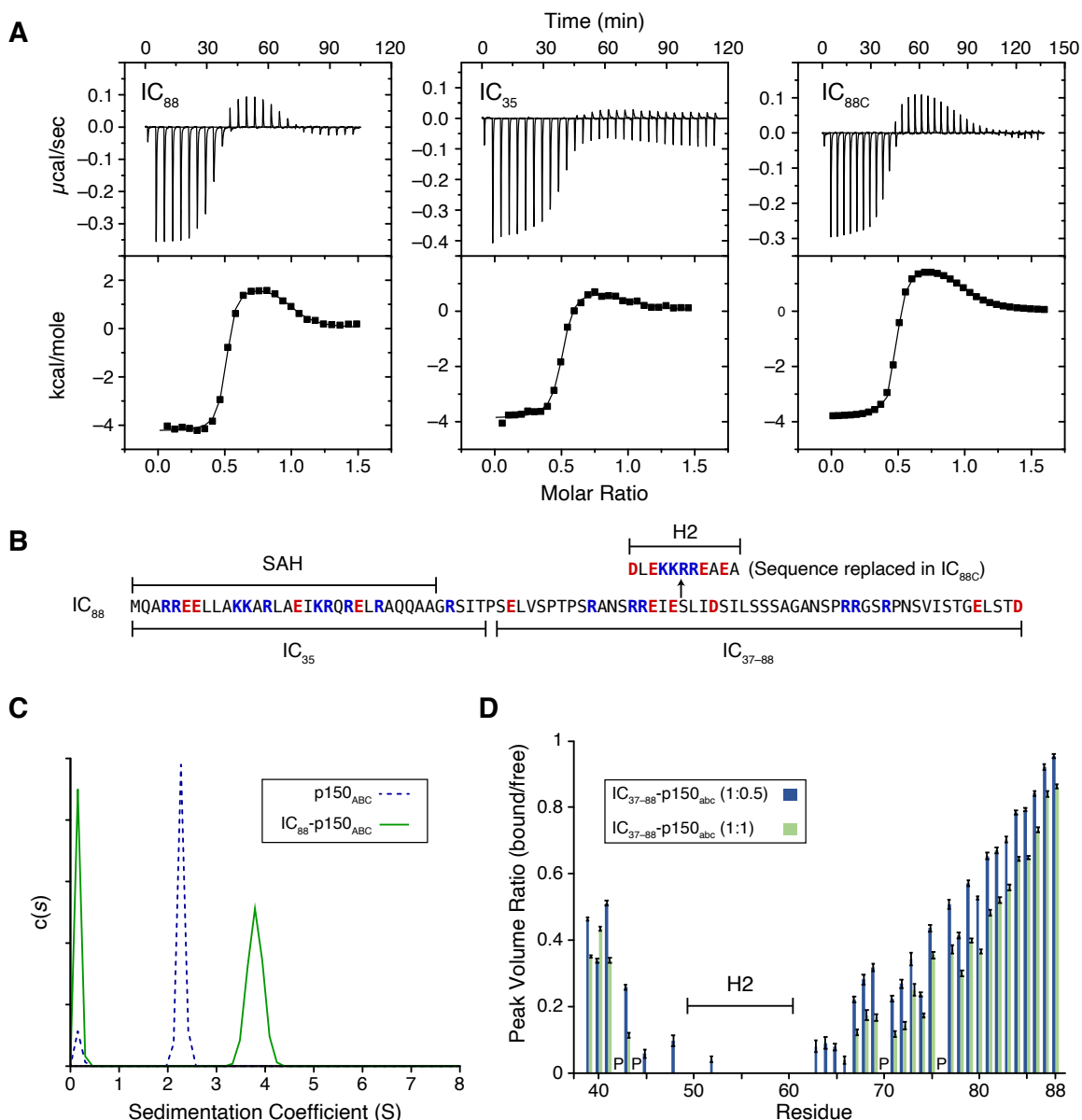


Figure 7: Interactions of CT IC constructs with p150_{ABC}

(A) Representative thermograms (top) and binding isotherms (bottom) from ITC titrations of IC₈₈ (left), IC₃₅ (middle), and IC_{88C} (right) with p150_{ABC} collected at 25°C (pH 7.5). (B) The sequence for IC₈₈ illustrating which amino acids in the H2 region are replaced in the IC_{88C} construct. The IC₃₅ construct consists of the N-terminal 35 amino acids of IC₈₈, and the IC₃₇₋₈₈ construct consists of the C-terminal 52 amino acids of IC₈₈. Basic residues (K, R) and acidic residues (D, E) are indicated in blue and red, respectively. (C) SV-AUC of free p150_{ABC} (80 μM, blue dashed line) and the SEC-purified IC₈₈-p150_{ABC} complex (60 μM, green solid line) shows that the IC₈₈-p150_{ABC} forms a stable complex with an apparent single oligomeric state. No data are collected for IC₈₈ for comparison because it has no absorbance at 280 nm. (D) ¹H-¹⁵N spectra peak volume ratios from titrations of 240 μM ¹⁵N-labeled IC₃₇₋₈₈ with unlabeled p150_{ABC} acquired at 600 MHz for ¹H. Samples were prepared with a substoichiometric (1:0.5, blue) or a stoichiometric (1:1, green) ratio of IC to p150_{ABC}. The location of the four prolines in IC₃₇₋₈₈ are indicated by "P".

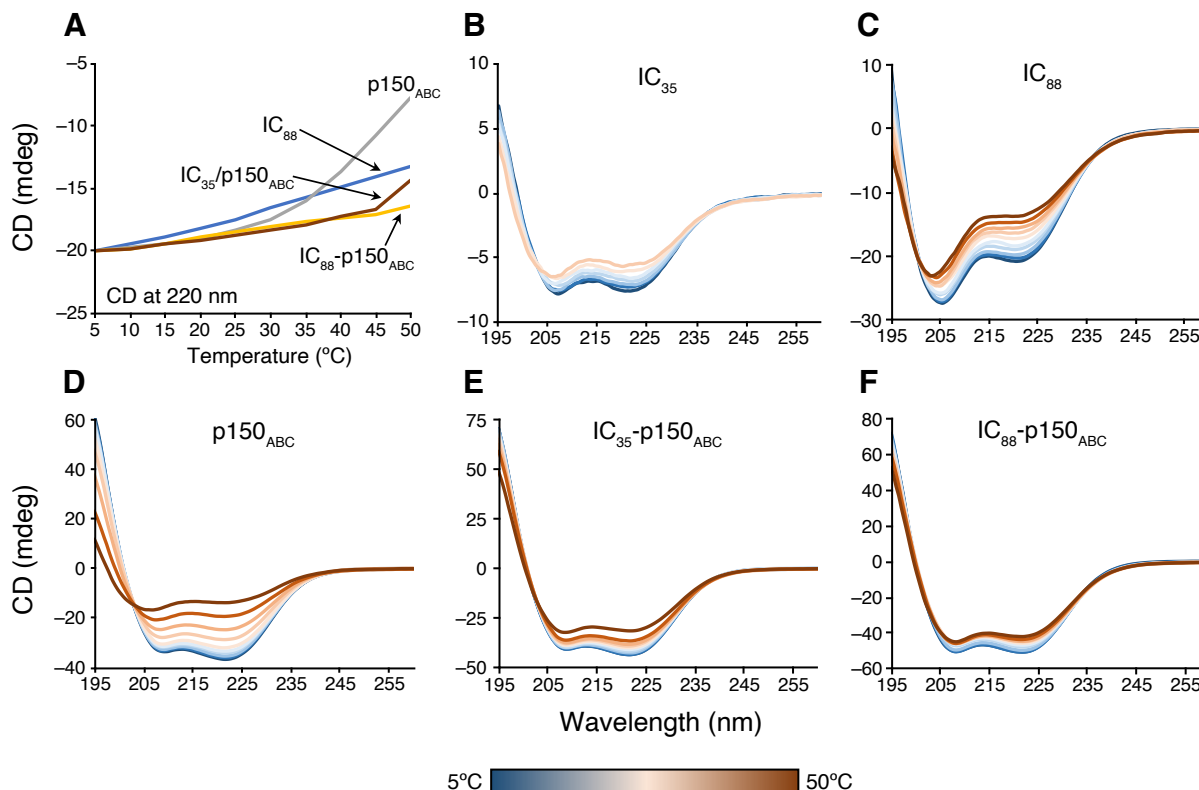


Figure 8: Complex-dependent secondary structure stability detected using temperature-dependent CD

(A). CD signal (in millidegrees, mdeg) as a function of temperature at 220 nm for IC_{88} , $p150_{ABC}$, IC_{88} bound with $p150_{ABC}$, and IC_{35} bound with $p150_{ABC}$. The curves on this graph are scaled so that they align at low temperatures.

(B-F) Temperature-dependent CD spectra of (B) IC_{35} , (C) IC_{88} , (D) $p150_{ABC}$, (E) $IC_{35}-p150_{ABC}$, and (F) $IC_{88}-p150_{ABC}$ collected at temperatures between 5 and 50 $^{\circ}C$.

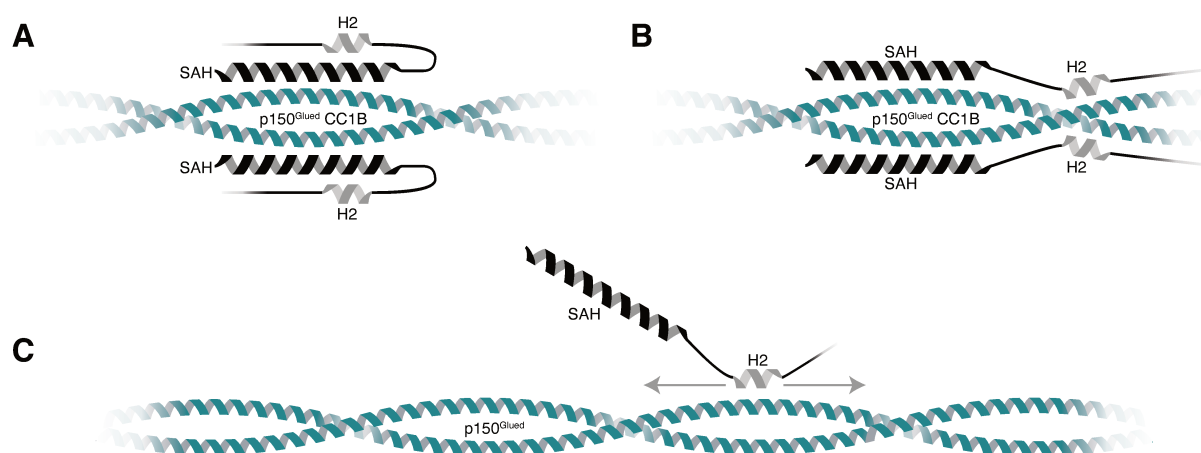


Figure 9. Models for the binding interaction between IC and p150^{Glued}

(A) Current model for IC-p150^{Glued} binding, in which only the SAH region interacts directly with the p150^{Glued} CC1B region (13, 21). (B) New model based on our NMR results for CT IC-p150^{Glued} binding, in which both the SAH and H2 regions directly interact with p150^{Glued}. For clarity, only a small portion of the ~200 amino acid long p150^{Glued} CC1B region is shown. (C) The observation of nonspecific electrostatic interactions between H2 and p150^{Glued} CC1B suggest a speculative model in which H2 is able to interact nonspecifically with the CC1B region of p150^{Glued} until the SAH region locks to a specific binding site, resulting in the configuration modeled in (B).

Supporting Material

Tables S1-S2. Figures S1-S2.

Supporting Citations

References (59, 60) appear in the Supporting Material.