Structure and dynamics conspire in the evolution of affinity between intrinsically disordered proteins

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In every established species, protein-protein interactions have evolved such that they are fit for purpose. However, the molecular details of the evolution of new protein-protein interactions are poorly understood. We have used nuclear magnetic resonance spectroscopy to investigate the changes in structure and dynamics during the evolution of a protein-protein interaction involving the intrinsically disordered CREBBP (CREB-binding protein) interaction domain (CID) and nuclear coactivator binding domain (NCBD) from the transcriptional coregulators NCOA (nuclear receptor coactivator) and CREBBP/p300, respectively. The most ancient low-affinity “Cambrian-like” [540 to 600 million years (Ma) ago] CID/NCBD complex contained less secondary structure and was more dynamic than the complexes from an evolutionarily younger “Ordovician-Silurian” fish ancestor (ca. 440 Ma ago) and extant human. The most ancient Cambrian-like CID/NCBD complex lacked one helix and several interdomain interactions, resulting in a larger solvent-accessible surface area. Furthermore, the most ancient complex had a high degree of millisecond-to-microsecond dynamics distributed along the entire sequences of both CID and NCBD. These motions were reduced in the Ordovician-Silurian CID/NCBD complex and further redistributed in the extant human CID/NCBD complex. Isothermal calorimetry experiments show that complex formation is enthalpically favorable and that affinity is modulated by a largely unfavorable entropic contribution to binding. Our data demonstrate how changes in structure and motion conspire to shape affinity during the evolution of a protein-protein complex and provide direct evidence for the role of structural, dynamic, and frustrational plasticity in the evolution of interactions between intrinsically disordered proteins.

INTRODUCTION
The web of protein-protein interactions that governs cell function is very complex, often involving competing interactions as well as spatial and temporal regulation of the proteins. Since most of this regulation has evolved over tens or hundreds of millions of years, it is likely that any new protein-protein interaction resulting from, for example, gene duplication, point mutation, horizontal gene transfer, or up-regulation of expression will interfere with the biochemistry of the cell in a non-beneficial way and disappear through purifying selection. However, sometimes, new interactions are sufficiently beneficial to be retained in the population by natural selection. This is what happened sometime between 600 and 540 million years (Ma) ago for two intrinsically disordered protein (IDP) domains: the nuclear coactivator binding domain (NCBD) from CREB-binding protein [CREBBP, also called CBP, and its paralog p300 (CREBBP/p300)] and the CREBBP interaction domain (CID) from the nuclear coactivator protein [nuclear receptor coactivator 1 (NCOA1), NCOA2, and NCOA3, also called Src1, Tif2, and ACTR, respectively]. The CREBBP/p300 and NCOA proteins contain histone acetyl transferase domains and are involved in transcriptional regulation, for example, by activating steroid receptors (1). The tighter association between NCOA and CREBBP/p300 likely resulted in a more efficient transcription, which was sufficiently beneficial to be retained by natural selection in an ancestral population leading to present-day deuterostome animals.

Little is known about the molecular details of protein evolution as it happened in nature. Today, the large number of genome and transcriptome sequencing efforts of extant species allows reconstruction of ancient proteins from evolutionary ancestors, which provides the possibility of studying the putative historical sequence of events leading up to present-day proteins (2–5). We recently published the reconstruction, biophysical characterization, and molecular dynamics simulation of CID and NCBD domains from animal species living approximately 390 to 600 Ma ago and found that the affinity between CID and NCBD was relatively low in an early deuterostome ancestor, which likely existed at some point 540 to 600 Ma ago (i.e., before or at the beginning of the Cambrian period). However, the affinity of the CID/NCBD protein-protein complex had probably increased, along with decreased conformational heterogeneity, to that of present-day CID/NCBD complexes already in an early ancestor of present-day fish living around or before 500 Ma ago during Cambrian and at the end of Ordovician/ beginning of Silurian at the time of the two whole-genome duplications in the vertebrate lineage around 440 Ma ago. This affinity was maintained in the bony fish/tetrapod ancestor 390 Ma ago and is retained in present-day species (6).

To gain a deeper insight into the molecular details shaping the evolution of this protein-protein interaction, we have used here nuclear magnetic resonance (NMR) to obtain structures of three historical CID/NCBD complexes and analyzed the dynamics of the respective interaction by Carr-Purcell-Meiboom-Gill (CPMG) relaxation dispersion NMR spectroscopy. We find that the most ancient Cambrian-like CID/NCBD complex lacks some of the structural features of the younger complexes and that there are a reduction and reshuffling of the
backbone dynamics as well as local frustration going from the low-
affinity ancestral to the higher Ordovician-Silurian/extant human
complexes. Furthermore, isothermal titration calorimetry (ITC) ex-
periments show opposing enthalpic and entropic contributions to the
total affinity. The structural heterogeneity and plasticity observed in
our evolutionary snapshots are consistent with an affinity sensitive
to changes in local frustration distribution and thermodynamics and
shed light on fundamental questions regarding the evolution of protein-
protein interactions.

RESULTS
Structures of CID/NCBD complexes through evolution
To investigate how structure and conformational dynamics modulate
protein function in natural evolution, we solved three protein complexes
of CID/NCBD domains spanning roughly 600 Ma of evolution (Protein
Data Bank codes 6ES5, 6ES6, and 6ES7). The ancient NCBD and CID
domain sequences were previously reconstructed, and the resurrected
proteins were subjected to a biophysical analysis (6). Using our previous
nomenclature, we denote these domains D/P NCBD, which was present
in the common ancestor of present-day Eubateria [i.e., deuterostome
(D) and protostome (P) animals], a creature that likely lived at some
point 540 to 600 Ma ago; 1R/2R NCBD and 1R CID, which were pres-
ent in fish that lived close to the transition between Ordovician and Si-
larian ~440 Ma ago (1R and 2R are the first and second round of the
whole-genome duplications that took place in the vertebrate lineage that
later diversified into present-day vertebrates such as jawed fish, reptiles,
and mammals) (7); and CREBBP NCBD and NCOA3 CID, which are
two extant human domains (Fig. 1A). Since the CID domain could not
be identified in species from protostome phyla (e.g., Arthropoda and
Mollusca) and sequences were too few and different in the deuterostome phyla Echinodermata (e.g., sea stars and sea urchins) and Hemichordata
(e.g., acorn worms), it was not possible to reconstruct a CID domain
older than 1R and as ancient as the D/P NCBD. It is also important to
point out that any reconstructed sequence is an approximation based on
gene variants from selected present-day species for which sequence
data are available. Nevertheless, on the basis of the data on alternative
ancient variants, we deem our overall conclusion robust, namely, that
the Cambrian-like complex has a significantly lower affinity than the
two younger complexes around neutral pH (K_D ~ 1 μM versus 100 nM;
table S1). Thus, the three complexes analyzed in the present work con-
sisted of the most likely CID and NCBD variants: (i) 1R CID with D/P
NCBD (most ancient Cambrian-like complex), (ii) 1R CID with 1R/2R
NCBD (the Ordovician-Silurian 1R/2R complex), and (iii) extant human
CID/NCBD complex (from NCOA3 and CREBBP). Note that the two
whole-genome duplications 1R and 2R occurred in a relatively short but
defined time span. Thus, while it is theoretically possible to reconstruct
two CID variants (since three of four NCOA genes are present today), in
practice, the 1R/2R should be treated as one evolutionary node. In ad-
dition, ITC binding data show that the affinities of 1R and 2R CID for
1R/2R NCBD are similar (6).

First, we determined the structure of the extant human CID/NCBD
complex by NMR using nuclear Overhauser effect (NOE) distance re-
straints, measured φ dihedral angles restraints from scalar couplings,
chemical shift–derived ω/ω dihedral angles, and 1D H-N residual dipolar
couplings (RDCs) (8). The use of RDCs in structure calculations improves the overall quality by restraining the relative orientation of the structural elements (9). Comparison to a previously published NCOA3
CID/CREBBP NCBD structure (10) shows that the domains fold into
similar overall structures with comparable compactness and total solvent-
accessible surface area (SASA) [5730 Å^2 (previous structure) versus 5940 Å^2 (our structure)]. A few minor differences between the structures
likely result from the different lengths of the CID and NCBD constructs
used in our structure compared to the previous one.

We then solved the structures of the most ancient Cambrian-like
complex and the Ordovician-Silurian 1R/2R complex to understand how the CID/NCBD interaction has evolved over time. The overall
topology of the resurrected ancient complexes was similar to that of the
extant human one (Fig. 1, C to E). Furthermore, all three complexes
had a rotational correlation time (τ_c) of approximately 5 ns, as expected
for a complex of this size and indicating that the domains gain structure
upon binding. This is markedly different from the free CID domains,
which had rotational correlation times of less than 1 ns, consistent with
a disordered structure (11). We also observe that the SASA decreased
from the Cambrian-like (7280 Å^2) to the Ordovician-Silurian complex
(6210 Å^2) and the extant human complex (5940 Å^2) (Fig. 2). This
change in compactness is associated with changes in the orientation
of the first and third α helices of NCBD (Nα1 and Nα3, respectively)
and the third α helix of CID (Ca3) (Figs. 1 and 2 and fig. S1). 1R CID
interacts with the Cambrian-like D/P NCBD using only Cα1 and Cα2, while, upon binding to Ordovician-Silurian 1R/2R NCBD, the
third helix Ca3 makes more contacts with Nα2 of NCBD, permitting
it to adopt a more helical structure (Figs. 1 to 3 and figs. S1 to S3). On
more detailed inspection, we find that the additional contacts in this
region in the extant human CID/NCBD complex result in an increase
in helicity of both Nα3 and Ca3 (Figs. 2 and 3 and figs. S1 to S3). These
additional structural elements increase the hydrophobic interaction
area between CID and NCBD and provide new networks of interactions
within the younger higher-affinity CID/NCBD complexes. For exam-
ple, in the most ancient complex, Leu^2047, Lys^2075, Ser^2076, Leu^2087,
and Ile^2101 of Cambrian-like NCBD make direct contacts (as measured
by NOEs) with Leu^1049, Ile^1050, and Ile^1073 of 1R CID. In the Ordovician-
Silurian 1R/2R complex, the number of detected direct NOEs tripled
to 18, notably interactions between Ile^2062, Leu^2067, Gln^2068, Lys^2072,
Gln^2095, Gln^2103, and Ala^2105 of Ordovician-Silurian 1R/2R NCBD
and Asp^1044, Gln^1045, Leu^1048, Leu^1056, Leu^1067, and Gln^1088 of 1R CID.
These interactions are maintained in the extant human complex except
Asn^1079 of NCOA3 CID taking the place for Gln^1088 of 1R CID. These
results are further supported by the change in the amide chemical shift
(ΔδH_i), which shows increased bending of the Nα3 and Ca3 helices as
they displayed alteration of the H_i^N shift in a wave-like manner (fig. S4).
Because bending will result in shorter hydrogen bonds between the car-
bonyl and amide groups of residues i, i + 4 at the concave side and longer
hydrogen bonds at the convex side, amides at the concave side will show
positive ΔδH_i while those at the convex side will show more negative
values (12). This behavior results in repeats of the amide proton secondary
chemical shifts with periodicity of three to four along the sequence.

Further interesting details are provided by the side chains of residues
1070 to 1080, which are partly solvent-exposed and remain partially
unstructured in the most ancient Cambrian-like complex (Figs. 2A
and 3 and fig. S1) but form part of the interaction surface in the
Ordovician-Silurian 1R/2R complex (Fig. 3H). The interaction surface
is further increased by interactions involving helix Nα3 of the two
younger NCBD domains. Specifically, Ala^2105 and Lys^2072 of 1R/2R
NCBD interact with Ile^1073 and Asp^1068 of 1R CID, respectively, and
Thr^2105 of human CREBBP NCBD forms an interaction with His^1053
of NCOA3 CID. In the most ancient complex, residues in Nα3 make
interactions with only part of Ca3 of 1R CID, while most of the Ca3

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Fig. 1. Reconstructed ancestral sequences, phylogenetic tree ancestral, and extant CID/NCBD complexes. (A) The sequences were previously reconstructed (6) using 181 (NCBD) and 184 (CID) protein sequences from present-day protostome and deuterostome species of the animal phyla, superclasses, and classes depicted in the simplified phylogenetic tree. (B) The complexes used in this study are (i) 1R CID with D/P NCBD (most ancient Cambrian-like complex), (ii) 1R CID with 1R/2R NCBD (the Ordovician-Silurian 1R/2R complex), and (iii) extant human CID/NCBD complex (from NCOA3 and CREBBP, respectively). Note that the timing of the whole-genome duplications 1R and 2R is not resolved and might predate the divergence of jawed and jawless vertebrates (25). Animals were downloaded from phylopic.org. (C) The most ancient Cambrian-like complex (red) contains D/P NCBD and 1R CID. (D) The Ordovician-Silurian 1R/2R complex (blue) contains 1R/2R NCBD and 1R CID. (E) The extant human complex (magenta) contains human CREBBP NCBD and NCOA3 CID. In the three left panels, the solved structures of NCBD domains are displayed in cartoon representation and colored, while the CID domains are cartoon and gray. In the three right panels, the solved structures of CID domains are displayed as colored cartoons, and the NCBD domains are gray cartoons. The complexes in the left and right panels are rotated 180° with respect to each other.
remains exposed to the solvent. Thus, the three historical complexes demonstrate how an extensive reorganization of structure and interactions, rather than specific side-chain substitutions in an intact structure, can lead to a higher affinity in a protein-protein complex.

Redistribution of motions accompanies the evolution of higher affinity in the CID/NCBD complex

Since dynamics on different time scales is an inherent property of proteins and a modulator of protein function, we next examined the evolution of dynamics within the three complexes using NMR relaxation dispersion CPMG experiments. These experiments can probe slow motions (i.e., in the microsecond-to-millisecond time scale) that are often relevant for function (13). Equilibrium interconversion between different protein conformations in this time window will frequently result in an exchange contribution to the transverse relaxation rate constant ($R_{\text{ex}}$), causing additional line broadening of the respective NMR spectral resonances with respect to the fast motion–driven $R_0$. The exchange contributions, $R_{\text{ex}}$, depend on the relative populations of the exchanging states, the chemical shift difference ($\Delta \delta$), and the rate constant of exchange ($k_{\text{ex}}$) (Eq. 1). We observed that the Cambrian-like complex exhibited a varying degree of slow motions over the entire protein sequences (Fig. 4). In the Ordovician-Silurian 1R/2R complex, slow motions were markedly reduced in CID with a few exceptions. In particular, helices Ca1, Ca2, and Ca3 contribute little or no slow motions. In the extant human complex, slow motions appear in other locations, resulting in conformational dynamics mostly in the loop region between Ca1 and Ca2 and in the entire Ca3 region, which experiences increased $R_{\text{ex}}$ values. The chemical shift–derived order

![Fig. 2. Evolutionary snapshots highlighting structural heterogeneity.](image-url)

(A) Overlay of the heteronuclear single-quantum correlation spectra for 1R CID bound to Cambrian-like NCBD (red) and Ordovician-Silurian NCBD (blue). Arrows indicate the peak shifts of the amino acid residues 1073 to 1079 corresponding to the CID Ca3 helix (for clarity, only the assignments of residues 1073 to 1079 are shown; complete assignments can be found in fig. S7). In the most ancient low-affinity complex with Cambrian-like NCBD, these residues remain unstructured. However, in the younger high-affinity Ordovician-Silurian 1R/2R complex, the increased number of interactions in the region leads to increased $\alpha$-helical content of Ca3 (see Fig. 4). The increase in dispersion for residues 1074 to 1077 is accompanied by an increase in the number of inter- and intramolecular NOEs ($i, i+3$, indicative of helices; figs. S3 and S4), indicating that this region becomes more structured. ppm, parts per million. (B) Root mean square deviations (RMSDs) between the combined H\textsuperscript{H}/N chemical shifts for the complexes of 1R CID with Cambrian-like D/P NCBD and Ordovician-Silurian 1R/2R NCBD, respectively. Large structural changes are seen for residues Leu\textsuperscript{1049} and Asp\textsuperscript{1050} located in Ca1 and for residues Asp\textsuperscript{1074}, Lys\textsuperscript{1075}, Leu\textsuperscript{1076}, and Val\textsuperscript{1077} in Ca3. sqrt, square root. (C) Superposition of structures of 1R CID bound to Cambrian-like NCBD (red) and Ordovician-Silurian NCBD (blue), respectively. (D) 1R CID bound to Cambrian-like NCBD (red) and Ordovician-Silurian NCBD (blue), and human NCOA3 CID bound to CREBBP NCBD (magenta). For clarity, only the CID domains are displayed in (C) and (D). (E and F) Plots of total SASA calculated from the respective complex and plotted separately for each domain, CID (E) and NCBD (F).
parameters, which are sensitive to motions faster than the inverse chemical shift difference in Hertz, (ca. 10 ms), also showed increased millisecond motions in this region (Fig. 3G). It is reasonable that such reshuffling of slow motions partly compensates for the loss of motion associated with the polypeptide going from disorder to order upon binding. In the two ancient complexes, the helix is not or only partially formed such that the conformational interconversion takes place in the fast-motion regime (faster than nanoseconds; Fig. 3G and fig. S4) (14). This process has been slowed down in the extant human complex because of the relatively stable Ca3 resulting in larger $R_{ex}$

![Fig. 3. Evolution of Ca3 and Nu3 helical content and conformational rearrangements within the Ca3 helix of CID domains during evolution.](image)
values (Fig. 4). The trends in dynamics are less clear for NCBD, but Nα1 and Nα2 display a loss in dynamics slower than nanoseconds when comparing the most ancient with the two younger complexes. 

\[ R_{ex} \] changes from the N- to the C-terminal half of the helix. Thus, along with formation of more intermolecular CID/NCBD interactions as measured by NOEs, there is a general trend toward less and more localized conformational heterogeneity in the CID/NCBD complex upon going from the low-affinity Cambrian-like complex to the higher-affinity younger complexes (Figs. 3 and 4). We like to point out that localization and redistribution of motions could be highly dependent on particular side chains. Therefore, any erroneously predicted amino acid residue in the ancestral reconstruction could significantly influence the dynamics. Nevertheless, by comparing the Ordovician-Silurian 1R/2R complex with the extant human one, it is clear that high affinity is compatible with different dynamics profiles. Our data suggest that redistribution of motions may play a role in fine-tuning affinity upon mutations in diverging sequences. It is important to note that faster motions (nanoseconds to picoseconds) have also been implicated in function (15, 16). However, it was not possible to perform model-free dynamic analysis of the free domains, which would be needed for a conversion of NMR relaxation data into thermodynamic parameters as the local motions cannot be easily separated from global tumbling. Nevertheless, we provide information on the backbone subnanosecond motions in terms of \( R_{ex} \) (Fig. S5). The results indicate that, mostly, the N-terminal, C-terminal, and loop regions with low \( R_{ex} \) values exhibit motions in the nanosecond-to-picosecond time scale.

**Thermodynamic basis for the different affinities of the CID/NCBD complexes**

To investigate how the observed changes in structure, dynamics, and affinity are reflected in the binding thermodynamics, we performed ITC experiments for the three complexes at different pH and temperature (Fig. 5, fig. S5, and table S1). ITC experiments directly measure the change in enthalpy (\( \Delta H \)) for the binding reaction and, from the shape of the titration profile, the equilibrium association constant (\( K_A \)). From these parameters, \( K_D = 1/K_A \), \( \Delta S \), and \( \Delta G \) can be calculated.
Fig. 5. The pH and temperature dependence of interactions for ancestral and extant NCBD/CID. (A) Examples of ITC experiments conducted at pH 7.7 and 25°C for the three different complexes. (B) Contribution from enthalpy ($\Delta H$, left) and entropy ($-T\Delta S$, middle) to the total free energy ($\Delta G$, right) at different pH values for formation of Cambrian-like (red), Ordovician-Silurian (blue), and extant human (magenta) CID/NCBD complexes, respectively. (C) The association ($K_A$, top) and dissociation ($K_D$, bottom) equilibrium constants plotted against pH and fitted to a two-state equation (Eq. 4 in Methods), which yielded the apparent $pK_a$ values listed in the table. Because of the large noise in the original data for the extant human complex at pH 5.2, these points were not included in the curve fitting but are shown in the figure. (D) Bar diagrams showing the contribution of enthalpy ($\Delta H$, left) and entropy ($-T\Delta S$, middle) to the free energy ($\Delta G$, right) for formation of the respective CID/NCBD complex at different temperatures and at two different pH values (6.5 and 7.5, respectively). To determine $\Delta C_p$, the $\Delta H$ values were plotted against temperature and analyzed according to Eq. 2 (fig. S5).
First, we investigated the pH dependence of the interaction at a constant ionic strength and temperature (25°C). Thus, ITC experiments were conducted at pH values ranging from 5.2 to 8.2 (Fig. 5 and table S1). The results show that there is a favorable enthalpic and unfavorable entropic contribution to the affinity at all pH values and for all variants (Fig. 5). Furthermore, the affinities of all complexes have a transition around pH 6.5 and reach a plateau at higher pH values (Fig. 5). The Cambrian-like complex and the Ordovician-Silurian complex displayed similar pH dependences, where the affinities of both protein complexes increased toward lower pH values, maintaining a 10-fold difference in $K_A$. In contrast, the extant human complex displayed an opposite pH dependence in which the affinity decreased toward lower pH values to equal that of the Cambrian-like complex around pH 5.2. $K_D$ and $K_A$ values were fitted to a two-state equation to estimate $pK_a$ values for the respective parameters in the different complexes. The pH dependence of $K_D$ reflects titrations of ionizable groups in the CID/NCBD complex, whereas the pH dependence of $K_A$ reflects titrations in free NCBD and CID (17). The data fitted reasonably well to the model, and the fitted $pK_a$ values for $K_A$ and $K_D$ were between 6.1 and 7.6 (Fig. 5). Except for one His in D/P NCBD, there are no obvious side chains with a $pK_a$ value in this range, and the most likely basis for the pH dependences of all three complexes is a general charge effect, corroborated by the relatively broad noncooperative transitions. These effects could lead to small structural perturbations of the molten globule-like NCBD domain, which, in turn, could modulate the affinity.

Next, we investigated the thermodynamic basis for the CID/NCBD affinity by performing ITC at different temperatures and at two different pH values, 7.5 (close to the affinity plateau) and 6.5 (in the pH transition region). We found that $\Delta H$ was negative (i.e., favorable) at temperatures above 285 K, whereas the entropy contribution −$\Delta S$ switched from being favorable (at lower temperatures) to nonfavorable between 289 and 297 K for the three complexes, both at pH 6.5 and 7.5 (Fig. 5). The calorimetric experiments show that the observed change in heat capacity upon binding $\Delta C_p$ (i.e., the temperature dependence of $\Delta H$) for all three complexes was identical within error at pH 6.5 (−850 to −900 cal K$^{-1}$ mol$^{-1}$) and in the same range at pH 7.5 (−720 to −950 cal K$^{-1}$ mol$^{-1}$; fig. S5). These numbers suggest that the contribution from the change in solvent entropy to the binding energetics (i.e., the hydrophobic effect) is substantial and similar for the three complexes (18). However, there is an offset in the values of $\Delta H$ for the Ordovician-Silurian and human complexes as compared to the Cambrian-like complex at pH 6.5 such that the enthalpic contribution, which mirrors bond strength, is weakened in the higher-affinity complexes. Instead, the thermodynamic basis for the higher affinity is a less unfavorable or even favorable entropic contribution to the overall affinity, a result that is qualitatively very similar to another IDP interaction—that between FCP1 and Rap74 (19). At pH 7.5, this offset is smaller, and all three complexes display a similar contribution from $\Delta H$.

In addition, the enthalpic contribution in the human complex is accentuated at the higher temperatures at pH 7.5, which might reflect the more well-formed Cn3. From the calorimetric experiments, we conclude that (i) the net effect of bond breaking and formation (ΔH) is favorable at supposedly physiological temperatures (>12°C); (ii) the hydrophobic effect makes a major and favorable contribution to the free energy of binding; (iii) the higher affinity observed for the Ordovician-Silurian and extant human complexes as compared to the Cambrian-like complex at pH 6.5 is due to entropic effects, which are likely not related to the hydrophobic effect since $\Delta C_p$ values are similar; and (iv) there is a delicate balance between factors contributing to affinity as shown by, for example, the large change in $\Delta H$ for the Cambrian-like complex at pH 6.5 compared to pH 7.5 and from the pH dependences of all three complexes. Although we cannot further dissect the entropic contribution in point (iii) into changes in rotational, translational, or conformational entropy (as explained above), the thermodynamic data underscore the impact of dynamics rather than noncovalent bonds for increasing the affinity of this interaction. In addition, we confirmed by urea and thermal denaturation experiments that the ancestral Cambrian-like complex was less stable as compared to the modern Ordovician-Silurian and extant human complexes, corroborating the affinity measurements by ITC (fig. S9).

**Change in frustration parallels the experimental data**

The concept of minimal frustration has been successfully applied to explain protein folding, and we were therefore interested in analyzing the evolution of frustration in the coupled binding-and-folding reaction of CID/NCBD. Frustration occurs when a physical system is not able to simultaneously achieve minimum energy for every part of it (20). It has been shown that, in protein-protein interactions, frustration of regions close to the binding site often changes upon association such that it is less frustrated in the complex, thus guiding specific association (20). It has been suggested that promiscuous IDP interactions display more frustration than those of well-folded proteins (21), but little is known, especially regarding evolution of frustration in IDPs. For our three CID/NCBD complexes, we therefore calculated and analyzed the mutational frustration index, for which the Frustratometer algorithm evaluates the energy of each contact of the native structure with respect to the distribution of the energies of decoys made by substituting residues at each position (Fig. 6) (22). It is apparent that there is a significant reduction and redistribution of highly frustrated interactions in the CID domain when comparing the most ancient Cambrian-like complex with the higher-affinity younger complexes (Fig. 6). In the most ancient Cambrian-like complex, the regions around residues 1056 to 1062 of the CID domain show a distinct frustrated patch, consistent with the location of a high-mobility region. It has been previously observed that regions of high local frustration correlate with extensive subnanosecond (23) and also slower time scale dynamics (24). In the extant human complex, the amount of frustration in this region is reduced and redistributed with respect to the ancient ones. Consistent with the pH dependence of the affinity, most of the frustration changes are related to electrostatic interactions, as the changes of frustration patterns are less evident when electrostatics is not considered in the calculation (figs. S6 and S9).

**DISCUSSION**

Vertebrates originated during Cambrian (485 to 540 Ma), and one lineage gave rise to fishes during the subsequent Ordovician period (440 to 485 Ma). Sometime around the end of Ordovician or beginning of Silurian, a dramatic event occurred that shaped much of the following vertebrate evolution, namely, the two whole-genome duplications 1R and 2R (7, 25). This resulted in four copies of each gene and possibilities of evolutionary innovation of new functions for the encoded proteins. As the ancestral vertebrate population diversified into cartilaginous fish, bony fish, and later tetrapods including present-day amphibians, reptiles, birds, and mammals, many of the duplicated genes were subject to neo- or subfunctionalization. The genome duplications paved the way for extensive evolution of protein-protein interactions, which play a major role in cell signaling and regulation and often involve intrinsically
disordered regions. Two examples are the protein domains involved in transcriptional regulation examined here—NCBD, which is present in extant CREBBP/p300 (two sister genes were lost during evolution) and CID, which is present in NCOA1 (Src1), NCOA2 (Tif2) and NCOA3 (ACTR) (one gene was lost). The analysis of this protein-protein interaction shed light on how a functional interaction can emerge from initially weak binding between IDPs and the molecular complexity of increasing affinity.

One of the fundamental differences of IDPs as compared to ordered proteins is their ability to adopt alternative bound conformations, which is advantageous when recognizing different protein ligands and which would facilitate neofunctionalization as for CID recruiting NCBD as binding partner (6). Not only the disordered C-terminal domain of p53 is a prime example of such conformational plasticity (26) but also the NCBD domain, which adopts a distinct structure upon binding to a protein domain from the interferon regulatory factor (27), as compared to binding the CID domain. However, little is known regarding the evolution of conformational plasticity and how it modulates affinity in a protein-protein interaction. Our three historical structural and dynamic snapshots of the CID/NCBD complex provide a unique example of how increased affinity can be attained.

Overall, our findings support the notion that the high conformational plasticity of IDPs contributes to their protein-protein interactions (28). In the present case, the Ca3 of 1R CID adopts a helical conformation in the high-affinity complex with 1R/2R NCBD but not in the low-affinity complex with D/P NCBD. We speculate that the initial ancestral interaction between CID and NCBD was of lower affinity than our most ancient Cambrian-like complex yet achievable through the N-terminal leucine-rich binding motifs. Our NMR structures, NMR dynamics and thermodynamic data, and previous molecular dynamics simulations (6) illustrate how higher affinity in IDP interactions can be achieved by lowering the entropic penalty and increasing the enthalpic contribution of complex formation through a combination of specific interactions, conformational rearrangements, and redistribution of dynamics and frustration patterns. The most ancient Cambrian-like complex is more dynamic, which is probably a reflection of the fewer
specific interactions and a very high local frustration not present in the higher-affinity variants. The high-affinity Ordovician-Silurian 1R/2R complex displays rearrangements of helices along with an increased hydrophobic interface. Thus, the conformational plasticity of the interacting IDPs permits formation of new favorable interactions between Ca3 and Na2, leading to higher affinity in the Ordovician-Silurian and the extant human complex. Why are these features not clearly reflected in a larger enthalpic contribution to binding for the high-affinity complexes? For example, the affinity of the Ordovician-Silurian complex is always 10-fold higher than the Cambrian-like complex, but the enthalpy (supposedly reflecting structure) is less favorable and the entropy (supposedly reflecting dynamics) is less unfavorable except at low pH. The answer must lie in the large and opposing overall contributions from enthalpy and entropy and the hard-to-predict effect of water. Thus, while the overall thermodynamics of this coupled binding-and-folding reaction, which includes formation of a well-defined hydrophobic core, follows the same principles as observed in protein folding studies (29), they cannot easily be correlated to structural details. This observation is likely a reflection of a general property of IDP interactions, namely, they are plastic and thereby able to adapt to its binding partner.

The evolution of new protein-protein interactions involving intrinsically disordered regions of proteins is very common in nature as evident from virus biology. Viruses often hijack host protein-protein interactions by simply mimicking the host’s disordered interaction motifs (30). Because of the high mutation rate and short generation time of viral genomes, such evolution happens on a time scale much

### Table 1. Structural determination statistics.

|                      | Most ancient | 1R/2R  | Extant human |
|----------------------|--------------|--------|-------------|
| **NMR distance and dihedral restraints** |              |        |             |
| **Distance restraints** |              |        |             |
| Total NOEs           | 620          | 700    | 550         |
| Intraresidue         | 222          | 265    | 213         |
| Sequential (| | 197    | 206    | 168         |
| Medium-range (1 < | | 140    | 164    | 97          |
| Long-range (>5)      | 45           | 55     | 38          |
| **Total dihedral angle restraints** | 91          | 85     | 79          |
| \(^{3}\)JHNHA scalar couplings | 70          | 63     | 67          |
| \(^{13}\)Ca chemical shifts | 138         | 142    | 140         |
| **Structure statistics** |              |        |             |
| Average CYANA target function value (Å²) | 7.9 ± 0.2    | 4.2 ± 0.30 | 2.2 ± 0.2 |
| Violations           |              |        |             |
| Distance constraints (≥0.5 Å) | 0           | 3      | 0           |
| Dihedral angle constraints (>5°) | 0           | 0      | 0           |
| **Deviations from idealized geometry** |              |        |             |
| Bond lengths (Å)     | 0            | 0      | 0           |
| Bond angles (°)      | 0            | 0      | 0           |
| Improper (°)         | 0            | 0      | 0           |
| Average pairwise RMSD (Å)* |              |        |             |
| Backbone             | 0.8 ± 0.2    | 0.6 ± 0.1 | 1.7 ± 0.6 |
| Heavy atoms          | 1.3 ± 0.2    | 1.1 ± 0.1 | 2.2 ± 0.5 |
| **RMSD of RDC-refined structure (Å)*** |              |        |             |
| Backbone             | 1.3 ± 0.3    | 0.8 ± 0.2 | 1.4 ± 0.7 |
| Heavy atoms          | 1.8 ± 0.2    | 1.3 ± 0.2 | 1.9 ± 0.6 |

*RMSD for residues 1042 to 1079 and 2065 to 2106.
shorter than that for animals. However, the general molecular principles governing emergence of new protein-protein interactions are expected to be general. Our results demonstrate how protein–protein complexes involving intrinsically disordered domains can achieve stronger binding via an intricate interplay between structural and dynamical rearrangements that cannot easily be predicted.

**METHODS**

**Protein expression**

The constructs for the CID domains contained the complementary DNA corresponding to residues 1040 to 1080 of human NCOA3, and the constructs for the NCBD domains contained residues 2062 to 2109 of human CREBBP NCBD, as described (6). All constructs contained an N-terminal expression tag (6x His and lipoyl domain), followed by a thrombin protease site and were expressed from a pRSET vector. The CID and the NCBD domains were expressed in *Escherichia coli* BL21 (DE3) pLysS cells either unlabeled (in terrific broth medium) or labeled for NMR experiments (in M9 minimal medium enriched with 15N ammonium chloride and 13C glucose) and purified, as previously described (6). Concisely, on day 1, BL21 pLysS cells containing transformed plasmid were grown overnight at 37°C on a plate containing ampicillin (100 μg/mL) and chloramphenicol (35 μg/mL). On day 2, transformed cells were grown in growth media containing ampicillin (50 μg/mL) at 37°C to an optical density of 0.6. Protein expression was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside, and cells were allowed to grow overnight at 18°C. On day 3, these cells were harvested by centrifugation and resuspended in purification buffer [20 mM tris/Cl (pH 8.0) and 200 mM NaCl]. The resuspended cells were then lysed by sonication and purified on a Ni Sepharose column (GE Healthcare). After desalting the partially pure fusion protein, ca. 0.5 units of thrombin protease per milligram of fusion protein was added to the sample and incubated at 37°C for 16 to 18 hours. The cleaved proteins were then passed through a second Ni Sepharose column, and unbound cleaved CID or NCBD was loaded on a reversed-phase C18 column equilibrated with 0.1% trifluoroacetic acid. Bound proteins were eluted with a gradient of 0 to 70% acetonitrile in 0.1% trifluoroacetic acid. The purity and identity of the proteins were confirmed by SDS–polyacrylamide gel electrophoresis and mass spectrometry. Pure proteins were lyophilized and stored at −20°C until use. Before NMR experiments, the lyophilized samples were dissolved in a buffer containing 10 mM NaPO4 (pH 6.8) and 150 mM NaCl and dialyzed against the same buffer overnight at 25°C.

**NMR spectroscopy**

Protein concentrations were estimated by absorbance measurement at 205 nm for all domains and double checked at 280 nm for 1R/2R NCBD and human CREBBP NCBD, which contain one tyrosine. The protein complexes were formed by adding saturating amounts of either unlabeled CID domain ([1H,15N]) with labeled NCBD domain ([15N,13C]) or vice versa. The final concentrations of the CID/NCBD complexes in the NMR tube ranged from 300 to 500 μM of the labeled protein and approximately a twofold excess of unlabeled protein. To these samples, 0.01% NaN2 and 5% D2O were added. All NMR experiments were recorded on Bruker spectrometers (600, 700, and 900 MHz, [1H frequencies], equipped with triple-resonance cryogenic temperature probes at 298 K. Assignment of the protein backbone was achieved by measuring two-dimensional (2D) [1H,15N]-HSQC (heteronuclear single-quantum coherence), 3D HNCA CB, and [1H,1H]-NOESY-[1H,15N]-HSQC experiments (31). Side chains were assigned from the following 3D experiments: [1H,15N]-HSQC- [1H,13C,13C]-TOCSY (total correlation spectroscopy; 60-ns mixing time), [1H,1H]-NOE-SY- [1H,13C]-HSQC, [1H,13C]-HSQC-[1H,13C,13C]-TOCSY (60-ns mixing time) in combination with 2D [1H,15N]-HSQC, and 2D [1H,13C]-HSQC-CT (constant time; fig. S7) (31). The φ angle–restraining 3JHNHα couplings were determined from a 3D HNHA-type experiment using quantitative J coupling intensity evolution (32). 2D and 3D in-phase and anti-phase [1H,15N]-HSQC–based and [1H,15N,13C] HNCO–based experiments were used for the determination of 1D HN RDCs in a sample, with proteins aligned by pf1 phage. The differences between the apparent scalar couplings in the absence and presence of alignment media yielded the RDCs (fig. S8). For the collection of distance restraints, the following NOESY (NOE spectroscopy) experiments and parameters were used: 3D NOESY-[1H,15N]-HMQC (heteronuclear multiple-quantum coherence) and NOE-SY-[1H,13C]-HMQCR with 60-ns mixing time and 128 (15N or 13C) × 200 (1H) × 2048 (1H, direct) number of points for intramolecular NOEs and a [14N],[13C,15N],[13C]-double-filtered NOESY-[1H,15N,13C]-HSQC for intermolecular NOEs (33). R1 and R10 relaxation rates were measured with different randomized relaxation delays in an interleaved manner, with a recovery delay of 3 s using TROSY (transverse relaxation optimized spectroscopy)–based pulse schemes (34). For R1, a spin lock of 2000 Hz was used. R10 values were then determined from R10 by correcting for the contribution of R2. The overall tumbling time τ 2 was then estimated from the ratio of R2 (R10)/R1. [1H,15N]-heteronuclear NOE experiments were recorded, with a 1H saturation time of 8 s with an equal recovery time for the reference experiment (34). CPMG dispersion profiles were determined from relaxation rates measured with a fixed CPMG transverse rotating-frame relaxation delay tCPMG (48 ms) with vCPMG frequencies of 83, 167, 250, 333, 417, 500, 583, 667, 750, 883, 917, and 1000 Hz in a randomized manner (35). The relaxation delay was set to 2 s. A reference experiment omitting the CPMG element was used to obtain the effective relaxation rate RCPMG from the expression –ln(I/I0) tCPMG, where I and I0 are the intensities at different CPMG times and reference intensities, respectively. All experiments were processed with NMRPipe (36) and analyzed with CCPNmr (37). Curve fitting was done in MATLAB, NMRPipe, and KaleidaGraph. Errors were determined from Monte Carlo simulations. In the fast exchange limit, the effective relaxation rate as a function of vCPMG can be described by the following equation (13)

\[
R_{\text{eff}} = R_0 + \left( (p_A p_B \Delta \omega^2) / k_B (1 - 4t_{\text{CPMG}}/k_B) \tanh (k_B / (4t_{\text{CPMG}})) \right)
\]

where, R0 is the transverse relaxation rate in the absence of exchange, pA and pB are the populations of the exchanging states, Δω is the chemical shift difference between the exchanging states, kB is the sum of the forward and reverse exchange rate constants, and tCPMG are the variable CPMG times. Chemical shift order parameters (S2) were predicted by submitting experimentally derived shifts to a web-based program from the Wishart laboratory (38). RCPMG was calculated by taking the difference between R2 (RCPMG) without and with a CPMG radio pulse applied at a frequency of 1000 Hz (fig. S9).

**Structure calculations**

Structure calculations were done using the CYANA 3.97 package in two steps. First, the NOE cross peaks were converted into upper distance restraints (fig. S7) following an automated process in CYANA. These
distance restraints, together with ϕ/ψ dihedral angles determined from Cα chemical shifts and D3JНИHA (fig. S8), were used as input for the initial structure calculations. The structures were calculated with 200,000 torsion angle dynamics steps for 100 conformers, starting from random torsion angles by simulated annealing. Initially, the structures of the CID and NCBD domains were determined separately without the intermolecular NOEs. In a second step, the calculations were repeated using the intermolecular NOE-derived distance restraints (table S1) and a linker with a weak harmonic well potential with a bottom width of 1.2 Å that kept the two domains together (39). The separate structures calculated in step 1 were used as the starting structures. For refinement and cross-validation, calculations were repeated with 1D1Н RDCs (fig. S8, F to K). The axially symmetric and rhombic components of the alignment tensor were optimized by an iterative process in CYANA in three steps. For representation and analysis, the 20 conformers with the lowest target function values were selected. The structural statistics, together with all input data for the structure calculations, are presented in Table 1 and figs. S8 and S9. The total SASA was determined using the program PyMol from the domains in the complex.

Isothermal titration calorimetry

ITC binding experiments were performed on an iTC200 (Malvern Instruments) and fitted using in-built software. Before performing the ITC experiments, all protein samples were dialyzed overnight in a buffer containing 20 mM sodium phosphate at pH 6.5 or 7.5. The protein samples were then filtered, and their concentrations were determined using absorbance at 205 nm. The ITC experiments were done at different temperatures (288, 293, 298, 303, and 308 K). Typically, 20 injections of 2 μl each of 300 mM CID solution were titrated into 280 μl of 30 μM NCBD domains. All samples were allowed to equilibrate for about 10 min at the experimental temperature before the actual measurements were initiated. For the determination of ΔCp, both ΔH and ΔS were plotted as a function of temperature following Eqs. 2 and 3.

\[
\Delta H(T) = \Delta C_p(T) \cdot T + \Delta C_p H
\]

\[
\Delta S(T) = \Delta C_p ln(T) + \Delta C_p S
\]

ΔCp is the specific heat capacity at constant pressure. ΔCp, ΔH, and ΔS are the temperature-independent contributions of enthalpy and entropy, respectively. The ITC measurements for pH dependence of affinity were carried out essentially as above, with the following differences: The protein samples were dialyzed against 20 mM sodium phosphate buffer (pH 8.2, 7.7, 7.2, 6.7, and 6.2) or, alternatively, 20 mM sodium acetate buffer (pH 5.7 and 5.2), and the ionic strength was adjusted with NaCl to 58 mM for all buffers. All measurements were conducted at 298 K. The measurement for the enthalp of the complex at pH 5.2 was repeated four times because of problems with noise in the isotherms, and these data points were excluded from the fitting of pKα values. The data were fitted to a two-state equation (Eq. 4) to extract apparent pKα values for the association (Kα) and dissociation (Kd) constants for the respective protein complex.

\[
K = (K_{\text{protonated}}10^{-pH} + K_{\text{deprotonated}}10^{-pKα})/(10^{-pKα} + 10^{-pH})
\]

Stability measurements

Urea and thermal denaturation experiments were performed on a JASCO J-1500 circular dichroism (CD) spectrophotometer, and the data were fitted using the program KaleidaGraph. All lyophilized protein samples were dissolved in a buffer containing 10 mM sodium phosphate (pH 6.8). For each complex, 40 μM of the CID domain was mixed with 15 μM NCBD domain. The temperature was set to 298 K for the urea denaturation experiments. The temperature was varied between 278 and 363 K for the thermal denaturation. The ellipticity at 222 nm was monitored for both measurements. The data were then fitted to the standard equations describing two-state unfolding by chemical denaturant and temperature, respectively (17).

**Frustration index calculations**

The frustration index was calculated for each contact in the complex using the Frustratometer, as described (22). A contact with an index of ≥0.78 is classified as minimally frustrated, contributing favorably to protein stability and affinity. On the other hand, interactions with a frustration index of ≤−1 are labeled as highly frustrated, and most substitutions at that location would be more favorable. In between these extremes, the interactions are labeled as neutral. Interactions are divided into classes according to this index and color coded in the figures. Considering that the CID/NCBD complexes are charged under the pH conditions used for the experiments, we computed the local frustration patterns using the electrostatics module of the Frustratometers, with an electrostatics k = 4.15 (22), and compared the results with calculations not considering the electrostatics.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/4/10/eaau4130/DC1

Fig. S1. Structural changes taking place and chemical shift restraints.

Fig. S2. Strips from [1H-1H]-NOESY-[1H-15N]-HSQC spectra showing resonances emanating from residues 1074 and 1075 of the respective CID domains.

Fig. S3. 15N relaxation data for the bound CID and NCBD domains and amide secondary chemical shifts of the complexes.

Fig. S4. Backbone subnanosecond motions and ΔCp for the three CID/NCBD complexes.

Fig. S5. Frustration in the CID/NCBD complexes without electrostatics.

Fig. S6. Backbone subnanosecond motions and ΔCp for the three CID/NCBD complexes.

Fig. S7. [1H-15N]-HSQC correlation spectra for bound and free CID and NCBD domains and the number of restraints in the structure determination as a function of amino acid residue.

Fig. S8. Urea and temperature denaturation experiments and Coulomb surfaces of the ancestral and extant CID/NCBD complexes.

Table S1. ITC parameters and intermolecular NOEs.

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