Published in final edited form as:

J Mol Biol. 2007 March 16; 367(1): 8–15.

Crystal structure at 2.8 Å of the DLLRKN-containing coiled-coil domain of Huntingtin-interacting protein 1 (HIP1) reveals a surface suitable for clathrin light chain binding

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Summary

Huntingtin interacting protein 1 (HIP1) is a member of a family of proteins whose interaction with Huntingtin is critical to prevent cells from initiating apoptosis. HIP1, and related protein HIP12/1R, can also bind to clathrin and membrane phospholipids and HIP12/1R links the CCV to the actin cytoskeleton. HIP1 and HIP12/1R interact with the clathrin light chain EED regulatory site and stimulate clathrin lattice assembly. Here we report the X-ray structure of the coiled-coil domain of HIP1 from 482–586 that includes residues crucial for binding clathrin light chain. The dimeric HIP1 crystal structure is partially splayed open. The comparison of the HIP1 model with coiled-coil predictions revealed the heptad repeat in the dimeric trunk (S2 path) is offset relative to the register of the heptad repeat from the N-terminal portion (S1 path) of the molecule. Furthermore, surface analysis showed there is a third hydrophobic path (S3) running parallel to S1 and S2. We present structural evidence supporting a role for S3 path as an interaction surface for clathrin light chain. Finally, comparative analysis suggests the mode of binding between *sla2p* and clathrin light chain may be different in yeast.

Keywords

HIP1 clathrin light chain interaction; HIP1; HIP12/1R; endocytosis; Sla2p

Introduction

Huntingtin interacting protein 1 (HIP1) is an essential obligate binding partner for Huntingtin (htt). Loss of the HIP1/htt interaction triggers a cascade of events that results in the apoptosis of neuronal cells and the onset of Huntington's disease¹. HIP1 and a related protein, HIP12/1R,

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have been found to participate in clathrin-mediated endocytosis^{2, 3}. HIP1 and HIP12/1R can exist as homodimers and there is evidence that HIP1 and HIP12/1R may also form heterodimers^{4, 5}. HIP1 and HIP12/1R interact with membrane phospholipids via an ANTH (AP180 N-terminal homology) domain at its N-terminus (see Fig. 1a)^{3, 6}. HIP1 and HIP12/1R also have a C-terminal THATCH domain, but only HIP12/1R has a C-terminal latch that activates its THATCH domain to tether CCVs to the cytoskeleton⁷. HIP1 has AP2-binding FXDXF and DPF motifs (X denotes any amino acid) that are both absent in HIP12/1R and a clathrin box (332LMDMD) that recognizes the clathrin N-terminal domain⁸. The clathrin light chain-binding site HIP1 was mapped to 484DLLRKN in HIP1 and 463ELLRKN in HIP12/1R⁶. Apparently L486 and R487 are critical because substituting these residues with alanine blocked the ability of HIP proteins to bind clathrin light chain⁵. Specifically, L486 and R487 interact with the regulatory 20EED sequence⁹ in clathrin light chains a and b (LCa and LCb)^{4, 5, 10}. Although the L486A or R487A mutant was sufficient to destroy light chain binding^{5, 10}, the ability of widely spaced single mutations in LCb to inhibit binding to HIP12/1R⁴ suggested to us that the interaction surface for clathrin light chain in the HIP proteins involved more than just the region around L486 and R487. To extend our understanding of the interaction between clathrin light chains and the coiled-coil domain of HIP1, we have determined the crystal structure of the coiled-coil domain of HIP1 that includes 484DLLRKN.

The HIP1 structure reported here to 2.8 Å is a partially coiled dimer that has a hinge region where the helical coils in the N-terminal region of the molecule separate from each other (Fig. 1). We found two closely spaced hydrophobic paths (S1 and S2) that can potentially be used to form a coiled-coil dimer interface. We also identified a third hydrophobic path (S3) that is on the solvent exposed side of each helix that may be involved in binding clathrin light chain.

Topology

Efforts to grow crystals of HIP1 constructs that contained the entire coiled-coil domain were disappointing, yielding only highly twinned crystals that diffracted poorly. We therefore made a series of shorter HIP1 sub-fragments that included 484DLLRKN, the sequence originally identified as being important for clathrin light chain binding, to see if resolution could be increased and twinning eliminated. We found that the HIP1 482–586 fragment (denoted by the yellow bar in the domain map in Fig. 1a, Panel c) gave large three-dimensional crystals, but these forms turned out to be very mosaic (~2 degrees) along the crystallographic c-axis, indicating there was a high degree of disordering in that direction. We tested about two hundred small molecule and detergent additives, but none increased crystal quality. We found, however, that dehydrating preexisting crystals improved the data sufficiently for MAD phasing. The refinement statistics ($R=29\%$, $R_{\text{free}}=31\%$) are higher because of the limited quality of the final dehydrated crystals. Nevertheless, the HIP1 crystal structure yields new information about the coiled-coil domain that includes 484DLLRKN. The numbering scheme from human HIP1 is used throughout (accession number NP 005329), but the first two residues in the HIP1 model are from the GST tag that was removed by thrombin. The last 5 residues (582–586) could not be built because of weak electron density. Note that we refer to the dimeric crystal structure as HIP1 482–586 even though the first two residues are not from HIP1 and the refined model ends at residue 581.

Figure 1a shows the parallel HIP1 482–586 dimer is Y shaped (~145 Å long) with a left-handed coiled-coil trunk region spanning residues 541–581 that is ~54 Å long. The splaying of Helix 1 from Helix 2 is restricted to one plane because rotating the open HIP1 dimer by 90 degrees around the long axis (Fig. 1a, Panel b) yields a model that appears entirely closed, with a ~70 Å node-to-node distance typical of coiled-coil proteins¹¹. Amino acid sequences giving rise to coiled-coils have a seven amino acid repeating unit (denoted *a-g*)^{12, 13}. Residues at the *a*- and *d*-positions are mostly apolar, while those at *e*- and *g*-positions are usually charged¹⁴,

¹⁵. The *a-b-c-d-e-f-g* heptad positions in the trunk of the model validated our COILS analysis of this region of the HIP1 482–586 sub-fragment. Fig. 1d shows that F570 (*a*), L573 (*d*), E574 (*e*) and R577 (*a*), in Helix 2 together form a pocket for L573 (*d*), protruding from Helix 1 (heptad positions predicted by COILS are in parenthesis). This observed heptad repeat is part of the *a-b-c-d-e-f-g* pattern that COILS predicts goes to the end of the coiled-coil domain at position 643.

A C α backbone template (aa547–581) from the trunk region was employed to evaluate if N-terminal segments of Helix 1 and 2 assumed normal left-handed coiled-coil topology. The superpositions revealed that most of the sections N-terminal to where the helices begin to separate were topologically very similar to the trunk domain of the HIP1 482–586 sub-fragment. The exception was a short segment between L532 and K539 (indicated by bar over the short ribbon in Fig. 1e). We have operationally defined this 532–539 stretch as the molecular hinge that opens the HIP1 structure. There is a cluster of hydrophobic residues (L532, V534 and L535) next to E530, E533 and E536 at one end of this 8-residue hinge segment. The juxtaposition of hydrophobic and acid clusters at the beginning of the designated hinge suggests any conformational change caused by repulsions between E530, E533 and E536 in Helix 1 and in Helix 2 would be mitigated by the natural tendency of L532, V534 and L535 from Helix 1 to pack against the same residues in the other helix to avoid contact with water. Any destabilizing impact of the hinge region (L532–K539) may also be sterically restricted by a cluster of charged residues at the end of the hinge. Fig. 1e shows the pattern of alternating positively and negatively charged residues (K539, E541, R547 and E548) punctuating the C-terminal end of the hinge. Specifically, K539 in Helix 2 lies almost directly across E541 in Helix 1 and R547 in Helix 2 is oriented opposite E548 in Helix 1.

Identifying parallel hydrophobic paths

The packing of HIP1 482–586 molecules in the crystal requires the partially splayed structure because the space between separated helices is used to build up a vertical stack of HIP1 molecules. Each HIP1 column is related to the next over by 2-fold symmetry to produce a lattice of alternating up/down (anti-parallel) columns. The molecular contact between anti-parallel HIP1 molecules that connect neighboring columns is defined by the S1 path shown in Helix 1 as a dotted line through H482, N489, V496, Q503 and K510 (Fig. 2a). An identical S1 path exists in Helix 2 (not shown). According to COILS, the S1 path is part of the heptad repeat that starts at the beginning of the HIP1 coiled-coil domain at position 389. When the trajectory of S1 was projected into the trunk region of the crystal structure, we observed an offset between S1 and the heptad repeat pattern shown in Fig. 1d. We therefore defined the S2 path in Fig. 2a and 2b. This path was drawn by continuing the register of the heptad repeat observed in the trunk into the open area (dashed line in Fig. 2a going through L485, V492, A499, L506 and L513). We emphasize the S2 path represents the heptad repeat of the trunk projected into the open region. We found that the observed S1 path and projected S2 path merge just before the designated hinge described above (merge point marked by an asterisk in the diagram in Fig. 2a and is labeled F in Fig. 2b). The S1 and S2 paths overlap to become a single path at point F. This single heptad repeat (S2 path) passes through the trunk region of our structure and continues on. The merging together of S1 and S2 raises the possibility that the single heptad repeat detected by COILS starting at position 389 (coincident with S1) gives rise to S2 at a branching point located in structure that is before our HIP1 model. In this scenario an upstream heptad discontinuity creates S2 off of S1, producing the double hydrophobic path in the region of HIP1 that contains ₄₈₄DLLRKN. The two paths coalesce at point F (Fig. 2b) and the resulting single S2 path continues on to the end of the entire coiled-coil domain. We point out that the S1/S2 offset may be functionally significant because heptad repeat discontinuities drive coiled-coils to under- or over-wind to produce loose or tightened coiled-coil structures ¹⁶.

The coiled-coil domain of HIP12/1R is straight and completely closed, but is sufficiently flexible to bend occasionally into a U shaped structure¹⁷. We anticipate the HIP1 482–586 sub-fragment in the full-length molecule will also be in a closed state because COILS predicts the sequence preceding our structure contains a strong heptad repeat. The proximity of S1 to S2 (Fig. 2a and 2b) may allow both of these hydrophobic paths to contribute to the dimerization interface between Helix 1 and Helix 2 in a closed conformation. This creates the possibility for movement between Helix 1 and Helix 2 because there would be more total surface area between the contacting helices for sliding against each other. There are three possible scenarios how the S1 and S2 paths in Helix 1 can align with the S1 and S2 paths in Helix 2. The S1 path of Helix 1 may interact with S1 of Helix 2, or the S2 path of Helix 1 can rest against S2 of Helix 2, or S1 of Helix 1 can contact S2 of Helix 2. We suggest that these different scenarios could control access to important elements in the S3 path, which may determine the binding of clathrin light chain. For example, it may be sufficient to regulate access to L or R in ₄₈₄DLLRKN to trigger the binding or release of clathrin light chain. The molecular hinge between position 532 and 539 that opens the structure would be a critical feature in any movement involving Helix 1 and Helix 2. We are currently generating point mutations in the hinge region to investigate if the interaction between HIP1 and clathrin light chain is regulated through molecular flexibility in the vicinity of the ₄₈₄DLLRKN region.

Surface suitable for clathrin light chain binding

We identified a path (S3) in the region of HIP1 that involves the ₄₈₄DLLRKN sequence (Fig. 3). Most of the S3 path is hydrophobic (L486, A483, A490, S497 and V504) and completely exposed to water and is therefore a potential surface for protein-protein interactions. The S3 path is short (~31 Å long) compared to S1 or S2, with an estimated surface area of ~150 Å². We stress that the S3 path does not have a heptad pattern. The S3 path is on the helix face opposite where S1 and S2 are located and is flanked by R487 (Fig. 3a). The presence of L486 and R487 at the beginning of S3 is significant because both L486 and R487 are essential for binding clathrin light chain⁵. Surface analysis in Fig. 3 reveals the S3 path is interrupted by K494, which is conserved in human and mouse HIP1 and HIP12/1R. Thus, S3 is subdivided by 2 positively charged regions, one close to the beginning involving R487, and the other in the middle, centered on K494.

HIP1 deletion studies conducted to find the clathrin light chain- binding region in HIP1⁵ provide information about the possible function of the S3 path. The binding of LCb to a HIP1 deletion construct missing V504 was slightly reduced (the 336–463 construct reported by Legendre-Guillemain *et al.*⁵ is numbered differently, but corresponds to 370–497 in our structure). However, deleting S497 and K494 (336–456 deletion construct) significantly weakened LCb binding⁵. These mutagenesis data suggest the hydrophobic region between S497 and V504 is involved in binding light chain. Moreover, the basic pocket in the crystal structure (Fig. 3b) may also participate in binding clathrin light chain because LCb binding was significantly decreased when K494 was removed⁵. Our HIP1 crystal structure also offers an explanation for why mutating L485 does not affect clathrin light chain binding⁵. L485 is located on the opposite face of S3 and is part of the S1 path that is shown in Fig. 2a. Therefore L485 would be in the hydrophobic core of a fully closed HIP1 dimeric molecule and would not contribute to binding light chain.

The determinants essential for binding HIP12/1R, and presumably HIP1, were found to depend on a variety of residues in the conserved region of LCa and LCb⁴. This work revealed that E20V, Q31R and I38M mutants weakened the binding of LCb to HIP12/1R⁴. Conversely, D22V, I35A and D39A mutants were found to completely inhibit the interaction⁴. Apparently E32A, S33A and E34A mutants have the opposite effect, enhancing the binding of LCb to

HIP12/1R⁴. The fact that the S3 path is very hydrophobic may explain why changing E32, S33 or E34 to alanine significantly increases binding⁴.

The two positively charged regions present in S3 (Fig. 3b) suggest R487 and K494 may be placed close to D22 and D39 when LCb (or LCa) complexes with HIP1 or HIP12/1R. There is evidence that D22, in the regulatory region of LCb⁹, interacts with R487 to stimulate clathrin assembly^{4,5}. Since there are no other basic residues in the immediate area, a possible contact model would position D39 in light chain proximal to K494 in HIP1 (or the corresponding lysine in HIP12/1R). We further assessed the likelihood of the D22/R487, D39/K494 contact model by calculating the C α -to-C α distance (~27 Å) between D22 and D39 in LCb, assuming helical conformation. This distance is 10 Å more than the distance between the η_1 and η_2 nitrogens of R487 and the ζ nitrogen of K494 (~17 Å), but the difference could be less if the oxygens from the γ carbons in the side chains of D22 and D39 point toward each other. However, we cannot rule out the possibility that the disagreement in the estimated distances is due to the assumption that the light chain is helical between D22 and D39. There is a proline at position 23 in both LCa and LCb immediately following the ₂₀EED regulatory region, which could discourage this part of light chain from folding into a helix.

Finally the HIP1 482–586 structure may be useful in thinking about how the yeast homolog of HIP1, *sla2p*, interacts with yeast clathrin light chain. *In vivo* data indicate that the association between *sla2p* and yeast clathrin light chain is absolutely required for invaginating the plasma membrane to form transport vesicles, but this interaction does not require the clathrin heavy chain¹⁸. In *s. cerevisiae*, *s. pombe* and *y. lipolytica* the KD(E)E(D)Q(L)I(L)K(T, E)N(A,S) sequence corresponds to ₄₈₃ADLLRKN (HIP1). The underlined amino acids (KDEQIKN) are from *s. cerevisiae* and those in parentheses are the changes in *pombe* or *lipolytica*. We determined where yeast (*s. cerevisiae*) residues (in parentheses in Fig. 3a) might be positioned in the S3-containing region of the crystal structure by aligning KDEQIKN against ₄₈₃ADLLRKN. In yeast, leucine also occupies position 486 (Fig. 3a), but R487 is not conserved (R->T). We also found that A483 is replaced by aspartic acid in yeast, which would make this region negatively charged. Lastly, a cluster of acidic residues in yeast replace the basic pocket centered on K494 (see Fig. 3a), suggesting the yeast clathrin light chain itself would have to be significantly substituted in order to compensate for the flipped electrostatic charges. This is consistent with the fact that the 22-amino acid region (aa20–41, LCb numbering) in LCa and LCb that is 100% conserved in all vertebrates and highly conserved in light chains from non-vertebrates¹⁹, is not conserved in yeast light chain. Only three residues (underlined) in the ₂₀EEDPAAAFL sub-region (from LCb) are identical in the corresponding yeast KKDDDTDFL light chain sequence. Thus, the mode of interaction between *sla2p* and yeast clathrin light chain may be different.

Conclusions

Mutational studies demonstrated that L486 and R487 in HIP1 are crucial for the binding of clathrin light chain⁵. These two HIP1 residues are revealed in our crystal structure and are part of the ₄₈₄DLLRKN region in HIP1 (ELLRKN in HIP12/1R) that was initially thought to interact with clathrin light chain⁴. We report here that the heptad repeat in the coiled-coil trunk region of our Y shaped HIP1 molecule is shifted from the heptad repeat coming from the N-terminus of the protein to produce two hydrophobic paths, S1 and S2, that ultimately merge together. The S3 path, solvent exposed and much shorter than S1 and S2, may function as a clathrin light chain-binding surface. Finally, the comparison of surface features suggests the interaction between *sla2p* and yeast clathrin light chain may be unique.

Protein Data Bank accession code

The coordinates have been deposited in the RCSB Protein Data Bank with accession code 2NO2. The coordinates will be released on October 24, 2007.

Acknowledgements

We thank Valerie Legendre-Guillemain and Peter McPherson for the pGST-Hip1h construct; Ed Westbrook at beamline 4.2.2 of the Molecular Biology Consortium at the Advanced Light Source for early data collection runs; Samantha Perez-Miller for comments on the manuscript. This work was supported by NIH grant RO1 GM064387 to J.A.Y.

References

1. Gervais FG, Singaraja R, Xanthoudakis S, Gutekunst C-A, Leavitt BR, Metzler M, Hackam AS, Tam J, Vaillancourt JP, Houtzager V, Rasper DM, Roy S, Hayden MR, Nicholson DW. Recruitment and activation of caspase-8 by the huntingtin-interacting protein HIP-1 and a novel partner HIPPI. *Nature Cell Biol* 2002;4:95–105. [PubMed: 11788820]
2. Engqvist-Goldstein AE, Kessels MM, Almers W. An actin-binding protein of the Sla2/Huntingtin interacting protein 1 family is a novel component of clathrin-coated pits and vesicles. *J Cell Biol* 1999;147:1503–1518. [PubMed: 10613908]
3. Mishra SK, Agostinelli NR, Brett TJ, Mizukami I, Ross TS, Traub LM. Clathrin- and AP-2-binding sites in HIP1 uncover a general assembly role for endocytic accessory proteins. *J Biol Chem* 2001;276:46230–46236. [PubMed: 11577110]
4. Chen C-Y, Brodsky FM. Huntington-interacting protein 1 (Hip1) and Hip1-related protein (Hip1R) bind the conserved sequence of clathrin light chains and thereby influence clathrin assembly in vitro and actin distribution in vivo. *J Biol Chem* 2005;280:6109–6117. [PubMed: 15533940]
5. Legendre-Guillemain V, Metzler M, Lemaire J-F, et al. Huntingtin interacting protein 1 (Hip1) regulates clathrin assembly through direct binding to the regulatory region of the clathrin light chain. *J Biol Chem* 2005;280:6101–6108. [PubMed: 15533941]
6. Legendre-Guillemain V, Wasiak S, Hussian NK, Angers A, McPherson PS. ENTH/ANTH proteins and clathrin-mediated membrane budding. *J Cell Sci* 2004;117:9–18. [PubMed: 14657269]
7. Brett TJ, Legendre-Guillemain V, McPherson PS, Fremont DH. Structural definition of the F-actin-binding THATCH domain from HIP1R. *Nature Struct and Mol Biol* 2006;13(2):121–130.
8. Brett TJ, Traub LM, Fremont DH. Accessory protein recruitment motifs in clathrin-mediated endocytosis. *Structure* 2002;10:797–809. [PubMed: 12057195]
9. Ybe JA, Greene B, Liu S-H, Pley U, Parham P, Brodsky FM. Clathrin self-assembly is regulated by three light chain residues controlling the formation of critical salt bridges. *EMBO J* 1998;17:1297–1303. [PubMed: 9482727]
10. Legendre-Guillemain V, Metzler M, Charbonneau M, et al. Hip1 and Hip12 display differential binding to F-actin, AP2 and clathrin. *J Biol Chem* 2002;277:19897–19904. [PubMed: 11889126]
11. Crick FCH. The packing of alpha-helices: simple coiled coils. *Acta Crystallogr* 1953;6:689–697.
12. Sodek J, Hodges RS, Smillie LB, Jurasek L. Amino acid sequence of rabbit skeletal tropomyosin and its coiled-coil structure. *Proc Natl Acad Sci USA* 1972;69:3800–3804. [PubMed: 4509342]
13. Cohen C, Parry DAD. a-helical coiled coil and bundles: how to design an a-helical protein. *Proteins: Structure, Function and Genetics* 1990;7:1–15.
14. Zhou NE, Kay CM, Hodges RS. The role of interhelical ionic interactions in controlling protein folding and stability. *J Mol Biol* 1994;237:500–512. [PubMed: 8151708]
15. Beck K, Gambia JE, Kamawal A, Bachinger HP. A single amino acid can switch the oligomerization state of the alpha-helical coiled-coil domain of cartilage matrix protein. *EMBO J* 1997;16:3767–3777. [PubMed: 9233786]
16. Brown JH, Cohen C, Parry DAD. Heptad breaks in alpha-helical coiled coils: stutters and stammers. *Proteins: Struct Func and Genetics* 1996;26:134–145.
17. Engqvist-Goldstein AEY, Warren RA, Kessels MM, Keen JH, Heuser J, Drubin DG. The actin-binding protein Hip1R associates with clathrin during early stages of endocytosis and promotes clathrin assembly in vitro. *J Cell Biol* 2001;154:1209–1223. [PubMed: 11564758]

18. Newpher TM, Idrissi FZ, Lemmon SK. Novel function of clathrin light chain in promoting endocytic vesicle formation. *Mol Biol Cell* 2006;17(10):4343–4352. [PubMed: 16870700]
19. Brodsky FM, Hill BL, Acton SL, et al. Clathrin light chains: Arrays of protein motifs that regulate coated vesicle dynamics. *Trends Biol Sci* 1991;16(6):208–213.
20. Metzler M, Legendre-Guillemin V, Gan L, Chopra VS, Kwok A, McPherson PS, Hayden MR. HIP1 functions in clathrin-mediated endocytosis through binding to clathrin and adaptor protein 2. *J Biol Chem* 2001;276:39271–39276. [PubMed: 11517213]
21. Hackam AS, Yassa AS, Singaraja R, Metzler M, Gutekunst C-A, Gan L, Warby S, Wellington CL, Vaillancourt J, Chen N, Gervais FG, Raymond L, Nicholson DW, Hayden MR. Huntingtin interacting protein 1 induces apoptosis via a novel caspase-dependent death effector domain. *J Biol Chem* 2000;275(52):41299–41308. [PubMed: 11007801]
22. McCann RO, Craig SW. Functional genomic analysis reveals the utility of the I/LWEQ module as a predictor of protein:actin interaction. *Biochem Biophys Res Commun* 1999;266:135–140. [PubMed: 10581178]
23. Harjes P, Wanker EE. The hunt for huntingtin function: interaction partners tell many different stories. *Trends in Biochem Sci* 2003;28(8):425–433. [PubMed: 12932731]
24. Van Duyne GD, Standaert RF, Karplus PA, Schreiber SL, Clardy J. Atomic structures of the human immunophilin FKBP-12 complexes with FK506 and rapamycin. *J Mol Biol* 1993;229:105–124. [PubMed: 7678431]
25. Terwilliger TC. Multiwavelength anomalous diffraction phasing of macromolecular structures: analysis of MAD data as single isomorphous replacement with anomalous scattering data using the MADMRG Program. *Methods in Enzymology* 1997;276(1):530–537. [PubMed: 9048380]
26. Terwilliger TC. Maximum likelihood density modification. *Acta Cryst* 2000;D56:965–972.
27. Jones TA, Zhou JY, Cowan SW, Kjeldgaard. Improved methods for binding protein models in electron density maps and the location of errors in these models. *Acta Crystallogr A* 1991;47(Pt 2):110–119. [PubMed: 2025413]
28. Sutton RB, Fasshauer D, Jahn R, Brunger AT. Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 Å resolution. *Nature* 1998;395(6700):347–353. [PubMed: 9759724]
29. Kabsch W. A solution for the best rotation to relate two sets of vectors. *Acta Cryst* 1976;A32:922–923.
30. Lupas A, Van Dyke M, Stock J. Predicting coiled coils from protein sequences. *Science* 1991;252(5010):1162–1164.

Figure 1.

Topology of the human HIP1 482–586 sub-fragment and domain map of full-length HIP1 and HIP12/1R. **a**, Helix 1 of the “Y” shaped HIP1 482–586 homodimer shows side chains (magenta), while Helix 2 shows only the backbone (grey). The N and C designate the N- and C-termini of the parallel dimer model. Helix 1 and Helix 2 in the trunk region are coiled together but start to separate a 1/3 of the way towards the N-terminus. **b**, The left-handed coiled-coil twist is evident when the molecule is rotated 90 degrees from the view in **a** and shows 2 nodes or points of intersection ~ 70 Å apart, which is typical of coiled-coil proteins¹¹. Structural images were prepared using PyMol (<http://www.pymol.org>). **c**, Domain organization of HIP1 and related protein, HIP12/1R (numbering from human HIP1 and HIP12/1R is used). ANTH domain (residues 47–159 in HIP1; 39–150 in HIP12/1R) recognizes inositol phospholipids^{3, 6}, AP2 binding sites in HIP1 only (residues 262–266 and 358–360^{3, 20}), CLTD region (residues 332–336, LMDMD in HIP1⁸) binds the N-terminal domain of clathrin, pDED is a pseudo-death effector domain that can bind HIPPI (residues 410–491 in HIP1; 393–470 in HIP12/1R^{1, 21}), LC is the clathrin light chain binding site (₄₈₄DLLRKN in HIP1; ₄₆₃ELLRKN in HIP12/1R⁵), USH is an upstream regulatory helix, which modulates affinity for actin (residues 780–805 in both HIP1 and HIP12/1R¹⁷), and an F-actin binding region (residues 813–1011 in HIP1 and HIP12/1R containing the actin-binding I/LWEQ motif²²). The Huntingtin binding site in HIP1 is indicated (residues 245–631)²³. The yellow bar indicates the position of the reported HIP1 482–586 crystal structure. *Methods*: HIP1 482–586 with an N-terminal GST tag was created by PCR mutagenesis starting with a construct of human HIP1 sub-fragment 371–645 inserted into pGEX-2T (pGST-HIP1h) (pGST-HIP1h was kindly provided by Valerie Legendre-Guillemain and Peter McPherson). Hip1 482–586 was over-expressed at 37°C in Rosetta 2 (DE3) pLysS bacterial cells (Novagen) in M9 minimal medium. After 10 hours of growth ($OD_{600} \sim 0.5$), selenomethionine was added as previously described²⁴ and IPTG (100 µg/ml, final concentration) was added to induce protein expression. Cells were then incubated for 15 hours at 30°C before being harvested and flash frozen for use. Cell pellets were thawed on ice for ~ 20 minutes, then gently resuspended in 45 ml of PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.3), supplemented with 0.25 ml DTT (1M stock), 0.25 ml protease inhibitor cocktail (Sigma), and 2 ml PMSF (17.4 mg/ml in 2-propanol). Before cells were sonicated, 2.5 ml of Triton X100 (20% stock) was added to facilitate lysis. The crude bacterial lysate was cleared by centrifugation and then mixed with ~ 5 ml glutathione Sepharose 4B (Amersham) resin suspended in PBS buffer. This slurry was rocked gently at room temperature for 2 hours before being transferred into a small column. The packed column was washed with 50 ml of PBS buffer until no more background protein was detected by Coomassie staining. The GST tag was removed by rocking the protein-charged resin overnight at room temperature with 30 units of sequencing grade thrombin (Novagen). The released HIP1 construct was eluted from the column with PBS buffer and 1/10th volume of 0.5 M EDTA at pH 8.0 was added before exhaustively dialyzing the sample against 10 mM Tris, 10 mM 2-mercaptoethanol, pH 7.9 buffer at 4°C. The partially purified sample was concentrated and then passed through a strong anion exchange column (POROS 20 HQ) equilibrated in 10 mM HEPES, 2 mM TCEP, 1% glycerol buffer at pH 7.9 (Buffer A). The target protein was eluted off with a linear gradient of Buffer B (10 mM HEPES, 500 mM NaCl, 2 mM TCEP, 1% glycerol, pH 7.9). As a final polishing step, the protein was passed through a Superose 6 gel-filtration column equilibrated with Buffer A. We confirmed the single selenomethionine substitution by electrospray mass-spectroscopy. The protein was crystallized by the hanging drop vapor diffusion method in reservoir buffer containing 25% (v/v) PEG 3350, 0.2 M Li₂SO₄ and 0.1 M Bis-Tris at pH 6.5. The crystals grew in the tetragonal space group $P4_22_12$ ($a = 54.2$ Å, $b = 54.2$ Å, $c = 152.2$ Å), with one monomer in the asymmetric unit. The crystals were highly mosaic (>2 degrees) along the c -axis. Exhaustive screening for alternative crystallization conditions or small molecule additives to improve the crystals did not impact quality. However, we found diffraction improved dramatically when preexisting crystals were slowly dehydrated and annealed. After treatment, crystals became much less mosaic (~ 1.2 degrees) and yielded data to ~ 2.6 Å sufficient for structure determination. *Data*

collection: The HIP 482–586 structure was solved by multi-wavelength anomalous dispersion (MAD). A 3-wavelength data set was obtained from a single crystal on beam line 4.2.2 at the Advanced Light Source, Lawrence Berkeley National Laboratory. The data were collected at 100K in 0.5 degree oscillations using a NOIR-1 CCD detector. Wavelengths λ_1 (0.97878 Å) and λ_2 (0.97863 Å) were at the peak and inflection, respectively, of the K-edge of selenium and λ_3 (0.96409 Å), was a high energy remote. Each wavelength was collected in a single sweep with an optimized kappa angle to prevent overlaps in the long axis. Data were integrated and scaled using D*TREK. Peak data set: R_{merge} , 0.091 [0.435]; I/σ , 12.3 [4.6]; Completeness, 100%. Inflection: R_{merge} , 0.088 [0.400]; I/σ , 12.9 [4.8]; Completeness, 100%. High energy remote: R_{merge} , 0.133 [0.597]; I/σ , 9.2 [3.4]; Completeness, 100%. Statistics for the highest-resolution shell are given in brackets. *Phasing and refinement:* A Bayesian approach²⁵ was used to phase the MAD data taking the high-energy remote (λ_3) as the ‘native’ wavelength and the other two as ‘derivative’ wavelengths. The single selenium site in each monomer was found by SOLVE (<http://www.solve.lanl.gov>) and the experimental map was improved using RESOLVE²⁶. Model building was carried out using O²⁷ and the model was refined using CNS²⁸. Alternating rounds of positional, grouped B factor and simulated annealing were performed in reference to $2F_o - F_c$ and $F_o - F_c$ maps and a bulk-solvent correction was applied near the end of refinement. The HIP1 482–586 structure refined against all the data from 30–2.8 Å with an R-factor of 28.6% and an R_{free} of 31.3% at 2.8 Å resolution. Ramachandran plot statistics: Most favored region, 97%; Additionally allowed regions, 1%; Generously allowed regions, 0%; Disallowed regions, 0%. Geometry statistics: B-values average, 77.4 Å²; *rmsd* bond distances, 0.008 Å; *rmsd* bond angles, 0.9 degrees. The high working and free R-values reflect the limited quality of our best crystals. *Structural analysis:* Superpositions of Ca traces in Figure 3 were carried out using Lsqkab²⁹. The coiled-coil analysis was performed by the program COILS³⁰. **d**, Heptad repeat in the coiled-coil trunk domain 541–581. The F570 (*a*), L573 (*d*), E574 (*e*) and R577 (*a*) residues in Helix 2 form a pocket for L573 (*d*) protruding from Helix 1. The crystal structure validates the heptad repeat *a*-, *d*- and *e*-positions predicted by COILS in parentheses. The N and C label the N- and C-termini and the image was prepared using PyMol (<http://www.pymol.org>). **e**, The dimer splay site contains a hinge region. The region indicated by the purple bar from L532 and K539 and highlighted by the short ribbon in Helix 1 designate a flexible hinge. A corresponding hinge region is present in Helix 2, but is not shown for the sake of clarity. The residues with yellow dotted surface in Helices 1 and 2 are L532, V534 and L535 that cluster next to an acidic path that surrounds the beginning of the hinge at L532. There is an alternating pattern of oppositely charged residues that punctuates the end of the hinge region at K539 in Helix 1 and 2. K539 and R547 in this region are shown as sticks, while E541 and E548 are shown with red dotted surface.

Figure 2a.

Multiple interaction interfaces exist in the open region of the HIP1 482–586 dimer. Top panel of the figure shows the Y shaped structure in the same orientation as in Fig. 1a with the N-termini of Helices 1 and 2 pointed to the left (labeled N). The bottom panel is a diagrammatic representation of Helix 1. Labeled circles indicate specific heptad positions that were predicted by COILS in the trunk region and validated by the crystal structure. In the trunk the heptad repeat *d*-positions, green side chains in Helix 1 in the top panel, are green circles labeled with “d”. The red circles labeled “a” are the heptad *a*-positions in Helix 1. Note that these *a*-position residues in the 2-D representation of Helix 1 are not shown in the model in the top panel for the sake of clarity. Instead we show the corresponding *a*-positions in the model that are in Helix 2. The amino acids corresponding to specific heptad repeat positions marked by color are indicated along the bottom edge of the diagram. The dashed line through the red and green circles represents the *a*-*d* coiled-coil interface between Helices 1 and 2 in the trunk. The box in the diagram shows the location of the hinge region (aa532–539). The continuation of the coiled-coil interface from the trunk into the open region is shown as dashed line labeled S2 passing through *a*- and *d*-positions indicated by grey and salmon colored circles. The anti-parallel crystal contact observed between neighboring HIP1 helices described in the text is shown as a dotted line labeled S1 and pass through *d*- and *g*-positions indicated by salmon and blue colored circles. The S1 residues in Helix 1 are also shown as blue colored side chains in the model in the top panel. The asterisk marks where S1 and S2 paths merge together (corresponds to point F in Fig. 2b). The S3 path described in the text is shown as a thin line going through *a*- and *e*-positions in the diagram. Note the yellow circles along S3 correspond to L486 (*a*), S497 (*e*) and V504 (*e*).

Figure 2b.

Surface analysis of Helix 1 shows the S1 and S2 hydrophobic paths. The S1 path is shown in blue and traced in dashed line to guide the eye. The S1 path is in yellow and lies very close to S1. The S1 and S2 paths merge at point F. The S3 path is not visible in this view and is on the other side of the helix, opposite to S1 and S2 and exposed to water. The N- and C-termini are labeled N and C, respectively. The HIP1 surface was rendered using PyMol.

Figure 3.

Surface analysis of the S3 path. **a**, N-terminal region of the HIP1 482–586 crystal structure containing $_{484}\text{DLLRKN}$ includes the hydrophobic S3 path (dotted trace) defined by the residues indicated in light yellow. The darker yellow surface (L486), critical for clathrin light chain binding⁵, is part of the S3 path. The red and blue colored regions denote the location of acidic and basic amino acids, respectively. The basic pocket centered on K494 (may also involve R500) divides the S3 path in half. Amino acids in parentheses are those in *S. cerevisiae* (see text) and were assigned by aligning KDEQIKN (yeast) against $_{483}\text{ADLLRKN}$. The N and C designate the N- and C-termini. The surface model was generated using PyMol. **b**, Surface potential shows two positively charged regions subdivide the S3 path. Model is oriented in the same as in (a) and the dotted tracing indicates the S3 path. Positive potential exists proximal to R487, a residue that is essential for clathrin light chain binding⁵. As shown in Panel a, the middle of S3 is marked by a basic pocket that is centered on K494. The surface potentials (blue, positive and red, negative) were calculated using PyMol.