Replacement of charged and polar residues in the coiled-coiled interface of huntingtin-interacting protein 1 (HIP1) causes aggregation and cell death

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1. Introduction

Huntingtin interacting protein 1 (HIP1) was first identified as an obligate binding partner of huntingtin (htt) [1,2]. The expansion of the polyglutamine (polyQ) tract of htt in the genetic neurodegenerative disorder, Huntington’s disease, severely weakens the interaction between htt and HIP1 [1,2]. HIP1 is a member of a family of coiled-coil proteins involved in clathrin-mediated endocytosis, including HIP1R (HIP1-related) and the yeast homolog Sla2p. The HIP family proteins share a domain structure that is divided into an N-terminal ANTH/ENTH domain, a central coiled-coil (CC) domain, and a C-terminal actin-binding (THATCH) domain [3]. The packing of heptad a- and d-positions in CCs are well defined (a-a’/d-d’ (parallel) or a-d’/d-a’ (antiparallel) [4]). Residues at the a-position are frequently leucine, isoleucine or alanine and the d-position is usually leucine or alanine [4,5]. Crystal structures from our laboratory reveal the CC region of a HIP1 sub-fragment is splayed opened, suggesting this coiled-coil region is plastic [6–8]. The HIP1 parallel CC has a number of a- and d-residues that are polar or charged. Previously, our laboratory argued the HIP1 CC was destabilized by the atypical interface between coils [7,8]. Understanding the unusual interface may provide new insights into why the polyQ expansion in mutated htt strongly compromises the htt/HIP1 complex. To test if charged or polar residues in the hydrophobic interface actually had an impact on the overall stability of the HIP1 CC, we mutated charged and polar residues in the opened region to leucine. Here we report that replacing threonine-528, glutamine-531 and lysine-510 with leucine alters the apparent thermal stability of the HIP1 coiled-coil domain. Confocal experiments show cells expressing HIP1 mutants contain aggregates that are detectable as early as 6 h post-transfection. The formation of HIP1 aggregations is correlated to loss of cell viability. Based on these data we conclude that the opened region of HIP1 coiled-coil domain is sensitive to mutation, suggesting the intrinsic plasticity of the CC is relevant for activity.

2. Materials and methods

2.1. Site-directed mutagenesis

Single, double and triple mutations were created by site-directed mutagenesis on pGEX2T1-HIP1h (aa373-647), pGEX2T-HIP1482-586, pCDNA3.1-HIP1 full length or pEGFP-C2 HIP1 full length. Primers used: T528L and Q531L 5′-AGGGCCAGCGGAAGCTTTCAAGAACTTCTGGAAGTTCTAGAGAGC-3′ and K510L 5′-TAGATTGGGAACGAGAGCTTAAAGAGCTGGAGGATTCG-3′. N489L: 5′-TGACCTGCTGCGGAAGCTTGCAGAGGTGACCAAACAGG-3′. All sequences were confirmed before use.

2.2. Circular dichroism

CD measurements were performed on 0.2 mg/ml HIP1h 373-647 (WT and mutations purified as in [7,8]) in 10 mM potassium...
phosphate pH 7.8, on a JASCO J-715 spectrophotometer. Protein concentration was determined using Bradford reagent. Spectra were collected on samples from 300 to 190 nm at 5 °C temperature increments from 5 °C to 80 °C. Experiments were performed in triplicate on three independent protein preparations. K2D2 was used for secondary structure analysis [9].

2.4. Cell culture and transfections

All cells were maintained at 37 °C in 5% CO₂. HEK293T cells were cultured in 90% DMEM (Gibco), 10% FBS (Sigma–Aldrich) and 100 U/ml penicillin/100 μg/ml streptomycin (Gibco). SH-SY5Y cells were cultured in 90% DMEM/F12 (Gibco), 10% FBS and penicillin/streptomycin. N2a cells were maintained in MEM (Gibco), 10% FBS, penicillin/streptomycin, and 2 mM GlutaMAX I (Gibco).

Transient transfections were performed in all cell lines with Fugene HD (Roche) or TransIT2020 (Mirus). Transfection complexes were added to cells directly after plating to and incubated for 6–48 h.

2.5. Confocal microscopy

Cells were plated onto poly-l-lysine coated coverslips and transfected with pEGFP-HIP1 full length (FL) WT and mutant constructs or pcDNA3.1-HIP1(FL) WT or 2M + K510L. Cells were fixed, permeabilized, and nuclei were stained with Draq5 (Cell Signal Technologies). For the untagged constructs, cells were incubated with anti-HIP1 (Novus Biologicals) and AlexaFluor-488 (Life Technologies) antibodies. Microscopy was performed on a Leica Sp5 scanning confocal microscope. Minimum of 75 cells per treatment over 5 independent experiments were analyzed.

2.6. MTS assays

Cells were plated in 96 well plates, transfected, and MTS assays (Promega) performed at 18, 24 and 48 h post-transfection. Experiments are minimum of six independent repeats per time point.

2.7. Flow cytometry

HEK293T cells were transfected for 18 h, trypsinized and collected by centrifugation. Cells were then washed in PBS and resuspended in 10 mM HEPES pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂. Cells were labeled with AlexaFluor-647-conjugated AnnexinV and SyTox AADvantage (Life Technologies), a 7AAD fluorophore. Cells were analyzed on a BD Biosciences LSRII flow cytometer. Gates were established for GFP-expressing cells only. Data were collected for six independent repeats.

2.8. Coimmunoprecipitation and Western blotting

HEK293T cells were co-transfected pcDNA3.1-23Qhtt-myc/pEGFP-HIP1(FL) WT or mutant constructs for 24 h. Cells were rinsed in PBS, then scraped in cold co-IP buffer (50 mM Tris–HCl, pH 7.5, 15 mM EDTA, 100 mM NaCl, 0.2% (v/v) Triton X-100, complete protease inhibitor cocktail (Roche)) and centrifuged. Supernatants were precipitated with Dynabeads ProteinA (Life Technologies) charged with anti-GFP antibody (ab290, Abcam).

Complexes were washed with co-IP buffer before elution by boiling in 1X SDS-buffer. Samples were run on 10% SDS–PAGE gels, transferred to PVDF (Millipore), probed with anti-HIP1 and anti c-myc (Novus Biologicals) primary antibodies and detected by chemiluminescence (ThermoFisher). Blots are representative of five independent repeats.

2.9. Statistics

Students t test or one way ANOVA tests were used to determine significance at 95% or greater confidence.

3. Results and discussion

3.1. Replacement of charged residues at a- and d-positions stabilizes the HIP1 coiled-coil

In this report HIP1 constructs with both T528L and Q531L are 2M, while HIP1 constructs that also include the K510L are designated 2M + K510L (Fig. 1A). To assess if the mutations impacted secondary structure or global stability of HIP1, far-UV CD data on HIP1h (aa 371-647) WT, K510L, 2M and 2M + K510L were acquired at different temperatures and compared (Fig. 1B–E). We analyzed the spectra from 240–190 nm to determine secondary structure content [9]. As the sample was heated, the characteristic helical CD profile (double minimum at 208, 222 nm and a peak ∼190 nm) was transformed (Fig. 1B–E). All four proteins had reversible melting curves (data not shown). We found that the mutations did not change the overall helical content, but altered the sensitivity of the structure to temperature. For WT and the single point mutation K510L, this sensitivity occurred around 40 °C. In contrast, the helical content of the double and triple mutations began to decrease at around 50 °C and 60 °C, respectively (see Supplementary Table 1). The 222 nm data plotted against temperature show mutations in HIP1h cause non-ideal features in the melting curves (Fig. 1F). We also observed that 80 °C is not sufficient to completely melt out secondary structure as we would expect the signal at 222 nm to be positive for aperiodic structure. To address the possibility that high temperature triggered dynamic protein aggregation, we monitored urea-induced unfolding at 222 nm. We find that HIP1h WT and the single point mutation K510L had a similar transition midpoint at 2M urea (data not shown). On the other hand, HIP1h 2M and 2M both had transition midpoints around ∼3.4 M urea. This suggests the double and triple mutation conferred more resistance to urea-induced unfolding than either WT or the single mutation K510L. Together, the CD data indicate secondary structure stability is enhanced when the charged or polar residues are removed from the specific locations within the CC interface.

3.2. Overexpression of full-length HIP1 mutants causes aggregation that increases htt immunoprecipitation

To investigate if the K510L, 2M, and 2M + K510L mutations impacted the binding of WT htt, we performed a series of co-IP experiments on HEK293T cells co-transfected with either full length (FL) GFP-HIP1 WT, K510L, 2M, or 2M + K510L and a 23 polyQ htt construct containing the first 900 amino acids of htt. After 24 h cells were harvested, and a co-IP was performed. As expected, GFP-HIP1 WT was able to co-IP 23Qhtt-myc (Fig. 2A, lane 3). We found that both GFP-HIP1 K510L and 2M constructs were able to co-IP a similar level of the 23Qhtt-myc construct (compare lanes 4.5 with lane 3; Fig. 2A). In contrast, the intensity of the band from the GFP-HIP1 2M + K510L co-IP experiment (lane 6) was very strong. One possible explanation is that there is increased interaction between
23Qhtt-myc construct and GFP-HIP1 2M + K510L. To determine if the enhanced coIP was due to a combination effect specific to K510, we repeated the co-IP on a different triple mutant, where N489 (the previous polar α-position) was mutated to leucine on
HIP1 2M (2M + N489L). The co-IP result (lane 7, Fig. 2A) shows this mutation did not produce an exaggerated htt precipitation, indicating the effect was specific to HIP1 2M + K510L.

To determine if subcellular localization could explain the co-IP results, we performed confocal microscopy experiments using GFP-HIP1 mutant constructs transfected into HEK293T cells. To compare the distribution pattern in a human neuronal cell, the experiment was also performed in SH-SY5Y cells. At 18 h, GFP-HIP1 WT was overexpressed in the cytosol and at the membrane but is non-nuclear, which agrees with previously published observations [10]. GFP-HIP1 2M and GFP-HIP1 K510L exhibited expression patterns that were similar to GFP-HIP1 WT (Fig. 2B). In contrast, a proportion of GFP-HIP1 2M + K510L cells (~30–40% of the total population) contained bright, cytoplasmic GFP-positive puncta (see arrows), indicating the accumulation of amorphous HIP1 protein aggregates. The size of aggregates ranged from a few nm to μm in diameter, and the effect was exaggerated at 24 h, when the proportion of cells with aggregated HIP1 increased to ~50%. By 48 h, we observed that nearly all of the GFP-HIP1 2M + K510L expressing cells were laden with aggregates (data not shown). We point out that GFP-HIP1 2M + N489L-expressing cells also had aggregates. However, in this case a smaller proportion of cells had aggregated HIP1, and the aggregates also tended to be smaller in diameter. To verify that the aggregation was not due to differential protein expression, we performed flow cytometry experiments to quantify the amount of GFP fluorescence in each cell. We found no significant difference between WT and GFP-HIP1 K510L, 2M or 2M + K510L (see Supplementary fig. S1).

To investigate the kinetics of aggregate formation, we analyzed GFP-HIP1 constructs at 6 h post-transfection (Fig. 2C). Even at this early time point, both triple mutants GFP-HIP1 2M + K510L and 2M + N489L-expressing cells contained aggregates (see arrows). In contrast, aggregates were not detected in cells overexpressing GFP-HIP1 WT, K510L, or 2M. This suggests aggregation is a direct effect of the triple mutation on structure.

To rule out the possibility that the aggregation effect was due to the presence of the GFP tag, cells were transfected with either pCDNA3.1-HIP1(FL)WT or 2M + K510L and visualized after 18 h using a HIP1 antibody. Aggregates (arrows) were only present in the cells overexpressing HIP1 2M + K510L (Fig. 2D). This is an

**Fig. 2.** Aggregation of FL HIP1 2M + K510L causes enhanced htt pulldown. (A) Co-IP of GFP-HIP1 constructs co-transfected with 23Qhtt-myc after 24 h transfection. All blots were balanced for protein load. Data are representative of five independent co-IP experiments. (B) Confocal microscopy of HEK293T and SH-SY5Y cells transfected with pEGFP-C2 vector only, GFP-HIP1 FL WT and mutations 18h post-transfection. (C) HEK293T transfected with pEGFP-C2 vector only, GFP-HIP1 FL WT and mutations 6 h post-transfection. (D) HEK293T transfected with pCDNA3.1-HIP1 FL WT or 2M + K510L after 18 h. Nuclei are counterstained blue in merged image. Arrows indicate aggregated GFP-HIP1. Scale bar is 10 μm. 75 cells over 5 independent transfections were analyzed. (E) Plot of dynamic light scattering data: mean particle size of HIP1482–586 WT, K510L, 2M and 2M + K510L. Data shown are mean ± SD (n = 3 *p<0.01).
indication that the aggregation effect is not caused by the GFP tag, but is the result of mutations in the opened region of the HIP1 CC.

To evaluate if the pro-aggregation property in Fig. 2 was due to the high level of overexpression in cells, we performed dynamic light scattering (DLS) experiments on short HIP1 CC constructs that had none, one, two, or all three mutations. The bar graph in Fig. 2E summarizes the DLS data on recombinant HIP1468-547 WT, K510L, 2M, and 2M + K510L. The DLS experiments indicated there were a mixed population of different sizes (polydisperse) in every sample measured. According to DLS, the mean particle size of HIP1 WT and 2M were similar (8.2 ± 1.2 nm and 7.3 ± 1.9 nm, respectively). On the other hand, both HIP1 2M + K510L and K510L constructs produced particle sizes that were significantly larger, with an average size of 205.5 ± 30.5 nm and 232.3 ± 41.8 nm, respectively (\( p < 0.01 \)). To characterize the aggregates caused by HIP1 2M + K510L, we performed negatively stained transmission electron microscopy on recombinant HIP1h 2M + K510L. We saw disordered aggregates and not ordered oligomers or protofibrils...
vector control at 24 h (Fig. 3C) show the different populations of living, necrotic, and apoptotic cells. The flow cytometry data indicate cells overexpressing GFP-HIP1 WT + K510L have significantly (**p < 0.001) increased proportions of late apoptotic (Annexin5/7AAD-positive, upper right quadrant) and necrotic (7AAD-positive only, upper left quadrant) populations compared to cells overexpressing either GFP-HIP1 WT, K510L, 2M or 2M + N489L (quantification shown in Fig. 3D). There is not a statistically significant difference in the percentage of cells staining for early apoptosis between WT and any mutant. This could be the case if 2M + K510L-expressing cells undergo apoptosis earlier than WT cells. Consistent with earlier apoptosis, cells expressing GFP-HIP1 2M + K510L exhibited membrane blebbing 12 h post-transfection. Consistent with earlier apoptosis, cells expressing GFP-HIP1 2M + K510L die more rapidly and involves necrotic and apoptotic pathways. Based on the confocal microscopy and DLS results, the significant loss of viability of HIP1 2M + K510L expressing cells can be correlated to the aggregates caused by the seemingly favorable replacement of charged/polar residues mutations in the open region of the HIP1 CC.

Inappropriate protein aggregates are present in over 40 different human diseases, including neurodegenerative and cardiac conditions, and are thought to mediate the pathology of these diseases. The most damaging of these protein aggregates are small amorphous aggregates associated with numerous deleterious effects that often lead to cell death [15–20]. The cytoplasmic aggregates in HIP1 2M + K510L overexpressing cells together with the DLS data indicate FL HIP1 2M + K510L exists as disordered particles with a significantly larger diameter than WT or 2M. This may explain why HIP1 2M + K510L expressing cells had decreased overall metabolic viability as well as increased late apoptotic and necrotic cell populations compared to GFP-HIP1 WT, K510L, or 2M expressing cells. Although GFP-HIP1 2M + N489L also formed aggregates, these were smaller and present in a fewer number of cells than GFP-HIP1 2M + K510L, which could explain why they did not provoke a similar toxic effect.

Recently, it has been shown that specific mutations that increase the coiled-coil propensity of Q/N rich yeast prions and polyQ proteins have the effect of increasing their aggregation properties and cellular toxicity [21]. Replacement of charged/polar residues at α- and β- positions in the interface of HIP1 would increase the coiled-coil propensity of the protein because they occupy positions that are typically reserved for hydrophobic residues. Therefore the HIP1 2M + K510L aggregation could be due to increased CC propensity of the protein, which then results in increased aggregation. The sum of our data presented here suggests that the location of the third mutation, in this case K510L, modulates the aggregation propensity and overall toxic effect on cells. Our results indicate this region of the HIP1 CC domain is sensitive to stabilizing mutations, suggesting the charged residues in the interface of the HIP1 coiled-coil domain are essential for its normal function. This information may yield new insights into understanding the relationship between HIP1 and htt in Huntington’s disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.07.011.

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