Structural Insights into the Specific Binding of Huntington Proline-Rich Region with the SH3 and WW Domains

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Summary

The interactions of huntingtin (Htt) with the SH3 domain- or WW domain-containing proteins have been implicated in the pathogenesis of Huntington’s disease (HD). We report the specific interactions of Htt proline-rich region (PRR) with the SH3GL3-SH3 domain and HYP-A-WW1-2 domain pair by NMR. The results show that Htt PRR binds with the SH3 domain through nearly its entire chain, and that the binding region on the domain includes the canonical PxxP-binding site and the specificity pocket. The C terminus of PRR orients to the specificity pocket, whereas the N terminus orients to the PxxP-binding site. Htt PRR can also specifically bind to WW1-2; the N-terminal portion preferentially binds to WW1, while the C-terminal portion binds to WW2. This study provides structural insights into the specific interactions between Htt PRR and its binding partners as well as the alteration of these interactions that involve PRR, which may have implications for the understanding of HD.

Introduction

Huntington’s disease (HD) is a dominant neurodegenerative disorder characterized by movement abnormalities, cognitive impairment, and psychiatric disturbances due to neuronal cell loss, especially in the basal ganglia and the cerebral cortex (Martin and Gusella, 1986; Vonsattel et al., 1985). Accumulating evidence supports the finding that a polyglutamine (polyQ) expansion tract in huntingtin (Htt), a ubiquitously expressed protein of yet unknown function, is the cause of this disease (MacDonald et al., 1993; DiFiglia et al., 1995).

Human Htt is a large multidomain protein of 3144 amino acid residues with a polyQ domain at the N terminus (MacDonald et al., 1993). The polyQ domain ranges from 11 to 34 glutamine residues in unaffected individuals, whereas that of HD patients extends to 37 or more glutamine residues (Bates et al., 2002). A proline-rich region (PRR) containing 40 residues (normally residues 41–80) directly follows the polyQ domain in sequence (Figure 1A).

Recently, many investigations revealed that mutant Htt with expanded polyQ impairs the normal interactions of several proteins involved in gene transcription, trafficking, endocytosis, and cell signaling (Goehler et al., 2004; Harjes and Wanker, 2003; Li and Li, 2004). Thus, it is speculative that HD neuropathology is related to the interference of the normal function of cellular proteins by the aberrant Htt protein (Landles and Bates, 2004; Li et al., 2003; Li and Li, 2004).

There are many Htt-interacting partners identified; some contain Src homology 3 (SH3) or tryptophan (WW) domains, such as SH3GL3/endophilin3 (Sittler et al., 1998), protein kinase C and casein kinase substrate in neurons 1 (PACSIN1/syndapin) (Modregger et al., 2002), HYP-A/FBP11 (Faber et al., 1998), PSD-95 (Sun et al., 2001), RasGAP (Liu et al., 1997), and CA150 (Holbert et al., 2001). Among them, SH3GL3 is reported to be preferentially expressed in human brain and testis, and its C-terminal SH3 domain is essential for the interaction with Htt PRR (Sittler et al., 1998). The characteristics of the interaction between SH3GL3 and Htt and the colocalization of these two proteins suggest that SH3GL3 could be involved in the selective neuronal cell death in HD (Sittler et al., 1998). SH3GL3 was also found to bind to the shell of the Htt body, suggesting that the SH3GL3-associated HD pathology may be caused by sequestering the Htt inclusion bodies (Qin et al., 2004). PACSIN1 has been implicated in clathrin-mediated endocytosis (DiProsero et al., 2004), and its abnormal binding behavior and altered intracellular distribution in pathological tissues suggest that it plays a role during the early stages of the selective neuropathology of HD (DiProsero et al., 2004; Modregger et al., 2002). Human HYP-A interacts with the N-terminal region of Htt protein through its tandem WW domains, as identified by yeast two-hybrid assay (Faber et al., 1998; Passani et al., 2000). FBP11, the murine ortholog of human HYP-A, is one of the several proteins that bind with forms involved in murine limb and kidney development (Bedford et al., 1997). FBP11 also participates in pre-mRNA splicing (Lin et al., 2004) and regulation of N-WASP localization (Mizutani et al., 2004).

The involvement of PRR in the pathological process of HD can also be observed from the fact that M1W7 scFv, a monoclonal antibody recognizing Htt PRR, significantly inhibits aggregation as well as the cell death induced by mutant Htt protein (Khosnian et al., 2002). Another observation indicates that removal of a series of prolines adjacent to the polyQ tract in Htt blocks formation of the shell of the Htt body and redistributes sequestered polyQ fragments (Li et al., 2004). A recent report suggests that SH3GL3-associated HD pathology may be caused by sequestering the Htt inclusion bodies (Qin et al., 2004). These findings imply that the PRR orients to the specificity pocket, whereas the N-terminal portion preferentially binds to WW1, while the C-terminal portion binds to WW2. This study provides structural insights into the specific interactions between Htt PRR and its binding partners as well as the alteration of these interactions that involve PRR, which may have implications for the understanding of HD.

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The typical PRR segment recognized by SH3 or WW domains contains less than 10 amino acid residues (Kay et al., 2000), and the molecular mechanism underlying the specific recognition has been elucidated and reviewed (Ilsley et al., 2002; Musacchio, 2002; Sudol, 1996; Zarrinpar et al., 2003). However, many proteins, such as Htt, formin (Bedford et al., 1997), and N-WASP (Bompard and Caron, 2004), contain PRR far longer than 10 residues. How these long PRRs recognize various SH3 and WW domains remains largely unknown. Obtaining the structural knowledge of the interaction between Htt PRR and these domains have different binding affinities for the SH3 domain of SH3GL3 or PACSIN1 and the tandem WW domains of HYPA.

Results

The Three Portions of Htt PRR Have Different Preferences for the SH3 and WW Domains

To identify which portion of the Htt PRR region (residues 41–80) is responsible for the interaction with the SH3 or WW domain, we subdivided the region into three peptide parts, corresponding to the N-terminal (Pept-1), central (Pept-2), and C-terminal (Pept-3) portions of Htt PRR, respectively (Figure 1A). The three segmental sequences were cloned and expressed as GST fusion proteins for pull-down assay. Figure 1B suggests that SH3GL3-SH3 and PACSIN1-SH3 only bind to Pept-2, not to Pept-1 or Pept-3, while HYPA-WW1-2 (the tandem domains of HYPA) binds to Pept-1 and Pept-3, but not to Pept-2. This is consistent with the previous study that an expanded Pept-2 specifically binds with PACSIN1-SH3 (Modregger et al., 2002). The results imply that the three portions of Htt PRR have different binding affinities for the SH3 domain of SH3GL3 or PACSIN1 and the tandem WW domains of HYPA.

Solution Structure of the SH3GL3-SH3 Domain

To study the interaction between SH3GL3-SH3 and Htt PRR in detail, we solved the structure of the SH3 domain in solution by heteronuclear multidimensional NMR techniques. A summary of the NMR experimental restraints for structure calculation and statistics is presented in Table S1 (see the Supplemental Data available with this article online). All ten of the lowest-energy final structures converge with an NOE or dihedral angle violation no greater than 0.3 Å or 5°, respectively. The average root-mean-square deviations (rmsds) for the ten structures for backbone and all heavy atoms are 0.64 and 1.76 Å, respectively. Figure 2A depicts a superimposition of the ten lowest-energy structures and a ribbon representation of one of the ten NMR structures. The canonical PxxP-binding pocket is indicated in a circle, while the specificity pocket between the RT loop and the n-Src loop is also highlighted.

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We have determined the solution structure of the SH3 domain of SH3GL3 and have assigned the backbone resonances of the WW domain pair of HYPA by heteronuclear NMR. Based on the structure, the binding specificities between Htt PRR and these domains have been elucidated in detail. This study reveals that Htt PRR recognizes the SH3 domain and the WW domain pair with high specificities, but with different mechanisms.

Figure 1. The N-Terminal, Central, and C-Terminal Portions of Htt PRR Have Different Binding Preferences for the SH3 and WW Domains

(A) The domain architecture of the N-terminal Htt protein and the amino acid sequence of the PRR region. Six peptides corresponding to different portions of Htt PRR were generated by peptide synthesis. Pept-1, Pept-2, and Pept-3 were also generated and purified as recombinant GST fusion proteins for pull-down assay. (B) GST pull-down assay showing that the tandem WW domains of HYPA-WW1-2 interact with GST-Pept-1 and GST-Pept-3, while SH3GL3-SH3 and PACSIN1-SH3 only interact with GST-Pept-2 in vitro. The “Input” lane represents the band from 10% of the amount of protein in each sample.

Figure 2. 3D Solution Structure of the SH3 Domain from SH3GL3

(A) Backbone atom superposition of the final ten structures. The structures are superimposed adopting residues 3–58. (B) Ribbon diagram representation of the SH3 domain of SH3GL3. The canonical PxxP-binding pocket is indicated in a circle, while the specificity pocket between the RT loop and the n-Src loop is also highlighted.
between these two loops. The canonical PxxP-binding pocket is located on the hydrophobic patch that contains a cluster of conserved aromatic residues (Tyr10, Phe12, Trp38, and Tyr54) that are arranged adjacent each other in a nearly parallel manner.

**Binding Specificity of SH3GL3-SH3 with Htt PRR**

To elucidate the binding specificity of SH3GL3-SH3 with Htt PRR, several truncated peptides of Htt PRR (Figure 1A) were tested for interaction with the SH3 domain by using the chemical shift perturbation method. Figure 3 shows the chemical shift mapping on the SH3 domain upon binding with various peptides. The effects on some residues, such as Glu15 and Phe49, which have amide resonances largely affected by the addition of Pept-1 (Figure 3A), Pept-2 (Figure 3B), or Pept-3 (Figure 3C), are very similar. The significant residues for these three titrations are mapped on the domain structure (Figure 3G), in which the significantly perturbed residues are located in the RT loop of the SH3 domain. The residues mostly affected by the addition of Pept-1-2 (Figure 3D) cover the canonical PxxP-binding site and the specificity pocket between the RT loop and the n-Src loop of SH3GL3-SH3 (Figure 3H). Moreover, Pept-2-3 also exhibits similar binding properties with Pept-1-2 (Figures 3E and 3I), though it possesses a different peptide sequence. The result that the three nonapeptides, though having different sequences, bind to the SH3 domain with similar binding behavior suggests that their binding abilities may originate from prolines, but not from the nonproline residues. A similar result is also obtained from the binding of two double-portion peptides (Pept-1-2 and Pept-2-3). It is likely that Pept-1-2 and Pept-2-3 bind to the SH3 domain with similar binding sites, but with different orientations. In addition, binding of these short peptides, whether single portion or double portions, to the SH3 domain is relatively weak, whereas binding of Pept-2 to the SH3 domain is a little stronger than that of Pept-1 and Pept-3 (Table S2). This may explain why only the binding of Pept-2 with the SH3 domain has been identified by the less sensitive experiment of pull-down assay (Figure 1B). These results are consistent with the studies that recognition of the SH3 domain by most short proline-rich peptides is moderately weak and poorly selective, and that the specificity pocket of the SH3 domain is favorable to proline-rich peptide recognition (Santamaria et al., 2003).

For this reason, we speculate that the binding of these short peptides to the SH3 domain is less specific, and that it is the proline residue that contributes to the binding.

As for the full-length PRR (Pept-1-2-3), its binding causes perturbation on some additional residues, such as Lys23, Glu24, and Asp26 (Figure 3F), which reside on the opposite surface of the PxxP-binding pocket on the SH3GL3-SH3 domain. Thus, the binding sites on the
domain structure cover the entire canonical PxxP-binding site and the specificity pocket and perhaps its opposite surface (Figure 3J). The saturation curves for the chemical shift changes of three representative residues in SH3GL3-SH3 upon titration with Pept-1-2-3 are well fitted with a 1:1 stoichiometry (Figure 3K) and give a dissociation constant \(K_D\) of \(0.68 \text{ mM}\). Mutation of Gln17 to Ala, which is located in the crucial RT loop, causes the binding ability to be reduced by \(3\)-fold \((K_D = 2.2 \text{ mM})\). Taken together, the chemical shift perturbation experiments indicate that the entire Pept-1-2-3 is necessary for its specific binding to the SH3 domain. When it is shortened, it would lose its binding affinity (Table S2) and specificity (Figure 3). This binding specificity of Htt PRR for the SH3 domain might be required for regulating SH3-mediated interactions (Li, 2005).

**Orientation of Htt PRR Binding on the SH3 Domain**

A spin-labeling NMR method (Mahoney et al., 2000) was applied to identify the orientation of Htt PRR binding on the surface of the SH3 domain. For ensuring that the binding sites on the SH3 domain are affected by the spin-labeled compound, we produced a pair of 22-residue peptides, which contain not only the entire central portion of Htt PRR, but also its several flanking amino acids (Figure 4A), that were spin labeled with MTSSL in either the N or C terminus. When the N-terminally labeled peptide Pept-22-N-MTSSL binds to the SH3 domain, two crosspeaks in the HSQC spectrum corresponding to Asp11 and Glu13 are nearly completely eliminated (Figure 4B), indicating that the N terminus of the peptide sterically contacts the SH3 domain near the Asp11 and Glu13 residues. As for Pept-22-C-MTSSL binding, many resonances, even including those of Asp11 and Glu13, are significantly affected by the spin label, but most of the residues are located in the proximity of the specificity pocket of the SH3 domain (Figure 4C). This observation demonstrates that the C terminus of the peptide contacts the SH3 domain near the specificity pocket (Figure 4D). Taken together, these findings indicate that Htt PRR exhibits a preferred binding mode on the SH3 domain (also see Figure 8A), and that the orientation of PRR is consistent with that of the Class II SH3 ligand (Mayer, 2001).

**Evidence for the Specific Binding from Intermolecular NOEs**

On the basis of filtered-NOESY analysis on the SH3GL3-SH3/Pept-1-2-3 complex (molar ratio of 1:1) (Figure 5A), several NOE peaks from 7 residues in the SH3 domain interacting with Pept-1-2-3 were unambiguously identified and assigned on the SH3 domain. Among the residues interacting with Pept-1-2-3 (Figure 5B), Gly8 and Asn53 are located in the typical PxxP-binding site, while Gln17 and Trp38 flank the specificity pocket. Surprisingly, the residues Cys6, Ile28, and Val59 have also been found to interact with Pept-1-2-3. These residues are located on a concave surface corresponding to the binding surface of Pex5p on the Pex13p-SH3 domain (Douangamath et al., 2002; Kami et al., 2002). There is a possibility that the extended residues in Pept-1-2-3 also contact the opposite surface of the SH3 domain when it specifically binds to the PxxP-binding site and the specificity pocket.
Backbone Dynamics of the HYPA WW Domain Pair

Since Htt PRR binds to the WW domain pair of HYPA, it will be interesting to study how Htt PRR interacts with this domain pair. The structure of the FBP11-WW1 domain has been elucidated (Pires, et al., 2005), but that of WW2 and the domain orientation in the domain pair remain unsolved. To explore whether these small WW domains are orientationally flexible with respect to each other in the WW domain pair of HYPA, we determined the relaxation rates ($R_1$ and $R_2$) and $^{1}H$-$^{15}N$ NOEs for the WW1-2 domain pair (Figure S1). Generally, large NOE values (>0.7) reflect slow internal motion, whereas small values indicate substantial internal motion and negative values are indicative of fully disordered regions (Bruschweiler, 2003). The average NOE value of WW1 (residues 13–42) is $0.52$, suggesting that WW1 in the free state is marginally stable, which has been proved by the following titration experiments. WW2 (residues 54–83) is more poorly folded than WW1, as indicated by its smaller NOE values (an average of 0.45). Moreover, the single WW1 domain is structured, as the $^{1}H$-$^{15}N$ HSQC spectrum shows wide dispersion; however, the single WW2 is fully disordered, as indicated by severe line broadening and peak overlapping (data not shown). The linker region (residues 43–53) exhibits even faster internal motions than both domains, and the average NOE value is only 0.33. A similar result can also be obtained from the relaxation data of the $R_2:R_1$ ratio (Figure S1). The different dynamics of WW1 and WW2 in the domain pair infers that they have different properties for binding with PRR-containing partners.

Binding Specificity of the WW Domain Pair with Htt PRR

To understand the binding properties of the HYPA WW domain pair with Htt PRR in detail, we studied binding of the three nonapeptides and Pept-1-2-3 from Htt PRR as well as peptide PPTPPPLPP from the PRR of formin (Bedford et al., 1997; Pires et al., 2005) by using the chemical shift perturbation method. The plots of the chemical shift changes versus residue number of the WW domain pair upon titration of various peptides at a peptide:protein molar ratio of 8:1 are displayed in Figure 6. The addition of increasing amounts of the peptide ligand PPTPPPLPP results in chemical shift changes of a number of residues dispersed in both the WW1 and WW2 domains (Figure 6A). During titration, the amide resonances of some residues exhibit a little broadening in the line widths. This line broadening may be due to the chemical exchange at an intermediate time regime between the ligand-free and -bound states. Notably, the chemical shift changes for the residues in WW1 are in some aspects similar to those for the corresponding residues in WW2 at every titration point. This indicates that both WW domains have similar binding capabilities for peptide PPTPPPLPP. In other words, the two WW domains have no selectivity for the peptide from formin, which is consistent with the previous alanine-scanning mutagenesis analysis (Bedford et al., 1997).

As for the peptides from Htt PRR, titration of Pept-1 causes chemical shift changes of a number of residues in both domains, but more prominently in the WW1 domain (Figure 6B). During titration, the amide resonances in WW1 exhibits large chemical shift changes with slight line broadening, in contrast to the large broadenings observed for the corresponding resonances in WW2 (Figure 7A). In particular, some amide resonances in WW2, such as Ser61, Asp62, Tyr69, and Arg77, rapidly disappear from the spectrum at the first titration point and reappear at a peptide:protein ratio higher than 4:1. Titration of Pept-2 results in only small or no chemical shift changes of the residues in both domains, even at a Pept-2:WW1-2 ratio of 8:1 (Figure 6C). There is no substantial line broadening in any of the resonances either (Figure 7B). This indicates that Pept-2 has no or very weak interaction with the WW1-2 domain pair, which is consistent with the result from the pull-down experiment (Figure 1B). Titration of Pept-3 also causes chemical...
shift changes of many residues in both domains (Figure 6E). The resonances of these residues in both domains rapidly disappear from the spectrum at a peptide:protein ratio below 1:1 and reappear at a ratio higher than 4:1 (Figure 7D). The intensities of these reappeared peaks at the last titration are significantly enhanced compared to the peaks in the ligand-free form. This indicates that binding of Pept-1-2-3 leads WW1-2, especially the WW2 part, to achieve a well-folded structure in the complex.

Upon binding with Pept-1, a number of resonances (e.g., Thr16, Ser20, and Tyr28) in WW1 experience an intermediate chemical exchange on the NMR timescale, while the corresponding resonances undergo fast exchange upon binding with Pept-3 (Figures 7A and 7C). As a rough approximation, tight binding might correlate with slow ligand exchange, and weak binding might correlate with fast exchange (Fielding, 2003). Moreover, the chemical shift changes of WW1 caused by Pept-1 binding are large, in contrast to the small chemical shift changes caused by Pept-3 binding in each titration step (Figure 7E). The titration curves of WW1 caused by Pept-1 binding show that the binding tends to reach saturation at a lower Pept-1 concentration than the concentration of Pept-3 in the curves of WW1 caused by Pept-3 binding. Collectively, these results demonstrate that Pept-1 binds to WW1 more tightly than Pept-3. Since WW2 is not as stable in the ligand-free state as WW1, which is indicated by the dynamics analysis (Figure S1), it is possible that WW2 undergoes conformational exchange on the NMR timescale even in the ligand-free state. Upon peptide binding, the chemical exchange between the ligand-bound and -free states makes it complicated to compare how WW2 binds to Pept-1 and Pept-3. Upon addition of Pept-1, a number of resonances in WW2 undergo chemical shift change and line broadening (Figure 7A). Some of them (e.g., Ser61 and Asp62) disappear and finally reappear at the later titrations; some (e.g., Tyr69 and Glu83) do not reappear even at the last titration point (peptide:protein ratio of 8:1). However, upon addition of Pept-3, the corresponding peaks in WW2 do not disappear, but their chemical shifts do change (Figure 7C). Interestingly, the intensities of these peaks increase considerably upon binding. This indicates that, in contrast to Pept-1, a small amount of Pept-3 could transform WW2 from a relatively unstable state to the rather stable bound state. From this aspect, Pept-3 is a preferential ligand for WW2 binding. The titration curves for chemical shift changes of WW2 upon addition of Pept-2 or Pept-3 clearly indicate that WW2 preferentially binds to Pept-3, not Pept-2 (Figure 7F). Taken together, these findings show that WW2 in the ligand-free state is less stable than WW1 in the form of tandem domains. Pept-1 is preferential to WW1 binding, while Pept-3 is preferential to WW2 binding; however, Pept-2 has no or very weak binding capability for both WW1 and WW2. Thus, Pept-1-2-3 should exhibit a preferred binding mode on the HYPA WW domain pair (also see Figure 8B).

Discussion

Multiple Binding Sites of the SH3GL3-SH3 Domain
Currently, three different ligand-binding sites on diverse SH3 domains have been identified. The first is the canonical PxxP-binding site that is constructed by the conserved aromatic residues. The second is the specificity pocket between the RT loop and the n-Src loop, such as the binding site of p67phox SH3(C) for the α-helical portion of the p47phox tail peptide (Kami et al., 2002). The third site, analogous to the binding site of Pex5p on the Pex13p-SH3 domain, is located on the opposite concave surface of the specificity pocket consisting of the β1 and β2 strands and the C terminus (Barnett et al., 2000; Douangamath et al., 2002; Kami et al., 2002). Our data provide convincing evidence to
support that Htt PRR specifically binds to the SH3 domain, and that the binding sites include the canonical PxxP-binding site and the specificity pocket (Figure 8A). Since the PRR chain is much longer than the canonical SH3 ligands, it is likely that it can contact the SH3 domain on the third site, as indicated by the results of the intermolecular NOE experiment. The C terminus of PRR orients to the specificity pocket, whereas the N terminus orients to the PxxP-binding site, which is consistent with what is observed in the Class II SH3 ligand (Mayer, 2001). This kind of binding mode may allow PRR motifs (generally in the PPII helix) to bind with various SH3 domains that are more diverse and selective.

Characteristics of the Interaction of the HYPA WW Domain Pair

Many proteins possess arrays of multiple WW domains; it is likely that the tandem WW domains cooperate with each other to achieve further specific recognition. To our knowledge, Htt PRR is the first example reported as a ligand for binding of the WW domain pair. The N-terminal portion of Htt PRR preferentially binds to WW1, while the C-terminal portion binds to WW2; however, the central portion remains unoccupied (Figure 8B). Up to date, only two structures of the tandem WW domain pair, Prp40 (Wiesner et al., 2002) and Su(dx) (Fedoroff et al., 2004), have been solved. In the case of HYPA, both

Figure 7. Summary of the Spectral Perturbations for Interactions between the HYPA WW Domain Pair and Various Peptides

(A) Overlay of a representative region of the $^{1}H$-$^{15}N$ HSQC spectra of HYPA-WW1-2 upon titration of Pept-1. The traces show the peak movements from free peptide (black) to a peptide:protein ratio of 8:1 (purple). The changes of some typical peaks in the WW1 and WW2 domains are indicated by labeling the residues. Most peaks in WW1 (such as T16, S20, Y27, and Y28) undergo chemical shift changes, while many peaks in WW2 (S61, D62, Y69, R77, and E83) tend to disappear.

(B) Titrated Pept-2 to HYPA-WW1-2. Pept-2 has no or very little effect on the peaks in both WW1 and WW2 domains.

(C) Titrated Pept-3 to HYPA-WW1-2. Some peaks in WW1 (T16 and S20) exhibit chemical shift changes, while some peaks in WW2 (S61, D62, Y69, and R77) exhibit line broadening.

(D) Titrated Pept-1-2-3 to HYPA-WW1-2. Many peaks in both domains are significantly affected by the addition of Pept-1-2-3. Most of the peaks first disappear and then reappear due to the intermediate exchange. The intensities of some of the reappeared peaks at the last titration (purple) are significantly enhanced compared to the peaks in the ligand-free form (black).

(E) Plot of weighted average chemical shift changes for three typical residues in WW1 (T16, S20, and Y28) versus the concentration of Pept-1 (red), Pept-2 (green), and Pept-3 (blue).

(F) Plot of average chemical shift changes for residues in WW2 (Y59, Y69, and R77) versus the concentration of Pept-2 (green) and Pept-3 (blue). Due to the large line broadening, the titration curve for chemical shift changes of the residues in WW2 upon the addition of Pept-1 to WW1-2 is not included.
Specific interactions is one of the substantial causes for HD pathology. Alteration of the interactions involving PRR has implications for the HD pathology. Since the aberrant interactions involved in Htt PRR and its SH3 domain- or WW domain-containing partners might be associated with the pathology of HD, it is interesting to know how the polyQ tract alters the normal interaction between Htt PRR and the SH3 or WW domain. Our current result showing that the N terminus of Htt PRR, occurring after the polyQ tract, is critical for binding to the SH3 domain and the WW domain pair implies that the specific interactions are susceptible to the length of the polyQ tract. In the Htt-interacting partners, all of the SH3 domain- or WW domain-containing partners, except PSD-95, bind more tightly to Htt with elongated polyQ stretch (Li and Li, 2004). It is possible that the elongated polyQ stretch makes the entire PRR region more accessible to these SH3 domain- or WW domain-containing partners and thus enhances their binding.

Since Htt PRR is proximal to the polyQ tract, it is likely that alteration of the interactions involving PRR is associated with the HD pathology. The expanded polyQ tract makes the PRR region more available to the WW-containing proteins or to MW7, a monoclonal antibody recognizing Htt PRR, and significantly inhibits aggregation as well as the cell death induced by the mutant Htt protein (Khoshravan et al., 2002). In addition, removal of a series of prolines adjacent to the polyQ region in Htt blocks formation of the shell of the Htt body and redistributes the sequestration of many vesicle-associated proteins, a process that may be related to neuronal dysfunction (Qin et al., 2004). Considering that interactions involving Htt PRR are susceptible to polyQ length (Feany and La Spada, 2003; Li et al., 2003; Sugars and Rubinsztei, 2003), we speculate that the alteration of these specific interactions is one of the substantial causes for HD pathology.
For pull-down assay, the GST fusion proteins were added to the glutathione Sepharose 4B beads (Amersham Biosciences) in a PBS buffer (10 mM sodium phosphate, 100 mM NaCl, 5 mM dithiothreitol), and the suspension was agitated at 4°C for 30 min. The GST fusion protein-bound beads were washed three times in the same buffer to remove any unbound protein. An equimolar amount of SH3 or WW1-2 was added, and the suspension was agitated at 4°C for an additional 30 min. The resin was recovered by centrifugation, and, after excessive washing, the sample was resuspended in the sample buffer and subjected to SDS-PAGE in a 15% gel, followed by Coomassie staining.

NMR Spectroscopy
To solve the solution structure of the SH3GL3-SH3 domain, a 15N/13C-labeled sample containing ~1 mM protein in a phosphate buffer (10 mM sodium phosphate, 100 mM NaCl, 5 mM dithiothreitol, and 0.05% w/v sodium azide [pH 6.0]) in 92% H2O/8% D2O or 100% D2O was used for NMR experiments. All NMR data acquisition was carried out at 27°C on a Varian INOVA 600 spectrometer equipped with three RF channels and a triple-resonance pulsed-field gradient probe. The backbone and side chain 1H, 15N, and 13C resonances were assigned based on the spectra of 3D HNCO, HN(CA)CO, HNCACB, CBCA(CO)NH and 3D 15N TOCSY-HSQC, HCC(CO)NH. NOE distance restraints for structure calculations were obtained from 3D 15N-edited NOESY and 13C-edited NOESY (aliphatic 13C regions). Intermolecular NOEs between SH3GL3-SH3 and Pept-1-2-3 were obtained from 2D-filtered/editied NOESY experiments as described (Peterson et al., 2004).

For HYP-WW1-2, 15N- or 13C-labeled sample containing ~1 mM protein in a phosphate buffer (10 mM sodium phosphate, 100 mM NaCl, 5 mM dithiothreitol, and 0.05% w/v sodium azide [pH 6.0]) in 92% H2O/8% D2O or 100% D2O was used for NMR experiments. The backbone resonances were assigned based on the spectra of 3D HNCO, HN(CA)CO, HNCACB, CBCA(CO)NH and 3D 13N TOCSY-HSQC, 15N-edited NOESY.

Structure Calculation and Analysis
The NMRPipe software suite (Delaglio et al., 1995) was applied to process the NMR data, and NMRView (Johnson and Blevis, 1994) software packages were used for resonance peak picking and data analysis. The CNS program (Brunger et al., 1998) with the ARIA module (Nilges et al., 1997) was adopted to assign NOE peaks and to calculate structures. The protein structures were assessed by using PROCHECK (Laskowski et al., 1996) and were displayed by the MOLMOL program (Koradi et al., 1996). Hydrogen bond restraints (two per hydrogen bond) were generated by a combination of H/D exchange data, medium-range NOEs, and chemical shift index. Backbone dihedral angle restraints (α and ω) were derived from the TALOS program (Cornilescu et al., 1999). For SH3GL3-SH3, the restraints used for structural calculation are summarized in Table S1. The structural calculation in combination with iterative NOE peak assignments was performed for 9 cycles, and a total of 200 structures were finally obtained. Ten structures of the lowest energies, which exhibit no NOE violation > 0.3 Å and no dihedral violation > 5°, were selected.

Determination of Backbone Relaxation Parameters
All 15N relaxation data for WW1-2 were acquired at 25°C by using 2D proton-detected heteronuclear NMR experiments implementing the standard pulse sequences (Farrow et al., 1994). A recycle delay of 1.5 s was used for measuring R2 and R1 relaxation rates. The spectra for measuring the (1H)15N heteronuclear NOEs were recorded with a 2 s relaxation delay, followed by a 3 s period of proton saturation. In the absence of proton saturation, the spectra were recorded by a relaxation delay of 5 s. The relaxation rates, R2 and R1, were obtained by fitting the measured peak intensities to a two-parameter function by using a nonlinear least-square fitting algorithm (Press et al., 1992) and were presented as a ratio of R2 to R1. The steady-state (1H)15N NOE enhancements were calculated as the ratio of peak intensity in spectra recorded with or without proton saturation.

NMR Titration
NMR titrations for SH3GL3-SH3 were performed at 27°C, and those for HYP-WW1-2 were performed at 25°C. 15N-labeled SH3 or WW1-2 was dissolved to a concentration of 0.25 mM in the above-mentioned NMR buffer, and the proline-rich peptides were added stepwise to give the peptide:protein molar ratios ranging from 0:1 to 8:1; each step was monitored by 2D 1H-15N HSQC spectrum. The combined average chemical shift changes were calculated as

\[
\Delta\delta_{21} = \frac{\delta_{2} - \delta_{1}}{\delta_{2} + \delta_{1}}
\]

where \(\Delta\delta_{21}\) and \(\Delta\delta_{12}\) are the chemical shift changes in the 1H and 15N dimensions, respectively. The titration curves were fitted assuming a bimolecular binding event as described (Liu et al., 1999). Dissociation constants (Kd) were obtained by analyzing the chemical shift changes of three typical residues for SH3GL3-SH3 or its Q17A mutant upon addition of each peptide.

Supplemental Data
Supplemental Data include one figure and two tables and are available at http://www.structure.org/cgi/content/full/14/12/1755/DC1/.

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The coordinates and structure factors for SH3GL3-SH3 are available in the Protein Data Bank under ID code 2EW3.