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Contributions of Conserved TPLH Tetrapeptides to the Conformational Stability of Ankyrin Repeat Proteins

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Abstract

Ankyrin repeat proteins are one of the most abundant classes of repeat proteins and involved in numerous physiological processes. These proteins are composed of various numbers of ankyrin repeat motifs (AR) stacked in a nearly linear fashion to adopt an elongated and nonglobular architecture. One salient feature prevalent in such a structural unit is the TPLH tetrapeptide or a close variant, T/SxxH, which initiates the helix-turn-helix conformation and presumably contributes to the conformational stability through a hydrogen bonding network. In the present study, we investigated the roles of T/SxxH motif in the stability, structure, and function of AR proteins by a systematic as well as rationalized mutagenic study followed by biochemical and biophysical characterization on gankyrin, an oncogenic protein composed of seven ARs and six T/SxxH tetrapeptides, and P16, a tumor suppressor with four ARs but no TPLH tetrapeptide. Our results showed that this tetrapeptide is ineffectual on the global structure and function, but contributes significantly to the conformational stability when its stabilizing potentials are fully realized in the local conformation, including (1) the intra-AR hydrogen bonding involving the hydroxyl group; (2) the intra- as well as inter-AR hydrogen bonds involving the imidazole ring; and (3) the hydrophobic interaction associated with the Thr-methyl group. Considering that the capping and close to capping units tend to have more sequence diversity and more conformational variation, it could be also generally true that a T/SxxH motif close to the terminal repeats contributes little or even negatively to the stability with respect to Ala substitution, but substantially stabilizes the global conformation when located in the middle of a long stretch of ARs.

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Keywords

ankyrin repeat; TPLH motif; gankyrin; P16; stability

Introduction

Ankyrin repeat proteins are one of the most abundant classes of repeat proteins that have been identified in the protein sequence data bank.^{1,2} Through mediating protein-protein interactions, they are engaged in innumerable physiological processes across most forms of life. For example, oncogenic gankyrin protein has been found to be involved in cell cycle progression, apoptosis, and proteasomal degradation via binding and modulating a number of proteins including pRb, MDM2, S6-ATPase, and Cyclin-dependent kinase 4 (CDK4)³⁻⁵; and P16, a well-known tumor suppressor⁶, shares considerable similarities with gankyrin in CDK4 binding and specifically inhibits CDK4-mediated pRb phosphorylation.⁷

Ankyrin repeat proteins comprise various numbers of structural unit – ankyrin repeat motif (abbreviated as AR hereafter), which is about 33-residue in length and stacked in a nearly linear fashion to adopt an elongated architecture possessing an extended and solvent-exposed surface highly versatile in protein binding.⁸ Whereas in globular proteins important contributions to stability mainly come from non-local interactions, in AR proteins with a non-globular fold, stabilization is largely a sum of local interactions between residues close in sequence.⁹

One of the structural features prevalent in AR proteins is the TPLH motif in specific or its variant T/SxxH in general. This tetrapeptide located at the 4th – 7th positions of an AR unit (Figure 1A) initiates the canonical helix-turn-helix conformation while generating a tight turn from the preceding AR unit.^{1,8} It was revealed previously by both crystallography⁹ and NMR¹⁰ that the Thr and His side chains could be engaged in several intra- as well as inter-repeat hydrogen bonds as depicted in Figure 1B. While the importance of this tetrapeptide to AR proteins has been suggested¹⁰, its impact on the (i) protein structure; (ii) conformational stability; and (iii) target binding property has never been reported. Toward this goal, we investigated the aforementioned two “contrasting” AR proteins: gankyrin with 6 T/SxxH in its 7 AR units, and P16 with no T/SxxH in its 4 AR units (Figure 2). We systematically perturbed the existing T/SxxH motifs in gankyrin through site-directed mutagenesis while introducing novel T/SxxH motifs into P16. The subsequent biochemical and biophysical characterization on these mutants showed that as a conserved structural motif, the T/SxxH tetrapeptide has little or marginal effect on function and global conformation. However, it contributes to the conformational stability but with different effects. The T/SxxH tetrapeptides in the middle of the gankyrin or P16 AR stretch comparatively play a major role in stabilizing the global structures, whereas the ones located close to both N- and C- termini contribute less or even negatively to the stability. From a structural perspective, this observation has been rationalized that the magnitude of contribution strongly depends upon the local conformation in which the following stabilizing potentials are realized: hydrogen bonds involving the hydroxyl group and imidazole ring, and the hydrophobic interactions involving the Thr-methyl group. Overall, our current work aims precisely to how a TPLH motif affects AR proteins and provides novel insights into natural AR protein engineering and *de novo* protein design for pharmaceutical application.

Results

In Silicon sequence analysis

Out of 5786 AR sequences in the Swiss-Prot database (<http://www.expasy.org/sprot/>), 2359 AR sequences (about 41%) contain the T/SxxH motif, reaffirming its nature of consensus. The

statistical analyses also show the T/SxxH motif exhibits a slight location preference to the middle of AR stretches (Figure 3). For example, in proteins composed of 4 ARs, the probabilities of a T/SxxH in AR2 and AR3 are notably higher than those in AR1 and AR4; in proteins containing 5 ARs AR2 is mostly favored with a probability greater than 50%, followed by AR3 around 50%. Considering that proteins with 6 or less repeats account for more than half of the total AR proteins, these analyses should be statistically significant. Furthermore, the ratio of TxxH to SxxH is roughly 3 to 1 (1801 vs 558), implying that Thr is more favored than Ser in the conserved T/SxxH tetrapeptides.

Contribution of T/SxxH motifs to gankyrin-CDK4 association

We first designed twelve gankyrin mutants (Table 1), in which either Thr or both Thr and His from GankTPLH1 to GankTPLH6 was replaced with Ala residues. It should be born in mind that GankTPLH3 has Ser instead of Thr, while GankTPLH6 has Gln instead of His. The results led to two more rationally designed S75T and H111Q. Except that GankTPLH4AB (Table 1) was insoluble during expression in bacteria, all the other mutants were subjected to further biochemical and biophysical characterization. We proceed with the report of the mutagenic effect on the CDK4 binding affinity.⁷ As shown in Figure 4A, no gankyrin WT was detected in the pull-down product in the absence of the (His)₆-tagged CDK4-cyclin D2 holoenzyme (lane 1). However, gankyrin WT was present in the pull-down product of the reaction mixture containing gankyrin WT and the CDK4-cyclin D2 holoenzyme at 2:1 ratio (lane 2), indicative of an in vitro gankyrin/CDK4 association. The binding assays performed on gankyrin mutants (lanes 3-7) revealed CDK4-binding abilities comparable to that of gankyrin WT, implying that mutations in TPLH motifs do not significantly impair the CDK4-binding ability of gankyrin.

Contribution of T/SxxH motifs to the conformational stability of gankyrin

The mutagenic impact on the gankyrin conformation stability was evaluated by chemical denaturation experiments. The GdnHCl-induced unfolding is highly cooperative, and denaturation curves can be fitted into a model of two-state transition between native and unfolded states (Figure 5A), indicating that all gankyrin mutants in their native states are compact and highly-folded.¹² First, as summarized in Table 1, a single mutation of Thr to Ala generally lowers stability, presumably due to the elimination of the intra-repeat Thr O^{γ1} – His (+3) H^N and Thr H^{γ1} – His N^{δ1} hydrogen bonds that may in turn further perturb the rest of the hydrogen bonding network. The largest drop (2.68 kcal/mol) of $\Delta G_d^{\text{water}}$ was observed in GankTPLH3A, followed by the one (1.30 kcal/mol) in GankTPLH4A. Comparatively lesser changes were observed in GankTPLH5A and GankTPLH6A. Surprisingly, there is some slight increase (0.31 kcal/mol) of stability in GankTPLH1A and a larger one (3.1 kcal/mol) in GankTPLH2A. The latter could be rationalized that unlike a Thr to Ala mutation that removes the hydroxyl group from the side chain, S75A mutation simultaneously introduces the missing hydrophobic interactions associated with A75 CH₃^β, rendering the interpretation of the change complicated. To address this issue, S75T was constructed and analyzed. As addressed below, our results verified the hydroxyl group's contribution to the stability enhancement with respect to S75A.

Secondly, additional Ala substitution of His residue in the corresponding T/SxxH motifs would supposedly eliminate the intra-repeat Ala H^N – His (+3) N^{δ1} as well as the inter-repeat His N^{ε2} – (His+30) O hydrogen bonds, and thus further destabilize the global structure. With the exception of GankTPLH1AB, this appears to be generally true. In particular, the H78 to Ala mutation compromised the stability gain in GankTPLH2A with a loss of ~1.5 kcal/mol (Table 1), whereas GankTPLH4AB could be viewed as an extreme case that further His144→Ala substitution in GankTPLH4A resulted in a protein too unstable to survive protein expression/purification. Notably, the largest stability drop in GankTPLH3A is merely followed by an additional loss of ~0.2 kcal/mol in GankTPLH3AB. One plausible explanation is that T108A

mutation itself may perturb H111 residue substantially. To further address this issue, we generated a single gankyrin mutant, H111Q, which completely abolishes the inter-repeat hydrogen bonding but may retain some of the reciprocal side chain-backbone hydrogen bonds. The resultant change in the stability of gankyrin H111Q is comparable with that of GankTPLH4A, indicative of a considerable contribution from the imidazole ring of H111 to the inter-repeat interactions.

The above results were further complemented by another two experiments. (i) In heat-induced unfolding experiments, the denaturation also follows a two-state transition (Figure 5B), and the relative order of T_m values of gankyrin mutants generally agrees with that of the conformational stability in GdnHCl-induced denaturation. For example, GankTPLH 3A, 3AB, and 4A all have T_m values at least 2.1 °C lower than that of WT. In contrast, the T_m value of GankTPLH2A is ~3.5 °C higher, and it is further increased by 5.6 °C in S75T, indicating that the contributions of the methyl and hydroxyl groups to the thermal stability are additive. (ii) The changes in the tertiary fold were monitored by 2D ^1H - ^{15}N HSQC upon the addition of GdnHCl to WT and three mutants.¹¹ On one hand, as self-evident in Figure 6, all proteins show an all-or-none behavior toward denaturation without detectable intermediates. On the other hand, the stability also follows the order of GankTPLH2A > GankTPLH1A ~ WT > GankTPLH3A, with their melting points falling within the range of 1.2-1.4, 1.4-1.6, and 1.6-1.8 M GdnHCl, respectively (data not shown).

Taken together, the above studies have demonstrated that the TPLH motifs in the middle of gankyrin (TPLH3 and TPLH4) contribute significantly to the conformational stability, while TPLHs at other locations have comparatively lesser (TPLH5 and TPLH6) or even negative (TPLH1 and TPLH2) contributions with respect to Ala substitution.

Contribution of T/SxxH motifs to the structure of gankyrin

GankTPLH 1A, 2A, and 3A with conformational stability comparable with, higher, and lower than that of WT, respectively, were further subjected to in-depth NMR studies. First, 3D ^{15}N -edited NOESY spectra were analyzed to evaluate overall structural perturbations.¹² NMR assignments were made starting from the terminal repeats gradually approaching the mutation site assuming that a perturbation propagates through the residues close in sequence. Aided with previous NMR studies on WT, this strategy has led to almost complete sequential assignment – 214 of 219 possible backbone NH protons – for each mutant (data not shown). The combined ^1H and ^{15}N pairwise chemical shift differences (Δppm) with respect to gankyrin WT were plotted in Figure 7. In consistence with the modular nature of AR proteins, all aforementioned mutations only induce localized perturbations. In specific, excluding the mutated residues, the average chemical shift changes are 0.03, 0.02, and 0.03 ppm, respectively, for GankTPLH 1A, 2A, and 3A. The largest ones are the residues around the mutation site, followed by a ripple effect on the loop residues preceding the next TPLH motif. It is interesting to note that S75 to Ala mutation introduced a relatively large perturbation on T108 NH (Δppm = 0.48 ppm), which could be attributed to perturbation relay of A75 methyl group interacting with the loop residues preceding TPLH3, e.g. A101. Additionally, the NOE patterns that entail three-dimensional conformational information were retained remarkably in 3D NOESY for the amide protons in all of these mutants. For example, the NOE between A108 H^{N} and H111 H^{N} in GankTPLH3A is also observed with comparable intensity as the counterpart in gankyrin WT. Taken together, these observations strongly support that gankyrin is fairly tolerated to the Ala mutation in a TPLH motif without global conformational changes.

Secondly, the downfield region of 2D ^1H -homonuclear NOESY spectra was analyzed that should provide more details pertaining to the local conformational changes around TPLH.¹² As shown in Figures 8B, 8C, and 8E, the five His $\text{H}^{\text{e}2}$ resonances from TPLH are clearly identified in these mutants, and all of them, including the $\text{H}^{\text{e}2}$ resonance in the mutated TPLH

motif, retain similar NOE patterns as those in gankyrin WT (Figure 8A). For instance, in GankTPLH2A, the NOE between H78 H^{ε2} and T108 H^α is also observed. This together with other closely-related long range NOEs, including H78 H^{ε2} – A101 H^β and H78 H^{ε2} – N103 H^N (labeled “e” in Figure 8C) in 2D and 3D NOESY, strongly suggests that the inter-repeat hydrogen bond between H78 N^{ε2} and C107 O has survived the S75A mutation. However, the H111 H^{ε2} resonance in GankTPLH3A has comparatively lesser and weaker cross peaks. The NOE between H111 H^{ε2} and T141 H^α (labeled “c” in Figure 8E), for example, is virtually at the noise level. This observation somewhat confirmed a more serious perturbation brought to H111 by T108 mutation, which in turn may well explain the aforementioned lesser stability drop from GankTPLH3A toward GankTPLH3AB. Additionally, it is noticeable that the Thr to Ala mutation in one TPLH motif has far less impact on the adjacent TPLH motifs, indicating the resilience nature of the hydrogen bonding network. In GankTPLH1A, this is evidenced by negligibly small (< 0.02 ppm) perturbation on H78 H^{ε2} resonance in contrast to ~0.17 ppm chemical shift change of H45 H^{ε2}.

Lastly, the change in conformational flexibility was probed by H²H exchange monitored with 2D ¹H-¹⁵N HSQC experiments.¹³ After 25-hour incubation at room temperature, all of the samples display virtually the same group of the slowly exchanging NH protons as gankyrin WT, located in the helices of AR2–AR6 (Figure 9) with the exception of A134 and A140 in the loop connecting AR4 and AR5 (Figure 10). It is worthwhile to note that among this group, the residues of L59 in GankTPLH1A, I79 in GankTPLH2A, and L126 in GankTPLH3A also experience relatively large chemical shift perturbations. Taken together, the results clearly reveal a stable hydrophobic core formed by the helical segments immune to the Thr to Ala mutation in a TPLH motif.

Local conformation and TPLH contribution

2D ¹H NOESY spectra were also recorded on S75T and H111Q mutants, further explore the structural roles of the hydroxyl group and imidazole ring of a T/SxxH motif, respectively. As anticipated, the spectral region of H^{ε2} resonances in S75T is barely perturbed, suggestive of an intact hydrogen bonding network in line with the retention of hydroxyl group; whereas in H111Q notable changes were observed on the adjacent H78 and H144 H^{ε2} resonances, in consistence with the fact that H111 is directly involved in the inter-repeat hydrogen bonding.

In summary, there is little doubt that the consensus T/SxxH motif plays a structural role and could notably contribute to AR protein conformational stability through hydrogen bonding and hydrophobic interactions. However, it is intriguing that the contribution of each T/SxxH varies with its location in gankyrin. In light of the above mutagenic and NMR analyses, we postulated that the magnitude of T/SxxH contribution might be modulated by the local conformation. In other words, the strength of a hydrogen bond depicted in Figure 1 can vary substantially depending on the context of its bond geometry. From this perspective, it is possible that TPLH motifs in the middle of gankyrin AR stretch (TPLH3 and TPLH4) may assume more ideal conformations than those in other locations, thus leading to stronger interactions. Consistent with this view, H111 was found more seriously perturbed by T108A mutation, and the loop between TPLH3 and TPLH4 was shown to have several residues undergoing comparatively slow amide H²H exchange.¹⁰ More importantly, the apparent anomaly of GankTPLH1A and GankTPLH1AB could be rationalized that an unusual 3¹⁰-helix in the spatial vicinity^{10, 14} could have altered the local microenvironment, rendering the hydrogen-bonding involving T42 and H45 weak or impossible. To further justify this point view, we intentionally introduced novel T/SxxH motifs into P16. As described previously,¹⁵ AR2 and AR3 of P16 are remarkably different from each other: while AR2 is atypical with a shorter helical version in its first helix (residues ⁴⁷RPIQV⁵¹), AR3 the latter assumes a typical conformation with a β-hairpin preceding the helix-turn-helix.¹⁵ We anticipated that introducing a TxxH motif into AR2 of

P16 would make little difference, whereas the same engineering on AR3 could increase the conformational stability substantially.

The impact of TxxH motifs on P16

Out of five P16 mutants we constructed, P16TPLH1 and P16TPLH1-2 mutants precipitated during protein expression and purification, indicating that R47T/Q50H mutations indeed significantly impair the stability and solubility of P16. The rest three mutants, P16TPLH2, P16TPLH3, and P16TPLH2-3, were soluble and exhibited no major structural perturbations as demonstrated in 1D and 2D ^1H NMR experiments. They also exhibited two-state transitions in GdnHCl- and heat- induced unfolding (Figure 5). Most importantly, a novel TxxH engineered into P16 AR3 with R80T mutation enhanced the stability by as high as ~ 2.3 kcal/mol. Apparently, it is the introduced hydroxyl group that confers the enhancement through the hydrogen bonding with H83. Figure 11 shows the downfield region of 2D NOESY spectra recorded on P16 WT and P16TPLH2 under identical experimental conditions. Evidenced by more and stronger NOEs associated with its $\text{H}^{\epsilon 2}$, H83 in P16TPLH2 appears to be better structured. Interestingly, P16TPLH3 has lower thermal stability than P16 WT, which can be attributed to the unfavorable D116H mutation (to be addressed below). Finally, P16TPLH3-R80T mutant has offset part of the gain by sole R80T mutation, further suggesting that TPLH contributions are somehow additive.

The CDK4-inhibitory activities of those soluble P16 mutants were also quantitatively evaluated by *in vitro* CDK4 kinase assays (Figure 4B). The IC_{50} values of P16TPLH2, P16TPLH3, and P16TPLH2-3 are determined to be 35 ± 10 nM, 290 ± 32 nM, and 110 ± 21 nM, respectively. In comparison, the IC_{50} value of P16 WT is 72 ± 15 nM. Since a two-fold change in IC_{50} is regarded as significant¹⁵, the CDK4-inhibitory activities of P16TPLH2 and P16TPLH3 are moderately increased and decreased, respectively, while P16TPLH2-3 exhibits a CDK4-inhibitory activity comparable to that of P16 WT.

Discussion

T/SxxH motifs mainly function to thermodynamically stabilize the global structure

The in-depth NMR characterization on representative gankyrin mutants demonstrates that the structural changes are localized around the mutation sites while the hydrophobic core between helices is virtually unperturbed. It may be safe to say that all gankyrin mutants except GankTPLH4AB retain global structures similar to that of gankyrin WT. This could be also true for P16 mutants with the exception of P16TPLH1 and P16TPLH1-2. Furthermore, biochemical characterization shows that all soluble gankyrin and P16 mutants exhibit little or modest change at most in CDK4 modulating. To the contrary, even a single substitution in specific TPLH motifs could bring substantial impact to the conformational stability, GankTPLH3A as a specific example. In this regard, GankTPLH4AB can be viewed as an extreme case, in which TPLH mutations are exceptionally deleterious to the stability thus resulting in the loss of the global structure. These findings strongly suggest that TPLH motifs likely function to impact the conformational stabilities of AR proteins.

The above conclusion can be rationalized from the perspective of an AR protein structure. It is arguably agreed that in most cases, the stability of a protein structure is dominated by its rigid parts and that the flexible, solvent-exposed parts contribute little.^{16, 17} As shown in Figure 1A, there are two conserved stretches in an AR motif, the TPLH motif in positions 4-7 and the V/I-V-X (hydrophilic)-L/V-L-L motif in positions 17-22.^{1,8} Both of them are important for the canonical helix-turn-helix conformation, with the first forming a tight turn and initiating the first α -helix, and the latter starting the second α -helix. Helices are tightly packed into α -helical bundles, and the loops connecting neighboring ARs are perpendicular to the helical

axes.¹⁵ The TPLH motif is located in the vicinity of the relatively flexible and solvent-exposed loop region. It is thus perceived that the TPLH contribution to the global structure could be next to the hydrophobic core between helices. Furthermore, it has been well demonstrated that most of the contacts between AR proteins and their targets are located in the helical regions as well as in the “tips” of the loops, which are defined as the first residue of an AR (position 1) and the last residue of the preceding AR (position 33 or 34).^{1,8} While TPLH tetrapeptides could presumably contribute to the target binding of an AR protein via moderating the contacts in their proximity or stabilizing the global structure, such indirect contribution is conceived to be modest at best.

One may assume that the six T/SxxH tetrapeptides in gankyrin have comparable contributions to the stability. However, our current study clearly shows that T/SxxH motifs in the middle contribute more to the stability; whereas those located at either at or close to N- and C- termini contribute little or even negatively to the stability. As described earlier, a TPLH motif needs ancillary interactions in its microenvironment to reach optimal hydrogen-bond geometry and to realize its stabilizing potentials. Since capping repeats shield the hydrophobic core of the AR proteins from the solvent, they tend to have higher sequence diversities as well as more variable local conformations, which in turn may further induce ripple effect on the AR units close to N- and C-termini. Consequently, AR proteins could behave in a way that the TPLH in the middle of AR stretches contribute the most. Therefore, while TPLH is consensus and could contribute significantly to the stability, it is critical to take into account the details of local conformations.

Thr over Ser in AR helix capping and unique role of His

In a TPLH motif, Thr is the helix *N*-cap characterized by non-helical ϕ and ψ angles, followed by helical residues Pro, Leu, and His labeled *N1*, *N2* and *N3* hereafter. It is well established that both Thr and Ser are among the group of four or five amino acids that generally have the highest *N*-cap preference, due to their possession of a hydroxyl group capable of accepting a hydrogen bond from the otherwise free backbone NH at *N3* position.¹⁸ While Ser has even better propensity than Thr, the sequence survey clearly shows a significantly higher statistical occurrence of Thr over Ser in natural AR sequences. Apparently, this difference should be attributed to the hydrophobic interactions associated with the Thr methyl group. The unexpected high stability of GankTPLH2A implies that these hydrophobic interactions such as A75 β CH₃ – A101 β CH₃ could outweigh the hydrogen bonding interactions associated with the hydroxyl group.

As reported previously¹⁹, Ala has the highest intrinsic preference for the interior of the helix, and a neutral His residue has the lowest helix propensity at *N3* position. Thus a helical His to Ala mutation does not necessarily reduce the stability in terms of the secondary conformation. This could explain the stability gain from GankTPLH1A to GankTPLH1AB. However, when a TPLH motif assumes an ideal conformation under the context of the tertiary fold, His at *N3* position not just can provide a reciprocal H-bonding chance to the capping residue Thr, but also directly mediates inter-repeat interactions using its imidazole ring. In this regard, His at *N3* position plays a unique role that could hardly be substituted by any other residue, and a mutation at this residue could cause more profound effect on the hydrogen-bonding network than mutations on the Thr residue as revealed in H111Q. In the C-terminal unit, however, this His would unlikely have another residue positioned to accept its N^{ε2} donor, and the bulky imidazole ring would be exposed to solvent resulting in an unfavorable contribution. Indeed, gankyrin has a ²⁰⁷TPL²¹⁰Q motif at the C-terminus, and a double mutant, T207A/Q210A (GankTPLH6AB) only slightly destabilizes the global structure with respect to T207A. More importantly, in P16, the D116H mutation has offset part of the gain by R80T in the mutant of P16TPLH2-3.

The pair of Thr and His is unique in AR proteins, and both residues can function to stabilize not just the secondary conformation but the tertiary fold. It is worthwhile to note that *N1* and *N2* residues could also impact the TPLH contributions to the stability. Pro is a popular *N1* residue²⁰, whereas Leu at *N2* is engaged in hydrophobic interactions with intra-repeat helical residues. Indeed, it has been reported that in I κ B α , single mutations at *N1* to conform to the consensus Pro lead to noticeable enhancement of thermal stability.²¹

Consensus design in AR proteins has drawn more and more attention in *de novo* design for biomedical applications partially due to the conserved nature and high sequence abundance of the AR motif.^{1,2,22} The efficacy of such approach has been well demonstrated in recent studies^{1,21,23-29}. On one hand, a group of “synthetic” AR proteins containing a common framework and novel binding specificities to clinically important targets have been generated from combinatorial libraries of consensus-designed ARs.^{1,26-30} On the other hand, it has been reported that the stability of a natural protein can be significantly improved by the addition of consensus AR motifs.^{24,25} With regard to the prevalence of TPLH in AR proteins, it is not surprising that this motif is present in all the designed AR proteins reported so far.^{9,22} Apparently, our present study provides structural insights underlying the roles of the conserved TPLH tetrapeptide, which are valuable in AR protein engineering. For example, TPLH modifications as well as other consensus-based mutations^{21,23} in natural AR proteins could increase the stability substantially while the global structures and functions of these proteins remain intact or slightly perturbed. In comparison, the insertion of exogenous consensus-designed ARs into an AR protein^{24,25} may influence its structure and function. Nonetheless, our findings do not compromise the significance of consensus design in protein engineering.

Materials and Methods

Database analysis

A total of 5786 AR sequences in 1008 proteins was downloaded from the Swiss-Prot database (<http://www.expasy.org/sprot/>) and analyzed using JAVA- or PERL-based programs.

Protein expression and purification

The cloning, expression, and purification of human gankyrin and P16 have been described previously.^{7,15} All mutants were generated using PCR-based site-directed mutagenesis (Stratagene), and were expressed and purified essentially the same way as the respective WT.

Conformational stability by circular dichroism (CD) analyses

Proteins were dissolved into 20 mM sodium borate buffer (pH 7.4) containing 40 μ M DTT. After dialyzing against the same borate buffer at 4 °C, samples of 7.5–10.0 μ M were then incubated with varying amounts of GdnHCl on ice overnight followed by equilibrating at 25 °C prior to CD analysis. The exact GdnHCl molarities were determined by refractive index measurements. The ellipticity at 222 nm, used to quantify the α -helical content, was measured three times and averaged for each sample on an AVIV far-UV spectropolarimeter using a 0.1 cm quartz microcell.¹² The values of $\Delta G_d^{\text{water}}$ (the denaturation free energy in water), $D_{1/2}$ (the denaturant concentration at the midpoint of transition), and the slope m were obtained on the basis of a two-state approximation.^{31,32} The latter is a constant related to a protein's susceptibility toward chemical denaturation.

Heat-induced unfolding experiments were performed using a 1-nm bandwidth and a 10-second response time.¹² The CD spectra were recorded at 222 nm by heating the samples from 5 to 65 °C at a rate of 1 °C per minute and a 1 °C interval followed by cooling down to 5 °C at the same rate. The thermal stability of a protein is gauged by T_m , the temperature at the midpoint of transition.

NMR analyses

Uniformly ^{15}N -labeled gankyrin WT, GankTPLH 1A, 2A, and 3A proteins were prepared as previously described.³³ NMR samples contained 0.4 mM protein, 5 mM HEPES, 1 mM DTT, 5 μM EDTA in 90% H_2O /10% D_2O at pH 7.5. 2D ^1H -homonuclear NOESY experiments were performed at 27 °C with a 150-ms mixing time on a Bruker DRX-800, while 3D ^{15}N -edited NOESY on a Bruker DRX-600 with the same experimental temperature and mixing time. Both spectrometers were equipped with a 5 mm cryoprobe and z-axis gradient. Data were processed with NMRPipe and analyzed using NMRView.

GdnHCl-induced gankyrin unfolding monitored by 2D ^1H - ^{15}N HSQC was carried out on a Bruker DMX-600 equipped with a 5 mm triple-resonance probe and three-axis gradients. About 0.2 mM lyophilized ^{15}N -labeled samples were reconstituted into a buffer containing 5 mM HEPES, 1mM DTT, 5 μM EDTA (pH 7.5), 90% H_2O /10% D_2O and various amounts of GdnHCl.¹¹ The GdnHCl concentration was determined as described above. The mixtures were incubated on ice overnight prior to NMR experiments at 20 °C.

2D NOESY on unlabeled P16 WT and mutants were recorded on the Bruker DRX-800 spectrometer at 18 °C with a 150-ms mixing time.¹¹ The samples contained 0.4 mM protein, 5 mM HEPES, 1mM DTT, 5 μM EDTA in 90% H_2O /10% D_2O (pH 7.5).

Gankyrin pull-down assay

The affinity of gankyrin for CDK4 was investigated by pull-down assay following a procedure described previously.⁷ Briefly, the reaction mixtures containing free gankyrin proteins and the (His)₆-tagged CDK4/cyclin D2 holoenzyme were incubated with TALON chelating resin. The elution by 1.0 M imidazole was analyzed by Western blot using a primary antibody against human gankyrin (PW8325, BIOMOL Int'l) and an AP-conjugated secondary antibody (sc-2034, Santa Cruz Biotechnology).

P16 *in vitro* CDK4 kinase assay

The *in vitro* CDK4 activity assay was performed as previously described.^{7,33} Briefly, each reaction mixture contained about 0.3 μg of recombinant CDK4/cyclin D2 holoenzyme, 50 ng of GST-Rb791-928, 5 μCi [γ - ^{32}P] ATP, and varying concentrations of P16 proteins. After incubation at 30 °C for 15 minutes, proteins in the reaction mixture were separated by SDS-PAGE, and the incorporation of ^{32}P into GST-Rb791-928³⁴ was evaluated using a PhosphorImager (Molecular Dynamics). The IC₅₀ value, the concentration of a kinase inhibitor required to achieve 50% of the maximal inhibition of CDK4, was obtained from the measurements performed in triplicate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AR	ankyrin repeat
BSA	bovine serum albumin

CD	circular dichroism
GdnHCl	guanidinium hydrochloride
HSQC	heteronuclear single-quantum coherence
NMR	nuclear magnetic resonance
NOE	nuclear overhauser effect
NOESY	NOE spectroscopy
WT	wild type

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Figure 1. The consensus sequence of an AR motif (A) and the TPLH-mediated hydrogen bonding network (B)

In (A), rectangles and arrows represent helices and β sheets, respectively. In (B), the potential hydrogen bonds (T108 H^N -H111 $N^{\delta 1}$, T108 $O^{\gamma 1}$ -H111 H^N , T108 $H^{\gamma 1}$ -H111 $N^{\delta 1}$, and H111 $H^{\epsilon 2}$ -A140 O) attributed to $^{108}\text{TPLH}^{111}$ of gankyrin¹⁰ are indicated by dashed magenta lines (see Figure 2B for the numbering of the entire amino acid sequence of gankyrin).

A

B

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P16      1 mepaagssmepsaDWLATAAARGRVEEVRAI~LEAGALPNAPNSYGRRPIQVMm~mGSAR 58
Gankyrin 1 ~~~~~megcvsnLMVCNLAYSgKLEELKEsIlADKSLATRTDQDSRTALHWAcsaGHTe 54

P16      59 VAELLLLHGAEPNCADPaTLTRPVHDAAREGFLDTLVVLHRAGARLDVRDAWGRLPVDLA 118
Gankyrin 55 IVEFLLQLGVPVNDKDD~AGWSPLHIAASAGRDEIVKALLGKGAQVNAVQNGCTPLHYA 113

P16      119 EELGHRDVARYLRAaaggt rgsnharidaaegpsdipd~~~~~ 156
Gankyrin 114 ASKNRHEIAVMLEgganpdakdhyatamhraaakgnlkmihillykastniqdtegn 173

P16      ~~~~~
Gankyrin 174 tplhlacdeerveeakllvsqgasiyienkeektplqvakggglglikrmveg 226

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Figure 2. The TPLH network in Gankyrin and P16

A, Tertiary structures of human gankyrin (left) and P16 (right). In both proteins, the TPLH tetrapeptides as well as its close variants are highlighted in yellow color. Helices are in green, and β sheets are in gold. **B**, Structure-based sequence alignments of human gankyrin and P16. The TPLH tetrapeptides and its close variants in P16 and gankyrin are highlighted in blue and red, respectively. Additionally, the two TxxH motifs presented in Figure 1B, $^{108}\text{TPLH}^{111}$ and $^{141}\text{TAMH}^{144}$, are underlined.

The Location of TPLH motifs in AR proteins

The Number of ARs in Proteins

Proteins	AR1	AR2	AR3	AR4	AR5	AR6	AR7	AR8	AR9	AR10	AR11	AR12
3-AR	0.266	0.385	0.294									
4-AR	0.107	0.420	0.411	0.286								0-0.09
5-AR	0.463	0.529	0.496	0.364	0.198							0.10-0.19
6-AR	0.278	0.351	0.304	0.201	0.438	0.366						0.20-0.29
7-AR	0.328	0.531	0.422	0.438	0.297	0.406	0.359					0.30-0.39
8-AR	0.125	0.281	0.156	0.313	0.438	0.344	0.469	0.438				0.40-0.49
9-AR	0.087	0.239	0.217	0.261	0.370	0.478	0.326	0.717	0.435			0.50-1.00
10-AR	0.269	0.231	0.385	0.423	0.077	0.192	0.385	0.462	0.423	0.115		
11-AR	0.045	0.136	0.136	0.364	0.182	0.091	0.045	0.227	0.136	0.091	0.227	
12-AR	0.167	0.000	0.083	0.083	0.583	0.167	0.083	0.250	0.500	0.167	0.083	0.167

Figure 3. Probability of T/SxxH appearing on each AR of proteins containing three to twelve ARs

The column represents the location of a T/SxxH motif in an AR protein, and the rows are different groups of AR proteins as represented by the number of ARs in these proteins. Probability at each location (AR) of a certain protein group was calculated by dividing the actual number of total T/SxxHs found on a specific location within the group by the total number of proteins in this group. The total numbers of proteins containing 3, 4, 5, 6, 7, 8, 9, 10, 11, and 12 ARs are 143, 112, 121, 194, 64, 32, 46, 26, 22, and 12, respectively. An example for data interpretation is as following: for a protein composed of three ARs, the probability of the T/SxxH presence in the first AR is 0.266. Of note, whereas proteins composed of more than 12 ARs are not included in this study due to the small population size in the aforementioned database, proteins containing 1 or 2 ARs are excluded since their amino acid sequences are deviated largely from the consensus AR sequence as described in Figure 1A.

A

B

Figure 4. Pull-down assays to assess the interaction between gankyrin proteins and the CDK4-cyclin D2 complex (A) and *in vitro* CDK4 kinase assay to evaluate the CDK4-inhibitory activities of P16 mutants (B)

Lanes in (A): 1, the control reaction only containing bovine serum albumin (BSA) and gankyrin WT; 2-7, the reaction mixtures containing the CDK4-cyclin D2 complex (1.0 μ M) and various gankyrin proteins (at the concentration of 2.0 μ M) as indicated. Of note, only results from selected gankyrin mutants are presented here. In (B), IC_{50} was defined as the concentration to achieve 50% of the maximal inhibition of the kinase activity, and a >2-fold change in the IC_{50} value was regarded as significant.¹⁵

A **B**

Figure 5. GdnHCl-induced (A) and heat-induced (B) unfolding of representative gankyrin and P16 mutants monitored by far-UV CD (190-260 nm)

The unfolding parameters were derived through a two-state transition approximation as previously described,^{23,32} and listed in Table 1. The lines represent the fitting curves.

Figure 6. 2D ^1H - ^{15}N HSQC spectra of gankyrin WT showing the all-or-none unfolding: (A) the overlap of the spectra recorded at 0 (black) and 1.4 M (red) GdnHCl, respectively; (B) the spectrum at 1.6 M GdnHCl (blue)

The sample still retains native fold at 1.4 M GdnHCl, but denatures at 1.6 M GdnHCl. The transition point is thus determined to be in the range of 1.4-1.6 M. Similar behavior was observed in gankyrin mutants GankTPLH 1A, 2A, and 3A, except with similar or different transition point.

Figure 7. Residue pairwise chemical shift difference between gankyrin mutant and WT: (A) GankTPLH1A, (B) GankTPLH2A, and (C) GankTPLH3A

The mutation site in each mutant is marked by asterisk, and T108 experiencing comparatively large perturbation in (B) is labeled. The combined ^1H and ^{15}N pair wise chemical shift differences (Δppm) with respect to the wild type were calculated as $[(\Delta\delta_{\text{HN}})^2 + (\Delta\delta_{\text{N}} \cdot \alpha_{\text{N}})^2]^{1/2}$, in which α_{N} is the scaling factor (0.17) used to normalize the ^1H and ^{15}N chemical shifts.

Figure 8. Selected spectral regions from 2D NOESY spectra on gankyrin (A) WT, (B) GankTPLH1A, (C) GankTPLH2A, (D) S75T, (E) GankTPLH3A, and (F) H111Q mutants
Data were recorded under identical experimental conditions. The cross-peaks designated “a”, “b”, and “c” are NOEs from His H ϵ^2 in a TPLH motif to His H ϵ^1 , His H δ^2 , and (His+30) H α , respectively. The cross-peaks designated “d” (in panel A and B) and “e” (in panel A and C) are NOEs of H45 H ϵ^2 – D68 H N , and H78 H ϵ^2 – N103 H N , respectively, further indicating the retention of a structured imidazole ring in T42A and S75A, respectively.

Figure 9. 2D ^1H - ^{15}N HSQC of gankyrin proteins after 25 hour $\text{H}/^2\text{H}$ exchange at room temperature: (A) WT; (B) GankTPLH1A; (C) GankTPLH2A; and (D) GankTPLH3A

The slow-exchanging backbone amide cross-peaks are labeled. It is clear from these results that the most stable hydrophobic core formed by the helical segments is hardly perturbed in these mutants.

Figure 10. Ribbon diagram showing the slow exchanging amide proton detected in gankyrin GankTPLH1A

No major differences were observed among gankyrin WT and three mutants, GankTPLH1A, GankTPLH2A, and GankTPLH3A, as evidenced in Figure 9.

Figure 11. Selected spectral regions from 2D NOESY spectra on (A) P16 WT, (B) mutant P16TPLH2

Data were recorded under identical experimental conditions. The cross-peaks designated a, b, c, d, e, f and g are NOEs from H83 H ϵ^2 to L113 H $^{\alpha}$, H83 H δ^1 , H83 H ϵ^1 , D108 H N , L113 H N , V106 H N , and R107 H N , respectively.

Table 1
Conformational stability of TPLH mutants of gankyrin and P16

Name ^a	Mutation	$\Delta G_d^{\text{water}}$ (kcal* mol^{-1}) ^b	$D_{1/2}$ (M) ^b	m (kcal* mol^{-1} *M ⁻¹) ^b	T_m (°C) ^c
Gankyrin	WT	3.99	1.61	2.47	46.8
GankTPLH1A	T42A	4.30	1.60	2.70	47.6
GankTPLH1AB	T42A/H45A	5.93	1.33	4.41	44.7
GankTPLH2A	S75A	7.09	1.75	3.99	50.3
GankTPLH2AB	S75A/H78A	5.53	1.53	3.54	47.2
GankTPLH3A	T108A	1.31	1.30	1.04	43.4
GankTPLH3AB	T108A/H111A	1.12	1.20	0.86	41.9
GankTPLH4A	T141A	2.69	1.40	1.87	43.8
GankTPLH4AB	T141A/H144A	N/A ^d	N/A	N/A	N/A
GankTPLH5A	T174A	3.23	1.39	2.27	47.6
GankTPLH5AB	T174A/H177A	3.24	1.55	2.05	46.5
GankTPLH6A	T207A	3.24	1.47	2.16	47.5
GankTPLH6AB	T207A/Q210A	2.86	1.53	1.89	47.1
Gankyrin S75T	S75T	6.83	2.01	3.39	55.9
Gankyrin H111Q	H111Q	2.69	1.26	2.13	45.8
P16 ^e	WT	1.94	0.6	3.23	46.0
P16TPLH1	R47T/Q50H	N/A	N/A	N/A	N/A
P16TPLH2	R80T	4.229	0.96	4.10	50.9
P16TPLH3	L113T/D116H	1.903	0.55	4.35	36.3
P16TPLH1-2	R47T/Q50H/R80T	N/A	N/A	N/A	N/A
P16TPLH2-3	R80T/L113T/D116H	2.487	0.62	4.13	44.4

^aThe location is defined as the *i*th TPLH motif in gankyrin or P16 from the N-terminus, while the *i*th TPLH motif is regarded as a part of the (*i*-1)th AR. For example, GankTPLH1A, T42A, is located within the first TPLH motif that belongs to the second AR of gankyrin.

^b $\Delta G_d^{\text{water}}$, $D_{1/2}$, and m values were calculated according to a two-state transition approximation,^{31,32} and the error limit in $\Delta G_d^{\text{water}}$ is estimated to be ± 0.5 kcal/mol.^{35,36}

^c T_m was defined as the temperature at the midpoint of transition, and the error is estimated to be ± 0.5 °C.^{12,37}

^dN/A, data not available mainly due to poor solubility during the process of expression, purification, and dialysis against the borate buffer.

^eData from previous studies.³⁶