Biological Regulation *via* Ankyrin Repeat Folding

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Noncovalent conformational changes have long been recognized as a means to control biological activity of proteins (*1, 2*). Conformational changes can influence ligand binding, enzymatic activity, and degree of polymerization. Although conformational changes that interconvert distinct, well-ordered tertiary structures were appreciated from the earliest protein crystal structures (*3*), the biological importance of larger-scale conformational changes between the folded and fully denatured conformations has recently been recognized (*4, 5*). Another well-established mechanism by which activity can be controlled is covalent modification, in particular, phosphorylation. The influence of a phosphate group on activity can be direct, acting as (or disrupting) part of a binding site, or it can be indirect, by coupling to a change between conformations with different activity. In this issue of *ACS Chemical Biology*, Löw *et al.* (DOI 10.1021/cb800219m) (*6*) use phosphomimetic site-directed mutagens in p19[INK4d], a regulatory ankyrin repeat protein, to explore the relationship between phosphorylation and regulation of biological function. Their findings highlight how partial unfolding transitions can play a significant role in protein function and, in this case, tumor suppression.

p19[INK4d] is a tumor suppressor composed of five ankyrin repeats. One of the main functions of p19[INK4d] (as well as other tumor suppressors in the INK4 family) is to bind to cyclin-dependent kinases (CDKs) 4 and 6. CDK4 and CDK6 are similar in se-quence, structure, and at least qualitatively, in interaction with INK4 family members. Here the two will be referred to jointly as CDK4/6, to distinguish them from other cyclin-dependent kinases of different specificity (e.g., CDK2). The binding of p19[INK4d] inhibits the cyclin−CDK4/6 interaction that would lead to progression through the cell cycle. This interaction, which is mediated primarily by ankyrin repeats 1 and 2 of p19[INK4d] (*7*), is depicted in Figure 1.

The inhibitory effect of p19[INK4d] on cell-cycle progression has been shown to be modulated, in a human osteosarcoma cell line, by ubiquitination at a lysine residue at position 62, followed by proteasomal degradation (*8*), and also by phosphorylation of serines at positions 66 and 76. Although all three of these putative regulatory sites are in (or near to) the ankyrin repeats that are involved in binding to CDK6, they are rather distant from the binding site, suggesting an alternative mechanism by which covalent modification controls activity.

By substituting Ser66 and Ser76 with glutamates, which provides a rough phosphomimetic, Löw and co-workers find clear evidence that phosphorylation leads to a partial unfolding reaction involving ankyrin repeats 1 and 2 and that this unfolding may be coupled to ubiquitination. The native state of the single point substitution S76E, at the start of the third ankyrin repeat, is significantly destabilized; at body temperature, this destabilization produces a wholesale shift in population to favor a partly folded intermediate. This stable equilibrium

ABSTRACT By mimicking the phosphorylation of p19[INK4d], a tumor suppressor containing five ankyrin repeats, the native state could be destabilized to such an extent that only a partially folded state is populated at physiological temperature. This partly folded state, which mimics an on-pathway folding intermediate lacking structure in ankyrin repeats 1 and 2, is more rapidly ubiquitinated than the parent construct. Thus, phosphorylation of p19[INK4d] is likely to regulate cell-cycle progression through both biochemical (proteasomal) and biophysical (folding and binding to cyclin-dependent kinases) mechanisms.

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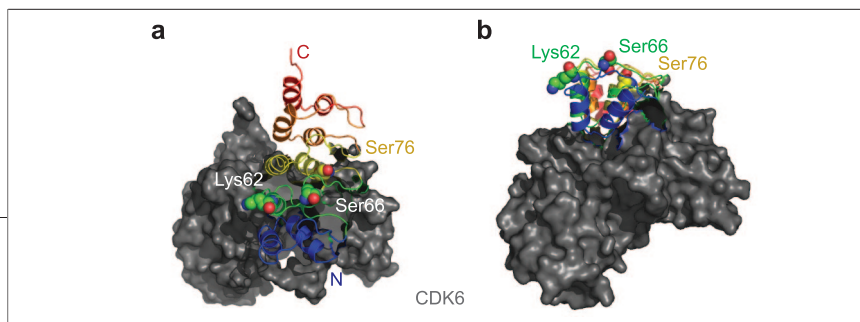
Figure 1. Interaction of p19^{INK4d} with CDK6. The five ankyrin repeats of p19^{INK4d} are shown as ribbons colored from blue to red from the N- to C-terminus; sites of phosphorylation (Ser66, Ser76) and ubiquitination (Lys62) are shown as CPK spheres. CDK6 is depicted using gray surface contours. The two views (panels a and b) differ by rotation of ~90° about the horizontal in-plane axis. As seen in panel a, contacts between p19^{INK4d} and CDK6 are largely made by p19^{INK4d} repeats 1 and 2 (with additional contacts from repeat 3). As seen in panel b, the sites of covalent modification are distant from the interface between the two proteins. This figure was generated from the coordinate file 1BLX.pdb (8) using MacPyMol (22).

intermediate strongly resembles a partly structured kinetic folding intermediate, with repeats 3−5 structured but repeats 1 and 2 unstructured, which has been characterized previously by the authors using biophysical methods (9, 10).

This shift in population is likely to have at least two effects on the activity of p19^{INK4d} (Figure 2, panel a). First, it is likely to disrupt interaction with CDK4/6 (Figure 1), since the native state (with repeats 1 and 2 folded) is thermodynamically disfavored at body temperature and binding free energy must be sacrificed to drive the folding reaction. Second, the population shift may increase accessibility of residues in repeats 1 and 2 to further modification. Candidate residues include additional phosphorylation of Ser66 and ubiquitination of Lys62, potentially facilitating proteasomal degradation and cell-cycle progression (8). Although the ubiquitin ligase that carries out this reaction is currently unknown, the authors demonstrate that the phosphomimetic S76E substitution promotes ubiquitination by a HeLa cell lysate, although curiously, this increased activity is also dependent on a second neighboring substitution, S66E, which does not in itself significantly shift the population from the native to the partly folded state. From these results, the authors suggest an ordered sequence of events in which phosphorylation of Ser76 leads unfolding of repeats 1 and 2, which promotes phosphorylation of Ser66, which in turn leads to ubiquitination of Lys62 (although it should be noted that the site of ubiquitination in the present studies remains to be determined).

The origin of the observed changes in folding free energy of p19^{INK4d} upon phosphorylation can be readily explained from the structure. Ser66, in the extended loop between repeats 2 and 3, is fully solvent-exposed, and acidic residues are most frequently observed at this position in ankyrin repeats (11). Thus, it is not surprising that S66E has little effect on conformational stability. However, Ser76, in the first helix of repeat 3, is substantially buried and is part of the highly conserved (T/S)PLH ankyrin consensus sequence. The hydroxyl group of the consensus serine/threonine side chain is often hydrogen-bonded to histidine at position +3, either to the main-chain NH, to the side-chain Nδ1, or in the case of Ser76, to the carbonyl oxygen of Val69 in the second repeat. Thus, substitution with a larger glutamic acid would be expected to be highly destabilizing. Consistent with this interpretation, the authors observed increased flexibility and a disruption of the interface between repeats 2 and 3 in molecular dynamics simulations (6).

Interestingly, there are considerable parallels between the current study and a mutational study from Li and co-workers (12) on gankyrin, an ankyrin repeat protein that is believed to bind (but not inhibit) CDK4 in the same mode as members of the INK4 family. By swapping residues from p16^{INK4a} into gankyrin in the same region of repeats 2 and 3, the activity of gankyrin was modified to inhibit CDK4, coincident with considerable destabilization of the fold (such that it also appears partly unfolded at body temperature) and rearrangement of the loops and (T/S)PLH regions of the N-terminal re-

peats (12). However, this mutated gankyrin retains its ability to bind to CDK4. In light of this observation, it would be worth examining whether the affinity of the phosphomimetic S76E p19^{INK4d} displays a decreased affinity for CDK4 (as would be expected from a partial unfolding model), especially given the observation by Thullberg and co-workers (8) that ubiquitination of p19^{INK4d} appears to require interaction with CDK4. Lys62 is highly solvent-exposed in the native state of p19^{INK4d} and is distant from the interface with CDK6 (7). Thus, although the partial unfolding model of Löw and co-workers may be expected to provide increased access to cognate E3-ubiquitin ligases, direct ubiquitination of a more folded (but perhaps significantly rearranged) state should not yet be ruled out.

Other Ankyrin Repeat Proteins That Undergo Partial Unfolding Transitions and/or Covalent Modifications. Ankyrin repeat proteins and repeat proteins in general are unique in that their tertiary structures are organized locally. Unlike globular proteins, there are no direct contacts between distant parts of the protein chain. Although many repeat proteins show surprisingly cooperative equilibrium unfolding transitions, it is clear that through local destabilizations and variations in stability on a medium length scale, partly folded states in which some repeats are ordered and others are disordered can be populated (see ref 13 for a review). The results of Löw and co-workers connect such a folding intermediate, characterized by biophysical studies, with important biological activities. Are there other ankyrin repeat proteins that show such connections?

IκBα, which contains six ankyrin repeats, shows a clear structural transition upon binding to its protein target, NF-κB. In solution, IκBα is partly folded, with structure in repeats 2−4, but with repeats 1, 5, and 6 significantly disordered. Upon binding to NF-κB, repeats 5 and 6 become structured, although repeat 1 appears to retain substan-
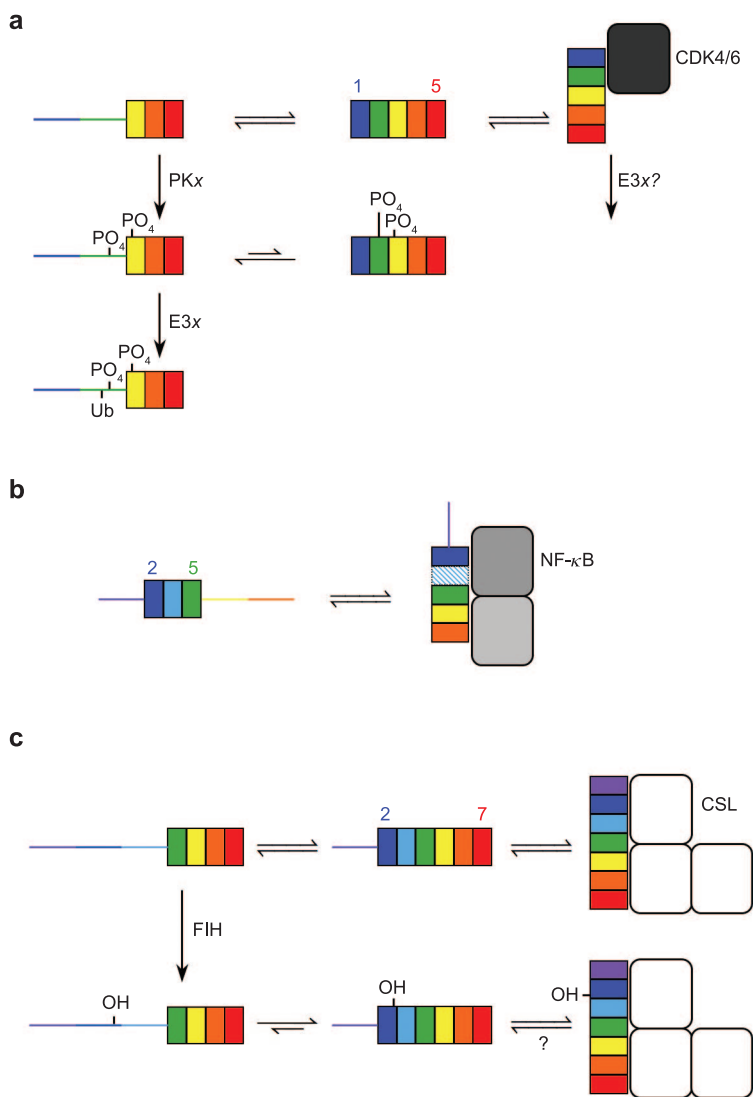
Figure 2. Coupling of folding/unfolding of ankyrin repeats to covalent modification and target protein binding. a) When unbound, the lowest free energy form of p19INK4d has all five ankyrin repeats folded (center top) and is competent for binding CDK4/6. A partly folded intermediate with repeats 1 and 2 unfolded is greatly stabilized by phosphorylation by an unidentified kinase (PKx) and shows increased reactivity to unidentified ubiquitin ligase (E3x; although CDK4/6 also appears to stimulate ubiquitination). b) When unbound, the lowest free energy form of IκBα has repeats 1, 5, and 6 unfolded. Binding to NF-κB is coincident with folding of repeats 5 and 6 and with a partial destabilization of repeat 3. c) When unbound, the lowest free energy form of the Notch ankyrin domain has repeats 2–7 folded (center). Binding to CSL is coincident with folding of repeat 1. FIH appears to hydroxylate an unstable form with repeats 2 and perhaps 3 unfolded. Hydroxylation further favors the folding of these repeats (lower line). Additional interactions stabilizing the Notch–CSL complex are omitted for clarity.

tial disorder. Surprisingly, repeat 3, in the center of the ankyrin repeat array, appears to become less ordered on binding to NF-κB (14). The potential that p19INK4d may engage its ubiquitin ligase through an altered structure on CDK4 may relate to the surprising recent observation by Komives and coworkers (15) that by using consensus sequence to stabilize the ankyrin repeat fold, IκBα affinity to NF-κB is decreased, suggesting that even within the "folded ensemble" of ankyrin repeat proteins there may be structural variations with significantly different biological activities and that this variation may provide another level of regulation.

Like IκBα, the ankyrin domain of the Notch receptor shows significant disorder (in the first of seven repeats) when unbound (16), but this repeat becomes ordered (along with a cryptic segment that adopts an ankyrin-like fold) upon binding to the transcription factor CSL (17, 18) (Figure 2, panel c). Recently, the Notch ankyrin domain was shown to be hydroxylated at one or more asparagine Cβs (19, 20). Although this modification is compatible with the native structure (19) and results in an increase in stability (21), crystallographic analysis of Notch target peptides in complex with FIH (the asparagine hydroxylating enzyme) indicates that the ankyrin domain must be transiently unfolded to be hydroxylated (19). Hydroxylation of the Notch ankyrin domain appears to affect aspects of both Notch signaling and the hypoxic response mediated by HIF-1α (20).

Although the mechanistic details by which hydroxylation regulates the function of ankyrin repeat domains are yet to be determined, it seems likely that conformational stability will play a significant role. The current study by Löw and co-workers on p19INK4d provides a clear example of how biophysical characterization of a protein folding intermediate can lead to insight into biological function. Given the rich spectrum of partly folded states available to ankyrin (and other types of) repeat proteins, it is

clear that detailed studies of folding of such proteins will be essential for understanding their function.

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