Current Topics

Regulation of Cell Division by Intrinsically Unstructured Proteins: Intrinsic Flexibility, Modularity, and Signaling Conduits[†]

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ABSTRACT: It is now widely recognized that intrinsically unstructured (or disordered) proteins (IUPs or IDPs) are found in organisms from all kingdoms of life. In eukaryotes, IUPs are highly abundant and perform a wide range of biological functions, including regulation and signaling. Despite an increased level of interest in understanding the structural biology of IUPs and IDPs, questions regarding the mechanisms through which disordered proteins perform their biological function(s) remain. In other words, what are the relationships between disorder and function for IUPs? There are several excellent reviews that discuss the structural properties of IUPs and IDPs since 2005 [Receveur-Brechot, V., et al. (2006) *Proteins 62*, 24−45; Mittag, T., and Forman-Kay, J. D. (2007) *Curr. Opin. Struct. Biol. 17*, 3−14; Dyson, H. J., and Wright, P. E. (2005) *Nat. Rev. Mol. Cell Biol. 6*, 197−208]. Here, we briefly review general concepts pertaining to IUPs and then discuss our structural, biophysical, and biochemical studies of two IUPs, p21 and p27, which regulate the mammalian cell division cycle by inhibiting cyclin-dependent kinases (Cdks). Some segments of these two proteins are partially folded in isolation, and they fold further upon binding their biological targets. Interestingly, some portions of p27 remain flexible after binding to and inhibiting the Cdk2−cyclin A complex. This residual flexibility allows otherwise buried tyrosine residues within p27 to be phosphorylated by non-receptor tyrosine kinases (NRTKs). Tyrosine phosphorylation relieves kinase inhibition, triggering Cdk2-mediated phosphorylation of a threonine residue within the flexible C-terminus of p27. This, in turn, marks p27 for ubiquitination and proteasomal degradation, unleashing full Cdk2 activity which drives cell cycle progression. p27, thus, constitutes a conduit for transmission of proliferative signals via post-translational modifications. The term "conduit" is used here to connote a means of transmission of molecular signals which, in the case of p27, correspond to tyrosine and threonine phosphorylation, ubiquitination, and, ultimately, proteolytic degradation. Transmission of these multiple signals is enabled by the inherent flexibility of p27 which persists even after tight binding to the Cdk2−cyclin A complex. Importantly, activation of the p27 signaling conduit by oncogenic NRTKs contributes to tumorigenesis in some human cancers, including chronic myelogenous leukemia (CML) [Grimmler, M., et al. (2007) *Cell 128*, 269−280] and breast cancer [Chu, I., et al. (2007) *Cell 128*, 281−294]. Other IUPs may participate in conceptually similar molecular signaling conduits, and dysregulation of these putative conduits may contribute to other human diseases. Detailed study of these IUPs, both alone and within functional complexes, is required to test these hypotheses and to more fully understand the relationships between protein disorder and biological function.

INTRINSICALLY UNSTRUCTURED (OR DISORDERED) PROTEINS

Many proteins, which play a wide range of biological roles, either entirely lack secondary and/or tertiary structure or possess long segments that lack secondary and/or tertiary structure, under physiological conditions (*3, 6–8*). These are commonly termed intrinsically unstructured (or disordered) proteins, abbreviated IUPs[1] (or IDPs). Bioinformatics analyses of whole genome sequences using disorder predictors (*9, 10*) indicated that 6−33% of proteins in bacteria and 35−51%

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of proteins in eukaryota contain disordered regions of 40 or more consecutive residues (*9, 11*). The greater abundance of IUPs in the latter was proposed to be due to the greater need for protein-mediated signaling, regulation, and control that is required in eukaryotes (*11*). It is now widely recognized that IUPs play broad biological roles in all kingdoms of life (*11, 12*).

Functions of Disordered Proteins. IUPs are involved in many cellular functions, including regulation of cell division, transcription and translation, signal transduction, protein phosphorylation, storage of small molecules, chaperone action, transport, and regulation of the assembly or disassembly of large multiprotein complexes, among many others (*13, 14*). Indeed, the majority of transcription factors (*15*) and proteins involved in signal transduction (*16*) in eukaryotes are predicted to be disordered or contain long disordered segments. Further, 79% of human cancer-associated proteins (HCAPs) have been classified as IUPs, compared to 47% of all eukaryotic proteins in the SWISS-PROT database (*16*). The latter observation highlights the importance of disorder in the function of proteins that regulate processes often dysregulated in cancer such as cell proliferation, apoptosis, and DNA repair.

Although many IUPs function by folding into an ordered conformation upon binding their biological targets, for many others, disordered conformations mediate biological function. For example, disordered segments serve as linkers in many IUPs [e.g., between folded domains in multidomain proteins such as p53 (*17*)]. In other cases, IUPs function as entropic bristles [e.g., NF-M and NF-H which serve a repulsive spacers in neurofilaments (*18*)], springs [e.g., titin, which induces passive tension in muscle filaments (*19*)], and semipermeable barriers [FG-domains within nucleoporins of the nuclear pore complex (*20*)]. In these examples, disordered polypeptide segments often occur in conjunction with other folded or partially folded domains.

Amino Acid Composition and Primary Structure of IUPs. The sequences of intrinsically unstructured proteins exhibit low complexity compared to those of globular proteins (*21*). Further, IUPs are depleted of hydrophobic amino acids (i.e., Val, Leu, Ile, Met, Phe, Trp, and Tyr) and correspondingly enriched with polar and charged amino acids (i.e., Gln, Ser, Pro, Glu, Lys, Gly, and Ala) relative to globular proteins (*21*). Consequently, IUPs occupy a different region of "charge−hydrophobicity space" compared to globular proteins (*22*) and lack tertiary structure because they possess too few hydrophobic residues to independently form a stable hydrophobic core. The term charge−hydrophobicity space refers to a two-dimensional graph of values of the mean net charge (*y*-axis) and mean hydrophobicity (*x*-axis) of proteins (*22*). Uversky and co-workers showed that the vast majority of IUPs exhibit values of these parameters which lie to the left on such a graph, and folded proteins to the right, of a boundary line (*22*). These sequence-based features have led to the development of algorithms that identify disordered segments within protein sequences with up to 80% accuracy (*10*).

While disordered segments within proteins can be reliably identified using these algorithms, it remains difficult to differentiate between disordered segments that, for example, play structural roles as linkers and those that mediate protein−protein interactions through folding upon binding. Such algorithms would need to be trained to recognize the sequence features of polypeptide segments with these distinct functions. While some progress has been made toward the goal of identifying interaction sites within IUPs (*23, 24*), only when a database of sequence−structure/disorder−function relationships based on detailed analysis of the conformational and functional properties of a wide variety of functional complexes of disordered proteins is established will more discriminating predictions of disordered protein function be possible.

How Disordered Are IUPs? Evidence of Partially Populated Secondary Structure within Certain IUPs. Structural analysis of IUPs is challenging because their polypeptide backbones exhibit a high degree of flexibility and disorder due to rapid interconversion among multiple conformers. This causes measurable parameters such as far-UV circular dichroism (CD) and NMR chemical shift values to be dynamically averaged among the interconverting states. Early studies of IUPs were performed using CD, NMR, and/or structural mapping using limited proteolysis (*25, 27*), and the results suggested that IUPs adopt conformations very similar to those of random coils. However, CD studies of the IUPs $p21^{Cip1}$ (p21) (*25*) and $p27^{Kip1}$ (p27) (*28, 29*) showed that these proteins possess nascent α-helical secondary structure. Detailed analysis using NMR spectroscopy revealed that several different segments of isolated p27 adopt secondary structure in solution; these were termed "intrinsically folded structural units", or IFSUs (*30*). In fact, NMR studies by many laboratories have now shown that IUPs exhibit varied degrees of nascent structure and disorder. Some IUPs completely lack secondary and tertiary structure (partial list in Table 1A), while others exhibit partial secondary structure (Table 1B).

Folding upon Binding. While IUPs are disordered in isolation under physiological conditions, they often perform their biological functions by binding specifically to other biomolecules through the process of folding upon binding. In general, folding-upon-binding reactions are enthalpically driven to overcome the accompanying large and unfavorable entropies of binding, as shown for protein−DNA interactions (*31*) and protein−protein interactions (*29, 32*). Due to the extended nature of many IUPs which fold upon binding their targets, the magnitudes of both the favorable enthalpy change for binding (ΔH) and unfavorable entropy change for binding (ΔS) are approximately proportional to the length of the disordered polypeptide segment involved in binding (*29*). This allows a range of different size binding sites to be targeted by IUPs through evolutionary tuning of the binding favorability and structural complementarity of IUPs and the protein surfaces they target. While the loss of conformational freedom due to folding upon binding (ΔS_{conf}) is entropically unfavorable, it is partially compensated by the entropically favorable release of bound water molecules (ΔS_{HE}) upon binding of an IUP to a protein surface (the hydrophobic effect). While some segments of the polypeptide backbone of IUPs involved in specific protein−protein interactions may become rigid after folding upon binding, other segments may

[1] Abbreviations: AUC, analytical ultracentrifugation; IDP, intrinsically disordered protein; IFSU, intrinsically folded structural unit; IUP, intrinsically unstructured protein; KID, kinase inhibitory domain; MD, molecular dynamics; NOE, nuclear Overhauser effect; PTM, post-translational modification; SAXS, small-angle X-ray scattering.

Table 1: Structural features of IUPs which either (A) lack or (B) exhibit some degree of secondary structure as determined at individual residue resolution using NMR spectroscopy.

protein name	protein length (no. of amino acids)	boundaries of unstructured region(s) (residue numbers)	structural observations [type of secondary structure observed (residues)]	refs
(A) Proteins Which Lack Secondary Structure under Physiological Conditions				
β-dystroglycan segment derived from dystroglycan precursor	893	654−750	highly disordered	*74*
IA3, inhibitor of yeast proteinase A	68	1−68	highly disordered	*75*
myelin basic protein, Golli isoform BG21	194	1−194	highly disordered	*76*
N protein of phage λ	107	1−53	highly disordered	*77*
p19^{Arf} tumor suppressor	169	1−37	highly disordered	*78*
SNARE (soluble NSF attachment protein receptor) protein, Snc1	117	cytoplasmic domain, residues 1−94	highly disordered	*79*
(B) IUPs Which Exhibit Nascent Secondary Structure under Physiological Conditions				
α-synuclein [β-synuclein and γ-synuclein]	140	1−140	α-helical propensity (∼10% α-helix, 18−31), slight α-helical propensity (1−100), possible β-turn (110−140) (data are also available for these alternative isoforms)	*80*, *81* and references therein; *82*
cAMP response element binding (CREB)	341	101−120 [CREB kinase-inducible activation domain (pKID)]	α-helix (119−130)	*83*
cyclin-dependent kinase inhibitor p27^{Kip1}	198	1−198, 22−105 [kinase inhibitory domain (KID)], 105−198 (C-terminal domain)	α-helix (37−59), β-hairpin (65−75), single turn of helix (87−90), highly disordered (105−198)	*29*, *30*, *70*
cyctic fibrosis transmembrane conductance regulator	1480	654−838 (R region)	α-helical propensity (>5% up to ∼30% α-helix; 654−668, 759−764, 766−776, 801−817), β-strand propensity (>5% up to ∼30% β-strand; 744−753)	*84*
dynein intermediate chain, IC74	640	84−143, 198−237	α-helical propensity (222−232), random coil (84−143)	*85*
fibronectin binding protein	1018	745−874 (fibronectin binding domains D1−D4)	α-helical propensity (773−778, 793−799, 811−816, 831−837)	*86*
histone mRNA binding protein [stem−loop binding protein (SLBP)]	276	1−175	α-helix (28−45, 50−57, 66−75, 91−96)	*87*
merozoite surface protein 2 (MSP2), isoform of *Plasmodium falciparum*	221	1−221	α-helical propensity (14−21, 140−150)	*88*
microtubule-associated protein tau, isoform tau-F	441	1−441	α-helix (253−267, 315−328, 346−361), β-strand (307−312)	*89*
negative regulator of flagellin synthesis (anti-sigma factor FlgM)	97	1−97	α-helix (60−73, 83−90)	*90*
Nrf2	597	1−98 (Neh2 domain)	α-helix (39−71), β-strand (74−76, 82−85)	*91*
potassium channel Shaker chain β1a	401	1−62	α-helix (2−10, 44−52, 56−61)	*92*
retinal phosphodiesterase inhibitory γ-subunit	87	1−87	α-helical propensity (∼50% α-helix; 68−84)	*93*
thymosin β4	44	1−44	α-helix (5−17)	*94*
titin	834	425−452 (elastic PEVK motifs)	polyproline II helix (425−429, 438−442, 445−449), highly disordered (430−437, 443−444, 450−452)	*95*
tumor suppressor protein p53	393	1−75 (transactivation domain), 1−73	α-helix (18−26), nascent turn (40−44, 48−53), α-helix (18−24), mixture of α-helix, β-strand, and random coil (39−59)	*96*, *97*

remain dynamic within complexes (*33*), mitigating to some extent the unfavorable ΔS_{conf}. Further, the methyl groups of either IUPs and/or their binding targets, which mediate intermolecular hydrophobic interactions, may experience motional restriction to different extents upon binding, providing an additional mechanism for modulating the ΔS of

binding (*34*). These two mechanisms allow tuning of the affinity of interactions (ΔG) through evolutionary variation of the associated entropy changes. Consequently, the values of dissociation constants (K_d) observed for IUPs binding their biological targets span a wide range, from low nanomolar values [tight binding; e.g., p27 binding to the Cdk2−cyclin A complex (*29*)] to high micromolar values [weak binding; e.g., WASP binding to Cdc42 (*35*)]. As a general rule, weak interactions involving IUPs involve relatively small amounts of buried surface area and tight interactions involve the burial of very large surfaces.

Functional Advantages of Disorder. The inherent flexibility of IUPs is thought to confer certain functional advantages over more highly structured proteins. First, some IUPs bind specifically to more than one biological target and thus exhibit diverse biological functions, often with involvement in signaling and regulation (*36*). For instance, p21 binds and regulates the catalytic activity of several cyclin-dependent kinase (Cdk)−cyclin complexes, an early example of "binding promiscuity" (*25*). Further, p21 and p27 bind additional partners in both the cell nucleus and cytoplasm, extending their functions to include regulation of apoptosis, cell motility, and transcription (*37* and references therein). Another example is the p53 tumor suppressor protein. While the DNA binding and tetramerization domains are folded, the N-terminal (residues 1−97) and C-terminal (residues 363−393) domains of p53 are intrinsically unstructured in isolation and mediate interactions with numerous binding partners that modulate p53 activity in diverse ways (*38* and references therein). Promiscuous binding activities allow p53 to regulate diverse cellular processes such as cell division, apoptosis, and DNA repair (*38*). Finally, it has been suggested that IUPs are specialized to function as hubs in protein interaction networks due to their propensity for promiscuous interactions (*39*); however, the generality (*40*) and validity (*41*) of this general concept have been challenged.

Second, because a large fraction of residues within IUPs are solvent-exposed, even within multiprotein assemblies, these sites are accessible for post-translational modification (PTM), allowing control of protein function, localization, and turnover. For example, the majority of known phosphorylation, acetylation, and ubiquitination sites in p53 occur within the disordered N- and C-terminal domains, and modification of these sites alters the function, localization, and turnover of p53 (*42*). PTM sites are often clustered within disordered polypeptide segments, affording accessibility not only to modification enzymes but also to other proteins that interact specifically with the modified sites to transduce biological signals. An example of this is the phosphorylation−ubiquitination cascade that regulates p27 function (*43*).

Third, disordered polypeptide segments within proteins are often highly susceptible to proteolytic cleavage in vitro, and this may be a factor which influences the rate of IUP degradation in cells. However, a recent study by Tompa and co-workers of >3000 yeast proteins showed that protein disorder was a poor predictor of the in vivo rate of protein turnover; hence, while it is intuitively obvious that polypeptide disorder is associated with proteolytic susceptibility, protein degradation in vivo is highly regulated and influenced by many other factors (*44*). For example, Shaul and coworkers discovered that p53 is degraded by the 20S proteasome via a "default" pathway, without the need for ubiquitination. These authors proposed that disordered segments of p53, and other proteins (*45*), are signals for 20S proteasome-mediated degradation and that the formation of multiprotein assemblies masks these signals and guards against degradation (*46*). This may represent a mechanism for sensing imbalances in the levels of subunits within multisubunit assemblies, allowing subunits present in excess to be degraded by default (*45*). Thus, the physical properties of disordered polypeptide segments allow proteins to be extensively regulated by PTM and provide the opportunity for rapid turnover and possibly quality control during assembly of multiprotein complexes.

Finally, the noncompact nature of IUPs may facilitate biomolecular interactions by increasing intermolecular association rates. Wolynes and co-workers (*47*) postulated that disordered proteins have a greater "capture radius" than compact, folded proteins. According to their so-called "fly-casting" mechanism, a segment of an extended, unfolded protein first binds relatively weakly to the surface of a target, followed by folding to reel in the target. By being extended, IUPs sample larger solution volumes, in a sense reducing the dimensionality of the search for their partners (*47*). For example, p27 binds via a sequential mechanism to the Cdk2−cyclin A complex, with an extended domain at the N-terminus binding first to a compact surface on cyclin A, followed by extensive folding of p27 and remodeling of Cdk2 as the inhibited p27−Cdk2−cyclin A complex is fully assembled (*29*). This fly-casting-like mechanism may facilitate assembly of Cdk−cyclin complexes under the low-concentration conditions found in cells. Another example of this mechanism was revealed recently by Wright and coworkers (*48*) in studies of the phosphorylated KID (pKID) domain of CREB binding to the KIX domain of the CREB binding protein (CBP). Intrinsically unstructured pKID was shown to initially dock nonspecifically on the surface of KIX, allowing rapid searching of the KIX surface for the specific binding site, followed finally by folding into the specific, high-affinity complex (*48*). IUPs often exhibit many, compact recognition elements throughout their sequences; the conformational disorder of IUPs may facilitate the assembly of multiprotein complexes by bringing together their many components via the fly-casting mechanism. Not only does intrinsic disorder promote association events, but Hilser and Thompson have proposed that linking ligand binding with disordered domain folding provides a mechanism for optimizing allosteric coupling in multidomain proteins (*49*).

INTRINSICALLY UNSTRUCTURED PROTEINS IN MAMMALIAN CELL CYCLE REGULATION

In eukaryotes, cyclin-dependent kinases (Cdks) are the master time keepers of cell division (*50*). Many proteins regulate the Cdks, both directly and indirectly, and in turn, the catalytic activity of the Cdks regulates the activity of myriad downstream targets (*51, 52*). While many of these regulatory proteins are folded, many others are intrinsically unstructured. In this review, we focus on a small subset of these IUPs: the cyclin-dependent kinase regulators (CKRs) p21, p27, and $p57^{Kip2}$ (p57) (*51*) that regulate cell division through direct interactions with Cdk−cyclin complexes (Figure 1A). Through binding promiscuity (*25*), the CKRs regulate Cdk−cyclin complexes that control (1) entry into

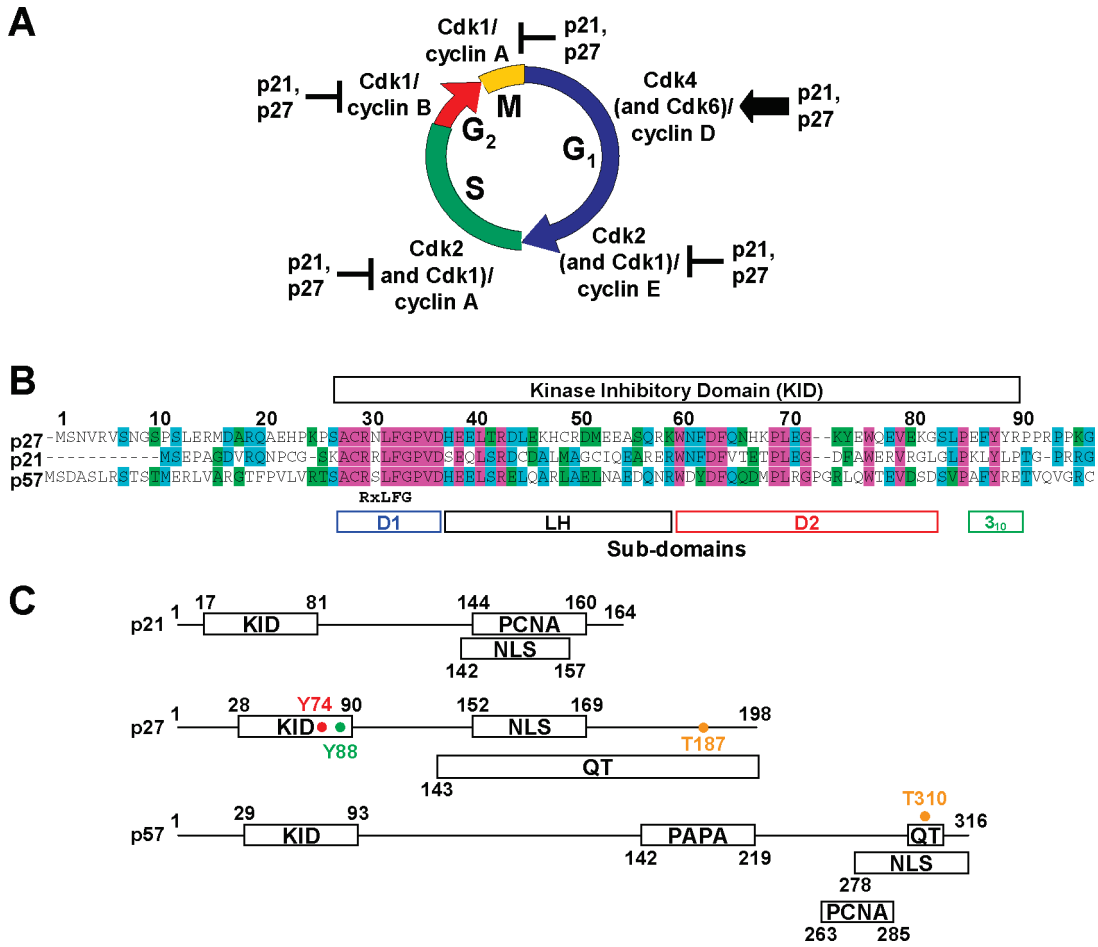

FIGURE 1: Regulation of the eukaryotic cell division cycle. (A) Illustration of the various stages of the cell division cycle and the Cdk−cyclin complexes that play roles in regulating progression through the different stages. Initiation of cell division in G_1 phase requires the activity of Cdk4−cyclin D and Cdk6−cyclin D complexes, and progression into S phase (when DNA synthesis or replication occurs, "S") requires Cdk2−cyclin E and Cdk2−cyclin A complexes (and similar complexes with Cdk1). The activity of Cdk1−cyclin B and Cdk1−cyclin A complexes is required for entry into mitosis ("M"). While initially thought to be universal inhibitors of these Cdk−cyclin complexes, p21 and p27 have been shown to activate Cdk4−cyclin D and Cdk6−cyclin D complexes under certain circumstances (indicated by the horizontal arrow). (B) Alignment of sequences of the kinase inhibitory domains (KIDs) of p27, p21, and p57. The boundaries of subdomains D1 (blue), LH (black), D2 (red), and 3_{10} (green) are indicated, as is the "RxL" motif which is recognized by cyclin A. (C) Illustration of the domain structure of p21, p27, and p57. PCNA, PCNA binding domain; NLS, nuclear localization signal; QT, QT domain which contains one or more QT motifs that are either known or putative phosphorylation sites; PAPA, domain with multiple repeats of the PAPA motif. The locations of known phosphorylation sites, Y74, Y88, and T187 in p27 and T310 in p57, are also indicated. Panel B was taken from ref *29*. Copyright 2004 Nature Publishing Group.

G_1 phase (Cdk4 and Cdk6 paired with D-type cyclins) and (2) progression from G_1 to S phase (Cdk2 paired with A- and E-type cyclins) (*51*). Further, p21 and p27 exhibit functional diversity by having seemingly opposite effects on these different Cdk−cyclin complexes, promoting the assembly and catalytic activity of some (e.g., Cdk4 paired with D-type cyclins) and potently inhibiting others (e.g., Cdk2 paired with A- and E-type cyclins) (*51*). In the following sections, we discuss results from our laboratory and others with respect to the structural and dynamics features of the CKRs and the relationship of these features to their diverse biological functions.

Domain Structure of CKRs. CKRs p21, p27, and p57 contain a conserved, 60-residue N-terminal kinase inhibitory domain (KID, residues 28−90 in p27) (Figure 1B) and nuclear localization signals (NLSs) (*53, 54*) within their C-terminal domains (Figure 1C). In addition, p21 and p57 contain a PCNA-binding domain within their C-termini that, when bound, inhibits the ability of PCNA to stimulate DNA synthesis (*55, 56*). Further, p27 and p57 possess a C-terminal QT domain that contains a critical threonine residue (T187 in p27 and T310 in p57) that, when phosphorylated by Cdk2, triggers ubiquitination of p27 (*57, 58*) and p57 (*59*) by SCF/Skp2. Human and mouse p57 have an additional domain comprised of multiple Pro-Ala repeats, and mouse p57 contains a segment rich in acidic residues; these domains were proposed to mediate protein−protein interactions (*60*).

Structure of p27 Bound to the Cdk2−Cyclin A Complex. Pavletich and co-workers showed that the KID of p27 binds in a highly extended conformation to both subunits of the Cdk2−cyclin A complex (Figure 2), burying more than 2000 Å^2 of solvent-exposed surface (*61*). Several subdomains within p27-KID, which possess many residues conserved among p21, p27, and p57 (Figure 1B), adopt secondary structure in the Cdk2−cyclin A complex-bound state (Figure 2): subdomain D1, containing the conserved RxL motif (*62*), binds in an extended conformation on the surface of cyclin A; subdomain LH forms a 22-residue α-helix that spans the nearly 40 Å gap between Cdk2 and cyclin A; subdomain D2 forms a β-hairpin and an intermolecular β-sheet (with

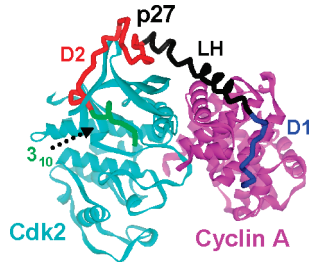

FIGURE 2: Structure of p27-KID bound to the Cdk2−cyclin A complex. Crystal structure of the p27-KID−Cdk2−cyclin A complex determined in 1996 by Pavletich and co-workers (PDB entry 1JSU). Subdomains p27, D1, LH, D2, and 3_{10} are indicated using the color scheme of Figure 1.

Cdk2) on the surface of the N-terminal lobe of Cdk2; and subdomain 3_{10} forms a 3_{10}-helix that inserts into the ATP binding pocket of Cdk2. p27 inhibits Cdk2 (1) by inserting subdomain 3_{10} into the active site and blocking access to ATP and (2) by remodeling the catalytic cleft through displacement of a β-strand of Cdk2 by subdomain D2 of p27 (*61*). Further, subdomain D1 of p27 blocks the substrate binding site on cyclin A which recognizes Cdk2 substrates possessing a RxL motif (*62*). These results can be extended to generally understand how the CKRs regulate the Cdks that control the G_1−S transition during cell division (*29, 63*). However, due to the inherent limitations of X-ray crystallography, this structural model does not provide insights into the role of p27's intrinsic flexibility in recognizing and binding to Cdk−cyclin complexes.

The CKRs Are IUPs. Analysis using proteolysis, CD, and NMR spectroscopy showed that p21, p27, and p57 are largely disordered, with ~15−20% α-helical content (*25, 28, 29, 64*). Secondary structure (*29*) and disorder prediction [using FoldIndex (*65*), IUPred (*66*), and PONDR (*21*)] also indicated that these proteins are predominantly disordered (Figure 3 shows results for p27). Two-dimensional (2D) $^1H-^{15}N$ HSQC NMR spectra for p21, p27, and p57 exhibited limited resonance dispersion (backbone amide protons resonate between 7.8 and 8.5 ppm), a feature typical of intrinsically unstructured proteins (*25, 29, 64*). However, despite being disordered, these proteins were shown to be potent inhibitors of various Cdks in vitro (*67, 69*) and in vivo (*51*). As early as 1996, structural data for p21 (*25*) and the previously identified heat-resistant nature of p27 (*69*) strongly suggested that polypeptide disorder was associated with the biological function of the CKRs.

The CKRs Exhibit Partially Populated Secondary Structure in Solution. Although p21, p27, and p57 can be categorized as IUPs on the basis of their lack of tertiary structure, CD spectra indicated the presence of a small amount of α-helical secondary structure (*25, 28, 29, 64*) within the KID of each protein (*25, 28, 64*). Subsequently, we used NMR spectroscopy to localize this secondary structure within p27-KID. Analysis of secondary $^{13}C_\alpha$ chemical shift values ($\Delta\delta^{13}C_\alpha$) indicated that the small amount of α-helical secondary structure observed using CD was localized to subdomain LH [~30% α-helical (Figure 4A)], while other subdomains of p27-KID appeared to lack secondary structure (*29*). To probe the dynamics of p27-KID, we measured $\{^1H\}-^{15}N$ heteronuclear NOE (hetNOE) values; this NMR relaxation parameter is sensitive to fluctuations of amide groups on the high

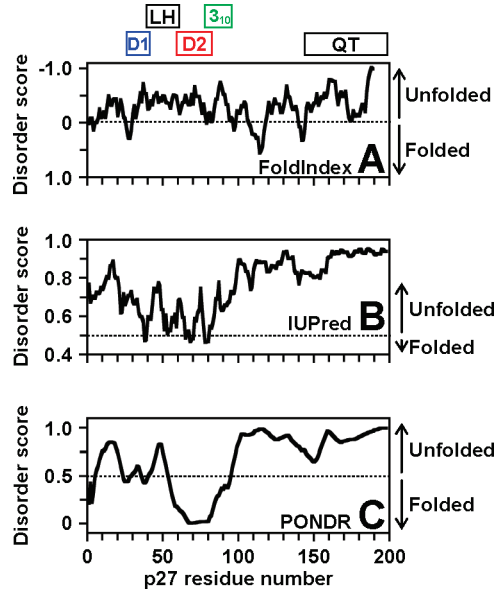

FIGURE 3: Disorder predictions for p27. Three disorder prediction programs were used to analyze the structure of p27, including FoldIndex (A), IUPred (B), and PONDR (C). The approximate locations of subdomains D1, LH, D2, and 3_{10} are indicated at the top, as is the QT domain.

picosecond to low nanosecond time scale. The hetNOE values observed for p27-KID (Figure 4B) indicated that the polypeptide backbone experiences intermediate dynamics, being less rigid than a folded protein but more rigid than a random coil. Interestingly, partially restricted motions were observed not only for subdomain LH but also for most residues in subdomains D2 and 3_{10}. In contrast, residues within subdomain D1 (residues 27−35) exhibited negative or near-zero hetNOE values, consistent with a high degree of flexibility.

More recently, we probed the structure and dynamics of p27-KID using a novel approach that utilized amide proton−amide proton ($^1H_N-^1H_N$) NOE data from NMR spectroscopy to provide insights into time-averaged structure and molecular dynamics (MD) computations to provide insights into how structure fluctuates with time (*30*). Interestingly, these studies revealed that several segments of p27-KID exhibited discreet structures which we termed intrinsically folded structural units (IFSUs) (Figure 4C−F). The IFSUs occurred within subdomains LH, D2, and 3_{10}, which also exhibited positive hetNOE values. Subdomain LH adopted helical structure, as expected from chemical shift analysis; subdomain D2 formed a β-hairpin and nascent helical structure, and subdomain 3_{10} formed a single turn of helix. These results indicated that p27-KID is quite rich in transient, discrete structures, in contrast to the picture which first comes to mind for an IUP. Importantly, with the exception of the nascent helical segment of subdomain D2, these structural features resemble those observed when p27-KID is bound to the Cdk2−cyclin A complex.

Finally, we recently characterized the solution structure of the ~100-residue C-terminal domain of p27 using NMR spectroscopy (*70*). This domain contains several phosphorylation sites, including T187 mentioned earlier and threonine 157 (T157) within the NLS which is phosphorylated by Akt in breast cancer (*71*), and several lysine residues that are

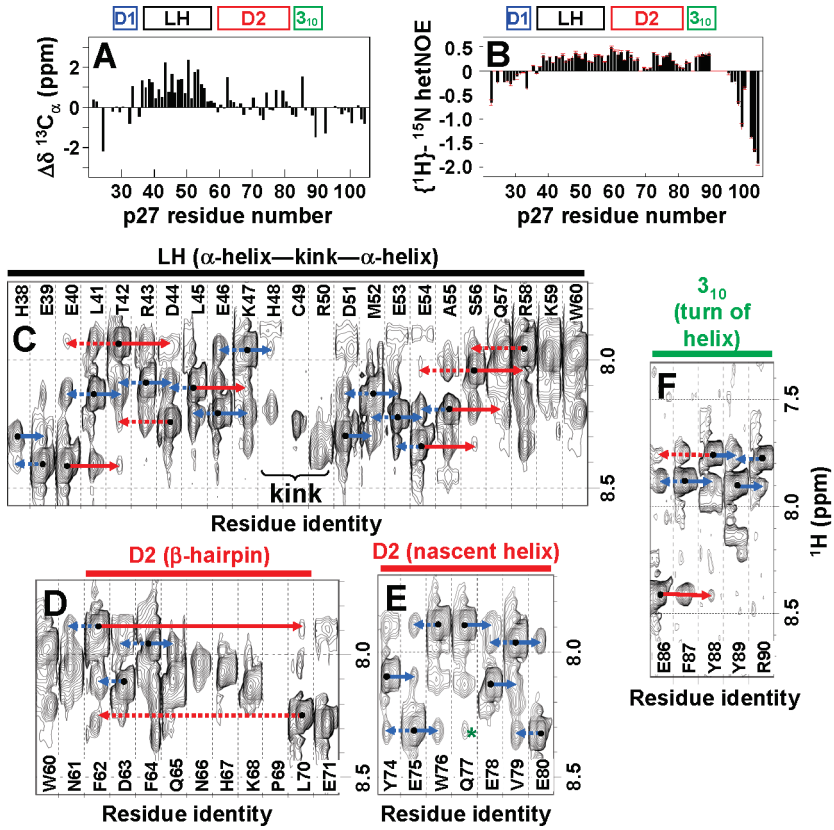

FIGURE 4: NMR results for the solution structure and dynamics of isolated p27-KID in solution. (A) Secondary $^{13}C_\alpha$ chemical shift values ($\Delta\delta^{13}C_\alpha$) and (B) $\{^1H\}-^{15}N$ heteronuclear NOE (hetNOE) values for p27-KID. (C−F) Patterns of $^1H_N-^1H_N$ NOEs which indicated the existence of partial secondary structure (as indicated) within subdomains LH (C), D2 (D and E), and 3_{10} (F) of p27-KID. Panels A and B were taken from ref *29*. Copyright 2004 Nature Publishing Group. Panels C−F were taken from ref *30*. Copyright 2005 Elsevier Ltd.

likely sites of ubiquitination. Earlier CD and NMR results suggested that this segment of p27 is highly disordered (*29*); these results were confirmed by the recent NMR studies which showed that this domain lacks tertiary and secondary structure on the basis of $\Delta\delta^{13}C_\alpha$ and hetNOE values (*70*).

The CKRs Fold Sequentially upon Binding Specific Cdk−Cyclin Complexes. In 1996, Kriwacki and Wright demonstrated that subdomains D2 and 3_{10} of p21 folded upon binding to Cdk2 using NMR spectroscopy and, through proteolytic mapping, deduced that subdomain D1 bound to cyclin A within the Cdk2−cyclin A complex (*25*). Later in the same year, Pavletich and co-workers demonstrated that p27-KID adopted a highly extended, folded conformation when bound to the Cdk2−cyclin A complex (*61*). As noted earlier, several of the IFSUs detected in p27-KID prior to binding were also observed in the Cdk2−cyclin A complex-bound state. For example, one part of subdomain D2 and subdomain 3_{10} maintained their β-hairpin and helical conformations, respectively, when bound to Cdk2. Importantly, the single turn of helix observed for apo subdomain 3_{10} occupied the ATP binding site of Cdk2 within the ternary complex, with tyrosine 88 (Y88) bound in place of the purine ring of ATP (*61*). Subdomain LH formed a 22-residue α-helix that linked subdomains D2 and D1, which were bound to Cdk2 and cyclin A, respectively. Subdomain D1 exhibited a high degree of disorder and flexibility in the free state and adopted an extended, rigid conformation upon binding to a pocket on the surface of cyclin A that is conserved in many cyclins that regulate cell division (*29, 61, 63*). Finally, another part of subdomain D2, which exhibited nascent helical features prior to binding, adopted an extended conformation and formed an intermolecular β-sheet upon binding to the N-terminal domain of Cdk2.

While the crystal structure defined the Cdk2−cyclin A complex-bound conformation of p27 and provided key insights into the molecular basis of specific recognition of Cdk−cyclin complexes (*61*), it does not explain why the CKRs have evolved to be disordered or how disorder plays a role in their biological functions. Answers to these questions came from studies that probed the mechanism through which p27 binds to the Cdk2−cyclin A complex (*29*). Isothermal titration calorimetry (ITC) was used to determine values of thermodynamic parameters (ΔG, ΔH and ΔS) associated with p27 binding to the Cdk2−cyclin A complex and to quantitatively characterize the polypeptide folding which accompanies binding (*31*). Further, surface plasmon resonance (SPR) was used to analyze the kinetics of association of p27 with and dissociation of p27 from the Cdk2−cyclin A complex. Results from these two methods, coupled with our knowledge of structure and dynamics, indicated that the subdomains of p27 bind to the Cdk2−cyclin A complex via a sequential mechanism; first, the highly flexible subdomain D1 binds cyclin A, followed by docking of helical subdomain LH and finally by docking and folding of subdomains D2 and 3_{10} to Cdk2 (Figure 5). In addition, other studies (*63*) suggested that subdomain D1 rapidly scans the surfaces of protein complexes for a conserved binding pocket, which was found on cyclin A and other cyclins that regulate cell division. When subdomain D1 encounters the cyclin pocket, p27 transiently binds, providing time for other

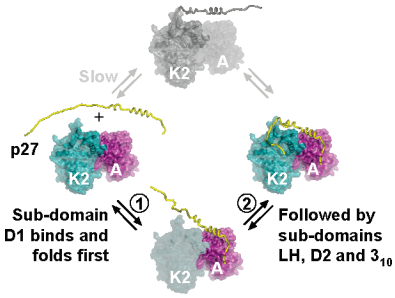

FIGURE 5: Subdomains of p27 sequentially fold and bind the Cdk2−cyclin A complex. NMR, ITC, and SPR results support a scheme in which the RxL motif within subdomain D1 of p27 binds first to cyclin A (1), followed by folding and docking of subdomain LH, followed by binding of subdomain D2 to Cdk2 (with extensive remodeling of Cdk2), followed finally by binding of subdomain 3_{10} in the ATP binding pocket of Cdk2 (2). This sequential scheme provides a mechanism for specificity toward Cdk−cyclin complexes which preserve the binding site for the RxL motif within subdomain D1 of p27. A movie depicting this sequential binding scheme is available at http://www.stjuderesearch.org/data/kriwackilab/p27.html. This figure was taken from ref *29*. Copyright 2004 Nature Publishing Group.

subdomains to sequentially dock and fold into the final, inhibited ternary Cdk2−cyclin A complex. Amino acids that comprise the cyclin A docking site for p27 are highly conserved in Cdk−cyclin complexes that directly regulate cell division and are inhibited by p21 and p27. However, these residues are not conserved in Cdk−cyclin complexes involved in other biological functions. Therefore, we proposed that the intrinsic disorder of p21 and p27 evolved to allow specific molecular recognition through sequential folding upon binding (*29, 63*). The extended character of p27 when bound to the Cdk2−cyclin A complex has evolved to accommodate the large distance (40 Å) between the specificity-determining site on cyclin A and the site of inhibition (ATP binding pocket) on Cdk2. It is possible that simultaneously engaging these two sites through interactions with the two ends of an extended polypeptide chain provided evolutionary advantages over an alternative scheme involving interactions mediated by multiple, folded protein domains.

Regulation of p27 Function through Post-Translational Modification: p27 as a Molecular Signaling Conduit. Post-translational modifications regulate the localization, turnover, and activity of p27 (*72* and references therein). For example, Akt-mediated phosphorylation of T157 within p27's NLS in breast cancer cells prevents its interactions with the nuclear import machinery and leads to cytoplasmic localization. p27, normally located in the nucleus, encounters new targets in the cytoplasm and exhibits a gain of oncogenic function. In a further example, phosphorylation of p27 on Ser10 promotes its interaction with the shuttling protein, CRM1, leading to export from the nucleus. Finally, cells entering the cell division cycle contain high levels of p27, and phosphorylation-dependent ubiquitination and degradation of p27 by the 26S proteasome are required for progression through the cell division cycle.

p27 degradation is regulated by two E3 ubiquitin ligases during cell division, KPC1 in the cytoplasm in G_1 phase and SCF^{Skp2} in the nucleus in S and G2 phases (*43* and references therein). While KPC1 ubiquitinates unphosphorylated and free p27, SCF^{Skp2} targets p27 that is phosphorylated on T187 and which is bound to the Cdk2−cyclin E or Cdk2−cyclin A complex. Ubiquitinated p27 is degraded by the 26S proteasome. The active cyclin E−Cdk2 complex can phosphorylate cyclin−Cdk complex-bound p27 on T187. However, while the free, active Cdk2−cyclin complex phosphorylates Cdk-bound p27 efficiently, structural and biochemical studies have demonstrated that p27-bound Cdk2 is catalytically inactive (*29*). This presented an apparent paradox since elimination of p27 appeared to require the activity of the enzyme (Cdk2) that p27 was known to potently inhibit. However, recent studies by Hengst, Kriwacki, and coworkers (*43*) have demonstrated that phosphorylation of p27, within the p27−Cdk2−cyclin A complex, at sites other than T187 provided a solution to this conundrum.

Preliminary in vivo studies indicated that p27 was phosphorylated on tyrosine 88 (Y88) by non-receptor tyrosine kinases, including Abl (*43*), Lyn (*43*), and Src (*5*). Phosphorylation of residue Y88 on p27 bound to the Cdk2−cyclin A complex relieved inhibition of Cdk2 and promoted Cdk2-mediated phosphorylation of T187 via a pseudo-unimolecular reaction mechanism (*43*). NMR studies showed that phosphorylation of Y88 (pY88) within the Cdk2 binding domain of p27 by Abl kinase led to ejection of the inhibitory 3_{10}-helix of p27 (subdomain 3_{10}) from the ATP binding pocket of Cdk2 while leaving other interactions between p27 and the Cdk2−cyclin A complex unperturbed (Figures 6 and 7, step 1). Surprisingly, Cdk2 retained significant catalytic activity even though pY88-p27 remained tightly bound to the Cdk−cyclin complex (*43*). Consequently, residue T187 within the intrinsically unstructured C-terminal domain of p27 could then be phosphorylated by the partially reactivated Cdk2 within the same pY88-p27−Cdk2−cyclin A ternary complex (*43*) (Figure 7, step 2). Although Cdk2 within this phosphorylated ternary complex exhibited submaximal catalytic activity, tethering of p27 to the cyclin A−Cdk2 complex strongly promoted the phosphorylation of T187 through the unimolecular mechanism (Figure 7B). p27 that has been phosphorylated on both Y88 and T187 (pY88/T187-p27) can be polyubiquinated by the SCF^{Skp2} ubiquitin ligase and degraded, resulting in complete reactivation of the Cdk2−cyclin A complex. The accumulation of the free, active cyclin A−Cdk2 complex may further promote Thr187 phosphorylation of p27 within remaining p27−Cdk2−cyclin A complexes and accelerate progression from G_1 to S phase of the cell division cycle.

We propose that the intrinsic disorder and flexibility of p27 are evolutionarily advantageous because they enable the structural fluctuations and post-translational modifications associated with the phosphorylation−polyubiquitination cascade that regulates p27 turnover at the G_1−S boundary during cell division. First, segmental flexibility between subdomains D2 and 3_{10} allows Y88 within subdomain 3_{10} to fluctuate between ATP pocket-bound and solvent-exposed conformations. This allows Abl and other NRTKs to access and phosphorylate otherwise occluded Y88. Notably, in addition to targeting Y88, Src also phosphorylates p27 on tyrosine 74 (Y74). This implies that the β-hairpin IFSU harboring Y74 within p27 subdomain D2 (Figure 7A) also fluctuates between bound and solvent-exposed conformations to provide Src access to Y74. Second, after Y88 (and sometimes Y74) has been phosphorylated and subdomain 3_{10} has been ejected from the Cdk2 active site, the extreme flexibility of the p27 C-terminus allows T187 to approach the substrate binding site of Cdk2 and be phosphorylated. This flexibility

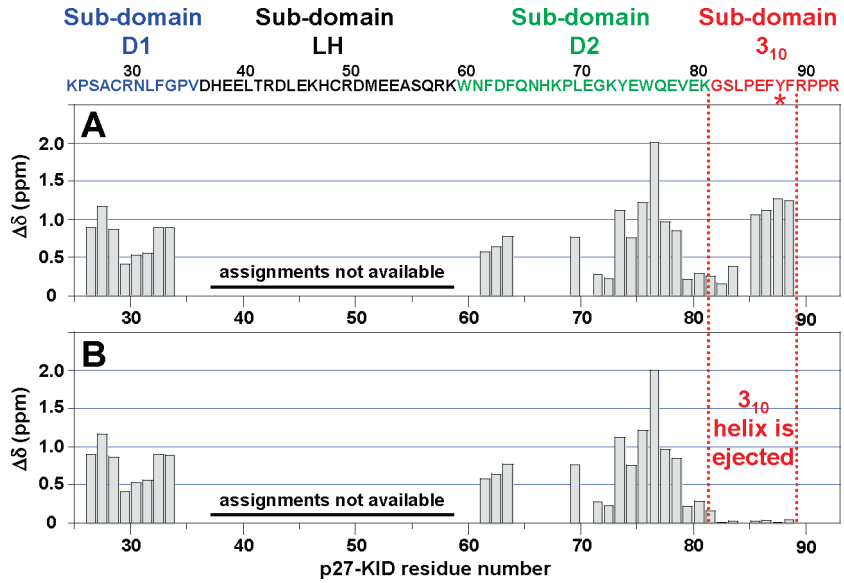

FIGURE 6: Phosphorylation of p27 on Y88 (pY88) ejects only the 3_{10}-helix (subdomain 3_{10}) from the ATP binding pocket of Cdk2. Other subdomains of p27 remain bound to the Cdk2−cyclin A complex. (A) NMR chemical shift differences ($\Delta\delta$) between (A) free p27-KID and p27-KID bound to the cyclin A−Cdk2 complex are compared with those for (B) free pY88-p27-KID and pY88-p27-KID bound to the cyclin A−Cdk2 complex. The locations of subdomains within p27 are shown at the top, as is the amino acid sequence of p27-KID. The asterisk indicates the location of Y88. Amino acid residues are indicated using the single-letter code. For technical reasons, a mutant form of p27-KID, with Y89 mutated to F, was used in these experiments. This figure was taken from ref *43*. Copyright 2007 Elsevier Ltd.

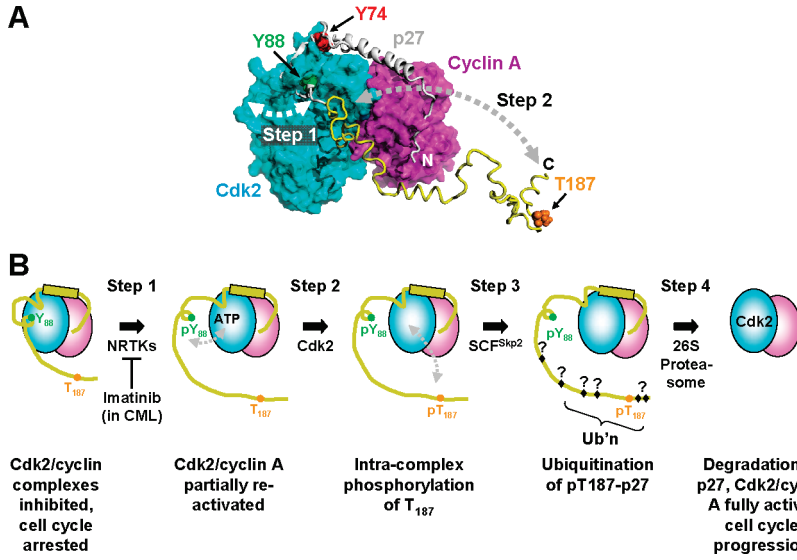

FIGURE 7: p27 is a signaling conduit. (A) A single snapshot from a 13 ns molecular dynamics trajectory illustrating the structure of p27 bound to the Cdk2−cyclin A complex (cyan and magenta, respectively). The 100-residue C-terminal domain of p27 (yellow tube), which contains T187 (orange), is intrinsically unstructured and highly dynamic in this trajectory. Also illustrated are two critical tyrosine residues, Y74 and Y88 (red and green, respectively), which are phosphorylated by non-receptor tyrosine kinases (NRTKs). Phosphorylation of Y88 (step 1), and possibly Y74, ejects subdomain 3_{10} from the ATP binding pocket of Cdk2 (indicated by the white arrow), allowing T187 within the flexible C-terminal domain to encounter the Cdk2 active site (step 2, indicated by the gray arrow) and be phosphorylated by Cdk2. (B) Scheme illustrating the two-step p27 phosphorylation mechanism involving Y88 and T187 which triggers p27 polyubiquitination and 26S proteasomal degradation in both normal and cancer cells. The pseudo-unimolecular nature of step 2 is illustrated. Panel A was taken from ref *70*. Copyright 2008 Elsevier Ltd. Panel B was taken from ref *98*. Copyright 2008 Nature Publishing Group.

also ensures that phosphorylated T187 is accessible for recognition by SCF^{Skp2} which is bound to the p27−Cdk2−cyclin A complex (or cyclin E). Third, the p27 C-terminus contains six lysine residues that are likely sites for polyubiquitination by SCF^{Skp2}. Intrinsic flexibility within this polypeptide segment not only will make these sites accessible for polyubiquitination but also ensures that covalently linked polyubiquitin chains are accessible for processing by the 26S proteasome and its various accessory proteins (*73*). Thus, the intrinsic flexibility of p27 critically mediates each step of this multistep PTM cascade. In view of this, we have proposed that p27 acts as a molecular signaling conduit which integrates proliferative signals from NRTKs (through phosphorylation on Y74 and Y88), participates in the processing of these signals (through reactivation of Cdk2 and phosphorylation of T187), and finally transduces these signals by triggering its own polyubiquitination and degradation (through phospho-T187-dependent interactions with SCF^{Skp2}). While p27 exhibits modular structure within the kinase inhibitory domain when free in solution, the segments connecting these

modules are highly flexible, allowing the individual modules to function independently as part of this signaling conduit. The flexibility and modularity which enable this structural independence allow signals to flow through the p27 conduit as a consequence of these sequential phosphorylation and ubiquitination modifications.

CONCLUSIONS

It is now well recognized that IUPs are highly abundant and that they play critical functional roles in biological systems, with many mediating signaling and regulation. Bioinformatics studies have dramatically enhanced our awareness of IUPs and their properties. However, structural, biophysical, and biochemical studies of IUPs have progressed at a slower pace, creating gaps in our knowledge of the relationship between the physical properties of these proteins and their wide-ranging biological functions. Our studies of the cell cycle regulators, p21 and p27, have revealed many interesting, often unexpected, results that have established important concepts that are likely to apply to many other, uncharacterized IUPs. These concepts include the existence of both highly disordered and partially folded modules (IFSUs) within IUPs prior to their interaction with their biological targets; the participation of these modules in highly specific, sequential binding events; the role of post-translational modification (PTM) of residues within these modules in regulating IUP function; and the linkage of multiple PTMs within individual IUPs into signaling conduits. While disorder prediction algorithms can accurately identify proteins, or protein segments, that are likely to exhibit extensive disorder, because relatively few IUPs have been structurally and dynamically characterized at atomic resolution, it is not yet possible to empirically predict the detailed structural features of IUPs. Several of the IFSUs identified within p27 play critical roles in interactions with Cdk−cyclin complexes; just as critical, however, is flexibility between these modules so that their functions can be regulated independently, for example, by PTMs. A challenge for the future is to characterize in detail additional IUPs, both with regard to their detailed structural features and with regard to the relationship(s) of these features to biological function. Multidisciplinary approaches are required to fully understand the physical properties of IUPs, for example, using NMR spectroscopy complemented with computational (MD), hydrodynamic (AUC), and scattering (SAXS) methods, as illustrated here for p27. When a larger body of biophysical data on IUPs is available in the future, we will gain a deeper understanding of relationships between their structural and dynamic features and biological functions and may be better positioned to broadly predict structure−function relationships for IUPs. As additional data are gathered, we will more clearly see that functionally relevant protein "structures" span a continuum which extends from near-complete disorder [e.g., FG-domains within nucleoporins (*20*)], to partial order (e.g., IFSUs within p27), to partial disorder [e.g., disordered N- and C-terminal domains of p53 (*38*)], and finally to near-complete order (e.g., many enzymes). We hope that this detailed review of the relationships between protein disorder and function for p21 and p27, and the experimental approaches used to gain these insights, will motivate others to undertake similar, detailed studies of other biologically important IUPs. Only through detailed study of the large number of intrinsically unstructured proteins evident in higher organisms will we begin understand why they have evolved to be ubiquitous.

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Due to space limitations, we could not discuss or cite many important reports related to IUPs; we apologize to authors whose work was overlooked. We acknowledge Limin Xiao and Cheon-Gil Park for technical support during the course of these studies, John Fisher for critical comments on the manuscript, and other members of the Kriwacki laboratory for stimulating discussion.

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