

3. Louise N. Johnson

Structural studies on protein kinases and regulatory molecules of the cell cycle

We are interested in the structural basis of the recognition processes that control intermolecular interactions in the cell cycle, especially those that mediate control by phosphorylation and by ubiquitylation. Current work includes studies on the regulatory kinases of the cell cycle (CDK2/cyclin A, CDK2/cyclin E, CDK7/cyclin H, CDK9/cyclin T and polo-like kinase) and those domains that mediate interactions of ubiquitylated proteins with the proteasome. Our X-ray crystallographic studies are complemented by a number of other biophysical and biochemical methods. For large macromolecular complexes, such as phosphorylase kinase and the anaphase promoting complex, we are also using electron microscopy (see Section 11 by Catherine Venien Bryan).

3.1 CDK7 (CDK Activating Kinase (CAK))

Graziano Lolli

CDK7, a member of the cyclin dependent protein kinase (CDK) family, regulates the activities of other CDKs through phosphorylation of their activation segment. CDK7 also assists in the regulation of transcription as part of the transcription factor TFIIF complex where it phosphorylates the C-terminal domain of RNA polymerase II. For maximum activity and stability, CDK7 requires association with cyclin H and a third protein, MAT1. In the absence of Mat1, CDK7 must be phosphorylated for activity. CDK7 poses many intriguing structural problems concerned with its recognition roles in the cell cycle and in transcription.

Last year we reported the crystal structure determination of CDK7 and characterisation of the kinase [1]. This has led to an inhibitor design project for inhibitors that target the ATP binding site. Such an inhibitor could be of value in the control of the cell cycle for cancer therapy and in cell biology where it is useful to be able to discriminate between the effects of CDK7 and CDK9 [2]. We are collaborating with Herbie Newell and Roger Griffin (University of Newcastle) where a survey of compounds related to the successful CDK2 inhibitor NU6102 [3] has identified possible compounds.

Co-expression of CDK7, cyclin H and Mat1 in baculoviral infected insect cells have led to the purified active ternary complex. Crystallisation trials are in progress.

We are interested in the mechanism by which kinases phosphorylate other kinases. Active CDK7 phosphorylates CDK2, an essential step in activation of CDK2 in the cell cycle. *In vitro*, active CDK2 phosphorylates CDK7. However neither CDK2 nor CDK7 will autophosphorylate. Attempts to form a complex between CDK2 and CDK7 showed that the association was not sufficiently high affinity to enable the two proteins to travel together under gel filtration and hence were unlikely to form a sufficiently stable complex for crystallisation. A structure for CDK2/CDK7 has been derived from a series of mutagenesis experiments and molecular modelling with the 3D-Dock suite. In the model, the two kinases are arranged in a quasi-symmetric head-to-tail arrangement in which the N-terminal lobe from one kinase docks against the C-terminal lobe from the other kinase and the activation segments are within reach of the opposite catalytic sites (Figure 1). The model has been tested by further mutagenesis and can explain why the two kinases are able to phosphorylate one another but not phosphorylate themselves. A manuscript has been submitted.

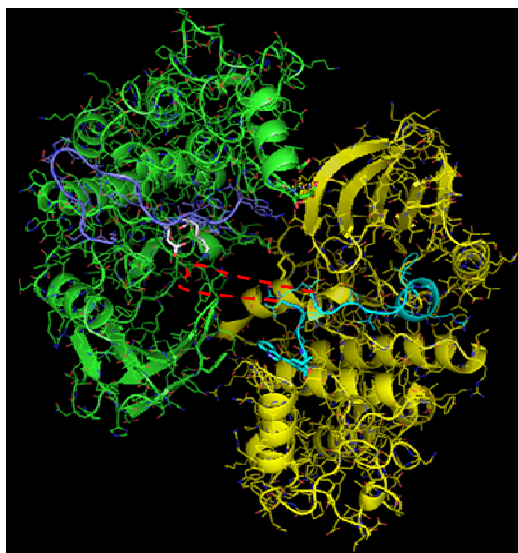


Figure 1. A model of CDK7 (as enzyme: green) and CDK2 (as substrate: yellow). CDK7 activation segment, in the active conformation, is in blue and CDK2 activation segment is in cyan. The red dotted curve indicates the path that CDK2 activation segment should follow to reach CDK7 active site.

In collaboration with Andre Furger (Department of Biochemistry) and Sasha Akoulitchev (Department of Pathology) we have begun structural studies on the interaction between cyclin H and the small nuclear RNA (snRNA) U1 that regulates mRNA splicing.

3.2 CDK2/cyclin E

Reiko Honda (until December 2004), Ed Lowe, Vicky Skamnaki, and Nick Brown

Cyclins play a key role in the orderly progression of the cell division cycle through their timed expression and their ability to bind, activate and enhance substrate affinity of their associated cyclin dependent protein kinases (CDKs). Cyclin E binds and activates CDK2 at the G1/S transition of the cell cycle. Shortly after entry to S phase cyclin E is degraded in response to auto- and other phosphorylation events that target cyclin E for ubiquitylation by the Skp1/cullin/Cdc4 F-box protein (SCF^{Cdc4}) complex and subsequent degradation by the proteasome. As cyclin E levels decline, those of cyclin A increase and it is the activity of the CDK2/cyclin A complex that drives cells through S phase. Cyclin E deregulation is directly implicated in cancer. Unusually high and persistent levels of cyclin E have been observed in human tumour cells, especially in the most aggressive cancers. Remarkably cyclin E deficient cells are resistant to oncogenic transformation by Ras.

Key experiments in 2003 with cyclin E knockout mice showed that cyclin E is essential for entry to the cell cycle from G0 quiescent phase, for the assembly of pre-replication complexes and for endoreduplication in megakaryotes and giant trophoblast cells but is not essential for cells that have entered the cell cycle. The most important role of cyclin E is to promote the assembly of the pre-replication complex, through the loading of CDC6 and Cdt1 to the origins of replication and recruitment of the Mcm2-7 proteins. Recent work has shown that a key role for CDK2/cyclin E is to phosphorylate CDC6 and thus protect CDC6 from ubiquitylation by the anaphase promoting complex, APC^{CDH1} complex.

Last year we reported the crystal structure determination of the pCDK2/cyclin E complex at 2.25 Å resolution [4]. The N-terminal cyclin box fold of cyclin E1 is similar to that of cyclin A and promotes identical changes in pCDK2 that lead to kinase activation. The C-terminal cyclin box fold shows significant differences to cyclin A. It makes additional interactions with pCDK2, especially in the region of the activation segment. Knockout experiments with CDK2 have raised the question of whether cyclin E has a CDK2 independent role since CDK2^{-/-} mice are normal (apart from defects in meiosis) and cyclin E^{-/-} mice die in utero. Cyclin E associates with centrosomes, an association that is important for cell cycle progression [5]. Remarkably the association is not dependent on CDK2, the first clear evidence for a CDK2-independent role for cyclin E. The cyclin E binding region is shown in the Figure 2. We hope to extend the observations to direct binding studies with the centrosomal protein.

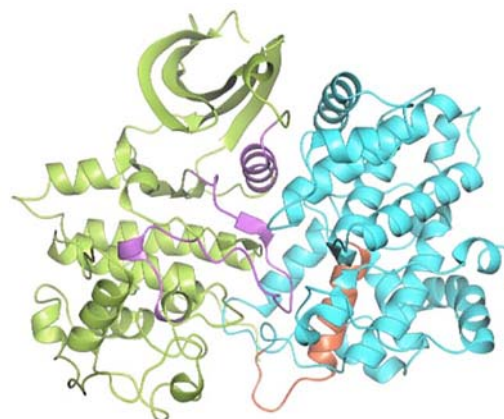


Figure 2. pCDK2 (green), with activation segment in magenta, in complex with cyclin E (cyan) with centrosomal binding region in red.

3.3 CDK2/cyclin A substrate recognition

Nick R. Brown, Vicky Skamnaki, Ed D. Lowe, Robert Cheng, Luke Kontogiannis in collaboration with Martin Noble and Jim McDonnell and in collaboration with Kui Shen and Philip Cole (Johns Hopkins) and with Giuliano Siligardi (Diamond Light Source).

Substrate specificity for phospho-CDK2/cyclin A (pCDK2/cyclin A) employs a dual recognition strategy. The preferred sequence that surrounds the phospho-acceptor site in CDK2 is S/T.P.X.R/K, (where S or T are the sites of phosphorylation, and X is any amino acid). Most of the important substrates of the cell cycle S phase contain an additional recruitment motif, RXL or KXL, which is

found at more than 18 residues away from the site of phosphorylation. The RXL motif allows relaxation of specificity at the catalytic site and non-optimal sequences S/T.P can be recognised without the downstream basic residue. The RXL recognition site is located in an exposed, hydrophobic region on the cyclin A molecule, that is conserved in cyclin E. The recruitment site is some 40 Å from the catalytic site of CDK2 [6,7]. We have used crystallographic binding studies together with kinetic and CD solution binding studies to investigate the relationship between the recruitment site and the catalytic site.

Kinetic studies with the peptide substrate **HHASPRK** compared with a modified peptide from the natural CDC6 substrate (sequence **HHASPRKQGKKENGPPHSHTLKGRRLVFDN**) showed that the presence of the RXL motif reduces the apparent K_m by x10. At the same time the k_{cat} is also reduced giving rise to a change in the specificity constant k_{cat}/K_m of about six fold. In contrast an RXL containing peptide that is not covalently linked to the substrate peptide gave no significant change in kinetic parameters. Such results are indicative of an entropic role for the RXL motif in helping to localise the substrate rather than conformational changes induced by binding at the RXL.

A bis-substrate analogue based on the CDC6 peptide was synthesised by Dr Kui Shen and Professor Philip Cole (Johns Hopkins University) in which the serine analogue aminoalanine is linked to ATP γ S via an acetyl bridge. Previous observations [8] showed that the covalent link of the peptide to the ATP provided very much tighter binding for a peptide substrate bound to the insulin receptor tyrosine kinase. Kinetic studies showed that the CDC6 bispeptide is a potent inhibitor of pCDK2/cyclin A activity against the short heptapeptide substrate ($K_i = 8$ nM vs. heptapeptide substrate, 6 nM vs. ATP) but is less effective against a substrate that contains an RXL motif ($K_i = 3.7$ μ M vs. p107 peptide, 1.9 μ M vs ATP). A bisheptapeptide that lacks the RXL motif did not inhibit. An RXL dodecamer peptide bound with a $K_d = 0.4$ μ M. These results show the power of the RXL motif when combined with substrate analogue peptide to produce a tight inhibitor. Both the CDC6 peptide and the CDC6 bispeptide have been co-crystallised with pCDK2/cyclin A. The results show binding of a dodecamer peptide at the recruitment site but no connection between the recruitment site and the catalytic site (Figure 3). We assume the intervening residues must be flexible. Substrate localization at the recruitment site leads to increased catalytic efficiency in which effects are exerted through localisation at the recruitment site but communicated to the catalytic site by a flexible linker peptide. The notion of a flexible linker, which must have more than a minimal number of residues in order to span the recruitment and catalytic sites, provides an explanation for recognition of, and discrimination against, different substrates that is a key component of cell cycle regulation.

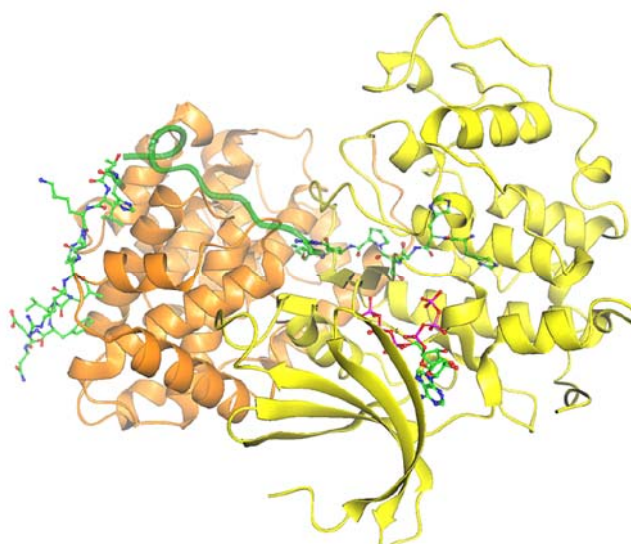


Figure 3. pCDK2 (yellow) in complex with cyclin A (orange) showing a 12 residue RXL CDC6 peptide bound at the cyclin A recruitment site (left), a heptapeptide, and the modified ATP from CDC6 bispeptide bound at the pCDK2 catalytic site. A possible path for the flexible linker is shown in green

3.4 Polo-like kinase

Robert Cheng, Vicky Skamnaki and Ed Lowe

Polo-like kinases (Plks) are a family of serine/threonine kinases related to the polo gene product of *Drosophila melanogaster*, originally identified from a mitotic mutant that displayed abnormal spindle poles. Plks play essential roles in several stages of mitosis and cytokinesis with a dynamic pattern of localisation to centrosomes, kinetochores and central spindle structures during the cell cycle. On entry to mitosis, Plks activate CDK1/cyclin B through phosphorylation of upstream regulators, promote centrosome maturation, disassembly of the Golgi complex and dissociation of cohesin from the chromosomes. Subsequently Plks help initiate anaphase through regulation of the anaphase-promoting complex (APC) and other proteins. Finally Plks promote cytokinesis. It is clear that Plks have multiple substrates and multiple roles. There is keen interest from pharmaceutical companies in targeting Plk1 for anti-cancer therapy.

Clues for the multiple roles of Plks are given by the organisation of the protein. Plks contain an N-terminal kinase domain (residues 49-310 in the human Plk1 sequence) with a requirement for phosphorylation of a threonine in the activation segment (Thr210) and a C-terminal region (residues 345-603) that contains the polo-box domain (PBD), so called because of conservation of two stretches of about 80 amino acids among the polo kinases from different species. The PBD plays a crucial role in sub-cellular localisation through recognition of phospho-peptide [9,10]). It is also an autoinhibitory domain and inhibition can be relieved by phosphorylation of Thr210.

We have determined the crystal structure of the PLk1 PBD with and without bound phospho-peptide [11]. We now focus on the kinase domain. Several different constructs have resulted in the expression of the kinase domain in *E. coli* but the purified protein readily aggregates, thus hampering crystallisation trials. CD and 1D NMR studies have indicated that the protein is folded. A new construct developed this year, with a double tag to facilitate purification, has led to very good expression levels. Further crystallisation attempts are in progress, in the presence and absence of Plk1 inhibitors. The report earlier this year that wortmannin was a potent Plk1 inhibitor ($K_i = 24$ nM) [12] stimulated us to model Plk (using the aurora kinase as start point, the closest kinase to Plk). Wortmannin is a classical phosphoinositide 3-kinase inhibitor where it reacts covalently with the reactive lysine at the ATP site. We are also seeking to characterise the kinetic properties of the Plk1 kinase domain, and its activation by PKA phosphorylation, with a natural substrate Translationally Controlled Tumour Protein (TCTP), a 172 amino acid microtubule binding protein which has a role in the formation of the mitotic spindle. In addition to other functions. In this natural substrate, whose structure is known from NMR, Plk1 phosphorylates two residues Ser46 and Ser64.

3.5 Regulators of transcription (CDK9/cyclin T)

Graziano Lolli and Ed Lowe

The cyclin dependent protein kinase complex CDK9/cyclin T regulates the elongation phase of RNA transcription. In concert with CDK7/cyclin H/Mat1, CDK9/cyclin T phosphorylates the C-terminal domain of RNA polymerase II at sites in the 52 heptad repeat motif. CDK9/cyclin T is also implicated in the transactivation of HIV transcription through recruitment of the complex with the TAT protein and TAR, a short RNA generated by initiation of transcription from the HIV promoter.

We have purified the Cdk9/CycT-298 complex after dual expression in baculoviral infected insect cells. CycT-298 is cyclin T residues 1-298, a fragment that contains both cyclin box folds and which was found to be a stable complex from limited proteolysis studies with full length cyclin T. Soluble CDK9/CycT-298 has been crystallised to yield very tiny crystals that diffract to 7-Å resolution. We have analysed the phosphorylation properties of the complex with a view to producing homogeneous material for crystallisation. LC-ESMS showed that CycT-298 was largely unphosphorylated (98%) and Cdk9 was present as mono-phosphorylated (80%) and unphosphorylated (20%). Tryptic digestion and peptide analysis by MALDI-MS and sequencing by MS/MS showed that phosphorylation is homogeneous on the activation segment T186. The Cdk9/CycT-298 complex is active and is able to autophosphorylate. We have carried out an analysis of the autophosphorylation sites and their effects on the activity of the complex in order to gain insight into the physiological role of autophosphorylation.

Autophosphorylation resulted in a Gaussian distribution of phosphorylated forms centred on 3P and 4P Cdk9 with the complete disappearance of the unphosphorylated form. CycT-298 was unaffected. Cdk9/CycT-298 activity on RNA polymerase II CTD was reduced after auto-phosphorylation. A time course study identified the new phosphorylation sites as preferential phosphorylation of S347 followed by phosphorylation of T362 or T363. During these experiments phosphorylation on T186 increased indicating that T186 is also a site of autophosphorylation and providing the first direct evidence that CDK9 does not require an activating kinase. Two mutants Cdk9-S347E and Cdk9-T362E exhibited low activities when compared to the WT-Cdk9 complex. Activity of S347E-Cdk9 increased after autophosphorylation, due to phosphorylation of T186, to levels comparable to the activity of autophosphorylated WT-Cdk9.

Our hypothesis is that Cdk9/CycT1 requires T186 phosphorylation for full activity and that activity is partially reduced by S347 phosphorylation. The effect of S347 phosphorylation is important in that it generates a conformational change needed to expose the TRM motif in CycT for RNA binding. The role of Ser347 phosphorylation in physiological conditions is then to configure Cdk9/CycT for HEXIM1/7SK binding and full inhibition. The inactive Cdk9/CycT1/7SK/HEXIM1 complex is no longer able to autophosphorylate. Reactivation is generated by phosphatase action, Ser347 dephosphorylation and release of HEXIM1/7SK. PPI is not able to dephosphorylate Thr186 and thus leaves the kinase active. Our studies indicate that T362 or T363 phosphorylation is able to substitute for S347 and these sites are also sensitive to phosphatase action. Phosphorylation of T186 activates the autophosphorylation activity of the kinase and thus favors inhibitor binding while dephosphorylation of S347 removes inhibition and reactivates the kinase.

3.6 Studies with Ubiquitin Domain Proteins (UDPs)

Nick Brown, Ed Lowe, Nail Hasan in collaboration with Jane Endicott, Martin Noble, Jean-Francois Trempe and Hideki Kobayashi (Kyushu University, Japan)

Ubiquitin (Ub) is used as a signal molecule for controlled targeting of proteins to the proteasome and in other signalling processes such as DNA repair and splicing. Ubiquitylation is a crucial regulatory process in the cell cycle. Proteins destined to be degraded by the proteasome are marked by the covalent attachment of poly-ubiquitin chains. Ubiquitylation allows the destruction of inhibitors such as p27 and the timely destruction of cyclins (cyclin E at the start of S phase, cyclin A at the start of mitosis and cyclin B on exit from mitosis).

The yeast *Saccharomyces cerevisiae* protein Dsk2 (373 amino acid) contains an N-terminal ubiquitin-like (UBL) domain and a C-terminal ubiquitin associated (UBA) domain separated by a region that contains repeat sequences. The Dsk2 UBL domain binds to the Rpn1 proteasomal subunit of the 19S regulatory particle. The bifunctional binding properties of Dsk2 have suggested a model whereby the UBA domain binds the poly-Ub moiety of an ubiquitinated substrate and the UBL domain binds to specific subunits of the proteasome, thus delivering the substrate protein to the proteasome for subsequent degradation.

The structure of the UBL domain has been solved at 1.15 Å resolution by molecular replacement using the UBL domain of the *Arabidopsis thaliana* protein Rub1 (34% sequence identity) as the search object. The UBA domain proved more challenging. The structure was solved at 2.6 Å resolution using selenium methionine SAD phasing and revealed a complex with 9 UBA molecules per asymmetric unit (Figure 4a). In the crystal, the Dsk2 UBA domains associate through electrostatic interactions to form 9-fold helical ribbons that leave the ubiquitin binding surface exposed. We next solved the structure of a UBA/UBL complex, which had 18 UBA domains per asymmetric unit. The structure showed the same 9-fold helical ribbon arrangement for the UBA domains, in which three UBL domains associated with the exposed Ub binding sites (Figure 4a) [13].

Measurements with surface plasmon resonance had shown that Dsk2 UBA bound to UBL ($K_d = 80 \mu\text{M}$) with a lower affinity than to Ub ($K_d = 8 \mu\text{M}$). The UBA/UBL complex shows that a key hydrophobic contact from Ub is lacking in the UBL complex and explains the reduced affinity of the UBA domain for UBL compared with Ub. This result has implications for the regulation of Dsk2 adaptor function during Ub-mediated proteasomal targeting. It suggests that unbound Dsk2 could exist primarily in a closed conformation in which the proteasomal-binding motif on the UBL domain is shielded by its association with the UBA domain as in the crystal structure. In the presence of a polyubiquitinated substrate, the Ub chains compete effectively with the UBL/UBA interaction and binding results in an opening of the full-length molecule permitting UBL/Rpn1 binding (Figure 4c).

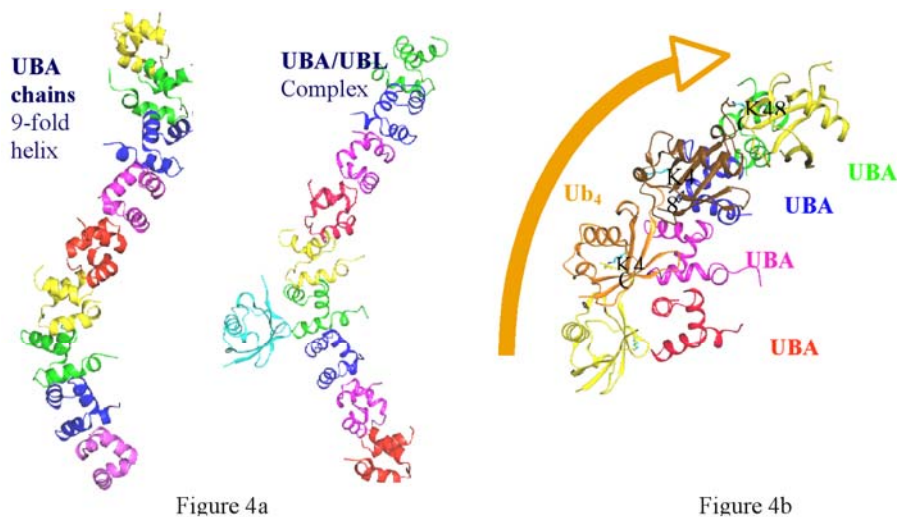


Figure 4a

Figure 4b

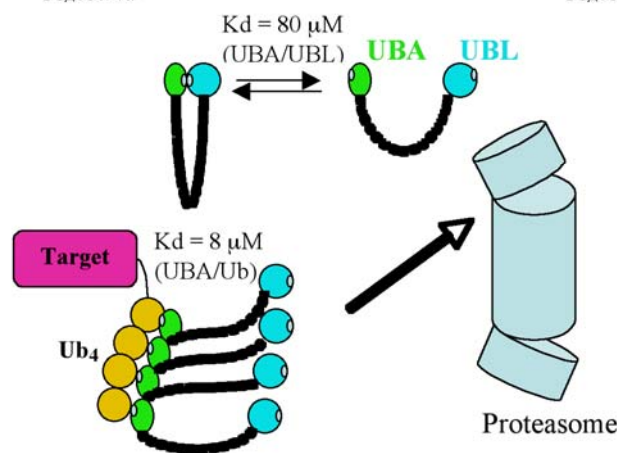


Figure 4c

Figure 4. The Dsk2 UBA structure. a) Dsk2 UBA domains associate into a ninefold helix in both the UBA and UBA–UBL crystals. The UBA domains are colored magenta, blue, green, yellow, red and then purple, cyan, pale green, orange so that similar colours repeating every five molecules indicate a rotation of 200. The UBL domain binds to one UBA in this chain. Most other UBL binding sites are blocked by the symmetry related molecules. b) A possible model for tetra-ubiquitin recruiting 4 UBA domains with the helical symmetry observed in the crystal structure. c) A possible model showing the role of the UBL domain to prevent the targeting when Dsk2 does not carry cargo and how the assembly of multiple UBA domains on the tetra-ubiquitin attached to the target molecule could facilitate transport of the target to the proteasome. (From Lowe et al. 2006).

The helical assembly of the Dsk2 UBA domains suggests a possible model for poly-Ub binding [13]. Four Ub molecules were modelled onto a UBA tetramer, by superimposing one of the Ub molecules onto the position of the UBL observed in the UBA/UBL complex structure, and generating successive Ub molecules by applying the 9-fold helical transformation. The model showed that such an assembly is stereochemically reasonable (Figure 4b). The UBA/Ub binding interfaces are maintained without any clashes and some favourable contacts between adjacent Ub molecules. In the model, the Ub-K48 side chain is close to the C-terminus of an adjacent Ub molecule and the flexible isopeptide link can be made. The side chains of other lysines (K6, K29, and K63) are distant from the C-termini and are unable to form the isopeptide bond. This model suggests a mechanism by which the Dsk2 UBA domains could selectively recognize K48-linked Ub molecules with high affinity. The model proposed for Dsk2 also implies that the association of UBA domains to Ub chains in the full length Dsk2 molecule would result in an effective increase in local concentration of the UBL domains thus enhancing the likelihood of interaction with the 19S regulatory particle and providing an opportunity for cooperative binding of target-loaded Dsk2 to the proteasome (Figure 4c).

In support of these proposals, we note that yeast two-hybrid studies have shown that Dsk2 associates to form homodimers via its C-terminal UBA domain and that association involves just those residues, which are important for UBA associations into the helical ribbons [14]. Moreover in an analysis of *in vivo* yeast extracts, Sasaki et al. report that higher molecular weight Dsk2 fractions are observed and that these fractions bind polyubiquitin while monomeric Dsk2 shows little binding of polyubiquitin and the higher molecular weight fractions did not bind monomeric Ub. These recent results are consistent with the model in which it is proposed that the ability of Dsk2 UBA domains to associate could be important for the biological function of Dsk2 in the delivery of polyubiquitinated targets to the proteasome. However we note that most UBA domains do not have charged residues corresponding to those that mediate the electrostatic stabilising interactions of the Dsk2 UBA helical structures. It may be that Dsk2 is unique. For these reasons we remain cautious about the universality of the proposed model. Structural and functional studies have been initiated to further explore the validity of the model.

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