

3. Louise N. Johnson

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

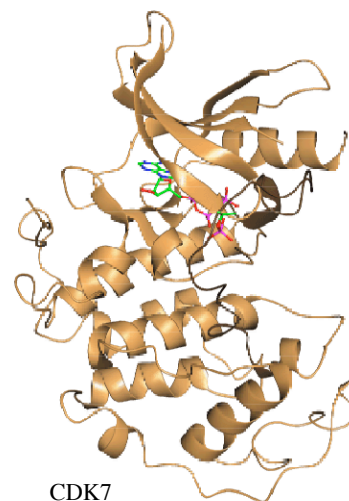
We are interested in the structural basis of the recognition processes that control biological interactions, especially those that mediate control by phosphorylation and by ubiquitination. Current work includes studies on the regulatory kinases of the cell cycle (CDK2/cyclin A, CDK2/cyclin E, CDK7/cyclin H, CDK9/cyclin T and polo-like kinase) and those domains that mediate interactions of ubiquitinated proteins with the proteasome. We continue our special interest with phosphorylase kinase and its regulation by calcium/calmodulin. Our major technique is protein crystallography. For large macromolecular assemblies we are also using electron microscopy (see Section by Catherine Venien Bryan).

CDK7 (CDK Activating Kinase (CAK))

Graziano Lolli, Ed Lowe and Nick R. Brown

CDK7, a member of the cyclin dependent protein kinase 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 TFIID complex where it phosphorylates the C-terminal domain of RNA polymerase II. For activity and stability, CDK7 itself requires phosphorylation and association with a cyclin, Cyclin H, and association with a third protein, MAT1. CDK7 poses many intriguing structural problems concerned with its recognition roles in the cell cycle and in transcription.

The crystal structure of CDK7 has been solved this year following data collection from very small crystals ($\sim 50 \times 10 \times 5 \mu\text{m}$) at beam lines ID29 and ID13 at ESRF [1]. The quality of the 3 Å resolution structure was enhanced by four fold averaging. The kinase is in the inactive conformation, similar to that observed for inactive CDK2. The activation segment is phosphorylated at Thr170 and is in a defined conformation that differs from that in phospho-CDK2 and phospho-CDK2/cyclin A. Comparison of the ATP binding sites for CDK2 and CDK7 showed some differences. These differences are currently being explored for the design of potent CDK7 inhibitors. Such an inhibitor could be of value in the control of the cell cycle in therapy and in cell biology where it is useful to be able to discriminate between the effects of CDK7 and CDK9. The CDK7 structure provides a lead for structural studies on the CDK2/CDK7 complex and the active CDK7/cyclin H and CDK7/cyclin H/Mat1 complexes.



We have investigated the recognition properties of the Kinase Associated Phosphatase (KAP) with CDK7. Our structure of the complex between KAP and pCDK2 [2] showed that recognition at the catalytic site was achieved solely through the pThr160 phosphate group of pCDK2 but that the major determinant of specificity was located about 20 Å from the catalytic site where the C-terminal helix of KAP interacted with residues from the G helix and the L14 loop of CDK2. The L14 region differs in CDK7. In order to test if these features destroy the docking site and abolish KAP activity we tested KAP activity against CDK7 and constructed KAP-interacting mutants (KM) for CDK2 and CDK7. These mutations create 2 chimeras: CDK2 with CDK7-L14 loop and CDK7 with CDK2-L14 loop. The L14 loop mutations in KM-pCDK2 abolish KAP activity against pCDK2. This result highlights the importance of the docking site for the regulation of KAP activity on pCDK2. KAP is not active against either CDK7 or KM-CDK7. Evidently the generation of a correct KAP docking site on CDK7 was not sufficient to make it a substrate. This may be because other effects are also important. For example the pCDK7 activation segment is considerably more rigid than the disordered activation segment in pCDK2, which may make it less amenable for conformational change to reach the KAP catalytic site.

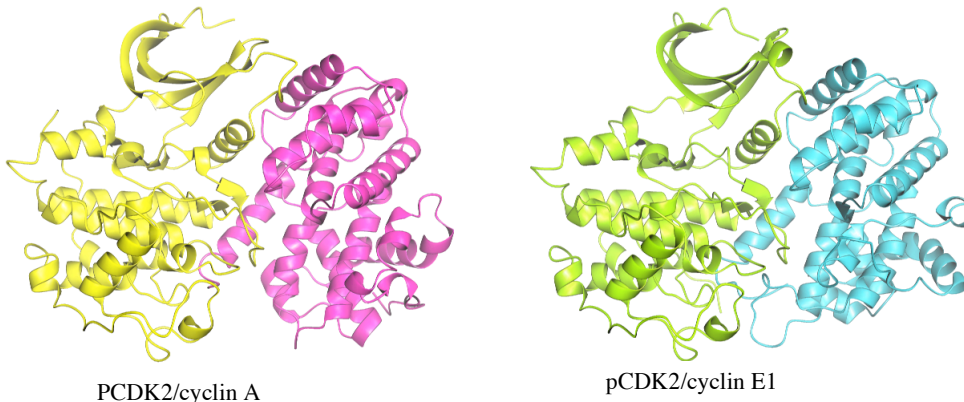
CDK2/cyclin E

Reiko Honda (from Jan 2004), Elena Dubinina (to Jan 2004), Ed Lowe, Vicky Skamnaki, Atlanta Cook 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 is an activator of cyclin dependent kinase 2 (CDK2). In mammalian cells, the mitogen stimulated activities of CDK4 or CDK6 in association with a D-type cyclin promote transcription of cyclin E during G1 phase and activation of CDK2/cyclin E kinase activity through the redistribution of cell cycle inhibitors. Levels of cyclin E decline during S phase in response to auto- and other phosphorylation events that target cyclin E for ubiquitination 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 thought that it is the activity of the CDK2/cyclin A complex that drives cells through S phase.

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. CDK2/cyclin E substrates also include proteins involved in the regulation of transcription (such as pRb and pRb related proteins), proteins involved in pre-mRNA splicing, histone biosynthesis, gene expression control, centrosome duplication, and cell cycle progression (for example p27^{Kip1} and CDC25). Although some substrates appear to be specific for CDK2/cyclin E *in vivo*, almost all can be phosphorylated by CDK2/cyclin A *in vitro*. This has raised the question of why cells need both cyclin E and cyclin A. In a dramatic development in 2003, it was shown from knock-out mouse embryo fibroblast cells, in which both cyclin E1 and cyclin E2 were ablated, that cyclin E is dispensable for cell cycling but is essential for cells to re-enter the cell cycle from the quiescent G0 state and for endoreduplication, highlighting the important role of cyclin E in the assembly of the pre-replication complex [3,4]. 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. The untimely presence of cyclin E has been shown to interfere with the replication complex assembly as cells exit mitosis. Remarkably cyclin E deficient cells are resistant to oncogenic transformation by Ras.

In order to understand the role of cyclin E in healthy cells and in cancer and to elucidate substrate specificity, we have determined the structure of pCDK2/cyclinE and compared it with pCDK2/cyclin A [5]. The crystal structure of truncated cyclin E (residues 81-363)/pCDK2 complex has been solved by molecular replacement at 2.25 Å resolution. 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, and contributes to CDK2-independent binding sites of cyclin E. Kinetic analysis with model peptide substrates show a 1.6 fold increase in k_{cat} for pCDK2/cyclin E1 (81-363) over k_{cat} of pCDK2/cyclin E (full length) and pCDK2/cyclin A. The structural and kinetic results indicate no inherent substrate discrimination between pCDK2/cyclin E and pCDK2/cyclin A with model substrates. We are currently probing the structural basis for substrate preferences of pCDK2/cyclin E and pCDK2/cyclin A with intact substrates such as nuclear lamin.



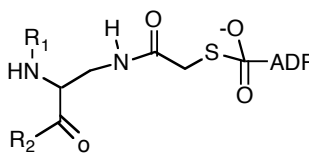
CDK2/cyclin A substrate recognition

Nick R. Brown, Vicky Skamnaki, Ed D. Lowe, Luke Kontogiannis in collaboration with Jim McDonnell and Martin Noble

We are pursuing a programme to identify how CDK2/cyclin A selects its substrates. Substrate specificity often employs a dual recognition strategy in which the sequence flanking the phospho-acceptor site (Ser.Pro.X.Arg/Lys) is recognised by CDK2 while the cyclin A component of the complex contains a site that binds Arg/Lys.X.Leu ("RXL" or "KXL") substrate recruitment motifs. The RXL recognition site is located in an exposed, hydrophobic region on the cyclin A molecule, that is conserved in cyclins A, B, D and E. The recruitment site is some 40 Å from the catalytic site of CDK2 [6,7].

We have used enzyme kinetics to compare the action of pCDK2/cyclin A on peptides lacking the RXL motif and those with the RXL motif. Comparison of the peptide substrate HHASPRK with a modified CDC6 peptide 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 the presence of 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 a conformational or a direct stereochemical path between RXL site and catalytic site. NMR studies are in progress in an attempt to distinguish further between the two mechanisms.

We have collaborated with Professor Philip Cole (Johns Hopkins University) who has synthesised for us a bis-substrate analogue based on the CDC6 peptide. Our kinetic studies have shown that the bis-substrate is a potent inhibitor of CDK2 activity ($K_i = 8$ nM vs. a model heptapeptide substrate, 6 nM vs. ATP and 3.7 μ M vs. p107 peptide containing an RXL motif). The bis-substrate analogue has been co-crystallised and the structure is currently being refined. 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 but this does not seem to be the case with CDK2/cyclin A..



R1 = AcNH-HHA-
R2 = -PRKGGKKENGPPHSHTLKGRRLVFDN-COO2

Polo-like kinase

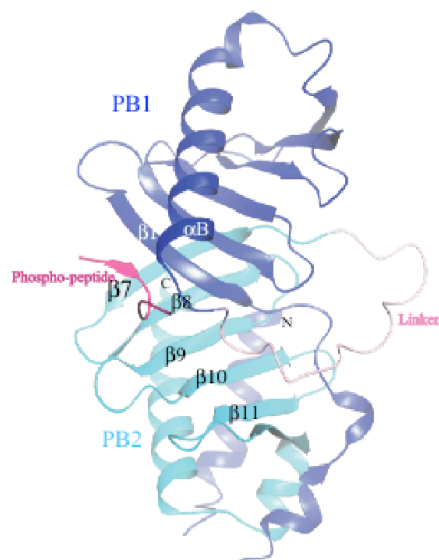
Robert Cheng, Ed Lowe in collaboration with Erich A. Nigg (Max Planck Institute for Biochemistry, Martinsried)

Polo-like kinase is an outstanding example of an enzyme controlled by remote site recognition and which acts as an integrator in signalling. 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. Plks have multiple substrates and multiple roles. 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.

Last year we reported on the crystal structure of the Plk1 PBD with and without bound phospho-peptide[11]. The two polo boxes of Plk1 PBD have identical folds based on a six-stranded anti-parallel β sheet and an α helix. The phospho-peptide binds at a site between the two polo boxes. Contact residues include many residues

that have been identified as important for function from mutational cell biology studies and the structure also defines new residues involved in phospho-peptide recognition. The structure of the PBD opens up the possibility of targeted mutations to probe cellular function and the design of peptidomimics that might inhibit the function of Plk in tumour cells. SiRNA experiments have shown that ablation of Plk1 sends tumour cells into apoptosis. There is keen interest from pharmaceutical companies in targeting Plk1 for anti-cancer therapy.

This year attention has focussed on the kinase domain, which has been expressed and purified. The kinase domain is temperature sensitive and so far has not crystallised in robot screens. We know that kinase domains are crystallisable. We are therefore using PCR to produce new constructs. We are also using phosphorylation by PKA to phosphorylate Thr210 and produce an active kinase and specific inhibitors to stabilise the domain.



Plk1 polo box domain

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 serine 2 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. Soluble CDK9/cyclin T has been produced from dual baculovirus infection in insect cells and crystallised to yield very tiny crystals that diffract to 7-Å resolution. Work is in progress to further characterise the complex and to improve the crystals.

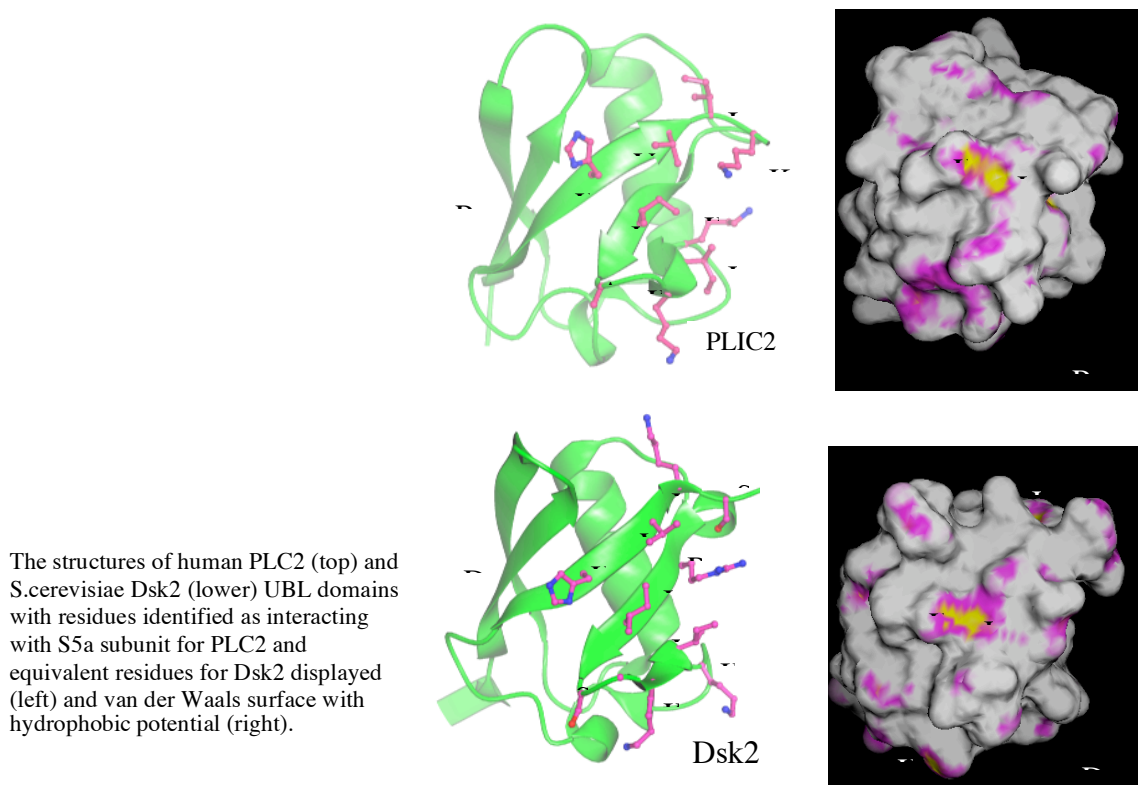
Studies with Ubiquitin Domain Proteins (UDPs)

Nick Brown, Ed Lowe, Nail Hasan (Royal Society International Exchange Fellow) in collaboration with Jane Endicott, Martin Noble and Jean-Francois Trempe.

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. Proteins that are destined to be degraded by the proteasome are marked by the covalent attachment of poly-ubiquitin chains. Ubiquitination 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). We are working with the yeast *Saccharomyces cerevisiae* protein Dsk2, a 373 amino acid protein that contains an N-terminal ubiquitin-like (UBL) domain and a C-terminal ubiquitin associating (UBA) domain separated by a region that contains repeat sequences. Reports [12,13] have shown that 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. We wish to understand the structural basis for the recognition properties of Dsk2 and relate these to function.

The UBL domain has been crystallised and the crystal structure solved at 1.15 Å resolution by molecular replacement. The structure was refined by REFMAC to a crystallographic R value 0.182 (R_{free} 0.193). The structure has a typical ubiquitin fold comprising a five stranded β sheet with one α helix and one 3_{10} helix

folded around a hydrophobic core. NMR studies on PLIC2 (the human homologue of Dsk2) UBL domain in association with the 19S proteasomal subunit S5a showed chemical shifts in key residues exposed on the surface of the β sheet (shown in figure) [14]. Display of these residues on the Dsk2 UBL surface showed that 7/10 of these residues are conserved in Dsk2 as is the hydrophobic potential. From a number of different structural studies, this surface has been identified as the common interaction surface for UB in complex with Ubiquitin Interacting Motifs (UIMs). In order to study the association of the UBL domain with the proteasomal subunit, several constructs of the yeast Rpn1 subunit (993 residues in total) have been engineered based on primary sequence alignments and on published results. The constructs will be tested for UBL binding properties to help understand which region(s) of Rpn1 are involved in UBL binding.



This year the Dsk2 UBA domain has been crystallised but these crystals proved challenging. A monoclinic C2 crystal form diffracted to 2.6 Å resolution but had > 8 molecules per asymmetric unit. A Se-methionine protein has allowed us to solve the structure by SAD methods and the results will be reported next year. We have measured the affinity of the UBA domain for ubiquitin (UB), di-ubiquitin linked through Lys48 (UB2) and tetraubiquitin (UB4) using BIAcore binding studies. We find K_d values of 8000 nM, 200nM and 5nM for UB, UB2 and UB4, respectively. This increase in affinity with chain length is in agreement with cell biology results that suggest a chain of at least four ubiquitin molecules is necessary before a modified protein can be targeted to the proteasome.

We also found that Dsk2 UBA binds to its UBL domain with $K_d = 80 \mu\text{M}$ suggesting a possible regulatory mechanism for Dsk2 function. Unbound Dsk2 could exist primarily in a closed conformation in which the proteasomal-binding motif on the UBL domain is shielded. 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. We have crystallised the UBA/UBL complex and knowledge of the UBA and UBL structures has allowed us to solve the UBL/UBA structure.

We are developing a preparation procedure for poly-ubiquitin to provide sufficient quantities for co-crystallisation. Recombinant mouse UB activating enzyme (E1) with an N-terminal His tag has been expressed from baculoviral infected insect cells (from a construct kindly supplied by Kazuhiro Iwai (Osaka)) and shown to be active. The human E2 UB conjugating enzyme (CDC34) expression constructs have been developed by Jane Endicott and Jan Gruber (see report by Jane Endicott). The enzyme has been expressed in soluble form in *E. coli* as both the full length CDC34 protein with a His tag and also as a truncated enzyme as a GST fusion protein. The truncated E2 is effective for the synthesis of milligram quantities of UB2. The synthesis of UB4 requires the full-length CDC34 enzyme and ion exchange chromatography to separate the different length UB chains from the reaction mixture. This procedure is currently being scaled up. These recent advances open the way for co-crystallisation trials with UBA/UB4, which will be guided by parallel mass spectrometry experiments using non-dissociating ESI conditions to measure the stoichiometry of the UBA interactions.

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